Oxidative Stress in Applied Basic Research and Clinical Practice

Maria Jose Alcaraz
Oreste Gualillo
Olga Sánchez-Pernaute *Editors*

Studies on Arthritis and Joint Disorders

Humana Press

Oxidative Stress in Applied Basic Research and Clinical Practice

Editor-in-Chief
Donald Armstrong

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Note from the Editor-in-Chief

All books in this series illustrate point-of-care testing and critically evaluate the potential of antioxidant supplementation in various medical disorders associated with oxidative stress. Future volumes will be updated as warranted by emerging new technology, or from studies reporting clinical trials.

Donald Armstrong
Editor-in-Chief

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Studies on Arthritis and Joint Disorders

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Preface

Rheumatisms are complex diseases in which the incidence of exogenous factors is determinant. Searching for an individual pathogenic factor is often useless in this kind of diseases, whereas targeting general cell processes might be a better strategy. Rheumatic diseases are highly heterogeneous in both causative mechanisms and lesion type, but in most cases their development and progression are characterized by a failure of resident cells to preserve tissue homeostasis.

The physiology of joints and connective tissues makes them particularly exposed to oxidative stress. Joint tissues have a high vascular supply and offer defensive cells the possibility of establishing contact with environmental factors. They also can act as receptacles for systemic inflammatory mediators, and even behave in the fashion of secondary lymphoid organs, in the setting of systemic autoimmune diseases. All these features make oxidation derivates especially harmful to joints and connective tissues. Additionally, joints are a major source of oxidative mediators because of their lipid-rich composition, their capacity to produce cytokines, and the presence of catalytic metals, such as iron and copper.

Both mechanical forces and chemical reactions can lead to the production of radical oxygen species (ROS) in joints, and render oxidative damage to the cartilage and the synovial tissue. Most frequently oxidative damage alters cell survival and growth, not only through a caspase-dependent death process, but also promoting mechanisms of senescence. Not surprisingly, the increased generation of ROS—or a deficient red-ox capacity—has been claimed as a major pathogenic factor in degenerative diseases. Generally speaking, accumulation of ROS provokes alterations in the structure of lipids, nucleic acids, and proteins, depletes the mitochondrial buffering reserve, and promotes protein misfolding.

Rheumatologists are used to cope with a highly popular alternative medicine promising cures for rheumatic ailments, without conducting a single experiment or clinical trial. This traditional medicine can be traced back to the Middle Ages, when both physicians and sorcerers administered a number of herb-containing potions and beverages. One of the most famous healing potions coming from the Classical Period was the "panacea," whose components are acknowledged for their antioxidative capacity and continue to be used nowadays with different clinical indications [1].

Only in the last decades scientific evidence is accumulating, supporting that various components of plants and dietary supplements are useful remedies against rheumatic diseases and offer the additional benefit of low toxicity. Most probably, in the next few years, medicines resembling antique remedies will be made. Eventually, chemical therapies will leave way to biologics, and these perhaps to nutraceuticals. Being realistic, the latter look valuable as adjuvant therapies helping keep tissue homeostasis. As has already been shown in cancer, some dietary products act as cell-conditioning agents and improve their response to cytostatics.

Joints and connective tissues are a rich milieu of cells and matrix components, not only including fibroblasts and vessels, but also bone marrow precursors, immune-specific cells, and adipocytes. Altogether they provide a highly versatile structure, accessible to therapeutic intervention. It looks as a good scenario for antioxidant drugs, but unfortunately the development of these compounds is confronted with a dreaded lack of efficacy. The search for panacea is still going on and its perfect recipe is yet far to be deciphered.

Several hurdles need to be overcome in order to establish the therapeutic capacities of nutraceuticals in rheumatic diseases. A major pitfall is that their in vitro antioxidative capacity does not correspond to an in vivo effect. This could depend on the daily dose of the nutrient, but also digestion seems to play a role in avoiding a direct effect of most dietary components. On the other hand, the beneficial effect for the joints of changing our dietary habits is quite clear, and it has been suggested that the effects might rely on the production of endogenous intermediates. Some of these controversies will be solved with the help of the new high-throughput technology, which makes possible to track the route that follows the administration of molecules with a medical intention.

Another handicap is the laborious clinical trials needed to assess efficacy in these typically heterogeneous and slowly progressive diseases. Joint replacement, bone erosion, fracture, or stroke are long-term efficacy measures and only valid when large cohorts are evaluated. Epidemiologic studies are usually confronted with numerous confounding factors, and the clinical assessment is often based on the measurement of nonobjective variables. In this sense, molecular biomarkers are attracting much interest as they could help selection of candidates and assessment of response, after their validation in large population studies.

This book offers a state-of-the-art overview of how oxidative stress participates in the most prevalent joint diseases, as discussed by experts working in the field from different approaches. From autoimmunity to senescence, and from bench to bedside, their acknowledged contributions to the field are sure to shed light on the complexity of the subject.

Burjassot, Valencia, Spain — Maria Jose Alcaraz
Santiago de Compostela, Spain — Oreste Gualillo
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Reference

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Part I
Biology of Oxidative Stress in Joint Disorders

Chapter 1
Soluble Proteomic Biomakers in the Management of Arthritis

Yves Henrotin and Ali Mobasheri

Abstract This chapter introduces the readers of this volume to arthritic diseases including osteoarthritis (OA) and rheumatoid arthritis (RA) before focusing on collagenous and non-collagenous biomarkers of these joint diseases. The main objective of this chapter is to focus on reactive oxygen species and in vivo biomarkers of oxidative stress. Such biomarkers may be early indicators of oxidative stress-induced tissue damage and could be used to identify patients at increased risk of developing joint disease.

Abbreviations

ACCP	Anti-cyclic citrullinated protein antibodies
ACPA	Anti-citrullinated protein antibodies
AGE	Advanced glycation end product
BMD	Bone mineral density
CDC	Centers for Disease Control and Prevention
CML	Carboxymethyl lysine
COMO	Cartilage oligomeric matrix protein
CRP	C-reactive protein
CTX-I	Carboxy-terminal cross-linked telopeptide of type I collagen

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CTX-II	Carboxy-terminal cross-linked telopeptide of type II collagen
DNA	Deoxyribonucleic acid
DTPA	Diethylene triamine penta-acetate
ECM	Extracellular matrix
EFSA	European Food Safety Authority
EGR-1	Early growth response protein 1
ELISA	Enzyme-linked immunosorbent assays
eNOS	Endothelial NOS
ESR	Erythrocyte sedimentation rate
ESR	Electron spin resonance
GC	Gas chromatography
GSH	Glutathione or gamma-L-glutamyl-L-cysteinylglycine
H_2O_2	Hydrogen peroxide
HA	Hyaluronic acid
HPLC	High-performance liquid chromatography
HRT	Hormone replacement therapy
IFN-γ	Interferon gamma
IGF-I	Insulin-like growth factor I
IGF-IR	Insulin-like growth factor I receptor
IL-1β	Interleukin 1 beta
IL-6	Interleukin-6
iNOS	Inducible NOS
JSW	Joint space width
LC	Liquid chromatography
MMP-13	Matrix metalloproteinase 13
MPO	Peroxynitrite
MRI	Magnetic resonance imaging
MS	Mass spectrometry
NADPH	Nicotinamide adenine dinucleotide phosphate
NALP	Pyrin-like protein containing a pyrin domain
NEN	Nonenzymatic nitrite
NF-κB	Nuclear factor kappa B
NIAMS	National Institute of Arthritis and Musculoskeletal and Skin Diseases
NO	Nitric oxide
NTX-I	Amino-terminal cross-linked telopeptide of type I collagen
OA	Osteoarthritis
PAS	Patient Activity Scale
PGE_2	Prostaglandin E_2
PKC	Protein kinase C
PMN	Polymorphonuclear leukocytes
PYCARD	Apoptosis-associated speck-like protein containing a caspase recruitment domain (CARD)
RA	Rheumatoid arthritis
RAGE	AGE receptor
RF	Rheumatoid factor
ROS	Reactive oxygen species
SOD	Superoxide dismutase

TBAR	Thiobarbituric acid reactants
TIINE	Collagen type II neoepitope
uPA	Urokinase or urokinase-type plasminogen activator
WHO	World Health Organization
WOMAC	Western Ontario and McMaster University Osteoarthritis Index
WT	Wild type
YKL-40	Cartilage glycoprotein-39

1.1 Introduction

Human life expectancy has consistently increased by a quarter of a year every year over the last 160 years [1]. It is predicted that life expectancy will continue to increase by 2.5 years each decade, meaning that the Western world's average life expectancy should reach and exceed 100 within the next 50 years [1]. This increase in life expectancy has been mainly due to the significant advances in medicine and healthcare. However, the increased life expectancy of human beings is accompanied by an increased prevalence of a range of arthritic, rheumatic, and musculoskeletal diseases.

Arthritic diseases of load-bearing synovial joints are leading causes of morbidity, disability, and loss of productivity throughout the world [2] (source: http://www.arthritis.org/).[1, 2] These are essentially "inflammatory" disorders. The term "arthritis" characterizes a group of conditions involving inflammatory damage to synovial joints [3]. According to the World Health Organization (WHO[3]), orthopedic, rheumatic, and musculoskeletal conditions comprise over 200 diseases and syndromes, which are usually progressive and associated with pain and disability. The most common form, osteoarthritis (OA), is one of the most prevalent and chronic diseases affecting the elderly [4]. The symptoms and signs characteristic of OA in the most frequently affected joints are heat, swelling, pain, stiffness, and limited mobility. OA is often a progressive and disabling disease, which occurs in the setting of a variety of risk factors, such as advancing age, obesity, and trauma, that conspire to incite a cascade of pathophysiological events within joint tissues [5]. Other sequelae include osteophyte formation and synovitis [6]. These manifestations are highly variable, depending on joint location and disease severity. Other forms of inflammatory arthritis include gouty arthritis, psoriatic arthritis, and rheumatoid arthritis (RA), an autoimmune disease in which the body's own immune system attacks synovial joints.

The aim of this chapter is to focus on biomarkers of joint disease by placing special emphasis on biomarkers of oxidative stress. We will briefly discuss the major forms of joint disease before discussing the biomarkers that can be used to diagnose them. This chapter also discusses the chemistry of reactive oxygen species (ROS) and antioxidants for targeting oxidative stress in joint diseases and attempts to link these back to biomarkers, focusing on biomarkers that may be early indicators of oxidative stress.

[1] http://www.who.int/healthinfo/statistics/bod_osteoarthritis.pdf

[2] http://whqlibdoc.who.int/bulletin/2003/Vol81-No9/bulletin_2003_81(9)_630.pdf

[3] http://www.who.int/en/

1.2 Osteoarthritis

OA is one of the most prevalent and chronic diseases affecting the elderly [4]. More than 20 million Americans are estimated to have OA.[4] Estimates from the Centers for Disease Control and Prevention (CDC[5]) suggest that OA is one of the top five causes of disability amongst nonhospitalized adults. The situation is similar in European countries. In 2006, it was estimated that around 35–40 million Europeans suffer from OA and nearly 25% of people aged 60 and above suffer from OA-induced disability. It is also anticipated that by the year 2030, 20% of adults will have developed OA in Western Europe and North America. Therefore, OA is expected to place a heavy economic burden on healthcare systems and community services throughout the world.

OA is the most common form of joint disease, with the majority of the population over 65 years of age demonstrating radiographic evidence of OA in at least one joint. Although it is rare in people under 40, it becomes much more common with age. The end-stage treatment for OA is surgery, either to modify or replace the joint. With increasing life expectancy, growth in the elderly population, and an alarming escalation of chronic, inflammatory, and age-related conditions (such as OA), there is increased demand for new treatments and preventative approaches. Although developing OA is a manifestation of aging, the disease may remain latent and asymptomatic, taking many years to reach clinical relevance. OA is not simply the common outcome of aging and joint injury; it is global, active, and inflammatory joint disease.

Although OA is primarily associated with aging, there are other important contributing factors [7]. These include obesity (which increases mechanical stress), underlying metabolic or endocrine disease, genetics and a history of joint trauma and instability, a history of joint trauma or repetitive use, genetics, heritable metabolic disorders, muscle weakness, underlying anatomical and orthopedic disorders (i.e., congenital hip dislocation), joint infection, crystal deposition, previous RA and various disorders of bone turnover, and blood clotting. The metabolic alterations that occur in obesity along with the pro-inflammatory factors produced by white adipose tissue in the chronically overweight are thought to be major factors in the progression of the disease [8].

Symptoms of OA in the most frequently affected joints include heat, swelling, pain, stiffness, and limited mobility. These manifestations are highly variable, depending on joint location and disease severity. OA can affect any synovial joint, but it primarily affects large load-bearing joints such as the hip and knee. The disease is essentially due to daily wear and tear of the joint. Its most prominent feature is the progressive destruction of articular cartilage [9]. It is generally accepted that OA begins in articular cartilage and eventually spreads to subchondral bone and other synovial tissues. However, there is the opposing view that suggests OA is a disease of subchondral bone and begins there. Despite the controversy regarding its initiation,

[4] http://www.niams.nih.gov/

[5] http://www.cdc.gov/

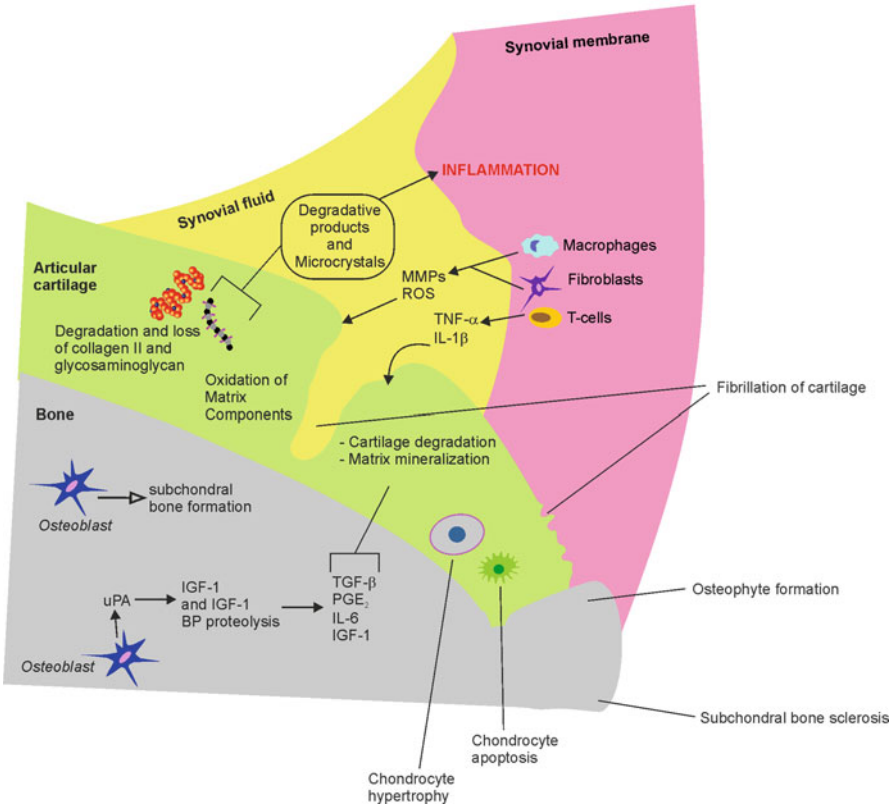

Fig. 1.1 The major molecular and cellular changes that occur in the synovial joint in OA. *MMPs* matrix metalloproteinases, *ROS* reactive oxygen species, *uPA* urokinase, *IGF-1* insulin-like growth factor-1, *IL-6* interleukin-6, *PGE_2* prostaglandin E_2, *BP* binding protein

the current consensus is that OA is a disease involving not only articular cartilage but also the synovial membrane, subchondral bone, and periarticular soft tissues [10]. OA may occur following traumatic injury to the joint, subsequent to an infection of the joint, or simply as a result of aging and the mechanical stresses associated with daily life. The synovitis that occurs in both the early and late phases of OA is associated with alterations in the adjacent cartilage—these changes are highly similar to those seen in RA. Catabolic and pro-inflammatory mediators such as cytokines, nitric oxide (NO), prostaglandin E2 (PGE_2), and neuropeptides are produced by the inflamed synovium, which alter the balance of cartilage matrix degradation and repair. These events lead to excess production of the proteolytic enzymes responsible for cartilage breakdown [11]. Cartilage alterations induce further synovial inflammation, creating a vicious circle. The progressing synovitis will then exacerbate clinical symptoms and joint degradation in OA [11]. Figure 1.1 outlines the major molecular and cellular changes that occur in the synovial joint in OA.

1.3 Rheumatoid Arthritis

Rheumatoid arthritis[6, 7, 8] (RA) is an "autoimmune" disease in which the immune system attacks synovial joints and other tissues. Most of the damage occurs to the joint lining (synovium) and cartilage, which eventually results in erosion of two opposing bones. RA is a painful, chronically disabling, and progressive disease affecting 0.8–1% of the adult population. The symptoms of RA usually vary over time. Sometimes, symptoms only cause mild discomfort. At other times, they can be extremely painful, making it difficult to move around and perform everyday tasks. When symptoms worsen, this is known as a flare-up or flare. A flare-up is impossible to predict, making RA difficult to live with. It can cause severe disability, which varies between individuals and depends on the severity of the disease. It can significantly affect a person's ability to carry out even the simplest of everyday tasks. The disease can progress very rapidly (again the speed of progression varies widely between individuals), causing swelling and damaging cartilage and bone around the joints. Any joint may be affected, but it is commonly the hands, feet, and wrists. RA is a systemic disease, which means that it can affect the whole body and internal organs such as the lungs, heart, and eyes. Furthermore, RA is associated with an increased risk of coronary disease, infection, and lymphoma, as well as reduced life expectancy [12–16]. RA affects approximately three times more women than men, and onset is generally between 40 and 70 years of age, although it can occur at any age. There are studies that suggest RA is also associated with sex hormone production in the body. The peak incidence of RA in women coincides with the perimenopausal age, and the juvenile form occurs mainly during puberty, suggesting a connection of RA with hormonal alterations [17]. Although controversial, several studies have reported on ameliorating effects on clinical measures of disease activity and inflammation, improved bone mineral density (BMD), and presented results pointing towards retardation of joint damage by hormone replacement therapy (HRT) [16, 18, 19].

The pathogenesis of RA is poorly understood. Smoking is an important risk factor and makes the outlook much worse, but there is no mechanistic insight to explain why this is the case. There is no cure for RA, and more information is needed to help understand about the inflammatory processes that occur in the disease and how to manage it. Uncontrolled RA increases mortality through an increased risk of cardiovascular disease such as heart attacks and strokes; again the need for early treatment is imperative. Therefore, we need new and safer drugs for RA and better ways to monitor the disease and avoid prevent noxious stimuli that may cause inflammatory "flare-ups" in the most susceptible individuals.

[6] http://www.nras.org.uk/

[7] http://www.nras.org.uk/about_rheumatoid_arthritis/what_is_ra/what_is_ra.aspx

[8] http://www.arthritisresearchuk.org/arthritis_information/arthritis_types__symptoms/rheumatoid_arthritis.aspx

1.4 Biomarkers of OA

A major focus of clinical research in recent years has been the identification of new disease markers that can facilitate early diagnosis and optimize individualized treatments. Such markers can also facilitate the drug discovery process by reducing the high levels of attrition in clinical trials. A biomarker is a *characteristic* that is objectively *measured* and *evaluated* as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention [20]. They are classified into "soluble" or "wet" biomarkers (i.e., proteins, peptides, metabolites) or as "dry" biomarkers including imaging (i.e., radiographs, magnetic resonance imaging (MRI), ultrasound), questionnaires, and visual analog scales. The National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS) established the OA Biomarkers Network to develop and validate standardized, sensitive biomarker assays in blood and urine to facilitate the diagnosis of the preradiologic stage of OA in humans and animal models. The objective is to identify markers that can help us understand the biological processes involved in disease progression and allow us to monitor the effects of lifestyle (i.e., drastic weight loss), surgical or pharmacological treatment, thus accelerating the pace of drug discovery. Such biomarkers could also potentially be used to identify patients at increased risk of developing OA.

1.4.1 Collagenous Biomarkers

The dry weight of articular cartilage is predominantly composed of collagen [21], and the remainder is mainly proteoglycans and glycoproteins [22]. Type II collagen is the major fibrillar collagen component of articular cartilage. Type II collagen is possibly the ideal source of markers for studying cartilage remodeling [23]. First, this protein is relatively specific to articular cartilage (although it is also present in the vitreous humor of the eye, the nucleus pulposus of vertebral discs, the meniscus, respiratory tract, and insertion sites of tendons and ligaments into bone [24]). Second, it makes up most of the collagen network in the ECM and is the most abundant protein in cartilage, representing 25% of the wet weight, 50% of the dry weight, and 90–95% of the total collagen content. Third, type II collagen makes up only 1% of all collagen in the body, and the normal turnover is low, suggesting that pathological turnover from a single joint might be expected to raise the systemic level epitopes significantly [25]. The breakdown of type II collagen is a characteristic feature of OA, and products of cartilage collagen metabolism can be detected in the blood, synovial fluid, and urine. Since type II collagen is specific for hyaline cartilage and is in high abundance in this tissue, the major biomarkers of collagen turnover in cartilage are epitopes derived from type II collagen [26]. Type II collagen biomarkers may be classified in four groups, according to the localization of the epitope in the molecule and the process that leads to expression of the epitope. The first group, cleavage neoepitopes, is localized to the cleavage site and includes

the following: C2C, C1, 2C, collagen type II neoepitope (TIINE), CIIM, Coll2-1/4N1, and Coll2-1/4N2. The second group includes the denaturation epitopes, which are localized to the triple helical domain and made accessible by unwinding of the triple helix: Coll2-1 and its nitrated form Coll2-1NO2, CB11 peptide (COL2-3/4m), AH8, AH9, and AH12. Epitopes localized to the telopeptides of the molecule represent the third group: Col2CTx and carboxy-terminal cross-linked telopeptide of type II collagen (CTX-II). The final group includes epitopes of propeptide protein fragments that are released during collagen synthesis: carboxyl propeptide and type IIA N-propeptide of type II collagen.

1.4.2 Non-collagenous Biomarkers

In OA proteoglycans are also degraded, and their fragments are released from the ECM into the synovial fluid, and from there they may be filtered to the circulation and urine [27].

1.4.2.1 Cartilage Oligomeric Matrix Protein

Cartilage oligomeric matrix protein (COMP) has shown promise as a diagnostic and prognostic indicator and as a marker of the disease severity and the effect of treatment [28]. It seems to be a good prospect for detecting early-stage OA. It has shown promise as a diagnostic and prognostic indicator and as a marker of the disease severity and the effect of treatment [28, 29]. Enzyme-linked immunosorbent assays (ELISAs) for the detection of this protein and its fragments in synovial fluid and serum have been developed at tested in patients with knee and hip OA [30–32], RA [33], and other forms of inflammatory arthritis [34]. Persistently, high serum levels of COMP have been detected in patients with traumatic knee injury and posttraumatic OA [35, 36]. Large-scale population studies (the Johnston County Osteoarthritis Project) have confirmed that serum COMP protein reflects presence and severity of OA [37]. Several other mesenchyme-derived cells including synoviocytes and dermal fibroblasts produce substantial amounts of COMP. These findings raise important concerns regarding the utility of measurements of COMP levels in serum or in synovial fluid as markers of articular cartilage degradation because of the likelihood that a substantial proportion of COMP or COMP fragments present in serum or synovial fluid may be produced by cells other than articular chondrocytes [38].

1.4.2.2 Hyaluronic Acid

Hyaluronic acid also known as hyaluronan, hyaluronate, or HA has been found to be elevated in plasma from patients with OA and RA [39]. Serum HA levels have been suggested to predict disease outcome in knee OA [40]. The higher

concentrations found in serum from OA patients suggest that there is a relationship between increased levels of HA and the increased risk for OA. Therefore, HA levels may have predictive value for the progression of knee and hip OA [41–43]. HA has also been evaluated as a biomarker for equine OA [44] and canine hip dysplasia and canine cruciate disease [45, 46]. Assays for serum HA and methods for its quantitation in biological fluids have existed for several decades [47, 48]. The rationale for developing such assays was the realization that HA is a potential diagnostic marker for cartilage breakdown in RA and OA [49, 50]. Indeed, quantitative analysis of HA in the synovial tissues of patients with joint disorders has confirmed this, particularly in RA and following joint injury [51]. More recent studies in African Americans and Caucasians in the Johnston County Osteoarthritis Project support a role for serum HA as a biomarker of radiographic OA [52]. Also, HA was found to be significantly higher in hand OA in the CARRIAGE family study [53] and an independent study in the Czech Republic [54]. The major problem with HA as a biomarker is that its levels may also change diurnally [55], with physical activity [56], posture, and different diets [57]. HA is ubiquitous in the body and not only found in joint tissues but also in other connective tissues. Furthermore, it has not been validated for the early stages of OA. A more sensitive biomarker might be hyaluronidase, the enzyme responsible for HA degradation. Zymographic examination of synovial fluid and serum hyaluronidase activity in RA and OA patients has shown that the expression of this enzyme and its activity could be used as a marker of synovial inflammation [58].

1.4.2.3 YKL-40 (Cartilage Glycoprotein-39)

YKL-40 or chitinase 3-like 1 (cartilage glycoprotein-39) is a biomarker that provides a snapshot of inflammatory events in joint tissues, potentially allowing rapid assessment of pharmacotherapy [59, 60]. Its presence in synovial fluid and serum may reflect articular cartilage degradation and the degree of synovial inflammation in the knee joint [61, 62]. This protein and related proteins may participate in cartilage remodeling and degradative processes in OA joints [63–66]. Furthermore, plasma levels of YKL-40 are raised in patients with RA and other inflammatory conditions [67]. Finally, serum or urinary determinations of these molecules are difficult to interpret adequately due to their complex metabolism in the body [59].

1.5 Biomarkers of RA

RA can be difficult to diagnose, and there are no blood tests that can definitively rule in or rule out the disease. A number of laboratory tests and biomarker assays have been developed and clinically validated to help to confirm the diagnosis of RA in human patients [68]. The rational use of laboratory testing and biomarkers for investigating early, undifferentiated joint pain also requires a detailed history and careful

physical examination of the patient [69]. Full blood cell count, serum uric acid, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), rheumatoid factor (RF), anti-citrullinated protein antibodies (ACPA), anti-cyclic citrullinated protein antibodies (ACCP), and antinuclear (ANA) antibody titers form a reasonable screening panel when RA is suspected based on the clinical manifestations.

The American College of Rheumatology has recently published recommendations for the use of RA disease activity measures in clinical practice [70]. They recommend the Clinical Disease Activity Index, Disease Activity Score with 28-joint counts (erythrocyte sedimentation rate or C-reactive protein), Patient Activity Scale (PAS), PAS-II, Routine Assessment of Patient Index Data with three measures, and Simplified Disease Activity Index because they are accurate reflections of disease activity; are sensitive to change; discriminate well between low, moderate, and high disease activity states; have remission criteria; and are feasible to perform in clinical settings [70].

1.6 Reactive Oxygen Species and Endogenous Antioxidants

Oxidation reactions in living cells produce free radicals, reactive oxygen species (ROS), and their derivatives. These dangerous and harmful chemical products can accumulate over time, causing extensive structural damage or even cell death. The cytotoxic effects of ROS can cause a variety of health problems including inflammatory disease, tissue necrosis, organ failure, atherosclerosis, infertility, birth defects, premature aging, mutations, and malignancy [71]. ROS production initiates an "inflammatory state" which unless quenched may result in chronic inflammatory disease states, e.g., arthritis, hepatitis, nephritis, myositis, scleroderma, lupus erythematosus, and multiple system organ failure [71]. ROS are involved in the initiation of inflammatory responses [72]. For example, ROS such as H_2O_2 can stimulate the transcription factor NF-κB, which is crucial for cellular processes such as inflammation, immunity, cell proliferation, and apoptosis [73]. Therefore, ROS-mediated upregulation of NF-κB can cause dysregulation of many inflammatory responses.

Living cells maintain a complex and interrelated protective system of endogenous antioxidant vitamins, minerals such as selenium and manganese as cofactors, and glutathione to protect themselves from the harmful effects of ROS [74, 75]. Cells also use a variety of antioxidant enzymes such as catalase, superoxide dismutase, and various peroxidases to quench and control cellular levels of ROS. Deficiency in antioxidants or inhibition of the antioxidant enzyme systems may cause oxidative stress and may damage or kill cells. Oxidative stress is an important component of many diseases. Therefore, the biology of ROS and antioxidants is widely investigated in the context of understanding the role of these chemicals in chronic diseases characterized by oxidative stress. The next two sections will discuss examples of endogenous antioxidants.

1.6.1 Catalase

Hydrogen peroxide (H_2O_2) is a harmful by-product of many normal metabolic processes. In the synovial joint, hydrogen peroxide is an important mediator of tissue damage [76], especially since its concentration is enhanced by pro-inflammatory cytokines [77]. In order to prevent damage to cells and tissues, hydrogen peroxide must be rapidly converted into other, less dangerous substances. Catalase is the ubiquitous enzyme that catalyzes the decomposition of hydrogen peroxide to water and oxygen. During acute inflammation, the production of hydrogen peroxide by polymorphonuclear cells is a suspected cause of cellular damage in the joint [78]. Hydrogen peroxide from polymorphonuclear cells plays an important role in cartilage degradation through direct damage to cartilage during inflammatory processes in the joint [79]. Chondrocytes within cartilage are sensitive to toxic oxygen metabolites [80] and have been shown to contain catalase and the glutathione peroxidase/reductase systems. These enzyme systems appear to be involved in the removal of hydrogen peroxide in these cells. Immunohistochemical studies in the rat have confirmed the presence of catalase within articular chondrocytes [81]. Interruption of either of these peroxide-metabolizing systems sensitizes cartilage to a greater inhibition of matrix synthesis and oxidative damage by hydrogen peroxide. Hydrogen peroxide suppresses chondrocyte proteoglycan synthesis as measured by ^{35}S sulfate incorporation assays and [82]. Inhibition of catalase with 3-amino 1,2,4 triazole or azide further inhibits matrix synthesis, possibly because of exposure to higher steady state levels of hydrogen peroxide [78].

1.6.2 Glutathione

Glutathione or gamma-L-glutamyl-L-cysteinylglycine (GSH) is one of the main endogenous antioxidants in cells and is involved in diverse functions including apoptosis, disulfide bond formation, detoxification, antioxidant defense, maintenance of thiol status, and modulation of cell proliferation. Increased oxidative stress with aging reduces chondrocyte survival, and this correlates with intracellular GSH levels [83]. Increased oxidative stress makes chondrocytes much more susceptible to oxidant-mediated cell death. This occurs through the dysregulation of the GSH antioxidant system [83]. The reduction in the capacity of antioxidant buffering systems such as GSH may represent an important contributing factor to the development of OA in older adults [83]. There have been a number of in vitro studies on GSH in chondrocytes. *N*-acetylcysteine, a precursor of GSH, has been shown to protect growth plate chondrocytes and temporomandibular joint chondrocytes from the effects of oxidative stress in vitro [84, 85]. *N*-acetylcysteine has been shown to prevent NO-induced chondrocyte apoptosis and cartilage degeneration in an experimental model of rabbit OA [86]. *N*-acetylcysteine also activates extracellular signal-regulated kinase signaling pathway in articular chondrocytes, which may provide a

mechanism for the promotion of chondrocyte survival by this thiol antioxidant [87]. When over-expressed, the enzyme glutathione-S-transferase can protect chondrocytes from the effects of oxidative stress [88]. GSH depletion and NO both decrease insulin-like growth factor I (IGF-I) receptor function chondrocytes in vitro [89]. Insulin-like growth factor I (IGF) helps maintain healthy articular cartilage; however, arthritic cartilage becomes less responsive to the anabolic actions of IGF-I. Thus, it is interesting that GSH depletion can reduce the responsiveness of chondrocytes to this important anabolic growth factor. ROS such as superoxide, hydrogen peroxide, and hydroxyl radical are typically produced in mitochondria as electrons leak from the electron transport chain and react with oxygen to form superoxide. It is estimated that 1–3% of oxygen reduced in cells may form superoxide in this way [90]. Hydrogen peroxide is formed from the dismutation of superoxide and by oxidases. These three reactive species are controlled via multiple-enzyme systems like superoxide dismutase (SOD), catalase, glutathione-S-transferase, and thioredoxin. SOD converts superoxide to hydrogen peroxide, which is then removed by glutathione peroxidase or catalase and therefore has the capacity to prevent the formation of highly aggressive ROS, such as peroxynitrite or the hydroxyl radical [91]. The production of hydrogen peroxide (H_2O_2) by inflammatory and synovial cells is an important cause of cellular damage during joint inflammation. Effective H_2O_2-metabolizing systems are important in the maintenance of normal biosynthetic rates in cartilage during inflammation. In addition to the antioxidant vitamins and GSH, chondrocyte antioxidant defenses include catalase, glutathione-S-transferase, and glutathione peroxidase. These enzymes afford protection against H_2O_2-dependent inhibition of proteoglycan biosynthesis [78]. Immunohistochemical studies have identified superoxide dismutases, catalase, and glutathione-S-transferases in rat joints [81]. Interestingly, there were no major age-related changes in antioxidant enzyme distribution in rat joints [81]. Microinjection of antibodies against superoxide dismutase and glutathione peroxidase has been shown to decrease their viability, whereas injection of control (nonimmune) has no effect [92]. These findings highlighted the importance of glutathione peroxidase as antioxidant and the relative efficiency of SOD according to the balance between the radical production and the activity of the other antioxidant systems in chondrocytes.

1.7 ROS and the Inflammasome

ROS are also linked to mitochondria and the inflammasome [93]. The inflammasome is a protein complex that stimulates caspase-1 activation to promote the processing and secretion of pro-inflammatory cytokines [94]. This multiprotein oligomer consists of caspase 1, PYCARD, NALP, and sometimes caspase 5 (also known as caspase 11 or ICH-3). Inflammasome-dependent inflammatory responses are triggered by a variety of stimuli including infection, tissue damage, and metabolic dysregulation [95]. Recent work suggests that mitochondria are involved in integrating distinct signals and relaying information to the inflammasome. Dysfunctional

mitochondria generate ROS, which is required for inflammasome activation. Interestingly, mitochondrial dysfunction has been linked to OA [96, 97]. Analyses of mitochondrial electron transport chain activity in cells from OA-affected cartilage show decreased activity of complexes I, II, and III compared to normal chondrocytes [98]. Therefore, it is possible that mitochondrial dysfunction in arthritis is exacerbated by ROS and catabolic processes that alter cellular metabolism. The inflammasome is negatively regulated by autophagy, which is a catabolic process that removes damaged or otherwise dysfunctional organelles, including mitochondria [95]. Autophagy has been shown to be a protective mechanism in normal cartilage, and its aging-related loss is linked with cell death and OA [99]. These studies suggest that the connections between mitochondria, metabolism, and inflammation are important for cell function and malfunctioning of this network is associated with many chronic inflammatory diseases. ROS generation and inflammasome activation are linked with mitochondrial dysfunction and may explain the frequent association of mitochondrial damage with inflammatory diseases.

1.8 Oxidative Stress in Joint Diseases

Oxidative stress, defined as an imbalance between oxidative processes and reduction equivalents (antioxidants), is involved in the development of degenerative joint diseases. There is a substantial body of published research that suggests that arthritic diseases are characterized by inflammation and oxidative stress. Oxidative stress produces reactive oxygen species (ROS) that play key roles in the development of OA. ROS are also involved in RA. In both diseases, metabolic reactions in chondrocytes and synoviocytes produce free radicals, ROS, and their derivatives. These dangerous chemicals accumulate in the synovial joint, causing extensive structural damage cell death and inflammation. For example, in OA, oxidative damage contributes to chronic inflammation and promotes age-related diseases [100]. This results in senescence-associated secretory phenotype, which has many of the characteristics of an "osteoarthritic chondrocyte" in terms of the cytokines, chemokines, and proteases produced [100].

1.9 In Vivo Markers of Oxidative Stress

Research on biomarkers of oxidative stress has the potential to develop a "fingerprint" or "chemical signature" for measuring and monitoring oxidative stress in health and disease. Many analytical techniques are available for the measurement of oxidative stress status in culture systems, animal models, and human subjects. The techniques are as diverse as blood tests for oxidized lipids and proteins indicative of oxidative damage, volatile hydrocarbons in exhaled breath, and oxidized DNA bases excreted in urine. The real challenge is to identify and validate measurable, sensitive, and

specific biomarkers for oxidative damage resulting from different types of oxidative insults and to understand the interrelationships among the markers identified in order to determine which of the available biomarkers of oxidative stress are the most specific, sensitive, and selective.

Free radicals can be measured directly using electron spin resonance (ESR), most often coupled to spin trapping to increase the sensibility of the method. However, ESR is difficult to use in human subjects in vivo [101]. Many indirect methods of RNOS measurements have been proposed, based on the use of antioxidants and enzyme inhibitors or on the measurement of stable compounds derived from ROS activity and considered as "markers of oxidant stress." Among the possible markers of oxidant stress, there are isoprostanes [102] hydroxynonenal and lipid peroxides, nitrated and oxidized proteins, chlorinated compounds, protein carbonyl, oxidized glutathione, and malondialdehyde (detected as thiobarbituric acid reactants; TBARs) [103]. These techniques, however, have limitations. They are not all specific for oxidative stress and are at risk of artifacts. For example, isoprostanes can be produced by platelets independently of oxidant stress [104, 105], and the chemical reaction of malondialdehyde detection is influenced by the presence of iron in the sample [106].

Moreover, these markers are not specific of one particular RNOS, and all RNOS do not generate one particular by-product. Therefore, it is speculated that probably more than one marker is needed to assess overall "oxidative stress." For example, nitrated proteins indicate that the "oxidative stress" involves peroxynitrite or MPO; nitrotyrosine shows that $^{\bullet}NO$ is involved and malondialdehyde that lipid radicals have been generated in the cell membrane. Further, these markers are not specific for the tissue in which oxidative stress occurs. For example, if the oxidative stress is located in the joint, biomarkers should be measured ideally in synovial fluid, but not in blood or urine.

There are two other popular ways to identify oxidant stress: the first is to measure the changes in the "antioxidant status" by estimating the consumption of the endogenous antioxidants or the changes in the activity or the expression of the antioxidant enzymes. The second is to measure the "total antioxidant capacity" of a biological sample (plasma, tissue extract) by testing the capacity of this sample to inhibit the transformation of a selected substrate by an in vitro generated free radical. These methods have encountered a great success, not only in severe diseases to identify oxidant stress, but also to evaluate the capacity of healthy humans to fight against a potential oxidative stress [107]. However, this method has some limitations. It does not allow the identification of the molecule(s) involved in the antioxidant status failure. The global antioxidant status is largely influenced by nutrient intake, physical activity, and other life conditions. There is no norm, and a longitudinal investigation is needed to research an individual variation. The RNOS-generating system used in this technique (hypoxanthine/xanthine oxidase) does not reproduce the complex RNOS-generating system involved in "oxidant stress" in vivo. Therefore, the capacity of a biological sample to scavenge $O_2^{\bullet-}$ does not necessarily reflect its capacity to scavenge RNOS in vivo. Another major problem is its application to plasma samples which naturally contain a high concentration of albumin, an excellent "antioxidant" barrier to RNOS activity. An increase in total antioxidant capacity is considered to be a poor marker for evaluating oxidative stress [108].

Table 1.1 Biomarkers of bone, cartilage, and synovial turnover

Tissue	Molecule	Markers of synthesis	Markers of degradation
Bone	Type I collagen		N-telopeptide (NTX-I) (s, u)
			C-telopeptide (CTX-I) (s, u)
	Non-collagenous proteins	Osteocalcin (s)	
Cartilage	Type II collagen	PIIANP (s)	
		PIINP (s)	
		NPII (p)	
		PIICP (s)	
			CTX-II (u, sf)
			Col2CTX (u)
			TIINE 45 mer or NET2C (u)
			Coll 2-1 (s, u)
			Coll 2-1 NO_2 (s, u)
			C2C (s, u)
			C1,2C (u)
			CIIM (u, s)
	Aggrecan	846 (cartilage matrix)	
			G1-G2 (s)
			342-FFGVG (s)
			374-ARGSV (u)
	Non-aggrecan and non-collagenous proteins		Pentosidine (s)
			COMP (s, sf)
	Proteases and inhibitors		MMP-13 (u)
Synovium	Type III collagen		III-Nys (synovial tissue)
	Non-collagenous proteins	Hyaluronic acid (HA) (p, s)	

s serum, *p* plasma, *u* urine, *sf* synovial fluid

Usually, OA progression is monitored by measurement of changes in joint space width on plain radiographs with a graduated magnifying lens or with a computer after digitization of the radiograph. This must be considered a rather indirect measure of cartilage integrity, as articular cartilage itself is invisible on the radiographs and thus has to be assessed indirectly from the spacing between the subchondral bone ends of the joint. Furthermore, joint space width does not allow detection of early structural damage, remains difficult to use in daily practice, and is poorly reproducible. It fails to measure a dynamic metabolic process and is confounded by the presence of meniscal lesions or extrusion. Its change overtime is very small, occurs in only a subset of patients (the progressors), and is not correlated with joint function and pain. Magnetic resonance imaging (MRI) is a promising noninvasive tool for evaluation of cartilage, but access to this technique is confined and very expensive. Further, MRI and radiographs provide a static picture of the cartilage lesion. These imaging techniques fail to explore the metabolic changes occurring in OA cartilage. Biochemical factors of bone synovium or cartilage turnover have been proposed as alternative diagnostic and prognostic tools for monitoring treatment efficacy (Table 1.1) [109–112]. The challenge is to identify tissue and disease-specific markers of oxidative stress. In order to address this aim, our group and others have developed a new generation of biomarkers useful for measuring the oxidative stress occurring in hyaline cartilage. These biomarkers have been validated in RA and OA.

1.9.1 Oxidant-Induced Changes in Collagens

1.9.1.1 Oxidative Cleavage

Collagen is the only protein susceptible to fragmentation by $O_2^{\bullet -}$ [113]. In comparison, proteins such as serum albumin or various enzymes are not degraded by $O_2^{\bullet -}$. This $O_2^{\bullet -}$-induced collagen degradation was characterized by the release of small 4-hydroproline-containing peptides, suggesting scissions in the triple helical part of the collagen molecule [114, 115]. This collagen oxidative degradation was inhibited by SOD but not by catalase or chelating agents such deferroxxamine or diethylene triamine penta-acetate (DTPA), confirming the key role that is played by $O_2^{\bullet -}$ in the process. Nevertheless, the action of $^{\bullet}OH$ on collagen remains questionable since it was demonstrated that its action is quite different in the absence or in the presence of O_2 [116]. In the presence of O_2, $^{\bullet}OH$ generated by gamma radiolysis released pattern of peptides different from that generated by $O_2^{\bullet -}$. OH-generated peptides are characterized by an increase of aspartic and glutamic acid residues and a decrease in the amount or 4-hydroproline and proline residues. In contrast, when irradiations of collagen are performed in the absence of oxygen, no collagen cleavage is observed but a polymerization of collagen.

Hypochlorite (HOCL/OCL−) within the predicted range generated by PMNs or monocytes at sites of inflammation (10–50 mM) does not cause fragmentation of collagen I or II [117]. Only the supra-physiological concentrations of 1–5 mM cause extensive fragmentation of collagen [118]. *N*-chloramine (5–50 mM) does not cause fragmentation but greatly increases the degradation of collagen by collagenase and elastase. The mechanism by which *N*-chloramines, and probably other oxidants, increase the proteolytic susceptibility of collagen is not clearly determined, although it is assumed that *N*-chloramines react with amino groups and disrupt the secondary and tertiary structures of collagen molecules [119]. Disruption of the tertiary structure of collagen by oxidation exposes hydrophobic regions and promotes the degradation of fibrillar collagens by proteases. Another explanation would be that oxidation and disruption of pyridinoline cross-links could result in the loss of functional interactions of collagen fibrils and consequently an increase in the susceptibility of collagen to proteolytic degradation.

Finally, exposure of collagen to ROS results in modification of the primary structure of collagen. Exposure of proline peptides to a Fenton system (Cu(II)/peroxide) results in conversion of some proline residues into hydroxyproline, along with formation of γ-aminobutyric acid [120].

Exposure of purified type II collagen to $FeSO_4$-EDTA ($^{\bullet}OH$ source) or xanthine oxidase-hypoxanthine system ($O_2^{\bullet -}$ source) also induced a cleavage of the proline producing more terminal glutamate residues, which are Ca^{2+} affinity ligand. These oxidative-induced changes promote crystal formation which is an important feature in some rheumatic diseases including OA, gut, or Kashin–Beck's disease [121].

1.9.1.2 Glycoxidation

The covalent binding of the aldehyde group of glucose to a free amino group of a protein (mainly lysine and arginine amino group) is called glycation. The initial reaction is the formation of Schiff base followed by a spontaneous Amadori rearrangement. Both the Schiff base and the Amadori products may subsequently undergo oxidation, particularly in the presence of metal ions (i.e., iron and copper ions) and RNOS (mainly $^{\bullet}OH$), and generate carbonyls and RNOS that lead to an extensive modification of the protein. This type of reaction has been referred to as glycoxidation. The stable end products of these reactions, mainly carboxymethyl lysine (CML) and pentosidine, are known collectively as advanced glycation end products (AGEs) [122]. CML can be formed in two pathways: oxidation of fructoselysine and reaction of protein with glycoxal, which is an autooxidation product of glucose or Schiff base adduct [123, 124]. Pentosidine is a cross-link between lysine and arginine residues resulting from the glycoxidation of Amadori products or the reaction of arabinose, which is an autoxidative product of glucose. Collagen is a protein with slow turnover and so is highly susceptible to glycoxidation in vivo. This is one reason for which collagen glycoxidation was commonly used as biological markers of disease progression, complication occurrence, or severity. Glycoxidation end products accumulation in cartilage is particularly well documented. Pentosidine levels are three- to fourfold higher in cartilage collagen than in skin collagen [125], probably as a consequence of the low turnover of type II collagen. The levels of pentosidine and CML have been shown to progressively accumulate with age in skin and cartilage collagens [126]. In contrast, in the age range 50–95 years, pentosidine concentration in bone trabeculae collagens did not depend on age [127]. After maturity (>20 years), pentosidine and CML accumulate linearly with age in human articular cartilage collagens. After maturity, their concentrations in cartilage collagen increase 33-fold and 27-fold with age, respectively. The rate of pentosidine accumulation in collagen is 0.32 nmol g^{-1} protein $year^{-1}$ or 0.64 mmol pentosidine mol^{-1} Arg $year^{-1}$ or 1.40 mmol pentosidine mol^{-1} Lys $year^{-1}$. In healthy adult cartilage, more than 80% of the pentosidine is present in collagen (84.4% at age 70) and less than 10% in aggrecan (9.0% at age 70) [128].

The glycation of collagen and accumulation of AGE contribute to the pathogenic mechanisms of many age-related diseases including atherosclerosis, diabetes mellitus, and OA. Glycation induced major changes in the physicochemical properties of collagen including stiffness, mechanical strength, temperature instability and insolubility, and high resistance to collagenase digestion. Further, glycated products are potentially active in the pathogenesis of some diseases. In bone, increased pentosidine concentration is associated with a decline in mechanical properties of bone [127–129], indicating that glycoxidation end-products' accumulation contributes to increase the propensity of individuals to microfracture. AGEs elicit a wide range of cell-mediated responses leading to cartilage degradation, vascular dysfunction, and atherosclerosis. Among these effects, glycoxidized collagen has been reported to increase the oxidation of unsaturated lipids and to lead to the formation of reactive products including malondialdehyde or 4-hydroxynonenal. The adhesion of PMNs

to in vitro-glycoxidized forms of collagen I and IV is significantly increased when compared with native collagens. Glycoxidized type I collagen increases the chemotactic properties of PMNs without significant stimulatory effect on respiratory burst, whereas preincubation of PMNs with glycoxidized type I collagen induced a priming on subsequent stimulation by *N*-formyl-methionyl-leucyl-phenylalanine. In contrast, glycoxidation of type IV collagen suppresses its inhibitory effect on further PMN stimulation or migration. These findings suggest that glycosidized type IV and I collagens act synergistically to increase extravascular migration and inappropriate activation of PMN [130]. The deleterious effects of glycoxidized products are mediated through receptors. At least four different AGE receptors have been characterized, of which two are scavenger receptors [131]. One AGE receptor, RAGE, which has a wide tissue distribution, when interacting with AGE, results in the generation of cellular oxidant stress manifested by the appearance of malondialdehyde and activation of NF-κB. AGE may also induce activation of DAG protein kinase C (PKC) pathway [132].

1.9.1.3 Chlorination and Nitration

In some inflammatory diseases, particularly in RA, HOCl/OCl−, mainly produced by activated PMNs, plays an important role in tissue destruction. HOCl/OCl− mediates some major type II collagen modifications including desamination with consecutive carbonyl group formation and transformation of tyrosine residues to dichlorotyrosine, fragmentation in small peptides (<10 kDa), and decrease of radius aggregates. These modifications were observed at low concentrations (less than 1 mM), suggesting that they can occur in synovial fluids in pathological circumstances. In contrast, *N*-chloramine (5–50 mM), which is formed by the reaction of a primary amine with HOCl/OCl−, does not cause direct fragmentation of type I or II collagens, but greatly increases their degradation by collagenase or elastase. Interestingly, pyridinoline cross-links, which determine the stability of the fibrillar collagen network, are also potential target of HOCl/OCl−. HOCl/OCl− reacts with the pyridinium ring structure and disrupts the ring structure, probably secondary to the formation of aldehydes. Although both collagen type I and II are oxidized by HOCl/OCl−, the reactions differ. Oxidized type II collagen shows greater amount of carbonyl formation and a decrease in fluorescence (325ex/400em), as compared with oxidized type I collagen.

An additional novel mechanism particularly relevant to inflammation-related diseases involves the nonenzymatic nitrite (NEN) modification of connective tissue proteins. Protein nitration is mediated by peroxynitrite, mostly by the derived nitryl radical ($NO_2^{\bullet -}$), at the level of aromatic amino acids (e.g., tyrosine). Recently, it was reported that NEN of fibrillar type I collagen inhibits the ability of primary adult cardiac fibroblasts to remodel type I collagen gels and reduces the deformability of type I collagen gels subjected to mechanical testing [133]. This latter finding correlates with the degree of cross-linking. Beside glycation, collagen nitration probably contributes to the alterations in the biomechanical properties of

collagen-containing tissues consistent with age-related functional decline observed in human disease.

1.9.2 Biomarkers of Oxidative Damage to Collagen

1.9.2.1 Glycoxidation Products

The Glycoxidation Products, Pentosidine, and CML

In cartilage, type II collagen turnover is very slow (half-life of type II collagen ~117 years), allowing AGE accumulation. Some groups have observed that pentosidine levels increase 50-fold from age 20 years to age 80 years [134, 135]. Recently, it was reported that AGE accumulation level in cartilage predispose to the development of OA in dogs with anterior cruciate ligament transaction [136]. Considering that one of the first characteristics of OA is damage to the collagen network, we can suggest the possibility that glycoxidized type II collagen peptides could be relevant biomarkers of early cartilage degradation.

1.9.2.2 Nitrated Peptides

Type II is possibly the ideal marker for studying cartilage remodeling. First, this collagen is relatively specific to articular cartilage, although it is also present in the vitreous humor of the eye and the nucleus pulposus region of the intervertebral disc. Second, it is the most abundant protein in cartilage, representing 25% of the wet weight, 50% of the dry weight, and 90–95% of the total collagen content. Recently, the Henrotin laboratory has published a method for the assessment of oxidative damage of type II collagen in cartilage and biological fluids (serum, urine, and synovial fluid). This original approach is based on the detection in biological fluids or in tissue of a nitrated peptide release from type II collagen during cartilage degradation. Our strategy was based on the following points: (1) type II collagen is specific for cartilage and is the most abundant collagen in the extracellular matrix; (2) peptide nitration results from the reaction of aromatic acid with –ONOO; (3) in pathological scenarios, chondrocytes and synovial cells (mainly macrophages) produce high levels of $^{\bullet}NO$ and $O_2^{\bullet -}$; and (4) type II collagen containing two tyrosine residues, but not other aromatic amino acid, one located in the triple helix and the other in the telopeptide of the C-terminal end. We have developed specific immunoassays, one for the peptide ^{108}HRGYPGLDG^{116} (Coll2-1) and the other for the nitrated form of this peptide ^{108}HRGY(NO2)PGLDG^{116} (Coll2-1NO$_2$) (Fig. 1.2). This strategy allows the calculation of the ratio Coll2-1NO$_2$/Coll2-1 that reflects the oxidative-related damage of the triple helical area of the molecule [103, 137]. These immunoassays have been validated in animal models and in human clinical surveys, with the following conclusions:

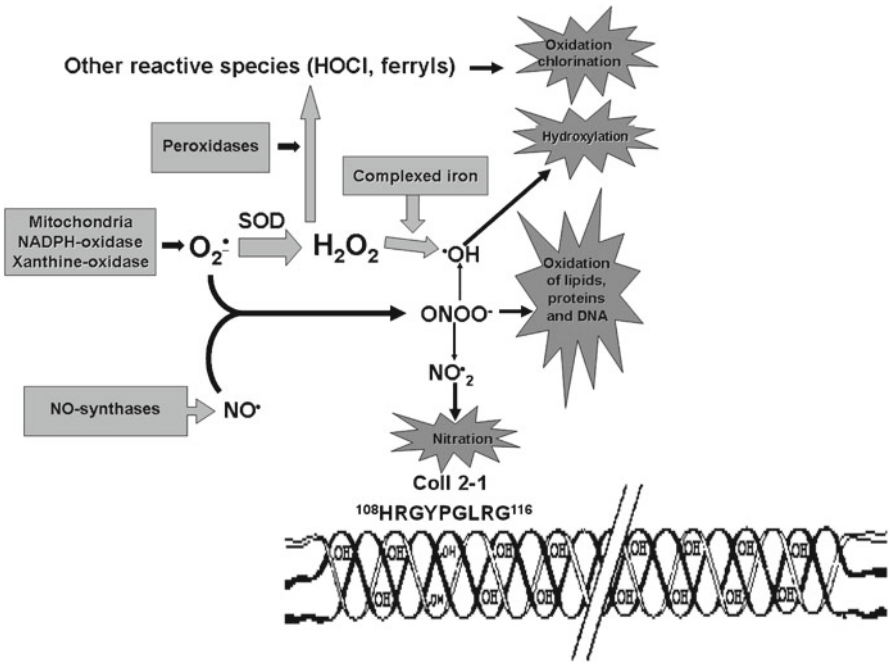

Fig. 1.2 Chemical pathway of type II collagen nitration

In mice, Coll2-1 levels were significantly higher in the serum of biglycan /fibromodulin double-deficient mice (DKO), which develop premature and severe knee OA compared to wild-type mice (WT). In contrast, Coll2-1NO_2 displayed cyclic variations with a DKO/WT ratio ≈1.60 at day 49 and 95 but =0.86 at intermediate time points (day 81 and 141). In both genotypes, immunostaining with Coll2-1 and Coll2-1NO_2 labeled some fibroblasts in tendons and menisci as well as the chondrocytes above the tidemark in articular cartilage, whereas chondrocytes in the growth plate were not immunoreactive. For the two biomarkers, extracellular staining was limited to fibrocartilage areas in tendons and menisci from both genotypes and to some of the focal lesions of the biglycan/fibromodulin deficient cartilage. No extracellular staining was observed in WT cartilage. The different DKO/WT ratios observed with Coll2-1 and Coll2-1NO_2 suggest that these serum biomarkers give complementary rather than redundant information on type II collagen catabolism [138].

In an experimental model of dog OA (induced resection of the cruciate ligament), Coll2-1NO_2 and the ration Coll2-1NO_2/Coll2-1 increased over time after surgery. Coll2-1NO_2 concentration were found to be significantly correlated with the size of osteophytes, and the ratio Coll2-1NO_2/Coll2-1 was correlated with the global macroscopic score, the size of the macroscopic lesions, and the total histological score but not with osteophytes [139].

In horses, plasma levels of Coll2-1 were significantly lower in animals with osteochondrosis compared to healthy horses. Osteochondrosis is a disease characterized

by endochondral ossification with further damage to the cartilage. In contrast, Coll2-1NO_2 levels were significantly higher, indicating an uncoupling variation of these two markers. This study demonstrates that the markers are able to differentiate healthy horses from those with osteochondrosis [140, 141].

In healthy humans, the concentration of Coll2-1 in serum remains stable throughout life. In contrast, Coll2-1NO_2 concentration is more elevated in young subjects (26–30 years old). These two markers were not affected by circadian rhythms [142, 143]. Furthermore, Coll2-1 and Coll2-1NO_2 concentrations were elevated in serum of patients with primary knee OA and early RA compared to healthy subjects. Interestingly, Coll2-1NO_2/Coll2-1 ratio was higher in RA (0.25) than in OA (0.15), suggesting that Coll2-1 nitration is related to the intensity of synovial inflammation. Also, Coll2-1NO_2, but not Coll2-1, was significantly correlated with C-reactive protein in serum of arthritic patients. These findings indicate that Coll2-1NO_2 could be a marker of arthritic disease activity and could replace other systemic inflammatory biomarkers for specifically monitoring joint inflammation and oxidative stress-related cartilage degradation.

These markers have also been measured in urine samples from a cohort of 75 patients with knee OA corresponding to the placebo arm of a large 3-year, randomized, double-blind study comparing the efficacy of glucosamine sulfate with placebo [144]. In this study, we have observed that elevated urinary levels of Coll2-1 and Coll2-1NO_2 at baseline were associated with a higher disability evaluated with the Western Ontario and McMaster University Osteoarthritis Index (WOMAC). This finding indicates that Coll2-1 and Coll2-1NO_2 in urinary levels reflect the clinical severity of OA disease. Another objective of this study was to investigate whether our biochemical markers could predict the radiological severity progression of OA. Interestingly, at baseline, significant correlation were found between the first-year change of these markers in urine and the 3-year change of the medial joint space width, suggesting that the increase of these peptides over 1 year could be predictive of the radiological progression of knee OA [145]. In a clinical study including 57 patients with erosive hand OA and 20 with non-erosive hand OA, myeloperoxidase serum levels discriminated the two subsets of patients, whereas trends were also observed with Coll2-1NO2 and the ratio Coll2-1NO2/Coll2-1 [139].

1.10 Concluding Remarks

Glycoxidation markers are potential markers of glycemic- and/or oxidant-related collagen network damage in diabetes and some age-related diseases including OA. The two main glycoxidation products investigated are pentosidine and CML. However, these markers are associated with the etiology of a variety of different diseases (i.e., cancer, diabetes), and their measurement in serum fails to reflect changes in one particular organ. For this reason, they are relevant as prognostic markers of disease progression or complication occurrence and severity and as markers of efficacy of intervention, but their use for the diagnosis or burden of

disease is questionable. To investigate problems in one particular tissue, these markers need to be measured in biopsies and require invasive biopsies.

Another limitation on the use of pentosidine or CML as disease markers is that protein glycoxidation results of a two subsequent mechanisms, the nonenzymatic glycation and the oxidation of glycated products by ROS. These mechanisms are not necessary coupled and may occur independently. This finding suggests that an increase of glycoxidation products may result in either an increase in glycation or an increase in ROS production. The increase in glycoxidation in one particular tissue may reflect a local disorder and/or systemic problems affecting the antioxidant status or the glucose level. Increased glycation may be the result of an increased glucose uptake or a high rate of glycolysis and seems to be dependent on protein turnover rate. In parallel, accumulation of oxidated glycation products may result in a decrease in antioxidant defenses or an increase of ROS. Serum protein-bound CML is likely to represent a mixture of oxidative and carbonyl stress originating in part from oxidized Amadori compounds. Cellular sources of these metabolites include peroxynitrites and hydroxyl radicals originating from Haber–Weiss cycle as well as glyoxal and glycoaldehyde originating from food and digestion. Therefore, these markers are not tissue specific; they are not disease specific either and are the result of different pathogenic mechanisms. There is a need to develop cell-based or specific matrix-bound AGE to improve the clinical relevance of these potential biomarkers.

The methods used to measure these markers are also questionable. In actual fact, pentosidine must be assayed by high-performance liquid chromatography (HPLC) because pentosidine enzyme-linked immunosorbent assays (ELISA) suffer from low sensitivity. These techniques need a prior acid extraction step to release pentosidine, which can generate artifacts. CML is most precisely assayed by gas chromatography/mass spectrometry (GC/MS) or liquid chromatography/mass spectrometry (LC/MS). However, both techniques are unsuitable for large numbers of samples. Various investigators have developed CML poly- and monoclonal ELISA using *N*-epsilon-(carboxymethyl)lysine as immunogen. Many of these assays are likely to suffer from lack of linearity, especially at lower CML concentrations, and perform poorly with intact proteins. Novel technologies are needed to determine glycoxidation products in tissues. One alternative would be to produce antibodies, which recognize tissue-specific molecular structures and neoepitopes modified by the oxidative process. This approach has been developed by the Henrotin laboratory, which has established new immunoassays for nitrated type II collagen.

Conflict of Interest This chapter was written by the authors within the scope of their academic and research positions at their host institutions. None of the authors has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of this chapter.

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Chapter 2
Regulation of T-Cell Functions by Oxidative Stress

Stuart J. Bennett and Helen R. Griffiths

Abstract The principal role of adaptive immunity is to distinguish between "self" and "nonself" and thus provide a highly specific line of immunological defence for the efficient removal of foreign material. It comprises of two arms: the effector B-cell arm and effector T-cell arm which act together to remove nonself. The balance between oxidising and reducing agents within these immune cells governs their redox state. This is important as transient controlled changes in the redox state, such as increased production of reactive oxygen species, are vital for signalling and induction of various biological processes, including cell growth and apoptosis. However, in chronic inflammatory diseases, the prolonged and persistent production of ROS, which overwhelms cellular antioxidant systems leading to oxidative stress, may influence T-cell function. This contributes to a T-cell phenotype which is hyporesponsive to growth and death signals and persists at the site of inflammation, perpetuating the immune response. The regulation of T-cell function by oxidative stress therefore has implications for rheumatoid arthritis.

Abbreviations

APC	Antigen-presenting cell
DC	Dendritic cell
DMARD	Disease-modifying antirheumatic drug
GSH	Reduced glutathione
GSSG	Oxidised glutathione
H_2O_2	Hydrogen peroxide
iGSH	Intracellular glutathione

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IL-1β	Interleukin-1β
IL-2	Interleukin-2
LAT	Linker for activation of T cells
MDA	Malondialdehyde
MTX	Methotrexate
NAC	*N*-acetylcysteine
NFκB	Nuclear factor kappa B
NO	Nitric oxide
NOS	Nitric oxide synthase
$O_2^{\bullet-}$	Superoxide anion radical
ONOO	Peroxynitrite
PBMC	Peripheral blood mononuclear cells
RA	Rheumatoid arthritis
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
TCR	T-cell receptor
TNFα	Tumour necrosis factor-α
Treg	Regulatory T cell
Trx	Thioredoxin
TXRX1	Thioredoxin reductase 1

2.1 Introduction

The immune system can be considered as two functional parts: innate and adaptive immunity. They act together to provide protection against invading foreign pathogens. Innate immunity is a fast acting, non-specific first line of defence towards microbial infection. Adaptive immunity is a highly specific line of defence which provides immunological memory allowing rapid and efficient removal of previously encountered pathogens. The principal role of adaptive immunity is to distinguish between "self" and "nonself". It comprises of two arms: the effector B-cell arm and effector T-cell arm, which together are responsible for removal of the "nonself" (i.e. pathogens). During the very early stages of T-cell development, self-reactive T cells, which recognise "self", are removed by apoptosis, and a subset of specialised regulatory T cells (Tregs) exist which inhibit autoimmune T cells and ensure self-tolerance is maintained [1, 2]. Aberrant T-cell function has been implicated in the development of autoimmune diseases such as rheumatoid arthritis (RA) [3]. RA is associated with intra- and extracellular oxidative stress: the imbalance between pro-oxidant (e.g. reactive oxygen species (ROS)) and antioxidant species in favour of the former [4]. In addition to specialised antioxidant enzymes, the most important intracellular low-molecular-weight antioxidant is glutathione (GSH), which has a thiol moiety and is reactive with pro-oxidant species. The importance of ROS in immune defence is exemplified by their generation and release in the form of an "oxidative burst" by phagocytic cells (e.g. neutrophils and macrophages, part of the

innate immune cell network) to effectively destroy pathogens and clear debris. It is also becoming increasingly apparent that altered redox states leading to changes in the oxidative environments at the surface of, and within, T cells are important for their function in health, ageing and disease. Due to their pivotal role in this adaptive immune response, the effect of the oxidative stress on T-cell biochemistry and function and its implications in the autoimmune disease RA will be the focus of this chapter.

2.2 T Cells in the Immune System

T cells originate in the bone marrow as haematopoietic stem cells, where they develop into progenitor cells and then migrate to the thymus via the bloodstream for maturation. Naïve T cells (i.e. mature T cells which have never previously been exposed to antigen) circulate to secondary lymphoid sites (e.g. lymph nodes, Peyer's patches, spleen and skin) where they become activated on recognising antigen-presenting cells (APC) displaying specific antigen–MHC molecule complexes. Dendritic cells (DC) are the principal APC, but macrophages and B cells can also perform this role. DCs originate in the bone marrow as haematopoietic stem cells, are released into the bloodstream where they travel to different sites and mature upon exposure to foreign pathogen. DCs internalise antigens and express them on MHC class molecules at their surface in the form of antigen. In this activated state, DCs travel to peripheral lymphoid organs and interact with T cells creating an immunological synapse and providing the necessary signals for T-cell activation: first, the T-cell receptor (TCR) engages with antigen-bearing MHC molecules; second, the co-stimulatory molecules, especially CD80 and CD86, bind co-receptor CD28 on surface of T-cells; and third, cytokines are produced to orchestrate the adaptive immune response [5, 6]. Once activated, T cells are retained in the lymphoid organ where they proliferate and differentiate into effector T cells under the control of local cytokines. The effector T-cell arm can be grouped into at least four subtypes: Th1, which mediate responses against intracellular pathogens and are involved in some autoimmune diseases; Th2, which are responsible for host defence; Th17, which promote bacterial immune responses and are responsible for autoimmunity; and Tregs, which make up 5–10% of total $CD4^+$ T cells and maintain self-tolerance [2]. With regard to RA, Th1 and Th17, effector T cells in particular have been implicated in the recruitment and activation of inflammatory cells and in mediating bone and cartilage damage associated with the disease [7].

The redox environment at the interface between APC and T cells within the immunological synapse also impacts on T-cell activation. The demonstration that upon activation T cells exhibit increased cell surface thiol levels suggests that a reduced extracellular environment is associated with T-cell activation [8]. Moreover, DCs have been shown to create this reducing environment by releasing cysteine into the extracellular space, thereby facilitating an immune response [9], and the mechanism by which Tregs exert their immunosuppressive effect has

been suggested as an interference with this process [10]. It is now therefore becoming increasingly recognised that changes in intracellular levels of ROS or altered redox state, as well as levels at the interface between APC and T cells at the cell surface, can impact on T-cell activation, proliferation and differentiation, thereby modulating their function. This has wider implications for ageing and disease which are associated with oxidative stress.

2.3 ROS and Redox State Are Central to Immunity and T-Cell Function

An imbalance between oxidising (e.g. reactive oxygen species (ROS)) and reducing agents (e.g. antioxidants) towards a pro-oxidant state leads to oxidative stress [11]. Prolonged and persistent oxidative stress can lead to macromolecular damage in the form of protein carbonylation, lipid peroxidation and DNA oxidation. This phenomenon has therefore historically been perceived as harmful and damaging; however, ROS are now more widely recognised as important signalling molecules [12] which exert a wide range of biological effects. With regard to the immune system, high levels of ROS can be beneficial; neutrophils generate ROS and release them intracellularly and extracellularly in the form of an "oxidative burst" to defend against and destroy pathogens, thus providing antimicrobial protection [13]. However, excessive ROS are generated in the presence of immune complexes with auto-antigens where further macromolecular damage is induced. Prolonged exposure to high ROS concentrations can inhibit T-cell proliferation and lead to apoptosis [14], and incubation of T cells with the reactive nitrogen species (RNS) peroxynitrite can also inhibit their proliferation [15]. It has also been observed that oxidative stress-induced modification to selective molecules involved in T-cell receptor (TCR) signalling is sufficient to render T cells hyporesponsive to activating stimuli [16]. Conversely small amounts of ROS have been shown to be important for T-cell function. Los and colleagues [17] reported that T-lymphoma cells exposed to low levels of hydrogen peroxide (H_2O_2) induced transcription of nuclear factor kappa B (NFκB) and gene expression of interleukin-2 (IL-2) and the IL-2 receptor chain-α [17]. The different T-cell responses to ROS production may be due to the extent of change to the cellular redox environment [18] (see Fig. 2.1).

T-cell differentiation is also affected by the redox environment. King et al. [19] stimulated peripheral blood mononuclear cells (PBMC) with the ROS generator 2, 3-dimethoxy-1, 4-naphthoquinone and reported that Th2 and Th1 phenotypes were promoted and inhibited, respectively. Moreover, in the absence of APC, reactive carbonyls including 4-hydroxy-2-nonenal and malondialdehyde (MDA), which are generated on proteins and lipids randomly in the presence of ROS, promote differentiation towards a Th2 phenotype [20]. These data emphasise that the importance of ROS homeostasis and flux in governing cell maturation and that the balance between oxidising and reducing agents is a delicate process which must

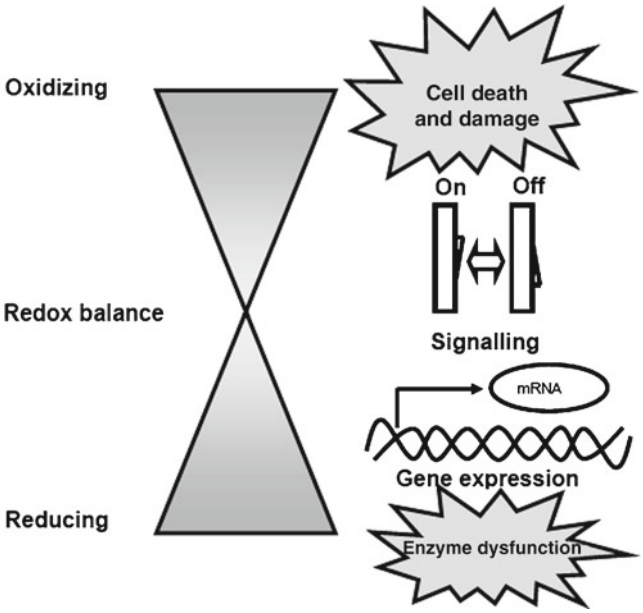

Fig. 2.1 Redox balance and T-cell function. Prolonged and persistent production of reactive oxygen and nitrogen species resulting in oxidative stress induces macromolecular damage, inhibits T-cell proliferation and leads to cell death. In contrast, small amounts of ROS are important for inducing transcription of NFκB and gene expression of cytokines and receptors essential for T-cell proliferation (e.g. IL-2 and IL-2 receptor), together highlighting an important role for cellular redox environment on T-cell function. Changes in levels of oxidative stress and therefore redox state can switch T cells towards a more hyporesponsive or proliferative phenotype

be tightly regulated and well managed, depending on whether the requirement is for protecting against bacteria, in an immune response, or requirements for T-cell signalling, activation and regulation of function.

2.4 Glutathione, Cysteine, Low-Molecular-Weight Thiols and T-Cell Function

GSH is a tripeptide consisting of amino acids glycine, glutamate and cysteine. It is synthesised in the cell by two ATP-dependent reactions: the first reaction, catalysed by γ-glutamyl cysteine ligase, combines glutamate and cysteine to form γ-glutamyl-cysteine; the second reaction, catalysed by GSH synthetase, combines γ-glutamyl-cysteine with glycine to form GSH [21]. The presence of a thiol group (–SH), provided by cysteine affords GSH its antioxidant activity in the form of radical scavenging. It is the major intracellular antioxidant and serves as a redox buffer cycling between its reduced and oxidised (GSSG) forms, and thus the ratio between GSH and GSSG serves as an indicator of intracellular oxidative stress [22].

In order for T cells to synthesise intracellular glutathione (iGSH), they require cysteine. However, given that circulating levels of cysteine are low, coupled to the fact that T cells are unable to import the oxidised form of cysteine, cystine, due to the lack of a cystine transporter, they rely completely on APC (e.g. dendritic cells) to provide them with cysteine [23]. DCs, in contrast to T cells, have the appropriate transporter required for cystine uptake, and once inside the cell cystine is reduced to cysteine which can then be secreted into the extracellular space. Cysteine is thus accessible both intra- and extracellularly for T cells and allows for their proliferation. The ability of DCs to control extracellular cysteine allows them to affect intracellular GSH levels and T-cell signalling [24].

Several studies implicate a role for GSH in T-cell function. Depletion of GSH in human and murine T cells, using the α-glutamylcysteine synthetase inhibitor L-buthionine-(S, R) sulfoximine, attenuates proliferative response to mitogenic and antigenic stimulation [25]. In addition, the reintroduction of exogenous GSH can restore a T-cell proliferative response [26] suggesting a direct relationship between iGSH levels and T-cell proliferation. Further investigation by Hadzic et al. [27] into the role of GSH and T-cell proliferation, using murine T cells, suggested that GSH is the rate limiting step for T-cell proliferation but only in the absence of other small molecular weight thiols. In addition depletion of GSH impaired T-cell function, as measured by IL-2 secretion, which was overcome by the addition of endogenous IL-2, suggesting that T-cell proliferation is regulated by a thiol-dependent pathway involving IL-2 [27].

A recent study by Checker et al. [28] supports the notion that thiols play an important role in T-cell function. In murine T cells treated with plumbagin, a naphthoquinone present in plants from Plumbaginaceae species, intracellular oxidative stress as measured by ROS production and GSH levels was increased and decreased, respectively. Proliferative responses to mitogenic stimulation as well as IL-2 production were also inhibited by plumbagin. The anti-proliferative effect and inhibition of IL-2 production was only prevented by thiol-containing antioxidants and not non-thiol antioxidants. The authors concluded that the anti-proliferative effects and reduced IL-2 production were due to the modulation of intracellular thiols, rather than altered ROS levels. Further support for the importance of thiols on T-cell function is demonstrated by studies which investigate the effect of selenium, an essential cofactor in GSH metabolic enzymes, on immune response; T-cell proliferation in response to antigenic stimulation in selenoprotein-deficient T cells isolated from mice is suppressed [29]. In a more recent study, Hoffman et al. [30] isolated $CD4^+$ T cells from mice fed either a diet of high, medium or low selenium for 8 weeks and investigated the dietary effect of selenium on T-cell function. $CD4^+$ T cells isolated from mice fed a diet high in selenium exhibited increased proliferation and expression of IL-2 and IL-2 receptor in response to antigenic stimulation compared to $CD4^+$ T-cells isolated from mice fed a low selenium diet, which was paralleled with a reduction in intracellular thiols and iGSH in diets low in selenium. The proliferative response to antigenic stimulation was rescued in $CD4^+$ T cells with the addition of *N*-acetylcysteine (NAC) suggesting that the effects of selenium on T-cell proliferation involve a pathway involving the modulation of free thiols. Taken together,

these data suggest an important role for iGSH and intracellular thiols in regulating T-cell activation and proliferation and highlight the importance of redox environment for T-cell function.

2.5 Oxidative Stress and Regulatory T Cells

Oxidative stress plays an important role in the function of regulatory T cells (Tregs). These specialised immunosuppressive T cells account for 5–10% of the total $CD4^+$ T-cell population [2]. One way in which Tregs exert their suppressive effect is by altering the redox state at the interface between DC and naïve T cells at the immune synapse, leading to reduced cysteine availability for naïve T cells [24]. In a recent study, Yan et al. [10] demonstrated that Tregs reduce extracellular cysteine concentration in an antigen-dependent but not antigen-specific manner, which requires cell to cell contact through the interaction between CLTA-4 on Tregs and CD80/CD86 on dendritic cells; this initiates an intracellular signalling response which inhibits DC iGSH synthesis and thus reduces extracellular cysteine generation [10]. In addition to this, Tregs compete with effector T cells at the immune synapse for extracellular cysteine, which they preferentially catabolise to sulphate, which therefore limits the amount of available cysteine for effector T cells [10]. As a consequence, iGSH levels in effector T cells are reduced and T-cell activation and proliferation is inhibited [10], and thus by altering the redox status at the immunological synapse, Tregs can have a profound impact on the functional response of effector T cells.

Tregs can actually withstand greater levels of oxidative stress than other $CD4^+$ T-cell subsets [31], which likely aids their ability to regulate the immune response. One factor which contributes to their increased tolerance to oxidative stress compared to other T-cell subsets is that they express and secrete greater levels of thioredoxin (Trx), a 12 kDa oxidoreductase enzyme substrate which contains a dithiol–disulfide, providing it with potential to scavenge ROS and metabolise H_2O_2 [32]. By blocking total and secreted Trx, Mougiakakos et al. [32] demonstrated that cell surface thiols on Tregs could be decreased and that their treatment with the thiol-depleting agent *N*-ethyl maleimide increased Treg susceptibility to H_2O_2-induced cell death. Moreover, they reported that treatment of Tregs with the inflammatory mediator tumour necrosis factor-α (TNFα) resulted in Trx release, with a paralleled increase of cell surface thiols and increased resistance to H_2O_2.

2.6 Ageing, Oxidative Stress and T-Cell Function

It is widely accepted that oxidative stress increases with age as evidenced by an increase in several stress markers, including protein carbonylation, lipid peroxidation and thiol to disulphide oxidation in plasma [22]. Studies of T-cell ageing reveal an association between increased oxidative stress and altered function. Early studies by Murasko et al. [33] and Franklin et al. [34] demonstrated that with age, T cells

exhibit a poor proliferative response to mitogenic stimuli. Moreover, GSH increases the proliferative response to mitogenic stimuli in older rats compared to young rats. Additionally, it has been reported that human lymphocytes exhibit reduced levels of iGSH and antioxidant enzymes in parallel with increased levels of protein carbonylation and lipid peroxidation, which correlates with age [35, 36]. Together, these data suggest that with age lymphocytes are under a state of increased oxidative stress which may lead to a poor response to mitogenic stimulus and thus affect T-cell function which may be attributed to either thiol loss or increased oxidants.

2.7 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a progressive, chronic inflammatory and autoimmune disease of joints which typically affects older adult. Histopathologically, RA is characterised by expansion and inflammation of the synovial membrane. In this state, chemokines are secreted from the synovial membrane leading to the recruitment and infiltration of diverse immune (e.g. $CD4^+$ and $CD8^+$ T cells and B cells) and inflammatory cells (e.g. neutrophils, monocytes and macrophages) [3]. $CD4^+$ T cells responsible for cell-mediated immunity activate monocytes, macrophages and synovial-like fibroblast cells to produce, amongst others, proinflammatory cytokines TNFα and interleukin-1β (IL-1β). TNFα and IL-1β, as well as T-cell-dependent cytokines, activate osteoclasts and chondrocytes to release proteolytic enzymes, ROS and RNS resulting in cartilage and bone destruction, characteristic of RA. Investigation of synovial tissue from RA patients confirms the presence of proinflammatory cytokines and metalloproteinases [37]. Moreover, synovial fluid from RA patients exhibits hallmarks of oxidative damage [38] suggesting an important role for oxidative stress in the pathogenesis of the disease.

2.7.1 Rheumatoid Arthritis: ROS and T-Cell Function

Several lines of evidence suggest that oxidative stress exists in rheumatoid arthritis (RA), these include reduced total antioxidant capacity and increased levels of lipid peroxidation and oxidative stress markers in plasma from RA compared to healthy control subjects [39, 40]; increased serum MDA levels in RA compared to osteoarthritis and healthy control subjects [41]; reduced glutathione peroxidase and catalase activity reported in erythrocytes isolated from RA patients [39]; increased oxidative DNA damage in PBMC and urine from RA subjects [42, 43]; increased plasma Trx levels [43]; and increased 3-nitrotyrosine in synovial fluid from RA joints compared to other noninflammatory joints [38]. More specifically in RA SF T cells, intracellular free-radical production and intracellular GSH are increased and decreased, respectively [44, 45]. In peripheral blood T cells, intracellular GSH levels are similar between RA and healthy controls [44] and steady-state ROS levels

in response to mitogenic stimulation are not altered compared to healthy controls [3]. These studies are independent and not undertaken in paired samples, but suggest the role of local niche environment in regulating T-cell oxidative stress.

Although an important role for low level ROS has been implied during the initial events for T-cell activation, prolonged and elevated levels such as those that are evident in the synovium from RA patients may counteract this benefit and lead to a hyporesponsive T-cell phenotype. Indeed, the elevated levels of ROS within the synovial T cells located at site of inflammation (RA joint) are produced intracellularly possibly through a mechanism involving the activation and inactivation of Ras and Rap1, respectively [45, 46]. Gringhuis et al. [47] suggested under conditions of oxidative stress, such as those reported at the site of inflammation in RA, phosphorylation of the adaptor protein linker for activation of T cells (LAT), responsible for TCR engagement, and T-cell activation becomes impaired and leads to hyporesponsiveness. This subsequently leads to its displacement from the plasma membrane, thus providing a molecular mechanism for impaired intracellular signalling and showing the importance of redox status. In the periphery, T cells show no increase in ROS when cultured in vivo in the presence or absence of a mitogenic stimulus compared to controls; one possible consequence of this observation is that intracellular ROS production required for TCR signalling coupled with the failure to elicit intracellular ROS leads to an unresponsive phenotype [3].

2.7.2 Rheumatoid Arthritis: RNS and T-Cell Function

Few studies have investigated the effect of the nitrogen radical nitric oxide (NO) on T-cell function in RA. The inflamed joint in RA is a major NO-producing site [48], as several cells capable of producing NO via nitric oxide synthases (NOS) including osteoblasts, macrophages, fibroblasts and endothelial cells are present [49]. Furthermore, inhibition of NOS in rats with experimental arthritis results in the reduction of synovial inflammation and tissue damage [50]. Recently, RA human peripheral blood T cells were reported to exhibit a twofold increase in NO production compared to healthy human T cells [51], which is of particular interest given that NO promotes a proinflammatory Th1 effector cell phenotype [52] which may perpetuate an autoimmune response. The presence of NO in synovial fluid may also lead to the production of peroxynitrite ($ONOO^-$) through reaction with the superoxide anion ($O_2^{\bullet -}$) generated in the respiratory burst. Indeed, 3-nitrotyrosine, a hallmark for the presence of peroxynitrite, is elevated in synovial fluid of RA subjects compared to individuals with noninflammatory arthritic disease [38]. A recent study by Kavic and colleagues [15] demonstrated that primary human T cells pretreated with peroxynitrite showed inhibition of T-cell activation and inhibition of migration directed by chemokines. Together, these studies implicate a role for NO in RA pathogenesis and infer that increased production of NO in the inflamed joint, coupled with $O_2^{\bullet -}$, leading to $ONOO^-$ production, may contribute to the hyporesponsive nature of T cells.

2.7.3 Rheumatoid Arthritis: Cell Surface Thiols

Cell surface thiols (–SH) and intracellular SH are redox buffers and protect T cells from oxidative stress. In mutant neutrophil cytosolic factor 1 (Ncf1) rats, deficient in p47phox, a component of the NADPH oxidase complex, which produce low levels of ROS compared to native Ncf1 rats, an increased susceptibility to pristane-induced arthritis is evident [53]. In addition to the observed decrease in ROS production, T cells possessed greater levels of cell surface SH; increasing cell surface SH increased the proliferative response to an immunodominant CII peptide in a mouse hybridoma T-cell line. Furthermore, naïve rats exposed to $CD4^+$ T cells, which were artificially treated with GSH to increase cell surface thiols to a comparable level to that of ncf1 mutant rats susceptible to arthritis, also developed arthritis. Reciprocal experiments in mutant rats, using GSSG to reduce SH levels, resulted in less severe arthritis [53]. Together these experiments suggest that T-cell surface thiol groups influence T-cell activation, proliferation and the development of arthritis. Intriguingly, in contrast, cell surface thiols and iGSH have been investigated in peripheral human T cells; $CD4^+$ T cells and $CD8^+$ T cells show lower cell surface SH and iGSH in RA, with the $CD4^+$ T-cell surface SH negatively correlated to age [54], implying that these T cells would generally be less sensitive to activation.

2.7.4 Rheumatoid Arthritis: Oxidative Stress and Apoptosis

Apoptosis or programmed cell death is a controlled, energy-requiring process which removes damaged or infected cells in a noninflammatory manner. Synovial RA T cells do not undergo apoptosis despite exhibiting proapoptotic characteristics [55], and inhibition of apoptosis in RA leukocytes appears to occur during the earliest stage of the disease [56]. The inability of T cells to undergo apoptosis in RA, resulting in their persistence and accumulation within the synovial joint, may provide one possible reason for disease progression. Oxidative stress and the subsequent changes to cellular redox environment have been implicated in apoptosis [57]. The association of increased ROS in RA synovium [45], coupled to unresponsive nature of synovial fluid (SF) T cells and their persistence in the joint due to their inability to undergo apoptosis [55], suggests a link between cellular redox state and apoptosis in RA. A recent study by Kabuyama and colleagues [58] reported enhanced oxidative stress and an up-regulation of antioxidant genes in RA synovial cells. More specifically, thioredoxin reductase 1 (TRXR1), an enzyme involved in antioxidant defence, was identified as up-regulated at the gene and protein levels. Treatment of RA synovial cells with 1-chloro-2, 4-dinitrobenzene, an inhibitor of TRXR1, led to a dose-dependent increase in cellular H_2O_2 and cell apoptosis. These data suggest an important role for TRXR1 in preventing ROS-driven apoptosis and enhancing survival of SF T cells [58]. It is feasible that increased levels of ROS at the earliest stages of RA could result in SF T cells which exhibit enhanced antioxidant levels and are more tolerant to oxidative stress. This may provide a reason for why these

synovial cells are less able to undergo apoptosis and accumulate and persist within the RA synovial joint. The demonstration that the RA synovial fluid is enriched with regulatory $CD25^{bright}CD4^+$ T cells [59], coupled to the report that regulatory T cells express and secrete greater levels of Trx-1 compared to $CD4^+$ T cells and exhibit greater tolerance to oxidative stress [32], further supports this notion. Collectively, these data highlight an important role for oxidative stress and apoptosis in RA which may be harnessed therapeutically.

2.7.5 Rheumatoid Arthritis: Therapies, ROS and T Cells

2.7.5.1 Methotrexate

Methotrexate (MTX), a folate antagonist originally developed for treatment of malignant disease, is an effective and widely used drug in the treatment of RA and other inflammatory disorders [60, 61]. It is one of a few known disease-modifying antirheumatic drugs (DMARDs) currently available for the treatment of RA. Several underlying mechanisms including production and release of the anti-inflammatory agent adenosine, inhibition of T-cell proliferation through altered purine and pyrimidine metabolism and prevention of T-cell cytotoxicity have been described for the anti-inflammatory and anti-suppressive actions of MTX [62]. The anti-inflammatory and anti-suppressive effects have also been studied in the context of cell apoptosis and cellular oxidative stress. Apoptosis allows for the controlled removal of damaged cells without causing inflammation, and studies have reported the induction of T-cell-dependent apoptosis by low-dose MTX in vitro and in primary $CD4^+$ T cells from active RA compared with nonactive patients [63–65]. A link between apoptosis and production of ROS has been reported. Phillips et al. [66] proposed that the immunosuppressive action of MTX is dependent on the generation of ROS and reported increased cytosolic peroxide levels, reduced iGSH concentrations and induction of growth arrest in Jurkat T cells exposed to MTX [66]. Later work by Herman et al. [64] showed that low-dose MTX induces apoptosis with the production of ROS in T cells, which is abrogated by pretreatment with the thiol antioxidant NAC. These observations suggest that the induction of apoptosis by MTX probably acts through oxidative stress and may underlie the anti-inflammatory and immunosuppressive action of MTX [64].

2.7.5.2 Novel Biologics

Additional DMARDs currently used to treat RA include the biologics infliximab and etanercept. These DMARDs target and block TNFα activity. Altered redox activity and ROS production within the RA synovial T cells [44, 45] which infiltrate the joint, coupled with the demonstration that RA synovial cells stimulated with TNFα enhance $O_2^{\bullet -}$ production [67], suggest a potential indirect mode

of action for DMARDs on oxidative stress in RA. Recent studies have indeed demonstrated beneficial effects of infliximab and etanercept on serum redox status in RA patients. Lemarechal et al. [68] reported that increased protein oxidation and decreased thiol levels evident in RA patients compared to healthy control patients were reduced and increased, respectively, after the short-term administration of infliximab. Furthermore, levels of the advanced glycation end product pentosidine, a product produced under conditions of oxidative stress, are reduced in serum from patients treated with infliximab and etanercept [69, 70]. Taken together, these data suggest that DMARD therapies which target TNFα may exert their beneficial effects indirectly through modulation of the redox environment.

2.8 Conclusion

T cells represent an important part of the adaptive immune response and are principally responsible for distinguishing between "self" and "nonself". Upon activation at the immunological synapse with DC, they proliferate and differentiate into various subsets (e.g. Th1, Th2, Th17 and Tregs) under the influence of the local cytokine environment and thus enable a competent immune response. ROS are additional molecules which are important in immune defence as demonstrated by their generation and release in the form of an "oxidative burst" by phagocytic cells to neutralise, destroy and clear pathogens [13] and in signalling which is required for normal T-cell function [17]. However, increased levels of oxidising agents, due either to their increased production or reduced removal by antioxidants, which is prolonged and persistent, can lead to oxidative stress [11]; in this state macromolecular damage can ensue. In the context of T cells, their activation leads to an increase in cell surface thiols, changes to intracellular GSH can affect their function [71] and exposure to ROS and RNS (e.g. peroxynitrite) inhibits their activation and proliferation [15] and thus highlights an important role for the cellular redox environment on their function. Autoimmune diseases such as RA are associated with altered levels of redox activity in the periphery [39, 40] but also in the synovium at the site of inflammation [3]. The latter represents a highly oxidative environment where reduced intracellular GSH and increased ROS production in synovial T cells and increased nitration in synovial fluid are evident [38, 44, 45]. Additionally, RA synovial T cells are also less responsive to antigenic stimulation and are unable to undergo apoptosis [47, 55] leading to a hyporesponsive T-cell phenotype which persists within the rheumatoid joint. These observations suggest a strong link between aberrant redox activity towards a more oxidative environment and a hyporesponsive RA T-cell phenotype. Effective treatments which target TNFα may also act indirectly through modulating the redox environment, and further work into the effect of these therapies on redox state of peripheral and synovial RA T cells may provide further insight into their clinical efficacy in different cases.

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Chapter 3
Nitric Oxide and the Respiratory Chain in Synovial Cells and Chondrocytes

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Abstract Mitochondrial characteristics, including mitochondrial respiratory chain (MRC) activity, ATP synthesis, and mitochondrial membrane potential ($\Delta\psi m$), are altered in osteoarthritis (OA) chondrocytes and the synovium of inflammatory arthritis, or in synovial cells under arthritic-like conditions, suggesting a role of mitochondrial dysfunction in these types of arthritis. Nitric oxide (NO) is an inflammatory mediator produced in large amounts at sites of inflammation, including rheumatic joint tissues. Most of the catabolic effects of NO in chondrocytes and synoviocytes are potentially related to the ability of NO to combine with superoxide anion (O_2^-) to generate peroxynitrite ($ONOO^-$), a very strong oxidant capable of inhibiting important enzymes and affecting mitochondrial integrity. Oxidative mtDNA damage caused by mutagens such as reactive oxygen and nitrogen species could potentially be a main source of mitochondrial genomic instability that leads to respiratory chain dysfunction. NO-induced mitochondrial damage may explain several of the events involved in the pathogenesis of chronic arthropathies such as OA and rheumatoid arthritis (RA).

Abbreviations

$\Delta\psi m$	Mitochondrial membrane potential
ADMA	Asymmetric dimethylarginine
CS	Citrate synthase
DDAH-2	Dimethylarginine dimethylaminohydrolase 2

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eNOS	Endothelial NOS
IL	Interleukin
iNOS	Inducible NOS
MMP	Metalloproteinase
MRC	Mitochondrial respiratory chain
mtDNA	Mitochondrial DNA
mtNOS	Mitochondrial NOS
NF-κB	Nuclear factor-kappaB
nNOS	Neuronal NOS
NO	Nitric oxide
NOS	NO synthase
NRF-1	Nuclear respiratory factor 1
NRF-2	Nuclear respiratory factor 2
O_2^-	Superoxide ion
OA	Osteoarthritis
$ONOO^-$	Peroxynitrite
PGC-1	Peroxisome-proliferator-activated receptor gamma coactivator 1
PGE_2	Prostaglandin E_2
PPi	Inorganic pyrophosphate
PsA	Psoriatic arthritis
RA	Rheumatoid arthritis
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SNP	Sodium nitroprusside
Tfam	Mitochondrial transcription factor A
TNFα	Tumor necrosis factor alpha

3.1 Mitochondrial Dysfunction in Chronic Arthropathies

Osteoarthritis (OA) and rheumatoid arthritis (RA) are the most common rheumatoid pathologies. OA is a slowly progressing degenerative disease characterized by matrix degradation and cell death resulting in the gradual loss of articular cartilage integrity [1, 2]. RA is a primarily inflammatory disease characterized by chronic cytokine-mediated inflammation of the synovial joints and by abnormal synovial hyperplasia associated with local infiltration of various types of immune and inflammatory cells [3]. Although several cell types are adversely affected by mitochondrial dysfunction in OA and RA, here we will focus on chondrocytes and synoviocytes because the OA disease process is defined by changes in chondrocytes [4–7], and RA synoviocytes are the drivers of inflammation and degradation of the joint, leading to cartilage and bone destruction [8].

In the last few years, the clinical importance of the mitochondrion has become apparent, with study results indicating that changes in mitochondrial proteins probably cause some human diseases [9–15]. Mitochondria are complex and dynamic organelles that coordinate numerous metabolic reactions, including oxidative

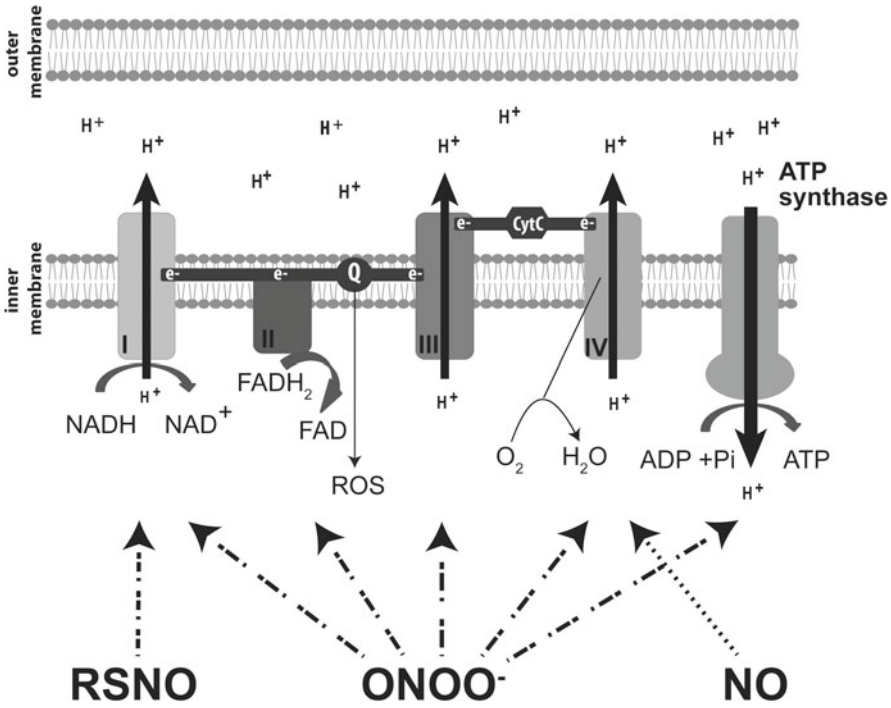

Fig. 3.1 Structure and function of mitochondria and the main effects of nitric oxide (NO) and reactive nitrogen species (RNS) on mitochondrial oxidative phosphorylation. A mitochondrion consists of inner and outer membranes composed of phospholipid bilayers and proteins. The outer mitochondrial membrane, through porins is permeable to molecules of 5 kDa or less. Larger molecules cross the outer membrane by active transport through mitochondrial membrane transport proteins. The inner mitochondrial membrane contains proteins (NADH dehydrogenase [complex I], succinate dehydrogenase [complex II], cytochrome *c* reductase [complex III], cytochrome *c* oxidase [complex IV], and ATP synthase [complex V]) that perform the oxidation reactions of the electron transport chain and ATP synthesis (oxidative phosphorylation). NO reversibly inhibits cytochrome *c* oxidase (complex IV), while $ONOO^-$ inactivates all respiratory complexes (I, II, III, and IV) and ATP synthase. Nitrosothiols (RSNO) inhibit complex I enzymes

phosphorylation [9, 16]. Mitochondria also participate in many other cellular events such as the production of reactive oxygen species (ROS), regulation of calcium homeostasis, and orchestration of apoptosis [17–19].

In mitochondrial oxidative phosphorylation, electron transport is coupled via four enzyme complexes (I, II, III, and IV) present in the mitochondrial inner membrane, with ATP synthesis from ADP by ATP synthase (complex V) (Fig. 3.1). The movement of donated electrons along the complexes of the mitochondrial respiratory chain (MRC) creates a proton gradient across the inner mitochondrial membrane, which drives ATP synthase to generate ATP [16, 20]. Leakage of electrons from the electron transport chain can result in the production of ROS (Fig. 3.1) [17]. Under physiological conditions, this process is efficient and only a small

number of electrons reduce oxygen to produce the free radical superoxide anions (O_2^-) as a by-product of the redox reactions of the MRC. In this framework, antioxidants and antioxidative enzymes are able to maintain the cellular defense mechanisms against oxidative stress. However, under pathological conditions, excess O_2^- ions are produced, and various antioxidant defenses could be insufficient to completely counteract this overload of ROS. Moreover, superoxide anions can combine with nitric oxide (NO), to produce peroxynitrite ($ONOO^-$), a very strong oxidant capable of inhibiting important enzymes and affecting mitochondrial integrity (Fig. 3.1) [21–23].

Mitochondrial functions, including MRC activity, ATP synthesis, and mitochondrial membrane potential (Δψm), are altered in OA chondrocytes [24–29] and synovium of patients with inflammatory arthritis, or in synovial cells under arthritic-like conditions such as hypoxia [30–33], suggesting the role of mitochondrial dysfunction in these types of arthritis. In particular, OA chondrocytes, compared with normal chondrocytes, showed a significant decrease in activity of the MRC complexes II and III and a reduction in Δψm [24] (Table 3.1). Moreover, OA chondrocytes showed a significantly increased activity of citrate synthase (CS, a marker of mitochondrial mass) as compared to that in normal cartilage cells, suggesting that mitochondrial mass is increased probably as a compensatory mechanism for the electron transfer deficiency and the consequent low production of ATP (decreased by 50%) [24, 34]. Mitochondrial function is also altered in articular chondrocytes of the endemic OA, Kashin–Beck disease. In these cells, a reduction has also been reported in the activities of complexes IV and V besides the alterations described above [26] (Table 3.1). In RA and systemic juvenile idiopathic arthritis, the synovium is shown to be deficient in cytochrome *c* oxidase subunit II (a subunit of MRC complex IV) [30, 31]. Furthermorβ) | m is significantly increased under 1% hypoxia in synovial cells [30]. Moreover, extracellular mtDNA has been detected in the synovial fluids of RA patients but not in those of control subjects, and mtDNA has been shown to be immunostimulatory and capable of inducing arthritis in mice [35]. Other cells such as T lymphocytes showed mitochondrial impairment in patients with RA and systemic lupus erythematosus [36, 37].

This mitochondrial dysfunction drives mitochondrial genome mutagenesis, affecting genes encoding respiratory chain complexes and transcription, which may lead to further mtDNA mutations causing a vicious cycle that may result in cell damage. Indeed, synoviocytes and chondrocytes from RA and OA patients, respectively, have been shown to exhibit significantly higher mtDNA mutation rates than synoviocytes or chondrocytes from normal individuals [11, 38, 39]. Mitochondrial dysfunction may influence several specific pathways involved in chronic arthropathies, including oxidative stress induction, increased apoptosis, and augmentation of inflammatory and matrix catabolic responses [25, 33, 34, 40–43]. Specifically to cartilage degradation, mitochondrial impairment may also induce defective chondrocyte biosynthesis and growth responses as well as cartilage matrix calcification.

Some of the single-nucleotide polymorphisms that characterize the different mtDNA haplogroups could alter mitochondrial oxidative phosphorylation coupling. Moreover, several common mtDNA haplogroups have been associated with several

Table 3.1 Mitochondrial parameters from osteoarthritis (OA) chondrocytes or normal chondrocytes or synoviocytes under nitric oxide (NO) or cytokine treatment

	OA chondrocytes [24–26]	NO-treated chondrocytes [97–99]	IL-1β-treated chondrocytes [101]	TNFα-treated chondrocytes [101]	NO-treated synoviocytes [102]
MRC					
Complex I	↓	↓	↓*	↓*	↑*
Complex II	↓*	=	↓	↓	=
Complex III	↓*	↓	=	=	↑*
Complex IV	=, ↓*	↓*	↓	=	↓
CS activity	↑*	↓	↑	↑	↓*
Δψm	↓*	↓*	↓*	↓*	↓*
ATP synthesis	↓*	↓*	↓*	↓*	↓*

*$p<0.05$ vs. control; MRC, mitochondrial respiratory chain; CS, citrate synthase; Δψm, mitochondrial membrane potential; ↓, decreased; ↑, increased; =, unchanged

diseases including joint pathology. In fact, haplogroup J exerts a protective role in the development of OA and psoriatic arthritis (PsA) and correlates with low oxidative stress levels; meanwhile haplogroup U is associated with an increase in the radiologic severity of OA [44–46].

Accumulating evidence indicates that NO and mitochondrial damage may play a significant role in the pathogenesis of joint diseases such as OA and RA. In this review, we examine the biochemical actions of NO on the mitochondria of chondrocytes and synovial cells, their signaling consequences, and their possible relationship with the physiology and pathophysiology of joint diseases.

3.2 NO in Joint Diseases

NO is a free radical messenger that mediates a variety of biological actions such as vasodilatation, inflammatory responses, and various effects on mitochondria [47–50]. NO is synthesized by NO synthase (NOS) enzymes that convert L-arginine to NO and L-citrulline [51]. The complex family of NOS enzymes is represented by three distinct cellular isoforms: two of these cellular isoforms—endothelial (eNOS) and neuronal (nNOS)—are constitutively expressed and regulated by second messengers (calcium/calmodulin and phosphorylation), producing relatively low levels of NO. The third isoform, namely, inducible NOS (iNOS), is encoded by an early response gene that is often rapidly induced in response to inflammatory stimuli and most likely responsible for the elevated NO levels at sites of inflammation, including human rheumatoid joints. All isoforms are tightly regulated by several transcriptional modifications, which control their localization within the cell, and substrates, and other proteins with which they form stable complexes [52, 53]. Some studies also described a mitochondrial NOS (mtNOS) isoform; however, its existence is controversial [23, 54]. Interestingly, recent in vitro findings identified, for the first time, the effect of interleukin (IL)-1β on the transport of dimethylarginine dimethylaminohydrolase 2 (DDAH-2) to the mitochondria of chondrocytes, a localization not previously described in any cell type. DDAH-2 can inhibit asymmetric dimethylarginine (ADMA), a natural inhibitor of NOS, leading to increased levels of NO [55]. mtNOS or forms of NOS associated with the outer mitochondrial membrane may play a specific role in the regulation of mitochondrial function [48, 52, 56].

Once formed, NO is chemically stable and can easily diffuse into the mitochondria, cell, or from one cell to another [52]. However, in the presence of other free radicals such as O_2^-, NO reacts quickly, forming the strong oxidant $ONOO^-$ [22], which can mediate the oxidation and nitration of membrane lipids, proteins, or DNA; however, NO cannot directly mediate any of these effects alone [52]. $ONOO^-$ is probably the major mediator of the pathological effects associated with NO, mainly inflammation. NO also reacts directly with oxygen to produce NO_2, which reacts with other NO molecules and forms N_2O_3, a nitrosating agent.

NO is produced in large amounts at sites of inflammation, including rheumatic joint tissues [50, 57–63]. Both OA and RA synovial cells spontaneously release NO [64]. Moreover, pathological articular cartilage/chondrocytes as well as normal cartilage/chondrocytes under cytokine stimulation, are among the most important sources of NO [65, 66]. Most researchers have found correlations between the disease activity or radiological progression of OA and RA and the levels of NO [58, 67–69], while others did not find such correlations [70]. In addition, NOS inhibition is reported to decrease disease activity in experimental RA and OA models [71, 72], and the NO levels in joint diseases are reportedly modulated by drugs used to treat chronic arthropathies [73–75]. Moreover, NOS polymorphisms could influence the risk of cardiovascular events in RA [76].

Overproduction of NO is likely induced by proinflammatory cytokines such as IL-1β and tumor necrosis factor (TNF) α in different articular cell types [77–81]. It has recently been shown that TNFα-stimulated normal human chondrocytes elevate the levels of the enzyme argininosuccinate synthase, which degrades ADMA, the natural inhibitor of NOS, probably leading to increase NO levels [82]. Moreover, mechanical loading of cartilage and synovial tissues increases the production of NO by upregulating iNOS [83–85]. This iNOS expression is upregulated by the redox-sensitive nuclear factor-κB (NF-κB) pathway, which is also activated by NO [85].

NO has been classically considered a deleterious mediator that contributes to chronic arthropathies by mediating a number of processes such as apoptosis and matrix degradation (by inhibiting proteoglycan and collagen synthesis and stimulating metalloprotease synthesis) and perpetuating the expression of proinflammatory cytokines both in chondrocytes and synovial cells [77, 80, 86–88]. However, recent evidence suggests that NO and its derivatives could have beneficial effects on several cell types, including articular cells such as osteoblasts and tendon cells [50, 52, 89–91]. In this sense, NO and the different reactive nitrogen species (RNS) have specific characteristics that could change with location, exposure, and concentration [23]. This could explain why NO may have opposing effects, that is, destructive and protective. In relation, chondrocytes from the superficial zone of normal human articular cartilage synthesize much more NO than cells from the deep zone, and this could be related with the role of NO as an endogenous modulator of the cartilage matrix turnover in different zones of the articular cartilage [92].

3.3 NO Regulates Mitochondrial Function in Joints

NO or its derivative RNS seem to play an important role in mitochondrial biology [21–23, 47, 48] (Table 3.2). One of the better described effects is that NO, at physiological concentrations, reversibly inhibits the activity of MRC complex IV (cytochrome *c* oxidase) by binding to this enzyme in competition with oxygen [93, 94]. This reversible inhibition of mitochondrial cytochrome *c* oxidase by NO may be a functional control of mitochondrial respiration [95]. Thus, NO could modulate oxygen distribution and consumption [96].

Table 3.2 Main actions of nitric oxide (NO) on mitochondria

Reversible inhibition of cytochrome c oxidase
Irreversible inhibition of mitochondrial respiration
Stimulates mitochondrial biogenesis
Stimulates ROS and RNS production from mitochondria
Modulates Δψm, mitochondrial calcium release, and mitochondrial permeability transition
Induces apoptosis and protection mediated by mitochondria
Modulates O_2 gradients and hypoxia-responsive targets
Activation of AMP kinase and modulation of glycolytic output

NO, nitric oxide; ROS, reactive oxygen species; RNS, reactive nitrogen species; Δψm, mitochondrial membrane potential; O_2, oxygen

3.3.1 Effect of NO on Mitochondrial Respiratory Activity in Chondrocytes and Synovial Cells

Several studies in chondrocytes have documented evidence for suppressed respiration and ATP generation by NO and $ONOO^-$ [34, 97–100]. In particular, NO induced a significant decrease in the activity of complex IV (by 33%) as well as a reduction in Δψm. Activities of complexes I and III were also reduced; however, the differences were not significant [98]. TNFα and IL-1β, both potent inductors of NO, also modify mitochondrial function through a decrease in the MRC complex I activity and ATP production as well as a reduction in Δψm [101] (Table 3.1). Complex I inactivation is one of the more rapid effects of RNS on respiration, possibly due to S-nitrosation of the complex [23]. Activities of complexes II and IV were also decreased; however, the differences were not significant. Notably, the modifications of MRC activities described in ex vivo OA chondrocytes were stable because they were detected in chondrocytes after several weeks of culture. These stable modifications can be the result of mitochondrial genome mutagenesis induced by ROS and RNS affecting the genes encoding respiratory chain complexes. To the best of our knowledge, only one in vitro study has analyzed the effect of exogenous NO on the enzymatic activity of MRC in human synoviocytes [102]; however, the effect on mitochondrial mutagenesis and dysfunction in inflammatory arthritis, the correlation of mitochondrial mutagenesis with the local inflammatory environment in arthritis, and the importance of normal Δψm have been evaluated [30, 33]. Synovial cells treated with NO showed decreased Δψm, ATP, and mitochondrial mass [102]. In contrast, the activities of complexes I and III were increased, possibly to compensate for the energy deficit (Table 3.1).

Considering the hypoxic environment of the articular cartilage and RA synovium, it should be noted that most of these in vitro studies were performed with chondrocytes/synoviocytes kept in standard media with high glucose concentrations and under aerobic conditions that could cause cells to obtain their energy predominantly from anaerobic glycolysis. For this reason, it is likely that the alteration of

mitochondrial activity observed in these studies in cultures maintained under a normal atmosphere is even more crucial in joint OA cartilage and/or RA synovium where the mitochondrial activity is probably lower (due to somatic mutations in mtDNA or the direct effects of proinflammatory stimuli on the MRC). In this sense, recent in vitro findings have shown that hypoxic conditions differentially modulate the chondrocyte proteome in normal and OA human chondrocytes, including some mitochondrial proteins [103]. A recent study has also shown that in vitro levels of mtDNA mutations, ROS, and $\Delta\psi m$ were higher in synoviocytes exposed to 1% hypoxia than in cells exposed to normoxia [30]. Moreover, it has been reported that NO-induced inactivation of complex I may be enhanced by hypoxia [104].

These in vitro results are consistent with the in vivo study performed by Terkeltaub and coworkers, in which progressive chondrocyte ATP depletion appeared to be responsible for the development of spontaneous knee OA in Hartley guinea pigs [25]. The authors described a marked spontaneous NO and glycosaminoglycan release from knee cartilage which increased as knee OA developed. Simultaneously, chondrocyte intracellular ATP levels declined by approximately 50%, despite the absence of mitochondrial ultrastructure abnormalities in knee chondrocytes. Concomitantly, lactate-to-pyruvate ratio increased, consistent with an adaptive augmentation of glycolysis to mitochondrial dysfunction. In relation, in vitro studies in chondrocytes treated with specific inhibitors of MRC similarly showed a progressive increase in lactate-to-pyruvate ratio.

3.3.2 Apoptosis, Matrix Synthesis, and Calcium Metabolism Mediated by the Effects of NO on the Mitochondria of Chondrocytes and Synovial Cells

Mitochondria play an important role during apoptosis. The classic signs of cell death are preceded by mitochondrial alterations, which include loss of $\Delta\psi m$, decrease in energy production, increase in the permeability of the mitochondrial membrane, alteration of MRC activities, release of pro-apoptotic factors such as cytochrome *c* and downregulation of apoptotic members such as bcl-2 and mcl-1, or activation of caspase pathways [105, 106]. All these alterations are modulated by the interaction between NO and MRC. In this sense, several studies suggest that NO induces apoptosis in chondrocytes and synovial cells by reducing the activity of complex IV and decreasing the $\Delta\psi m$ [88, 102].

$\Delta\psi m$ could reflect the functional status of the mitochondria. Although some studies have reported that the inhibition of MRC by NO could induce a temporary small increase in $\Delta\psi m$ associated with the initiation of apoptosis [107], most reports suggest that NO causes mitochondrial depolarization and the ensuing mitochondrial calcium release would facilitate mitochondrial permeability transition opening and apoptosis. Human chondrocytes and synoviocytes treated with the NO donor, sodium nitroprusside (SNP), showed reduced survival and increased cell death, with

morphological changes characteristic to apoptosis. Furthermore, it was shown that SNP induces the mRNA expression of caspase-3, caspase-7, and caspase-9 and reduces the synthesis of anti-apoptotic molecules bcl-2 and mcl-1 [88, 98, 102]. In contrast, a recent study on peroxynitrite-mediated chondrocyte apoptosis showed that the predominant mode of cell death involved calcium-dependent cysteine proteases, known as calpains, and that peroxynitrite-induced mitochondrial dysfunction in cells leads to caspase-independent apoptosis [108]. Moreover, some studies have reported that NO protects cultured chondrocytes and synoviocytes from cell death depending on its concentration and the oxidative milieu [109, 110].

This disparity in the results is probably due to the lack of uniformity between different types of NO donor compounds and because, as mentioned, NO can interact with other ROS possibly concomitantly produced as a consequence of NO itself. NO and different RNS have specific characteristics that could change with concentration, location, and exposure. Interestingly, some studies have proposed that the balance between intracellular NO and ROS may determine the type of chondrocyte death, with low ROS concentrations promoting apoptosis in the presence of NO and high ROS concentrations promoting necrosis [111–113]. In this sense, the increase of oxidative stress with aging makes chondrocytes more susceptible to NO-mediated cell death through the dysregulation of the glutathione antioxidant system, suggesting an important contributing factor to the development of OA in older adults [114].

NO can also irreversibly inhibit respiration for a long duration, probably through the strong oxidant $ONOO^-$ which inactivates all respiratory complexes (I, II, III, and IV) and ATP synthase (Fig. 3.1). One approach to explain the functional significance of NO-induced mitochondrial dysfunction is to determine the effects of MRC inhibition and to compare them with the findings of joint disease. In this sense, to determine whether the cell death caused by the NO donor is directly mediated by the inhibition of complex IV, a mitochondrial inhibitor of complex IV (NaN_3) was used, and cytotoxicity and mitochondrial activity were evaluated. The results showed that NaN_3 altered $\Delta\psi m$ and induced apoptosis. Interestingly, apoptosis was greater when glucose was absent, as observed in the deeper layers of the cartilage. Inhibition of complex IV alone is possibly not enough to induce apoptosis; other cellular events as reduction in glucose intake may be necessary [98]. However, rotenone (mitochondrial inhibitor of complex I) decreased $\Delta\psi m$ but increased the bfl-1 and mcl-1 mRNA expression and bcl-2 protein in concordance with the failure to induce cell death [101].

Microcalcifications are present in OA cartilage. Because mitochondria play a crucial role in calcium homeostasis, the implication of NO-induced mitochondrial dysfunction in this process has been suggested [34]. In this sense, chondrocyte mitochondrial impairment facilitates matrix mineralization to reduce the content of inorganic pyrophosphate (PPi, a critical inhibitor of hydroxyapatite deposition). Furthermore, cartilage matrix calcification is stimulated by apoptosis, and chondrocyte apoptosis co-localizes with cartilage matrix calcification in OA cartilages [28].

Moreover, NO-induced mitochondrial damage could regulate matrix loss through attenuated collagen and proteoglycan synthesis [34, 97]. It was reported that mitochondrial impairment induced by inhibition of complexes III and V

markedly decreased proteoglycan and collagen synthesis [34]. Moreover, rotenone (a complex I inhibitor) reduced the proteoglycan content in the superficial and middle zones, increasing the release of glycosaminoglycan from the cartilage to the supernatant [101].

3.3.3 Inflammatory Response Induced by the Effects of NO on the Mitochondria of Chondrocytes and Synovial Cells

Mitochondria are the major source of ROS production [17]. It has been described that inhibition of the activities of complexes III and V induces ROS production and NF-κB activation in human articular chondrocytes [40] and synovial cells in vitro (MN Valcárcel-Ares, unpublished data) as well as low-grade inflammatory and matrix degradation processes through the synthesis of proinflammatory stimuli such as prostaglandin E_2 (PGE_2), chemokines IL-8 and monocyte chemotactic protein-1, and metalloproteinase (MMP)-1, MMP-3, and MMP-13 [40, 41, 115]. In addition, this decline in mitochondrial function could increase the inflammatory responsiveness of chondrocytes and synoviocytes to cytokines, accelerating the mechanism that may contribute to the impairment of the structure and function of joints.

Interestingly, in vitro results have shown that the production of PGE_2 induced by mitochondrial dysfunction is partially blocked by caspase inhibition in normal human chondrocytes. This finding suggests that mitochondrial dysfunction is implicated in both apoptosis and inflammatory pathways (C Vaamonde-García, unpublished data).

3.4 Potential Beneficial Effects of NO on the Mitochondria in the Physiology of Cartilage and Synovial Cells

Articular cartilage chondrocytes must preserve tissue integrity in an avascular environment. Oxygen and glucose concentrations in cartilage are characterized by asymmetry with a gradient from the superficial to the deep zones because chondrocytes in articular cartilage obtain nutrients only by diffusion from the synovium [116, 117]. Cells at the superficial area are considered to be exposed to 5–7% oxygen (versus 13% in arterial blood), whereas chondrocytes in the deepest area of the cartilage are exposed to low oxygen tension (<1%). Chondrocytes from deep and superficial zones may require adaptively increased anaerobic glycolysis and aerobic respiration, respectively, to support ATP synthesis. Mitochondria under the influence of NO may be implicated in the attenuation of adaptive responses to low oxygen because a major function of NO-mediated inhibition of respiration is to modulate oxygen gradients and hypoxia-responsive targets in cells by inhibiting oxygen consumption in respiring mitochondria. A consequence of this blockage would be to extend the oxygen gradient within tissues and limit hypoxia [52, 96]. Chondrocytes

from the superficial zone of normal human articular cartilage synthesize much more NO than cells from the deep zone [92], which could extend the oxygen gradient to the deepest cartilage and inhibit hypoxia.

NO can also stimulate mitochondrial biogenesis in several cell types [48, 118]. This effect occurs through increased expression of peroxisome-proliferator-activated receptor gamma coactivator 1 (PGC-1, the principal regulator of mitochondrial biogenesis), nuclear respiratory factor (NRF)-1, NRF-2, and mitochondrial transcription factor A (Tfam). This increasing mitochondrial mass generates functionally active mitochondria capable of generating ATP through oxidative phosphorylation. Thus, as mentioned before, OA chondrocytes showed a significant increase in mitochondrial mass that may be induced by the high NO level found in OA joints. In contrast, in vitro studies on chondrocytes or synoviocytes treated with an NO donor did not show any modification in the CS activity. A possible explanation for these results is that high levels of NO produced by NO donors acutely inhibit cell respiration by binding to cytochrome *c* oxidase. Conversely, chronic and small increases in NO levels observed in OA joints could stimulate mitochondrial biogenesis [48]. Consistent with this finding, when cells were treated with the cytokine IL-1β or TNFα for a long duration, an increase in CS activity was observed. Whether defective mitochondrial biogenesis is a hallmark of the joint disease regulated by NO should be elucidated.

3.5 Conclusions

iNOS induction during inflammation led to high levels of NO, which, together with ROS, could be responsible for the MRC inhibition and mtDNA damage described in chronic arthropathies such as OA and RA. This mitochondrial damage could affect genes encoding respiratory chain complexes, which may lead to further mtDNA mutations, causing a deleterious mitochondrial spiral. This NO-induced mitochondrial dysfunction may explain several of the events involved in the pathogenesis of OA and RA.

As the accumulation of mtDNA mutations correlates with the decline in mitochondrial function observed in disorders such as OA and RA, an ongoing debate over various therapeutic strategies utilizing antioxidant compounds is well underway. Preserving mitochondrial function could reduce oxidative stress and may represent a novel therapeutic approach for patients with these diseases. In this sense, natural antioxidants such as resveratrol have been shown to improve mitochondrial function by preventing oxidative stress. In particular, resveratrol protects against chondrocyte apoptosis via effects on mitochondrial polarization and ATP production [119]. We also demonstrated that supplementation with resveratrol in chondrocytes and synovial cells significantly reduced IL-8, PGE_2, and MMP-1 and MMP-3 levels induced by the combination of mitochondrial dysfunction and cytokines [115, 120, 121].

Several common mtDNA haplogroups have been linked to the prevalence of joint diseases. In fact, haplogroup J exerts a protective role in the development of OA and

PsA [44, 45]. Moreover, chondrocytes from individuals carrying haplogroup J exhibit lower NO production than non-J carriers [122]. Additionally, NOS polymorphisms could influence the risk of cardiovascular events in RA [76]. This may point to the importance of studying interactions between NOS gene polymorphisms and mtDNA haplogroups in terms of risk for developing joint diseases.

Lastly, NO has been classically considered a catabolic factor in joint diseases. However, recent studies suggest that it may have beneficial effects on joint tissues. More research is needed to elucidate the role of NO-induced mitochondrial modulation on both normal and pathological joint tissues.

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Chapter 4
Adipokines, Molecular Players at the Crossroad Between Inflammation and Oxidative Stress: Role in Arthropathies

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Abstract Oxidative stress is becoming to be recognized as a relevant process at play in rheumatic diseases. Certain novel biochemical players were identified and appear to be related with the severity and progression of the different rheumatic diseases. Adipocytes synthesize and secrete a wide variety of factors called adipokines, which are involved in many different physiological processes and diseases. These adipose tissue-derived factors seem also to be involved in the pathophysiology of rheumatic disorders by regulating also oxidative stress at vascular and joint tissues level.

In this chapter, we discuss the relationships between adipokines and different arthropathies and their complications, as well as their liaison with oxidative stress.

Abbreviations

AdipoR1	Adiponectin receptor 1
AdipoR2	Adiponectin receptor 2
ADSF	Adipocyte-secreted factor
ALP	Alkaline phosphatase
AS	Ankylosing spondylitis

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CART	Cocaine- and amphetamine-related transcript
CRP	C-reactive protein
CVD	Cardiovascular diseases
eNOS	Endothelial nitric oxide synthase
FIZZ3	Inflammatory zone 3
GH	Growth hormone
IGF-1	Insulin growth factor-1
IL	Interleukin
IL-1Ra	IL-1 receptor antagonist
IR	Insulin resistance
LDL	Low-density lipoprotein
MCP-1	Monocyte chemotactic protein 1
MMP	Matrix metalloproteinase
Nampt	Nicotinamide phosphoribosyltransferase
NO	Nitric oxide
NPY	Neuropeptide Y
OA	Osteoarthritis
PAI-1	Plasminogen activator inhibitor-1
PBEF	Pre-B-cell colony-enhancing factor
PBMCs	Peripheral blood mononuclear cells
PGE_2	Prostaglandin E_2
RA	Rheumatoid arthritis
RLM	Resistin-like molecules
ROS	Reactive oxygen species
sdLDL-C	Small dense low-density lipoprotein subclasses
SF	Synovial fluid
sIL-6R	Soluble IL-6 receptor
SLE	Systemic lupus erythematosus
TGF-β	Transforming growth factor-β
TNF-α	Tumor necrosis factor-α
WAT	White adipose tissue

4.1 Introduction

Inflammation is a complex mechanism of cell/tissue responses to injuries triggered by multiple causes including trauma, pathogens (viral or bacterial), or autoimmune abnormal responses. Oxidative stress is another face of inflammatory response and is involved in the pathogenesis of several chronic diseases including cardiometabolic diseases (hypertension, dyslipidemia, diabetes, and obesity) but also chronic rheumatic diseases. In the last years, a novel line of thought is emerging by giving a more holistic vision of chronic rheumatic diseases by linking cardiometabolic diseases with chronic arthropathies through a recent identified group of molecules called adipokines. Actually, most of these recently identified factors, produced

prevalently by white adipose tissue but also produced by cells of the joints (chondrocytes and synovial fibroblast) and immune cells, play a significant role in chronic inflammation, by oxidative stress-induced damage to protein, lipids, and DNA. Thus, adipokine deregulation has emerged as a common characteristic of chronic inflammation in rheumatic diseases in particular when obesity or, more precisely, adipose tissue dysfunction is associated with rheumatic diseases. Hence, in this chapter, we discuss the role of adipokines in chronic arthropathies providing an updated overview of their potential pathophysiological roles and potential use as therapeutic targets.

4.2 Leptin

Leptin is a 16 kDa non-glycosylated hormone encoded by the gene *ob*, the murine homologue of the human gen *LEP* [1]. It belongs to the class I cytokine superfamily, consisting of a bundle of four α-helices. Leptin is mainly produced by adipocytes, and circulating levels are correlated with white adipose tissue (WAT) mass. It decreases food intake and increases energy consumption by acting on specific hypothalamic nuclei, where leptin induces anorexigenic factors such as the cocaine- and amphetamine-related transcript (CART) and suppresses orexigenic molecules such as neuropeptide Y (NPY) [2]. Leptin levels are mostly dependent on the amount of body fat, but its synthesis is also regulated by inflammatory mediators [3]. Leptin exerts its biological actions through the activation of its cognate receptors, which are encoded by the diabetes gene (*db*) and belong to the class I cytokine receptor superfamily. There are six different isoforms of leptin receptors, but only the long isoform is functional (Ob-Rb). Several tissues produce leptin and express its receptors, including those of the cardiovascular system such as blood vessels and cardiomyocytes [4]. Leptin gene expression is mainly regulated by food intake, energy status, hormones, and also by inflammatory mediators [5, 6]. Genetic deficiency in the gene encoding for leptin or its receptors provokes severe obesity and diabetes mellitus.

4.2.1 Leptin and Atherosclerosis

Leptin is associated with cardiovascular diseases (CVD) (Fig. 4.1). In fact, elevated serum concentrations of this adipokine are related with myocardial infarction and stroke independently of traditional cardiovascular risk factors [7]. Moreover, it has been proposed that leptin plays a pathogenic role in atheromatous plaques, due to its positive association with C-reactive protein (CRP) and soluble IL-6 receptor (sIL-6R) [8], two inflammatory mediators involved in the pathogenesis of atherosclerosis [9, 10]. The proatherogenic actions of leptin are supported by several experimental observations demonstrating that this adipokine induces hypertrophy of vascular

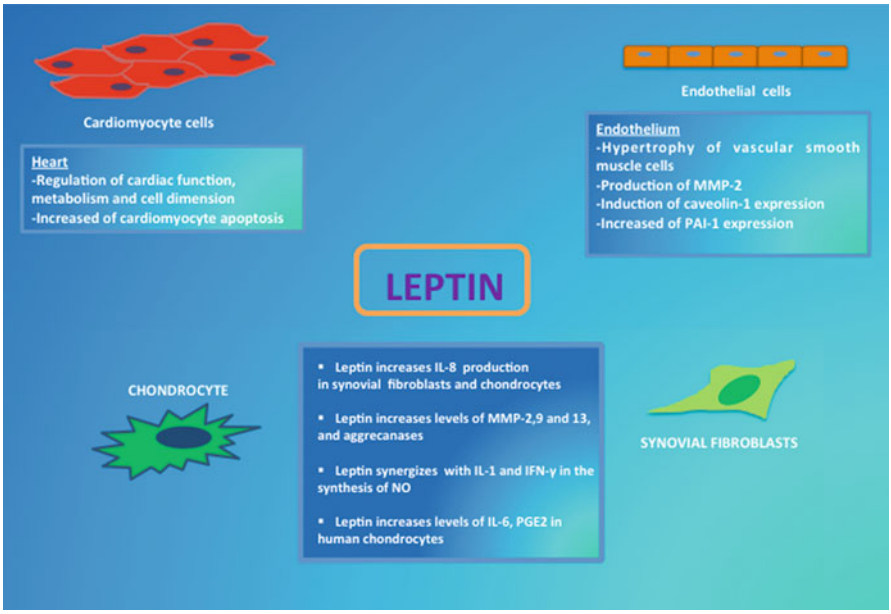

Fig. 4.1 Schematic representation of leptin implication in cardiomyocyte, endothelial, chondrocyte, and synovial fibroblast cells

smooth muscle cells [11] and the production of matrix metalloproteinase (MMP)-2 [12]. The latter exerts important actions in plaque rupture [13]. Also, leptin could stimulate vascular remodeling by promoting profibrotic cytokine production [14]. Apart from this, leptin increases the secretion of proatherogenic lipoprotein lipase by cultured human and murine macrophages [15], enhances platelet aggregation [16, 17], and induces CRP expression in human coronary artery endothelial cells [18].

It has been described that leptin induces caveolin-1 expression in endothelial cells [19]. Caveolin-1 plays a relevant regulatory role in the development of atherosclerosis, promoting the transcytosis of low-density lipoprotein (LDL) to the subendothelial space and inhibiting endothelial nitric oxide synthase (eNOS) function [20, 21]. This represents a novel mechanism through which hyperleptinemia contributes to the development of atherosclerosis. Recently, it has been reported that leptin was able to increase plasminogen activator inhibitor-1 (PAI-1) expression in human coronary artery endothelial cells [22]. PAI-1 plays an important role in the development and progression of atherosclerosis [23, 24], and its deficiency has been described to protect against atherosclerosis progression [25]. Indeed, in human atherosclerotic arteries, PAI-1 production and enhanced expression appear to be directly related with the degree of atherosclerosis [26].

Leptin has an important role in obesity-induced oxidative stress. Increased leptin levels have been associated with oxidative stress and endothelial dysfunction in early obesity. The hormone directly stimulates ROS production such as H_2O_2 and hydroxyl radical [27].

4.2.2 Leptin and Rheumatic Diseases

In addition to its well-known actions on the immune system, leptin has also been associated with rheumatic diseases due to its ability to modulate bone and cartilage metabolism [28, 29].

Leptin can play a significant role in certain autoimmune diseases such as rheumatoid arthritis (RA). This idea is supported by several in vitro and in vivo studies. Serum leptin levels were increased in RA patients compared to healthy controls [30, 31]; however, other studies reported unchanged levels [32]. Moreover, several authors suggested that a correlation between the RA disease activity and leptin levels might exist [33–35]. To note, synovial/serum leptin ratio was correlated with disease duration and erosion parameters in RA patients [36], whereas other authors did not find any correlation between leptin levels and disease activity [37]. In patients undergoing antitumor necrosis factor-α (TNF-α) therapy because of severe disease refractory to conventional therapy, there was a positive correlation between body mass index of RA patients and serum level of leptin [37]. Interestingly, in these patients, there was a correlation between leptin levels and vascular cell adhesion molecule-1 [37]. This is of potential relevance as biomarkers of endothelial dysfunction (or endothelial cell activation) have been found elevated in patients with RA, and anti-TNF-α blockade improved endothelial dysfunction [38] and also decreased the levels of some of these endothelial cell activation biomarkers [39]. However, in different studies assessing the influence of genetic polymorphisms, both inside and outside the MHC region, in the increased cardiovascular risk of RA [40–42], no associations with leptin (LEP) have been found [43].

Low leptin levels, related to food restriction, have been linked to CD4+ lymphocyte hyporeactivity and increased interleukin (IL)-4 secretion [44]. Leptin was involved in RA-induced hypo-androgenicity, due to the fact that leptin levels were negatively correlated to androstenedione [45]. Then, since leptin acts as a pro-inflammatory factor and androgens are commonly considered as anti-inflammatory agents, the preponderance of leptin and hypo-androgenicity may help to perpetuate chronic rheumatic diseases such as RA. In addition, TNF-α blockers such as infliximab or adalimumab did not modify serum leptin levels [37, 45, 46]. Several studies carried out in arthritis animal models, as well as in vitro studies, support the involvement of leptin in RA [47].

Leptin stimulation increases IL-8 production in RA synovial fibroblasts via leptin receptor/JAK2/STAT3 pathway [48]. However, the effects of leptin in RA are not only related to articular tissues. Leptin also modulates the activity of multiple immune cells, including regulatory T cells, which are potent inhibitors of autoimmunity [49]. The ability of leptin to induce regulatory T-cell anergy and T-cell receptor hyporesponsiveness has gained much interest since altered functioning of this cell type was described in RA [50].

Leptin has also been related with osteoarthritis (OA) and cartilage metabolism. It is known that chondrocytes from human OA cartilage produce much more leptin

than chondrocytes from normal cartilage [51]. Moreover, leptin was found in synovial fluid from OA affected joints [51, 52]. In addition, the expression pattern of leptin was related to the grade of cartilage destruction [51] and with the severity of the disease, with the highest levels of leptin being in the advanced stages of the disease [53, 54].

Recently, it has been reported that extreme obesity due to the impaired leptin signaling induces alterations in subchondral bone morphology but without increasing the incidence of OA [55]. These results suggest that obesity, per se, is not sufficient to induce OA, leptin being necessary in the development and progression of OA associated with obesity. In vitro experiments also pointed a role of leptin in OA. Leptin increases IL-8 production by OA synovial fibroblasts and chondrocytes [48, 56]. In human cultured chondrocytes, leptin synergizes with IL-1 and interferon-γ in the synthesis of nitric oxide [29, 57]. In addition, this adipokine enhances MMP-9, MMP-13, prostaglandin E_2, and IL-6 production in human chondrocytes [54, 58]. Leptin has also been related with bone metabolism; actually it has been suggested that abnormal leptin production by OA osteoblasts could be responsible for an altered osteoblast function in OA [59].

Regarding the role of leptin in systemic lupus erythematous (SLE), some contradictory data exist. Nowadays, most of the studies suggest a role for leptin in this disease. Several authors found higher leptin levels in SLE patients compared with healthy controls, even after BMI correction [60–64]. Interestingly, in some of these studies, the hyperleptinemia was associated with cardiovascular diseases and with several features of the metabolic syndrome [63, 64]. Indeed, using a lupus animal model, it was determined that leptin enhanced the pro-inflammatory high-density lipoproteins scores and atherosclerosis induced by a high-fat diet [65], indicating that factors related with metabolic syndrome can accelerate the disease and its cardiovascular complications. On the other hand, other groups have described lower or unchanged circulating leptin levels in SLE patients compared to healthy control [66, 67].

The role of leptin in ankylosing spondylitis (AS) is still unclear, and the data available are limited. For instance, certain studies have not found any correlation between serum leptin concentrations and markers of disease activity [68, 69]. However, other authors determined an association among serum leptin levels, CRP, IL-6, and markers of disease activity [70, 71]. In addition, it has been also reported that peripheral blood mononuclear cells (PBMCs) from the patients express higher amounts of leptin compared with PBMCs from controls [72], and exogenous administration of leptin exacerbates the production of pro-inflammatory cytokines in these cells as compared with those from controls [72] (Fig. 4.1).

4.3 Adiponectin

Adiponectin, also known as GBP28, apM1, Acrp30, or AdipoQ, is a 244-residue protein with structural homology to types VIII and X collagen and complement factor C1q, whose major source is adipose tissue. Adiponectin circulates in the blood

in large amounts and constitutes approximately 0.01% of the total plasma proteins, and it is secreted from adipocytes as different molecular forms (trimers, hexamers, and also 12–18-monomer forms) [73, 74]. It increases fatty acid oxidation and reduces the synthesis of glucose in the liver and other tissues [73]. Ablation of the adiponectin gene has no dramatic effect on knockout mice on a normal diet, but when placed on a high-fat/high-sucrose diet, animals develop severe insulin resistance and exhibit lipid accumulation in muscles [75]. Circulating adiponectin levels tend to be low in morbidly obese patients and increase with weight loss and with the use of thiazolidinediones (insulin-sensitizing drugs) which enhance sensitivity to insulin [73, 76].

Adiponectin acts via two receptors, one (AdipoR1) found predominantly in skeletal muscle and the other (AdipoR2) in liver. Transduction of the adiponectin signal by AdipoR1 and AdipoR2 involves the activation of AMPK, PPAR-α, PPAR-γ, and other signaling molecules [73]. To note, targeted disruption of AdipoR1 and AdipoR2 causes abrogation of adiponectin binding and all its metabolic actions [77]. The gene that codes for human adiponectin is located on chromosome 3q27, a locus linked with susceptibility to diabetes and cardiovascular diseases [78].

4.3.1 Adiponectin and Atherosclerosis

Unlike most other adipokines, plasma levels of adiponectin are decreased in obesity and related pathologies, including type 2 diabetes and cardiovascular diseases [79]. Adiponectin possesses multiple healthy effects on obesity-related metabolic complications, dyslipidemia, nonalcoholic fatty liver disease, and several types of cancers [80]. It has been suggested that hypoadiponectinemia is an independent risk factor for hypertension [81] and has a detrimental effect on aortic stiffness [82]. Furthermore, subjects carrying the genetic variants that are related to lower plasma levels of adiponectin have a higher risk of hypertension [83, 84]. Several studies have shown that dyslipidemia is also associated with low circulating levels of adiponectin, even in the absence of other metabolic syndrome risk factors [85]. Hypoadiponectinemia has been linked to inflammatory atherosclerosis, suggesting that normal adiponectin levels are required to maintain a noninflammatory phenotype on the vascular wall [5].

Adiponectin might regulate many steps in the atherogenic process, such as anti-apoptotic actions on endothelial cells and angiogenic effects on the vasculature [86]. Antiatherosclerotic effects of adiponectin are exerted through multiple actions on several vascular cell types, such as cardiomyocytes, endothelial cells, and endothelial progenitor cells. Particularly, adiponectin inhibits neointimal formation by suppressing proliferation and migration of vascular smooth muscle cells [87–89], blocks inflammation and foam cell formation from macrophages [90, 91], and stimulates the production of the anti-inflammatory cytokine IL-10 and of IL-1 receptor antagonist (IL1Ra) by macrophages [92]. Adiponectin was also able to inhibit the production of reactive oxygen species (ROS) in cultured endothelial cells [93–95]. In addition to its effects on the vasculature, several

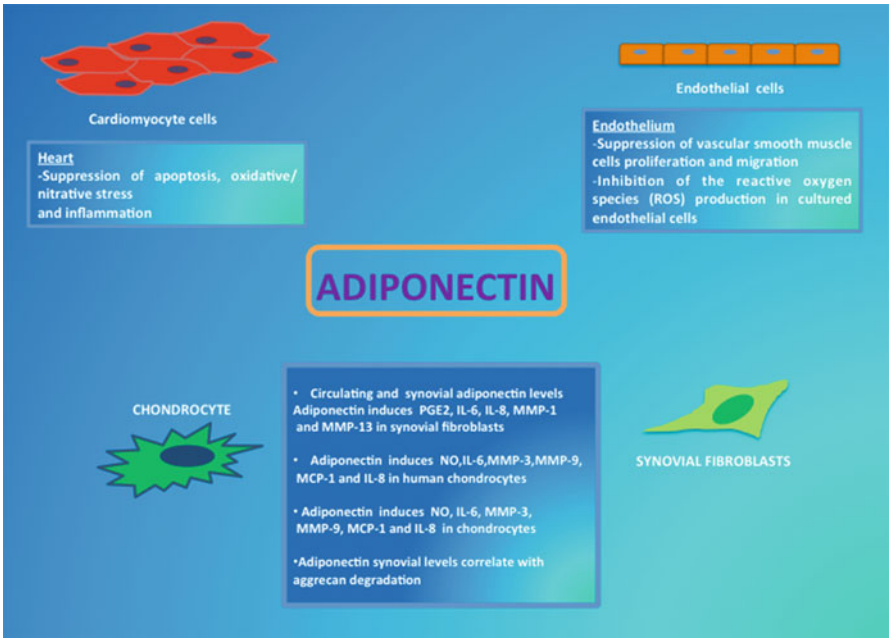

Fig. 4.2 Schematic representation of adiponectin implication in cardiomyocyte, endothelial, chondrocyte, and synovial fibroblast cells

studies in vitro and in vivo demonstrated that adiponectin acts directly on cardiomyocytes to protect the heart from ischemic injury, hypertrophy, cardiomyopathy, and systolic dysfunction [96]. In particular, the cardioprotective effects of adiponectin are attributed to its ability in suppressing apoptosis, oxidative/nitrative stress, and inflammation in cardiomyocytes [97]. Also, high plasma adiponectin levels are associated with a lower risk of myocardial infarction in men [98], a reduced coronary heart disease risk in patients with diabetes mellitus [99], and a lower risk of acute coronary syndrome [100] (Fig. 4.2). The globular isoform of adiponectin inhibits cell proliferation and production of ROS during atheromatous plaque formation [101]. In addition, the plasma adiponectin levels correlated inversely with systemic oxidative stress [102]. In general, adiponectin deficiency results in NO reduction in the vascular walls and promotes leukocyte adhesion, causing chronic vascular inflammation [103].

4.3.2 Adiponectin and Rheumatic Diseases

In contrast to its protective role in cardiovascular diseases and obesity, described above (Sect. 4.3.1), there is evidence supporting that adiponectin acts as a pro-inflammatory factor in the joints and it could be involved in matrix degradation.

Adiponectin levels have been found to be higher in RA patients than in healthy controls [30, 104–108]. Recently, it has been reported that adiponectin and adiponectin receptor 1 expression is higher in synovial fluids and the synovial tissues of patients with RA compared to controls, confirming the correlation of circulating adiponectin levels with the severity of RA [109]. In the patients under the anti-TNF-α agent infliximab therapy because of severe disease, high-grade inflammation was negatively correlated with circulating adiponectin concentrations; nonetheless, low adiponectin levels clustered with metabolic syndrome features such as dyslipidemia and high plasma glucose levels that have been reported to contribute to atherogenesis in RA [110]. However, the interaction of high-grade inflammation with low circulating adiponectin in RA is probably not mediated by TNF-α mediated in RA [110]. Besides, no association between adiponectin and carotid intima-media wall thickness, a surrogate marker of cardiovascular events in this disease [111], was observed in patients with RA [112]. In keeping with these negative results, no associations between functional adiponectin ADIPOQ rs266729 and ADIPOQ rs1501299 polymorphisms and cardiovascular disease were found in patients with RA [113].

Several studies supported the catabolic role for adiponectin. It has been reported that adiponectin is able to stimulate the production of PGE_2, IL-6, IL-8, vascular endothelial growth factor, and MMP-1 and MMP-13 in RA synovial fibroblasts [53, 114–116]. In addition, in cultured human chondrocytes and synovial fibroblasts, adiponectin induces the production of NO, IL-6, MMP-3, MMP-9, monocyte chemotactic protein 1 (MCP-1), and IL-8 [56, 117–119]. Adiponectin has a similar behavior in other cell types involved in RA, such as lymphocytes and human macrovascular endothelial cells. This adipokine promotes inflammation through increased TNF-α, IL-6, IL-8, and RANTES secretion by human primary lymphocytes. Moreover, it induces IL-6, IL-8, MCP-1, and RANTES secretion by human macrovascular endothelial cells [120, 121].

Concerning the role of adiponectin in SLE, several studies have showed elevated levels of this adipokine in patients with SLE [61, 64, 66]. Nevertheless, other authors did not find any difference in adiponectin levels between SLE patients and controls [63, 122]. However, the same authors found a positive correlation of leptin with vascular stiffness parameters, whereas adiponectin inversely correlates [123].

In the study by Rovin et al. [124], the authors showed that serum adiponectin levels are higher in patients with renal SLE than in healthy controls and in patients with nonrenal SLE. In addition, lower levels of adiponectin were present in SLE patients with insulin resistance (IR) compared to SLE subjects without IR [61]. It has also been reported that mice with experimental lupus that lack adiponectin develop more severe disease than wild-type mice, suggesting the involvement of adiponectin in regulating disease activity [125].

In addition, very recently, McMahon and colleagues have demonstrated that leptin levels confer increased risk of atherosclerosis in women with systemic lupus erythematosus and that there is no significant association between adiponectin and atherosclerotic plaques in SLE [126].

Little is known about the role of adiponectin in other rheumatic diseases, such as AS and Sjögren's syndrome. However, it has been reported that serum adiponectin

levels are not different between AS patients and healthy controls [69]. Regarding Sjögren's syndrome, it has been described that adiponectin is expressed in salivary gland epithelial cells, and this expression is higher in patients with Sjögren's syndrome [127]. Moreover, the same group demonstrated that adiponectin was able to protect salivary gland epithelial cells from spontaneous and IFN-γ-induced apoptosis [128] (Fig. 4.2).

4.4 Visfatin

Visfatin, also called pre-B-cell colony-enhancing factor (PBEF) and nicotinamide phosphoribosyltransferase (Nampt), is a protein of approximately 471 amino acids and 52 kDa [129]. It is a hormone that originally was discovered in liver, bone marrow, and muscle, but it is also secreted by visceral fat [129, 130].

It has been reported that visfatin is increased in obesity. Moreover, leucocytes from obese patients produce higher amounts of visfatin compared with lean subjects, and specifically, granulocytes and monocytes are the major producing cells [131, 132]. Macrophages have been described as a source for visfatin production too [133].

It is supposed that visfatin has insulin mimetic properties; however, the role of this adipokine in glucose metabolism is still unclear [130, 134]. Visfatin is upregulated in models of acute injury and sepsis [135], and its synthesis is regulated by other factors such as glucocorticoids, TNF-α, IL-6, and growth hormone (GH). Moreover, visfatin has been shown to induce chemotaxis and the production of IL-1β, TNF-α, and IL-6 in lymphocytes [138].

4.4.1 Visfatin and Atherosclerosis

The role played by visfatin in atherosclerosis is still unclear, but some studies recognize the involvement of this adipokine in atherosclerotic processes. In patients with metabolic syndrome, serum visfatin concentrations were higher if they had carotid atherosclerotic plaques compared to those without [136]. Moreover, visfatin expression was found to be increased in symptomatic plaques, while asymptomatic plaques presented lower visfatin expression [137]. Recently, it has been described that visfatin pericoronary fat expression was positively correlated with coronary atherosclerosis [138]; in addition, CRP and the atherogenic small dense low-density lipoprotein subclasses (sdLDL-C) levels were increased in individuals with higher visfatin levels [139]. All of these data suggest that visfatin develops certain actions in the progression of atherosclerosis, probably related to the fact that visfatin acts as an inflammatory mediator.

In vitro experiments support a pro-inflammatory role of visfatin. This adipokine induces MCP-1 expression in human endothelial cells via NF-κB and

phosphatidylinositol 3-kinase [140]. In line with this, macrophage foam cells from coronary atherosclerotic lesions produce visfatin, and this is able to enhance inflammatory factors synthesis such as IL-8, TNF-α, or MMP-9 in the monocytic cell line THP-1 and in PBMCs [137]. These results indicate strong pro-inflammatory effects of visfatin, which could be related with atherogenesis and plaque destabilization.

Another study reveals that visfatin could improve endothelial function by increasing eNOS expression [141].

4.4.2 Visfatin and Rheumatic Diseases

Serum visfatin levels were also increased in patients with RA compared with healthy controls [30, 106, 142]. This adipokine has important pro-inflammatory and catabolic roles in RA pathogenesis, and it is now being intensively studied as a potential target in this illness. In fact, serum and synovial visfatin concentrations were associated with the degree of inflammation, with clinical disease activity, and with increased radiographic joint damage [106, 143, 144]. However, in a study which included RA patients with severe disease undergoing anti-TNF-α infliximab therapy, visfatin levels were not associated with inflammation or metabolic syndrome and infliximab infusion did not show significant changes in visfatin levels [145]. Another study showed that prolonged anti-TNF-α treatment may reduce visfatin levels [145, 146]. Brentano et al. reported an interesting study, in which high levels of visfatin were observed in the synovial lining layer and at sites of cartilage loss [143]. In addition, the authors demonstrated that visfatin induced IL-6, IL-8, MMP-1, and MMP-3 production in RA synovial fibroblasts as well as IL-6 and TNF-α in monocytes. Notably, visfatin knockdown in RA synovial fibroblasts significantly reduced the synthesis of IL-6, IL-8, MMP-1, and MMP-3 [143].

Other authors identified visfatin as a key component of the inflammatory processes leading to arthritis, since visfatin inhibition significantly reduced inflammation, cartilage damage, and the severity of arthritis in a collagen-induced arthritis animal model [147]. Moreover, the inhibition of this adipokine reduced the circulating levels of TNF-α [147]. Anyway, the mechanisms by which visfatin exerts its pro-inflammatory and catabolic functions in the arthritic joint are not well understood; therefore, the use of visfatin as a therapeutic target needs to be studied more in depth.

Visfatin is encoded by the NAMPT gene. Studies on the potential influence of functional NAMPT gene polymorphisms in the risk of cardiovascular disease of RA were conducted. However, no significant association of NAMPT rs9770242 and rs59744560 polymorphisms with disease susceptibility and cardiovascular risk in patients with RA was observed [148].

At cartilage level, human OA chondrocytes produce visfatin, and similar to IL-1β, visfatin is able to enhance the synthesis of PGE_2 [149]. This adipokine also

increases the expression of ADAMTS 4, ADAMTS5, MMP-3, and MMP-13, which are very relevant cartilage degradative enzymes [149]. To note, OA patients had higher synovial fluid visfatin concentrations, which are correlated with degradation biomarkers such as collagen type II and aggrecan [150]. Taken together, these data indicate that visfatin develop catabolic functions at cartilage level and it could play an important role in the pathophysiology of OA.

Studies performed in SLE and AS patients present conflicting results. Some of them showed higher visfatin levels in patients with SLE patients than in healthy controls [64], but others did not find any variation between patients and controls [151]. Similarly, there was no association between visfatin levels and disease activity in both SLE and AS [68, 151].

4.5 Resistin

Resistin, known as adipocyte-secreted factor (ADSF) or found in inflammatory zone 3 (FIZZ3), was discovered in 2001 and was proposed as potential link between obesity and diabetes [152]. It is secreted by adipose tissue but has been found also in macrophages, neutrophils, and other cell types. Serum resistin levels increase with obesity in mice, rats, and humans [153, 154]. Resistin belongs to a family of resistin-like molecules (RLM) with distinct expression patterns and biological effects [155].

In animal models, resistin promotes insulin resistance, while the evidence for this effect in humans is less clear [152, 156]. Also, it was observed that resistin production is restricted to adipocytes in mice, while in humans, it is mainly derived from circulating monocytes and macrophages [157].

4.5.1 Resistin and Atherosclerosis

Increasing evidence indicates that resistin might play important regulatory roles apart from its role in insulin resistance and diabetes, in a variety of biological processes such as atherosclerosis and cardiovascular diseases. Several studies suggested that resistin was involved in pathological processes leading to CVD including inflammation, endothelial dysfunction, thrombosis, angiogenesis, and smooth muscle cell dysfunction [158, 159]. Several studies have showed that CVD is accompanied by changes in serum resistin levels [160]. Moreover, a similar study demonstrated a significant increase in plasma resistin levels in patients with unstable angina when compared with patients with stable angina or control patients [161]. Resistin levels were elevated in ACS, which has been hypothesized to be due to release of resistin from atherosclerotic plaque during plaque rupture [162]. In addition, the group of Jung has showed that macrophages infiltrating atherosclerotic aneurysms were able to secrete resistin, which, in turn, affects endothelial function and vascular smooth muscle cell migration, thus contributing to atherogenesis [163]. Resistin also might be involved in the maintenance of epithelial cell barrier function, a physical barrier

between blood and the arterial wall. In fact, it has been reported that high concentrations of resistin generated in conditional media from epicardial adipose tissue of patients with ACS increase endothelial cell permeability [164]. Very recently, a novel role of resistin has been described in modulating serum low-density lipoproteins and, thereby, atherosclerotic CVDs in obese humans [165].

4.5.2 Resistin and Rheumatic Diseases

There are several demonstrations that resistin may be involved in the pathogenesis of RA. Increased levels of this adipokine have been previously observed in synovial fluid from patients of RA compared to patients with noninflammatory rheumatic disorders [104]. Resistin may be a significant mediator in the inflammatory process of RA. In fact, serum resistin levels are associated with disease activity and acute phase reactants, including CRP and IL-1Ra antagonizing IL-1β [166, 167]. However, resistin-RTN rs1862513 polymorphism was not found to be a genetic risk factor either for clinically evident cardiovascular disease or subclinical atherosclerosis in a large series of patients with RA [168].

Resistin has been found in the plasma and synovial fluid from RA patients, and injection of resistin into mice joints induces an arthritis-like condition, with leukocyte infiltration of synovial tissues, hypertrophy of the synovial layer, and pannus formation [167, 169]. Bokarewa et al. have showed that resistin induces and is induced by several pro-inflammatory cytokines, such as TNF-α or IL-6, in peripheral blood mononuclear cells, via nuclear factor-κB pathway, indicating that resistin can increase its own activity by a positive feedback mechanism [169]. This group has recently demonstrated that resistin utilizes IGF-1R pathway in RA synovium [170].

Increased serum resistin in patients with rheumatoid arthritis correlated with both CRP and DAS28, suggesting a role of this adipokine in the pathogenesis of rheumatoid arthritis [167]. Gonzalez-Gay et al. have confirmed this association between laboratory markers of inflammation, particularly CRP and resistin levels, and have showed that anti-TNF-alpha therapy results in a rapid reduction of serum resistin levels in patients with RA [171].

Recent experimental data suggest that resistin, in the presence of dendritic cells, might induce the expansion of functional regulatory T cells [172].

The pro-inflammatory profile of resistin, together with its association with obesity, suggests that this adipokine might be another potential mediator that links OA with inflammation and obesity.

In addition, resistin has a role as a marker of inflammation in other rheumatic diseases, such as SLE [173]. In fact, Almehed et al. have demonstrated a positive correlation between serum resistin levels, inflammation, bone mineral density, and renal functions in patients with SLE [174].

In a very recent study, higher serum resistin levels have been reported in patients with AS compared to healthy subjects giving clues that resistin could have also a role in the pathogenesis of AS [175].

4.6 Conclusions

Adipose tissue-derived factors, called adipokines, are now being considered to play multiple and relevant roles in the body, including a complex adipokine-mediated interaction among white adipose tissue, cardiovascular disorders, and rheumatic diseases. The chronic increase of the inflammatory tone is generally associated with an increased risk for the development of cardiovascular diseases, and the pro-inflammatory environment presented in rheumatic diseases contributes to the increase of severe cardiovascular disorders. In addition, the inflammatory functions exerted by adipokines in certain rheumatic diseases could explain some of their associated cardiovascular comorbidities, suggesting a potential therapeutic role for these molecules.

Anyway, the main causes of abnormal fat mass accumulation and adipokine dysfunction are bad nutritional and lifestyle habits, such as overeating and physical inactivity. Therefore, the first therapeutic approach for cardiovascular disorders in rheumatic diseases should be the correction of these bad habits.

In summary, modification in the lifestyle, as well as other therapeutic interventions leading to reduce fat mass, and its associated dysfunction, might improve cardiovascular mortality in patients with rheumatic diseases.

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Chapter 5
Regulation of Cartilage and Bone Metabolism by Oxidative Stress

Anja Niehoff and Christoph Ziskoven

Abstract The overproduction of reactive oxygen species (ROS) causes an imbalance between pro-oxidants and antioxidants in cells and the extracellular matrix (ECM) resulting in oxidative stress, which has been linked to ageing and a number of pathological conditions. In recent years, a pathomechanism involving ROS was also implicated in arthritis and degenerative joint diseases. Osteoarthritis (OA) is the most common form of joint degeneration and affects all joint structures including cartilage, subchondral bone, synovial joint lining and adjacent supporting connective tissue. Oxidative stress influences the cartilage metabolism by disrupting fundamental cellular processes that control apoptosis, senescence, differentiation, survival, growth and protein synthesis, and this stress leads to an imbalance in matrix synthesis and degradation resulting in the functional failure of the joint. A role of oxidative stress in the regulation of subchondral bone metabolism was speculated, and earlier studies support this hypothesis. Oxidative stress in cartilage tissue has also been shown to affect the metabolism of the underlying subchondral bone either direct or indirectly. The influence of ROS and reactive nitrogen species (RNS) on the function of chondrocytes and bone cells is very complex, and mechanical loading of the joint may also be involved in the pro-oxidant–antioxidant homeostasis in the different tissues of a joint.

Abbreviations

AGEs	Advanced glycation end products
ASK	Apoptosis signal-regulating kinase
BMU	Bone multicellular unit
ECM	Extracellular matrix

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IGF	Insulin-like growth factor
IL	Interleukin
iNOS	Inducible NOS
JNK	c-Jun N-terminal kinase
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteinase
MRI	Magnetic resonance imaging
NAC	*N*-acetylcysteine
NF-κB	Nuclear factor κB
nNOS	Neuronal NOS
NO	Nitric oxide
NOS	Nitric oxide synthase
OA	Osteoarthritis
OANOS	Osteoarthritis-affected NOS
OSF	Osteoblasts-stimulating factor
PTH	Parathyroid hormone
PTH-R	Parathyroid hormone receptor
PTHrP	Parathyroid hormone-related peptide
RA	Rheumatoid arthritis
RAGE	Receptors for advanced glycation end products
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TGFβ	Transforming growth factor-β
TNFα	Tumour necrosis factor-α
VEGF	Vascular endothelial growth factor

5.1 Introduction

The increased intrinsic production or increased external exposure to reactive oxygen species (ROS) causes an imbalance between pro-oxidants and antioxidants in cells and the extracellular matrix (ECM), resulting in oxidative stress . Increased oxidative stress has been linked to ageing [1, 2] and a number of pathological conditions including neoplasias [3–5], neurodegenerative diseases [6–11] and cardiovascular diseases [12–14]. In recent years, a pathomechanism involving ROS has also been proposed for several degenerative diseases of the musculoskeletal system. Here, an imbalance between oxidants and antioxidants has been related to arthritis and joint diseases [15, 16]. Osteoarthritis (OA) is the most common form of arthritis and is considered as the uniform end state of numerous pathologic conditions of the joint such as incongruence, anatomic deformities and mechanical overload. Within ageing, a number of patients develop so-called ‘primary’ OA without a definable underlying pathology. The incidence and extent of OA is influenced by multifactorial inheritance [17]. Oxidative stress has been related to cartilage degeneration by its influence on the homeostasis of the synthesis and degradation of ECM

proteins [18]. However, OA has been identified not only as a disease of articular cartilage but also one that affects all joint structures including subchondral bone, synovial joint lining and adjacent supporting connective tissue [19]. For example, cartilage degeneration in OA is associated with subchondral bone sclerosis and formation of osteophytes [19, 20]. Previous studies have demonstrated that ROS and reactive nitrogen species (RNS) are involved in both cartilage and bone metabolism by having an essential influence on the cell function [18, 21, 22], but little information exists on the role of oxidative stress on the metabolism of periarticular bone in OA.

Cartilage is an avascular, aneural and alymphatic tissue. Only a small quantity of total tissue volume that amounts to about 5% consists of tissue-specific cells, mainly chondrocytes [23]. In contrast, the relative cell volume in bone is much higher. The essential cell types for regulation of bone growth and maintenance are osteoblasts, osteoclasts and osteocytes. The influence of ROS and RNS on the function of these different cells during arthritis and joint diseases seems to be very complex. The objective of this chapter is to summarise the actual findings on the regulation of cartilage and bone metabolism by oxidative stress in relation to arthritis and degenerative joint diseases.

5.2 Cartilage Metabolism and Oxidative Stress

The important role of cartilage degradation in the pathogenesis of degenerative and inflammatory joint diseases is clear. Here, recent data elucidate on the roles of oxidative and nitrosative stress in addition to other factors in the pathogenesis of OA [24], as well as in the immune pathogenesis of rheumatoid arthritis (RA) [25]. As evidence suggesting the involvement of free radicals in chondrocyte function impairment increases, the contribution of oxidative imbalance in degenerative joint diseases has become the focus of more detailed investigation [18]. ROS can directly damage nuclear DNA, intracellular structures and the extracellular matrix of chondrocytes [26]. In the healthy joint, the rate of ROS production and clearance by scavenging mechanisms tends to maintain a stable state known as redox homeostasis. If an increase in ROS level occurs, the resulting antioxidative response is sufficient to reset the original balance in healthy tissue. If this fails because of a mismatch between the presence of higher free radical levels and the antioxidative capacity of tissue, a quasi-stable state will develop, adjusted to the presence of higher levels of oxidative agents [27]. Yet, the consequences of an increased oxidative burden resulting in a more oxidative state are not limited to pathological conditions; increased oxidative burdens have been reported to be associated with the physiological process of ageing. In pathological conditions, the perpetual generation and elimination of ROS can come out of equilibrium, leading to oxidative imbalance by two major mechanisms. First, imbalance occurs when antioxidative capacity is depleted. The resulting more oxidative intracellular state causes pathological alterations in osteoarthritic cartilage [21]. Second, a rise of the free radical burden that exceeds the capacity of free radical elimination of tissue can result in oxidative

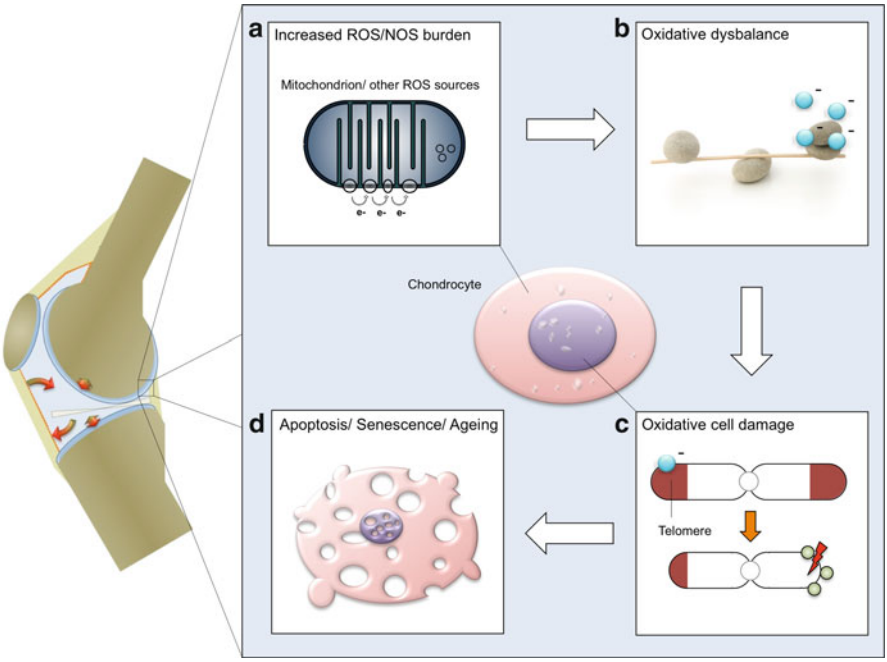

Fig. 5.1 In joints, increased ROS burden from intrinsic and extrinsic sources (**a**) leads to oxidative imbalance (**b**). The intracellular damage in chondrocytes (**c**) by telomere length shortening and protein damage leads to impaired cell function and eventual apoptosis (**d**)

imbalance and concomitant impairment of cell function. Cellular senescence, apoptosis and the effects of ageing promoted by ROS/RNS activity threaten chondrocyte survival and impair the ability of chondrocytes to maintain the structural integrity of the joint [28–30] (Fig. 5.1).

Yet, the increasing knowledge about the involvement of ROS and RNS in tissue homeostasis indicates that free radicals have, in addition to their adverse effects, many important features for cell function. The differential role of ROS and RNS in joint and cartilage metabolism becomes obvious because ROS produced by oxidative metabolic turnover in cartilage tissue are a substantial factor in the maintenance of intracellular signalling [31, 32]. The involvement of free radicals in signal transduction supports the hypothesis that during evolution, higher life forms took advantage of the ability of ROS molecules to mediate signals across cell boundaries in systems consisting of discrete compartments, such as the joint [31, 33].

In particular, the controlled cell death by free radical damage-induced apoptosis represents a beneficial free radical-mediated mechanism, which provides a strategy to ensure the survival of the organism by scavenging damaged cells. Cysteine–aspartic-acid proteases (caspase) activation initiates the apoptosis cascade, a process that was for a long time thought to be irreversible once started. Free radical species promote apoptosis [28] by the activation of c-Jun N-terminal kinases (JNK) and p38 mitogen-activated protein (MAP) kinases, via apoptosis signal-regulating

kinase (ASK) 1 [34]. Interestingly, free radicals act as second messengers within this pathway that amplify ASK1 activation, which leads to cell death even at ROS concentrations not normally sufficient to initiate apoptosis [35]. Exogenous and endogenous nitric oxide (NO), in addition to other stimuli, have been shown to induce chondrocyte apoptosis through intracellular induction of ROS [29]. This cytotoxic effect is a result of an increased ROS level and the formation of cytotoxic peroxynitrite by direct reaction of NO and ROS [36, 37]. In contrast, chondrocyte apoptosis induced by toxins can be suppressed by hypoxia, which shifts chondrocytes to a less oxidative intracellular state [38]. A recent study showed the anti-apoptotic effects of *N*-acetylcysteine (NAC) in isolated chondrocytes [39].

Linear chromosomes are capped by repetitive nucleoprotein structures called telomeres, and each cell division results in the progressive shortening of telomeres [40] that, below a certain threshold, promotes genome instability, cell senescence and apoptosis. Telomeres are highly sensitive to ROS-induced instability due to their high guanine content, which leads to faster telomere length reduction during oxidative stress [41]. Interestingly, evidence exists that cell senescence can also be induced by extrinsic factors in chondrocytes, which is known as so-called free radical-induced 'stress senescence' [42]. In fact, irreversible telomere length shortening was proposed as a specific marker of chronic exposure to oxidative stress [26, 43]. These facts imply a connection between oxidative stress and joint disease. Still, to date, data on the impact of telomere length shortening under in vivo conditions, in tissues other than the hematopoietic system, are limited.

In addition, a recent study showed that mitochondria are not only a major source of free radicals but are also a target of oxidative damage themselves. Disruption of chondrocyte respiration by NO-induced inhibition of mitochondrial electron transport has been proposed to be centrally involved in chondrocyte functional compromise [44]. Mitochondrial DNA damage mediated by RNS via tumour necrosis factor-α (TNF-α) and interleukin (IL)-1 has been hypothesised to be an important factor involved in chondrocyte functional compromise and induction of apoptosis [45].

5.2.1 Free Radicals in RA

RA is a chronic autoimmune disease characterised by joint inflammation and following impairment of structural integrity of the affected joint. In RA, numerous pathogenic mechanisms have been identified that are related to the ROS/RNS action. In RA pathogenesis, modification of native proteins by ROS/RNS action results in more effective binding of autoantibodies. The generation of neo-antigens by oxidative stress that triggers the autoimmune dysregulation is thought to be a central mechanism in RA pathophysiology [46, 47]. Within RA, chemokine secretion by resident synoviocytes of the synovial lining that occurs along with expansion of the synovial membrane attracts inflammatory cells [48–50]. Infiltration and persistence of immune cells through support from cytokines, chemokines, angiogenic factors and antigen-presenting cells [48] in synovial tissue lead to local

hyper-cellularity with the concomitant increase in the production of pro-inflammatory cytokines and increased production of ROS/RNS. The increased formation of ROS/RNS in combination with the release of proteolytic enzymes by recruited immune cells in primary inflammatory joint diseases is a possible pathomechanism for cartilage and bone destruction resulting joint deformity [51, 52].

Nutrition for articular cartilage, which is avascular tissue containing chondrocytes embedded into a large volume of extracellular matrix, depends on diffusion from synovial and subchondral bone compartments [53]. Thus, oxygen and metabolic end products must diffuse over relatively long distances, resulting in low O_2 tension in cartilage tissue [54]. Despite synovial hypervascularity, the arthritic joint shows hypoxia-induced biochemical alterations due to an increased metabolic rate, increased intraarticular pressure due to joint effusion and relatively reduced capillary density [55, 56]. A limitation of the effect of ROS and RNS is the very short life span of these highly reactive molecules. Nevertheless, the dependency on O_2 tissue concentration, at least of NO life span, is described, allowing for a life span up to 2 s in avascular tissues. Conditions of lowered oxygenation, as in cartilage and especially in primary inflammatory disease, enhance and prolong the bioactivity of NO [57].

The induction of NO production by chondrocytes after exposure to cytokines involved in inflammatory response like IL-1 and TNF-α was proven in the early 1990s [58, 59]. An NO-dependent increase in lactate production of chondrocytes after IL-1 exposure proved the efficacy of intracellularly released NO as a signal transduction molecule in chondrocytes [60]. In contrast to the uncoupling effects mediated by ROS in synovium inflammation [21], ROS have been shown to down-regulate expression of pro-inflammatory genes in chondrocytes [61]. NO is released, along with several pro-inflammatory factors, by synovial cells as a result of an inflammatory transition [62]. Nuclear transcription factor κB (NF-κB), which is involved in the upregulation of several inflammatory genes [63], is an important factor in ROS-/RNS-induced inflammatory transitions in synovial membranes.

Furthermore, the involvement of ROS in the regulation of haemapoietic cell function came into focus regarding RA disease development. The role of altered presentation of ROS-modified proteins by anti-gene presenting cells in RA is currently considered contradictory [64–66]. Monocytes and macrophages contribute directly to important pathophysiological features of RA such as synovial inflammation, hyperplasia of the synovial lining and destruction of bone and cartilage. In addition, they serve as the main source of pro-inflammatory TNF-α and IL-1β [67].

5.2.2 Free Radicals in Osteoarthritis

Although the heterogeneity of OA is associated with a variety of etiopathogenic factors, a common thread is emerging regarding the uniform presentation of end-stage OA [68, 69].

Chondrocytic death in OA has been examined in various studies, most of which report increased apoptotic cell death [70]. With respect to intracellular modifications

caused by free ROS, accumulation of cartilage matrix proteins in the endoplasmic reticulum and Golgi apparatus, in chondrocytes modified by oxidant stress during ageing, has been proposed as a cause of decreased cartilage matrix proteins synthesis and eventual chondrocyte apoptosis in degenerative joint diseases [71]. A major role in OA development is attributed to the effects of ageing, since OA shows a clearly age-dependent incidence. Because of their slow metabolism and low proliferation rate, cartilage tissue chondrocytes and cartilage extracellular matrix collagens lack the turnover displayed by most tissues. Therefore, cartilage is highly sensitive to the cumulative effects of extrinsic factors such as oxidative stress [72]. In fact, evidence exists that nonenzymatic glycated and oxidised proteins so-called advanced glycation end products (AGEs) accumulate in joint cartilage [73, 74]. The production of AGEs is elevated in diabetic individuals [75]. The influence of AGE accumulation on chondrocyte function is mediated by specific receptors for advanced glycation end products (RAGE), which are located on chondrocyte membranes. Expression of these receptors is increased during ageing and OA development [76], and receptor stimulation leads to the increased expression of matrix metalloproteinases (MMPs), resulting in increased catabolic function in chondrocytes [77]. Intracellular messaging mediated by RAGE was shown to be ROS dependent soon after their discovery, and RAGE activation, in turn, induces oxidative stress [78], illustrating once more the involvement of oxidative stress in pathological alterations leading to joint degradation.

As OA progresses, differential roles of ROS and especially RNS have become apparent. In a study comparing the effects of NO and oxidative imbalance in OA and RA, NO was hypothesised to play a major role in the modulation of chondrocyte function in OA [79].

With respect to radical nitrosative species, NO has been the subject of extensive research. Interestingly, several nitric oxide synthase (NOS) isoforms have been identified in OA joint tissue. Furthermore, the functional relevance of constitutively expressed neuronal NOS (nNOS) [80] and inducible NOS (iNOS) [81] in OA pathogenesis has been demonstrated. Moreover, an osteoarthritis-affected NOS (OANOS), which is only detectable in OA cartilage, has been described [82] and displays properties similar to nNOS and iNOS. Evidence for NOS-mediated signalling in OA was detected in cartilage [83] and the synovium [84], in which a relative deficit in the production of natural IL-1 receptor antagonists has been demonstrated, and this signalling could possibly be related to excess production of NO in OA tissues [85]. In cartilage tissue, chondrocyte IL-1-converting enzyme and IL-18 levels have been shown to be mediated by NO [86, 87]. Other studies have demonstrated that proinflammatory mediators, such as IL-1, in turn give rise to NO formation in inflamed joints [59, 88]. The correlation between NO production and upregulation of iNOS is a well-known feature of IL-1 [89], while NO is also suspected to play a major role in the influence of mitochondrial function in OA.

Patients suffering from OA often show a discrepancy between objective findings gathered in X-ray or magnetic resonance imaging (MRI) examinations and patient-reported pain and functional restrictions [90]. This sudden onset of disabling joint pain and articular effusion is a typical clinical feature of decompensation and is

known as activated OA. On a cellular and subcellular level, pro-inflammatory agents, such as NO, IL-1 and TNF-α, are overexpressed in chondrocytes and joint stromal cells in OA [91]. Recently, NO-dependent intracellular signalling in OA nociception has been the subject of investigation [92].

In summary, the investigation of the cellular action of ROS and RNS gives new insights into the pathophysiology of RA and OA. The clinical application of this knowledge to date is limited. Few therapeutic approaches found their way into clinical practise, mainly via OA therapy. Reviewing the clinical study situation in the appropriate area of application, reliable data regarding the effectiveness in pain reduction and functional improvement in OA derived from controlled double-blinded clinical trials exist only for avocado/soybean unsaponifiables [93]. The effectiveness of n-3 polyunsaturated fatty acids, collagen hydrolysates and vitamins via ROS/RNS modification in symptomatic OA was postulated. Yet, there is no proper evidence for clinical influence of administration of these medications on pain and function. Therefore, the clinical application is not recommendable to date [93]. Further investigation of the pathophysiology of RA and OA is necessary to gain an improved understanding of RNS and ROS action.

5.3 Subchondral Bone Metabolism and Oxidative Stress

The subchondral bone and the overlying articular cartilage form a functional unit to withstand the mechanical requirements acting on a joint [94]. Strong evidence exists that the subchondral bone is involved in the pathogenesis of OA [19, 95–98]. Several studies have demonstrated that in OA, the subchondral bone becomes sclerotic and hypomineralised with a lower stiffness [19, 20, 96, 99, 100]. Furthermore, bone cysts develop and osteophytes are formed at the joint margins [95, 101, 102]. However, it is not clear which molecular mechanisms account for these alterations in the subchondral bone and if these changes precede or are a consequence of cartilage degeneration [95, 103–109].

Bone is a dynamic tissue and continuously underlies a remodelling process. Under healthy conditions, a balance exists between bone formation and resorption. This remodelling is essential for bone adaptation in response to the mechanical stimuli of the environment to adjust tissue function, and it influences both bone mineral density and strength. Bone consists of 90–95% osteocytes, 5% osteoblasts and less than 1% osteoclasts [110], with osteoblasts and osteoclasts working tightly together in a coupled functional unit called the bone multicellular unit (BMU) [111]. A disturbance in the BMU function can lead either to a loss or a gain of bone tissue. The osteocytes orchestrate the bone modelling and remodelling process by sensing the magnitude and distribution of strain in bone and responding to it [112, 113]. They control and regulate bone mineral homeostasis, detect microdamage and regulate both osteoblast and osteoclast activities [110, 114–116].

It has been proposed that an accelerated bone remodelling process is the reason for alterations observed in subchondral bone during the progression of OA [96, 117].

The increased rate of bone remodelling is associated with the accumulation of osteoid and decreased mineralisation [118]. Growing evidence from recent studies suggests that the function of subchondral osteoblasts may be perturbed in OA. The osteoblasts from the sclerotic zones of human osteoarthritic subchondral bone showed a phenotype with increased synthesis of matrix proteins, growth factors and cytokines. Thus, increased levels of osteocalcin, osteopontin, C-terminal type I procollagen propeptide, MMP-13, IL-1β, IL-6, IL-8, transforming growth factor (TGF)-β1, vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF)-1 and urokinase plasminogen activator have been demonstrated in sclerotic osteoblasts. In addition, the increased activity of alkaline phosphatase and transglutaminases was detected indicating a decreased capacity for matrix mineralisation [119–122]. Furthermore, an abnormal collagen I metabolism of subchondral cancellous bone was found in OA [123], whereas the quality of the collagen is modified by the formation of type I homotrimer α1 [124]. A recent study showed that the elevated TGF-β1 level partially accounts for the abnormal ratio of COL1A1 and COL1A2 and for the irregular production of collagen I, which lead to a hypomineralised bone matrix [118]. In addition, OA subchondral osteoblasts are partially resistant to parathyroid hormone (PTH) stimulation [122], which could also explain the abnormal bone remodelling. However, it is not completely understood whether these alterations in subchondral bone remodelling are initiated through abnormal osteoblast metabolism or systemic regulation.

There have been two major mechanisms for the changes in subchondral OA bone suggested. First, they are induced either through the higher mechanical loading of the subchondral plate due to the loss of articular cartilage. Second, bone sclerosis preceding cartilage degeneration may enhance bone remodelling by abnormal OA bone cells that initiate events that trigger cartilage damage [98, 125]. However, besides these mechanisms, oxidative stress may also contribute to the imbalance in the homeostasis of the subchondral bone metabolism in OA. Strong evidence comes from osteoporosis research demonstrating that oxidative stress can affect bone metabolism [126–128]. Both ROS and RNS play an important role in bone homeostasis with respect to the differentiation and survival of osteoblasts, osteoclasts and osteocytes [129]. For a long time, an oestrogen-dependent mechanism has been assumed to be involved in the pathogenesis of osteoporosis, but research in recent decades indicates that age-related mechanisms, such as oxidative stress, are contributory [114, 129, 130]. The loss of sex steroids accelerates the effect of bone ageing and decreases the defence against oxidative stress [114]. Hence, oxidative stress seems to influence osteoblast–osteoclast interaction [131], because overproduction of ROS by osteoclasts can destabilise the balance between osteoclasts and osteoblasts activities of the BMU resulting in bone reduction [127]. Furthermore, the superoxide anion can stimulate the formation and differentiation of osteoclasts [132]. Ageing leads to a decrease in osteocyte numbers that is associated with an increase in bone remodelling and a disruption of several signals in bone metabolism [114, 130]. Both ageing and the associated changes in hormone levels promote osteocyte apoptosis. Recently, it has been demonstrated that bone and bone cells, similar to other tissues, possess a system of enzymatic and nonenzymatic antioxidants to

protect themselves against oxidative stress and to re-establish redox homeostasis [133]. Postmenopausal loss of oestrogen initially leads to a reduction of the levels of glutathione and thioredoxin in bone [126]. Interestingly, the increase in osteocyte apoptosis in ovariectomised mice is prevented when treated with antioxidants [130]. The fact that oxidative stress has an important effect on bone metabolism in osteoporosis indicates that ROS and RNS may also be involved in altered bone remodelling in OA. However, little information exists regarding the regulation of subchondral bone metabolism by oxidative stress. Increased nitrotyrosine levels were detected in the subchondral bone of osteoarthritic equine metacarpophalangeal joints compared to normal healthy joints, which may indicate that oxidative stress in the subchondral bone is related to OA [134]. Furthermore, other findings reveal that NO has the potential to be an important mediator of the subchondral bone changes in OA, because treatment with a NO donor increased subchondral bone sclerosis during subchondral remodelling following ovine meniscectomy [135]. Treatment with avocado/soybean unsaponifiables decreased the level of inducible nitric oxide synthase in cartilage accompanied by a reduced loss of subchondral bone volume and calcified cartilage thickness in experimental canine OA [136]. These first studies strongly indicated that oxidative and nitrosative stress are most likely involved in abnormal subchondral bone metabolism. However, further research is necessary to unravel the exact mechanisms by which oxidative stress can influence the metabolism of subchondral bone in OA.

Interestingly, articular cartilage and subchondral bone are not only a mechanical functional unit but also a biological functional unit. Previous studies have demonstrated that subchondral bone cells can influence chondrocyte metabolism [137–139]. Osteoblasts secrete molecules that could affect the chondrocyte in the overlying cartilage through microcracks or blood vessels in the calcified layer of articular cartilage [95]. Such morphological alterations of the joint in OA are believed to enhance the capacity for a crosstalk between subchondral bone and articular cartilage [140]. Recently, it was found that cells derived from OA bone in human hip and knee joints changed the chondrocyte metabolism by increasing the glycosaminoglycan release from cartilage [139]. Furthermore, osteoblasts from sclerotic zones of human OA subchondral bone induced a significant inhibition of aggrecan gene expression and production and an increase in MMP-3 and MMP-13 gene expression by chondrocytes in alginate beads [137]. In addition, the subchondral osteoblasts from sclerotic zones decreased the SOX9, collagen II, parathyroid hormone-related peptide (PTHrP) and parathyroid hormone receptor (PTH-R) gene expression in chondrocytes but increased that of osteoblasts-stimulating factor (OSF)-1 [138]. Because both oxidative and nitrosative stress have been shown to play an important role in articular cartilage in OA [18], it is likely that through an imbalance in pro-oxidants and antioxidants, the affected chondrocyte metabolism can influence bone cell metabolism. It has been demonstrated that the diffusion of several molecules into OA articular cartilage is possible from the synovium as well as through the calcified cartilage from the subchondral bone because of the enhanced permeability of the bone–cartilage unit through angiogenesis and microcracks [94, 141, 142]. However, it is not known if oxidative

stress in articular cartilage could also affect the metabolism of subchondral bone cells either directly or indirectly. Much more research needs to be performed to gain insight into this speculation.

Although a few indications exist that oxidative stress may have an effect on bone metabolism and seems to play a role in the pathomechanism of OA, more evidence is necessary to establish the validity of this hypothesis.

5.4 Effects of Mechanical Loading on Cartilage and Bone Oxidative Stress

The morphology and composition of healthy articular cartilage is optimised to ensure its mechanical functions and displays profound viscoelastic properties under pressure and shear stress. These biomechanical properties of cartilage are based on the composition of the matrix, which consists mostly of collagen type II and the proteoglycan aggrecan [143, 144]. Articular cartilage is stable under both permanent compression and shear stress, conditions that would be harmful for most other tissues [145]. Mechanical loading is even essential for normal cartilage metabolism and maintenance [146–149]. Unloading of rat hind limbs resulted in a reversible increase of NO production and consecutive chondrocyte apoptosis [150].

Evidence exists that mechanical loading through physical activity also has an influence on cellular redox homeostasis [151–154]. It has been suggested that prolonged strenuous exercise disturbs pro-oxidant–antioxidant homeostasis resulting in oxidative stress [153, 155]. For example, elevated levels of oxidative stress in muscle tissue have been shown after strenuous exercise [153, 155]. In support of this finding, a recent study demonstrated that running exercise increased oxidative stress in the cartilage of a heterozygous superoxide dismutase (SOD)2-deficient mice ($SOD2^{+/-}$) model [156]. Furthermore, some in vitro studies have found that extreme mechanical loading leads to an increase of oxidative stress in chondrocytes [157, 158]. Excessive repetitive loads have been related to chondrocyte apoptosis, highlighting the relevance of injurious cartilage compression in OA pathogenesis [159, 160]. In fact, mechanical loading that exceeds the tolerance of the articular surface was found to indirectly cause oxidative stress by decreasing the rate of cell death following administration of antioxidants [161, 162]. Furthermore, some data indicate that RNS act as mediators of cell death after mechanical overuse and injury to cartilage [163]. Together, these findings are in accordance with the observation that excessive shear stress can induce chondrocyte death mediated by ROS [164]. In contrast, for the first time, we show that physical exercise can actually reduce oxidative stress in joints in an experimental OA animal model [165].

Once produced in response to mechanical overload, free radical diffusion into all joint compartments is promoted by cyclic compression during gait. Fluctuations of synovial fluid and squeezing of fluid from cartilage tissue during static pressure phases cause a constant interchange of fluid between cartilage tissue and the

synovial compartment [166]. During joint inflammation, it is very likely that this mechanism increases the exposure of cartilage to ROS.

Little information exists on the effect of mechanical loading on redox homeostasis in bone, especially in the subchondral bone compartment. The first studies suggested that there is a relationship between mechanical loading and the pro-oxidant–antioxidant balance in bone. An in vitro study detected that mechanical strain beyond physiological levels has been shown to enhance ROS synthesis and SOD activity in osteoblast-like cells [167]. Furthermore, NO release from osteoblast-like cells was affected through cyclic tensile stretch [168]. In contrast, moderate physical activity might protect osteocytes in cortical bone against oxidative stress in heterozygous SOD2-deficient mice [156]. However, the functional consequence and the underlying mechanism of the relationship between mechanical loading and redox homeostasis in cartilage and bone are still unclear.

5.5 Summary and Conclusions

Arthritis and degenerative joint diseases affect all joint structures including cartilage, subchondral bone, synovial joint lining and adjacent supporting connective tissue. The synthesis and degradation of matrix molecules is disturbed resulting in the functional failure of articular cartilage. Oxidative stress has been shown to play a critical role in initiating degenerative joint diseases by influencing the cartilage and bone metabolism. Several studies have demonstrated that the accumulation of ROS and RNS induces both apoptosis and senescence of chondrocytes and impairs their function to maintain the structural integrity of cartilage. First studies strongly indicated that oxidative and nitrosative stress may also contribute to the imbalance in the homeostasis of the subchondral bone metabolism in OA. This is also supported by osteoporosis research, which has already demonstrated that both ROS and RNS influence the differentiation and survival of osteoblasts, osteocytes and osteoclasts. However, further research is necessary to unravel the exact mechanism detailing how an imbalance in pro-oxidant–antioxidant homeostasis can influence the metabolism of subchondral bone and to determine whether oxidative stress in articular cartilage could also affect the metabolism of subchondral bone either directly or indirectly. In addition, mechanical loading has been shown to modulate cartilage and bone oxidative stress.

In summary, the regulation of cartilage and subchondral bone metabolism by oxidative stress is very complex, and the molecular mechanisms that control redox homeostasis in cartilage and subchondral bone are poorly understood. More research is required to elucidate the complex relationship between oxidative stress, ageing and cartilage, as well as subchondral bone metabolism in the pathomechanism of arthritis and degenerative joint diseases. New studies will produce insightful data that may allow a better understanding of the contribution of ROS and RNS in initiating arthritis and degenerative joint diseases.

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Chapter 6
Role of Oxidative Stress in Bone Ageing

Sergio Portal-Núñez and Pedro Esbrit

Abstract Oxidative stress (OS) plays a major role in ageing process. Aerobic metabolism involves the production of reactive oxygen species (ROS) whose accumulation produces irreversible cell damage. Nature has developed various antioxidant mechanisms as defence against oxidative stress, but their efficacy decreases with ageing, which leads to homeostasis alterations. There is evidence showing that OS plays a major role in the development of age-related osteopenia. In bone, loss of oestrogen production and chronic illnesses such as diabetes mellitus contribute to the increased ROS levels with age. However, typical antioxidants such as *N*-acetyl cysteine or catalase are not effective to prevent the deleterious effects of high OS in this tissue, probably due to their concomitant anti-remodelling action. Of interest, it has very recently been reported that parathyroid hormone, the only currently available anabolic agent for osteoporosis, exerts various osteogenic effects including anti-OS features. Unravelling the mechanisms underlying the increase of OS and its relation to bone loss with age would be important to design novel strategies to prevent the development of osteoporosis in ageing subjects.

Abbreviations

BMD	Bone mass density
DM	Diabetes mellitus
ERK	Extracellular signal-regulated kinase
FoxO	Forkhead box O transcription factors
Gadd45	Growth and DNA Damage 45

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GPx	Glutathione peroxidase
GRase	Glutathione reductase
GSH	Glutathione
JNK	c-Jun N-terminal kinase
LRP5	Low-density lipoprotein receptor-related 5
Mst-1	Mammalian sterile 20-like kinase-1
NAC	*N*-acetyl cysteine
NF-κB	Nuclear factor-κB
OPG	Osteoprotegerin
OS	Oxidative stress
PTH	Parathyroid hormone
PTHrP	PTH-related protein
RANKL	Receptor activator of NF-κ-B ligand
ROS	Reactive oxygen species
Runx2	Runt-related transcription factor 2
SERMs	Selective oestrogen receptor modulators
SOD	Superoxide dismutase
T1D/T2D	Types 1 and 2 diabetes mellitus
TCF/LEF	T-cell factor/lymphoid enhancer factor

6.1 Introduction

The advance in life expectancy in developed countries is related to the increase of incidence of chronic illnesses such as cardiovascular diseases, cancer, diabetes and osteoporosis. In fact, the latter has been considered as a silent epidemic ravaging the elder population as a consequence of multiple morbidities associated with age. In humans, the peak of bone mass density (BMD) is reached in adulthood and bone loss starts at the 3rd decade of life. Several factors, including nutritional, hormonal and genetic factors, cause and/or influence this decline.

Oxidative stress (OS) plays a pivotal role in the development of a wide variety of diseases including osteoporosis [1] and is highly responsible for the ageing process itself [2]. OS is the result of the aerobic metabolism that takes place in the mitochondria, which is accounted for by an imbalance between the production of reactive oxygen species (ROS) and the cell capacity to detoxify ROS. When the latter production overwhelms the physiological defence system, the cell machinery starts to deteriorate eventually leading to cell death. Nature has developed an intricate system in order to keep the aforementioned balance to avoid cellular damage. In bone, sex steroid depletion that occurs in women after menopause and in men with ageing dramatically hampers the ROS defence system because both oestrogens and androgens play a major role in this regard. Other chronic illnesses such as diabetes mellitus (DM), in which inflammation is an important component favouring OS, may also contribute to the development of osteopenia in this scenario.

We will here review the current knowledge pertaining OS as a major pathogenetic cause of skeletal ageing. In particular, we will show different mechanisms by which OS affects bone cells, namely osteoblasts, osteocytes and osteoclasts, as well as the influence of sex hormone depletion and DM as OS-inducing conditions. In addition, putative strategies targeting OS to prevent bone loss in ageing subjects will be revised.

6.2 Ageing Process and Oxidative Stress

Ageing is not a disease itself but is rather a progressive decrease of multi-organ function that impairs the capacity of the organism for adequately preserving homeostasis [2]. The ageing process is commonly thought to occur as a consequence of the overproduction of free radicals causing an increase in ROS [3, 4]. During aerobic metabolism in the mitochondria, generated free electrons combine with O_2 to yield ROS, namely superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\bullet-}$) [5]. ROS are essential for the normal cell function because they can act as second messengers to modulate different physiological processes [6, 7]. On the other hand, OS builds up in situations in which an excess of ROS production occurs that cannot be compensated for by cell detoxifying mechanisms. ROS can interact with different cell molecules (DNA, lipids and proteins), giving rise to their oxidation and damage, eventually causing cell death [8]. Cells have developed different antioxidant systems in order to keep ROS under control levels (Fig. 6.1).

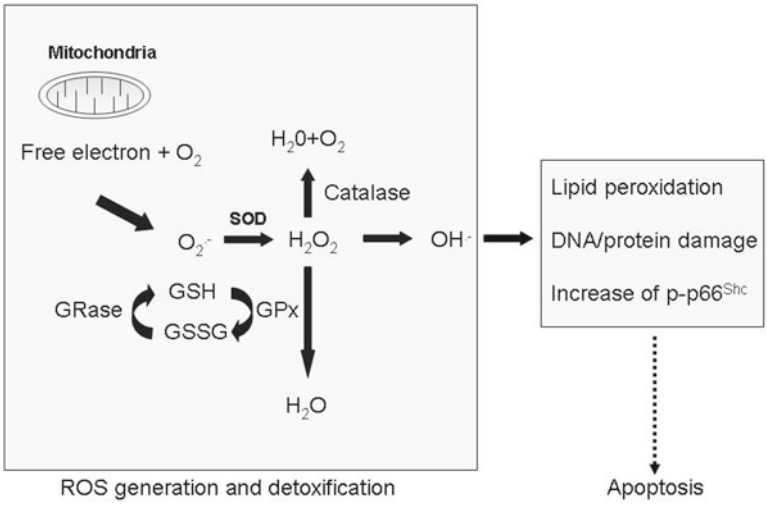

Fig. 6.1 ROS production and cell damage mechanism. Aerobic metabolism in the mitochondria produces ROS, namely anion superoxide ($O_2^{\bullet-}$), H_2O_2, and $OH^{\bullet-}$. In conditions in which detoxifying mechanisms are overwhelmed by an excess of ROS production, cell damage arises leading to apoptosis. *SOD* superoxide dismutase, *GRase* glutathione reductase, *GSH* total glutathione (reduced), *GSSG* oxidised glutathione, *GPx* glutathione peroxidase

Changes in some enzymatic activities arise as an important cell response to prevent ROS overproduction. Superoxide dismutase (SOD) is an enzyme which converts $O_2^{\bullet-}$ to H_2O_2, whereas glutathione peroxidase (GPx) degrades peroxides in a reaction whereby glutathione (GSH) becomes oxidised as disulfide glutathione (GSSG), which can be converted back to GSH through the activity of glutathione reductase (GRase) [9]. In physiological conditions, there is a fine balance and a good coupling action of these enzymes. It has been demonstrated that the ability of these enzymes to clear ROS is reduced with age, contributing to tissue ROS accumulation [10].

The forkhead box O (FoxO) transcription factors are a major family of transcription factors with multiple functions [11], since they are implicated in the control of glucose metabolism [12], tumorigenesis [13, 14] as well as the cell defence against OS [15, 16]. There are four major FoxOs, namely FoxO1, FoxO3, FoxO4 and FoxO6 [17]. FoxOs 1, 3 and 4 are all expressed in bone, whereas FoxO6 is restricted to brain [18]. The activity of FoxOs can be mainly inhibited by promoting extrusion from the nucleus where they exert their action. Growth factors, e.g., insulin-like growth factor I, which activate Akt kinases can phosphorylate FoxOs at specific sites (e.g., Thr^{32}, Ser^{253} and Ser^{315} in FoxO3) and thus retain them in the cytoplasm [19], eventually leading to their degradation [20]. Activation of extracellular signal-regulated kinase (ERK) pathway also promotes phosphorylation of FoxOs, favouring their ubiquitination and destruction [21, 22].

On the other hand, in response to OS, activation of both c-Jun N-terminal kinase (JNK) and the protein mammalian sterile 20-like kinase-1 (Mst-1) induces phosphorylation of FoxOs at sites different from those targeted by Akt and ERK pathways [23, 24]. This triggers their nuclear translocation and binding to specific sequences in the promoter region of genes related to cell cycle and DNA repair (i.e., growth and DNA damage 45 (Gadd45) [25]) and antioxidant enzymes (i.e., catalase [26] and SOD [27]). There is compelling evidence that FoxOs play a major role in skeleton maintenance [28–31]. FoxOs implication in bone mass regulation will be described in detail further ahead in this chapter (Sect. 6.4).

6.3 Ageing-Related Bone Loss

Skeleton is under constant remodelling which demands a fine regulation between the different cell types involved in this process. The remodelling process starts with osteoclast recruitment and activation (resorption phase), followed by a phase of bone formation carried out by osteoblasts. Recent data indicate that recruitment of osteoblast precursors to the bone remodelling unit is dependent on signals such as sphingosine 1-phosphate and cardiotropin 1 from active osteoclasts [32, 33]. In the end, the balance between osteoclast-dependent resorption and osteoblast-mediated bone formation determines the overall bone status [34]. OS is now known to be one of the factors which alter the bone resorption/bone formation balance towards bone loss. Before describing the specific effects of OS on bone remodelling, we will deal with the main structural and functional changes that occur in ageing bone.

It has been sustained for years that peak bone mass, which occurs in humans around the third decade of life [35–37], remains stable until menopause in women and the 5th decade in men. However, more recent evidence indicates that bone loss already starts in the third decade of life in both women and men [38, 39]. Traditionally, this bone loss (involutional osteoporosis) has been related to three main causes, namely nutritional, genetic and environmental.

In women, menopause plays an important role in the rapid development of osteoporosis, and administration of oestrogens prevents menopause-associated fractures [40, 41]. Sex steroid deficiency is related to osteoporosis in males too, although bone loss is not as sharp as in postmenopausal women [42–44]. Indeed, the classical link between oestrogen deficiency and osteoporosis [42, 45] has been challenged by some recent studies showing the key role of OS in the pathogenesis of involutional osteoporosis in both women and men [1]. The recent development of quantitative computed tomography versus classical 2D densitometry has allowed clinicians and biomedical researchers to gain knowledge about the status of both cortical and trabecular bone compartments. Using this methodology it has been demonstrated that trabecular bone loss starts in young adults before a decrease of sex steroid levels [46]. Thus, a new paradigm has emerged establishing that oestrogen depletion is just one among many deleterious manifestations related to the high OS status that occurs with age.

Ageing has also been related to a deterioration of bone vascularity which is essential in maintaining skeletal homeostasis and during bone regeneration [47, 48]. One of the mechanisms whereby vascularity decreases with age implicates the concomitant increased endogenous production of glucocorticoids [49].

6.4 OS and Bone Metabolism

As mentioned above, OS is generally increased with ageing, but the consequences of this increase in bone metabolism have only recently deserved attention. There is evidence indicating that the presence of OS markers such as 8-iso-prostaglandin F (2α) in the urine inversely correlates with BMD [50]. In addition, antioxidant levels in plasma are decreased in aged osteoporotic women [51]. Furthermore, an increase in the intake of vitamin C, an antioxidant, has shown to improve BMD and to decrease fracture risk [52–55]. These data support the contention that OS may influence bone status. In the following paragraphs, putative mechanisms by which OS might affect bone physiology will be described.

The osteoblast is the cell type in charge of forming new bone, and a decrease in its function leads to a deficit of bone formation and bone loss. In fact, the latter is frequently associated with a high rate of osteoblast apoptosis [56] and/or an altered osteoblastogenesis from mesenchymal precursors [57]. In osteoblasts, OS induces the phosphorylation of $p66^{shc}$ which is then released from the mitochondria inner wall to promote H_2O_2 formation in a reaction carried out by cytochrome c, resulting in apoptosis [58]. Both H_2O_2 and xanthine/xanthine oxidase induction by OS impair

viability of preosteoblastic cells and inhibit osteoblast differentiation [59, 60], which can be reversed by pyrrolidine dithiocarbamate and trolox, two well-known antioxidants [61, 62]. Furthermore, compromised osteoblast viability in this setting may be recovered at least in part by antioxidants such as glutamate and high doses of adenosine [63]. It has also been reported that metallothionein (a protector against OS) was efficient in protecting bone marrow stromal cells from H_2O_2-induced inhibition of osteoblastic differentiation [64].

OS triggers different molecular pathways which are known to have an impact on bone cells. One of them is activation of the nuclear factor (NF)-κB pathway [65], which transactivates the expression of a plethora of genes related to inflammation through the highly conserved DNA-binding/dimerization Rel domain [66]. Indeed, Almeida et al. [67] have described that OS induces NF-κB activation by a protein kinase C- and $p66^{shc}$-dependent mechanism in mouse osteoblastic cell lines UAM-32 and OB-6 and in the pluripotent cell line C2C12 [68]. Both NF-κB and ERK activation by OS inhibit the differentiation of rabbit bone marrow stromal cells, as shown to occur by using inhibitors of these pathways, namely PD98059 and caffeic acid phenethyl ester [69]. In addition, it has been reported that H_2O_2 inhibits mouse osteoblastic MC3T3-E1 cell proliferation by inducing cell arrest in G2 phase related to a decrease of cyclin B1 [70].

As mentioned above, FoxOs transcription factors play a major role in the tissue defence against OS, and bone tissue is not an exception in this respect. Several reports indicate that FoxO1 and FoxO3 are the most widely expressed FoxOs in osteoblasts [29, 71]. FoxO1-specific deletion in these cells produces a decrease in cell proliferation without affecting apoptosis [71]. On the other hand, FoxO3 overexpression is associated with an increased lumbar spine BMD related to a decreased osteoblast apoptosis [29]. Of interest, sirtuins (a family of deacetylases [72]) are known to increase bone mass through interaction with oestrogen receptors [73] but also by promoting FoxO deacetylation and thus enhancing its transcriptional activity [74].

Perhaps the effect of OS with a major impact on osteoblast function might be impairment of the canonical Wnt/β-catenin pathway, which plays a key role in bone formation and regeneration [75, 76]. This pathway is activated upon binding of different Wnt proteins to a complex formed by frizzled membrane receptors and low-density lipoprotein receptor-related 5 or 6 (LRP5/6) as co-receptors [77, 78]. This binding prevents β-catenin phosphorylation and thus its proteasomal degradation by a complex formed by axin, adenomatous polyposis coli, casein kinase Iα and glycogen synthase kinase 3 β [79]. Stabilised β-catenin translocates to the nucleus where it associates with T-cell factor (TCF)/lymphoid enhancer factor (LEF) transcription factors and activates the transcription of different osteoblastic genes such as runt-related transcription factor 2 (Runx2) [80] and osteoprotegerin (OPG) [81]. Moreover, β-catenin is required for FoxOs transcriptional activity [82, 83]. Indeed, in osteoblasts under OS conditions, β-catenin forms a complex with FoxOs to drive the expression of genes involved in the cell defence against OS, and this prevents the regular function of the Wnt pathway [30].

The final fate of osteoblasts is either apoptosis or terminally differentiation into osteocytes. Approximately 10–30% of all osteoblasts become osteocytes [84]. The role of the latter cells has been recently redefined from an early consideration of a merely "buried" cell in the mineralized bone matrix to a true "orchestrator" of bone metabolism [85, 86]. The effect of OS on osteocytes has not yet been properly evaluated, but OS has been reported to induce apoptosis in some osteocytic cell preparations [87].

In mice, it has been shown that the number of osteoclast precursors increases with age [88]. In fact, OS can positively interact with osteoclasts in various manners. Receptor activator of NF-κB ligand (RANKL) (an important promoter of osteoclast maturation) produces an increase of ROS in osteoclasts which is abolished by the antioxidants *N*-acetyl cysteine (NAC) and GSH [89]. Furthermore, OS reduces the expression of thioredoxin binding protein 2, an endogenous inhibitor of thioredoxin-1 activity—a protein that translocates to the nucleus upon OS and helps to activate activator protein-1 (another transcription factor) and NF-κB, thus favouring osteoclast differentiation [90]. In addition, treatment of osteoclasts with H_2O_2 further increases OS [91, 92].

FoxOs activation in osteoblasts appears to negatively affect the osteoclast. In this regard, an increased or a decreased number of osteoclasts occurs in mice with osteoblastic deletion of FoxO1 or overexpression of FoxO3, respectively [29, 71]. However, FoxO1 deletion is expected to increase β-catenin-dependent transcriptional activation of OPG, which would decrease osteoclastogenesis. One possible explanation for this counterintuitive finding comes from a recent report showing that FoxO1 is able to directly induce OPG expression in osteoblasts [93]. Since overexpression of FoxO3 did not affect OPG expression, its observed deleterious effect on osteoclast number would be independent of OPG changes. Moreover, osteoclast apoptosis is induced by activation of Mst-1 which, as mentioned above, activates FoxOs [94]. On the other hand, RANKL, which promotes osteoclastogenesis, activates Akt pathway that in turn produces inactivation of FoxOs [95, 96].

6.5 Oestrogen Depletion as an OS-Inducing Factor

Oestrogens protect osteoblasts and osteocytes while promoting apoptosis of osteoclasts [97–99]. Thus, oestrogens can promote osteoblast survival by a nongenomic mechanism involving ERK activation upon binding to specific receptors in the cell membrane [100]. Ovariectomised animals have a decrease in bone mass [101], associated with high levels of H_2O_2 and an increased lipid peroxidation together with a reduction of the capacity of defence enzymes against OS [102]. As additional proof that oestrogens may act as a natural antioxidant, bone loss in ovariectomised animal models is reversed by NAC administration [91, 103]. The mechanisms whereby oestrogens protect against OS are poorly understood. Of interest in this regard, a similar increase of $p66^{shc}$ phosphorylation and ROS and a decreased GRase

activity have been reported to occur in both young ovariectomised mice and old normal mice [103]. In addition, thiol antioxidants appear to play a role in bone loss related to oestrogen deficiency. Thioredoxin system is a major defensive mechanism against OS. Thioredoxin is oxidised upon OS conditions and can be reduced back to its original form by thioredoxin reductase whose activity is diminished in ovariectomised mice; administration of 17-β estradiol to these mice normalised the latter enzyme activity [91]. Furthermore, osteoclasts of these mice showed increased TNF-α, a pro-inflammatory cytokine implicated in bone loss, and this increase was abolished by 17-β estradiol administration [91]. In contrast to the protective effect of oestrogens on osteoblasts, oestrogen depletion promotes osteoclast survival [104]. As mentioned above, osteoclasts require RANKL and the presence of ROS for their maturation and avoiding apoptosis; oestrogens would inhibit both processes by counteracting OS. The mechanism by which estradiol produces osteoclast apoptosis involves upregulation of GRase by a mechanism apparently dependent on the activation of mitogen-activated protein kinase [103].

6.6 Role of OS in Diabetic Osteopenia

Types 1 and 2 DM (T1D and T2D) are conditions associated with bone fragility and high fracture risk. A reduction in BMD frequently occurs in T1D [105], whereas T2D is not often characterised by low bone mass but a poor bone quality [106]. In the latter disease, OS might contribute to the formation of advanced glycation end products that would exert deleterious effects on collagen structure and bone matrix quality [107].

Animal models for both types of DM present low bone turnover and an increase of OS markers [108–110]. In T1D, hyperglycaemia affects normal cell physiology in several ways, including an increase in ROS production due to a dramatic mitochondrial activity [111]. As a consequence of OS, JNK phosphorylates the insulin receptor substrate-1 avoiding its binding to the insulin/insulin receptor complex thus preventing Akt-dependent FoxOs inactivation and reducing insulin sensitivity [112, 113].

6.7 Anti-OS Therapies for Age-Related Osteopenia

As discussed above, oestrogen depletion is a major determinant of ROS increase with age, but long-term substitutive therapy with oestrogens is not advisable due to observed side effects such as coronary disease and invasive breast cancer [114]. Selective oestrogen receptor modulators (SERMs) are a suitable alternative in this respect; they are tissue specific and are devoid of the aforementioned side effects. In addition, these oestrogen analogues might also have an antioxidant action in bone.

Thus, raloxifene—a widely used SERM for osteoporosis treatment—has proven to protect osteocytes from damage induced by H_2O_2 [115].

On the other hand, "true" antioxidant agents such as aforementioned NAC and catalase are not a promising tool for counteracting the deleterious effects of OS in ageing bone. This is because of several unwanted features: (1) these agents can block osteoclastogenesis hampering the initiation of the remodelling cycle; and even more important, (2) they impair the Wnt-signalling pathway by promoting the binding of nucleoredoxin to dishevelled, a component of the protein complex affecting β-catenin destabilisation [116]. Thereby, these agents inhibit a key process in osteoblastic function.

Interestingly, an agent that combines a high osteogenic capacity and antioxidant features is parathyroid hormone (PTH). As administered intermittently (daily)—the only currently available therapy approved for osteoporosis treatment [117, 118]—PTH (1-34) reduces osteoblast apoptosis and improves matrix mineralization [119]. Moreover, this peptide can induce Wnt pathway activation through various mechanisms including increasing the expression of various key components of this canonical pathway [120] and formation of a complex between its type 1 receptor and LRP6 [121]. Furthermore, it has also been recently described that PTH (1-34) displays anti-OS features when administered to old mice [31]. In fact, it was shown that this peptide exhibited a higher bone anabolic capacity in the latter mice, related to the concomitant OS, than in younger mice. In addition, Schnoke et al. [122] have demonstrated that PTH (1-34) promotes DNA repair in osteoblastic cells upon H_2O_2 treatment. Also of note is the recent observation that PTH-related protein (PTHrP), which can display bone anabolic actions through its N-terminal PTH-like and C-terminal domains [123–125], might also have antioxidant potential. Hence, PTHrP was shown to reverse the decreased alkaline phosphatase activity produced by H_2O_2 in mouse mesenchymal C3H10T1/2 cells cultured in an osteogenic medium [126].

6.8 Summary and Conclusions

OS arises as a consequence of impaired regulatory mechanisms that control ROS production. Current findings support the notion that the latter plays a major role in age-related bone loss. In bone, OS impairs the proliferation and differentiation of osteoblasts and increases osteoclastogenesis. These effects of OS are enhanced in the setting of oestrogen deficiency and chronic illnesses such as DM, which contribute to the development of osteopenia with ageing (Fig. 6.2). Recent studies have begun to disclose an unexpected action of PTH by targeting OS as an important feature related to its bone anabolic action. Thus, new approaches specifically addressing OS as a key pathogenetic factor in involutional and primary osteoporosis are expected to add novel agents to the scarce therapeutic armamentarium against this growing epidemic.

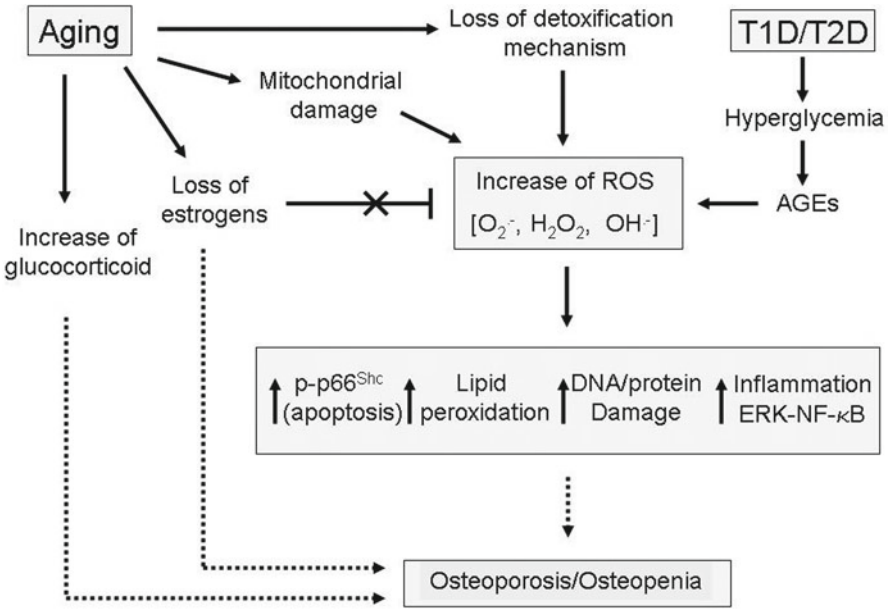

Fig. 6.2 Influence of OS in ageing-related osteopenia. The ageing process is characterised by an increased ROS production and a decrease in the efficacy of detoxifying ROS mechanisms and aggravated by concomitant loss of oestrogens and increased production of endogenous glucocorticoids. These factors altogether contribute to alterations of bone turnover leading to bone loss. *T1D* type 1 DM, *T2D* type 2 DM, *AGEs* advanced glycation end products

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Part II
Clinical Aspects of Oxidative Stress in Joint Disorders

Chapter 7
Genetic Component of Oxidative Stress in Rheumatoid Arthritis

F. David Carmona, Jose-Ezequiel Martin, and Javier Martin

Abstract There are increasing evidences pointing to a crucial role of the redox control in the maintenance of self-tolerance, and oxidative stress is emerging as a key factor contributing to susceptibility and progression of a wide spectrum of autoimmune diseases. For instance, oxidative stress at sites of chronic inflammation has been associated with the course of rheumatoid arthritis (RA) patients. Nevertheless, very little is known about the possible genetic background of oxidative stress that leads to pathologic conditions like RA despite the large genome-wide association studies performed during the last years. There are some genes that clearly represent good candidates to be important players in the pathophysiology of diseases influenced by oxidative stress. These include nuclear genes like *HIF1A*, *MSRA*, *HO1*, *p53*, *NCF1*, and *NOS2A* but also mitochondrial genes. Here we shall review the current knowledge about the molecular mechanisms affected by oxidative stress situations and will give some clues of how these changes may contribute to the development and progression of RA.

Abbreviations

ACPA	Anti-citrullinated protein autoantibodies
ASK1	Apoptosis signal-regulating kinase 1
ATP	Adenosine triphosphate
DNA	Deoxyribonucleic acid
GWAS	Genome-wide association study
HIF-1α	Inducible factor-1 α
HIPK1	Homeodomain-interacting protein kinase 1
HO-1	Heme oxygenase-1

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LDL	Low-density lipoprotein
MSRA	Methionine sulfoxide reductase A
mtDNA	Mitochondrial deoxyribonucleic acid
NADH	Nicotinamide adenine dinucleotide
NCF1	Neutrophil cytosolic factor 1
NF-κB	Nuclear factor-κB
RA	Rheumatoid arthritis
Redox	Reduction–oxidation
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SENP1	Sentrin-specific protease 1
SUMO	Small ubiquitin-like modifier
TNFR	Tumor necrosis factor receptor
TNF-α	Tumor necrosis factor alpha

7.1 Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease of complex etiology, with an incidence of around 1% in Western countries, that affects many tissues and organs but especially the synovial joints [1]. RA develops as a consequence of a cascade of immunologic and physiologic abnormalities caused by the interaction of multiple genetic and environmental factors. Based on familiar studies, including twin studies, the heritability of this disease is estimated to be around 50% [2, 3]. Thanks to the advent of new technologies such as genome-wide association studies (GWASs), the knowledge of the genetic component of RA has been vastly improved. These novel techniques allowed the identification of many relevant genetic risk factors, including the *HLA*, *PTPN22*, *IRF5*, *TNFAIP3*, *STAT4*, *PXK*, and *REL*, to name a few [1, 4–11]. Due to the increasing number of described susceptibility *loci*, the genetic background of RA has been widely reviewed in the last years [1, 12]. However, it is known that external factors including food, smoking, environmental pollutants, or infections, which produce molecular mimicry, are also important players contributing either to RA susceptibility or to worsen the symptoms [13–15].

In the recent years, there is increasing evidence that reactive oxygen species (ROS) and reactive nitrogen species (RNS), highly active molecules which are increased after stress situations at sites of inflammation, have genotoxic effects in this type of inflammatory disorders [16]. However, very few associations with RA of genes involved in the redox regulation have been reported so far. This could be due to the highly significant thresholds of the GWASs, in which statistical significance is only considered when the P values are lower than 5×10^{-8}. In any case, some clear evidence of the genetic component relevance in the RA pathology related to oxidative stress has been reported lately.

In this chapter, we will summarize the recent findings about the molecular mechanisms that may underlie the dysregulation of the redox homeostasis leading to pathologic conditions in rheumatic disorders, focusing on the RA pathophysiology.

7.2 Oxidative Stress as Pathogenic Factor in Human Diseases

Oxidative stress represents an imbalance between the production and manifestation of ROS and the biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage by these defense mechanisms [17]. In this abnormal situation, ROS exceed the antioxidant capacity of the organism, leading to toxic effects through the production of peroxides and free radicals that may damage all components of the cell, including proteins, lipids, and DNA [18, 19]. Chemically, oxidative stress is associated with increased production of oxidizing species or a significant decrease in the effectiveness of antioxidant defenses, such as glutathione reductase [20]. The effects of oxidative stress depend upon the size of these changes, with a cell being able to overcome small perturbations and regain its original state. However, more severe oxidative stress can cause cell death, and even moderate oxidation can trigger apoptosis, while more intense stresses may cause necrosis [21].

Oxidative stress is thought to be involved in the development or progression of many human diseases, such as cancer [22], Parkinson's disease, Alzheimer's disease [18], atherosclerosis, heart failure [23], myocardial infarction [24], schizophrenia [25], bipolar disorder [26], fragile X syndrome [27], sickle cell disease [28], chronic fatigue syndrome [29], and RA [30].

7.2.1 Protein Damage by Reactive Oxygen Species

Although the exact molecular mechanisms have not been fully identified, it is clear that ROS are relevant components of the pathogenic network underlying RA. For instance, it has been observed that ROS can alter the structure of collagens and proteoglycans creating new antigens that may facilitate inflammation and autoimmune responses in the joints. Hence, ROS are considered to be extremely harmful during oxidative stress at the inflammatory site [31]. In addition, high levels of ROS are detected in arthritic joints by excessive ROS production of antigen-presenting cells during antigen presentation. It has been hypothesized that this may affect the function of T cells (which intracellular ROS levels are higher than those of the peripheral T cells in RA patients), thus promoting RA development [16, 31, 32].

7.2.2 Lipid Peroxidation

There are several studies that evidenced the potential pathologic effect that ROS may cause in the synovial joints of RA patients, including products of lipid peroxidation and oxidation damage to hyaluronic acid, glucose, and LDL proteins [33–36]. Lipid peroxidation refers to the oxidative degradation of lipids and has been related to cancer, atherosclerosis, and inflammatory arthritis. This pathogenic process affects mainly polyunsaturated fatty acids of the cell membranes, which are oxidized to produce lipid peroxyl radicals that in turn lead to further lipid oxidation,

originating a free radical chain reaction mechanism. *In vitro* studies have reported that the chondrocyte-derived ROS produced by lipid peroxidation lead to cartilage matrix protein degradation by oxidation. Interestingly, addition of vitamin E seemed to prevent this situation [37]. Cartilage injury by ROS and RNS has been also described as a consequence of damage to intracellular and matrix components. Articular cartilage is an avascular tissue, so oxygen supply is reduced and depends on diffusion from the synovial fluid to fulfill its metabolic requirements. The chondrocytes are very sensitive to O_2, and it has been proposed that abnormal ROS levels caused by pathologic O_2 tensions occurring in RA may interfere with cell morphology and function, including synthesis of matrix components [38, 39]. Other studies showed that ROS and RNS may also impair the migration of chondrocytes to injured areas and their interactions with the extracellular matrix [40].

It is likely that lipid oxidation is crucial for the accelerated atherosclerosis observed in some RA patients [41, 42]. On the other hand, significantly elevated levels of lipid peroxidation have been correlated with disease activity and C-reactive protein levels in patients with RA [43, 44]. Hence, lipid peroxidation markers are considered to be good potential markers for disease activity [45, 46].

7.2.3 Oxidative DNA Damage

Finally, another harmful effect of ROS and RNS is the oxidative DNA damage, leading to significantly increased reaction products in lymphocytes and synovial fluid of RA patients in comparison with healthy controls [47, 48]. It was suggested that these cytotoxic and mutagenic endogenous nucleic acid compounds may contribute to the inflammation process of the joints by enhancing the proinflammatory cytokine synthesis by immune cells [48]. Furthermore, purified human and murine oxidatively damaged mitochondrial DNA (mtDNA) induced arthritis when injected intra-articularly in mice, suggesting that mtDNA has inflammatory properties as a result of the presence of unmethylated CpG sequences and its oxidative status [49]. ROS can also produce mutations in master genes of the cell cycle control like *p53* [50]. The severe effects of this deleterious situation are reviewed in the subsequent section.

7.2.4 Hypoxia in Rheumatoid Arthritis

As stated above, alterations in oxygen tension, which may lead to oxidative stress, have been postulated to contribute to RA pathophysiology [38]. The term hypoxia refers to reduced levels of oxygen, either in air, blood, or tissue. Tissue hypoxia represents a serious complication since it can affect cell function, leading in some cases to cell death. The potential consequences of tissue hypoxia have been largely studied in tumors, which are in hypoxic microenvironments due to the high metabolic

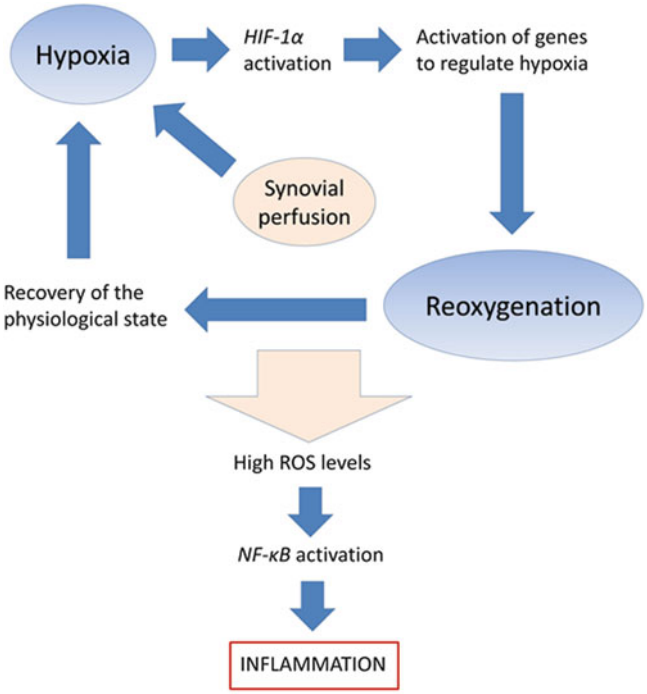

Fig. 7.1 Relation between prolonged cycles of hypoxia and reoxygenation as a consequence of synovial perfusion in patients with rheumatoid arthritis. Low oxygen tensions activate hypoxia-inducible factor-1α (HIF-1α), which is a master factor of the cellular mechanisms that regulate hypoxia. Reactive oxygen species (ROS) are produced in every cycle that upregulate eventually the nuclear factor-κB (NF-κB) pathway

demands of the tumor cells [51, 52]. A master regulator of the cellular and systemic homeostatic response to alterations in oxygen tension is the hypoxia-inducible factor 1α (HIF-1α), a subunit of the heterodimer HIF (the other component, HIF-1β, is expressed constitutively in the nucleus) [53]. Activation of its encoding gene modifies significantly the expression profile of the cell. As a consequence of *HIF1A* expression, there is upregulation of genes involved in energy metabolism, angiogenesis, apoptosis, and pathways that lead to increased oxygen delivery, which helps the cell to adapt and survive hypoxia [54].

During the course of RA, the synovial expansion is thought to influence the oxygen supply, thus generating areas of hypoxia, with the consequent activation of HIF-1α [55–57]. Several cycles of hypoxia and reoxygenation would lead to cycles of activation of the HIF transcription factor signaling cascade, which produces repetitive bursts of ROS formation. These high levels of ROS may cause an inappropriate activation of another master protein, the nuclear factor-κB (NF-κB), which, in turn, upregulates multiple proinflammatory genes that promote inflammation, angiogenesis, and cell survival, thus worsening the RA clinical phenotype [58–60] (Fig. 7.1).

7.3 Genetic Background of the Pathologic Redox Control in Rheumatoid Arthritis

RA has a complex genetic component that is estimated to represent half of the factors contributing to the development and course of the disease [2, 3]. Candidate gene studies and GWASs have identified numerous *loci* that are clearly associated with RA. These include certain frequently occurring *HLA-DRB1* alleles but also non-HLA genes involved in the immune system, such as *PTPN22*, and *STAT4*, among others (Table 7.1).

However, despite the large number of firmly described associations, there is still a considerable missing heritability, since even large GWASs are underpowered to detect associations of polymorphisms with reduced minor allele frequencies [61]. Additionally, very few associations have been reported in pathways involving redox regulation [12]. The following are some examples of the possible genetic component of the RA pathology caused by oxidative stress.

7.3.1 Methionine Sulfoxide Reductase A

Through pathway analyses to prioritize regions in GWAS data, Martin et al. [62] identified methionine sulfoxide reductase A (*MSRA*) as a novel susceptibility *locus* for RA, and further studies related this association with an increased risk to develop cardiovascular events, in particular ischemic heart disease, in RA patients [63]. This gene encodes an enzyme that has been demonstrated to repair oxidative damage to proteins in the skin using the thioredoxin reductase/thioredoxin system [64]. However, it is thought that it also controls the redox state in several cell types through the mechanism summarized in Fig. 7.2.

During T cell maturation in the thymus, apoptosis induction of autoreactive T cells prevents the loss of tolerance against self-antigens. Although the mechanisms by which this is accomplished are not yet fully understood, it is likely that this process is mediated by the tumor necrosis factor-α (TNF-α) apoptosis pathway, among other signals [65, 66]. MSRA seems to play an important role in the control of this signaling pathway, since the activation of several mediators depends on the redox state of the cell. For instance, the small ubiquitin-like modifier-specific protease sentrin-specific protease 1 (SENP-1) in its inactive form is conjugated with thioredoxin [67, 68], a component of the system by which cells maintain redox equilibrium [69]. In addition, SENP-1 translocation to the nucleus is also ROS dependent [67]. Consequently, polymorphisms within the *MSRA* gene may have a profound impact on the thymic selection of T cells.

Interestingly, the authors found that the risk caused by genetic variants in *MSRA* was most probably confined to the subgroup of RA patients which presented the shared epitope HLA variations [4]. The presence of the shared epitope variations

Table 7.1 Genetic associations with rheumatoid arthritis in Caucasian population organized by year of identification

Year of publication	Susceptibility *loci*
1978	*HLA-DR4*
1987	SE hypothesis
2003	*PADI4*
2004	*PTPN22*
2005	*CTLA4*
2007	*TNFAIP3*, *STAT4*, *TRAF1-C5*, *IL2-IL21*
2008	*CD40*, *CCL21*, *CD244*, *PIP4K2C*, *IL2RA*, *IL2RB*, *PRKCQ*, *AFF3*, *TNFRSF14*
2009	*REL*, *BLK*, *TAGAP*, *CD28*, *TRAF6*, *PTPRC*, *FCGR2A*, *PRDM1*, *CD2-CD58*
2010	*SIAE*, *SPRED2*, *RBPJ*, *CCR6*, *IRF5*, *PXK*, *IL6ST*
2011	*MSRA*, *MMEL1*, *TAGAP*, *SH2B3*, *DDX6*, *CD247*, *UBE2L3*, *UBASH3A*
2012	*CIITA*, *HLA-B*, *HLA-DPB1*, *IL23R*

Adapted from Gregersen et al. [12]

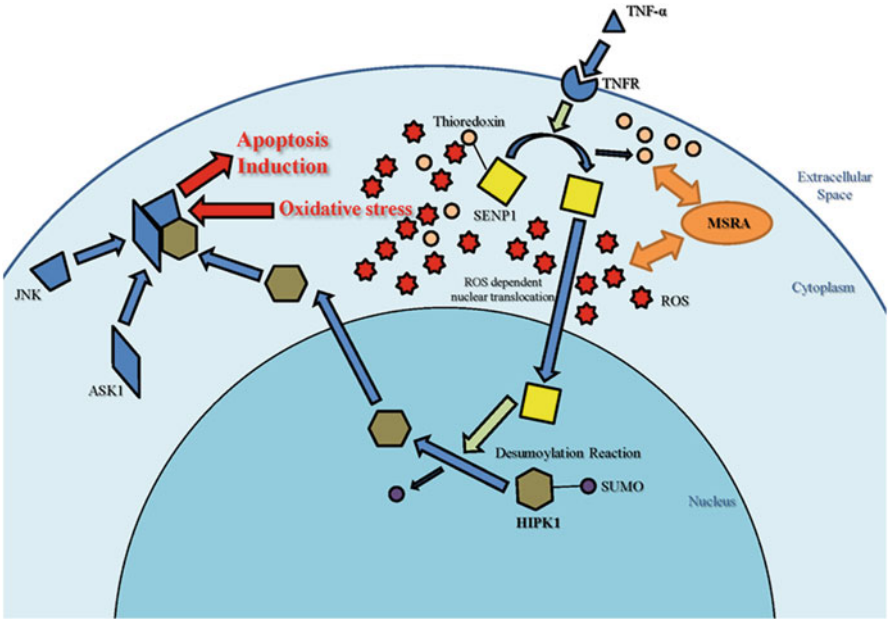

Fig. 7.2 Role of MSRA in apoptosis under oxidative stress. Risk MSRA genetic variants may lead autoreactive T cells to avoid clonal deletion by apoptosis during thymic maturation. ASK1 apoptosis signal-regulating kinase 1, HIPK1 homeodomain-interacting protein kinase 1, ROS reactive oxygen species, SENP1 sentrin-specific protease 1, SUMO small ubiquitin-like modifier, TNF-α tumor necrosis factor alpha, TNFR tumor necrosis factor receptor (adapted from Martin et al. [62])

in the HLA molecules is the main genetic risk factor for RA and is also highly correlated with the presence of anti-citrullinated protein autoantibodies (ACPA) in these patients [70]. Reciprocally, it has been suggested that most, if not all, of the observed association in the HLA region in RA is found in the ACPA positive subset of patients [71]. Taken together, this suggests a role for oxidative stress, at least for the involvement of *MSRA*, only in the ACPA positive subset of patients. All these bind the role of the oxidative stress in RA to HLA genes in this disease while giving some insight of the future directions to further elucidate the importance of oxidative stress in RA.

MSRA has been associated with other autoimmune diseases, such as vitiligo and systemic sclerosis (SSc) [72, 73]. Interestingly, presence of anti-MSRA antibodies with the ability of inhibiting MSRA enzymatic activity was detected in the sera of SSc patients, and this was related with a worse disease phenotype as a consequence of enhanced oxidative stress [73].

7.3.2 Heme Oxygenase-1

The inducible protein heme oxygenase-1 (HO-1) is an essential stress response enzyme in heme catabolism to biliverdin, carbon monoxide, and free iron, which is activated by different exogenous and endogenous factors, including heavy metals, inflammatory mediators, oxidative stress, and hypoxia [74, 75]. It has been reported that the activation of HO-1 assists different antioxidant systems to prevent oxidative damage by decreasing ROS production [76].

Studies in animal models of acute and chronic inflammation showed that HO-1 protects cells from oxidative damage during stress situations, playing a key role in the resolution of inflammation. It has been observed that inhibition of this protein in mice worsens chronic inflammation, while its induction is related with an improvement of the inflammatory response [77–83]. On the other hand, the activation of HO-1 in experimental models of collagen- and TNF-mediated arthritis decreases proinflammatory cytokine levels [84]. Similarly, HO-1 induction in mouse models of lupus nephritis leads to a reduction of the glomerulus damage by decreasing the production of local nitric oxide synthase (NOS) [85].

All these data clearly suggest that HO-1 is an important regulator against the chronic inflammation associated with autoimmunity processes, and it is likely that it exerts its function by modulating the oxidative damage. Indeed, HO-1 is strongly induced by inflammatory mediators as an adaptive response in the synovial tissue of RA patients, in which it seems to confer protective effects against ROS production [86, 87]. Hence, it is considered to be a sensitive and reliable indicator of cellular oxidative stress.

The promoter region of its encoding gene, *HMOX1*, contains a guanine–thymidine (GT)n microsatellite of variable length that it has been reported to influence HO-1 levels. Homozygous individuals for short (GT)n alleles (lower than 25 repeats) show a higher *HMOX1* expression than those with large (GT)n alleles (more than 25 repeats) [88].

Interestingly, the (GT)n microsatellite has been associated with susceptibility to several pathologic disorders in which the inflammatory process is directly involved, including coronary artery disease, emphysema, nephritis, cancer, and RA [89, 90]. In relation to the latter, long (GT)n alleles, which lead to reduced HO-1 induction, have been associated with RA susceptibility in the Spanish population [90], and Dutch RA patients with short (GT)n repeats have been reported to exhibit less progression of joint damage [91].

The functional significance of the HO-1 system has been considered for the development of novel gene therapeutic strategies based on HO-1 gene transfer in a variety of clinical conditions [76].

7.3.3 p53 Tumor Suppressor Gene

As previously indicated, ROS and RNS have the capacity to harm nucleic acids [92, 93]. Furthermore, oxidatively damaged DNA seems to be an important player in the synovial injuries of RA patients [47, 48]. When the DNA is affected by diverse cellular stresses, there is an upregulation of the tumor protein p53, which regulates target genes that induce cell cycle arrest, senescence, DNA repair, changes in metabolism, and apoptosis [94, 95]. Overexpression of *P53* has been observed in several inflammatory diseases including RA. In this regard, elevated P53 levels have been detected in the synovial tissue of RA patients [96, 97]. However, among the many genes that might be affected by oxidative stress, mutations in *P53* itself can also occur. Indeed, higher rates of DNA fragmentation have been observed in the inflamed synovial tissue from RA patients compared with controls [98]. Taking into account that *P53* mutations are dominant negative, mutant alleles of *P53* may suppress endogenous wild-type P53 function [99], leading to a reduced apoptosis as a consequence of oxidative stress. This, in turn, would allow a selective advantage of mutated cells, since mutations in *P53* allow the mutant cell to develop resistance to P53-dependent apoptosis. It has been suggested that this positive feedback may help to convert inflammation into chronic disease (Fig. 7.3) [50, 100].

7.3.4 Mitochondrial Mutations

An important role for the mitochondria is the generation of ATP, which is used by the cells as a source of biological energy. In this process, the redox energy from NADH molecules is transferred to oxygen in the mitochondrial electron transport chain. This generates small amounts of ROS as by-products that may cause oxidative stress if they exceed the capacity of the antioxidant defense system, which may occur under inflammatory and pathological conditions [101]. mtDNA is especially sensitive to oxidative damage by ROS because it lacks the

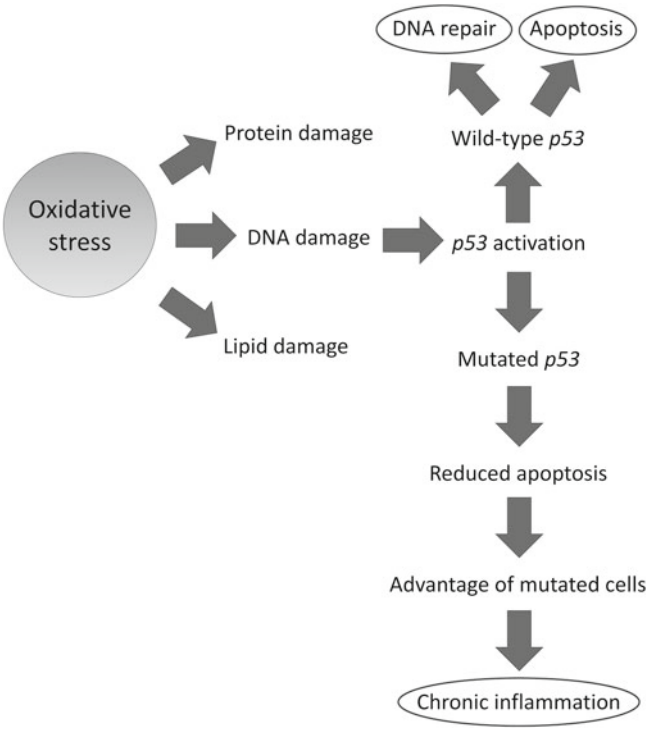

Fig. 7.3 Effects of oxidative stress on nuclear DNA. The tumor suppressor gene *P53* is upregulated after DNA damage and can either activate DNA repair proteins or induce apoptosis if the damage is excessive. However, mutations may also affect *P53,* affecting its function and leading to chronic inflammation (adapted from Tak et al. [50])

repair mechanisms of nuclear DNA. As a consequence, the mutation rate is higher than that of the nuclear DNA, and this may alter mitochondrion function, leading to multiple pathologic conditions [102, 103]. In a recent study, Harty and colleagues reported that mitochondrial mutations are significantly higher in the inflamed synovium of RA patients than in healthy controls. These mtDNA mutations were also associated with proinflammatory cytokine production, thus suggesting that oxidative damage of mtDNA may play a relevant role in RA pathophysiology [104].

7.3.5 Neutrophil Cytosolic Factor 1

Studies in animal models also support that susceptibility *loci* may predispose to oxidative stress during the development of RA. A functional mutation in the neutrophil cytosolic factor 1 gene (*NCF1*), a subunit of the NADPH oxidase complex,

has been reported to produce a reduced oxidative burst capacity that leads to an enhance arthritis susceptibility and severity in rodents. Interestingly, addition of substances that activate the NADPH oxidase complex, which is mainly expressed in phagocytes and produces ROS in phagosomes and the extracellular space upon activation, seemed to ameliorate the arthritis [105, 106]. The authors of those papers proposed that the physiological production of ROS by phagocytes in response to antigen affects T cell–antigen interactions and may induce apoptosis of autoreactive arthritogenic T cells, thereby preventing autoimmune responses. In humans, *NCF1* is redundant and a complete loss of function is associated with chronic granulomatous disease that has increased susceptibility to microbial infections. The associations of *NCF1* with other experimental autoimmune conditions suggest that polymorphisms within this gene might be important for autoimmunity in general [107].

7.3.6 Nitric Oxide Synthase

Nitric oxide synthases (NOSs) are a family of enzymes that metabolize L-arginine to produce nitric oxide (NO), a RNS that plays a crucial role in many biological processes. In humans, there are three different genes encoding NOS enzymes, the neuronal (*NOS-1*), the cytokine-inducible (*NOS2A*), and the endothelial (*NOS-3*) [108]. NOS-2 is a relevant player in the immune system, being involved in NO production during the immune response. In this regard, different inflammatory mediators regulate the activation of *NOS2A*, and its encoding protein, iNOS, is only present in the tissues after cytokine induction [109, 110].

High NO levels produced by iNOS represent an important component of the defense systems against microbial infections and tumoral cells. Indeed, NO has been described as key mediator of the Th1/Th2 balance in autoimmunity. However, since NO is a reactive free radical, uncontrolled activation of *NOS2A* may lead to several pathogenic conditions, including autoimmune disorders like asthma, multiple sclerosis, colitis, psoriasis, and RA but also to tumor development, transplant rejection, or even septic shock. Specifically, increased NO levels, as a consequence of enhanced *NOS2A* expression, correlate with disease activity in RA patients [111].

Because of the above, *NOS2A* has a very tight regulation at both transcriptional and posttranscriptional levels [112]. The promoter of this gene contains a high number of polymorphic elements. Interestingly, a multiallelic repeat in the promoter region, (CCTTT)n, has been associated with RA susceptibility in a Spanish population [113], and interactions between some gene polymorphisms and *HLA-DRB1* alleles seem to confer risk to develop cardiovascular events in RA patients [114]. Although it still needs to be confirmed, it is possible that these susceptibility variants may be influencing the disease phenotype by generating oxidative stress.

7.4 Conclusions

Recent studies have described oxidative stress as a crucial pathogenic factor for RA. Although ROS are important regulators of many physiological processes, the excessive ROS production generated under oxidative stress may affect relevant signaling pathways, thus contributing to the chronic inflammatory state of synovial tissues. Understanding the mechanisms that underlie the causes and consequences of oxidative stress would represent a substantial step forward for the development of novel and more effective therapeutic strategies. However, this is a rather challenging issue because of the large variety of routes in which redox molecules are involved. The advent of new technologies is allowing better approaches that may help to increase the current knowledge about RA pathophysiology. Consequently, it is expected that many gaps of the RA puzzle will be filled in during the next few years, and the elucidation of the genetic background underlying oxidative stress may shed light into the obscure path leading to this goal.

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Chapter 8
Oxidative Stress in Rheumatoid Arthritis

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Abstract Redox imbalance has long been recognised to be a factor in the pathology of rheumatoid arthritis. There is increasing evidence that reactive species of oxygen, nitrogen and sulphur play biphasic roles in inflammation and may have disease aggravating or ameliorating effects, depending on the dose, tissue compartment and disease phase. A promising target both for therapeutic purposes and as disease markers is the thioredoxin family of redox enzymes, including thioredoxins, thioredoxin reductases and peroxiredoxins. Through its cytokine-like properties, thioredoxin has been proposed to be pro-inflammatory in rheumatoid arthritis. Yet, administration of recombinant thioredoxin appears to ameliorate the disease. We demonstrated recently that protein levels of peroxiredoxin 2 are increased in peripheral blood lymphocytes in rheumatoid arthritis compared with healthy subjects. Therapeutically targeting peroxiredoxins in rheumatoid arthritis provides a new avenue for biomedical research.

Abbreviations

GSH	Glutathione
GSSG	Glutathione disulphide

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H_2O_2	Hydrogen peroxide
HOCl	Hypochlorous acid
IL	Interleukin
LPS	Lipopolysaccharide
NF-κB	Nuclear factor kappa-B
$^{\bullet}NO$	Nitric oxide
NOS	NO synthase enzyme
$^1O_2$	Singlet oxygen
$^1O_2^{\bullet-}$	Superoxide
$^{\bullet}OH$	Hydroxyl radical
$ONOO^-$	Peroxynitrite
oxLDL	Oxidised low-density lipoprotein
Prdx	Peroxiredoxin
RA	Rheumatoid arthritis
RNS	Reactive nitrogen species
$ROO^{\bullet}$	Peroxyl radical
ROS	Reactive oxygen species
RSS	Reactive sulphur species
SNO-MBL	S-nitrosated mannose binding lectin
SOD	Superoxide dismutase
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Trx	Thioredoxin
2D-PAGE	2-Dimensional polyacrylamide gel electrophoresis

8.1 Background: Disease Aetiology

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disorder which affects about 1% of the population worldwide [1]. RA is a multifactorial disease, and it is thought that genetic, environmental and host factors all contribute to the disease. Thus, the disease develops as a result of environmental insults on a susceptible phenotype, and together these lead to immunological dysfunction and chronic inflammation [2].

Although RA mainly affects joints, it also has significant co-morbidities such as an increased risk of cardiovascular complications (e.g. vasculitis, atherosclerosis) [3]. At the cellular level, RA is characterised by synovial inflammation; infiltration of the synovium by T cells, B cells, neutrophils and macrophages; germinal centre formation; and hyperplasia of the synovial membrane, leading to the formation of the invasive tissue: *pannus* [2] (Fig. 8.1). RA is a disabling disease, and it is generally estimated to decrease the life expectancy of affected individuals by 3–10 years, mostly due to cardiovascular, infectious, respiratory, gastrointestinal and renal complications [4], exacerbated by long-term medication. The prognosis of RA depends on the extent to which the disease is aggressive. Current treatment options do not

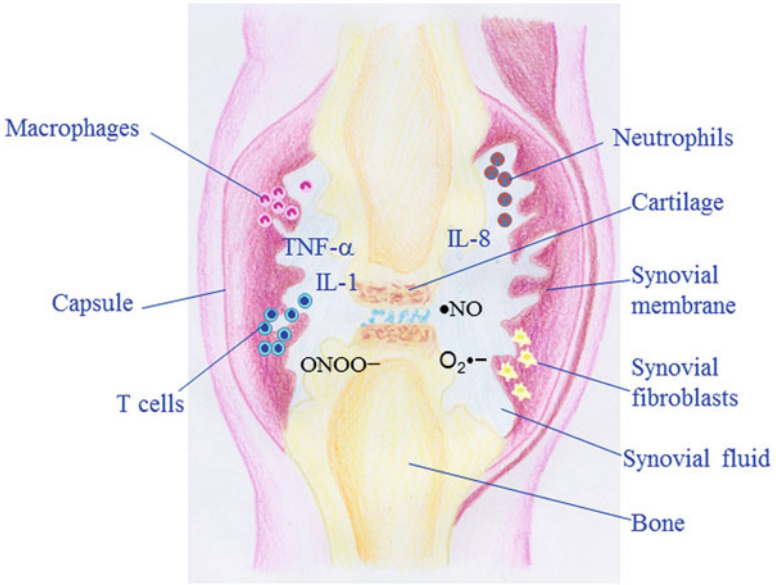

Fig. 8.1 Schematic drawing of a rheumatoid arthritis joint. The joint is infiltrated by inflammatory cells such as macrophages, neutrophils and lymphocytes and characterised by increased levels of reactive species (such as $^{\bullet}NO$, $O_2^{\bullet-}$, $ONOO^-$) and inflammatory cytokines (such as IL-1, IL-8 and TNF-α). The synovial membrane forms an invasive tissue growth with an increased number of synovial fibroblasts which, together with damage from reactive species and chronic inflammation, lead to the erosion of the joint

offer a cure but rather the management of the disease, with the main goal being to ensure minimal patient discomfort and maximal patient function. Therefore, gaining a deeper understanding of RA and identifying new therapeutic targets are still paramount, especially so in an ageing population. Studying the redox aspects of the disease contributes to a better understanding of the disease and may also lead to the identification of new therapeutic targets.

8.2 Origins of Oxidative Stress and Redox Imbalance in RA

Redox processes are crucial regulators of the immune system, and redox imbalance is a factor in RA development and maintenance [5–7].

Oxidative stress is the imbalance of pro-oxidants and antioxidants in favour of pro-oxidants [8]. Thus, "oxidative stress" is not an inevitable consequence of the mere production of reactive oxygen species (ROS) such as, for example, superoxide and peroxynitrite. The formation of ROS is, however, an inevitable consequence of oxidative metabolism as the respiratory transport chain leaks electrons, which leads to the formation of ROS.

8.2.1 The Formation of ROS, RNS, RSS

Reactive oxygen species comprise both oxygen-containing radicals and non-radical oxygen derivatives, such as superoxide ($O_2^{\bullet-}$), hydroxyl radical ($^{\bullet}OH$), peroxyl radicals ($ROO^{\bullet}$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), singlet oxygen ($^1O_2$) and peroxynitrite ($ONOO^-$) [9]. In mammals, superoxide dismutase enzymes (SODs) catalyse the conversion of $O_2^{\bullet-}$ to the less reactive H_2O_2 via an electron transfer reaction where one $O_2^{\bullet-}$ molecule acts as a reductant and the other as an oxidant ($O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$). Three human SOD forms are known: SOD1 (cytosolic or Zn, Cu-SOD), SOD2 (mitochondrial Mn-SOD) and SOD3 (extracellular Zn or Cu-SOD) [9]. Unlike superoxide, H_2O_2 can diffuse across cell membranes via aquaporin channels [10, 11]. H_2O_2 is a by-product of oxidative metabolism, but it is also actively produced in response to various growth factors and cytokines, for example, epidermal growth factor (EGF) [12], platelet-derived growth factor (PDGF) [13], transforming growth factor β1 (TGFβ1) [14], interleukin 1 (IL-1), tumour necrosis factor-α (TNF-α) [15], insulin and angiotensin II [16, 17], via NADPH oxidase (Nox) activation and also NADPH oxidase-independent mechanisms [18]. Hydrogen peroxide acts as a signalling molecule by reversibly modifying amino acids—often cysteine residues—on proteins. Hydrogen peroxide is removed by a range of redox regulator enzymes such as catalases, peroxiredoxins and glutathione peroxidases. Catalase dismutates H_2O_2 to molecular oxygen and water. Glutathione peroxidase reduces H_2O_2 to H_2O with the aid of glutathione (GSH) which is oxidised to glutathione disulphide (GSSG) in the process. The reaction centre of catalase contains haem, while glutathione peroxidase—like thioredoxin reductase—is a selenoprotein [9].

Nitric oxide ($^{\bullet}NO$) is a multifunctional molecule that plays a crucial role in several physiologic functions, such as apoptosis, inflammation, mitochondrial biogenesis and the regulation of blood vessel tone [19, 20]. $^{\bullet}NO$ regulates signal transduction by regulating Ca^{2+} signalling [21]. It is synthesised from L-arginine by $^{\bullet}NO$ synthetase enzymes (NOS): neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS). Nitric oxide regulates the cytoplasmic redox balance through the generation of peroxynitrite ($ONOO^-$) following its reaction with superoxide ($O_2^{\bullet-}$).

Hydrogen sulphide (H_2S) is a recently recognised physiological gaseous mediator which bears relevance to oxidative processes. Hydrogen sulphide is synthesised from the amino acids L-cysteine, L-homocysteine and L-cystathionine and can be oxidised to sulphite and sulphate by activated neutrophils [22]. Furthermore, crosstalk exists between different reactive species, such as H_2S and $^{\bullet}NO$ [23].

8.2.2 Sources of Reactive Species of Oxygen, Nitrogen and Sulphur in RA

ROS are reactive molecules which can cause oxidative damage to the surrounding molecules, and, importantly, oxidative DNA damage, which in turn leads to mutations. In order to prevent this damage from happening, organisms relying on

oxidative metabolism have developed an array of antioxidant enzymes such as, superoxide dismutases, catalases, thioredoxins and peroxiredoxins. In addition, dietary antioxidants such as vitamin C (ascorbic acid), vitamin E (α-tocopherol) and β-carotene help to neutralise the effects of free radicals [24].

ROS are also produced in the human body as part of a physiological defence against infections—neutrophils and macrophages kill invading pathogens by releasing these noxious ROS substances [25], although there is evidence that the role of ROS is more complex in phagocytes than oxidative destruction, and they may also regulate pH and the release of proteolytic enzymes within phagosomes [26].

The main sources of reactive oxygen species and oxidative stress in RA are (1) chronic inflammation which leads to the constant production of reactive oxygen species by activated leucocytes [27]; (2) repeated hypoxia–reperfusion cycles, superimposed on a hypoxic, ROS-rich, environment in the RA synovial joint [6]; (3) an increased metabolic rate of the synovial tissue [27]; and (4) free transition metal ions and transition metal ion-containing molecules (e.g. haem) released in the course of tissue injury which act as catalysts of free radical reactions [28]. Hypoxia–reperfusion also appears to activate the pro-inflammatory transcription factors hypoxia inducible factor-1α and nuclear factor-κB, thereby contributing to the maintenance of an inflammatory loop [29]. Together these mechanisms contribute to a vicious circle where the enzymatic and nonenzymatic antioxidant defence is overwhelmed and the excess ROS can cause a state of oxidative stress. This in turn can trigger further inflammatory signalling. However, as we will see below, RA is not necessarily characterised by constant oxidative stress at all sites and at all phases of the inflammatory response. Instead, these mechanisms are restricted in time and place, and occasionally, a more reducing rather than more oxidising environment may occur. Therefore, although oxidative stress is undoubtedly a contributing factor in RA, perhaps a more accurate concept is "redox imbalance". Furthermore, although some of the described mechanisms mainly take place in the arthritic joint, RA is a systemic disease which is also characterised by systemic inflammation and systemic redox imbalance [30].

8.3 Evidence for, and Consequences of, Oxidative Stress and Redox Imbalance in RA

Most ROS are transient chemical species, and their direct detection in biological systems is generally not possible. Instead, in human samples, the "footprints" of ROS can be detected in the form of oxidatively modified molecules [31]. Among the consequences of ROS production and oxidative stress in RA are (1) direct damage to molecules and tissues, (2) the formation of DNA mutations, (3) neoepitope formation and (4) disturbance of redox signalling pathways. Besides reactive species of oxygen, sites of inflammation are also characterised by the presence of reactive species of nitrogen and sulphur, and footprints of these species, or the species themselves, are detectable [32].

8.3.1 Direct Damage due to ROS

There is plenty of evidence for the existence of oxidative stress and the detrimental effects of ROS in RA: the presence of oxidatively modified/damaged protein, lipid and DNA products can be detected in the RA joint, for example, carbonylated proteins, oxidised low-density lipoprotein and oxidised hyaluronic acid [6, 33]. Moreover, the level of protein carbonyl groups—an indicator of oxidative stress—in the serum of RA patients also appears to be increased compared with healthy control subjects [34].

However, given the evidence listed above for the presence of oxidative stress in RA, it is important to remember that increased amounts of oxidised products may not only be due to increased levels of production but also decreased rates of removal of these products [30].

8.3.2 The Formation of Mutations due to ROS

In line with the systemic nature of chronic inflammation and oxidative stress in RA, Bashir et al. [35] detected increased levels of the mutagenic 8-hydroxy-deoxyguanosine in RA peripheral blood lymphocytes compared with healthy controls.

Mutations in the tumour suppressor gene p53 are also well documented in RA synovium and contribute to the pathogenesis of the disease by reducing apoptosis and contributing to the development of a transformed, neoplastic synovial fibroblast phenotype [27, 36].

8.3.3 Neoepitope Formation due to ROS and RNS

Oxidative modification can alter the structure and function of affected proteins [29]. Such posttranslational modifications of proteins and modifications of lipid/carbohydrate components of the body can result in the formation of "altered self" and have the potential to trigger an autoimmune response. A diagnostically relevant posttranslational modification in RA is citrullination of proteins [37]. However, oxidative modifications are also important since they have the capacity to trigger autoimmune reactions. For example, oxidatively damaged IgG and IgM can form advanced glycation end products, and serum autoantibodies against these products are associated with RA [29]. Oxidised low-density lipoprotein (oxLDL) was detected in RA synovial fluid but not in matched plasma samples [38]. The formation of oxidised low-density lipoprotein (oxLDL) upon oxidative stress in RA may partly explain the link between RA and the increased incidence of atherosclerosis, since oxLDL leads to the formation of foam cells and atherosclerotic plaques [39]. Nissim et al. [40] demonstrated that autoantibodies in sera from RA patients reacted to oxidatively modified type II collagen but only to a very small extent, compared to the native protein.

Several lines of evidence in patients with RA support an increased •NO synthesis: according to our earlier data, T cells from RA patients generate >2.5 times more •NO than healthy donor T cells, and increased •NO production is associated with increased cytoplasmic Ca^{2+} concentrations [41]. Although •NO production is significantly increased in RA, there is little information on nitrative/nitrosative modifications in this disease. The plasma levels of S-nitrosated mannose binding lectin (SNO-MBL) were higher in RA patients than in healthy control subjects, and the carbohydrate binding ability of MBL was modified by S-nitrosation. Interestingly, levels of anti-MBL autoantibodies against SNO-MBL in RA plasma samples were higher compared with normal MBL [42]. Circulating T cells in RA were reported to be responsive to IgG which had been modified by peroxynitrite [43].

8.3.4 Disturbance of Redox Signalling Pathways

Redox regulation plays an important role in inflammation and immune function. A few examples are the following: (1) H_2O_2 acts as a paracrine chemotactic signal in injured tissues (as a result of mechanical injury-activated dual oxidase activity, H_2O_2 is generated upon injury in the injured tissue, which is rapidly followed by the recruitment of leucocytes to the site of the injury) [44], (2) this H_2O_2 is detected by a single cysteine residue on the Src family kinase Lyn on neutrophils [45] and (3) the redox-sensitive cation channel TRPM2 inhibits ROS generation by phagocytes thereby decreasing inflammation [46].

A number of cell signalling pathways involve ROS, and H_2O_2 appears to be a universal second messenger [47]. An especially important mechanism of this redox regulation is the oxidative inhibition of protein tyrosine phosphatases: the catalytic cysteine residue of tyrosine phosphatases may either be directly oxidised to cysteine sulfenic acid by H_2O_2 or glutathiolated [48]. Oxidative inhibition of protein tyrosine phosphatases results in the phosphorylation and activation of downstream proteins such as the tyrosine kinases Lck, Fyn, Syk and ZAP70 (all involved in lymphocyte signal transduction).

The MAP kinases p38 and JNK and protein kinase C are also activated by ROS, and the ROS-induced activation of the transcription factors AP-1 and NF-κB is often the direct consequence of upstream oxidative regulation [48]. The redox regulation of NF-κB and AP-1 by thioredoxin is well known [30, 49]. Different receptors on T and B cells also induce intracellular ROS production. In B cells, CD40-induced activation of the JNK, p38 and Akt pathways is ROS-dependent, while BCR signalling functions even in the presence of antioxidants [50]. T cell signalling via TCR engagement also proceeds via ROS production [51].

All these pathways may be affected by alterations in redox balance as is the case in RA. Interestingly, the intracellular production of ROS appeared impaired in RA T cells upon stimulation ex vivo compared with healthy controls [30].

Levels of H_2S were elevated in RA synovial fluid compared with paired plasma and also synovial fluid from osteoarthritis patients [23]. Intriguingly, the levels were

in negative correlation with white blood cell and neutrophil count in the samples, potentially explained by the fact that neutrophils oxidise H_2S to sulphite and sulphate. Somewhat simplified, based on experimental evidence accumulated so far, H_2S appears to play a biphasic role in inflammatory signalling whereby low concentrations generally appear to inhibit inflammation, while high (potentially supraphysiological) concentrations promote inflammation [52]. However, the effects of H_2S also seem to be dependent on tissue site and disease [52, 53].

The redox regulation of immune signalling pathways relevant in RA has been discussed in great detail by Phillips et al. [30] and is also discussed in Chap. 2 of the present volume.

8.3.5 The Role of Peroxiredoxin 2 in TLR4 Signalling and Potential Links with RA

Peroxiredoxins—one of the major antioxidant enzyme families in humans [54]—have recently been implicated in Toll-like receptor signalling. Toll-like receptors (TLRs) are type I integral membrane glycoproteins, crucial for innate immune defence. They belong to the TLR/IL-1R (interleukin-1 receptor) superfamily, the members of which are characterised by a conserved intracellular TIR (Toll/IL-1R) domain [55]. The inappropriate activation of Toll-like receptor signalling, especially TLR4 signalling, has been suggested to contribute to RA [56]. TLR4 may be induced by endogenous ligands, such as damaged extracellular matrix products, present in the rheumatoid joint space, and therefore, inappropriate activation of TLR4 may contribute to maintaining arthritis [57]. Inhibition of TLR4 suppressed the disease (e.g. marked by reduced swelling, reduced IL-1β expression) in mouse models of arthritis [57].

Lipopolysaccharide (LPS) is the main activator of the TLR4 signalling pathway. Asehnoune et al. [58] examined downstream signalling events in LPS-activated neutrophils. Asehnoune et al. found that LPS-induced TLR4 signalling is ROS-dependent, since antioxidant treatment inhibited the nuclear translocation of NF-κB, production of inflammatory cytokines and a number of downstream signalling molecules such as IKK, p38, Akt, IRAK1 and IRAK4. Prdx2 appears to be a negative regulator of the LPS/TLR4-induced activation of MAPK and NF-κB pathways: LPS stimulation resulted in increased intracellular H_2O_2 levels and $^{\bullet}NO$ production in Prdx $2^{-/-}$ murine bone-marrow-derived macrophages compared with wild-type bone-marrow-derived macrophages. The production of inflammatory cytokines such as TNF-α, IL-6 and COX2 was also enhanced in $Prdx2^{-/-}$ bone-marrow-derived macrophages [59]. Prdx 2 appears to negatively regulate the downstream NF-κB signalling pathway: IκBα levels decreased while IKKα/β phosphorylation increased in Prdx2 double-negative transfected Raw264.7 cells (commercially available mouse macrophage cell line) and Prdx $2^{-/-}$ bone-marrow-derived macrophages [59].

8.3.6 ROS: Good or Bad? Lessons from Animal Models

An important and often underappreciated role of antioxidant enzymes is their role in cellular redox control. A host of processes in the human body involve redox reactions, and redox enzymes are so important in the regulation of these that, for instance, thioredoxin (Trx)-knockout mice die in utero [60]. The group around R. Holmdahl advocates a role of ROS in protecting from autoimmune diseases [26]. One of the first pieces of evidence to demonstrate that ROS can be protective came from an Ncf1 mutant mouse model (Ncf1 is the gene encoding the P47phox subunit of the NADPH complex NOX2)—characterised by decreased ROS production—which displayed increased susceptibility to severe arthritis [61]. NOX2 is expressed on phagocytes and antigen-presenting cells (APCs) and seems to play an active role in the immunological synapse [26]. T cells isolated from rodents with impaired ROS production had more surface thiols, and a lack of ROS in mice also resulted in breaking T cell tolerance to collagen type II [26]. This all appears to tie in well with the observation that T cell activation requires a reducing environment and APCs release Trx in the context of the immunological synapse in order to activate T cells. It is therefore possible that ROS are responsible for terminating an immunological synapse/T cell activation by oxidising surface thiols on the T cell surface and in the immediate proximity of the synapse. A possible role for cell surface Trx (and other reduced thiols) could then be to remove ROS, thus enabling T cell activation. Subsequently, when all reduced thiols are oxidised, ROS would be able to terminate the immunological synapse. This idea is in accordance with the observation of the negative regulatory effect of ROS on T lymphocyte activity [33]. Hitchon and El-Gabalawy [29] suggested that ROS production by phagocytes induces apoptosis of autoreactive T cells. Dendritic cells with impaired ROS production secreted increased levels of IL-1β, IL-6, TNF-α and TGF-β and induced activated T cells to secrete IFN-γ and IL-17, thus promoting the development of a Th17 phenotype [62].

However, the redox regulation of immune responses is likely to be cell-specific, that is, regulatory T cells (Tregs) are almost certainly under a redox control which is different from that in effector lymphocytes. Indeed, this is supported by the finding that different leucocyte subsets have different levels of exofacial thiols [63, 64]. Furthermore, Tregs have been shown to have reduced sensitivity to oxidative stress-induced cell death [65] which is due to an increased production of thioredoxin-1 in these cells [66]. The experimental conditions different authors use to describe different phenomena can also have an enormous impact on the findings. To demonstrate the complexity of redox control in RA, another mouse model, the Nrf2-knockout mouse which is characterised by a defective antioxidant response and hence increased levels of ROS, had more severe cartilage injury compared with wild-type mice [67, 134]. Still, the Nrf2-knockout mouse model [67] of redox control in RA is not necessarily in contradiction with the Ncf1 knockout described above. Indeed, Gelderman et al. [68] suggested a "Yin–Yang" model for the role of ROS in RA whereby ROS produced at low levels before disease onset may be preventive, whereas ROS produced in great quantities as a result of ongoing inflammation might have damaging properties.

8.4 Redox Control Enzymes in RA with an Emphasis on the Thioredoxin System and Peroxiredoxins

Since ROS, RNS and RSS can lead to damage and often to cell death if not properly controlled, aerobic organisms have evolved mechanisms to neutralise reactive species and to utilise these species for physiological processes. In humans, the major redox control enzyme systems—as also mentioned above—are superoxide dismutase [69], catalase [70], glutathione peroxidase [71], thioredoxin [72] and peroxiredoxin [73, 74].

The data on the activity of antioxidant enzymes in RA are conflicting [75]. Levels of some antioxidant enzymes and dietary antioxidants are decreased in both the plasma and synovial fluid of RA patients [6]. RA synovial fluid contains a low activity and concentration of extracellular SOD. Also, leucocytes from RA patients are deficient in Mn-SOD [76], and the concentration of catalase is extremely low in RA synovial fluid [76].

On the other hand, certain antioxidant enzymes appear to be overexpressed in RA. There is agreement in the literature about the upregulation of thioredoxin 1 (Trx1) in different compartments in RA such as plasma and synovial fluid. Thioredoxin—part of the thioredoxin system—is a multifunctional protein with a wide subcellular distribution: Trx1 is abundant in the cytosol [77], but it also translocates to the nucleus [78], associates with the cell membrane [79] and is secreted extracellularly [80]. Yoshida et al. [81] found that serum Trx1 levels were elevated in RA patients compared with osteoarthritis patients and healthy controls. Moreover, Trx1 concentration was higher in synovial fluid than in plasma in RA. Trx1 appeared to have a pro-inflammatory role as treatment of synovial fibroblast cultures with Trx1 augmented IL-1 and IL-6 production upon TNF-α stimulation. Therefore, the authors suggested that Trx1 contributes to the maintenance and aggravation of inflammation in RA. Jikimoto et al. [82] found very similar results, whereby plasma and synovial fluid Trx1 were elevated in RA patients compared with osteoarthritis patients and healthy control subjects, and the Trx1 concentration was higher in synovial fluid than in plasma. These authors also found that plasma Trx1 concentrations correlated with disease activity assessed by rheumatoid factor (RF), C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), morning stiffness, grip strength and articular index. Synovial fluid Trx1 concentrations also correlated with CRP and the number of infiltrating leucocytes. Importantly, the concentration of plasma Trx1 decreased when the disease was well managed [82], suggesting that Trx may be used as a disease activity marker. Similarly, Maurice et al. [83] described increased concentrations of extracellular Trx1 in the synovial fluid of RA patients compared with healthy control subjects and gout or osteoarthritis patients. The authors were also able to show the expression of Trx1 in infiltrating leucocytes in the synovial joints and synovial tissue. Synovial fluid Trx1 concentration was in positive correlation with the number of infiltrating leucocytes, and higher Trx1 concentrations were observed in erosive joints compared with non-erosive joints. Furthermore, TNF-α and H_2O_2 increased production of Trx1 at the protein level in synovial fibroblasts [83]. Taken together, these results all support the idea that Trx1 contributes to rheumatoid inflammation and thus has a pathophysiological role in RA.

A recent study investigated the expression in RA of Trx80 [84], which is a truncated form of Trx 1 and acts as a mitogenic cytokine and also induces a Th1 type response via the induction of IFNγ production [84–86]. RA synoviocytes expressed Trx80 without any stimulation but increased its expression and extracellular release upon TNF-α and IL-1β stimulation. Trx80 also stimulated the proliferation of lymphocytes. Thus, Trx80 also seems to have a pathophysiological role in RA via the induction of cell proliferation (synovial hyperplasia) and a pro-inflammatory effect [84].

A few studies have looked at the role of thioredoxin reductase 1 (TR1) in RA. Kabuyama et al. [87] found that TR1, along with other antioxidants such as peroxiredoxin 2 (Prdx2), catalase, superoxide dismutase 2, glutaredoxin, glutathione peroxidase 2, was upregulated at the mRNA level in RA synovial fibroblasts compared with osteoarthritis synovial fibroblasts. These authors also concluded that TR1 acts as an anti-apoptotic molecule and prevents cell death of the inflammatory synovial cells via suppressing H_2O_2 levels. Inhibition of TR1 via 1-chloro-2,4-dinitrobenzene resulted in increased H_2O_2 production in the fibroblasts and increased apoptosis in a dose-dependent manner [87].

Levels of the multifunctional redox enzyme, Prdx2, also appear to be elevated in inflammatory joint disease: Prdx2 protein expression was increased in RA synovial tissue compared with osteoarthritis tissue [88], and Prdx2 concentration was also increased in RA synovial fibroblasts compared with both osteoarthritis and healthy control fibroblasts [89]. Prdx2 expression was also elevated at the protein level in peripheral blood lymphocytes of RA patients compared with healthy subjects [64] (Fig. 8.2).

In further support of the above findings, where certain antioxidant enzymes were overexpressed in RA, an increased activity of Nrf2 was detected in the synovial cells and in subintimal adipocytes in the joints of patients with RA compared with healthy donors [67]. This suggests increased expression of certain antioxidant enzymes. However, the net effect on inflammation of such an increase is difficult to estimate, since certain antioxidant enzymes—thioredoxin being a prime example—are known to have additional functions, such as cytokine properties. Furthermore, as described above, thioredoxin has been suggested to enhance the inflammatory loop in RA. Therefore, increased oxidative stress due to ongoing inflammation in RA may trigger the increased production of antioxidant enzymes. These enzymes, however, are multifunctional and so the ongoing increased expression of, for example, thioredoxins may have some inflammatory functions, and yet at the same time may also have anti-inflammatory functions. Therefore, it is difficult to calculate the net effect of antioxidant enzymes in RA, and protective vs. pathological functions are likely to be specific to subcellular compartments and cells/tissues.

8.5 Cell Surface and Extracellular Thiols in RA

Although the extracellular environment is generally oxidising, reduced thiols are still present on mammalian cell surfaces [63], and the importance of the oxidation state of surface thiols (thiol or disulphide) for their functional characteristics was

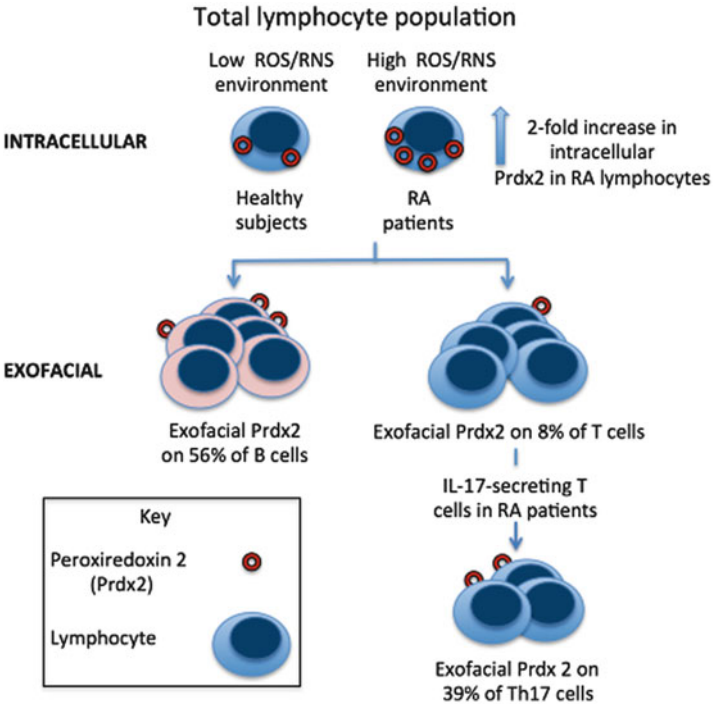

Fig. 8.2 A basic summary of some of the major lymphocyte subsets in relation to the levels of intracellular and exofacial Prdx2 in RA patients [64]. The diagram summarises the key results found in a recent study by Szabó-Taylor et al. [64], comparing healthy subjects with RA patients. For simplicity, a number of subtypes of T cell have been omitted from the figure. Prdx2 is schematically shown as a *red ring*, representing the crystal structure of the decameric human red blood cell Prdx2 [133]. It is unknown if the exofacial Prdx2 is in the decameric or a different oligomeric form. Intracellular Prdx2 protein content was elevated in RA lymphocytes compared with lymphocytes from healthy subjects. The median proportion of B cells displaying exofacial Prdx2 and Trx1 was higher compared with $Prdx2^{+ve}$ and $Trx1^{+ve}$ T cells in both the RA and healthy subjects. The indicated values of 56% $Prdx2^{+ve}$ B cells and 8% $Prdx2^{+ve}$ T cells are median values of percentages obtained from all the analysed peripheral blood lymphocyte preparations ($n = 10$; made up of the pooled values from the 5 RA patients and 5 healthy subjects). The median proportion of Th17 cells displaying exofacial Prdx2 was higher compared with the median proportion of $Prdx2^{+ve}$ cells in the total lymphocyte population in RA patients (*Th*: T helper cell). Reproduced from Szabó-Taylor et al. [64] with permission

recognised decades ago [90]. Many protein thiols on the cell surface are exposed, and their oxidation state—which affects their function—is highly sensitive to exposure to oxidising or reducing agents [91]. The redox state of surface thiols also appears to bear relevance to RA. Lawrence et al. [63] used a thiol-reactive reagent coupled with a fluorescent dye to detect differences between lymphocyte subsets with regard to their surface thiol levels: $CD19^{+ve}$ cells had highest surface thiol levels, followed by $CD8^{+ve}$ T cells, and then by $CD4^{+ve}$ T cells. Furthermore, mitogenic

activation increased surface thiol levels. Notably, an increase in surface thiol levels was not a consequence, but a prerequisite for increased cell proliferation [63]. Indeed, addition of *N*-ethylmaleimide before or simultaneously with mitogens such as phytohaemagglutinin or concanavalin A was able to completely inhibit stimulation of T lymphocytes [92].

Intriguingly, although an increase in surface thiol levels has been associated with cell activation, Pedersen-Lane et al. [93] showed that surface thiol levels of leucocytes in peripheral blood were significantly lower in RA patients compared with healthy controls. In contrast, Gelderman et al. [94] found that impaired ROS production contributed to an increase in leucocyte surface thiol levels and increased the susceptibility of rats to develop severe arthritis. Although superficially contradictory, these findings are not mutually exclusive. One of these studies [93] looked at primary leucocytes from RA patients with a disease duration of at least 6 months, while the other study [94] investigated the sensitivity of rodents to develop arthritis but did not follow up the surface thiol status of leucocytes once the disease developed. Moreover, the RA patients in one study [93] were treated with different RA drugs, while the experimental rodents were untreated. It is known that some RA drugs, notably methotrexate, induce oxidative stress [95], which could lead to a depletion of leucocyte surface thiols. Therefore, although there is plenty of evidence for oxidative damage in RA, at the same time, certain inflammatory cell populations are activated, which is associated with a reducing environment.

Surface thiols not only contribute to the maintenance of redox balance on the cell surface but are also necessary for cell signalling and cell proliferation, and certain surface molecules such as CD4 and the IL-2 receptor are thiol modulated [63].

Thioredoxin has been identified on the surface of different cell types such as a human monocytic cell line, human lymphocytes, macrophages, fibroblasts and endothelial, epithelial and neuronal cells [79]. In another study [96], lymphocyte, granulocyte and monocyte cell lines expressed both full-length Trx and a truncated Trx species both intracellularly and on their surface, but the truncated form was mostly localised on the cell surface. Truncated Trx (also known as Trx 80) has cytokine-like functions whereby it enhances eosinophil cytotoxicity and it also stimulates B cell activity [96]. Trx 80 activates monocytes and induces the formation of a special type of monocyte, termed the thioredoxin 80-activated monocyte (TAM). TAMs have higher levels of exofacial pathogen recognition receptors and T cell activating molecules. They also display increased pinocytosis and release pro-inflammatory cytokines. TAMs are more efficient at inhibiting intracellular pathogens than monocytes not primed by Trx 80, due to their increased capacity for phagocytosis and lysosomal degradation [97].

Full-length redox-active exofacial Trx1 on endothelial cells appeared to play a role in the attachment of leucocytes to endothelial cells, as an anti-Trx1 antibody and a redox-active—but not a redox-inactive (Cys35Ser mutant)—recombinant Trx1 inhibited the attachment of activated leucocytes to endothelial cells [98]. Therefore, cell surface Trx1 seems to play a role in the inflammatory response, and the active site cysteine residue is crucial for this process [98].

Importantly, Prdx2 was also found on the exofacial surface of lymphocytes from healthy human subjects and in RA patients. A high proportion of IL-17-secreting cells displayed exofacial Prdx2 compared with the total lymphocyte

population, suggesting that Prdx2 may be involved in the activity of these inflammatory cells [64].

Not only surface thiols but also the redox potential of the extracellular environment is important in leucocyte function. Lymphocytes need free thiols in their extracellular environment for both activation and proliferation, and although CysSS is normally abundantly available in the extracellular space, lymphocytes lack an efficient CysSS transporter [99]. Mature dendritic cells generate free thiols (mainly through the reduction of CysSS) even in resting state, but their thiol release increases several fold during antigen-specific interaction with T lymphocytes [99]. Therefore, extracellular redox-active molecules are implicated in inflammation and rheumatoid arthritis.

Trx is secreted from different cell types (e.g. fibroblasts, airway epithelial cells, certain lymphoblastoid cell lines, activated T and B cells) using a leaderless secretory pathway [100]. Similarly, TR was also reported to be secreted into the plasma but using the classical Golgi pathway [101]. Except for Prdx4, Prdxs lack known secretory signals and seem to be secreted from cells using a nonclassical secretory pathway. This is supported by the fact that the secretion of Prdx1 could not be inhibited by brefeldin A, an inhibitor of the classical ER/Golgi secretory pathway [102]. A recent study proposed that Prdx1 and Trx1 are secreted in a caspase-1-dependent manner [103]. Upon cellular stress (e.g. UV radiation, injury), inflammasome-induced activation of the "inflammatory" caspase, caspase-1, induces the secretion of a number of proteins, most of which are involved in inflammation, cytoprotection and tissue repair such as IL-1α and fibroblast growth factor 2 [103]. Therefore, it is feasible that Prdxs and Trx1 are also secreted in an inflammatory context [103].

A number of roles have been assigned to extracellular Trx1. Secretory Trx has a synergistic effect on mitogen/cytokine-induced lymphocyte proliferation [104]. It is necessary for the transmigration of neutrophils across airway epithelium [105] and interacts with several plasma proteins. One of these is complement factor H, an inhibitor of complement [106]. Secreted Trx 1 is a chemoattractant for neutrophils, monocytes and T cells [107], but it also acts as a growth factor on different cell types, including lymphocytes and certain tumour cells [108]. The presence of an excess of extracellular Trx—in contrast to normal (lower) plasma concentrations—inhibited the extravasation of neutrophils [106]. Trx acted by interfering with the function of endothelial cell surface Trx, which normally acts to aid neutrophil adhesion [106]. In the context of an immunological synapse, dendritic cells activate T cells, and T cells induce dendritic cells to release Trx [99]. Trx1 regulates CD4 on the surface of Th lymphocytes [109]: the D2 domain of CD4 is redox-sensitive, and Trx1 secreted by CD4 T cells regulates its activity. Locking both CD4 and Trx in the reduced state inhibited the entry of HIV-1 to the cells [109]. Extracellular Trx also binds to the TNF receptor superfamily member CD30 and interferes with the binding of the CD30 ligand CD30L [110].

Very little is known about secreted Prdxs with regard to RA. However, secretion of Prdxs has been described in other inflammatory diseases, for example, HIV infection [111], severe acute respiratory syndrome patients [112] and lung cancer [113]. Prdx2 was detected in the plasma and synovial fluid of RA patients, but its role is presently unknown [64].

8.6 Redox Processes and Apoptosis in RA

The lack of apoptotic clearance of inflammatory immune cells and synovial fibroblasts has been implicated in RA [114], resulting in synovial hyperplasia and chronic inflammation. RA fibroblast-like synoviocytes undergo tumour-like transformation (or "stable activation" using the term of Korb et al. [115]), contributing to disease by the formation of an aggressive tissue growth. These cells are able to sustain an activated, inflammatory phenotype even in vitro and go on to produce inflammatory cytokines and growth factors [115]. These RA fibroblasts are also resistant to apoptosis which considerably contributes to disease pathology. High levels of endogenous Fas-induced apoptosis inhibitors such as $^{\bullet}$NO, sentrin and matrix metalloproteinase-3, as well as high local concentrations of anti-apoptotic cytokines, such as IL-1, TGFβ1, bFGF and TNF-α, all contribute to the abnormality of the Fas-induced apoptosis pathway in RA [116].

$^{\bullet}$NO is a bifunctional regulator of apoptosis. Both pro-apoptotic and anti-apoptotic effects of $^{\bullet}$NO are known [117, 118]. $^{\bullet}$NO inhibits mitochondrial respiration (possibly by binding to the oxygen binding site of cytochrome oxidase). Thereafter, $^{\bullet}$NO exposure may lead to decreased intracellular ATP levels, leading to necrosis rather than apoptosis [119]. Peroxynitrite also inhibits mitochondrial respiration and induces mitochondrial permeability transition. Consequently, the release of intracellular structures (such as citrullinated proteins) during necrosis may trigger autoimmune reactions leading to chronic inflammation in RA.

To underline the importance of redox status with regard to apoptosis in RA, a remarkable study by Cross et al. [120] investigated the apoptosis of neutrophils in RA. These authors found that the rates of apoptosis of RA joint neutrophils were accelerated ex vivo. They also found that RA synovial fluid contained a mixture of pro- and anti-inflammatory cytokines, which had a pro-apoptotic effect on neutrophils ex vivo under normoxic conditions (21% O_2). However, when added to neutrophils under hypoxic (1% O_2) conditions (which are known to exist in the RA joint), synovial fluid was anti-apoptotic. This study suggests that local oxygen tension in the RA joint is crucial for neutrophil survival [120].

Enzymes of the peroxiredoxin-based system are often considered anti-apoptotic, and numerous studies have looked at the roles of the Prdx-based system in apoptosis. Prdx2-transfected Molt4 leukaemia cells were resistant to etoposide or serum deprivation-induced apoptosis, while non-transfected cells readily underwent apoptosis, suggesting that Prdx2 acts as an anti-apoptotic factor [121]. In a similar experiment, using 2D-PAGE, Wang et al. [122] showed that radiation-resistant MCF-7 breast cancer cells overexpressed Prdx2 at the protein level. By knocking down Prdx2 in the resistant cells, using siRNA, they were able to induce partial radiation sensitivity. At the same time, by transfecting radiation-sensitive MCF-7 cells with Prdx2, they were able to induce apoptosis resistance in these cells [122]. Moon et al. [123] showed that Prdx2 protects cells from oxidative stress-induced apoptosis by forming high-molecular-weight chaperone complexes. In Prdx2-transfected endothelial cells, Prdx2 protected these cells from a chemotherapeutic agent (CT-2584) that stimulates ROS-mediated apoptosis [124].

RNA interference-induced depletion of Prdx3 resulted in increased H_2O_2 levels and enhanced sensitivity to TNF-α and staurosporine-induced apoptosis in HeLa cells [125]. In an earlier study, the abrin a-chain was shown to interact with Prdx3, inhibiting its antioxidant activity and leading to increased cytochrome c release, the activation of the caspase cascade and apoptosis [126]. In Fas- and/or TNF-α-treated Jurkat cells, the majority of the Prdx3 pool underwent oxidation in the early phase of apoptosis, resulting in an increase of mitochondrial ROS [127]. Wonsey et al. [128] found that knockdown of Prdx3 in the MCF7/ADR breast cancer cell line led to reduced mitochondrial membrane potential and increased c-Myc-mediated apoptosis upon glucose deprivation.

These studies raise the possibility that the targeting of Prdxs may be harnessed for therapeutic purposes in RA.

8.7 Therapeutic Targeting of Redox Control in RA

8.7.1 Antioxidant Supplementation

Based on the above evidence, a number of studies proposed that chronic inflammation in RA—by inappropriately producing large quantities of ROS—leads to the exhaustion of antioxidants. Indeed, lower ascorbic acid and α-tocopherol levels were detected in the plasma and synovial fluid of RA patients compared with healthy control subjects [24]. This led to the idea of dietary antioxidant supplementation in RA patients by nutraceuticals containing large doses of vitamins C and E. However, scientists had to reconsider this idea as recent studies concluded that there is little evidence to support the therapeutic benefits of such antioxidant supplementation in RA, and it is also still unclear whether supplementation with large doses of a single antioxidant can restore physiological antioxidant function [24]. A handful of studies suggested that there is a causative relationship between low antioxidant levels and RA development [24]. However, it is also feasible that low levels of antioxidants are the result of the disease process. Some studies even suggested that antioxidant supplementation can be harmful by generating reactive radicals and interfering with cell signalling pathways [24].

8.7.2 Thioredoxin Administration

Thioredoxin overexpression appears to have a pro-inflammatory effect in RA (see above). Counter-intuitively, however, in several animal model studies of inflammatory diseases, the administration of extracellular, recombinant, redox-active Trx actually improved the condition of the animals [129–132]. Therefore, Trx might be considered as a potential therapeutic tool for rheumatoid arthritis

(reviewed by Nakamura et al. [106]). Thioredoxin is a functionally versatile molecule and appears to fulfil vastly different roles, depending on its tissue and subcellular localisation, redox state, alternatively spliced forms and posttranslational modifications. Therefore, statements in the literature such as "Trx is pro-inflammatory" or "anti-inflammatory" can be misleading. It is important to point out that recombinant Trx administered intravenously into experimental animals did not appear to be metabolised and was eventually excreted into the urine [106]. Therefore, any therapeutic effects of exogenous Trx are caused by the increased levels of circulating Trx. Nakamura et al. [106] suggested that the beneficial effects of exogenously administered Trx may be caused by its ability to inhibit complement activation (since Trx interacts with complement factor H to suppress C3a production). Administered Trx competitively inhibits endogenous exofacial thioredoxin, inhibiting macrophage migration inhibitory factor (since the ability of MIF to enter cells depends on exofacial Trx), and leucocyte adhesion in the circulation. In addition, it appears that although lower (physiological) concentrations of circulatory Trx act as a chemotactic signal for leucocytes, higher concentrations have the opposite effect [106].

8.7.3 Oxidative Burst-Inducing Substances

As mentioned above, the group of Holmdahl advocates a role for ROS in preventing the development of RA [26]. Indeed, the administration of phytol—an oxidative burst-inducing substance—in rodents had a prophylactic effect and inhibited development of pristane- or collagen-induced arthritis when administered before onset of the disease. Furthermore, administering the substance to rodents with ongoing disease had the capacity to decrease disease severity [68].

8.8 Outlook

The redox dysfunction in RA is intricate and complex, and while antioxidant therapies in RA have been disappointing, we now appreciate that redox processes drive immune responses, the cell cycle and apoptosis. A better understanding of redox dysregulation in RA may result in direct therapeutic benefits by selectively targeting these processes. A novel and promising target in this quest is the peroxiredoxin enzyme family. Prdx2 has recently been shown to be overexpressed in RA [64]. Future studies will explore the potential therapeutic value of this finding.

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Chapter 9
Oxidative Stress and Premature Atherosclerosis in Rheumatoid Arthritis

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Abstract In rheumatoid arthritis (RA), there is an imbalance between the antioxidant defensive system and the production of reactive oxygen species (ROS) that leads to a state of oxidative stress. Increased oxidative stress is hypothesized to both initiate and propagate inflammation in RA. On the other hand, premature atherosclerosis is responsible for an excessive cardiovascular morbidity and mortality in these patients. Moreover, recent studies show that RA, by itself, is an important and independent cardiovascular risk factor. The oxidation hypothesis of atherosclerosis seems to play an important role in the development of cardiovascular disease (CVD). In the later years, several oxidation-related pathways have been identified in chronic and autoimmune inflammatory diseases addressing this hypothesis. Thus, and beyond traditional risk factors, oxidative stress is proposed as one of the mechanisms underlying premature atheroma formation in RA.

Abbreviations

AGE	Advanced glycation end-products
Anti ox-LDL	Antibodies against ox-LDL
β_2-GPI	β_2-glycoprotein I
cIMT	Carotid intima–media thickness
CRP	C-reactive protein
CV	Cardiovascular
CVD	Cardiovascular disease
DM	Diabetes mellitus
esRAGE	Endogenous secretory RAGE
HDL	High-density lipoprotein

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LDL	Low-density lipoprotein
Lp(a)	Lipoprotein (a)
Lp-PLA_2	Lipoprotein-associated phospholipase A_2
ox-LDL	Oxidized LDL
ox-Lp(a)	Oxidized Lp(a)
RA	Rheumatoid arthritis
RAGE	The receptor for advanced glycation end-products
ROS	Reactive oxygen species
sRAGE	Soluble RAGE
Tx	Thromboxane

9.1 Introduction

Rheumatoid arthritis (RA) is a systemic inflammatory autoimmune disease that affects approximately 1–2% of the population worldwide. Among extra-articular manifestations, premature atherosclerosis is responsible for an excessive rate of cardiovascular morbidity and mortality [1, 2]. This high risk of cardiovascular disease (CVD) has led RA to be considered as a new, independent cardiovascular risk factor similar as that of diabetes mellitus (DM) [3–5] even suggesting that specific target values for traditional risk factors should be established in RA patients. The inflamed rheumatoid synovium and the unstable atherosclerotic plaque share some local and systemic features like the accumulation of inflammatory monocytes, macrophages and T-cells or the production of inflammatory cytokines like TNF-α and IL-6 [6]. When phagocytic cells, such as macrophages and neutrophils, are activated, they undergo an oxidative burst that produces highly toxic reactive oxygen species (ROS) aimed at killing the invading pathogens. The term oxidative stress is used to describe situations in which the organism production of oxidants exceeds the capacity to neutralize them. Finally, oxidative stress and inflammation are deeply interrelated, as different oxidant-free radicals are generated by phagocytic cells in response to inflammatory stimuli: both are related to endothelial dysfunction, as the endothelium is a source and a target of oxidants and participates in the inflammatory response (Fig. 9.1).

9.2 Clinical and Subclinical Atherosclerosis in RA

Several studies have shown that RA by itself should be considered as an independent risk factor for cardiovascular disease (CVD). Overall, a recent meta-analysis of 24 studies showed a 50% increased risk of CV death [7]. Del Rincon et al. showed a high incidence of cardiovascular events in an RA cohort even after adjusting by traditional cardiovascular risk factors [8]. In the Nurses Health Study, traditional cardiovascular risk factors were similar between RA patients and the control group,

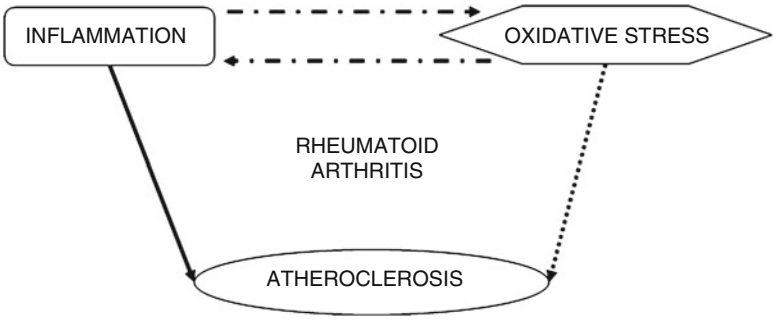

Fig. 9.1 Inflammation and oxidative stress are interrelated in RA atherogenesis

but the adjusted relative risk for myocardial infarction was 2.0 [2]. In a Dutch study, nondiabetic RA patients ($n=294$), patients with type-2 DM ($n=194$), and nondiabetic individuals ($n=258$) were compared. The prevalence of CVD was higher in RA and diabetic patients compared to nondiabetics (13% and 12%, respectively, versus 5%). The gender and age adjusted odds ratios for CVD were 3.1 in RA patients and 2.3 in subjects with DM in comparison to nondiabetics [9]. RA patients have also shown a 2-fold increased risk of congestive heart failure, a 2-fold increased risk of sudden death, and a 1.7-fold increased risk of strokes [1, 10, 11]. Moreover, in contrast to the general population, the risk of CVD in RA patients is particularly increased in younger patients and females [2, 12].

Subclinical early and accelerated atherosclerosis has also been detected as evidenced by carotid intima–media thickness (cIMT) and coronary calcium [13–17]. Finally, RA atheroma also shows distinct histologic features than plaques from non-RA controls like a higher degree of inflammation and instability [18].

9.3 Systemic Inflammation and Oxidative Stress in RA-Accelerated Atherosclerosis: Dependent or Independent Mechanisms?

Beyond traditional risk factors for CVD seen in the general population, both inflammation and oxidative stress seem to play a central role in the development of the premature atherosclerosis seen in RA patients [8]. Increased oxidative stress is hypothesized to be important in the pathogenesis of RA [19]. Furthermore, patients with active RA are found to be exposed to greater amount of oxidative stress [20–26]. There is growing evidence, from experimental and clinical studies, that oxidative stress may also be implicated in the pathogenesis of the atherosclerotic CVD. In the general population, increased concentrations of F2-isoprostanes are associated with coronary artery calcification and cIMT and also with the presence and severity of coronary artery disease [27–29]. Indeed, even when RA patients have the disease well controlled, oxidative stress may still be increased and contribute to the development

of atherosclerosis. Rho et al. recently reported increased F2-isoprostane excretion in RA patients which was independent of oxidative stress-related risk factors and was associated with a greater severity of coronary calcification [30].

9.4 Oxidative Stress Contributes to Accelerated Atherosclerosis in RA Through Several Mechanisms

9.4.1 Interaction with Lipoproteins' Physiological Role

9.4.1.1 Oxidation of Lipoproteins

Chronic local and systemic production of inflammatory cytokines promotes lipolysis, and the systemic release of free fatty acids contributes to the dyslipidemia seen in RA. On the other hand, oxidative stress arising from inflammatory reactions leads to the oxidative modification of lipoproteins which seems to play a pivotal role in the pathogenesis of the atherosclerotic lesion [31]. Several lines of evidence showed that oxidized low-density lipoprotein (ox-LDL) and oxidized lipoprotein (a) [ox-Lp(a)] induce intracellular cholesteryl ester accumulation in macrophages which leads to their transformation into foam cells after being taken up by the scavenger receptor pathway [31, 32]. Both of the oxidized lipoproteins induce adhesion molecules expression on monocytes, promoting their recruitment and adhesion to the endothelium [31] contributing to functional impairment of the vasomotor properties of involved arteries [33]. Susceptibility of LDL to oxidation has been found to be increased in patients with RA. Ox-LDL has been detected in the synovium and synovial fluids of RA patients [34]. Also, increased ox-LDL plasma concentrations have been identified in these patients as a risk factor for cardiovascular disease [35].

Lipoprotein(a) [Lp(a)] is an atherogenic particle that structurally resembles a low-density lipoprotein (LDL) particle but contains a molecule of apolipoprotein(a) [apo(a)] attached to apoB-100 by a disulfide bond, so elevated plasma concentrations of Lp(a) have been shown to be one of the independent risk factor for atherosclerosis [36]. Ox-Lp(a) has been reported to play more potent role than its native counterpart in atherosclerosis. In addition, *in vitro* oxidative modification increases the inhibitory effect of Lp(a) on plasminogen binding to cell surfaces, which could attenuate fibrinolytic activity by reducing plasminogen activation [37]. Increased plasma levels of both Lp(a) and ox-Lp(a) have been found in active RA suggesting their involvement in RA atherothrombogenesis [38].

Recently, it has been reported that ox-LDL interacts with β_2-glycoprotein I (β_2-GPI), a plasma protein with anticoagulant properties and also a major antigen for anticardiolipin antibodies. They form stable and possibly pathogenic β_2-GPI-ox-LDL complexes in the arterial intima of atherosclerotic lesions and are avidly taken up by macrophages via anti-β_2-GPI autoantibody-mediated phagocytosis [39]. Increased concentrations of β_2-GPI-ox-LDL complexes have been found in the sera of patients

with atherosclerosis and some inflammatory diseases and also in atherosclerosis-prone mice with apoE−/− and LDL−/− genotypes, especially those fed a high-cholesterol diet [40–42]. Their presence in RA patients is controversial. One study found undetectable β_2-GPI-ox-LDL plasma concentrations while the concentrations of IgM antibody against β_2-GPI-ox-LDL were significantly increased [43]. However, another study found increased β_2-GPI-ox-LDL together with increased ox-LDL concentrations, and both exhibited a positive correlation with CRP, suggesting an active disease.

Lp(a) and mainly ox-Lp(a) also form complexes with β_2-GPI *in vivo*, and serum β_2-GPI-Lp(a) complexes are increased in patients with systemic lupus erythematosus, an autoimmune disease with a high incidence of CVD [44]. Increased β_2-GPI-Lp(a) complex concentrations have also been found in RA patients supporting its hypothetical involvement in RA CVD.

9.4.1.2 Pro-inflammatory HDL

More than lipid levels, lipoprotein content is important in the development of inflammatory vascular lesions [45]. Normal HDL removes ROS from LDL, preventing both the oxidation of LDL and the recruitment of inflammatory mediators [46, 47]. During inflammation, serum HDL can become pro-inflammatory [45] with replacement of its antiatherogenic components such as apolipoprotein-AI (Apo-AI) or paraoxonase 1 by proatherogenic components such as serum amyloid A, ceruloplasmin and oxidized lipids [48]. Pro-inflammatory HDL does not protect LDL from oxidation. Recently, this dysfunctional form of HDL has been associated with the increased risk of coronary atherosclerosis seen in rheumatic diseases [47]. Furthermore, McMahon et al. reported the presence of pro-inflammatory HDL in 45% and 20% of SLE and RA patients, respectively [49].

On the other hand, the effects of oxidative processes on HDL cholesterol are less well defined. Increased levels of HDL along with elevated urine F2-isoprostane excretion has been reported to be associated with coronary calcification in RA patients, suggesting that oxidative stress may counteract the protective effect of HDL against atherosclerosis [30]. Besides, recent studies have shown that HDL is the major lipoprotein carrier of F2-isoprostanes [50]. Therefore, further clinical studies are needed in order to define the relationship between increased oxidative stress, CVD and dysfunctional HDL in patients with RA.

9.4.2 Contribution to Autoimmune-Mediated Atherosclerosis: Antibodies against Oxidized Lipoproteins

The adaptive response is enhanced by oxidative processes which increase antibody and T-cell dependent immune response. On the other hand, autoimmune diseases are characterized by the abrogation of self-tolerance and the presence of autoantibodies

serve in most of them as markers of this state. Oxidative phenomena are implicated not only in the atherogenicity but also in the immunogenicity of lipoproteins. The process of lipid peroxidation releases aldehydic products of lipid peroxidation that can form adducts with amino acids. Aldehyde-modified proteins are highly immunogenic. Thus, oxidation of LDL induces a specific immune response, and autoantibodies directed against epitopes in the ox-LDL molecule have been found in human sera [51]. These autoantibodies have been related to the atherosclerotic process, and specific immunoglobulins against ox-LDL have been found in atherosclerotic lesions in man [52]. Furthermore, antibodies against ox-LDL (anti ox-LDL) have been described in a variety of diseases with several manifestations of atherosclerosis [53, 54].

Anti ox-LDL has been found in RA as a risk factor for cardiovascular disease [55]. Higher levels of these antibodies have been detected in early RA patients with carotid plaques with respect to those without [56]. CV risk linked to anti ox-LDL in RA may both depend on the degree of inflammation and/or the presence of a pro-oxidative status. Thus, anti ox-LDL levels have shown a strong positive correlation with CRP levels, an acute phase reactant which is also known to be involved in plaque generation [57]. In addition, early RA patients have shown elevated anti ox-LDL associated with low activity of the total plasma lipoprotein-associated phospholipase A_2 (Lp-PLA_2) also known as platelet-activating factor acetylhydrolase [58]. This enzyme seems to be involved in inflammatory reactions, oxidative modulation and atherosclerosis [59]. Monocytes are the main cellular source of plasma Lp-PLA_2 which is secreted during their differentiation into macrophages and inhibited by most of the inflammatory mediators [60, 61]. During LDL oxidation, Lp-PLA_2 catalyzes the hydrolysis of LDL content in oxidized phospholipids. Finally, LDL-associated Lp-PLA_2 activity together with the extent of LDL oxidation has shown to significantly influence anti ox-LDL titres in patients with stable angina [62]. Whether antibodies reacting against oxidatively modified LDL might directly modulate the atheromatous process or whether in turn just be a marker of the presence of ox-LDL in vivo is yet to be clarified.

9.4.3 The Receptor for Advanced Glycation End-Products

Oxidative stress is associated with sequential processes that generate advanced glycation end-products (AGE) that are damaging to and may modify proteins like IgG. Pentosidine, an AGE modification product which cross-links lysine and arginine, was elevated in 50% of patients with RA, and these increased serum and urine pentosidine levels correlated with clinical disease activity [63]. A novel pathway which seems to be involved in the pathogenesis of both autoimmune inflammatory diseases and atherothrombosis is the receptor for advanced glycation end-products (RAGE). The ligand-RAGE axis is a critical amplifier of the inflammatory response. RAGE signaling results in enhanced generation of ROS and further pro-inflammatory cytokine transcription [64]. Soluble (s) RAGE isoforms including the endogenous

secretory (es) RAGE may counteract RAGE-mediated pathogenesis by acting as decoys. sRAGE and esRAGE are inversely associated with atherosclerosis and cardiovascular risk [65, 66]. Reduced plasma sRAGE and esRAGE levels have been seen in RA possibly reflecting some degree of inflammation [67, 68].

On the other hand, platelets are an important source of inflammatory mediators involved in RA pathogenesis [69], and activated platelets seem also to play a key role in the development of atherothrombosis [70]. In addition, lipid peroxidation and inflammatory mediators have been shown, in several clinical settings, to be critical determinants of *in vivo* thromboxane (TX) biosynthesis [71]. This TX-induced biosynthesis may be dependent on platelet activation and/or inflammatory cells COX-1 and COX-2 production. The hypothesis of enhanced oxidative stress and platelet activation in RA has been separately addressed by several studies [72, 73]. More recently, Ferrante et al. have found *in vivo* a significant direct correlation between enhanced TX biosynthesis and increased markers of lipid peroxidation in patients with RA [68]. In the same study, authors described an inverse association with esRAGE levels. Taking all this into account, they hypothesized that RAGE hyperactivity and enhanced Tx biosynthesis both associated with oxidative stress may provide a link between chronic inflammation and atherothrombosis in RA.

9.4.4 Hyperhomocysteinaemia

Congenital homocystinuria associated with severe hyperhomocysteinaemia is associated with early atherosclerosis and extensive arterial thrombosis [74]. Several potential mechanisms have been identified including impairment of endothelial function [51], production of ROS and consequent oxidation of low-density lipoproteins [75, 76], increased monocyte adhesion to the vessel wall [77], increased lipid uptake and retention [77], activation of the inflammatory pathway [78], stimulatory effects on smooth-muscle proliferation [77], thrombotic tendency mediated by activation of coagulation factors [79], hypofibrinolysis [80], and platelet dysfunction [81].

Hyperhomocysteinaemia is among the factors believed to contribute to accelerated atheroma formation in patients with RA. Moderate homocysteine concentrations have been reported in 20–42% of patients with RA [82–85] and are associated with an increased risk of coronary heart disease [82, 83, 86]. Hyperhomocysteinaemia is usually associated with folate and vitamin B_{12} deficiency in these patients. In addition, treatment with methotrexate, frequently used in RA, may increase homocysteine by interfering with folate metabolism [87].

Immune activation might contribute to hyperhomocysteinaemia. Homocysteine has been found to accumulate in supernatants of stimulated peripheral blood mononuclear cells [88]. Furthermore, association of higher homocysteine and lower folate concentrations with increased levels of immune activation markers have been found in RA patients [84]. In fact, enhanced demand for B vitamins is produced during proliferation of immunocompetent cells. Also, increased consumption of B vitamins including folate is produced by ROS formed by macrophages during Th1-type

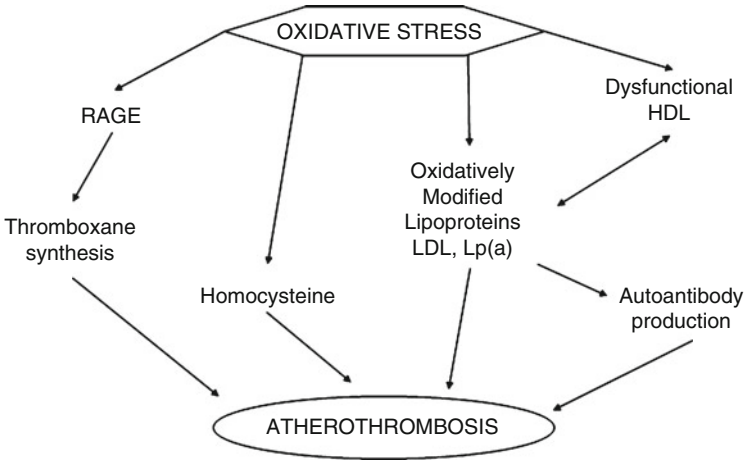

Fig. 9.2 Oxidation-dependent mechanisms in RA atherogenesis

immune response [89]. Interestingly, RA patients under regular treatment with non-steroidal anti-inflammatory drugs show higher concentrations of vitamin B_{12} than those untreated [84]. Therefore, hyperhomocysteinaemia-related atherosclerosis in RA might well reflect a depletion of B vitamins more due to a pro-inflammatory and pro-oxidative status than to a reduced intake or a metabolic blockade.

9.5 Summary and Conclusions

Oxidative stress has been shown to play a pivotal role in the initiation and development of rheumatoid arthritis, a chronic inflammatory autoimmune disease with an excess cardiovascular morbidity and mortality. Oxidative phenomena are also involved in the development of atherosclerosis. Inflammation and oxidation have shown to be deeply interrelated, but even when the disease activity is under control, some degree of oxidative stress escapes the loop and can be detected. Several atherothrombotic pathways linked to oxidation have been identified in RA, supporting the role of oxidative phenomena in the pathogenesis of the vascular damage seen in this disease. Thus, oxidative modification of lipoproteins increases their atherogenicity by interfering with their physiological role and inducing an autoimmune-specific response. AGE generation and RAGE hyperactivity facilitates thromboxane synthesis and may facilitate acute cardiovascular event. Finally, B vitamin depletion by ROS increase homocysteine levels favoring CVD (Fig. 9.2). In conclusion, therapeutic strategies aimed at correcting not only the inflammatory cascade but also the disturbance between the prooxidant–antioxidant balance might counteract the mechanisms involved in the development of RA premature atherosclerosis.

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Chapter 10
Role of Oxidative Stress and Reactive Oxygen Radicals in the Pathogenesis of Systemic Sclerosis

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Abstract Systemic sclerosis (SSc) is an autoimmune connective tissue disease which causes progressive fibrosis of skin and numerous internal organs. Although the etiology of SSc is unknown and the detailed mechanisms responsible for the fibrotic process have not been elucidated, there is strong evidence to support the concept that oxidative stress mediated by an excessive generation of oxidative free radicals plays a crucial role. Elevated levels of markers of oxidative stress and reduced levels of antioxidants have been found in SSc patients, and the most commonly studied animal models of SSc are induced by chemical agents that generate oxidative stress. In this chapter, the available evidence for the participation of oxidative stress in SSc pathogenesis will be reviewed emphasizing the link between free radicals and the process of fibrosis and the potentially beneficial effects of antioxidant treatment for the disease.

Abbreviations

ADA	Adenosine deaminase
Col I	Type I collagen
ECM	Extracellular matrix
ECS	Extract from fruits of *Capparis spinosa* L.
EGCG	Epigallocatechin-3-gallate
ERK1/2	Extracellular signal-regulated kinase 1/2
ET-1	Endothelin 1
IPF	Idiopathic pulmonary fibrosis
NAC	*N*-acetylcysteine

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NOX	NADPH oxidase
PDGFR	Platelet-derived growth factor receptor
PG	Prostaglandin
((PHTE)(2)NQ)	2,3-bis(phenyltellanyl)naphthoquinone
PTP1B	Protein tyrosine phosphatase 1B
ROS	Reactive oxygen species
SOD	Superoxide dismutase
SSc	Systemic sclerosis
TGF-β1	Transforming growth factor-β1

10.1 Introduction

Systemic sclerosis (scleroderma, SSc) is an autoimmune inflammatory disease of unknown etiology characterized by pronounced fibroproliferative alterations in the microvasculature, cellular and humoral immunity abnormalities, and an often progressive severe fibrotic process affecting the skin and many internal organs [1–3].

The pathogenesis of SSc is complex, and despite numerous studies that have examined several aspects of its intricate picture, the exact mechanisms involved are not well understood. However, it is apparent that its most severe clinical and pathologic manifestations are the result of a fibrotic process characterized by the excessive and often progressive deposition of collagen and other connective tissue macromolecules in skin, heart, lungs, and many other internal organs. The cells responsible for this process are activated fibroblasts also known as myofibroblasts. These cells display an upregulated expression of genes encoding various collagens and other extracellular matrix (ECM) proteins in affected organs. This is the most important difference that distinguishes normal fibroblasts that promote normal wound healing from SSc fibroblasts which display exaggerated and uncontrolled collagen and ECM production resulting in pathologic organ fibrosis [3, 4]. Regardless of the etiologic event, the accumulation of activated fibroblasts or myofibroblasts in affected tissues and the persistence of their elevated biosynthetic functions are responsible for the extent and rate of progression of the fibrotic process which is the main determinant of SSc's clinical course, response to therapy, prognosis, and mortality. Although the mechanisms involved in the initiation and progression of the remarkable fibrotic process in SSc remain largely unknown, oxidative stress has been implicated in SSc pathogenesis. Numerous studies have demonstrated that oxidative stress participates in some aspects of each of the three crucial pathological processes of SSc, i.e., the fibroproliferative vasculopathy, the cutaneous and visceral fibrosis, and the cellular and humoral immunity abnormalities. This review will focus on the studies examining the role of oxidative stress in the pathogenesis of the fibrotic process in SSc.

10.2 Evidence of Oxidative Stress in SSc

Following the early work of Murrell [5] suggesting that oxidative stress may play a role in SSc pathogenesis, a number of studies have confirmed these initial observations and have explored the mechanisms involved [6, 7]. Owing to the crucial role of the fibrotic process in SSc clinical manifestations, most of these investigations have focused on the involvement of oxidative stress in the pathogenesis of tissue fibrosis. It has been shown that oxidative stress in SSc is the result of an imbalance between the production of oxidative stress producing systems and their antagonist antioxidant mechanisms. Indeed, reduction of endogenous antioxidants and a parallel overproduction of reactive oxygen species (ROS) have been documented in SSc, although increased generation or overproduction of ROS appears to be the main mechanism whereas ROS inactivation is believed to play a less important role. Several studies demonstrated decreased levels of antioxidants in serum of SSc patients [8, 9] although other studies have not confirmed these observations [10]. Furthermore, numerous others have shown increased ROS production [11–14] providing strong evidence to the important contribution of oxidative stress in the pathogenesis of the disease.

Numerous oxidative stress-related products have been detected in various biological fluids from SSc patients supporting the concept of increased oxidative stress in SSc, including increased urinary 8-oxodG levels [15] or presence of increased levels of isoprostanes in SSc serum [16, 17]. Isoprostanes such as F2-isoprostanes are markers of lipid peroxidation (for review, see ref. [18]) produced in vivo in humans by the non-cyclooxygenase, free radical-catalyzed, peroxidation of arachidonic acid and have been shown to be a reliable measure of lipid peroxidation in vivo. Increased levels of urinary F2IP-M, a bioactive prostaglandin (PG)F_2-like compound, produced by this pathway was also found to be elevated in urine from SSc patients [19]. Another isoprostane, 8-isoprostane, was described as a biomarker of oxidative stress in interstitial lung diseases [20]. The level of oxidative stress has also been shown to be enhanced in patients with interstitial lung diseases such as fibrosing alveolitis associated with SSc as reflected by increased concentrations of 8-epi-$PGF_{2\alpha}$ in bronchoalveolar lavage fluid [21]. Other markers of oxidative stress in SSc include increased serum levels of N(epsilon)-(hexanoyl)lysine [22] and elevated serum levels of heat shock protein 70 [23].

Clastogenic activity measured in plasma has also been evaluated as a biomarker of oxidative stress in SSc [24]. Inosine triphosphate, the deamination product of ATP, is one of the clastogenic and superoxide generating components of clastogenic plasma factors. Clastogenic activity is found not only in the plasma but also in the supernatants of fibroblast cultures obtained from SSc patients. This activity was correlated with an increase of adenosine deaminase (ADA), an ubiquitous enzyme involved in purine metabolism. ADA catalyzes the irreversible deamination of adenosine or deoxyadenosine leading to the formation of inosine triphosphate and inosine diphosphate, which are abnormal nucleotides with clastogenic and superoxide stimulating properties.

10.3 Reactive Oxygen Species

ROS are a group of oxygen-derived molecules characterized by high chemical reactivity. ROS play both a deleterious and a beneficial dual role. Under physiological conditions, ROS perform important functions in cellular signaling pathways and host defense, but in pathological states, they can induce oxidative stress causing damage to proteins, lipids, and DNA, as well as activating various redox-sensitive cell signaling pathways [25]. ROS include the free superoxide ($^{\bullet}O_2^-$) and hydroxyl ($^{\bullet}OH$) radicals and the non-radical species, hydrogen peroxide (H_2O_2), hypochlorous acid (HClO), and peroxynitrite ($ONOO^-$). Although several enzyme systems are capable of producing ROS including the mitochondria electron transport chain, lipoxygenases, cyclooxygenases, xanthine oxidase, cytochrome P450, uncoupled eNOS, and peroxisomes, numerous studies have shown that NADPH oxidases are the primary enzymes responsible for inducible ROS formation. NADPH oxidase generation of ROS has been documented in many systems and organs including the vascular system [26–28], lungs [29], kidneys [30], and neurologic system [31].

10.3.1 ROS in the Pathogenesis of SSc

Recent studies have implicated excessive oxidative stress and the generation of deleterious ROS in the pathogenesis of SSc [7, 14, 32]. Although ROS are produced by normal fibroblasts and are essential for numerous intracellular functions including fibroblast proliferation, there is evidence that SSc dermal fibroblasts produce increased ROS levels [14] and that elevated ROS may be involved in the increased collagen expression in these cells. Also, monocytes from SSc patients spontaneously release increased amounts of superoxide anion in vitro [11]. These studies demonstrated that increased ROS production by either untreated or phorbol 12-myristate 13-acetate-stimulated monocytes was mediated through the activation of NADPH. These results are in agreement with previous studies that suggested that NADPH oxidases are the most important mediators of ROS production by fibroblasts. Other studies in support of the potential role of ROS in the pathogenesis of fibrotic and vascular lesions in SSc included the demonstration that sera from patients with SSc induce high production of ROS by endothelial cells and fibroblasts in vitro and that the levels of induction are correlated with the severity of clinical involvement [13]. It is of substantial relevance that ROS participate in important signaling cascades and pathways in numerous types of cells including those that are activated in SSc such as fibroblasts, myofibroblasts, endothelial cells, or pericytes. It has also been recently shown that ROS may cause the oxidation of DNA-topoisomerase-1 and that through this mechanism, ROS can also mediate the induction of immunologic intolerance to this nuclear antigen [33]. Anti-DNA-topoisomerase-1 antibody, also known as Scl-70 antibody, the prototypic marker autoantibody in diffuse SSc, is found in serum of patients with diffuse cutaneous SSc but is not detected in

patients with limited cutaneous SSc or other connective tissue disorders. In these studies which employed a novel SSc animal model, it was demonstrated that HOCl or •OH induced high levels of oxidized DNA-topoisomerase-1 and that this protein modification increased DNA-topoisomerase-1 antigenicity, a required initial step for the development of the autoantibody.

A recent study described a novel mechanism by which ROS may promote a profibrotic phenotype in SSc fibroblasts [34]. This mechanism involves the oxidative inactivation of protein tyrosine phosphatase 1B (PTP1B) leading to pronounced platelet-derived growth factor receptor (PDGFR) activation. This study also provided a novel molecular mechanism by which therapy with *N*-acetylcysteine (NAC), a thiol antioxidant, may be beneficial for SSc by acting on ROS and PTP1B. ROS production and expression of type I collagen (Col I), a fibrotic marker gene, were significantly higher in SSc, and these alterations were accompanied by significantly lower amounts of free thiols compared to normal fibroblasts. In this study, it was also shown that following PDGF stimulation, PDGFR and extracellular signal-regulated kinase 1/2 (ERK1/2) were phosphorylated to a greater extent, whereas the ability to produce PTP1B was substantially diminished in SSc fibroblasts. Furthermore, PTP1B activity was significantly reduced in SSc fibroblasts, most likely as a result of increased cysteine oxidation caused by higher levels of ROS. Confirmation of the important role of PTP1B on the regulation of the fibrotic process was obtained from studies showing that decreased PTP1B expression in normal fibroblasts led to increased Col I. Treatment of SSc cells with NAC restored the low PTP1B activity, improved the profile of p-PDGFR, decreased the amounts of tyrosine-phosphorylated proteins and Col I, and scavenged ROS in SSc fibroblasts [34]. These studies collectively provide strong support for the participation of ROS not only in the development of exaggerated fibrotic responses in SSc but also in the generation of disease-specific autoantibodies and other immunologic alterations characteristic of the disease.

10.4 NADPH Oxidases

The NADPH oxidase (NOX) family of enzymes, which catalyze the reduction of O_2 to form ROS, are likely to have crucial roles in normal cellular physiology as evidenced by the remarkable increase in the number of NOX enzymes during eukaryotic evolution with seven distinct NOX isoforms identified in mammals [35–37]. The role of NOX enzymes in a variety of human disorders is currently the focus of intense investigation, and there is strong experimental evidence to support their participation in the fibrotic process. Transforming growth factor-β1 (TGF-β1), the most important pro-fibrogenic growth factor, has been shown to be a potent inducer of ROS generation in myofibroblasts, the cells responsible for exaggerated ECM production in SSc. Furthermore, the NOX4 isoform, one of the seven members of the NOX family, has been identified as a source of ROS generation in cardiac and lung myofibroblasts, and it has also been implicated in the process of TGF-β-induced differentiation of

quiescent cardiac fibroblasts to myofibroblasts [38, 39]. NOX4 is upregulated in lungs of patients with idiopathic pulmonary fibrosis (IPF) and in lung mesenchymal cells, and NOX4-dependent generation of H_2O_2 is required for TGF-β1-induced myofibroblast differentiation and increased ECM production [39]. Furthermore, abrogation of NOX4 has been shown to inhibit fibrogenesis in two different murine models of lung injury [39]. Generation of H_2O_2 by lung myofibroblast NOX4 mediates additional fibrogenic effects by inducing epithelial cell apoptosis [40]. Therefore, numerous studies have provided strong supporting evidence for a role of NOX4 in normal myofibroblast tissue repair functions and in abnormal, exaggerated fibrogenesis. Thus, there is a very cogent and strong rationale for therapeutic targeting of NOX4 in abrogating lung fibrosis and other fibrotic disorders such as SSc. The development of small molecule inhibitors and/or other strategies targeting NOX4 offers substantial promise for the treatment of currently incurable fibrotic disorders, such as IPF and SSc.

10.4.1 Regulation of NOX Activity

The mechanisms involved in the regulation of NOX activity are quite complex, and it is likely that they vary depending on a specific cellular and functional context. There are four general mechanisms of NOX regulation: (1) regulation of expression of NOX genes or of the genes encoding regulatory subunits of the corresponding NOX isoenzymes including modulation of mRNA translation or stability, (2) regulation of NOX protein stability and turnover, (3) regulation of enzymatic activity, and (4) feedback or other endogenous inhibitory mechanisms. Among all the NOX enzymes, it appears that NOX4 is the most likely member of this extended family of ROS-generating enzymes to play a role in some of the complex molecular pathways involved in the SSc fibrotic process. It is important to emphasize that, in contrast with all other NOX enzymes, NOX4 does not require other protein subunits for its activity, and, therefore, enzyme activity is dependent on the expression level of its corresponding gene. Given the important functions of NOX4 in a variety of physiological processes and in the pathogenesis of numerous diseases, there has been intense interest in unveiling the intimate mechanisms of its regulation.

Although the exact mechanisms involved in the regulation of NOX4 levels in normal cells are becoming unveiled, the possible alterations responsible for the constitutive elevation in NOX4 levels and activity in SSc cells are not known. Multiple growth factors and related polypeptides which contribute to the activation of NADPH oxidases have been shown to participate in SSc pathogenesis. Among these are the following: (a) TGF-β, a well known cytokine implicated in the fibrotic process [4, 41] which stimulates transmembrane serine/threonine kinases activating NOX4 [38]; (b) PDGF which also plays an important role in the pathogenesis of SSc [42] has been extensively studied following the initial demonstration that this growth factor was capable of potent stimulation of fibroblast proliferation as well as of causing increased production of ECM by mesenchymal

cells and activation of NOX1 and NOX4 [43, 44]; (c) angiotensin II, which has recently been implicated in fibrosis through its ability to induce oxidative stress [45, 46]; and (d) endothelin-1, another potent profibrotic factor able to cause elevated ROS production; indeed, it has been found that endothelin-1 (ET-1) induces NADPH oxidase in human endothelial cells [47].

Recent studies have shown that antibodies to PDGFR also induce the production of ROS [48, 49]. This is a novel mechanism that provides a cogent link between the recently identified PDGF antibodies in the sera of SSc patients [49] and the pathogenesis of the fibrotic process. Studies of the mechanisms of PDGF-induced stimulation of ROS production have shown that PDGF increased ROS, and through this effect, it may regulate Ras protein levels via ERK1/2. These studies found that PDGF induced posttranscriptionally Ha-Ras by stimulating ROS and ERK1/2. Activation of ERK1/2 and high ROS levels were shown to stabilize Ha-Ras protein, by inhibiting its proteasomal degradation. The increased levels of ROS induced by PDGF resulted in increased Ha-Ras and active ERK1/2 and subsequently in stimulation of collagen synthesis, DNA damage, and accelerated senescence.

Thus, stabilization of Ras protein by ROS and ERK1/2 in primary fibroblasts induced by PDGF amplifies the response of the cells to growth factors, and this mechanism may represent a critical factor in SSc onset and progression [50].

10.5 Animal Models of Oxidative Stress-Induced Tissue Fibrosis

Animal models are extremely valuable to examine and validate various hypotheses about the pathogenesis of human diseases and, most importantly, to develop and test potentially effective therapeutic interventions before they can be applied to humans. Several animal models have been developed to examine various aspects of SSc pathogenesis or of the fibrotic process associated with SSc and other fibrotic disorders [51–55].

Although most of the animal models for SSc do not reproduce the entire spectrum of pathogenic mechanisms proposed for the disease, there are several induced animal models that reproduce some of the most relevant pathophysiologic alterations of the disease. Among these, the most commonly and extensively utilized animal model is the one induced by intratracheal or subcutaneous injections of bleomycin, a free radical generator [56–60]. Bleomycin-induced skin fibrosis mimics early changes in SSc, and it has been suggested that one of the most likely mechanisms involved is the increased production of ROS which then damage the surrounding cells, such as endothelial cells.

Another recently described animal model of SSc is induced by repeated and prolonged (daily for 6 weeks) intradermal injections of HOCl, which generate hydroxyl radicals and result in the development of severe fibrosis in skin and lungs with characteristics resembling those of diffuse cutaneous SSc in humans [33]. The fibrosis persists for at least 10 weeks following the last administration

of HOCl. The skin of these mice contained large numbers of myofibroblasts with increased rate of proliferation and increased production of collagen and α-smooth muscle actin, the molecular marker of their transdifferentiation into activated myofibroblasts. The serum of these mice was found to harbor increased levels of IgG and IgM and anti-DNA-topoisomerase-1 antibodies. In a variation of this animal model, the authors also described that treatment of mice with agents capable of generating peroxynitrite anions induced changes consistent with limited cutaneous SSc, including the absence of lung involvement and the production of anti-centromere binding protein-B antibodies, which are serologic markers considered to be highly specific for the limited form of SSc.

10.6 Antioxidant Systems

Mammalian cells contain numerous and quite efficient systems capable of counteracting the potent and pleiotropic effects of oxidative stress. These include antioxidant enzymes such as catalase, glutathione peroxidase, and superoxide dismutases (SOD), which are found in mitochondria, cytosol, plasma membranes, and the extracellular space. SOD, which converts superoxide radicals to hydrogen peroxide, functions as a highly efficient defense mechanism against ROS effects. The important role of SOD in the abrogation of ROS-mediated profibrotic mechanisms has recently been supported by interesting studies describing a novel autoantibody to Cu/Zn SOD present in sera of patients with localized scleroderma [61]. These authors hypothesized that the autoimmune background in localized scleroderma induced anti-Cu/Zn SOD autoantibodies that inhibited SOD activity and thereby contributed to fibrosis by increasing ROS. In other studies, it was found that alterations in SOD3 expression and activity were associated with SSc fibrosis [62]. The increased expression of SOD3 mRNA and enzymatic activity detected in SSc fibroblasts, as compared to control healthy fibroblasts in these studies, may have represented a protective mechanism attempting to balance the elevated ROS effects.

10.7 Antioxidant Treatments

Owing to the importance of the oxidative process in SSc pathogenesis and to the observations that patients with SSc have reduced serum concentrations of the natural antioxidants, ascorbic acid, α-tocopherol, and β-carotene, as well as low values of selenium, a large number of antioxidants have been used with therapeutic purposes [63]. The therapeutic application of various antioxidant agents for SSc has been supported by provocative in vitro results with SSc fibroblasts [34] as well as studies in various SSc animal models. In one of these studies, edaravone, a free radical scavenger, has been used in two different mouse models of SSc and a significant

inhibitory effect on fibrosis was observed [64]. In another recent study, the efficiency of a tellurium-based catalyst, 2,3-bis(phenyltellanyl)naphthoquinone ((PHTE)(2)NQ), in the treatment of HOCl-induced murine model of SSc was demonstrated. (PHTE)(2)NQ efficiency is linked to the selective pro-oxidative and cytotoxic effects of this compound on hyperproliferative fibroblasts [65]. Another putative antioxidant molecule, α-melanocyte-stimulating hormone, suppressed bleomycin-induced oxidative stress, reduced skin fibrosis and collagen content, and increased tissue levels of superoxide dismutase 2 (SOD2) and heme oxygenase-1 in a murine model of SSc and was, therefore, suggested as a potential therapy for SSc [66].

Despite these highly encouraging results from animal models, the clinical observations employing various antioxidant agents, however, have not consistently demonstrated beneficial effects. For example, a short-term treatment with vitamin E in SSc patients failed to decrease the basal rate of lipid peroxidation and did not improve microvascular perfusion following cold exposure in these patients [67]. However, in related studies, Allanore et al. found acute and sustained effects of dihydropyridine-type calcium-channel antagonists on oxidative stress in SSc [68], and nifedipine, one of the most commonly used dihydropyridine-type calcium-channel antagonist, was found to protect against overproduction of superoxide anion by peripheral blood monocytes from SSc patients. Further studies on this topic showed that this beneficial property of nifedipine appeared to be mediated both by calcium-channel cellular action and by the inhibition of protein kinase C activity [12].

Besides its potent effect causing reduction of disulfide bonds in susceptible proteins, the thiol compound NAC exerts a strong antioxidant effect. Therefore, there has been intense interest in the possible beneficial effects of NAC in the treatment of SSc. One of the earliest studies of NAC in SSc was conducted by Furst et al. [69] in 22 SSc patients. The parallel, double-blind, placebo-controlled prospective trial of 1 year in duration failed to show any significant differences in various relevant parameters between the placebo and the NAC-treated patients. A subsequent study by Sambo et al. [70] evaluated the effects of a short-term (5 days) intravenous infusion of NAC in patients with SSc-associated Raynaud's phenomenon. Although there was a reduction in the number of digital ulcers and in the recovery time following cold challenge, it is difficult to interpret the validity of the results because the study was not placebo-controlled. A more extensive study of intravenous NAC therapy was reported by Salsano et al. in 2005 [71]. These authors examined the effects of intravenous infusion of NAC given daily for 5 h for 8 months, during the winter (from October to May) for 2 years. The results showed increased hand perfusion assessed by laser Doppler perfusion imaging. However, owing to the fact that the study was uncontrolled, the beneficial effects reported need to be interpreted cautiously. Three recent studies from the same group of investigators examined the effects of a similar therapeutic regime of intravenous NAC on kidney and liver perfusion in SSc patients showing beneficial effects with an improvement in hepatic perfusion [72] and a reduction in renal artery resistance index [73]. Highly encouraging results were also obtained in a long-term prospective study of 50 patients with a previous history of severe Raynaud's phenomenon with digital ulcers who had

failed to respond to calcium-channel blocker therapy. The patients received NAC intravenously for a duration of 5 h every 14 days for an average of 4.2 ± 2.11 years. In this study, there was a substantial reduction in the numbers of digital ulcers and in the frequency and severity of Raynaud's phenomenon episodes. An assessment of the long term effects of NAC intravenous therapy on various parameters of lung function in the same cohort of SSc patients was recently published [74]. The results indicated that NAC caused stabilization or minimal improvement in pulmonary function studies with stabilization of in the parenchymal abnormalities assessed by high-resolution chest CT scans. Although the results of the later studies are encouraging, the lack of a placebo control group suggests that these conclusions will need to be validated in properly controlled studies.

Numerous recent studies have focused on novel classes of antioxidant compounds with encouraging results. One study showed that the antioxidant epigallocatechin-3-gallate (EGCG) was able to reduce ECM production by dermal fibroblasts from SSc patients in culture, modulating collagen type I and fibronectin gene expression, reducing the fibrotic marker connective tissue growth factor, and inhibiting collagen gel contraction. EGCG was also able to suppress intracellular ROS, ERK1/2 kinase signaling, and nuclear factor-κB activity [75].

It has been suggested that an extract from fruits of *Capparis spinosa* L. (ECS) protects against oxidative stress in SSc dermal fibroblasts. ECS exhibits a notable activity in protecting against oxidative stress and interrupting the ROS-ERK1/2-Ha-Ras signal cascade in SSc fibroblasts, suggesting that it may exert a potential protective effect against the development of skin sclerosis. ECS significantly reduced the production of O_2^-, H_2O_2, and ROS in SSc fibroblasts and minimized the loss of cell viability and apoptosis induced by H_2O_2 in normal and SSc fibroblasts [76].

Iloprost, a stable synthetic analogue of prostacyclin, currently employed in the treatment of SSc vascular features, also possesses strong anti-oxidative properties beside its prostaglandin-like vasodilatory and platelet antiaggregation effects. Indeed, it has been shown that a standard course of iloprost therapy was capable of acutely reducing oxidative stress in SSc patients. This effect appeared to be more consistent in patients with the early phases of SSc and in the limited subset of disease [77, 78]. However, earlier studies on the effects of iloprost in vivo have shown that its strong vasodilator effect did not reduce oxidative status because urinary 8-isoPGF$_{2\alpha}$ did not diminish following its administration to SSc patients [79]; thus, the role of iloprost as an antioxidant and its potential therapeutic benefits in SSc remains to be conclusively determined.

Among other potential antioxidants that may have beneficial effects in the therapy of SSc patients is activin, a grape seed-derived proanthocyanidin extract, which has been found to reduce plasma levels of oxidative stress markers in SSc. Indeed, malondialdehyde, a marker for oxidative stress which was increased in the plasma of the SSc patients, was significantly reduced by the administration of activin [80].

Although the numerous studies discussed above suggesting that various antioxidants may be of benefit for the therapy of SSc patients have strong support from

in vitro and in vivo studies employing experimental animal models, their beneficial effects need to be conclusively demonstrated in rigorously controlled clinical trials in SSc patients.

10.8 Conclusion

There is strong experimental evidence to suggest that oxidative stress contributes to the progression of fibrosis in SSc and that these effects appear to be caused by excessive and unbalanced generation of ROS in affected tissues. Therefore, the potential use of antioxidants in the treatment of SSc has been suggested. However, clinical trials with some of these antioxidant agents have failed to show conclusively documented positive results. Thus, it will be necessary to perform further in vitro studies and well-controlled placebo-matched clinical trials to document conclusively any beneficial effects. Furthermore, it is possible that the use of combined antioxidants with other drugs may result in improved therapy for this disabling and frequently fatal disease.

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Chapter 11
Antioxidant and Antiinflammatory Properties of Heme Oxygenase-1 in Osteoarthritic Articular Cells

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Abstract Heme oxygenase-1 (HO-1) is induced in cells by various stimuli as a defense system against oxidative stress. It is known that reactive oxygen species (ROS) participates in the initiation and progression of osteoarthritis (OA) and several antioxidant systems may protect cartilage components. HO-1 induction or CO release from CORM-2 counteracts oxidative stress and protects against proinflammatory and catabolic effects of interleukin-1β in OA chondrocytes, osteoblasts, and synoviocytes as well as in OA osteochondral explants. Both approaches have been able to downregulate the production of mediators such as reactive oxygen species, nitric oxide, matrix metalloproteinases, prostaglandin E_2, cytokines, or chemokines accompanied by inhibition of cartilage degradation and improved aggrecan synthesis. Therefore, HO-1 or CO would be active on cell metabolism alterations, cartilage degradation, and synovitis. Understanding the mechanisms responsible for these effects may lead to novel strategies to prevent or treat joint destruction.

Abbreviations

ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
CCL	Chemokine (C-C motif) ligand
CoPP	Cobalt protoporphyrin IX
COX-2	Cyclooxygenase-2
DAPI	4′,6-diamidino-2-phenyl indol

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EGR-1	Early growth response-1
ERK1/2	Extracellular signal-regulated kinase 1/2
HIF	Hypoxia-inducible factor
HMGB1	High-mobility group box 1
HO	Heme oxygenase
IGF-1	Insulin-like growth factor-1
IGFBP3	IGF-binding protein-3
IL	Interleukin
iNOS	Inducible NO synthase
IκBα	Inhibitor of κBα
LV	Lentiviral vector
MAPK	Mitogen-activated protein kinases
MMP	Matrix metalloproteinase
mPGES-1	Microsomal prostaglandin E synthase-1
NF-κB	Nuclear factor κB
NO	Nitric oxide
OA	Osteoarthritis
PGE_2	Prostaglandin E_2
RANKL	Receptor activator of NF-κB ligand
ROS	Reactive oxygen species
TIMP	Tissue inhibitor of metalloproteinases
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α

11.1 Introduction

Heme oxygenase (HO) activity catalyzes the degradation of the heme group to iron, carbon monoxide (CO), and biliverdin which is converted to bilirubin by biliverdin reductase [1]. Both the constitutive (HO-2) and the inducible (HO-1) isoforms act as a defense system against oxidative stress, and they also play an important role in the maintenance of iron homeostasis [2–4]. HO activity can provide protection against oxidative stress through the degradation of the prooxidant heme and the generation of CO, biliverdin, and bilirubin which can inhibit the generation of reactive oxygen species (ROS) or inactivate them. Direct and indirect antioxidant activities of bilirubin would be higher than that of biliverdin. In addition to its antioxidant properties, CO interacts with different signaling pathways and binds to heme group in heme proteins leading to enzyme activity regulation [5–11]. Various stimuli such as ROS, nitric oxide (NO), radiation, cytokines, metals, heat shock, hypoxia, or hyperoxia induce HO-1 contributing to the regulation of different cell functions such as proliferation, differentiation, and apoptosis (reviewed in [12]).

There is compelling evidence supporting a key role of HO-1 in oxidative stress. Thus, the presence of polymorphisms in the HO-1 gene promoter is associated with susceptibility to oxidative-stress-mediated diseases [12–14], and genetic

deficiency in HO-1 results in the production of oxidative damage and hepatic and renal iron accumulation [15, 16]. In contrast, HO-1 upregulation results in antiinflammatory and antioxidant effects in a wide range of experimental models of diseases such atherosclerosis [17], cardiac ischemia/reperfusion injury [18], diabetes [19], inflammatory bowel disease [20], or rheumatoid arthritis [21]. HO-1 exerts immunomodulatory effects through the inhibition of T cell proliferation and apoptosis [22, 23]. In addition, this protein is able to inhibit apoptosis in other cell types such as endothelial cells [24] and enterocytes [25]. In mouse models of arthritis, HO-1 induction or administration of the CO-releasing molecule CORM-3 exerted antiinflammatory effects and reduced cartilage erosion [21, 26, 27], whereas it suppressed osteoclastogenesis and bone destruction in tumor necrosis factor-α (TNF-α)-mediated arthritis [28]. HO-1 is present in synovial tissues from rheumatoid arthritis patients, and its overexpression in cellular lines derived from synoviocytes inhibits the production of proinflammatory cytokines [29]. Recent studies have demonstrated that HO-1 polymorphisms are relevant in rheumatoid arthritis (see Chap. 7).

Osteoarthritis (OA) is the most prevalent articular disease and a leading cause of disability in the elderly [30]. Recent studies have shown that ROS may participate in the initiation and progression of OA [31, 32]. Antioxidant systems may protect cartilage components against damage, as it has been proposed for extracellular superoxide dismutase, which is present in large amounts in cartilage. Nevertheless, an association between OA and a decrease in the levels of this enzyme has been reported, supporting a role for ROS in this condition [33]. Although OA has been traditionally considered as noninflammatory, recent evidence supports the contribution of synovial inflammation to OA pathogenesis (reviewed in [34, 35]). ROS and NO may be mediators of mechanical stress and inflammatory cytokines in OA. These agents are released during the inflammation of the synovial membrane and can activate transcription factors such as nuclear factor κB (NF-κB) contributing to cartilage degradation [36]. Therefore, oxidative stress induced by a wide range of factors such as overload, trauma, or inflammation promotes cartilage destruction and also the transition from a clinically silent process to apparent OA [32]. At present, OA is considered to be a disease of the whole joint [37], and as such, different cell types are involved and complex relationships among them can be established. In this chapter, we summarize recent findings on the influence of HO-1 on ROS production and the progression of inflammatory and degradative responses in different articular cells from OA patients.

11.2 Heme Oxygenase-1 in Chondrocytes

Activation of the HO-1 pathway has been shown to elicit antioxidant and antiinflammatory effects in vitro and in vivo (reviewed in [38]). Concerning OA articular tissues, our results in ex vivo cultures of human OA chondrocytes or osteochondral explants have demonstrated the antiinflammatory and anti-catabolic

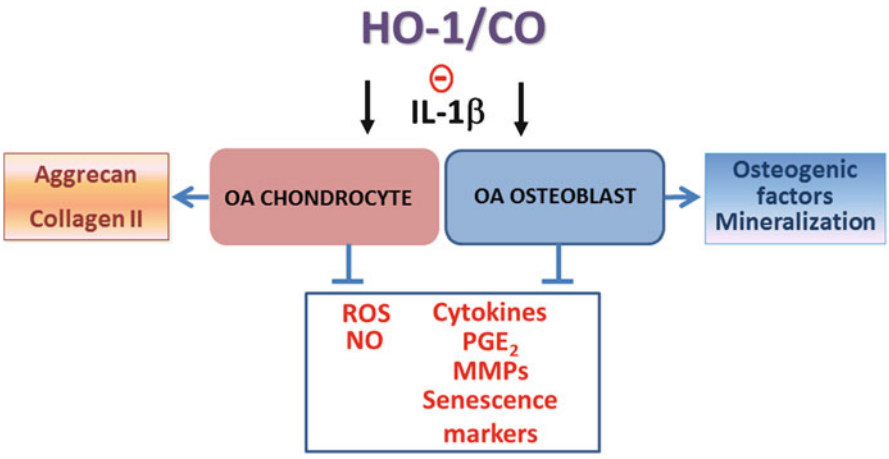

Fig. 11.1 Summary of HO-1 (or CO) effects on OA chondrocytes and osteoblasts

properties of HO-1 induction by cobalt protoporphyrin IX (CoPP), HO-1 transduction with a lentiviral vector (LV-HO-1), or CO released by CORM-2 [39, 40] (Fig. 11.1). HO-1 is increased in human OA cartilage with respect to normal samples, and its expression is regulated by proinflammatory and antiinflammatory mediators. Therefore, treatment of human OA chondrocytes in primary culture with proinflammatory cytokines such as interleukin (IL)-1β, IL-17, or TNF-α downregulates the expression of HO-1, whereas the antiinflammatory cytokine IL-10 enhances it [41]. Proinflammatory cytokines may play an important role in OA progression. Superficial areas of cartilage are in contact with high levels of cytokines present in synovial fluid which may activate signaling pathways such as mitogen-activated protein kinases (MAPK) and NF-κB, leading to the production of many inflammatory and degradative mediators including cytokines, chemokines, NO, ROS, and matrix metalloproteinases (MMPs) [42].

The intracellular oxidants generated could function as second messengers involved in mitochondrial dysfunction [43, 44], chondrocyte apoptosis [32, 45] and senescence [46], and induction of inflammatory mediators and degradative enzymes [47]. ROS may also cause inhibition of proteoglycan synthesis [48]. Several studies show that NO generated in joints inhibits respiration in chondrocytes [49], enhances the sensitivity of these cells to oxidative stress [50], and promotes cell death by several mechanisms [45, 51, 52], while ROS such as superoxide can modulate NO effects in chondrocytes stimulated with IL-1 [53]. In human OA chondrocytes, HO-1 induction protects against apoptosis induced by high doses of sodium nitroprusside [54]. Induction of HO-1 would also play a role in the protection by cilostazol against NO-induced apoptosis in chondrocytes and cartilage destruction in a model of OA [55]. NO is an inducer of HO-1 [56] which may represent a feedback mechanism to control NO effects as HO-1 in turn downregulates inducible NO synthase (iNOS) expression and NO production in OA chondrocytes

[57]. Our results suggest that inhibition of ROS generation by HO-1 induction or CORM-2 may contribute to the antiapoptotic effects observed in primary OA chondrocytes [58]. It is known that CO exerts regulatory effects on redox signaling, and it has been reported that exogenous CO from CO-releasing molecules or HO-1 stimulation can inhibit NADPH oxidase and cytochrome oxidase in some cell types (reviewed in [59]).

Our work has shown that HO-1 may function to regulate the modifications in chondrocyte metabolism produced by proinflammatory cytokines. HO-1 induction reduced the production of oxidative stress and apoptosis in IL-1β-stimulated chondrocytes. We provided evidence of a protective role for HO-1 showing that HO-1 can inhibit IL-1β-induced proteoglycan breakdown and decreased proteoglycan synthesis as well as the production of a number of mediators responsible for cartilage degradation [40]. In OA, loss of aggrecan precedes collagen degradation [60] which can be mediated by IL-1 and TNF-α and the collagenases MMP-1 and MMP-13 [61–63], with an important role of aggrecanases such as a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)-4 and ADMTS-5 in the breakdown of aggrecan [64] and other components of the extracellular matrix [65]. In human OA chondrocytes stimulated with IL-1β, mRNA levels and activity of collagenase enzymes are strongly elevated, and HO-1 can decrease cartilage degradation through the inhibition of MMP-13 expression at the protein and mRNA levels. The induction of HO-1 by CoPP and the liberation of CO by CORM-2 not only inhibits the degradation of extracellular matrix but also increases the synthesis of proteoglycan and collagen II in OA chondrocytes [40, 66]. Our studies have shown the inhibitory effects of CORM-2 on MMP-3, MMP-10, and MMP-13, indicating that this molecule could act in different levels of the cascade of reactions that result in extracellular matrix degradation. In addition, CORM-2 is able to enhance the production of IL-1 receptor antagonist (IL-1Ra) in primary OA chondrocytes stimulated with IL-1β. IL-1Ra may block small amounts of IL-1 [67], and therefore, this activity of CORM-2 may contribute to limit the effects of this cytokine.

IL-1β induces in chondrocytes large elevations of iNOS, cyclooxygenase-2 (COX-2), and microsomal prostaglandin E synthase-1 (mPGES-1), leading to increased levels of NO and prostaglandin E_2 (PGE_2). Both mediators are proinflammatory and can participate in increased proteoglycan loss and decreased proteoglycan synthesis during OA progression [68–70]. Large amounts of NO are associated with matrix degradation due to the suppression of glycosaminoglycan and collagen synthesis, expression of MMPs, and activation of proenzymes [49, 71]. NO mediates MMP expression [72] and the suppression of cartilage proteoglycan synthesis induced by IL-1 [73] as well as the synthesis of type II collagen [74].

PGE_2 exerts catabolic effects through the enhancement of MMP production or the inhibition of tissue inhibitor of metalloproteinases (TIMP) synthesis [75, 76], leading to proteoglycan degradation [70]. In contrast, low concentrations of PGE_2 may downregulate collagenases, proinflammatory cytokines, and collagen 2A1 cleavage [77]. In addition, PGE_2 may enhance NO-induced chondrocyte death [78]. COX-2 and mPGES-1 are significantly increased in OA cartilage [79] and chondrocytes [80]. We have demonstrated the downregulation of iNOS and

mPGES-1 gene expression by HO-1 which results in reductions in NO and PGE_2 in OA chondrocytes treated with IL-1β. We have found that HO-1 or CORM-2 inhibits the expression of mPGES-1 protein and to a lesser extent of COX-2, which would mediate the inhibitory effects observed on PGE_2 production [39, 81].

NF-κB is a key intracellular signal pathway for inflammatory and catabolic gene expression in articular chondrocytes [82, 83]. Therefore, the inhibition of NF-κB activation may provide a basis for the observed reductions in the expression of iNOS, COX-2, and key degradative enzymes such as MMP-1, MMP-3, MMP-10, MMP-13, and aggrecanase [66]. The phosphorylation of inhibitor of κB-α (IκBα) and subsequent proteosomal degradation are critical steps in NF-κB activation [84]. Our data indicate that the inhibition of IκBα phosphorylation by HO-1 or CORM-2 would result in the prevention of NF-κB nuclear translocation. In addition, the reduction in extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation [66, 85] may contribute to the inhibition of inflammatory gene expression.

We have also shown that HO-1 increases the production of insulin-like growth factor-1 (IGF-1) [40], an important anabolic factor for cartilage as it stimulates proteoglycan synthesis and slows proteoglycan catabolism (reviewed in [86]). In OA, there is a reduced responsiveness of chondrocytes to IGF, which may be related to overexpression of IGF-binding proteins or defects in IGF receptor binding or signaling [87, 88]. Our data indicate that HO-1 induction decreases the high levels of IGF-binding protein-3 present in OA chondrocytes [89–91], which may facilitate the interaction of IGF-1 with its receptors [88].

Articular chondrocytes are adapted to live in conditions of low O_2 [92]. Such hypoxic conditions in the avascular cartilage play an important role in extracellular matrix synthesis and survival of chondrocytes. However, the influence of O_2 tension on HO-1 function in healthy and OA chondrocytes remains unknown as most studies have been performed in normoxia. Therefore, we wanted to compare the antiinflammatory and chondroprotective effects of HO-1 on OA chondrocytes in normoxia (20 %) or hypoxia (1 % O_2). We have shown [93] that HO-1 protein was expressed in both healthy and OA chondrocytes and IL-1β downregulated HO-1 expression in all conditions, whereas CoPP treatment counteracted this effect. CoPP also decreased the production of NO induced by IL-1β in healthy and OA cells at both O_2 concentrations which was accompanied by a reduction in iNOS expression at 24 h. Interestingly, HO-1 induction in hypoxic conditions reduces the levels of TNF-α and MMP activity after IL-1β stimulation of chondrocytes. TNF-α induces inflammatory mediators and catabolic enzymes in OA tissues [94], and its blockade inhibits extracellular matrix cleavage while increasing glycosaminoglycan content in OA cartilage [95]. Therefore, the observed reduction in TNF-α production would be relevant for HO-1 antiinflammatory effects. We have also shown that IL-1β induces hypoxia-inducible factor (HIF)-1α expression irrespective of O_2 tension, whereas HO-1 induction by CoPP downregulates HIF-1α expression in OA chondrocytes only. In hypoxic conditions, HIF-2α and SOX9 expression was decreased by IL-1β in both healthy and OA chondrocytes. However, HO-1 induction was able to reverse this effect and prevented the decrease in type collagen II expression. Interestingly, HO-1 induction in primary chondrocytes cultured in

hypoxic conditions resulted in stronger antiinflammatory and chondroprotective effects compared with normoxia. These results suggest that the antioxidant enzyme HO-1 could be a physiologically important chondroprotective factor.

The affinity of CO for transition metal atoms leads to the inhibition of different metalloproteins or to the direct regulation of phosphorylation–dephosphorylation of MAPK [96]. The activation of MAPK signaling seems to mediate the expression of MMPs and the regulation of extracellular matrix components in response to IL-1β stimulation in normal or OA chondrocytes [42]. In particular, ERK1/2 is a negative regulator of the chondrogenesis and the differentiation of chondrocytes [97]. Our results show that the induction of HO-1 or CORM-2 inhibits the phosphorylation of ERK1/2 and p38 [40, 66] which could contribute to the maintenance of extracellular matrix and chondrocyte phenotype. Possible mechanisms of MAPK modulation may include the inhibition of NADPH oxidase [59] and ROS production in mitochondria [98]. The inhibitory effects of HO-1 induction or CORM-2 on p38 and ERK1/2 phosphorylation could contribute to reduce mPGES-1 expression [85]. In addition, the inhibition of NF-κB, HIF-1α, and early growth response-1 (EGR-1) transcription factors by CoPP and CORM-2 could mediate mPGES-1 downregulation [39, 99].

11.3 Heme Oxygenase-1 in Osteoblasts

In addition to cartilage degradation, several lines of evidence indicate the involvement of bone in OA [100, 101]. The calcified cartilage and subchondral zone are two areas with low O_2 tension and intense cellular remodeling with important mechanisms to control ROS production. Lipid peroxidation products are abundant in human OA osteoblasts where they could activate signal transduction pathways leading to alterations in the osteoblastic phenotype and production of inflammatory mediators [102].

Early bone remodeling plays a key role in the development of OA [103]. Bone is sclerotic in subchondral areas but mechanically weaker because of under mineralization and increased collagen metabolism [104, 105]. There are a number of interactions between chondrocytes and subchondral osteoblasts which can contribute to cartilage degradation by stimulating chondrocytes to produce MMPs and by inhibiting aggrecan synthesis [106]. Therefore, the modification of bone abnormal metabolism may be a therapeutic strategy in OA [107]. Abnormal bone tissue remodeling in OA can be dependent on the imbalance between osteoblast and osteoclast functions [108–110]. Proinflammatory cytokines play an important role in bone remodeling in both physiological and pathological conditions [111] as they inhibit osteogenic differentiation of mesenchymal stem cells [112] and activate osteoblasts to produce inflammatory and degradative mediators in OA [107, 113] involved in the local regulation of bone metabolism and osteoclast activation [114–116]. We have shown that HO-1 upregulation counteracts the effects of IL-1β on OA osteoblasts [117]. As a consequence, HO-1 induction or

transduction of OA osteoblasts with LV-HO-1 promoted gene expression of collagen I, osteocalcin, bone morphogenetic protein 2, and Runx2 as well as the mineralization process (Fig. 11.1).

The imbalance between osteoprotegerin and receptor activator of NF-κB ligand (RANKL) results in bone loss (reviewed in [118, 119]). IL-1β decreases the ratio osteoprotegerin/RANKL in OA osteoblasts, while HO-1 induction in these cells reverses this effect and also reduces the production of proinflammatory cytokines such as TNF-α and IL-6, whereas IL-10 levels are enhanced [117]. IL-6 plays an important role in osteoclast recruitment and bone resorption [120] besides the inhibition of human osteoblast differentiation and mineral deposition [121]. Our results also indicate that HO-1 induction decreases PGE_2 production in OA osteoblasts stimulated with IL-1β, an effect dependent on the inhibition of COX-2 and mPGES-1 mRNA expression. This prostanoid exhibits a dual role as a mediator of both bone resorption and bone formation [122] by mechanisms mediated by EP2/EP4 [123] or EP4 receptors [124], respectively. Of note, PGE_2 contributes to osteoclast-like cell formation induced by proinflammatory cytokines [125, 126], enhances IL-6 production and MMP-1 in osteoblasts [127, 128], and suppresses osteoprotegerin production [129].

IL-1β and oxidative stress elicit premature senescence [130]. Interestingly, we have shown that HO-1 downregulates senescence markers in OA osteoblasts, thus suggesting that HO-1 could be a strategy to prevent senescence-associated osteoblast alterations. The role of MMPs in bone metabolism can be complex [131], and MMP-2 may be involved in bone resorption induced by IL-1β [132]. We have shown that HO-1 induction downregulates the expression of MMP-1, MMP-2, and MMP-3 stimulated by IL-1β. These effects of HO-1 in OA osteoblasts may be mediated, at least in part, by the inhibition of NF-κB activation induced by IL-1β [117].

11.4 Heme Oxygenase-1 in Synoviocytes

Several lines of evidence have demonstrated that synovitis reflects cartilage degradation and is associated to clinical symptoms of OA such as joint swelling, synovitis, and inflammatory pain. These findings have prompted interest in synovium-targeted therapies [34]. The inflamed synovium produces a wide range of proinflammatory and catabolic mediators, leading to cartilage matrix degradation and amplification of synovial inflammation [34, 133–135]. OA synovial cells are activated by proinflammatory cytokines to release ROS, cytokines and chemokines, proteases, and other mediators that sustain the inflammatory response [136] and cooperate to degrade the components of joint tissues [137].

Our results indicate that HO-1 protein is regulated by cytokines in OA synoviocytes similarly to chondrocytes [41]. HO-1 is present in human OA synoviocytes and downregulated by proinflammatory cytokines such as IL-1β, TNF-α, and IL-17, in a concentration-dependent manner, while the antiinflammatory cytokines, IL-10 and IL-13, increase its expression [138].

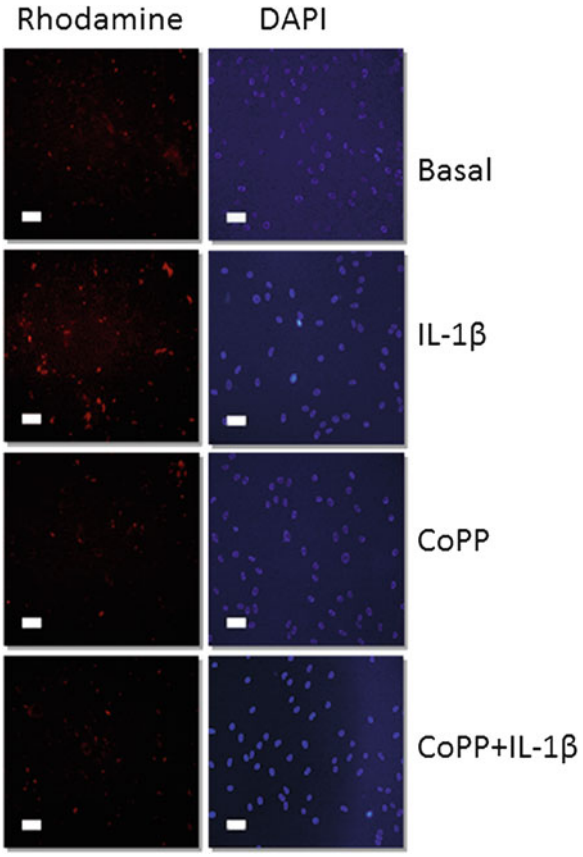

Fig. 11.2 Effect of HO-1 induction by CoPP on oxidative stress in OA synoviocytes. All studies were performed under the University of Valencia Ethical Committees approved protocol. Synoviocytes were isolated as previously described [139]. Cells were incubated with CoPP (10 μM) in presence or absence of IL-1β (10 ng/ml), and the evaluation of oxidative stress was determined with dihydrorhodamine-123 [139]. The nucleus was labeled with 4′,6-diamidino-2-phenyl indol (DAPI). The figure is representative of three different assays with cells from three OA patients. Original magnification ×200

We have shown that induction of HO-1 or treatment with CORM-2 reduces the production of relevant mediators of joint inflammation and destruction in OA synoviocytes in primary culture. Induction of HO-1 by CoPP (Fig. 11.2) or CORM-2 administration [139] decreases ROS production in OA synoviocytes stimulated with IL-1β. Our studies demonstrate the antioxidative capacity of HO-1 in these cells, as ROS production was significantly reduced (% of positive cells; basal cells, 36.05 ± 4.06; IL-1β, 55.19 ± 3.13, $p < 0.05$ versus basal cells; CoPP, 27.83 ± 2.81; CoPP + IL-1β, 39.89 ± 5.77, $p < 0.05$ versus IL-1β).

The inhibitory effect of HO-1 on ROS production is accompanied by the down-regulation of catabolic and inflammatory mediators. We have shown previously

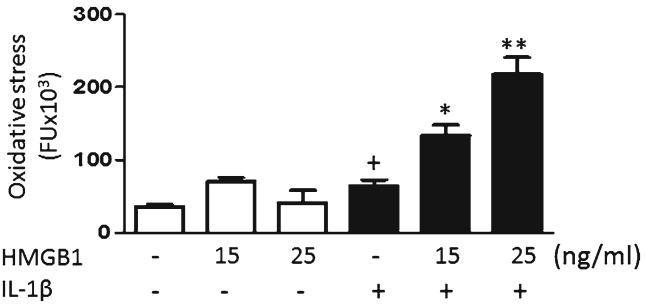

Fig. 11.3 Effect of HMGB1 on oxidative stress in OA synoviocytes. Human OA synoviocytes, isolated as in Fig. 11.2, were incubated with different concentrations of HMGB1 in the presence or absence of IL-1β (10 ng/ml) for 30 min, and the evaluation of oxidative stress was determined by laser cytometry with dihydrorhodamine-123 as we previously described [39]. Data are expressed as mean ± S.E.M. of independent cultures with cells from 3 different donors. $+p<0.05$ with respect to nonstimulated cells; $*p<0.05$, $**p<0.01$ with respect to IL-1β. One-way analysis of variance followed by Bonferroni's test

that HO-1 induction by CoPP, transfection of OA synoviocytes with LV-HO-1, or treatment with CORM-2 decreases the production of MMPs [138, 139]. It is interesting to note that both strategies significantly decrease high-mobility group box 1 (HMGB1) secretion [138]. This nuclear protein is released by necrotic cells or secreted in response to stimuli such as oxidative stress [140] or proinflammatory cytokines which stimulate its translocation into the cytoplasm and extracellular release (reviewed in [141, 142]). Extracellular HMGB1 binds to receptor for advanced glycation end products and Toll-like receptors (TLR) including TLR-2 and TLR-4 [141] to activate cells like monocytes, macrophages, and dendritic cells. In addition, the interaction of HMGB1, with phosphatidylserine on the cell surface, inhibits the phagocytosis of apoptotic neutrophils by macrophages [143], which may retard the resolution of inflammation. HMGB1 exerts proinflammatory effects mainly through binding to IL-1β and other inflammatory mediators [144]. We have shown that HMGB1 distribution is similar in healthy and OA synovial membrane, with positive staining in the intimate and subintimate layer cells and in cell infiltrate. Nevertheless, in OA synovium, HMGB1 is overexpressed as a result of synovial thickening and the high number of infiltrate cells, and its location is primarily cytosolic and extracellular. Incubation of HMGB1 with primary human OA synoviocytes does not result in the induction of any inflammatory or catabolic effect, but in the presence of IL-1β, HMGB1 potentiates the production of inflammatory and catabolic mediators. However, HMGB1 enhances the production of oxidative stress (Fig. 11.3), MMPs, chemokines leading to increased cellular migration [145], and PGE_2 which would be dependent on COX-2 and mPGES-1 expression (unpublished results).

Among inflammatory mediators, chemokines are thought to be important to attract and activate inflammatory cells in the joint. Over 50 chemokines have been characterized and classified into different families (CXC, CC, C, CX3C) according

to the arrangement of the first two cysteine residues (reviewed in [146]). Different chemokines have been found in the synovial fluid from OA patients [147, 148]. Experimental evidence suggest that chemokine (C-C motif) ligand (CCL)2 and IL-8 can play a major role in attracting monocytes to the synovium [149]. Interestingly, chemokine receptors are present not only in cells of the immune system but also in OA chondrocytes and synoviocytes [150]. The expression of chemokines is increased during OA in both chondrocytes [151] and synoviocytes [152], suggesting a role in the pathogenesis of this condition [153]. These mediators can induce the release of catabolic enzymes such as MMPs [151] and activate apoptotic pathways in OA chondrocytes [154]. Chemokines may also contribute to synovitis and joint damage through the migration and activation of synovial fibroblasts which can adhere to cartilage and bone matrix proteins [133].

HO-1 overexpression may regulate chemokine production and cell migration in OA synoviocytes. Incubation of OA synoviocytes with IL-1β for 24 h strongly stimulated the release of IL-8, CCL2, and CCL20 into the culture medium, whereas in cells transduced with LV-HO-1, the production of these chemokines was significantly decreased at both protein (Fig. 11.4) and mRNA (Fig. 11.5) levels. We next examined the functional consequences of these changes in chemokine production. For this purpose, we assessed the effects of LV-HO-1 or CoPP on synoviocyte migration. Experiments were performed in transwell chambers to measure the migration of OA synoviocytes towards the compartment containing supernatants harvested from cells incubated with CoPP or transduced with LV-HO-1, in the presence or absence of IL-1β. As shown in Fig. 11.6a, the percentage of cell migration was significantly lower against supernatants from cells incubated with CoPP + IL-1β compared with those from control incubations with IL-1β. Similar results were obtained with LV-HO-1 (Fig. 11.6b).

Proinflammatory cytokines could amplify the inflammatory response in OA synoviocytes. However, IL-1β is a strong stimulus for chemokine production in synovial cells [152, 155]. In particular, CCL2 may play an important part in joint degradation as this chemokine induces MMP-3 [151], inhibits proteoglycan synthesis, and enhances proteoglycan release from OA chondrocytes [156]. Our data indicate that HO-1 exerts inhibitory effects on the expression of IL-8, CCL2 and CCL20 in OA synoviocytes stimulated with IL-1β, which is in line with previous studies showing inhibitory effects of HO-1 on CCL2 production in U937 cells [155] and NRK-52E cells [157]. Little is known of the possible role of CCL20 in OA. This chemokine may be involved in subchondral bone remodeling [158] and mechanical responses in chondrocytes [159]. Interestingly, in rheumatoid arthritis, CCL20 production by synovial cells seems to be critically involved in the disease process and is significantly downregulated by anti-TNF-α treatments [160]. CCL2, CCL20, and IL-8 are chemoattractant for leukocytes and synovial macrophages and fibroblasts in the rheumatoid joint [149, 161, 162]. Therefore, the downregulation of these chemokines by HO-1 may be responsible for the lower migratory ability of OA synoviocytes.

Several studies have demonstrated the participation of NF-κB in the regulation of inflammatory mediators in arthritic and osteoarthritic synoviocytes [163, 164]. The results of our previous work suggest that CORM-2 inhibitory effects on MMP

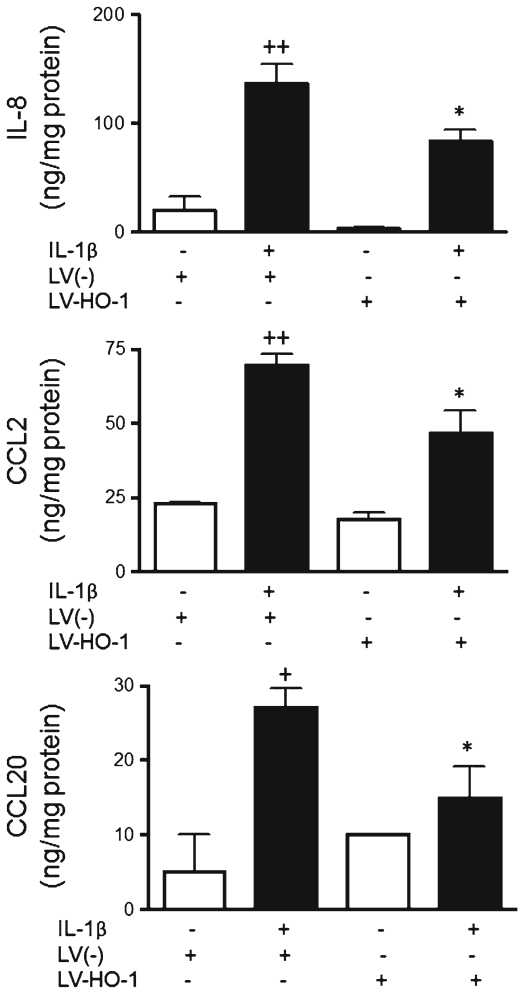

Fig. 11.4 Effect of HO-1 on chemokine protein expression in OA synoviocytes. Cells were stimulated with IL-1β (10 ng/ml) for 24 h in the presence or absence of synoviocytes transduced with lentiviral vector HO-1 (LV-HO-1) or empty vector (LV(−)) [138]. Protein levels were determined in cell supernatants by ELISA. IL-8 and CCL2 levels were determined by specific ELISA from eBioscience (San Diego, CA, USA) with sensitivities of 4 and 7 pg/ml, respectively, and CCL20 by specific ELISA from R&D Biosystems (Abingdon, UK) with sensitivity of 0.47 pg/ml. Data are expressed as mean ± S.E.M. Duplicate samples from 6 patients were used. $+p<0.05$, $++p<0.01$ with respect to nonstimulated cells transduced with LV(−). $*p<0.05$ with respect to LV(−)-transduced cells stimulated with IL-1β. One-way analysis of variance followed by Bonferroni's test

and chemokine production by OA synoviocytes are mediated by the downregulation of NF-κB and the inhibition of MAPK activation [139]. Figure 11.7a shows that CoPP treatment significantly reduced NF-κB binding to DNA induced by IL-1β. In addition, CoPP decreased the effects of this cytokine on IκBα phosphorylation

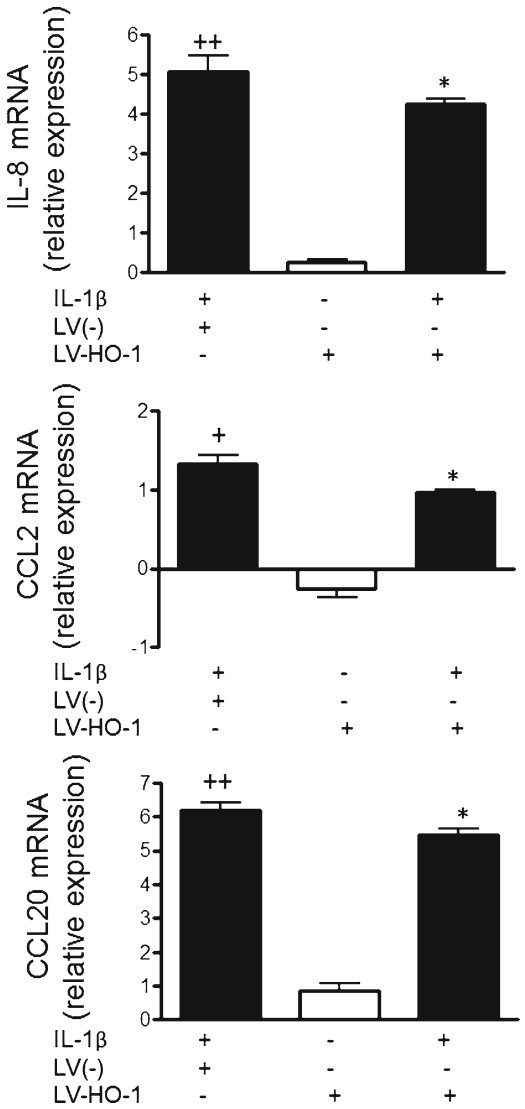

Fig. 11.5 Effect of HO-1 on chemokine mRNA expression in OA synoviocytes. Cells transduced with LV-HO-1 or LV(−) were stimulated with IL-1β (10 ng/ml) for 16 h. mRNA expression was determined by real-time PCR [139]. Data are expressed as mean ± S.E.M. Duplicate samples from 3 different patients were used. $+p<0.05$, $++p<0.01$ with respect to nonstimulated cells transduced with LV(−). $*p<0.05$ with respect to LV(−)-transduced cells stimulated with IL-1β. One-way analysis of variance followed by Bonferroni's test

(Fig. 11.7b). Our results thus suggest that the observed inhibitory effects of HO-1 on chemokine and MMP expression could be related to the downregulation of NF-κB activation in OA synoviocytes stimulated with IL-1β which may be dependent on the reduction in IκBα phosphorylation, leading to a reduced nuclear translocation of NF-κB and thus preventing the binding to this transcription factor to DNA. The effects of HO-1 on OA synoviocytes are summarized in Fig. 11.8.

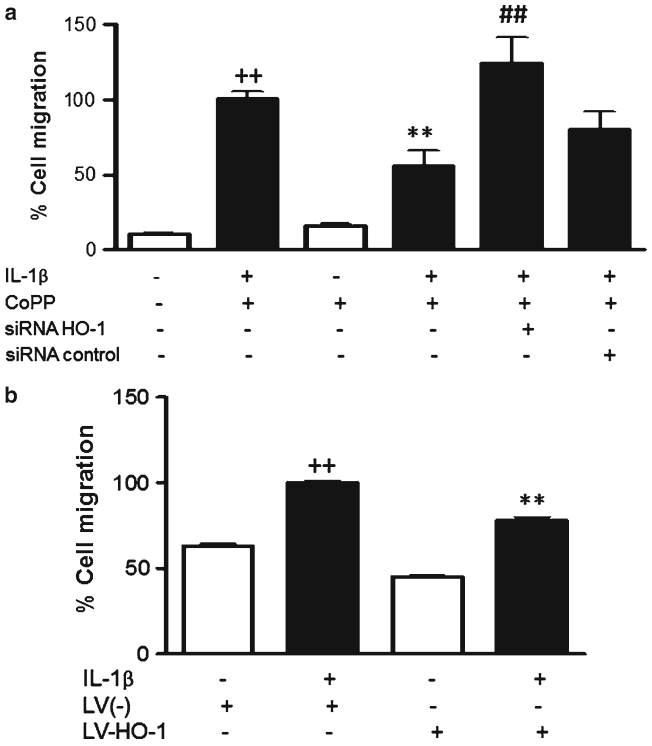

Fig. 11.6 Effect of HO-1 on synoviocyte migration. (**a**) Cells were stimulated with IL-1β (10 ng/ml) for 24 h in the presence or absence of CoPP (10 μM) and a siRNA specific for HO-1 or siRNA control (100 nM). Transwell chambers were kept in culture for 24 h. The upper compartment was detached, and cells migrated to the lower side were counted in 6–8 microscopic power fields. Data are expressed as mean ± S.E.M. of cells migrated to the lower compartment, considering 100 % the migration induced by IL-1β. Duplicate samples from 3 different patients were used. $++p<0.01$ with respect to nonstimulated cells. $**p<0.01$ with respect to IL-1β. $\#\#p<0.01$ with respect to IL-1β + CoPP. (**b**) Cells transduced with LV-HO-1 or LV(−) were incubated in the presence or absence of IL-1β (10 ng/ml) for 24 h. Duplicate samples from 3 different patients were used. $++p<0.01$ with respect to nonstimulated cells transduced with LV(−). $**p<0.01$ with respect to LV(−)-transduced cells stimulated with IL-1β. One-way analysis of variance followed by Bonferroni's test

11.5 Conclusions

Oxidative stress is involved in the generation of key mediators involved in the progression of articular degradation and OA. HO-1 represents a protective strategy against stress with antioxidant, antiinflammatory, and anabolic properties. Our results suggest that HO-1 effects in OA articular cells occur, at least in part, via its ability to attenuate oxidative stress.

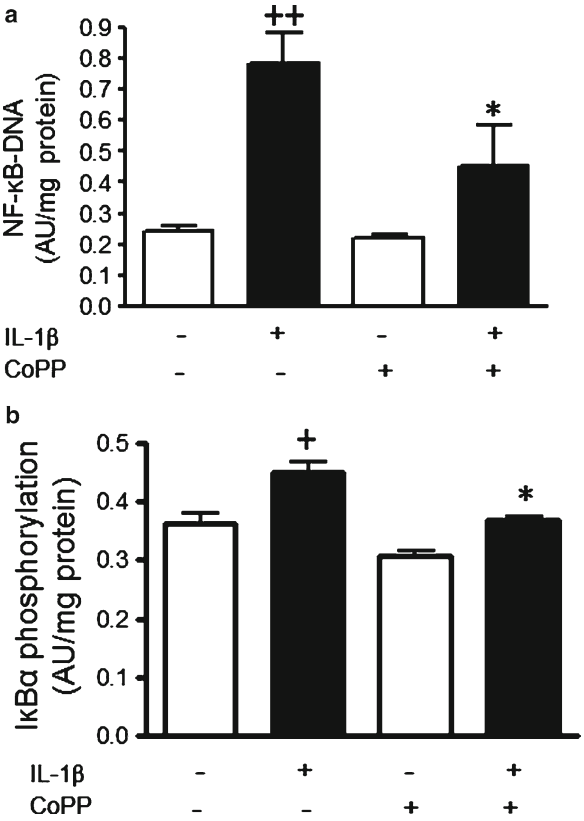

Fig. 11.7 Effect of CoPP on NF-κB activation in OA synoviocytes. (**a**) NF-κB binding to consensus oligonucleotide in DNA. (**b**) IκBα phosphorylation. Cells were stimulated with IL-1β (10 ng/ml) for 1 h in the presence or absence of CoPP (10 μM). IκBα phosphorylation and NF-κB binding to DNA were determined by ELISA in cytosolic and nuclear fractions, respectively. AU (arbitrary units). Data are expressed as mean ± S.E.M. of independent cultures with cells from 6 different donors. $+p<0.05$, $++p<0.01$ with respect to nonstimulated cells. $*p<0.05$, with respect to IL-1β. One-way analysis of variance followed by Bonferroni's test

Therefore, HO-1 or CO acts on OA chondrocytes, osteoblasts, synoviocytes, and osteochondral explants counteracting cytokine-induced inflammatory and catabolic effects on cell metabolism, cartilage degradation, and synovitis which may preserve joint integrity. These properties of HO-1 or CO may lead to novel therapeutic targets and approaches to prevent or treat joint destruction.

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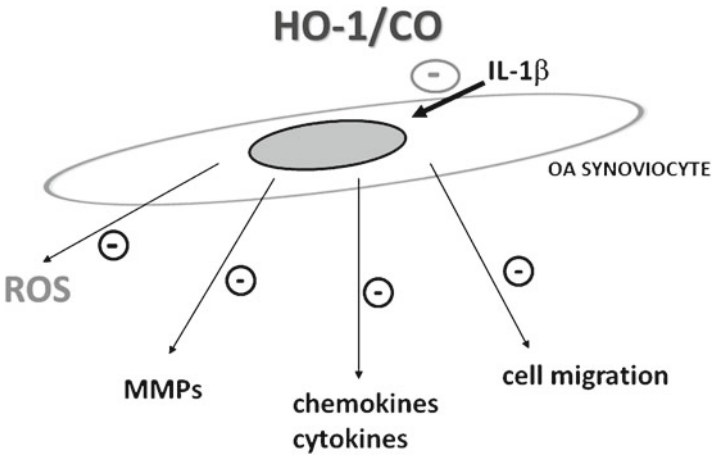

Fig. 11.8 Summary of HO-1 (or CO) effects on OA synoviocytes

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Chapter 12
Oxidative Stress at the Crossroads Between Osteoarthritis and Metabolic Syndrome

James D. Katz and Manuel T. Velasquez

Abstract This chapter focuses on the role of oxidative stress in osteoarthritis and metabolic syndrome with discussion of mechanisms and commonalities. We address the molecular biology of chondrocytes, synoviocytes, adipocytes, and mitochondria as they relate to these two disorders. The final discussion concerns oxidative stress and epigenetic modulation as a target for therapy of osteoarthritis. In short, oxidative stress plays a role in the generation of the osteoarthritis changes seen in cartilage. As outlined below, oxidative stress may adversely impact not only the loss of maturational arrest of chondrocytes but the extracellular joint matrix as well.

Abbreviations

AP-1	Activator protein-1
EC-SOD	Extracellular superoxide dismutase
eNOS	Endothelial nitric oxide synthase
FRZB	Frizzled-related protein
HDL-C	High-density lipoprotein cholesterol
IL	Interleukin
LDL	Low-density lipoprotein
MetS	Metabolic syndrome
MMPs	Matrix metalloproteinases
MPCs	Mesenchymal progenitor cells
MRC	Mitochondrial respiratory chain
NF	Nuclear factor
NO	Nitric oxide
OA	Osteoarthritis

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ROS	Reactive oxygen species
SNPs	Single-nucleotide polymorphisms
TAC	Total antioxidant capacity
TGFα	Transforming growth factor-α
TLR-4	Toll-like receptor-4
TNFα	Tumor necrosis factor-α
TP	Total peroxide

12.1 Introduction

Osteoarthritis (OA) is one of the most common disabling joint diseases affecting individuals across populations with increasing prevalence worldwide [1]. OA is characterized pathologically by the progressive loss of articular cartilage and by abnormalities of many parts of the joint structure including chondrocytes, synovium, and subchondral bone. The disease is marked by local ischemia, inflammation, cartilage degradation, osteophyte formation, subchondral sclerosis, and subchondral cyst formation that together result in pain, decreased function, and sometimes deformity of the affected joints. Although mechanical loading of joints, age, obesity, and heredity are well-recognized risk factors for OA, the mechanisms or mediators involved in its pathogenesis are complex and still incompletely understood.

In recent years, a large body of evidence has accumulated that suggests that OA is not simply a local disease related to mechanical "wear and tear" of joints but rather a part of a multisystem disorder in which various interrelated metabolic, hormonal, humoral, cellular, and molecular mediators contribute to the initiation and progression of the disease process [2]. Recent epidemiological and population studies have shown an association of OA with systemic conditions or disorders such as diabetes, dyslipidemia, hypertension, and insulin resistance. This clustering of metabolic and hemodynamic abnormalities, commonly referred to as the "metabolic syndrome" (MetS), in association with OA, suggests an underlying common pathogenic mechanism or pathway.

12.2 Oxidative Stress: A Shared Mechanism in MetS and OA

Oxidative stress may be viewed as a reactive response of cells in which the production of endogenous reactive oxygen species (ROS) or "free radicals" exceeds the capacity of cells to produce and neutralize reactive metabolites (antioxidant activity) thereby leading to damage. Oxidative damage from excessive ROS production has been implicated in numerous conditions including aging, inflammation, cancer, hypertension, diabetes, obesity, atherosclerosis, and the metabolic syndrome itself. Although OA has traditionally been considered a noninflammatory (or degenerative) disorder, evidence also exists suggesting that inflammation, in general, and ROS, specifically, both play important pathophysiological roles in the development of OA.

In a clinical assessment of several hundred patients with knee osteoarthritis, the relationship with oxidative stress was affirmed [3]. In order to best appreciate the significance of this finding, it is necessary to not only assess the impact of proinflammatory cytokines on joint health but also to consider the local tissue milieu (e.g., adipokines), differentiation signals and transcription factors, extracellular matrix remodelers, hypoxia-inducible transcription factors, lipids, advanced glycation end products, and miRNAs [4]. For example, given the possible increased incidence of diffuse idiopathic skeletal hyperostosis (a degenerative arthritis disorder abbreviated DISH) in patients with diabetes mellitus, it has been postulated that the bone-promoting effects of glucose-sensitive factors are pathogenic (e.g., insulin-like growth factor, growth hormone, and insulin itself) [5]. Moreover, besides glucose-sensitive factors, among the inflammatory cytokines known to be elevated in degenerative (as well as inflammatory) arthritides are interleukin (IL)-1β, tumor necrosis factor-α (TNFα), and IL-6 [6]. Not surprisingly, these same cytokines may play pathogenic roles in the end-organ damage seen in diabetes mellitus [7]. Observations such these compel search for a linkage between metabolic syndrome and OA.

12.3 Chondrocytes

Under normal conditions, chondrocytes show little metabolic activity with low turnover of matrix components. However, under conditions of mechanical or metabolic stress, chondrocytes are capable of producing a variety of mediators that are associated with cellular growth and proliferation. They are also capable of producing prostaglandins, nitric oxide (NO), and inflammatory cytokines such as interleukin-1 and tumor necrosis factor-alpha, which in turn stimulate the production of ROS. Chondrocytes express receptors for these mediators, which can act in an autocrine–paracrine fashion to regulate chondrocyte behavior.

In the setting of OA, chondrocytes have been shown to produce extracellular-matrix-degrading proteins such as the matrix metalloproteinases (MMPs) and proinflammatory cytokines (predominantly interleukins and tumor necrosis factors). They also produce prostaglandins, NO, and other ROS. The clinical relevance of this is an ongoing thrust of investigation. For example, the successful downregulation of transforming growth factor-alpha (TGFα)-induced production of MMP-13 in rat chondrocyte explants treated with endothelin receptor antagonist demonstrates a novel potential therapeutic target for intervening in the OA process [8].

Chondrocyte differentiation and chondrocyte sensitivity to mechanical stress are two other areas of potential therapeutic intervention. Adult bone marrow-derived mesenchymal progenitor cells (MPCs), which are able to differentiate along various mesenchymal cell lineages (including chondrocytes, osteocytes, and adipocytes), have been isolated from articular synovium. These MPCs appear to participate in local cartilage regeneration during arthritic diseases [9]. In patients with advanced OA, however, MPCs display reduced chondrogenic activity [10]. Furthermore,

in vitro data confirms that chondrocytes subjected to mechanical (high fluid shear) stress adopt a gene expression profile akin to that seen in OA [11], and of course, prominent among the various responses by chondrocytes to mechanical loading is the production of reactive oxygen species [12]. Therapeutic intervention at this phase of disease is, nevertheless, possible. In a porcine model, adverse effects of mechanical stress can be ameliorated by exposing cartilage to sublethal concentrations of peroxide. Presumably, peroxide-induced changes in gene expression render chondrocytes more resistant to oxidative damage [13].

12.4 Synoviocytes

Synovial cells (or synoviocytes) participate in the development and progression of OA. Indeed, several studies indicate that in the setting of OA, synovial cells are activated by proinflammatory cytokines resulting in release of chemokines, ROS, and other mediators that likely sustain the inflammatory response [14], as well as proteases such as MMPs, which act in a synergistic manner to degrade the components of connective tissue. Moreover, the proinflammatory cytokines produced by chondrocytes are also produced by synoviocytes [15]. In particular, IL-1β is one such cytokine that activates a broad array of signaling pathways in joint tissues and ultimately shifts cartilage homeostasis toward catabolism [16, 17].

Like bone marrow-derived mesenchymal stem cells, synovial fibroblasts from OA patients' evidence reduced chondrogenic differentiating potential. Evidence suggests that treatment with the proinflammatory cytokine, TNF-α, leads to upregulation of surface and secreted molecules, including CD54, CD106, membrane IL-15, CCL2, and CCL5. This upregulation of adhesion molecules seems to parallel fibroblast activation including both differentiation and IL-6 production [18]. However, under TGF-β1 treatment (and adipogenic culture conditions), synovial fibroblasts display chondrogenic and adipocytic activities that are reduced in OA compared with non-inflamed synovium [19]. In particular, they demonstrate reduced ability to produce cartilage-like matrix. Synovial fibroblasts are therefore likely candidates for potentiating joint damage under proinflammatory conditions.

Finally, high levels of NO have been measured not only in cartilage but also in synovial membrane tissues from patients with rheumatoid arthritis (RA) and OA, alike. Available data suggests that NO induces synoviocyte apoptosis [20]. Cultured synovial fibroblasts in particular appear to be sensitive to the adverse effects of reactive oxygen species [21]. Moving out of the basic science and into the clinical realm, the overall thesis that this type of oxidative stress adversely impacts fibrovascular synovial tissue has borne out on analysis of subsynovial tissue obtained during carpal tunnel surgery [22]. Specifically, fibrovascular proliferation parallels an increase in histological staining for endothelial nitric oxide synthase (eNOS), nuclear factor (NF)-κβ, and TGF-β receptor I.

12.5 Adipocytes

The local (to joints) adipose milieu is capable of promoting synovial inflammation. Activated adipose tissue synthesizes proinflammatory cytokines that in turn stimulate neuropeptides. This adversely impacts cartilage homeostasis as well as overall organismal metabolism [23]. Consequently, this finding has compelled some authors to refer to cartilage degeneration as a "metabolic disease" [24].

Not only adipose tissue but regional activated macrophages may play a role in the degenerative process. Human adipose tissue contains macrophages that, when activated, demonstrate proangiogenic and matrix remodeling/fibrotic phenotypes with the ability to produce TGF-β [25]. It is likely that this pro-fibrotic phenotype is supported by a synergistic effect between TGF-β and Toll-like receptor-4 (TLR-4) recognition [26]. At least in vitro it appears that the inflammatory local environment of joints is augmented by stimulating macrophages via TLR-4 [27]. Indeed, TLR-4 is a key receptor involved in enhancing the induction of the TH1 immune response [28]. Along these lines, it is not surprising that preliminary evidence suggests that uric acid-mediated changes are in part due to activation of TLR-4 [29]. Ultimately, when TH1 cytokines are upregulated, insulin receptors on both muscle and fat are rendered insensitive. From this, it can safely be surmised that obesity itself serves to amplify the overall problem [30]. Indeed, a candidate cytokine mediator of the TH1 inflammatory response is the hormone leptin [31]. Of course, leptin can also induce the production of ROS, and recently, experimental evidence now points mechanistically to enhanced adipocyte maturation from mesenchymal stem cells in the setting of elevated levels of ROS [32].

12.6 The Biochemical and Molecular Environment

12.6.1 Circulating Markers

Paraoxonases are enzymes well known to reduce oxidative stress in serum and tissues and have been reported to have protective effects against cardiovascular disease, particularly atherosclerosis. Decreased paraoxonase and arylesterase activities have been implicated in the pathogenesis of atherosclerosis, and decreased serum levels of these enzymes may be found in patients with osteoarthritis [33].

Serum paraoxonase and arylesterase activities and serum levels of high-density lipoprotein cholesterol (HDL-C) levels are significantly lower in OA patients than those in the controls, whereas low-density lipoprotein (LDL) levels are significantly higher. The lower serum paraoxonase-1 activity with the lower level of HDL-C appears to be related to increased oxidative stress in these patients [34]. In a study of serum markers of oxidative stress assessed by total

peroxide (TP) and lipid hydroperoxide, it was found that these markers are increased in OA patients. At the same time, when antioxidative status was assessed by total antioxidant capacity (TAC), it was found that thiol levels and catalase enzyme activity were reduced in OA. Serum prolidase enzyme activity (a measure of collagen metabolism) was also observed to be significantly lower in OA patients than in controls. In addition, prolidase activity was negatively correlated with TP and positively correlated with TAC. These data support the thesis that OA patients are exposed to a systemic environment of increased oxidant stress with a resultant adverse impact on overall cartilage metabolism [3]. Such data has also been confirmed clinically in the situation of patients undergoing joint replacement surgery for OA [35].

12.6.2 Synovial Fluid

Joint fluid levels of vitamin E are reduced among some arthritis patients [36]. In contrast, protein carbonyl content is elevated as compared to controls [37]. This latter finding is a marker of free radical attack and is associated with OA per se [38]. More significantly, a reduction of superoxide dismutase has been documented among the earliest findings for osteoarthritis [39]. Extracellular superoxide dismutase (EC-SOD) is the major scavenger of ROS in extracellular spaces and fluids. It is also decreased in late-stage OA joint fluid compared to fluid from injured/painful joints with intact cartilage [40]. While injured joints may be able to increase or maintain secretion of EC-SOD, it appears that late-stage OA joints fail to do so in spite of the increased oxidative stress seen in the disease. Compounding the problem, associated age-related declines in glutathione and ascorbate may be potentiating factors. The net effect of these observed changes in joint fluid antioxidants is likely to accelerate the damaging oxidant effects on extracellular matrix stability within cartilage tissue [40].

IL-18 is emerging as a biomarker for metabolic syndrome and increased risk for cardiovascular disease [41]. In particular, changes in IL-18 are robust to changes in waist circumference and lifestyle modifications. At the cellular level, isolating monocyte-derived macrophages from subcutaneous adipose tissue biopsies has confirmed a significant relationship between in vitro hyperglycemia-induced expression of IL-18 and established metabolic syndrome [42]. Finally, concurrent with increased circulating levels of IL-18, it is possible to identify increased levels of IL-18 within the unstable atherosclerotic plaque itself [43]. From a rheumatological perspective, it is noteworthy that IL-18 is capable of regulating chondrocytes and of mediating cartilage degradation [44]. For example, IL-18 mRNA is induced by IL-1β within chondrocytes, and in the setting of rheumatoid arthritis, elevated levels of IL-18 may be found in joint fluid [45]. Preliminary studies also suggest that therapeutically targeting IL-1β can ameliorate osteoarthritis. Ultimately, it is likely that this effect relates in part to a downregulation of matrix metalloproteinases [46].

12.6.3 Nitric Oxide and Osteoarthritis

The pathogenesis of OA includes increased NO as produced by chondrocytes and synovial cells. This is a consequence of the upregulation of NO synthase (NOS). Such upregulation, in turn, is induced by IL-1β, TNF-α, and other factors [47]. NOS has been of interest in the pathogenesis of OA because of its role in chondrocyte and synoviocyte death [48]. Along these lines, it has been shown that NO donors have potent effects at both the synovial and cartilage levels. Specifically, NO donors may enhance hyaluronic acid synthesis or impact apoptosis of synovial fibroblasts [49, 50]. However, the exact mechanism by which an imbalance in NO/NOS within the joint impacts the development of arthritis remains to be elucidated. Certainly, one mechanism of joint damage, the accumulation of advanced glycation end products, is intimately tied to the NO/NOS balance within joints [51]. Perhaps more importantly, as will be explored next, NO is also known to modulate various cellular pathways and thus inhibits the activity of the mitochondrial respiratory chain (MRC) of chondrocytes.

12.6.4 Radical Oxygen Species

The role of oxidative stress as a mechanism in obesity-associated metabolic syndrome (including individually diabetes, hyperlipidemia, hypertension, and the development of cardiovascular complications) has been extensively investigated in both animals and humans. In both situations, fat accumulation closely correlates with the markers of systemic oxidative stress. In addition, increased production of ROS in accumulated fat resulting from increased NADPH oxidase and decreased antioxidative enzymes has both local and systemic adverse effects. It is thought that ROS participate in the various stages of inflammation by virtue of activating multiple intracellular signaling molecules and transcription factors associated with the inflammatory response, namely, NF-κβ and activator protein-1 (AP-1).

In the setting of OA, a slew of transcription signals may trigger NF-κβ production. These may include not only proinflammatory cytokines but actual mechanical stress and the presence of matrix degradation products [52]. In theory, targeting NF-κβ-activating kinases could be a therapeutic target for preventing the loss of maturational arrest that is so characteristic of OA chondrocytes.

12.7 Mitochondria

The mitochondrion is a complex organelle that plays an important role in various cellular processes, such as regulating energy production, apoptosis, and the oxidative state of the cell. In vascularized aerobic tissues, it provides metabolic fuel to

cells by generating ATP. It is now known that the release of caspase activators by the mitochondrion is central to apoptotic pathways [53].

In contrast to highly vascular tissues, articular chondrocytes survive and maintain tissue integrity in a low-oxygen environment, with asymmetric, decreasing oxygen and glucose concentration gradients from the superficial to the deep zones. Thus, chondrocytes in the deeper zones may require adaptively increased anaerobic glycolysis, while those in superficial areas use aerobic respiration to support ATP synthesis. Recently, the redox state of the mitochondrion in chondrocytes has been implicated in OA pathogenesis. Alterations in ATP production and modulation of calcium levels could explain some mechanisms that induce cartilage degradation by chondrocytes during OA.

Research suggests that mitochondrial activation, and/or dysfunction, may contribute to the development of OA. In vitro data reveals that under mechanical loading, mitochondria release ROS thereby resulting in chondrocyte death [54]. At the same time, clinical data from a detailed investigation of endemic degenerative osteoarthritis confirms the presence of mitochondrial dysfunction in human OA chondrocytes by virtue of decreased activity of respiratory chain enzyme complexes [55]. Here, it should be acknowledged that mitochondrial dysfunction may affect other pathways that have been implicated in cartilage degradation, such as defective chondrocyte biosynthesis and growth responses, increased cytokine-induced chondrocyte inflammation and matrix catabolism, and cartilage matrix calcification.

Speculating upon the various potential etiologies of mitochondrial dysfunction brings up the possibility of somatic mutations in the mtDNA itself or the adverse direct effects of proinflammatory mediators such as cytokines, prostaglandins, ROS, and NO. If somatic mtDNA mutations are at play, then polymorphisms in mtDNA may become useful as biomarkers for the diagnosis and prognosis of OA. In fact, a point mutation in mtDNA of chondrocytes has been reported [56]. This supports the concept that mtDNA haplogroups may define specific OA phenotypes. Going one step further, the hypoxia-related resistance to apoptosis of chondrocytes may even be a therapeutic target for OA disease modulation [57].

On the organismal level, the generation of reactive oxygen species by the mitochondrion may be an intriguing clue to the intimate relationship between OA and MetS. In the rat model, allopurinol effectively inhibits the clinical manifestations of oxidative stress [58]. This is striking because of the involvement of endothelin receptor-modulated ROS that are derived from xanthine oxidase and from other mitochondrial oxidative enzymes within arteries from DOCA-salt rats. Moreover, from a mutagenesis standpoint, treatment with antioxidants rescues the cell from hypoxia-induced mitochondrial dysfunction [59].

12.8 Gene Level

Because osteoarthritis is a state of increased oxidative stress, superoxide dismutase, itself, has been proposed as a negative serum biomarker for the disease [60]. Indeed, gene expression profiling performed in normal and OA cartilage shows increased

expression of many anabolic and catabolic matrix genes. However, in the animal model, SOD appears to be downregulated within late-stage OA tissue [39]. Specifically, the expression of oxidative defense genes, namely, the genes for SOD 2 and SOD 3 and for glutathione peroxidase 3 is reduced [61].

12.8.1 Genetic and Epigenetic Modulation

The influence of genetic factors on the development of OA is well documented in a variety of studies, including epidemiological studies of familial clustering, twin studies, gene association studies using single-nucleotide polymorphisms (SNPs), and genome-wide scans [62]. Genetic association studies have identified an increasing number of SNPs that are associated with the pathology of OA. These include, among others, SNP rs143383 [63] and frizzled-related protein (FRZB) gene polymorphisms [64]. However, it appears that so far these identified SNPs contribute to only a small genetic component of OA.

Evidence has also emerged that suggests that epigenetic modifications (i.e., changes in gene expression that do not involve a change in genomic DNA sequence) play a crucial role in many common chronic diseases. Epigenetic markers not only differ between individuals but also as a result of disease and environmental interactions. Changes in epigenetic events, such as aberrant DNA methylation (a leading mechanism for regulation of gene expression), have been amply described in many diseases including cancer, diabetes, atherosclerosis, autoimmune diseases, and other chronic inflammatory states. Inflammatory signaling may be one mediator driving epigenetic modulation in chronic diseases [65]. Interestingly, the nuclear transcription factor, (NF-κβ), appears to be involved in this epigenetic dysregulation. For example, histone deacetylase inhibitors, which are known to possess anti-inflammatory activities, directly inhibit NF-κβ-induced transcription. This effect is mediated by inhibiting recruitment of RNA polymerase II [66].

There is evidence that suggests that epigenetic events may play an important role in chondrocyte biology and the gene expression changes observed in OA cartilage. For example, DNA methylation is an important factor for tissue- and cell-specific differentiation during chondro-neogenesis, indicating that genes involved in chondrocyte development and function may be subject to epigenetic regulation [67]. In studies of chondrocytes harvested from OA subjects, Roach et al. found a close association between the abnormal expression of matrix-degrading enzymes by OA chondrocytes and demethylation of specific CpG sites in the promoter regions [68]. However, Poschl et al. did not find an association between hypermethylation and silencing of aggrecan expression in OA chondrocytes [69].

Previous studies of human articular cartilage have shown that the aggrecanase, ADAMTS-4, contributes to cartilage degradation in human OA. For example, Cheung et al. found that expression ADAMTS-4 was upregulated in human OA cartilage but was virtually absent in control cartilage [70]. Specifically, ADAMTS-4 immuno-positive chondrocytes were present in OA cartilage, and their numbers

increased with disease severity. Moreover, DNA methylation was lost at specific CpG sites in the ADAMTS-4 promoter in OA chondrocytes, suggesting that the increased gene expression involved loss of DNA methylation at specific CpG sites, resulting in a heritable and permanent expression of ADAMTS-4 in OA chondrocytes. These results suggest that ADAMTS-4 is epigenetically regulated and plays a role in aggrecan degradation in human OA.

Other evidence for epigenetic modulation in OA chondrocytes is provided by the studies of Iliopoulos et al. which showed that the adipokine leptin can also be regulated by epigenetic mechanisms in OA [71]. In their study, leptin was found to be methylated in normal chondrocytes and unmethylated in mildly and severely affected osteoarthritic chondrocytes and also that the methylation status correlated with gene expression levels. Using the chromatin immunoprecipitation approach, these investigators observed that leptin was regulated by histone acetylation in its promoter region. An intriguing finding in this study is that leptin's epigenetic regulation also affected its downstream catabolic target (MMP-13). Finally, treatment with siRNA against leptin transfected into OA chondrocytes, together with liposomes, downregulated MMP-13 expression and had no effect on the expression levels of other metalloproteinases. Taken together, these series of observations suggest that leptin is epigenetically regulated in OA and that it directly affects MMP-13 expression levels. Since epigenetic changes are potentially reversible, epigenetic therapy using small interference RNA could be a new molecular target for therapeutic intervention, especially in the early stage of OA.

12.9 Conclusion

Evidence has accumulated that OA is not simply a local disease caused by mechanical "wear and tear" of joints but rather a part of a generalized metabolic disorder in which various interrelated humoral, metabolic, cellular, and molecular mediators or factors contribute to the progressive degradation of articular cartilage, which is the pathological hallmark of OA. Recent epidemiological and population studies have shown a strong and independent association of OA with several components of the metabolic syndrome, namely, obesity, diabetes, dyslipidemia, hypertension, and insulin resistance, suggesting an underlying common mechanism or pathway.

Oxidative stress resulting from increased generation of ROS has been implicated in many common conditions or diseases, including aging, inflammation, cancer, hypertension, diabetes, obesity, and atherosclerosis. In vivo and in vitro evidence also exists to suggest that ROS play an important pathophysiological role in the development of OA. Increased ROS and markers of oxidative stress have been observed in chondrocytes, synoviocytes, and adipocytes in the setting of OA. The signaling mechanisms or pathways that ultimately lead to cartilage damage in OA are complex and include participation of hormonal mediators, growth factors, proinflammatory cytokines, adipokines, as well nuclear transcription factors that promote cartilage degradation.

Finally, emerging evidence has been presented that suggests that epigenetic modifications (i.e., changes in gene expression that do not involve a change in DNA sequence) may play an important role in chondrocyte biology and the gene expression changes observed in degenerative joint disease. More importantly, epigenetic processes alter gene function and are potentially reversible. Thus, such processes may provide new molecular targets for therapeutic intervention, especially in the early stage of OA. More research is needed to explore epigenetic and other novel factors or mechanisms that cause joint damage in patients with OA.

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Chapter 13
Metabolic Syndrome and Gout

Eswar Krishnan

Abstract The association between gout and the phenotype that we now recognize as metabolic syndrome has been recognized for thousands of years. With aging population, increasing prevalence of risk factors for metabolic syndrome, the number of people in the general population with metabolic syndrome and gout is large and is rising over time. This chapter summarizes contemporary literature on this topic as well as provides the latest data on the incidence and prevalence of metabolic syndrome in gout in the US. The potential pathophysiological links between metabolic syndrome and gout such as oxidative stress, and inflammation is discussed in the context of the epidemiologic data.

Abbreviations

BC	Before Christ
CHAOS	Coronary artery disease hypertension, atherosclerosis, obesity, and stroke
CKD	Chronic kidney disease
CKD EPI	Chronic Kidney Disease Epidemiology Collaboration
LDL	Low-density lipoprotein
MRFIT	Multiple Risk Factor Intervention Trial
NCEP	National Cholesterol Education Program
NHANES	National Health and Nutrition Examination Survey
SIR	Standardized incidence ratios

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13.1 Gout

Gouty arthritis (gout) is one of the best known types of arthritis. Gout manifests as an abrupt onset of excruciating pain and swelling and can affect almost any joint in the body. The underlying reason for such inflammation is the deposition of urate crystals within the joints. This type of arthritis is, fortunately, episodic in most patients, and most flares self-resolve over a week or less. With some exceptions, however, the arthritis is recurrent over time.

Gout is an ancient disease. Urate crystals have been identified in the mummified joints of Egyptians dating back to 2000 BC [1], although it is not known if gout was recognized as a distinct arthritis at that time. The earliest written description of gout is attributed to Hippocrates. Since then many physicians and researchers have examined patients with this disease and have documented the central role of urate in the causation of gout.

The fundamental pathophysiologic phenomenon that precedes the onset of gout is the accumulation of excess total body urate, manifested as elevated serum urate levels [2]. Increases in serum urate concentrations are associated with increased incidence of gout (Fig. 13.1). Gout can occur where serum uric acid levels are as low as 6 mg/dL (~357 μmol/L); however, an individual can have serum values as high as 9.6 mg/dL (~565 μmol/L) and not have gout [3]. Women tend to have a different distribution of serum urate and hence develop gout at lower serum urate concentrations than men (Fig. 13.2).

In humans, the hepatic end product of purine metabolism is uric acid. The kidneys eliminate two-thirds, and the gastrointestinal tract eliminates one-third of the uric acid load. Urate is secreted by the kidney's proximal tubules via transporters URAT1, OAT1, OAT2, OAT3, OAT4, and OAT10; the multidrug resistance proteins ABCC4 and ABCG2; the glucose transporters GLUT 9a and GLUT 9b; and others [4].

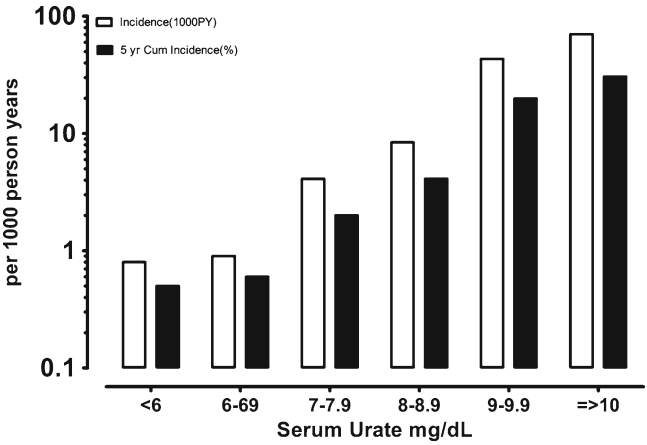

Fig. 13.1 Serum urate concentrations and incidence (*open bars*) and cumulative incidence (*closed bars*) of gout (data from the Normative Aging Study [2]. PY, person years; Cum Incidence, cumulative incidence)

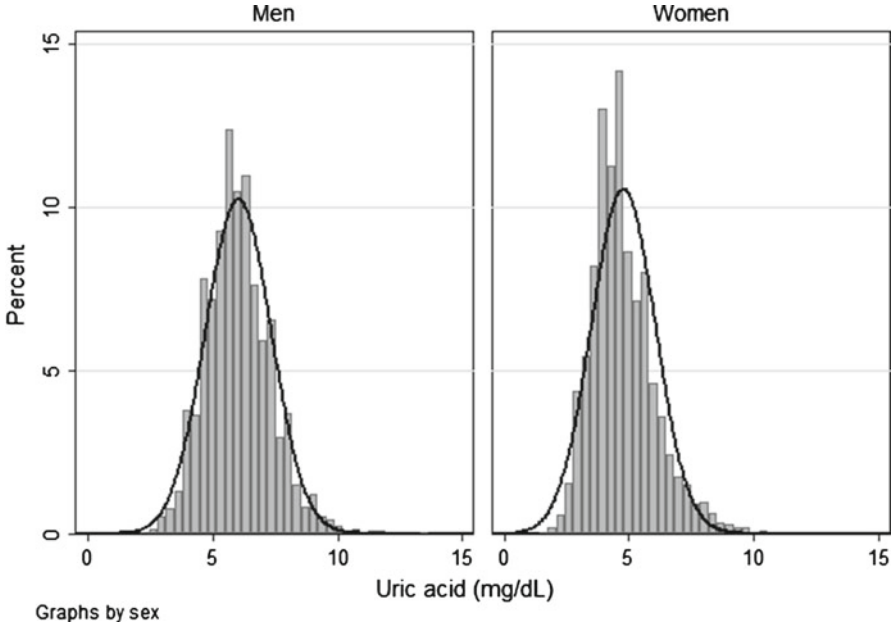

Fig. 13.2 Population distribution of serum urate in the USA in 2009–2010. The age-standardized, mean serum urate was 6.1 and 4.9 mg/dL for men and women, respectively

13.2 Metabolic Syndrome and Gout

The term metabolic syndrome has been used to describe clustering of multiple cardiovascular risk factors within each individual. This specific phenotype has been called metabolic syndrome X, cardiometabolic syndrome, syndrome X, insulin resistance syndrome, Reaven's syndrome, deadly quartet and insulin resistance syndrome, and CHAOS. One of the earliest descriptions of this phenotype was by two Viennese physicians Hitzenberger and Richter-Quittner in 1921 [5]. Others made similar reports at about the same time. Interestingly, in those days, hyperuricemia was part of the "hypertension–hyperglycemia–hyperuricemia syndrome" [5]. Camus in France and Hanefeld in Germany had recognized gout as an integral component of the metabolic syndrome by the 1960s [5, 6].

The most common criteria cited in the literature for metabolic syndrome are found in the National Cholesterol Education Program (NCEP) definition in the USA and in the International Diabetes Federation and the World Health Organization definition [7, 8]. The recent definitions, however, do not consider either hyperuricemia or gout to be "official" components of metabolic syndrome, although their prevalence is very high among patients with the syndrome. There are numerous criteria for defining this phenotype, and there is no consensus on an ideal definition. Nevertheless, these criteria have more in common with each other than differences. The choice of the criteria does influence prevalence rate estimates of metabolic syndrome in populations, however [9].

13.3 Metabolic Syndrome, Hyperuricemia, and Gout: Recent Data

The common denominator for gout and metabolic syndrome is the presence of elevated serum urate. Indeed, hyperuricemia was one feature of the original description of metabolic syndrome [10]. We have observed that the patients with metabolic syndrome generally have greater serum urate concentrations than those without [11].

Metabolic syndrome is prevalent among patients with gouty arthritis. A recent study of 12,179 men from the Hoping Hospital Database, Taiwan, found high prevalence of obesity, triglyceridemia, and diabetes among patients with gout [12]. In a carefully collected series of 64 consecutive cases of primary gout, 83% of patients had metabolic syndrome that met the NCEP criteria [13]. Data patients identified in Korean hospitals indicate that the prevalence of metabolic syndrome in patients with gout was much higher than that in the general Korean population (44% vs. 5%). Among those in the US population who participated in the Third National Health and Nutrition Examination Survey, the prevalence of metabolic syndrome was about 63% among those with a self-reported physician diagnosis of gout, whereas the prevalence among those who did not report gout was about 25% [14].

Although these data provide useful insights into the links between metabolic syndrome and gout, several important pieces of information are missing. For instance, what is the prevalence of gout among those with and without metabolic syndrome in the contemporary USA? Are there gender disparities in such prevalence? And is metabolic syndrome a risk factor for gout?

We assessed the first question using data from the 2009–2010 cycle of the National Health and Nutritional Examination Survey (NHANES). The sampling frame of participants in the NHANES is all noninstitutionalized adults and children in the US population. An exhaustive description of the survey design, data collection strategies, and instruments are available online [15]. Briefly, this survey is a complex multistage sample of the US population where the basic geographic unit is the county. The survey deliberately oversamples difficult-to-enroll patient subgroups. The survey had three major data collection components: (1) a telephone interview, (2) an in-person study visit with additional questionnaires, anthropometry, and other biometric measurements, and (3) laboratory testing including a fasting phlebotomy. Self-reported use of diabetes and hypertension medication was available although specific medication data were not available for the present analyses. All adults (age > 20) who completed a household interview and laboratory visit and who were not pregnant or breast feeding were included. A very small number of participants (<22) had a self-report of receiving dialysis. These were not excluded. Fasting serum specimens are processed, stored, and shipped to the Collaborative Laboratory Services for analysis. Detailed specimen collection, assays, standardization, calibration, and processing protocols are described in the NHANES Laboratory/Medical Technologists Procedures Manual [15, 16]. Serum creatinine was assayed using the Jaffe rate method, and urate was assayed by uricase method.

We used the standard NHANES case definition for gout [17]. All participants completing the medical history questionnaire were informed that "Gout is one of the most painful forms of arthritis. It occurs when too much uric acid builds up in the body. For many people, the first attack of gout occurs in the big toe. Often, the attack wakes a person from sleep." Subsequently they were asked: "Has a doctor or health professional ever told you that you have gout?" Those who responded "yes" were assessed as having gout. Methodology similar to this has been validated and found reliable in large population-based studies [18, 19]. Hyperuricemia was defined as a serum urate of >7.0 mg/dL among men and >6 mg/dL for women, similar to the definition used in other studies [20, 21]. Serum urate concentration can be converted to SI units (μmol/L) by multiplying by 59.48. Estimated glomerular filtration rate (eGFR) was calculated per the Chronic Kidney Disease (CKD) Epidemiology Collaboration (CKD-EPI) creatinine equation as previously described [22]. Urinalysis results were not available for the participants in the survey. Based on eGFR, we categorized participants as normal (≥90 mL/min per 1.73 m^2) or as having mild CKD (60–89 mL/min per 1.73 m^2), moderate CKD (30–59 mL/min per 1.73 m^2), or severe CKD (<30 mL/min per 1.73 m^2). For analyses where CKD was dichotomized, we classified subjects with eGFR ≥ 90 mL/min per 1.73 m^2 as normal and the rest as suffering from CKD.

Hypertension was defined as a mean blood pressure of ≥140 mmHg or a diastolic blood pressure of ≥90 mmHg [23]. Current use of antihypertensive drugs categorized the individual as hypertensive regardless of the actual blood pressure measurement. Diabetes was defined as a fasting glucose concentration of ≥126 mg/dL or current use of antidiabetic medications [24]. Metabolic syndrome was defined per the ATP guidelines described by Grundy et al. [8]. In patients where waist circumference was not available, we considered a body mass index of ≥30 kg/m^3 as meeting the waist circumference criterion for metabolic syndrome. Hyperlipidemia was defined as presence of one or more of the following serum measures: total cholesterol > 200 mg/dL, triglycerides > 200 mg/dL, high-density lipoproteins < 40 mg/dL, and low-density lipoproteins > 130 mg/dL. Current use of cholesterol-lowering medications classified an individual as hyperlipidemic. Alcohol consumption was the self-reported number of days in the prior month when the participants drank alcohol. Blood lead concentrations, known to be associated with gout, were measured by inductively coupled plasma mass spectrometry using standard NHANES protocol [25, 26]. This measure was log-transformed as the distribution was skewed. We used participants' self-reported ethnicity, and there were too few participants in the categories outside whites, African Americans, and Hispanics to be analyzed individually. Individuals of Mexican and non-Mexican origins were combined into a single Hispanic category.

All analyses were performed as per the NHANES guidelines [27]. Unless specified otherwise, all analyses were performed using the survey suite of commands in Stata 11 (SVY, StataCorp). These analyses incorporated the study visit weights, primary sampling unit, and stratification design of the study enabling estimation of the number of people in the USA with gout. Rates were calculated as the proportion of participants with gout in each category. Age standardization was performed with the year 2000 census standard [28].

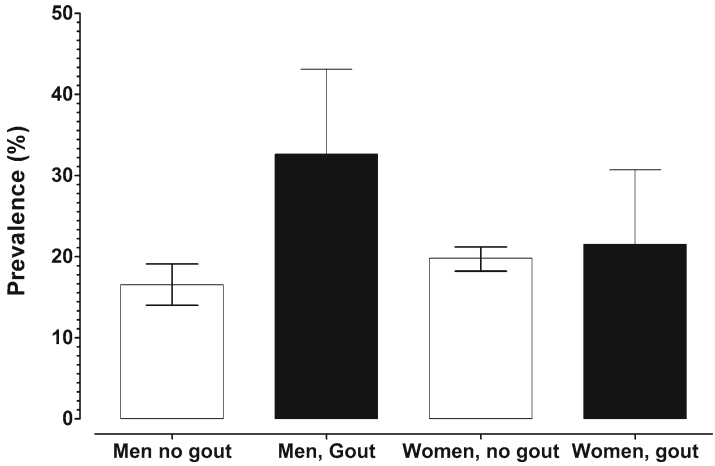

Fig. 13.3 Age-standardized prevalence of metabolic syndrome (NCEP criteria) with 95% confidence interval by gout status among men and women. The prevalence of metabolic syndrome was greater among men with gout than without, whereas differences in women with and without gout were not significant (data from NHANES 2009–10)

In men, 31% (24–38%) of those with gout also had an age-standardized prevalence of metabolic syndrome; 19% (17–20%) had metabolic syndrome but not gout (Fig. 13.3). The metabolic syndrome prevalence rates did not differ significantly between women with and without gout. Increasing the number of components of metabolic syndrome was associated with an increasing prevalence of gout (Fig. 13.4). Among men and women with gout, the proportion of people with one or more components of metabolic syndrome was greater among those with gout than those without (Table 13.1).

13.4 Metabolic Syndrome and the Risk for Incidence of Gout

We assessed the relationship between presence of metabolic syndrome and the risk for incident gout using data from a large prospective trial of 12,886 men at high cardiovascular risk but free of diabetes and clinical cardiovascular disease. From 1973 to 1975, 356,222 men aged 35–57 years, who were free of a history of hospitalization for myocardial infarction, were screened by the Multiple Risk Factor Intervention Trial (MRFIT) recruitment effort at 22 clinical centers in 18 cities in the USA. From this sampling, 12,886 men who were free of clinical evidence of coronary heart disease and diabetes were randomized equally to usual care and a program of stepped-up care (special intervention group). These men had levels of cigarette smoking, serum cholesterol, and diastolic blood pressure sufficiently high to place them in the upper portion—initially the upper 15%, subsequently the upper 10%—of the distribution of a risk score derived from the Framingham Heart Study. Men were ineligible for MRFIT for any of the following reasons: serum cholesterol

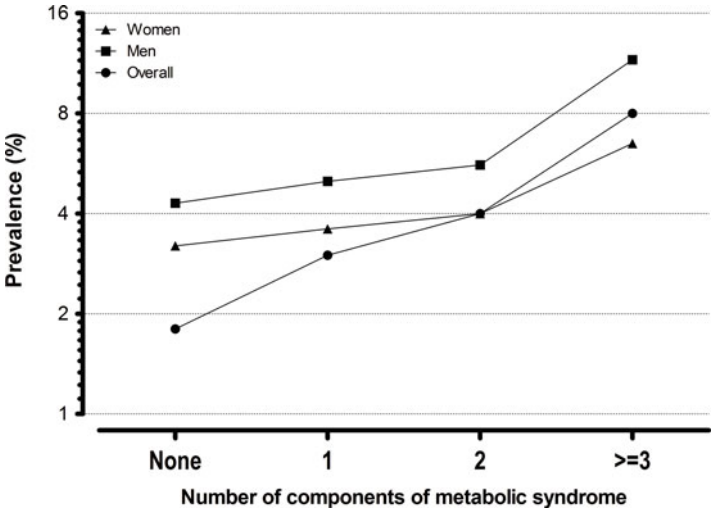

Fig. 13.4 The age-standardized prevalence of gout increased with increasing number of components of metabolic syndrome ($p<0.001$ for both genders and overall) (data from the NHANES 2009–10)

Table 13.1 Age-standardized distribution of components of metabolic syndrome by gender and gout status (NHANES 2009–10). These distributions were statistically different between those with and without gout and among men and women

	Proportion of participants in each category (%)[a]			
Number of components of metabolic syndrome	Men without gout	Men with gout	Women without gout	Women with gout
None	29	18	27	22
1	32	34	29	33
2	23	18	26	25
3	12	19	14	12
4	4	10	4	6
5	<1	1	1	3

[a]The column proportions do not add up to 100 because of rounding

of ≥350 mg/dL; diastolic blood pressure of ≥115 mmHg; body weight≥150% of desirable weight; diabetes mellitus requiring medication or untreated symptomatic diabetes; treatment with guanethidine, hydralazine, insulin, oral hypoglycemic agents, or lipid-lowering drugs; illnesses or disabilities likely to impair full participation in the trial; diets incompatible with the MRFIT food pattern; and intention to leave the clinic's geographic area in the near future. For the present analyses, all those randomized in the trial with a baseline history of gout were excluded.

All randomized participants were assessed in three baseline visits and subsequent annual visits. At each of these visits, participants completed a medical questionnaire that assessed presence of gout, a dietary questionnaire that assessed alcohol use, and provided

a fasting blood sample and a urine sample. The participants were interviewed by a study physician, and weight and height were measured. Two resting blood pressure measurements using Hawskley's random zero sphygmomanometers were performed at all clinic visits, and the mean of these two measurements was used for statistical analyses. Alcohol consumption was assessed as the number of drinks per day and the frequency of alcohol consumption. From this number of grams of alcohol per day was computed. Although the study was designed to include data from six annual visits, some participants who entered the study early in the enrollment phase were followed for 7 years. Interval medical history and cardiovascular events were assessed at each annual visit. Additional information about the MRFIT design has been published [29–31].

The primary case definition of gout was the study physician's diagnosis. The MRFIT study protocol did not specify any criteria for this clinical assessment, and the determination was left to the clinical judgment of the study physician. Hyperuricemia was defined as a serum urate > 7.0 mg/dL (>417 μmol/dL) consistent with prior analyses [11, 32–34]. Glomerular filtration rate was estimated using the CKD-EPI equation [22]. CKD was defined as an eGFR < 60 mL/min per 1.73 m^2 and/or the presence of renal damage as assessed by a urinary dipstick protein concentration of >30 mg/dL. Data were analyzed as a prospective observational study of two separate cohorts, the usual care group and the special intervention group. These data were combined for final analyses. For each participant, the observation started at baseline and ended at the study visit gout was first reported. The observations of those who dropped out of the study or died were censored at the last documented visit date.

Renal function is analyzed as a continuous measure (eGFR), stratified variable (>90, 0–90, <60 mL per 1.73 m^2), and as a dichotomous variable that defined CKD as an eGFR < 60 mg per 1.73 m^2 or any proteinuria > 30 mg/dL by spot urine dipstick measurement. Incidence rates were calculated as the number of events per 1,000 person years of follow up. Standardized incidence ratios (SIR) were computed using the age-specific incidence data for the year 1977–1978 from the Rochester Epidemiology Project program, a contemporaneous incidence study that used the 1997 American College of Rheumatology criteria for defining gout [35, 36]. We observed that the incidence rate of physician diagnosis of gout was 15.9 per 1,000 person years, whereas the corresponding rate among those without metabolic syndrome was 7 per 1,000 person years. The more components of metabolic syndrome, the greater was the incidence rate of gout (Fig. 13.5). Participants with metabolic syndrome had higher serum urate concentrations than those without in both genders (Fig. 13.6).

13.5 Gout, Hyperuricemia, Metabolic Syndrome, and Oxidative Stress

The links among hyperuricemia, gout, and oxidative stress are somewhat murky. Like Janus the Roman god, urate has both antioxidant and prooxidant effects. Peroxynitrite is one example of a toxic molecule produced by the reaction of superoxide and nitric oxide (NO) [37]. Urate plays an important role as an antioxidant,

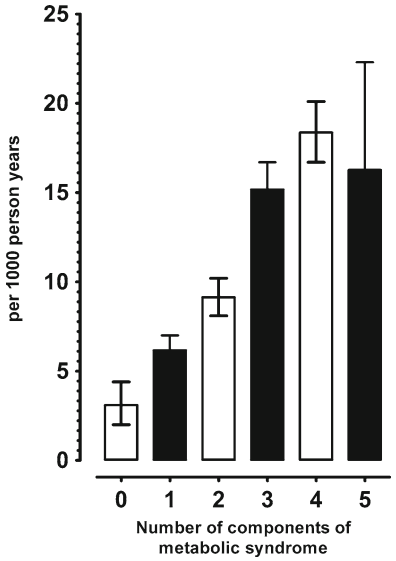

Fig. 13.5 Incidence rates (95% confidence interval) of physician-diagnosed gout increased with the number of preexisting components of the metabolic syndrome in the Multiple Risk Factor Intervention Trial (MRIFT; $n = 12.886$)

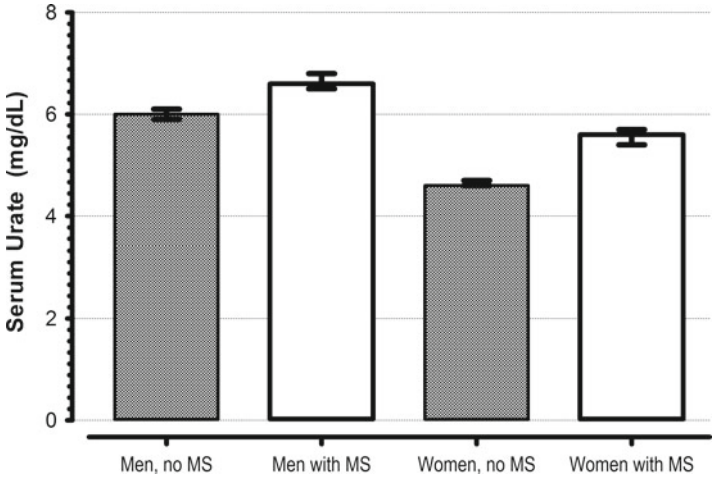

Fig. 13.6 Comparison of mean serum rate by metabolic syndrome (MS) status in the Multiple Risk Factor Intervention Trial ($n = 12,886$) population. The p value for differences in the mean urate was significant at $p < 0.001$ among both men and women

neutralizing the impact of the oxidate stress from peroxynitrite by scavenging the radicals produced from the reaction of peroxynitrite and carbon dioxide. Some in vivo studies suggest that urate can induce endothelial dysfunction by inhibiting the endothelial proliferation and reducing NO production [38–40] This effect is thought to reflect the prooxidant effect of urate on vascular cells, which boosts the rate of lipid oxidization and impairs the (healthy) endothelium-dependent vasodilation [39, 41, 42].

Allopurinol, an inhibitor of xanthine oxidase, was observed to improve endothelial function in a small study of 11 patients (mean age 68 years; 10 men, 1 woman) with chronic heart failure [43]. Two later studies demonstrated a dose-dependent improvement in endothelial dysfunction with increasing doses of allopurinol but not with probenecid, a uricosuric drug [44, 45]. The authors concluded that the improvements in endothelial function were a result of a reduction in oxidative stress rather than the urate lowering itself [44].

On the other hand, the oxidative stress environment in gout and hyperuricemia may explain some of the excess cardiovascular risk observed in patients with these two conditions. Such a link might be mediated through oxidized low-density lipoprotein (LDL). In animal models, consumption of oxidized dietary lipids, including oxidized LDL, promotes formation of fatty streak lesions in the aorta [45]. Oxidized LDL antibodies are elevated in patients with gout; urate-lowering treatment can mitigate such elevations [46]. Urate's dual nature with regard to oxidative stress is reflected in its effect on oxidized LDL: Urate can act both as an antioxidant and a prooxidant [42]. Patterson et al. demonstrated that urate is an antioxidant when it encounters native LDL, but in the presence of oxidized LDL, it behaves as a prooxidant. Thus we see that the links among hyperuricemia, gout, metabolic syndrome, oxidative stress, and poor outcomes are complicated.

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Part III
Antioxidative Approach in Joint Disorders

Chapter 14
Natural Antioxidants in the Pharmacological Treatment of Rheumatic Immune and Inflammatory Diseases

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Abstract Natural antioxidants, both supplements and diets, have long be advocated for the treatment of a wide range of serious diseases, including rheumatoid arthritis, osteoarthritis, and other inflammatory and immune diseases. Anyway, their therapeutic role has been questioned on the bases of their inefficacy or even for their detrimental effects [1]. Moreover, it is unrealistic to expect that natural antioxidants by themselves could solve the basic disease problem, since reactive oxygen species represent only one subset of a vast panorama of mediators involved in the pathogenesis of inflammatory and rheumatic disorders. On the other hand, for the majority of the natural antioxidants, a long-term "curative" effect has been not proven and controlled clinical studies on these compounds are still lacking. This overview suggests that several encouraging evidence exists for some of the natural antioxidants reported as treatment of common rheumatic conditions, but it also highlights the relative paucity and/or incompleteness of these studies. Anyway, it is conceivable to argue that the association of standard therapies and natural products with antioxidant activity could better control inflammation in rheumatic pathologies and could constitute a valuable support for the patients.

Abbreviations

ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
AP-1	Activator protein 1
CIA	Collagen-induced arthritis
CoQ_{10}	Coenzyme Q_{10}
COX	Cyclooxygenase
EGCG	Epigallocatechin 3-gallate
ENA-78	Epithelial neutrophil-activating peptide 78

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GRO-α	Growth-regulated oncogene
iNOS	Inducible nitric oxide synthase
JNK	c-Jun-N-terminal kinase
Mcl-1	Myeloid cell leukemia-1
MCP-1	Monocyte chemotactic protein-1
MDA	Malondialdehyde
MMP	Metalloproteinase
NFAT	Nuclear factor of activated T-cells
NF-κB	Nuclear factor-κB
NO	Nitric oxide
NSAID	Nonsteroidal anti-inflammatory drugs
OA	Osteoarthritis
PGE_2	Prostaglandin E_2
RA	Rheumatoid arthritis
RANKL	Receptor activator of nuclear factor kappa-B ligand
RANTES	Regulated upon activation, normal T-cell expressed, and secreted
ROS	Reactive oxygen species
SOD	Superoxide dismutase

14.1 Role of Oxidative Stress on Rheumatic Immune and Inflammatory Diseases

A large body of evidence implicates reactive oxygen species (ROS) as mediators of inflammation and/or tissue destruction in inflammatory and arthritic disorders [2].

Excess ROS, such as superoxide, hydrogen peroxide, hydroxilic radicals, and nitric oxide (NO), not properly eliminated, accelerate inflammatory responses and aggravate the disease, activating cellular redox sensitive transcription factors involved in inflammation [3]. Among ROS-sensitive transcription factors, activator protein 1 (AP-1) induces the gene transcription of collagenases, TNF-α, IL-8, IL-9, IL-3, and IFN-γ, and adhesion molecules, while nuclear factor-κB (NF-κB) activates the promoters of the genes for IL-1, IL-6, IL-8, and TNF-α, and for enzymes, such as inducible form of nitric oxide (iNOS) and cyclooxygenase (COX)-2, and proteins including E-selectin, vascular cell adhesion molecule-1, class I and II human leukocyte antigens, IL-2 receptors, and several acute phase proteins [4].

Under normal circumstances, chondrocytes in the cartilage make extracellular matrix components, such as aggrecan and type II collagen, as required in response to mechanical pressure [5]. Under abnormal or diseased conditions, however, chondrocyte metabolism is altered under the influence of the increased influx of proinflammatory cytokines and reactive mediators, which activate matrix-degrading enzymes and promote cartilage degradation [6]. Matrix metalloproteinases (MMPs) are a large group of enzymes that play a crucial role in tissue remodeling as well as in the destruction of cartilage in arthritic joints due to their ability to degrade a wide variety of extracellular matrix components. Among these enzymes, the collagenases are of particular importance in joint disorders due to their ability to efficiently cleave type II collagen [7].

The elevated levels of ROS of synovial fluid of rheumatoid arthritis (RA) patients are thought to be generated by activated macrophages, monocytes, and granulocytes, following hypoxic reperfusion injury from elevated synovial cavity pressure during joint movement [8, 9]. Moreover, another source of free radical generation in the inflamed rheumatoid joints is activated neutrophils that also liberate elastase, hypochlorous acid, and eicosanoids [10].

Although the exact pathogenesis of RA remains to be fully delineated, the balance between pro- and anti-inflammatory cytokines due to chronic inflammation is a substantial feature of the disease [11]. Clinical evidence has suggested oxidative stress is elevated in RA and osteoarthritis (OA) patients [12, 13]: in particular patients with juvenile RA revealed higher level of plasma malondialdehyde (MDA) in conjunction with low antioxidant levels in terms of superoxide dismutase (SOD) activity and vitamin E concentrations [8]. Other studies demonstrated a similar profile in adult patients with RA [13]. Anyway, the decreased activity of SOD and the elevated levels of MDA in RA patients were not accompanied by catalase activity alteration [14]. These clinical evidences showed that in RA patients the reduced activity of SOD, responsible for removing superoxide anion in serum and synovial fluid, was inadequate to ensure effective antioxidant protection, suggesting that increased free radical formation and reduced antioxidative protection may be both contributing factors in the oxidative stress, as part of the causation of RA.

14.2 Treatment of Arthritis: Approaches and Options

Conventional disease-modifying antirheumatic drugs, such as methotrexate, have long been the mainstay of RA treatment as first-line option in newly diagnosed RA patients; anyway, these drugs lose efficacy over time, and only a minority of patients achieve disease remission from their use [15]. TNF-α inhibitors, as first-generation biologics, have radically changed the treatment of patients with refractory RA. Among RA patients unresponsive to methotrexate, only two-thirds respond to TNF-α inhibitors, opening the option of combination therapy [15, 16]. As a result, newer approaches have resulted in the development of next-generation biologics during the past few years, including abatacept, rituximab, and tocilizumab [16]. Pharmacological management of OA includes analgesics and nonsteroidal anti-inflammatory drugs (NSAID). Unfortunately, these drugs can precipitate severe adverse reactions, provide only symptomatic relief from pain, and showed no effect on the progress of OA [17]. In addition, increased rates of cardiovascular events associated with COX-2 inhibitors and some conventional NSAID have made the treatments inappropriate for long-term use by OA patients with high risk of heart disease or stroke [18]. In this scenario, substances of natural origin possessing anti-inflammatory and/or antioxidative properties have been neglected for rheumatic disorders, since they failed to show good efficacy in monotherapy compared to standard antirheumatics; anyway despite their incapability to reduce clinical symptoms, they demonstrated to improve many biochemical disease markers.

More recent emerging data underlined the experimental efficacy of several antioxidants in combination therapy, demonstrating a higher potency than when administered alone [19]. Considering the long-term therapy needed for rheumatic diseases and hence the occurrence of side effects and/or ineffectiveness of treatment, combinatory therapy is expected to have a higher efficacy with beneficial effect.

14.3 Antioxidants

An antioxidant has been defined by Halliwell and Gutteridge as "any substance that delays, prevents or removes oxidative damage to a target molecule" [20], including either small molecules, such as uric acid, or large molecules like albumin. Since ROS are by definition very reactive towards other molecules, most chemical compounds can react with and neutralize them. However, a good antioxidant is a molecule that reacts with the ROS at low concentrations, and the product of its oxidation is either a stable chemical or can be easily recycled back to an active antioxidant. Other vital characteristics for a good in vivo antioxidant are its ability to achieve sufficient concentrations at sites, where it is supposed to act, and the solubility profile [21].

Antioxidants could be divided to endogenous molecules that are naturally synthesized in the human body or exogenous compounds that are mostly produced in plants and are taken up by humans from the diet. Although, the presence of antioxidants has been claimed by many to be responsible for the beneficial effect of vegetables and fruits, it has also been postulated that low content of fat in these foods may be the responsible cause. Most of these studies generally agree on the notion that antioxidants are much more effective in prevention of disease, rather than in the treatment of an already established active pathology.

14.4 Flavonoids

Flavonoids are a large group of naturally occurring phenolic compounds, which are present at high levels in human diet. They have been extensively studied for their vast antioxidant properties in vitro [22] and for the immunomodulating effects on acute and chronic inflammation [23, 24]. The term "flavonoids" generically covers more than 8,000 compounds, which have a C6-C3-C6 carbon (A-C-B rings) structure and are widely distributed in the plant kingdom. While flavonoids usually occur as glycosides, they are based on the fundamental chemical structure of the corresponding aglycones having only the ring structure, e.g., into flavones, flavonols, flavanones, flavanonols, isoflavones, catechins, chalcones, anthocyanidins, proanthocyanidins, and others [25].

Epidemiological studies have shown that higher dietary intake of flavonoids is protective against cardiovascular diseases, certain cancers, and some other chronic diseases [26].

The inhibitory effects of representative flavonoids and other components, present in green tea, grapes, oranges, olives, and vegetables, on RA and other rheumatic immune and inflammatory diseases have been extensively evaluated [27]. The pharmacokinetics of flavonoids is also an important issue to take into consideration. Flavonoids are extensively metabolized in the liver and gastrointestinal tract, and their hydroxyl group is usually blocked by methylation, glucuronidation, and sulphation. The unconjugated form of flavonoids in the systemic circulation rarely reaches the concentration of 1 μM, and the conjugated forms are much less potent antioxidants; therefore, it is plausible not to expect powerful systemic activity after their consumption [28].

14.4.1 Green Tea Catechins

Epigallocatechin 3-gallate (EGCG) is the ester of epigallocatechin and gallic acid and is a type of catechins, which is also commonly used to refer to the related family of flavonoids and the subgroup flavan-3-ols (or simply flavanols). EGCG is the most abundant catechin in tea, whose potent antioxidant activity is used for therapeutic applications in the treatment of many disorders. It is found in green but not black tea because during black tea production, the catechins are converted to the aflavins and the arubigins. A cup of green tea typically provides 60–125 mg catechins, including EGCG [29].

Extensive studies in the past decade have verified the cartilage-preserving and chondroprotective action of EGCG. Studies on the benefits of EGCG on progressive cartilage degradation and repair processes, using chondrocytes derived from OA cartilage, showed a decrease in IL-1β-induced iNOS and COX-2 expression and activity, which further resulted in reduced NO and prostaglandin E_2 (PGE_2) synthesis [30, 31]. The molecular mechanism of EGCG's regulation of iNOS expression is the inhibition of IL-1β-induced phosphorylation and proteasomal degradation of IκB-α, thereby suppressing NF-κB nuclear translocation [31].

Moreover, Singh and colleagues [32] showed that EGCG selectively inhibited the p46 isoform of c-Jun-N-terminal kinase (JNK) induced by IL-1β. This resulted in the reduced accumulation of phosphorylated JNK and AP-1 DNA binding activity and a reduction of AP-1-mediated inflammatory responses in OA chondrocytes.

The protective effect of EGCG on cartilage explants from IL-1β-induced release of cartilage matrix proteoglycans was also determined in human OA chondrocytes; EGCG pretreatment was shown to significantly inhibit the expression and activities of MMP-1 and MMP-13 in a concentration-dependent manner [33].

Other studies found that catechins from green tea inhibited the degradation of human cartilage proteoglycan and type II collagen and selectively inhibited the proteases ADAMTS-1, ADAMTS-4, and ADAMTS-5 (*a d*isintegrin *a*nd *m*etalloproteinase with *t*hrombospondin *m*otif*s*) involved in connective tissue organization [34, 35]. Further studies showed that EGCG ameliorated IL-1β-mediated

suppression of TGF-β synthesis and enhanced type II collagen and aggrecan core protein synthesis in human articular chondrocytes [36]. These results were further supported by a recent in vitro study showing the protective effect of EGCG on advanced glycation end product-induced MMP-13 production in human OA chondrocytes [37].

The chondroprotective effects of EGCG in arthritis were previously shown preincubating collagen with EGCG. A circular dichroism spectral analysis of the triple-helical structure of EGCG-treated and untreated collagen showed a higher free radical scavenging activity in EGCG-treated collagen, inducing a remarkable resistance against degradation by bacterial collagenase and MMP-1 [38].

Recent studies, evaluating the cartilage-preserving property of EGCG, showed that articular cartilages (preserved in a storage solution containing EGCG for up to 4 weeks) showed a higher degree of chondrocyte viability and proteoglycan content of the extracellular matrix, at least in part, by reversibly regulating the cell cycle at the G0/G1 phase and NF-κB expression [39, 40]. These findings provide a scientific rationale for the efficacy of EGCG in protecting cartilage breakdown during the progress of joint disorders and could be utilized in other chronic ailments where integrity of the collagen is compromised in tissue destruction or remodeling.

EGCG has been shown to also have a bone-preserving activity, modulating the balance between bone formation and resorption, whose impairment leads to skeletal abnormalities.

Hafeez and colleagues [41] showed that green tea polyphenols triggered caspase-3-dependent apoptosis in osteoclasts by regulating the constitutively active NF-κBp65 to induce DNA fragmentation and apoptosis in osteosarcoma SaOS-2 cells. Another study, using human osteoblastic cells, evaluated the inhibitory effect of EGCG on oncostatin M-induced monocyte chemotactic protein-1 (MCP-1)/CCL2 synthesis in human osteoblastic and MG-63 cells, reducing c-Fos synthesis [42]. Moreover, EGCG dose-dependently inhibited IL-6 synthesis, partially through suppression of ERK 1/2 and p38 MAPK pathways in osteoblastic cells, suggesting a reduction of IL-6-induced bone resorption and osteoclast formation [43]. A recent study by Kamon et al. [44] showed that EGCG reduced osteoclast formation, inhibiting the differentiation of osteoblasts, without affecting their viability and proliferation.

The molecular mechanism underlining EGCG inhibition of osteoblast differentiation was ascribed to an anti-osteoclastogenic effect by the inhibition of receptor activator of nuclear factor kappa-B ligand (RANKL)-induced activation of JNK and NF-κB pathways, thereby suppressing the gene expression of c-Fos and nuclear factor of activated T-cells (NFAT)c1 in osteoclast precursors [45].

Moreover, EGCG is able to regulate synovial fibroblast activity. In particular, it has been demonstrated that EGCG pretreatment of RA synovial fibroblasts significantly inhibited the production of several factors, such as chemokine MCP-1/CCL2; the regulated upon activation, normal T-cell expressed and secreted (RANTES/CCL5); growth-regulated oncogene (GRO-α/CXCL1); and epithelial neutrophil-activating peptide 78 (ENA-78/CXCL5) and the activation of MMP-2 [46]. Moreover, it has also been shown that EGCG was effective in

inhibiting IL-1β-induced MMP-1, MMP-3, and MMP-13 in human tendon fibroblasts [47] and inhibited IL-1β-induced IL-6 and VEGF synthesis in RA synovial fibroblasts [46]. In a more recent study, Yun and colleagues [48] showed that EGCG treatment resulted in dose-dependent inhibition of TNF-α-induced production of MMP-1 and MMP-3 in RA synovial fibroblast by inhibiting AP-1 DNA binding activity. In this cell population, EGCG inhibited constitutive and TNF-α-induced antiapoptotic myeloid cell leukemia-1 (Mcl-1) protein expression [49], whose overexpression has linked to RA synovial fibroblast resistance to apoptosis [50]. Moreover, EGCG specifically abrogated Mcl-1 expression in RA synovial fibroblasts and affected Mcl-1 expression to a lesser extent in OA synovial fibroblasts, normal synovial fibroblasts, and endothelial cells. All these findings suggest that EGCG may selectively induce apoptosis and further sensitize RA synovial fibroblasts to TNF-α-induced apoptosis, regulating their invasive growth in RA.

In vivo studies have confirmed the beneficial effect of EGCG in collagen- or adjuvant-induced arthritis models. The consumption of EGCG-containing green tea extract in drinking water ameliorated collagen-induced arthritis (CIA) in mice [51], accompanied by a marked inhibition of COX-2, IFN-γ, and TNF-α content in arthritic joints and lower serum levels of total and type II collagen-specific immunoglobulin G.

Interestingly, some recent studies confirmed the effect of EGCG or green tea in bone resorption [42, 52, 53]. In particular, EGCG treatment ameliorated arthritis and reduced bone resorption activity and osteoblast-specific gene expression of the transcription factor NF-ATc1 in CIA mice [53]. Moreover, EGCG reduced macrophage infiltration and the amount of MCP-1/CCL2-synthesizing osteoblasts [42].

It has been demonstrated that the administration of EGCG (100 mg/kg, intraperitoneally daily), during the onset of arthritis in rats, results in a specific inhibition of IL-6 levels in serum and joints of arthritic animals. Moreover, EGCG enhances the synthesis of soluble gp130 protein, an endogenous inhibitor of IL-6 signaling and trans-signaling, and reduces MMP-2 activity in the joints [54].

Green tea intake also induced changes in arthritis-related immune responses in Lewis rats [55]. In fact, it induced a lower concentration of the proinflammatory cytokine IL-17 but a greater concentration of the immunoregulatory IL-10 than controls. Green tea feeding also suppressed the antimycobacterial heat-shock protein 65 antibody response.

Chemokine receptor overexpression with reduced chemokine production can be considered another potential mechanism of green tea extract to limit the overall inflammation and joint destruction in RA, demonstrated both in human RA synovial fibroblasts and rat adjuvant-induced arthritis [56]. In animal models of arthritis, the expression of CC/CXC chemokines generally coincides with the onset of clinical symptoms; in this chapter, green tea significantly inhibited the expression of MCP-1/CCL2 and GRO-α/CXCL2 in arthritic rat. Additionally, it blocked IL-1β-induced MCP-1/CCL2, RANTES/CCL5, GRO-α/CXCL2, and IL-8/CXCL8 production by RA synovial fibroblasts in a concentration-dependent manner and increasing chemokine receptor expression. These two potential

mechanisms could be suggested for these unique differential effects induced by green tea. First, by inhibiting chemokine synthesis, green tea directly reduces the chemokine pool available for CC/CXC receptor binding. Second, by increasing chemokine receptor expression, it maximizes chemokine ligand-receptor binding that consequently reduces the amount of free chemokine available for binding to leucocytes or monocytes/macrophages, thus acting as a chemokine trap and reducing the chemotactic environment [57].

14.4.2 Soy Isoflavones

Soy isoflavones are an important class of flavonoids, and among these, genistein and daidzein, classified as phytoestrogens, have been extensively studied for their beneficial effects in several physiopathological conditions [58]. It was recently shown that genistein has adipogenic and anti-inflammatory effects on synovial fibroblasts, transforming synovial fibroblasts into adipocytes, expressing perilipin-A and adiponectin, but not leptin [59]. Furthermore, genistein-enhanced glucocorticoid-mediated synovial fibroblast adipogenesis, downregulated glucocorticoid-induced leptin and leptin receptor and a role for PPAR-γ, and tyrosine kinase in genistein-induced adipogenesis were reported. Endogenous and TNF-α-induced expressions of IL-6, IL-8, p38, p65, and C/EBP-β were also downregulated by genistein, confirming its anti-inflammatory properties [59].

Several studies on the mechanism of action of herbal medicine, containing isoflavones and traditionally used for the treatment of rheumatic diseases, have been performed on plant extracts, addressing their activity on the major flavonoids therein contained.

A recent study suggests a potential beneficial effect of root ethanol extracts of *Glycine tomentella* Hayata that have long been used to treat a variety of rheumatic diseases, including RA and OA in Taiwan, and known to have anti-inflammatory and antioxidative activities [60]. Actually, these authors associated the therapeutic effects of the ethanol extract of *Glycine tomentella* Hayata to its major components, daidzein (42.5%), epicatechin (28.8%), and naringin (9.4%), in suppressing proinflammatory cytokines (IL-1β and IL-6) and MMP-9 activity and enhancing apoptotic cell uptake and clearance.

14.4.3 Quercetin

Quercetin is one of the representative flavonoids possessing potent biological and pharmacological activities due to the strong inhibition of phospholipase A2 [61]. Oral administration of quercetin to arthritic rats significantly attenuated the severity of clinical signs of the disease [62]. Another study was focused on *Taxillus liquidambaricola* used to treat rheumatic arthralgia and hypertension in the Chinese

traditional medicine [63]. The findings suggest that the ethanolic extract rich in polyphenols, flavonoids, and quercetin, as reference compound, showed anti-inflammatory effects in vitro and in vivo. The anti-inflammatory effects, evaluated in several experimental models of inflammation, have been related to iNOS and COX-2 reduction and reduced excess of TNF-α generation. Moreover, the antioxidant effects have been related to the increase in the activities of antioxidant enzymes and to radicals scavenging.

14.4.4 Micronized Purified Flavonoids Fraction

Purified micronized flavonoid fraction, also known as Detralex® (Les Laboratoires Servier, France) containing two flavonoids: diosmin (90%) and hesperidin (10%), has been shown to have antioxidant properties due to the scavenger activity of ROS in vitro and in vivo. It has been shown that not only it mildly reduced some of the clinical symptoms of adjuvant arthritis, such as hind paw edema and arthritic score in the rat, but it also increased the efficacy of basal methotrexate therapy in arthritic rats [64].

14.4.5 Hesperidin and Nobiletin

Hesperidin, a flavanone glycoside found abundantly in citrus fruits, acts as an antioxidant according to in vitro studies [65]. Several experimental and clinical studies showed its beneficial effects in rheumatic diseases. Successive oral administration of hesperidin caused a decreased incidence of clinical scores in CIA mice either after the secondary antigenic stimulation or from the logarithmic phase of the development of disease onwards [66]. Moreover, hesperidin significantly attenuated secondary paw swelling and reduces the arthritis index in rats with adjuvant arthritis.

A double-blind and placebo-controlled trial was performed on RA patients, receiving standard therapy and beverages containing 3 g glucosyl hesperidin or placebo, every morning for the duration of the trial. It was shown that food containing glucosyl hesperidin plus standard anti-rheumatoid drugs improved the quality of life compared to RA patients receiving standard therapy and placebo [67].

The citrus polymethoxy flavonoid, nobiletin, was studied in vivo and administered intraperitoneally in arthritic mice-suppressed mRNA expression of aggrecanase-1/a disintegrin, MMP with thrombospondin-like motifs (ADAMTS)-4 and aggrecanase-2/ADAMTS-5 in the joint tissues [68]. Moreover, nobiletin was reported to reduce whole bone mineral density in ovariectomized mice by 61%, which was comparable to or exceeding the efficacy of 17β-estradiol and markedly decrease the incidence of arthritis into CIA mice [69].

14.5 Other Polyphenolic Compounds

14.5.1 Resveratrol

Resveratrol, a polyphenolic phytoalexin found at high levels in grape skin and red wine, has been reported to have many biological activities and to protect several inflammatory diseases, showing antioxidant, anti-cancer, anti-inflammatory, and immunomodulatory properties [70]. The anti-inflammatory effects of resveratrol have been demonstrated by suppression of carrageenan-induced paw edema, an effect attributed to suppression of PG synthesis and COX-2 expression and specific inhibition of NF-κB activation induced by TNF-α- and IL-1β [71–73]. Elmali et al. [74] have studied the in vivo effects on cartilage destruction and synovial inflammation following intra-articular injections of resveratrol in an experimental inflammatory arthritis model in rabbits, showing a reduced severity of cartilage lesions and synovial inflammation.

Very recently, this approach has been re-proposed to treat rheumatoid-related bone lesions using resveratrol and structurally related compounds due to their anti-osteoclastogenic effects, which are most likely mediated by the suppression of RANKL-p300-NF-κB activation and the target genes regulating osteoclastogenesis [75].

14.5.2 Curcumin

Curcumin, a yellow pigment present in the rhizomes of turmeric (*Curcuma longa*), has long been used as a food additive and spice in India and elsewhere in the world. Many years ago, preclinical studies had shown that curcumin had antioxidant and anti-inflammatory properties [76]. However, curcumin has limited bioavailability due to poor absorption and rapid metabolism [77].

Curcumin was evaluated in RA patients in a randomized clinical trial [78]. Patients were assigned to curcumin (1,200 mg) or phenylbutazone (300 mg). In both groups there was a significant improvement in morning stiffness, walking time, and swelling compared to baseline. Thereafter, another clinical trial was performed in OA patients, combining turmeric with *Boswellia carteri* [79]. After 2 or 3 months, those taking the herbal remedy had significantly better outcomes compared to placebo in terms of pain, degree of tenderness, and knee effusion. Anyway, the relieving effect of Borrelia species on OA symptoms per se compromised the determination of turmeric's contribution to these results.

The well-known antioxidative mechanism of action of curcumin alone, as an antitumour or antiarthritic agent, appears to be substantially augmented by coordination to the vanadyl ion. Pharmacological effects that curcumin and vanadium have in common are the improvement of oxidative stress/antioxidant balance and the inhibition of specific protein tyrosine phosphatases in the signal transduction cascade, both effects relevant for cell proliferation and apoptosis [80]. Vanadyl curcumin was more

effective in inhibiting synoviocyte proliferation than curcumin alone, suggesting striking potential as a particularly nontoxic treatment for RA [81].

14.6 Sesquiterpene Lactones

Sesquiterpene lactones contained in herbal preparations from Asteraceae plants were commonly used in alternative medicine for RA. In particular, *Arnica* preparations, as well as *Tanacetum parthenium*, have long been used for the symptomatic treatment of rheumatic complaints. In vitro experiments had demonstrated that sesquiterpene lactone parthenolide selectively inhibits activation of the NF-κB pathway by targeting IKK [82] and/or preventing the degradation of IκB-α and IκB-β without interfering with ROS formation [83].

The efficacy of Arnica is presumed to be mainly due to its anti-inflammatory properties and inhibition of the transcription factor NF-κB [84]. Arnica preparations suppress MMP1 and MMP13 mRNA levels in bovine and human articular chondrocytes in a concentration-dependent manner and in a low concentration range. This suppression may be due to inhibition of DNA binding of the transcription factors AP-1 and NF-κB. Interestingly, sesquiterpene lactones present in the preparations were always more active than the pure compounds, demonstrating the advantage of using plant preparations [84].

The use of topical preparations for symptom relief is common in OA. A randomized, double-blind study receiving ibuprofen (5%) or arnica (50 g tincture/100 g, DER 1:20), as gel preparations, was performed in 204 patients with active osteoarthritis of interphalangeal joints of hands. There were no differences between the two groups in pain relief and hand function improvements [85]. Thereafter, the similar activity of arnica and ibuprofen was confirmed in patients with OA of the hands [86].

14.7 Capsaicin

Capsaicin (trans-8-methyl-*N*-vanillyl-6-nonenamide), which is derived from *Capsicum frutescens*, the common pepper, is thought to work by depleting the small, unmyelinated, type C sensory neurons of substance P, the neuropeptide implicated in the transmission and modulation of pain and the pathogenesis of various arthritic conditions. It further inhibits substance P reaccumulation from cell bodies and nerve terminals, resulting in analgesia. Touch, temperature, and proprioception are unaffected [87].

Capsaicin has been used to treat several painful disorders, including postherpetic neuralgia, postmastectomy pain, cluster headaches, diabetic neuropathy, and phantom limb syndrome [88]. More than 20 years ago, four randomized placebo-controlled studies had shown significantly better pain and tenderness relief with topical capsaicin cream than with placebo [89–92] and a 40% improvement in dolorimeter scores

in OA. For the most part, no effect was demonstrated on joint swelling, grip strength, duration of morning stiffness, or functional capacity. The effects in patients with RA, who were only evaluated in two of the four trials, have been inconsistent, and the number of patients was too small to make meaningful comparisons.

Capsaicin cream is especially useful in patients with OA of the hands and knees and purchased over the counter. Beneficial effects are usually seen after 3–7 days of application. Although it is quite safe, effective use of capsaicin is limited by the local stinging or burning sensation that typically dissipates with regular use after the first 7–10 days of therapy. Initially thought to contribute to a patient's perception of effectiveness (and resulting in unblinding of trials), the response of capsaicin-treated patients who had the burning sensation was slightly less than those who did not have the burning sensation [90].

Recently, a Cochrane Database study on pain management in RA patients by neuromodulators including capsaicin confirmed a significantly greater reduction in pain favoring topical capsaicin over placebo at 1 and 2 weeks; however, 44% of patients developed burning at the site of application and 2% withdrew because of this [93]. Drying of the cream and inhalation have been also reported to result in coughing [89, 92] and, anecdotally, asthma.

14.8 Vitamins A, C, and E

The most extensively studied antioxidants are vitamins. Under physiological conditions, antioxidant vitamins, including vitamins A, C, and E, are very useful for different functions in the human body and have the advantage of being able to be recycled back to an antioxidant molecule after the reaction with ROS. Systematic reviews and meta-analysis performed by Cochrane group investigators have given a significant contribution to our knowledge about the efficacy of vitamin supplementation for primary and secondary prevention of disease, analyzing the effect of antioxidant and on all-cause mortality [94].

It is well known that people fed with antioxidant-deficient diet presents an increased risk of OA and RA [95, 96]. The use of vitamin E in OA and RA and the combination with vitamin E, C, and A in RA were recently reviewed [95, 97]. Many years ago, five clinical trials tested vitamin E in inflammatory arthritis. Two of these used placebo controls [98, 99] and three compared vitamin E with diclofenac [100–102]. The placebo-controlled studies, both short term and of relatively poor quality, reported contradictory findings. The first is very poorly reported but found no clear effect of vitamin E on clinical status [98]. The other found significant positive effects of high-dose vitamin E treatment on pain but not on parameters related to inflammation, and this treatment did not reduce the use of NSAIDs or disease-modifying antirheumatic drugs (DMARDs) or analgesic medication in the treatment of RA [99]. All three randomized clinical trials comparing vitamin E with diclofenac reported similar findings and, taken together, provide very little convincing evidence of a positive effect of vitamin E in inflammatory arthritis.

Of the seven clinical trials of vitamin E in OA, four compared the treatment with placebo [103–106], two against diclofenac [107, 108], and one against vitamin A [109]. Of the placebo-controlled trials, two suggested effectiveness of vitamin E for pain, but one of these [103] was methodologically very weak. The second, more robust trial [104] indicated greater effectiveness for both the whole patient sample and for a subgroup with OA of the knee and hip. This latter finding somewhat contradicts the results of the other two placebo-controlled trials [105, 106], which both specifically evaluated OA of the knee and both of which had largely negative results. These latter negative studies had larger sample sizes and relatively long treatment periods of 6 months [106] and 2 years [105] compared with previous trials. The two equivalence trials comparing vitamin E treatment with diclofenac [107, 108] suggest similar effectiveness for the two treatments, and one reported a statistically significant superiority of vitamin E over diclofenac on the Keitel Function Test [108]. The findings of the final trial [109] comparing vitamin E with vitamin A and the combination of the two vitamins are difficult to interpret because of the absence of either a placebo control or a standard comparator treatment and because of inadequate statistical analysis and poor reporting.

The combination of vitamin E, C, and A was examined in a case-control trial with 40 patients newly diagnosed with RA and divided in two groups, one receiving standard therapy and another receiving standard therapy (NSAIDs and steroids) plus a fixed dose combination of the antioxidant vitamins [110]. A significant decrease of the disease state severity in both the antioxidant and standard treatment groups was shown. Moreover, in the same group, the concentration of oxidative stress markers changed significantly after the antioxidant treatment compared to baseline values.

In conclusion, there is no robust evidence that antioxidant vitamins are effective in any type of arthritis since published trials are methodologically weak and results are contradictory; whether any of these supplements can be effectively and safely recommended to reduce NSAIDs or steroid usage is unclear and requires more high-quality research [94, 97].

14.9 Other Endogenous Antioxidants: Coenzyme Q_{10} and Carnosine

Coenzyme Q_{10} (CoQ_{10}), or ubiquinone (oxidized form) or ubiquinol (reduced form), is an endogenous lipid that takes part in the transport of electrons in the mitochondria during the process of respiratory chain reactions [111]. CoQ_{10} has been suggested for treatment of a variety of diseases including heart failure, hypertension, and neurodegenerative diseases [112].

The antirheumatic activity of CoQ_{10} supplementation was tested not only as to its capability to suppress the inflammation edema of the arthritic animals but also to improve biochemical parameters as markers of inflammation and

oxidative stress, and of mitochondrial bioenergetics [113, 114]. A significant protective effect was observed on the level of mitochondrial energetic and antioxidant disbalance. The effect of CoQ_{10} supplementation was confirmed in combinatory therapy with a classical antirheumatic, methotrexate, in adjuvant-induced arthritis in rats [114, 115], where the association suppressed arthritic progression more effectively than did MTX alone. In particular, CoQ_{10} potentiated both the antiarthritic and the antioxidant effect of methotrexate on the level of oxidation of proteins as well as lipoperoxidation. Moreover, the combination therapy improved the functionality of peripheral blood neutrophils in arthritic rats, with a balancing effect on the immunosuppression caused by MTX monotherapy.

Carnosine, another essential endogenous molecule, has many physiological functions: radical scavenging, pH buffering, heavy metal chelating, anti-glycating, and neutralizing toxic aldehydes. It was found to have neuroprotective, hepatoprotective, cataract treating, and antiaging abilities [116], and its anti-inflammatory potential in autoimmune systemic inflammatory diseases, as RA, has been recently investigated [117]. In this study, carnosine inhibited degradation of hyaluronan induced by free radical processes in vitro and improved the redox imbalance in adjuvant arthritis in vivo, delaying the disease onset, but it was not so effective later on when adjuvant arthritis was fully developed.

14.10 Olive Phenolic Hydroxytyrosol

The olive fruit and compression-extracted olive oil is the essential component of the Mediterranean diet, a nutritional regimen gaining ever-increasing renown for its beneficial effects on inflammation, cardiovascular disease, and cancer. Its anti-inflammatory and protective properties are linked to the large presence of omega-3 polyunsaturated fatty acids, vitamins, but especially to the constituents of extra virgin olive oil: oleic acid, phenolic compounds, and recently discovered oleocanthal.

It has been shown that the Mediterranean diet can reduce disease activity, pain, and stiffness in patients with inflammatory arthritis and may thus constitute a valuable support for patients suffering from these diseases [118]. In particular, a clinical study on RA patients showed that the supplementation with olive oil could improve clinical and laboratory parameters of disease activity when patients were already using fish oil [119].

A unique characteristic of olive oil is its enrichment in oleuropein, a member of the secoiridoid family, which converts to hydroxytyrosol after sequential digestion by glycosidase and esterase. The phenolic compounds are hydroxytyrosol, tyrosol, and oleuropein, which occur in highest levels in virgin olive oil, and have demonstrated antioxidant activity [120]. Antioxidants are believed to be responsible for a number of the biological activities of olive oil [121]. Hydroxytyrosol, which is abundant in the aqueous fraction of olive pulp, is a simple phenolic compound and possesses a marked antioxidant activity. In combination

with glucosamine, olive vegetation water containing hydroxytyrosol inhibits LPS-induced release of NO and TNF-α in BALB/c mice [122] and in human monocytic THP-1 cells [123]. Hydroxytyrosol-20 (HT20), a preparation containing 20% hydroxytyrosol, significantly decreased clinical scores in CIA mice when given orally at a dose of 100 mg/kg, although its efficacy was weaker than that of 1 mg/kg prednisolone.

It has been demonstrated that both the enantiomers of oleocanthal, the dialdehydic form of deacetoxy-ligstroside aglycone, are both potent nonsteroidal anti-inflammatory agents and strong antioxidants and that the levorotary enantiomer is the agent responsible for the back of the throat irritant properties often experienced upon ingestion of extra virgin olive oils. In this regard, it has been recently disclosed that (−)-oleocanthal, the naturally occurring irritant, is both a potent nonsteroidal anti-inflammatory agent, similar to that of ibuprofen, sharing the capability to inhibit both COX-1 and COX-2 activity [124] and a powerful antioxidant similar to α-tocopherol [125]. Recently, we demonstrated that oleocanthal was able to decrease lipopolysaccharide-induced iNOS synthesis in chondrocytes, evidencing an additional mechanism of action in reducing NO which has been associated with cartilage degradation and OA progression [126].

14.11 Conclusions

This chapter summarizes beneficial effects of naturally occurring antioxidants which are obtained from intake of commonly available foods (see Table 14.1). There is increasing evidence that several potential candidates might be useful for preventive or therapeutic treatment of disease related to oxidative stress. Anyway, ROS represent only one subset of a vast panorama of mediators of pathogenetic importance in inflammatory (and/or degenerative) arthritis, such as RA, OA, and other rheumatic disorders.

Cells and tissues involved in this pathology probably generate radicals in excess, but also other inflammatory and immune mediators; likewise, it is unrealistic to expect that an agent which detoxifies some of these mediators will itself solve the basic disease problem. On the other hand, for the majority of the natural antioxidants, information about their pharmacokinetic, efficacy, toxicity, or the long-term beneficial effect is still lacking. This overview suggests that encouraging preclinical evidence exists for some of the natural antioxidants reported as treatment of common rheumatic conditions, but it also highlights the relative paucity and often disappointing quality and/or results of the clinical studies. Anyway, it is conceivable to argue that the association of standard therapies with natural products having antioxidant activity could better control inflammation in rheumatic pathologies. This novel and alternative approach could constitute a valuable support for patients suffering from these pathologies, allowing to reduce the dosage of standard drugs and their toxicity during long-term administration.

Table 14.1 Natural antioxidants useful in the treatment of rheumatic inflammatory diseases

Active principles	Food sources	Studies and references
Flavonoids		
Catechins	Green tea, chocolate, red wine, apples, and berries	In vitro [30–49, 54] In vivo [51, 53–56]
Isoflavones		
Genistein Daidzein	Lupin, fava beans, soybeans, kudzu, and psoralea	In vitro [59]
Quercetin	Green tea, capers, lovage, apples, onion, red grapes, citrus fruit, tomato, broccoli and other leafy green vegetables, berries	In vivo [62]
Hesperidin	Citrus fruits	In vivo [66, 67] Clinical study [67]
Nobiletin	Citrus fruit	In vivo [68, 69]
Polyphenolic compounds		
Resveratrol	Grape skin, red wine	In vitro [71, 75] In vivo [74]
Curcumin	Turmeric	In vitro [81] Clinical studies [78, 79]
Sesquiterpene lactones		
Parthenolide	Many plants (i.e., lettuce, spinach, sunflower, artichoke, chamomile)	In vitro [82–84] Clinical studies [85, 86, 90]
Capsaicin	Pepper	Clinical study [88–91]
Vitamin A, C, E	Carrots, peanuts, seeds, vegetable oils, whole grain, peppers	Clinical study [8, 95, 96, 98–108, 110]
Coenzyme Q_{10}	Cereals, nuts, soy, oily fish (such as salmon and tuna), organ meats (such as liver)	In vivo [19, 113–115]
Carnosine	Protein-rich food (meats, milks, eggs, and cheese)	In vivo and in vitro [117]
Hydroxytyrosol	Olive pulp, olive oil	In vitro [122, 123] Clinical study [119]
Oleocanthal	Olive, olive oil	In vitro [126]

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Chapter 15
Antioxidants in the Treatment of Osteoarthritis and Bone Mineral Loss

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Abstract The concept that inflammatory diseases of bones and joints can be postponed or even prevented naturally by consuming certain foods or food-derived substances is currently eliciting considerable interest from researchers, clinicians and patients. Oxidative stress results in the production of reactive oxygen species (ROS), which play important roles in the development of many diseases including those relating to bones and joints. Metabolic reactions in osteoblasts, osteoclasts, chondrocytes and synoviocytes produce free radicals, ROS and their derivatives. These dangerous chemicals can accumulate in bones and synovial joints, and in combination with inflammatory mediators they can cause extensive structural damage, inflammation and cell death. Antioxidants are naturally occurring reducing agents capable of inhibiting ROS formation, scavenging free radicals and removing ROS derivatives. Antioxidant vitamins have major roles in modulating oxidative stress, regulating immune responses and contributing to cell differentiation. Vitamin C (ascorbic acid), vitamin E, thiols (glutathione) and plant polyphenols have the

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capacity to neutralize ROS in joints and decrease the oxidative stress associated with the progression of arthritis. This chapter focuses on antioxidants and their potential for the treatment of diseases of bones and joints, particularly focusing on osteoarthritis (OA) and bone mineral loss.

Abbreviations

AP-1	Activator protein 1
AR	Androgen receptor
BMD	Bone mineral density
CBP	CREB-binding protein or CREBBP
CDC	Centers for Disease Control and Prevention
cGMP	current Good Manufacturing Practices
COX	Cyclooxygenase
EFSA	European Food Safety Authority
EGR-1	Early growth response protein 1
eNOS	Endothelial NOS
EpRE	Electrophile-responsive element
FA	Fatty acids
FDA	Food and Drug Administration
GSH	Glutathione or gamma-L-glutamyl-L-cysteinylglycine
H_2O_2	Hydrogen peroxide
IFN-γ	Interferon gamma
IGF-I	Insulin-like growth factor I
IGF-IR	Insulin-like growth factor I receptor
IL-1β	Interleukin 1 beta
iNOS	Inducible NOS
JSW	Joint space width
LOX	Lipoxygenase
LPS	Lipopolysaccharides
NAC	*N*-acetylcysteine
NADPH	Nicotinamide adenine dinucleotide phosphate
NALP	Pyrin-like protein containing a pyrin domain
NF-κB	Nuclear factor kappa B
NIAMS	National Institute of Arthritis and Musculoskeletal and Skin Diseases
NO	Nitric oxide
NOS	Nitric oxide synthase
NRF-2	Nuclear respiratory factor 2
NSAID	Nonsteroidal anti-inflammatory drug
OA	Osteoarthritis
p300	E1A binding protein p300
p53	Protein 53 or tumour protein 53
PGE_2	Prostaglandin E_2

PPAR-γ	Peroxisome proliferator-activated receptor gamma
PYCARD	Apoptosis-associated speck-like protein containing a caspase-recruitment domain (CARD)
RA	Rheumatoid arthritis
RANK	Receptor activator of nuclear factor kappa B
RANKL	Receptor activator of nuclear factor kappa B ligand
ROS	Reactive oxygen species
Sirt-1	Sirtuin (silent mating type information regulation 2 homolog) 1
SLE	Systemic lupus erythematosus
SOD	Superoxide dismutase
STAT	Signal transducer and activator of transcription
TGF-β	Transforming growth factor beta
UN	United Nations
WHO	World Health Organization

15.1 Introduction

The incidence of chronic age-related diseases is steadily rising, seriously affecting the health of millions of people around the world. According to the United Nations (UN)[1] and the World Health Organization (WHO)[2], arthritic and rheumatic conditions are leading causes of morbidity and disability throughout developed and developing nations, giving rise to enormous healthcare expenditures and loss of work [1] (source: The Arthritis Foundation[3]). Many types of arthritic and rheumatic diseases are essentially age-related and "inflammatory" disorders where the inflammation actively facilitates the progression of disease. The term "arthritis" characterizes a group of conditions involving inflammatory damage to synovial joints [2]. Arthritic diseases encompass a wide variety of over 200 different syndromes and disorders involving inflammation of the joints. Pain, redness, heat, swelling and other harmful and degradative effects of inflammation within synovial joints characterize the majority of these diseases. However, the most common and important form of arthritis is osteoarthritis (OA).

15.1.1 Osteoarthritis

OA is an extremely common type of degenerative joint disease and a major cause of pain and chronic disability in older individuals [3]. It is estimated that more than 20

[1] http://www.un.org/

[2] http://www.who.int/en/

[3] http://www.arthritis.org/

million Americans suffer from OA.[4] A 2005 study in the USA estimated that OA is one of the top five causes of disability amongst nonhospitalized adults (source: Centers for Disease Control and Prevention (CDC[5]), USA). The disease is also highly prevalent in Europe. In 2006, it was estimated that around 35–40 million Europeans suffer from OA and nearly 25% of people aged 60 and above suffer from OA induced disability. The majority of the human population over 65 years of age demonstrates radiographic evidence of OA in at least one joint. However, in most cases active OA is present in multiple joints along with co-morbidities that exacerbate the disease. Although OA is rare in people under 40, it becomes much more common with age. The end-stage treatment for the most debilitating forms of OA is surgery to modify or replace the joint. With increasing life expectancy, growth in the elderly population and an alarming escalation of chronic, inflammatory and age-related conditions, there is increased demand for new treatments and preventative approaches. It is anticipated that by the year 2030, 20% of adults in Western Europe and North America will have developed OA. Therefore, OA is expected to place a heavy economic burden on healthcare systems and community services globally.

15.1.2 OA Risk Factors

Ageing is a major contributor to the development of OA [4, 5]. Age-related changes in articular cartilage contribute to the development and progression of OA. Although the degeneration of articular cartilage is not simply the result of ageing and mechanical wear, ageing nevertheless modifies the chemical structure and biological function of the tissues that make up the articular joint including cartilage, subchondral bone, muscle, soft tissues, synovial membrane and synovial fluid [4, 6]. Although older age is the greatest risk factor for OA, OA is not an inevitable consequence of growing old [7]. The mechanisms for the link between ageing and OA are thus far incompletely understood.

Although OA is primarily associated with ageing, there are other key contributing factors [8]. These include obesity (which increases mechanical stress), underlying metabolic or endocrine disease, genetics, a history of joint trauma, instability or repetitive use, heritable metabolic disorders, muscle weakness, underlying anatomical and orthopaedic disorders (i.e. congenital hip dislocation), joint infection, crystal deposition, previous rheumatoid arthritis and various disorders of bone turnover and blood clotting. The metabolic alterations that occur in obesity along with the proinflammatory factors produced by white adipose tissue in the chronically overweight are thought to be major factors in the progression of the disease [9]. Symptoms and signs in the most frequently affected joints include heat, swelling, pain, stiffness and limited mobility. OA is often a progressive and disabling disease, which occurs due to a variety of risk

[4] http://www.niams.nih.gov/

[5] http://www.cdc.gov/

factors, such as advancing age, obesity and traumatic joint injury [10]. Other sequelae include osteophyte formation and synovial inflammation (synovitis) [11]. These manifestations are highly variable, depending on joint location and disease severity. OA can affect any synovial joint, but in humans it primarily affects large and load-bearing joints such as the hip and knee. The disease is essentially due to daily wear and tear of the joint. Its most prominent feature is the progressive destruction of articular cartilage, the main tissue involved in OA [12]. It is generally accepted that OA begins in articular cartilage and eventually spreads to subchondral bone and other peri-articular tissues. However, there is also the opposing view that suggests OA is a disease of subchondral bone and begins there. Despite the controversy regarding its initiation the current consensus is that cartilage damage is a core feature. In reality OA is a disease of the whole joint, involving not only articular cartilage but also the synovial membrane, subchondral bone and peri-articular soft tissues [13]. Pain is a constant and daily feature in well-established forms of OA and occurs due to the inflammation that occurs around and within the joint. Disability in patients with OA is a consequence of degeneration in the joint and surrounding tissues and is further enhanced by inflammation-induced pain [14]. Aside from analgesics, there are currently no effective pharmacotherapies capable of restoring the structure and function of the damaged synovial tissues in any form of arthritis.

15.1.3 Loss of Bone Mineral Density

Bone mineral density (BMD) refers to the amount of calcified mineral in bone. BMD is used in clinical medicine as an indirect indicator of osteoporosis and fracture risk. Osteoporosis[6] is a bone disease that makes bones weak and more susceptible to fracture. It is characterized by low bone mass and structural deterioration of bone tissue, leading to bone fragility and an increased risk of fractures of the hip, spine, and wrist. Although anyone can develop osteoporosis, the disease is most common in older women. Risk factors include old age, being small and thin and having a family history of osteoporosis or having osteopenia, which involves a decrease in the amount of calcium and phosphorus in the bone, causing weakness and increasing the likelihood of fracture. Men as well as women are affected by osteoporosis. As many as half of all women and a quarter of men older than 50 will break a bone due to osteoporosis in their lifetime. The risk of osteoporosis is significantly higher in women over 65 and men over 70. Osteoporosis is a disease that can be prevented and treated, particularly through nutrition and exercise. In the United States, more than 40 million people either already have osteoporosis or are at high risk due to low bone mass.

[6] http://www.niams.nih.gov/Health_Info/Bone/Osteoporosis/overview.asp

15.1.4 Chapter Aims

The main objective of this chapter is to discuss the role of oxidants in the pathogenesis of arthritis and explore the potential for using antioxidants in the treatment of OA and bone mineral loss. It summarizes the information about antioxidants and their potential for preventing and treating arthritic diseases such as OA. The expectation is to relate the potential importance of dietary antioxidants and their supplementation in OA patients. This chapter will also include a brief discussion of the potential for the antioxidant resveratrol in the context of treating bone mineral loss.

15.2 Oxidative Stress in Arthritis

Oxidative stress, defined as an imbalance between oxidative processes and reduction equivalents (antioxidants), is involved in the development of degenerative diseases. There is a substantial body of published research that suggests that arthritic diseases are characterized by inflammation and oxidative stress (Fig. 15.1). Oxidative stress produces ROS that play key roles in the development of OA. ROS are also involved in RA. In both diseases, metabolic reactions in chondrocytes and synoviocytes produce free radicals, ROS and their derivatives. These dangerous chemicals accumulate in the synovial joint, causing extensive structural damage, cell death and inflammation. In OA oxidative damage contributes to chronic inflammation and promotes age-related diseases [15]. This results in senescence-associated secretory phenotype, which has many of the characteristics of an "osteoarthritic chondrocyte" in terms of the cytokines, chemokines and proteases produced [15]. Therefore, developing OA is a manifestation of ageing. The disease may remain latent and asymptomatic, taking many years to reach clinical relevance. However, OA is not simply the common outcome of ageing and joint injury; it is an insidiously active and inflammatory joint disease. The synovitis that occurs in both the early and late

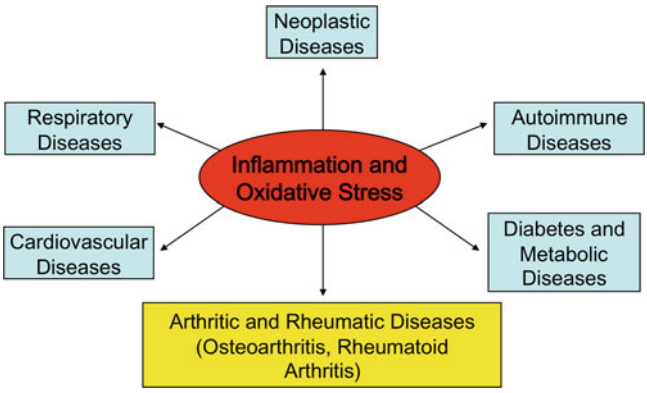

Fig. 15.1 The central role of inflammation and oxidative stress in chronic disease

phases of OA is associated with alterations in the adjacent cartilage—these changes are highly similar to those seen in RA. Catabolic and proinflammatory mediators such as cytokines, nitric oxide (NO), prostaglandin E2 (PGE_2) and neuropeptides are produced by the inflamed synovium and alter the balance of cartilage matrix degradation and repair. These events lead to excess production of the proteolytic enzymes responsible for cartilage breakdown [16]. Cartilage alterations induce further synovial inflammation, creating a vicious circle. The progressing synovitis will then exacerbate clinical symptoms and joint degradation in OA [16].

15.3 Reactive Oxygen Intermediates

Oxidation reactions in living cells produce free radicals, ROS and their derivatives. These dangerous and harmful chemical products can accumulate over time, causing extensive structural damage or even cell death. The cytotoxic effects of ROS can cause a variety of health problems including inflammatory disease, tissue necrosis, organ failure, atherosclerosis, infertility, birth defects, premature ageing, mutations and malignancy [17]. ROS production initiates an "inflammatory state" which unless quenched may result in chronic inflammatory disease states, e.g. arthritis, hepatitis, nephritis, myositis, scleroderma, systemic lupus erythematosus (SLE) and multiple system organ failure [17]. ROS are involved in the initiation of inflammatory responses [18]. For example, ROS such as H_2O_2 can stimulate the transcription factor NF-κB, which is crucial for cellular processes such as inflammation, immunity, cell proliferation and apoptosis [19]. Therefore, ROS-mediated upregulation of NF-κB can cause dysregulation of many inflammatory responses. ROS are also linked to mitochondria and the inflammasome [20]. The inflammasome is a protein complex that stimulates caspase-1 activation to promote the processing and secretion of proinflammatory cytokines [21]. This multiprotein oligomer consists of caspase 1, PYCARD[7] (apoptosis-associated speck-like protein containing a caspase-recruitment domain (CARD)), NALP[8] (pyrin-like protein containing a pyrin domain) and sometimes caspase 5 (also known as caspase 11 or ICH-3). Inflammasome-dependent inflammatory responses are triggered by a variety of stimuli including infection, tissue damage and metabolic dysregulation [22]. Recent work suggests that mitochondria are involved in integrating distinct signals and relaying information to the inflammasome. Dysfunctional mitochondria generate ROS, which is required for inflammasome activation. Interestingly, mitochondrial

[7] PYCARD promotes caspase-mediated apoptosis. Its proapoptotic activity is mediated predominantly through the activation of caspase 9. It may be a component of the inflammasome and part of a protein complex whose function would be the activation of pro-inflammatory caspases.

GeneCards entry: http://www.genecards.org/cgi-bin/carddisp.pl?gene=PYCARD

[8] NALP (NLR family, pyrin domain containing 1) is a protein involved in activation of caspase 1 and caspase 5 as part of the NALP1 inflammasome complex which leads to processing and release of IL-1β and IL-18.

GeneCards entry: http://www.genecards.org/cgi-bin/carddisp.pl?gene=NLRP1

dysfunction has been linked to OA [23, 24]. Analyses of mitochondrial electron transport chain activity in cells from OA-affected cartilage show decreased activity of complexes I, II and III compared to normal chondrocytes [25]. Therefore, it is possible that mitochondrial dysfunction in arthritis is exacerbated by ROS and catabolic processes that alter cellular metabolism. The inflammasome is negatively regulated by autophagy, which is a catabolic process that removes damaged or otherwise dysfunctional organelles, including mitochondria [22]. Autophagy has been shown to be a protective mechanism in normal cartilage, and its ageing-related loss is linked with cell death and OA [26]. These studies suggest that the connections between mitochondria, metabolism and inflammation are important for cell function and malfunctioning of this network is associated with many chronic inflammatory diseases. ROS generation and inflammasome activation are linked with mitochondrial dysfunction and may explain the frequent association of mitochondrial damage with inflammatory diseases.

15.4 Antioxidants

Antioxidants are naturally occurring reducing agents capable of inhibiting ROS formation, scavenging free radicals and removing ROS derivatives. They are capable of inhibiting the oxidation of biological molecules. Antioxidants are thought to interfere with inflammatory reactions by being oxidized themselves. Antioxidant vitamins have major roles in modulating oxidative stress, regulating immune responses and contributing to cell differentiation. Vitamin C (ascorbic acid), vitamin E, thiols (glutathione) and plant polyphenols have the capacity to neutralize ROS in joints and decrease the oxidative stress associated with the progression of arthritis. Data from epidemiological studies suggest that a high dietary intake of antioxidants may indeed protect against several degenerative diseases. Antioxidant vitamins may prevent cardiovascular disease, with the clearest effect reported for vitamin E [27]. Evidence supporting the beneficial or harmful effects of antioxidants in other diseases is either emerging or highly controversial. Antioxidants, present in fruits and vegetables and edible oils, may afford protection from long-lasting effects of oxidative stress.

15.4.1 Endogenous Antioxidant Defences

Living cells maintain a complex and interrelated protective system of endogenous antioxidant vitamins, minerals such as selenium and manganese as cofactors and glutathione to protect themselves from the harmful effects of ROS [28, 29]. Cells also use a variety of antioxidant enzymes such as catalase, superoxide dismutase and various peroxidases to quench and control cellular levels of ROS. Deficiency in antioxidants or inhibition of the antioxidant enzyme systems may cause oxidative stress and may damage or kill cells. Oxidative stress is an important component of many diseases. Therefore, the biology of ROS and antioxidants is widely investi-

gated in the context of understanding the role of these chemicals in chronic diseases characterized by oxidative stress.

Endogenous antioxidants are also important for cartilage biology. Chondrocytes exist in an avascular microenvironment, with low nutrient and oxygen levels [30, 31]. Although the majority of metabolic functions in chondrocytes rely on glycolysis [32], some of the metabolic functions of these cells are oxygen dependent [33, 34]. Oxygen is mainly supplied by diffusion from the synovial fluid [30, 35]. Consequently, the lack of oxygen means that chondrocytes display a metabolism adapted to anaerobic conditions [33, 34, 36].

There is relatively little published information in the literature about the regulation of antioxidant enzymes within cartilage. Equally little is known about the transport of antioxidants from the circulation to chondrocytes. However, transport of nutrients, oxygen and antioxidants to chondrocytes is thought to occur by diffusion from subchondral bone [37] and the synovial microcirculation [38]. The role of subchondral bone in the pathogenesis of cartilage damage has been underestimated [37]. There is increasing evidence that vascular pathology plays a role in the initiation and/or progression of OA [39]. In pathological conditions, oxygen tension in synovial fluid is subject to fluctuation as blood flow may be reduced by venous occlusion and stasis, vascular shunt and fibrosis in synovium and/or the development of microemboli in the subchondral vessels [39]. In response to oxygen variations induced through ischemia/reperfusion injury, mechanical stress and immunomodulatory and inflammatory mediators, chondrocytes produce abnormal levels of ROS that are generally produced by immune cells [33, 34, 40]. The main ROS produced by chondrocytes are NO and superoxide anion that generate derivative radicals, including peroxynitrite and hydrogen peroxide (H_2O_2) [41–43]. NO is synthesized by nitric oxide synthase (NOS) enzymes. Chondrocytes express both endothelial (eNOS) and inducible (iNOS) forms of the enzyme. NO production is stimulated by cytokines such as interleukin 1β (IL-1β) and tumour necrosis α (TNF-α), interferon γ (IFN-γ) and lipopolysaccharides (LPS). In contrast NO production is inhibited by growth factors such as transforming growth factor β (TGF-β). The enzyme complex nicotinamide adenine dinucleotide phosphate (NADPH), which catalyses the reduction of molecular oxygen to superoxide anion radicals, produces superoxide anion radicals. Articular chondrocytes express cell-specific components of NADPH oxidase complex such as p22phox (also known as cytochrome b-245 light chain), p40phox (also known as neutrophil cytosol factor 4), p47phox, p67phox and gp91phox [44].

Responses to ROS generation are dependent on the cellular redox status. When the oxidant level does not exceed the reducing capacities of cells, ROS are strongly involved in the control of cellular functions including signal transduction. In contrast, in some pathological situations, when the cellular antioxidant capacity is insufficient to detoxify ROS, oxidative stress may occur that degrades not only cellular membranes and nucleic acids but also extracellular components including proteoglycans and collagens. Furthermore, ROS can modify proteins by oxidation, nitrosylation, nitration or chlorination of specific amino acids, leading to impaired

biological activity, changes in protein structure and accumulation of damaged proteins in the tissue. A further point that needs to be made in connection with oxidative stress is the fact that redox-sensitive transcription factors (e.g. NF-κB) are upregulated, which might result in an uncontrolled inflammatory response. Oxidative stress may also cause cell death and release of cellular content into extracellular environment. Altogether, degradation products and cellular content containing oxidized molecules may contribute to the exacerbation of synovial inflammation and form a vicious circle, constituted by newly formed ROS and further degradation products.

This is still a relatively new area of research, and very few studies have been conducted on the effectiveness of antioxidant vitamins, antioxidant drugs and nutritional supplements containing combination products for relieving the symptoms of arthritis and slowing down disease progression. Nevertheless, because of the clear connection between oxidative stress, ROS and the pathogenesis of both OA and RA, patients with either form of arthritis are advised to maintain a healthy diet that consists of the naturally occurring antioxidants such as vitamin C and vitamin E and supplements such as *N*-acetylcysteine (NAC) that may support glutathione antioxidant levels.

15.4.2 Glutathione

Glutathione or gamma-L-glutamyl-L-cysteinylglycine (GSH) is one of the main endogenous antioxidants in cells and is involved in diverse functions including apoptosis, disulfide bond formation, detoxification, antioxidant defence, maintenance of thiol status and modulation of cell proliferation. Increased oxidative stress with ageing reduces chondrocyte survival, and this correlates with intracellular GSH levels [45]. Increased oxidative stress makes chondrocytes much more susceptible to oxidant-mediated cell death. This occurs through the dysregulation of the GSH antioxidant system [45]. The reduction in the capacity of antioxidant buffering systems such as GSH may represent an important contributing factor to the development of OA in older adults [45]. There have been a number of in vitro studies on GSH in chondrocytes. *N*-acetylcysteine, a precursor of GSH, has been shown to protect growth plate chondrocytes and temporomandibular joint chondrocytes from the effects of oxidative stress in vitro [46, 47]. *N*-acetylcysteine has been shown to prevent NO-induced chondrocyte apoptosis and cartilage degeneration in an experimental model of rabbit OA [48]. *N*-acetylcysteine also activates extracellular signal-regulated kinase signalling pathway in articular chondrocytes, which may provide a mechanism for the promotion of chondrocyte survival by this thiol antioxidant [49]. When over-expressed, the enzyme glutathione-S-transferase can protect chondrocytes from the effects of oxidative stress [50]. GSH depletion and NO both decrease insulin-like growth factor I (IGF-I) receptor (IGF-IR) function in chondrocytes in vitro [51]. Insulin-like growth factor I (IGF-I) helps maintain

healthy articular cartilage; however, arthritic cartilage becomes less responsive to the anabolic actions of IGF-I. Thus, it is interesting that GSH depletion can reduce the responsiveness of chondrocytes to this important anabolic growth factor. ROS such as superoxide, hydrogen peroxide (H_2O_2) and hydroxyl radical are typically produced in mitochondria as electrons leak from the electron transport chain and react with oxygen to form superoxide. It is estimated that 1–3% of oxygen reduced in cells may form superoxide in this way [52]. Hydrogen peroxide is formed from the dismutation of superoxide and by oxidases. These three reactive species are controlled via multiple enzyme systems like superoxide dismutase (SOD), catalase, glutathione-S-transferase and thioredoxin. SOD converts superoxide to (H_2O_2), which is then removed by glutathione peroxidase or catalase and therefore has the capacity to prevent the formation of highly aggressive ROS, such as peroxynitrite or the hydroxyl radical [53]. The production of H_2O_2 by inflammatory and synovial cells is an important cause of cellular damage during joint inflammation. Effective H_2O_2-metabolizing systems are important in the maintenance of normal biosynthetic rates in cartilage during inflammation. In addition to the antioxidant vitamins and GSH, chondrocyte antioxidant defences include catalase, glutathione-S-transferase and glutathione peroxidase. These enzymes afford protection against H_2O_2-dependent inhibition of proteoglycan biosynthesis [54]. Immunohistochemical studies have identified superoxide dismutases, catalase and glutathione-S-transferases in rat joints [55]. Interestingly, there were no major age-related changes in antioxidant enzyme distribution in rat joints [55]. Microinjection of antibodies against superoxide dismutase and glutathione peroxidase has been shown to decrease their viability, whereas injection of control (nonimmune) has no effect [56]. These findings highlighted the importance of glutathione peroxidase as antioxidant and the relative efficiency of SOD according to the balance between the radical production and the activity of the other antioxidant systems in chondrocytes.

15.4.3 Vitamin C

Vitamin C (also known as ascorbic acid or dehydroascorbic acid) is a soluble vitamin and a potent antioxidant. Vitamin C reacts with free radicals and acts as a cofactor for hydroxylase enzymes in the synthesis of collagens in cartilage, intervertebral disc, skin, tendons, ligaments and blood vessels. Vitamin C is needed for the growth and repair of tissues. It helps heal wounds and form scar tissue. It is also needed for the repair and maintenance of cartilage, bones and teeth. It also promotes iron absorption. In humans vitamin C must be ingested for health and survival. Vitamin C is an electron donor, and this property accounts for all its known functions. As an electron donor, vitamin C is a potent water-soluble antioxidant in humans. Antioxidant effects of vitamin C have been demonstrated in many experiments

in vitro. In chondrocyte cultures, ascorbate has a general anabolic effect. It stimulates and enhances matrix formation and assembly through mechanisms other than its redox function.

Theoretically, antioxidants such as vitamin C should protect cartilage and bone from accumulating damage induced by oxidative stress. Indeed, there is evidence to suggest that systemic oxidative stress is associated with reduced vitamin C levels. In critically ill patients, the rapid restoration of depleted vitamin C levels with high-dose parenteral vitamin C has been proposed to reduce circulatory shock and improve fluid homeostasis [57]. However, high-dose intravenous vitamin C is not associated with an increase of pro-oxidative biomarkers [58], and the impact of vitamin C supplementation on oxidative stress-related diseases is moderate because of its limited oral bioavailability and rapid clearance [57]. It is therefore possible that vitamin C supplementation could help OA patients with other co-morbidities.

15.5 Antioxidant Phytochemicals

Current treatments for OA are associated with unwanted side effects and are expensive. Natural products do not have such disadvantages and offer alternative treatment options for OA [59, 60]. Traditional and complementary medicine is known to be fertile ground for the source of modern medicines [61]. In many different chronic diseases (including OA) in which inflammation is known to play a central role, plant-derived phytochemicals (i.e. curcumin and resveratrol, Figs. 15.2 and 15.3) have been shown to exhibit therapeutic potential. The main objectives of OA therapy are to counteract the local chronic inflammation and associated inflammatory symptoms in the joints, delay joint degradation, reduce and minimize disability and provide a better quality of life for patients. It is recognized that current treatments for arthritis are inefficient, cause substantial side effects and tend to be expensive (especially when the cost of treatment is calculated and spread over the long time course of the disease). However, natural products do not have such disadvantages

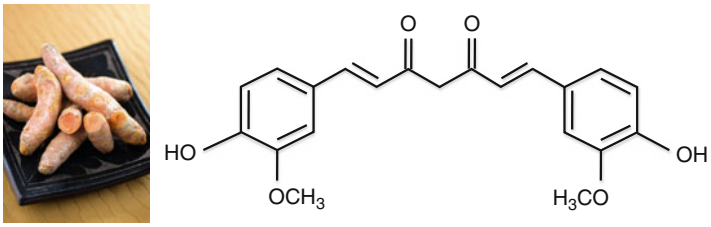

Fig. 15.2 Curcumin (diferuloylmethane) is one of the major curcuminoid constituents of turmeric. It is a modulator of NF-κB with potent antioxidant and anti-inflammatory properties

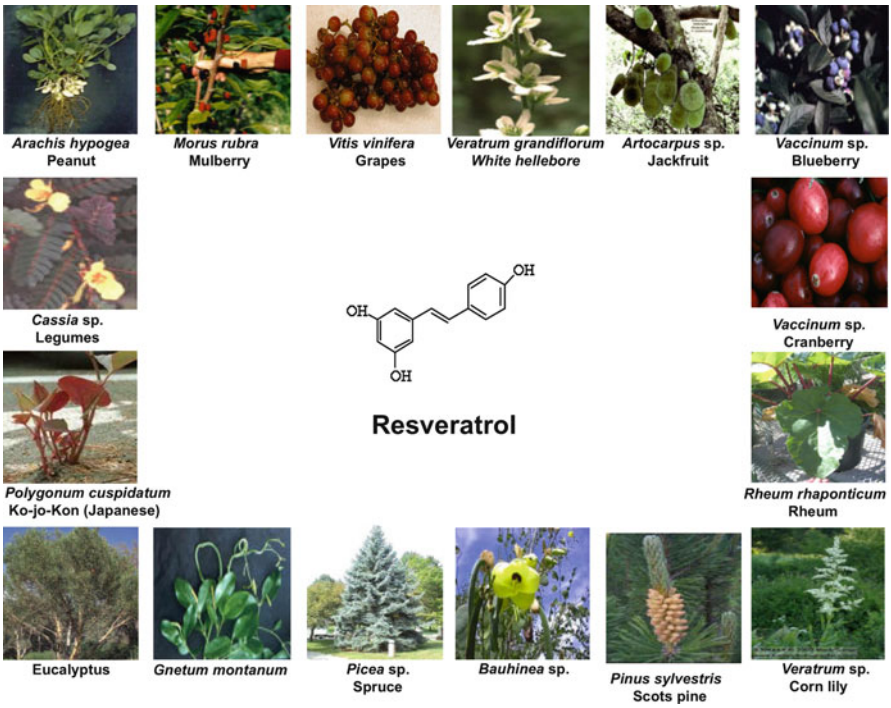

Fig. 15.3 Resveratrol (trans-3,5,4′-trihydroxystilbene) is a polyphenolic, antifungal natural phytoalexin found in grapevines (*Vitis vinifera*) and a variety of other plants

[59, 60]. A number of natural substances have been investigated for their anti-inflammatory capabilities, including omega-3 fatty acids (FA) [62], curcumin [63], resveratrol [64], the polyphenolic green tea catechins [65, 66] and various flavonoids [67, 68]. Many of them have the ability to interfere with inflammatory processes and their mediators. Their use along with nonsteroidal anti-inflammatory drugs (NSAIDs) may reduce inflammation and damage to joint tissues and could be of prophylactic and therapeutic value. Therefore, naturally occurring compounds capable of blocking NF-κB-mediated catabolic activity may prove to be promising therapeutic agents for the treatment of OA and other inflammatory conditions. This realization has resulted in the proliferation of new research aimed at understanding how nutrients and genes interact and the creation of an exciting field known as nutrigenomics. Studies on curcumin and resveratrol in the literature have highlighted how these compounds target transcription factors such as nuclear factor kappa B (NF-κB), activator protein 1 (AP-1), early growth response protein 1 (EGR-1), signal transducer and activator of transcription (STAT), peroxisome proliferator-activated receptor gamma (PPAR-γ), β-catenin, nuclear respiratory factor 2 (NRF-2), electrophile-responsive element (EpRE), protein 53 (p53), CREB-binding protein (CBP), androgen receptor (AR) and AR-related cofactors [69].

15.6 Resveratrol for Treating Bone Mineral Loss

Resveratrol is a naturally occurring polyphenol and has been shown to exhibit anti-inflammatory and antioxidant effects in various cell types. It has also been suggested to possess therapeutic potential for treatment of various diseases, including osteoporosis. As outlined earlier, osteoporosis is characterized by a negative balance of bone metabolism, which leads to a decreased bone mass with subsequent thinning of the trabecular and cortical bone and a significantly greater risk of bone fractures. Therefore, it is of critical interest to study the mechanism by which resveratrol activates osteoblasts. It has been reported that resveratrol acts as a phytoestrogen and may exhibit variable degrees of oestrogen receptor agonism. The similarity in structure between resveratrol and the synthetic oestrogen diethylstilbestrol has encouraged research on resveratrol and bone mineral loss. Resveratrol might therefore exhibit estrogenic activity, a property that is known to produce a protective benefit in osteoporosis. As a phytoestrogen, resveratrol is a potent activator of Sirt-1 deacetylase activity. In a recent study [70], we found that both p300 acetyltransferase and Sirt-1 deacetylase are expressed in bone-derived cells. In addition, we have shown that RANKL activates p300 acetyltransferase activity. Resveratrol can exert anti-osteoclastogenic effects, which is most likely mediated through the suppression of RANKL-p300-NF-κB activation (see Fig. 15.4). These recent observations provide the theoretical basis for a novel approach to treating osteoporosis and rheumatoid-related bone lesions using resveratrol and structurally related compounds. Resveratrol may become a useful adjunct in the prevention and treatment of osteoporosis and OA. The following section reviews data from some recent clinical trials of plant antioxidants.

15.7 Clinical Trials of Plant Antioxidants

"Curcumin in Rheumatoid Arthritis" (ClinicalTrials.gov [71] Identifier: NCT0075 2154[72]) is a clinical trial registered on the ClinicalTrials database. The study is sponsored by University of California at Los Angeles. It is a randomized, placebo-controlled crossover study in which 40 subjects will receive a total of 4 g of curcumin per day (capsule form, precise composition not disclosed) and then switch to placebo. The subjects' participation may last up to 8 months. By completion of the study, all 40 subjects will have taken curcumin and placebo for 4 months each. Subjects will have blood tests and complete questionnaires and be seen by the study doctor. At the present time, the status of this study is unknown and it would appear that the original completion deadline will not be met.

Limbrel® [73] is a prescription medical food product for the clinical dietary management of the metabolic processes of OA. Limbrel® was developed and formulated specifically for patients with OA. Although it is not a NSAID nor a COX-2 selective inhibitor, it is proposed to function as an antioxidant as well as being a dual inhibitor of the cyclooxygenase (COX) and lipoxygenase (LOX) enzymes of arachidonic

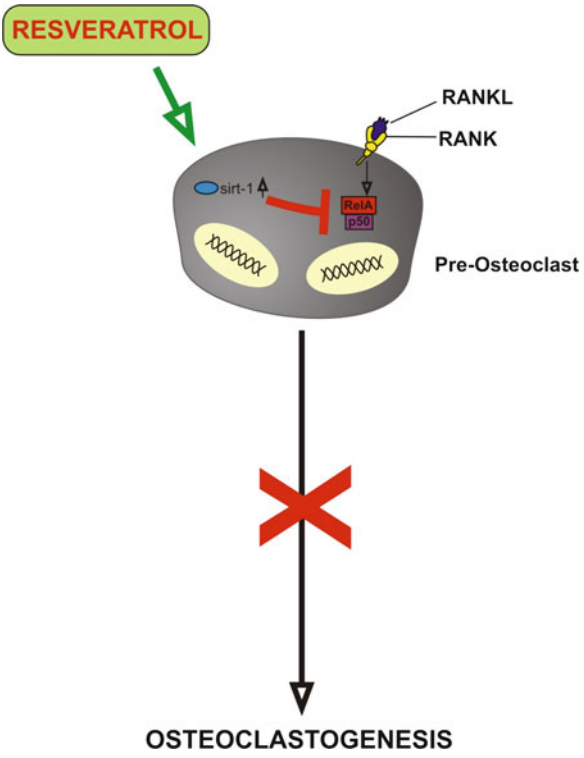

Fig. 15.4 The effects of resveratrol on osteoclastogenesis. *RANK* receptor activator of nuclear factor kappa B, *RANKL* receptor activator of nuclear factor kappa B ligand

acid metabolism. Limbrel® is manufactured according to Food and Drug Administration (FDA[9]) current Good Manufacturing Practices (cGMP). It contains flavocoxid, a proprietary blend of natural ingredients from phytochemical food source materials. Flavocoxid is comprised primarily of the flavonoids such as baicalin and catechin. These or similar ingredients can be found in common foods such as soy, peanuts, cauliflower, kale, apples, apricots, cocoa and green tea. The fact that these and similar ingredients have been widely researched and used in medicinal products in other countries also supports baicalin and catechin's safety and effectiveness. It is claimed that Limbrel® provides levels of these flavonoids needed to meet the distinctive nutritional requirements of people with OA and cannot be obtained through simply changing the diet. A recently conducted clinical trial of Limbrel® (ClinicalTrials.gov [71] Identifier: NCT00928837) [74] has shown Limbrel® to be effective in safely managing the unique nutritional needs of OA with side effects comparable to placebo. The primary and secondary outcome measures were to compare the efficacy, safety, quality of life and economic impact of Limbrel® compared to the NSAID naproxen and placebo. Although the clinical trial of Limbrel® has been completed by Primus Pharmaceuticals, no links to publications have been provided by Primus Pharmaceuticals, the National Institute of Arthritis

[9] http://www.fda.gov/

and Musculoskeletal and Skin Diseases (NIAMS) or on any of the associated clinical trials on the ClinicalTrials.gov website [71].

15.8 EFSA Guidelines for Health Claims Related to Nutraceuticals and Implications for Developing Nutritional Supplements for the Treatment of Osteoarthritis and Bone Mineral Loss

The European Food Safety Authority (EFSA) [75] based in Parma, Italy, has issued new guidelines and proposed new scientific requirements for health claims related to the maintenance of joints and to the reduction of the risk of developing OA. EFSA has proposed that clinical trials of functional foods and nutraceuticals should be designed in new and innovative ways to demonstrate a "beneficial physiological effect" on healthy joints. For example, new guidelines have been introduced for the substantiation of health claims related to glucosamine alone or in combination with chondroitin sulphate and maintenance of joints [76]. According to regulation EC 1924/2006 a "beneficial physiological effect" has specific meanings for function and disease risk claims:

- For function claims: To maintain or to improve a function
- For reduction of disease risk claims: To reduce a risk factor for the development of a human disease (not reduction of the risk of the disease)—a risk factor that may serve as a predictor of development of that disease

According to these new guidelines, only clinical trials designed to demonstrate a beneficial physiological effect on joints or a reduction in joint degradation in people without OA should be accepted as indicative. These guidelines present some major new challenges to the scientific and clinical communities. Furthermore, they create a number of opportunities for new types of clinical trials. Since the maintenance of a "normal joint" is considered to be a beneficial physiological effect, possible outcomes related to joint structure and function may include changes in:

- Joint space width (JSW) on radiographs
- Mobility
- Stiffness
- Joint discomfort (i.e. pain)

Studies performed in non-diseased (including high risk) population subgroups in which the incidence of OA is the outcome measure could be used for substantiation of health claims relating to the normal maintenance of the joint. Whilst attempting to address these requirements, we need to discriminate between food and nonfood supplements. Studies dealing with "nonfoods" will require a much more traditional pharmacological design compared to studies on "foods". Clearly, addressing this issue requires new strategies and large-scale clinical studies lasting several decades.

Such new trials will require radical rethinking of the concept of clinical trials in the OA research community. Human studies appear to be central for substantiation of clinical data, and study groups should be representative of the entire population. Hierarchy of evidence is also considered; for example, interventional studies are of greater significance compared to observational studies and reproducibility of the effect must be demonstrated. In addition, demonstrating efficacy of food supplements to EFSA will also require data on tolerance and safety, specifically gastric tolerance, hepatotoxicity, renal toxicity and allergenicity. Once these important obstacles have been overcome and new clinical trials have been carried out, curcumin and resveratrol may become useful alternative adjuncts to the NSAIDs that are currently used for the treatment of OA and loss of bone mineral.

15.9 Concluding Remarks

Antioxidants are currently a key focus of nutritional research and are widely used as ingredients in dietary supplements intended to support health. There is a pressing need to understand the contribution of antioxidants to OA, because they may provide important insight into ameliorating the initiation and progression of the disease. This knowledge will improve the design of future clinical trials and interventional studies on OA and related cartilage and bone diseases. Arthritic diseases are characterized by oxidative stress. Therefore, the idea of using dietary antioxidants for treating arthritis remains rational and realistic. However, there is a fundamental problem associated with the use of antioxidants for the treatment of OA; our diet is not able to separate antioxidant vitamins into independent entities. Many fruits and vegetables are sources of vitamins C and A. Clearly, a healthy and balanced diet is unlikely to require supplementation with these vitamins. If antioxidants possess genuine benefits for bones and joints in arthritic diseases such as OA, the primary emphasis should be prevention rather than treatment. Realistically, the use of antioxidants as adjunct treatments for OA could potentially extend to both prevention and treatment as they have the capacity to neutralize ROS in joints and decrease the oxidative stress associated with the progression of OA.

Antioxidants are thought to play a crucial role in oestrogen-deficiency bone loss [77]. Oestrogen deficiency causes bone loss by lowering thiol antioxidants in osteoclasts, and this directly sensitizes osteoclasts to osteoclastogenic signals and entrains ROS-enhanced expression of cytokines that promote osteoclastic bone resorption [77]. Therefore, combinations of plant-derived antioxidants may have long-term benefits for treating bone mineral loss in postmenopausal women. Similar approaches may also help babies, children and older men with osteopenia.

Conflict of Interest This chapter was written by the authors within the scope of their academic and research positions at their host institutions. None of the authors has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of this chapter.

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Chapter 16
Anti-cytokine Agents to Combat Oxidative Stress

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Abstract Oxidative stress induced by reactive oxygen species (ROS) is a key underlying mechanism of destructive and proliferative synovitis in rheumatoid arthritis (RA). Abundant ROS have been detected in the synovial fluid of inflamed joints. ROS are also important mediators of cardiovascular degeneration. Patients with RA have a higher risk for cardiovascular events. Recent studies reported the significant suppression of ROS and oxidative stress in the serum of patients with RA by biologics targeting the cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-6. Indeed, serum ROS levels in patients with RA treated with an IL-6-blocking drug are significantly suppressed. Therapy blocking IL-1 also suppresses oxidative stress-induced changes in tissues of patients with RA. Taken together, these findings suggest that anti-cytokine therapies may not only reduce joint damage but vascular degeneration in patients with RA. Given that recent reports have pointed to the beneficial effects of anti-cytokine agents on cardiovascular degeneration, the observed beneficial effects in RA may rely in part on the reduction of oxidative stress.

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Abbreviations

AGE	Advanced glycation end products
CAVI	Cardio-ankle vascular index
CFR	Coronary flow reserve
CRP	C-reactive protein
DMARDs	Disease-modifying antirheumatic drugs
ET	Endothelin
FMD	Flow-mediated dilatation
FN	Interferon
IL	Interleukin
LDL	Low-density lipoprotein
MMP	Matrix metalloproteinases
MMR	Mismatch repair
mtDNA	Mitochondrial DNA
MTX	Methotrexate
NAC	*N*-acetylcysteine
NO	Nitric oxide
O_2^-	Superoxide
OA	Osteoarthritis
·HO	Hydroxyl radicals
$ONOO^-$	Peroxynitrite
RA	Rheumatoid arthritis
RNS	Reactive nitrogen species
ROM	Reactive oxygen metabolites
ROS	Reactive oxygen species
TNF	Tumor necrosis factor

16.1 Introduction

Oxidative stress induced by reactive oxygen species (ROS) is an important mechanism that underlies destructive and proliferative synovitis in rheumatoid arthritis (RA) [1–4]. Reactive oxygen species also play a central role in cardiovascular degeneration, which can be considered a major extra-articular manifestation of RA. Indeed, patients with RA have a higher risk of cardiovascular morbidity and mortality that cannot be explained entirely by traditional cardiovascular risk factors [5]. Proinflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, and IL-6 are known to induce the production of ROS in the joints of patients with RA. Therefore, anti-cytokine therapies that target proinflammatory cytokines involved in synovitis may not only suppress RA progression but also prevent ROS-induced cardiovascular disease associated to RA. In this chapter, we discuss recent insights and evidence regarding the use of anti-cytokine agents to combat oxidative stress.

16.2 Oxidative Stress in Rheumatoid Arthritis

RA is a chronic inflammatory disease of unknown etiology that affects synovial joints, leading to cartilage breakdown and subchondral bone erosion [6]. Many studies have demonstrated that oxidative stress induced by ROS contributes to autoimmune diseases, such as RA [1–4]. Reactive oxygen species are normally produced during oxidative phosphorylation in phagocytic cells when stimulated by infection, ultraviolet radiation, and pollutants. Overproduction of ROS above physiological levels leads to oxidative stress and has destructive effects in cells. Studies with synovial fluid and tissues from patients with RA have revealed the presence of oxidative damage of hyaluronic acid [7], lipid peroxidation products [8, 9], oxidized low-density lipoprotein (LDL) [10], and increased carbonyl groups, which are all indicative of oxidation damage [10, 11].

In addition to direct effects on joint destruction by ROS, recent reports show that atherosclerosis associated with RA is accelerated by lipid oxidation [12–14]. Persistent local and systemic elevation of inflammatory cytokines in RA promotes lipolysis and systemic release of free fatty acids, which is directly linked to dyslipidemia. Oxidative stress resulting from persistent inflammatory reactions oxidizes local LDL, which promotes further inflammatory changes. These include local upregulation of adhesion molecules and chemokines that attract monocytes and macrophages. Monocytes and macrophages engulf large amounts of oxidized LDL and become lipid-filled foam cells. These cells are a major component of atherosclerotic plaques and have also been found in the synovial fluid [10] and the synovium [15] of patients with RA.

Reactive oxygen species also contribute to oxidative damage of cartilage extracellular matrix, such as collagens, as well as of chondrocyte DNA [16, 17]. Both ROS and reactive nitrogen species (RNS) inhibit the synthesis of matrix components produced by chondrocytes and cause indirect damage to components of the chondrocyte extracellular matrix by activating and upregulating matrix metalloproteinases (MMPs). Reactive oxygen species have also been shown to fragment hyaluronan and chondroitin sulfate [18, 19] and damage the hyaluronan-binding region of the proteoglycan core protein, thereby interfering with proteoglycan–hyaluronan interactions [20].

Regarding DNA damage, ROS and RNS directly alter the molecule by inducing DNA strand breaks at a nucleotide level and also inhibit DNA repair mechanisms. Chondrocytes are not the only cells targeted by this oxidative lesion. The levels of DNA products formed by reactions between deoxyguanosine and hydroxyl radicals (·OH) are increased in leukocytes and in sera from patients with RA [21, 22]. There is evidence supporting that mutations observed in RA tissues are a result of defective DNA mismatch repair (MMR). Mutations may also occur in genes encoding functional proteins important in repairing mutations, such as the p53 tumor suppressor gene. Positively, somatic mutations of p53 have been identified in RA synovium and in cultured RA fibroblast-like synoviocytes [23–26], and it is thought that they might directly contribute to the pathogenesis of inflammatory arthritis [27]. In particular, mutations in p53 appear to be concentrated in RA synovial tissues [28]. Mitochondrial DNA (mtDNA) is particularly susceptible to oxidative stress, and prolonged exposure to oxidative stress causes mtDNA damage without effective

repair, loss of mitochondrial function, cell growth arrest, and apoptosis [17]. Interestingly, a relationship between mtDNA and inflammatory arthritis was revealed from murine studies demonstrating that arthritis results from oxidative damage to mtDNA [29, 30]. mtDNA has similarities to bacterial DNA and differs from eukaryotic nuclear DNA (nDNA). mtDNA-induced nuclear factor-κB stimulation results in the production of TNF-α. In addition, extracellular mtDNA is detected in the synovial fluids of RA patients but not of control group. Endogenous mtDNA displays inflammatory properties, as a result of its content of unmethylated CpG motifs and oxidatively damaged products.

Oxidative stress can induce T cell hyporesponsiveness in RA through effects on proteins and proteasomal degradation [31]. Hyporesponsive T cells often appear to display impaired expression of some (e.g., TCR-ζ, $p56^{lck}$, and LAT) but not all (e.g., TCR-αβ and CD3-ε) crucial TCR-proximal signaling molecules. Oxidative stress-exposed proteins are differentially susceptible to proteasomal degradation: whereas modified TCR-ζ is relatively resistant, reactive oxygen species (ROS)-altered LAT and $p56^{lck}$ are much more susceptible. T cell hyporesponsiveness is strongly correlated with ROS-dependent protein alteration, since inhibition of proteasomal degradation does not restore function.

In addition, antioxidants and oxidative enzymes have been shown to ameliorate arthritis in animal models [32–34]. Moreover, abundant amounts of ROS have been detected in the synovial fluid of inflamed rheumatoid joints [35]. Reactive oxygen species are thought to act as second messenger to stimulate nuclear factor κB-dependent expression of proinflammatory cytokines, forming an amplified feedback loop that induces further ROS production. This hypothesis is supported by several studies showing increased oxidative enzyme activity correlating with decreased antioxidant levels in serum and synovial fluid from patients with RA [9, 36–39].

Several lines of evidences suggest a role for oxidative stress in RA pathogenesis. Epidemiologic studies have revealed an inverse relationship between dietary antioxidant intake and the incidence of RA [40–43], while an inverse relationship between antioxidant levels and the severity of inflammation has also been confirmed [44, 45]. These studies suggest that an imbalance between oxidative stress and antioxidant levels is not only related to the severity of inflammation but also triggers the onset of RA. In a collagen-induced arthritis (CIA) rat model, oxidative modification of type II collagen induced a higher immune response, resulting in more severe synovitis and joint destruction [30]. Although it is not likely that oxidative stress by itself causes the disease, reduction of the oxidative load may help suppress inflammation and destruction of joints in patients with RA. Furthermore, the possibility of preventing the progression to severe RA makes oxidative stress an attractive therapeutic target.

Finally, oxidative stress taking place during inflammation can cause nonenzymatic damage of proteins by glycoxidation. This process primarily involves lysine and arginine residues and ultimately results in the generation of advanced glycation end products (AGE). Immunoglobulin G can undergo glycoxidation to generate AGE-IgG. In the context of inflammatory arthritis, the formation of AGE-IgG is related to the severity of the systemic inflammatory response, which is not specific

to RA, whereas the presence of antibodies against AGE-IgG has been specifically associated with RA [46, 47].

On the other hand, oxidative stress contributes to the body's immune defenses against pathogens. Activated phagocytes of the immune system produce ROS and RNS with lethal reactive effects to kill pathogens. These include superoxide (O_2^-), nitric oxide (NO), and the highly reactive peroxynitrite ($ONOO^-$) [48]. Although these lethal oxidants damage host tissues, their nonspecificity is also advantageous since they damage almost every part of the target cell [49], thereby preventing pathogens from evading the immune response through mutation of a single molecular target.

16.3 Oxidative Stress and Cytokines

TNF-α, IL-1β, and interferon (IFN)-γ exert multiple effects on cellular processes including gene expression, host defense reactions, and immune response. TNF-α increases mitochondrial ROS production in tumor cells, endothelial cells, and hepatocytes [50, 51]. In inflammatory arthritides, including RA, TNF-α has been shown to induce ROS production in neutrophils [52, 53]. Similarly, IL-1β is able to stimulate ROS production in multiple cell types. In patients with RA, IL-1β production and oxidative [54] and nitrosative [55] stress markers are increased. IL-1β amplifies the inflammatory process through the induction of IL-6, C-reactive protein (CRP) [56–58], and endothelin-1 (ET-1) [59] and upregulates the release of superoxide anion [60]. IL-1 also causes excessive release of NO^- [61], which reacts with superoxide anion to form peroxynitrite [62]. Peroxynitrite increases the generation of nitrotyrosine, a marker of nitrosative stress [55, 62], which plays an important role in endothelial dysfunction [63]. IFN-γ induces an immediate and marked augmentation of intracellular ROS in transformed lymphoblast cell lines [64, 65]. IFN-γ also induces ROS and endoplasmic reticulum stress during IFN-γ-induced apoptosis of hepatocytes [66]. IL-6 is another pivotal proinflammatory cytokine in RA that induces ROS production in RA synovial fibroblastic cells [67]. IL-6 stimulates the proliferation of fibroblast-like synoviocytes, and this IL-6-induced proliferation is inhibited with methotrexate (MTX) or *N*-acetylcysteine (NAC, 1 mM). Furthermore, ROS production in fibroblast-like synoviocytes is increased significantly by IL-6, and this effect is also abrogated by MTX or NAC.

16.4 Oxidative Stress After Administration of Anti-cytokine Agents

Recently, several anti-cytokine agents targeting TNF-α, IL-6, and IL-1 and T cell activation have been established. These biological agents have strong efficacy not only in ameliorating signs and symptoms of RA but also in preventing progression of structural joint damage.

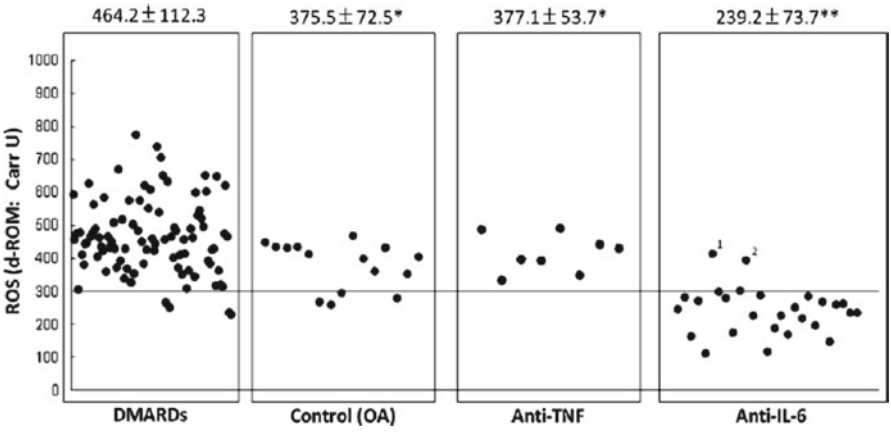

Fig. 16.1 Serum levels of ROS (d-ROM: Carr U) in control (OA), DMARDs, anti-TNF-blocking, and anti-IL-6-blocking groups. Normal values: <300 Carr U. *$P<0.05$, **$P<0.01$, significantly lower d-ROM levels compared to the DMARDs group. Results are expressed as mean ± standard deviation (SD). Intergroup differences were assessed by one-way ANOVA. $P<0.05$ was considered statistically significant [70]

Multiple lines of evidence support the hypothesis that selectively blocking cytokines inhibits ROS generation. Treatment with infliximab, a monoclonal antibody against TNF-α, reduces oxygen stress markers, including pentosidine and 8-hydroxy-2′-deoxyguanosine [68]. Decreased carbonyl groups and increased thiol groups found on proteins in patient serum after infliximab treatment indicate suppression of serum protein oxidation. Etanercept is a decoy receptor that blocks TNF-α signal by a slightly different mechanism of action than that of infliximab. Similar to the infliximab effect, etanercept also reduces oxidative stress marker levels in patients with RA [69].

Like TNF-α, IL-6 is a pivotal proinflammatory cytokine in RA. Therefore, IL-6-blocking therapies may also suppress oxidative stress in patients with RA. In our recently published study [70], patients with RA treated with tocilizumab, a monoclonal antibody against IL-6 receptor, exhibited significantly lower levels of serum oxidative stress markers than those of RA patients treated with disease-modifying antirheumatic drugs (DMARDs), TNF-α antagonists, and patients with osteoarthritis (OA). Due to the biochemical instability of ROS, their levels were determined by measuring reactive oxygen metabolites (ROM). As shown in Fig. 16.1, control patients with OA ($N=15$) showed 375.5 ± 72.5 Carr U of ROM, and patients with RA treated with DMARDs ($N=91$) showed significantly higher ROM levels (464.2 ± 112.3 Carr U). The value of 375.5 ± 72.5 Carr U in the OA group was also higher than normal values (<300 Carr U) and may be influenced by the older mean age of the OA cohort [71, 72]. Furthermore, as shown in Fig. 16.1, serum levels of ROM in eight patients with RA treated with TNF-α-blocking agents (infliximab or etanercept) showed 377.1 ± 53.7 Carr U ROM, a level similar to the OA control group (375.5 ± 72.5 Carr U), indicating that anti-TNF-α biologics effectively reduce oxidative stress in patients with RA, similar to what was described previously in TNF-α antagonist studies.

Table 16.1 Patient characteristics

	Control (OA) $N=15$	RA		
		DMARDs $N=91$	Anti-TNF $N=8$	Anti-IL-6 $N=26$
Age (years)	68.1 ± 14.5	63.1 ± 12.1	58.8 ± 3.81	58.0 ± 8.0
Duration of disease years)	–	12.6 ± 10.7	10.3 ± 4.5	13.0 ± 8.5
Body weight (kg)	–	51.4 ± 9.6	54.9 ± 7.9	52.8 ± 7.7
Prednisolone (mg/day)	0	2.9 ± 3.1	2.8 ± 3.2	2.0 ± 3.0
CRP (mg/dL)	–	1.12 ± 1.76	0.42 ± 0.37*	0.03 ± 0.14**
WBC (counts)	–	8,090 ± 2,914	7,746 ± 2,779	6,302 ± 2,866
ROM (d-ROM: Carr U)	375.7 ± 72.5*	464.2 ± 112.3	377.1 ± 53.7*	239.2 ± 73.7**

Results are expressed as mean ± SD. Intergroup differences were assessed by one-way ANOVA. $P<0.05$ was considered statistically significant [70]
*$P<0.05$
**$P<0.01$

Finally, serum levels of ROM in 26 patients with RA treated with tocilizumab showed 239.2 ± 73.7 Carr U of ROM, with only two cases above the normal range (409 and 394 Carr U). While CRP levels of these two cases remained slightly elevated (0.7 and 0.1 mg/dL), CRP levels of the other 24 cases showing normal levels of ROM were completely suppressed (<0.04 mg/dL). These findings support the idea of the suppression of oxidative stress by IL-6 inhibition in RA (Table 16.1) [70].

There was no significant difference in disease duration, body weight, daily dose of prednisolone, and WBC counts among the three treatment groups. CRP levels were significantly lower in the anti-IL-6 group compared to both the DMARDs and anti-TNF groups. CRP levels of the anti-TNF group were significantly lower than in the DMARDs group and significantly higher than in the anti-IL-6 group. *$P<0.05$, **$P<0.01$ significantly lower CRP and d-ROM compared to the DMARDs group. Results are expressed as mean ± SD. Intergroup differences were assessed by one-way ANOVA. $P<0.05$ was considered statistically significant [70].

At present, there are no reports on the effects of anakinra (a recombinant human IL-1 receptor antagonist) on serum levels of oxidative stress in patients with RA. However, anakinra reduced IL-1-mediated oxidative tissue damage in a rat model of lung injury [73]. Moreover, IL-1 receptor antagonist treatment reduced islet endothelial activation and oxidative stress-induced gene expression in rat model of type 2 diabetes [74]. These observations suggest that IL-1-blocking therapies may also be effective against oxidative stress.

16.5 Oxidative Stress and Extra-articular Disease in RA

Reactive oxygen species-induced oxidative stress is an important mechanism that underlies destructive and proliferative synovitis [1–4], but it is also an important mediator of extra-articular manifestations of RA. In particular, RA is associated

with increased cardiovascular morbidity and mortality [75]. Patients with RA have a higher risk for cardiovascular events that cannot be explained entirely by traditional cardiovascular risk factors [5], implying that cardiovascular disease is an extra-articular manifestation of RA [76]. In patients at increased risk of or diagnosed with cardiovascular disease, the pathology of arterial atherosclerosis can be characterized by increased arterial stiffness, increased carotid intima–media thickness, and endothelial dysfunction [77, 78]. These pathological changes in the cardiovascular system are also observed in patients with RA [79, 80], a fact that may explain why cardiovascular disease is one of the leading causes of death in these patients [81]. As discussed above, oxidative stress is an important trigger of arterial atherosclerosis. Therefore, the suppression of oxidative stress may decrease the cardiovascular mortality risk of patients with RA by decreasing atherosclerosis and calcification of arteries and lower the risk for cardiovascular events.

TNF-α is a cytokine that promotes vascular disease [87, 88]. Indeed, patients with RA treated with TNF-α-blocking biologics show reduced oxidative stress and improvements in vascular stiffness [82, 83]. Other studies have also suggested that TNF-α-blocking drugs may reduce mortality [84] and cardiovascular disease in patients with RA [85, 86]. These studies suggest that TNF-α-blocking drugs may reduce cardiovascular degeneration in part by decreasing oxidative stress.

The link between cardiovascular degeneration and oxidative stress is further supported by studies in patients treated with the anti-IL-6 antibody, tocilizumab, which significantly decreased the level of oxidative stress [70]. It is reasonable to expect that tocilizumab may improve intra-articular synovitis and joint destruction associated with RA, as well as arterial atherosclerosis. Multiple reports have associated high circulating concentrations of IL-6 with an increased risk of coronary heart disease [89–91]. Although few studies have reported the effects of reducing IL-6 on vascular stiffness in patients with RA, one report suggests that the reduction of arterial stiffness by tocilizumab monotherapy, as measured by the cardio-ankle vascular index (CAVI), is as effective as etanercept or adalimumab [92]. Furthermore, large-scale genetic and biomarker data provide strong evidence that IL-6 receptor signaling may have a causal role in the development of coronary heart disease in humans, suggesting that IL-6 receptor blockade could provide a novel therapeutic approach for coronary heart disease prevention [93, 94]. Additional studies on the effects of IL-6-blocking drugs on vascular degeneration and incidence of cardiovascular events in patients with RA are required to demonstrate the efficacy of this therapeutic approach in lowering cardiovascular morbidity and mortality.

As mentioned above, IL-1 also plays a pivotal role in RA pathogenesis by upregulating IL-6. Thus, inhibiting IL-1 may have a similar effect on vascular degeneration and cardiovascular events. Indeed, patients with RA treated with anakinra revealed rapid beneficial effects on vascular and left ventricular function [95]. Measures of endothelial dysfunction, as assessed by flow-mediated dilation (FMD), coronary flow reserve (CFR), aortic distensibility, and aortic strain were clearly improved 3 h after anakinra administration, correlating with reduced oxidative stress. These results

indicate that IL-1 activity is tightly linked to oxidative stress and influences acute cardiovascular events. Collectively, these findings suggest that anti-cytokine therapies targeting TNF-α, IL-6, and IL-1 suppress oxidative stress and have beneficial effects on reducing cardiovascular degeneration in patients with RA.

16.6 Summary and Conclusions

Oxidative stress induced by ROS is not only an important mechanism that underlies destructive and proliferative synovitis in RA but also an important mediator of cardiovascular degeneration, which is considered a major extra-articular manifestation of RA. Several recent studies have shown that serum ROS levels and oxidative stress are significantly suppressed by anti-TNF-α and anti-IL-6 biologics. Anakinra, an IL-1-blocker, also suppresses oxidative changes in tissue samples from patients with RA. These findings suggest that anti-cytokine therapy not only reduces joint damage but also prevents vascular degeneration in patients with RA. Recently, many reports have pointed to the beneficial effects of anti-cytokine agents on cardiovascular degeneration. Accordingly, their beneficial effects in RA may rely in part on the reduction of oxidative stress.

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Chapter 17
Oxidative Conditioning and Treatment for Osteoarthritis

Prem S. Ramakrishnan, Marc J. Brouillette, and James A. Martin

Abstract Oxidative stress is associated with aging and is also implicated as a contributing factor in osteoarthritis, a degenerative joint disease resulting mainly due to progressive degradation of the articular cartilage. Avascular and aneural in nature, articular cartilage is a unique tissue thriving in a mechanically active environment that requires physical stimuli to maintain tissue health. Cartilage adaptation is achieved by modulating matrix synthesis and other protective biological features in an effort to tolerate increased mechanical demands. One potential mechano-transductive pathway that regulates functional adaptation is believed to be driven by oxidative stress, but the exact mechanisms of this phenomenon are not clear. As an important rate-limiting factor for cartilage metabolism, sublethal levels of oxidants play a protective role against injurious mechanical loads, probably by modulating multiple biochemical pathways that increase stress tolerance thresholds of cartilage. Antioxidant status, nuclear factor (NF-κB), and hypoxia-inducible factor (HIF-1α) are potential factors that may play a role in oxidant conditioning and cartilage adaptation.

Abbreviations

ATP	Adenosine triphosphate
HIF-1α	Hypoxia-inducible factor-1α
NF-κB	Nuclear factor-κB
OA	Osteoarthritis
PTOA	Post-traumatic osteoarthritis
ROS	Reactive oxygen species

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17.1 Introduction

Aging and joint overuse are significant risk factors for osteoarthritis (OA). A sizable subset (~12%) of the OA population develops the disease as a result of prior joint trauma, and this condition is referred as post-traumatic OA (PTOA). A recent review estimated that a serious ligamentous or capsular injury increases the risk of OA as much as 10-fold and that most articular fractures increase the risk of OA more than 20-fold [1]. A significant portion of patients who suffer from PTOA are young, and current therapies of joint replacement and joint fusion are not deemed desirable. Moreover, nonsurgical therapies such as anti-cytokine/anti-inflammatory treatment growth factors target a variety of symptoms observed in OA, but their effectiveness in mitigating the severity of the disease is modest at best. There is a significant need to develop alternative therapeutic strategies that may forestall the development of OA.

Cartilage health and function are strongly influenced by mechanical stimuli. Physiological mechanical stresses stimulate matrix synthesis and also support cartilage homeostasis, whereas supra-physiological stresses contribute cartilage dysfunction, degeneration, and development of OA [2]. In non-phagocytic cells, low levels of ROS contribute to intracellular signaling mechanisms, modulate gene expression, and regulate the activation inflammatory mediators, whereas high levels lead to apoptosis and senescence [3].Chondrocytes produce reactive oxygen species (ROS) in response to mechanical stress, but their role in cartilage biology and mechanotransduction is complex and not completely understood [4]. Majority of mechanically induced ROS originate from the mitochondria, suggesting their potential role in cartilage mechanotransduction in both health and disease conditions [5, 6]. Although elevated levels of ROS are deleterious and are implicated in aging and OA, cellular energy production in chondrocytes relies on the availability of mitochondrial ROS to produce adenosine triphosphate (ATP) [7, 8].

Taken together, ROS levels in cartilage are in part regulated by the mechanical environment in a dose-dependent manner. The interrelationship between mechanically induced ROS and cartilage metabolism suggests that periodic changes in physiological joint loading induce sublethal levels of ROS that may have potential implications in cartilage biology. ROS generated by these elevated mechanical demands may influence cartilage growth and response to injury by modulation of antioxidant defenses, bioenergetics, and numerous other cell processes. In vivo studies suggest that high-intensity physical exercise as well as a sedentary lifestyle increases the risk of cartilage degeneration and high levels of systemic oxidative stress in the long term, whereas moderate intensity of physical training leads to healthy cartilage with lower ROS levels[9]. It is reasonable to propose that nonlethal levels of oxidative stress primarily produced by mechanical stimuli condition the cartilage by activating specific biochemical pathways that (1) elevate intracellular antioxidant availability, (2) increase matrix synthesis, (3) increase metabolism, and (4) suppress inflammation. Upon activation, such preemptive oxidant conditioning can provide adequate basis to tolerate future

degenerative effects of oxidant assaults. We will focus on key cell-signaling pathways that are influenced by oxidative stress, namely, nuclear factor-κB (NF-κB), hypoxia-inducible factor-1α (HIF-1α), and redox signal transcriptions, and how these signals may potentiate oxidant conditioning in cartilage.

17.2 Role of Oxygen and ROS in Cartilage Metabolism

17.2.1 Oxygen

It is estimated that oxygen availability varies from 6% at the surface of the cartilage (closer to the synovial fluid) to 1–2% at the deep zone (subchondral interface). Hence, chondrocytes are well adapted to hypoxia by producing most of their chemical energy source—ATP with substrate level phosphorylation (glycolysis) [10]. Chondrocyte adaptation to hypoxia is evident in its low density of mitochondria (1–2% of the cell volume when compared to 15–20% in other cell types) [11]. Chondrocytes are very sensitive to changes in oxygen status, affecting proteoglycan synthesis [10, 12, 13], growth factor expression [14], glucose uptake [15, 16], ATP levels [10, 17], and focal adhesion constituents [12, 18]. Oxygen diffusion in cartilage is regulated by mechanical stimuli, indicating that joint loading exerts control over several oxygen-dependent pathways that influence cartilage pathology [19]. Oxygen tension can also influence the production of inflammatory mediators in response to mechanical compression in cartilage explants [20]. Matrix turnover is altered by oxygen tension, affecting material properties of articular cartilage [21, 22] and in turn response to mechanical stress [23, 24]. Taken together, changes in structural (cracks, fissures, cartilage delamination) and mechanical environments (trauma, weightlessness) can influence cartilage pathology by disturbing nutrient (including oxygen) availability for chondrocyte metabolism.

17.2.2 Oxidants and Cellular Redox System

The majority of available oxygen in cartilage is utilized to produce energy for cell processes. Molecular oxygen is also processed to form ROS that are involved in both intracellular signaling and also in cellular destruction. In addition to the mitochondria, peroxisomes, lipoxygenases, cytochrome P450, and NADPH oxidase family are other potential cytosolic ROS-generating systems. A variety of external agents (or stimuli) such as ionizing radiation, heat, mechanical stress, ultraviolet light, and chemical and environmental toxins can also modulate cellular processes by initiating intracellular oxidant production. Nitric oxide (NO), superoxide anion ($O_2^{\bullet -}$), and hydroxyl radicals (OH^-) along with hydrogen peroxide (H_2O_2) are the most extensively studied among the diverse chemical species of ROS. As a balancing

act, cells possess numerous enzymatic (catalase, superoxide dismutase, and glutathione peroxidase) and nonenzymatic (glutathione; vitamins A, C, and E; pyruvate; flavonoids; and carotenoids) antioxidant defense systems to counter and regulate ROS levels in physiological conditions. The balance between ROS production and availability of antioxidants would dictate the degree of oxidative stress. The term "oxidative stress" does not necessarily imply deleterious effects to a cell's survival as certain levels of oxidative stress are essential multiple cellular processes. In general, when ROS levels fall below a physiologic set point, impaired host defense mechanism and decrease in physiological functions can be observed. High levels of ROS cause oxidation of cellular components, proteins, lipids, DNA, and extracellular millieu that are followed by the initiation of multiple signaling pathways that modulate a cell's fate of survival, senescence, or apoptosis [3].

Chondrocytes primarily produce NO and $O_2^{\bullet -}$ along with derivative radicals including peroxynitrite ($ONOO^-$) and H_2O_2 [25]. Oxidants play a crucial role as a replacement to molecular oxygen in stimulating glycolysis in chondrocytes [7]. ROS are required in regulating gene expression profiles of cytokines, matrix metalloproteinases (MMP), adhesion molecules, and matrix components along with achieving ionic homeostasis [26–30]. Chronic increase in ROS occurs in the event of depleted or inhibited antioxidant resources and is known to cause mitochondrial damage [31], cell death [26, 32], senescence, sensitivity to mediators [33], cartilage degradation [34, 35], matrix stiffness, and brittleness [36–39]. ROS levels are also modulated by inflammatory and immunomodulatory mediators [40–46] that can lead to a synergistic effect. Recent reviews have concluded that ROS act as integral factors of intracellular signaling mechanisms although their equally significant contribution to cartilage degradation cannot be discounted [25, 47].

17.3 Mechanical Regulation in Cartilage Pathophysiology

Since the primary function of cartilage is to transfer mechanical load, physical stimulation has strong influence on metabolic activities of chondrocytes and turnover rate of the extracellular matrix [48]. The location of the subchondral growth front during joint development is believed to be the influenced by local balance between intermittent shear stress and hydrostatic pressure within the deep zone cartilage [49]. Clinical and animal studies provide definitive evidence on the causal relationship between joint loading and cartilage health and function. An in vivo study with female beagles that underwent 10 weeks of moderate running regiment showed increased cartilage thickness and augmented proteoglycan content [50, 51]. Cartilage physical quality in terms of its mechanical stiffness increased with moderate exercise [52], whereas strenuous exercise led to cartilage lesions [53]. Also, decreased or complete unloading of joints caused atrophy of the cartilage, characterized by cartilage thinning [54] and decrease in proteoglycan content [55]. Surprisingly, cartilage weakening was

observed to be reversible, and remobilization can almost completely restore most of the structural and biochemical changes associated with joint disuse [56, 57].

Joint trauma is strongly associated with the incidence of OA-like conditions in cartilage, but the contributing factors are not completely understood. Upon joint injury, several folds of increased mitotic activity have been observed in chondrocytes, indicating that repetitive joint injury can result in a condition of replicative senescence possibly by telomere erosion [58–60]. Abnormal loads may act in synergy with other mediators and promote cartilage degeneration [61–63]. These studies indicate that mechanical inputs that are within a certain threshold are essential for cartilage homeostasis and fluctuation within these limits seem to improve tissue structure and function.

Physiological loading that is experienced by cartilage is complex. Joint loading causes articular cartilage to undergo matrix deformation, interstitial fluid flow, and hydrostatic pressure. Chondrocytes respond to these stimuli through mechanotransduction, and their response varies with the mechanical environment presented to them [64–66]. Cartilage matrix is a very challenging environment, and chondrocytes thrive in an environment that is hyperosmotic, acidic, and avascular. Lack of blood supply renders the chondrocytes to mainly rely on the synovial fluid (and subchondral bone to a lesser extent) for oxygen and nutrients. Cartilage composition is dense, and its relative thickness restricts free diffusion of nutrients and waste products suggesting that periodic mechanical stimuli may in part regulate transport of solutes and metabolic waste. As a general consensus, static stimuli are considered injurious to cartilage since it results in the inhibition of solute diffusion [67], whereas dynamic stimuli encourage favorable solute transport [68]. Reviews on mechanical regulation of cartilage physiology are available for further reading [69, 70].

17.4 Mechanical Regulation of ROS in Chondrocytes

17.4.1 Source of Oxidants in Cartilage

In vitro studies show that ROS are regulated by physical forces that are sensed by chondrocytes [4, 71]. Mechanical regulation of nitric oxide (NO) has been widely investigated for its role in cartilage biology. Nitric oxide is biosynthesized endogenously from L-arginine, oxygen, and NADPH by various nitric oxide synthase (NOS) enzymes. Among the three isoforms of NOS, endothelial and neuronal NOS are calcium dependent, whereas the inducible form is regulated by various sources that include inflammatory cytokines. A variety of mechanical stimuli such as intermittent mechanical compression [72, 73] and shear stress [72–76] can stimulate NO production in a dose-dependent manner [20, 24, 77, 78]. Potential pathways that regulate mechanotransduction of NO seem to involve stretch-activated ion channels and purinergic receptors [79, 80]. Nonphysiological mechanical stresses may induce excessive oxidative stress that can be blocked by exogenous application of antioxidants [81–83].

Besides NO, mitochondria are a significant source of ROS involved in cartilage biology. Chronic oxidative stress leads to mitochondrial DNA damage causing phenotypical signs of aging and degeneration in cartilage such as dysregulation of antioxidant defenses, senescence, and cell death [38, 84]. The mitochondria are mechanically linked to the chondrocyte cell membrane via f-actin, and this mechanical linkage ensures movement and distribution of mitochondria within the cytosol [85]. The interaction of the mitochondria and the cytoskeleton influences energy and ROS production [86] along with calcium homeostasis [87] and mitochondrial apoptotic factors [88]. Under mechanical stress, deformation of the extracellular matrix is transmitted to the chondrocytes via membrane-bound receptors called integrins [89]. Subsequently, chondrocytes undergo deformation causing conformational changes in cellular organelles including the mitochondria [90]. Mitochondrial distortion regulates mitochondrial functions [90–93] including availability of mitochondrial ROS in chondrocytes.

Interestingly, oxidants derived from the mitochondria play a critical role by maintaining availability of ROS to maintain glycolytic function. During cellular metabolism, mitochondrial superoxides are generated at complex I and complex III of the electron transport chain via formation of the intermediate free radical semiquinone. Age-associated mitochondrial damage and disruption of the electron transport chain cause sporadic release of ROS from the mitochondria, affecting cellular ATP production and exposes cells to further oxidative damage [94]. The end result of these irreversible changes forces the mitochondria to initiate apoptosis or a state similar to replicative senescence [95]. One among many prevailing hypotheses on early onset of osteoarthritis suggests that mitochondrial damages are accelerated by excessive oxidative stress induced as a result of joint trauma. In vitro explant studies from our investigations observed a burst of ROS after a single blunt impact injury resulting in 60% cell death, indicating that mechanical trauma can induce lethal levels of oxidative stress [5]. The mitochondrial origin of ROS was confirmed when injured explants pretreated with rotenone, an electron transport chain complex I inhibitor, showed decreased production of superoxide, saving cells from trauma-induced death [6]. These studies clarify the detrimental effects of mitochondrial-derived ROS in response to injurious loading and supporting antioxidant therapies as a viable option to treat joint trauma.

17.4.2 Mitochondrial ROS and ATP Synthesis in Response to Mechanical Stimuli

Our previous investigations focused on injurious effects of mitochondrial ROS in the aftermath of joint trauma. Although our findings indicated that dangerous levels of ROS are produced under extreme mechanical conditions, it was not clear whether ROS availability could also be mechanically regulated under physiological conditions. We hypothesized that mechanical stimuli (in terms of stress and

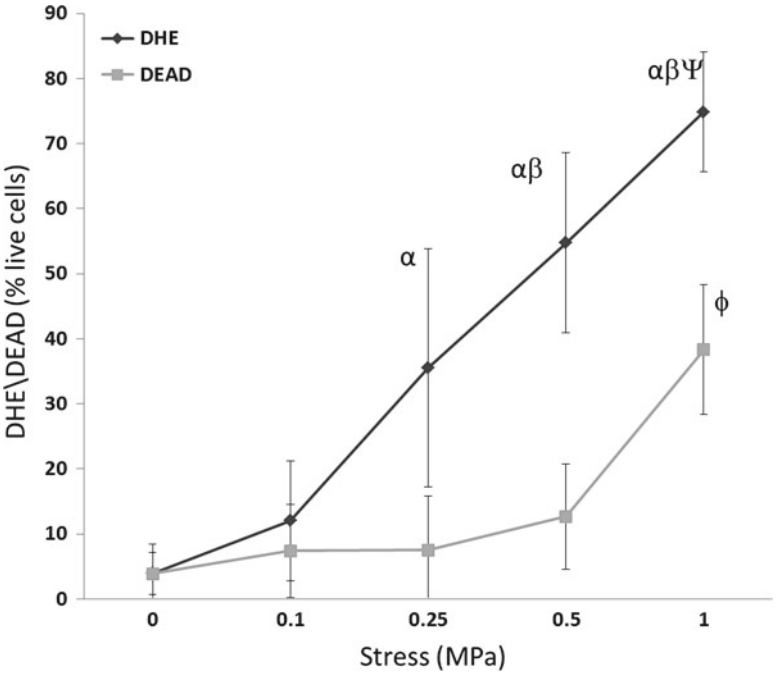

Fig. 17.1 Percentage of cells stained with oxidative marker dihydroethidium (DHE) and cell death percentage (DEAD) under various static compressive stresses applied. Statistical significance $p<0.05$ is represented as follows (α vs. 0 MPa, β vs. 0.1 MPa, Ψ vs. 0.25 MPa, and φ vs. 0.5 MPa)

deformation) would induce mitochondrial ROS release in a dose-dependent fashion. Deformations (determined as normal tissue strain) were measured in osteochondral explants subjected to compressive stresses of 0, 0.25, 0.5, and 1.0 MPa using a customized mechanical stimulation device under standard low-oxygen (5% O_2) culture conditions . Cell death and ROS were determined by confocal imaging with ethidium homodimer (EtHD2, Invitrogen®) and dihydroethidium (DHE, Invitrogen®), respectively. ROS release increased along with increasing stress application. Oxidative stress and cell death increased appreciably at stresses > 0.5 MPa (Fig. 17.1). Linear regression analysis showed high positive correlation between ROS release and cartilage strain (Fig. 17.2; $R^2=0.8$, $p<0.05$). Significant cell death was observed at strains > 40%, indicating a potential physiological limit. These findings provided the first proof that ROS in cartilage can be regulated by mechanical stress in a dose-dependent manner and magnitude of oxidant production may be closely associated with chondrocyte deformation.

Our successive study was to determine whether mechanical loading stimulates ATP synthesis for chondrocyte function by regulating mitochondrial ROS. One hour of cyclic loading on osteochondral explants under 0.25 MPa at 0.5 Hz deformed resulted in mean cartilage strains of ~ 0.21 with ~4-fold increase in ATP content

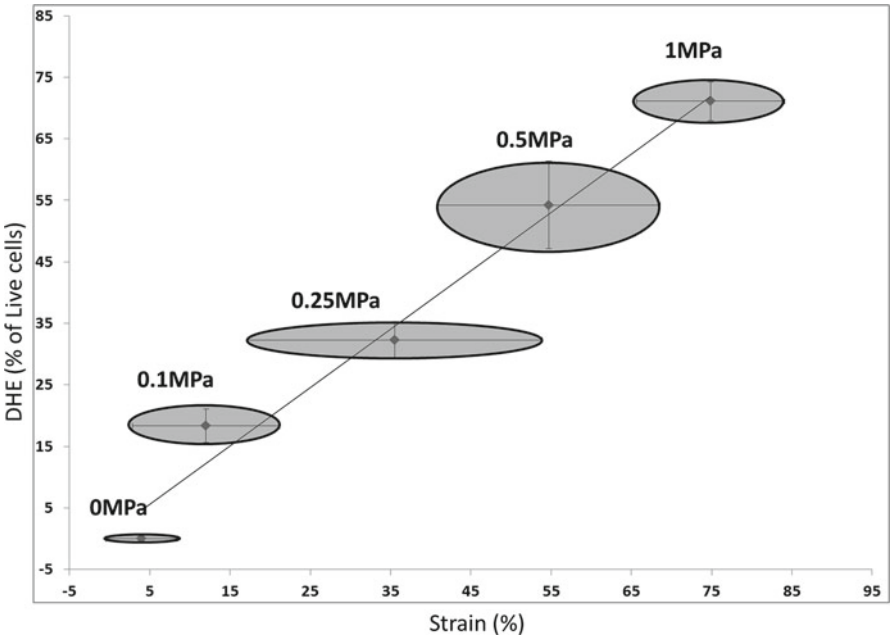

Fig. 17.2 DHE staining vs. mechanical strain under various mechanical stresses applied. The *gray ellipse* denotes the general distribution of data under applied mechanical stress. In general, cumulative mechanical strain endured by cartilage increased with increasing stress magnitude with standard deviations represented as the major axis of each ellipse. The minor axis of each ellipse shows the spread of oxidative stress data at the applied stress. Linear relationship between DHE and strain showed good linear correlation with $R^2 = 0.8$

(luciferase-based ATP assay, Sigma-Aldrich®) when compared to non-stimulated cartilage. Electron transport inhibitor rotenone and mitochondrial ROS scavenger MitoQ significantly suppressed mechanically induced ROS production (measured using DHE) and ATP synthesis, all indicating that the majority of the ROS required for chondrocyte ATP synthesis originate from the mitochondria. When under hydrostatic pressure, minimal normal strains are experienced in cartilage even under higher magnitude of stress (1 MPa) correlating well with very low oxidative stress and corresponding low ATP synthesis (Table 17.1).

Our findings indicated that mitochondrial ROS are generated in a strain-dependent phenomenon and are essential for chondrocytes metabolism. Tissue strains > 40% were associated with cell death, indicating a threshold effect in mechanically regulated ROS. This observation agrees with in vivo findings on cartilage deformations when the knee is undergoing physiological activities [96]. Hence, excessive cartilage deformation can, in part, lead to chondrocyte death and development of OA via overproduction of ROS. Taken together, ROS is involved in the mechanically induced maintenance of cartilage health. However, the extent and degree of its involvement in regulation of cartilage adaptation needs further elucidation.

Table 17.1 Results of oxidative stress and corresponding ATP contact of explants under subjected to mechanical stress

Treatment	% DHE positive cells (SD)	ATP in nmol/mg of tissue (SD)
Dynamic loading alone	43.11 (18.83)[a]	0.45 (0.14)[b]
Dynamic loading + Rotenone	6.09 (7.79)	0.15 (0.07)
Dynamic loading + MitoQ	4.65 (4.32)	0.04 (0.003)
No loading	1.87 (2.26)	0.12 (0.07)
Hydrostatic loading	7.98 (9.11)	0.15 (0.07)

When treated with inhibitors of oxidative stress such as rotenone and mitoQ, oxidative stress (% DHE of live cells) and ATP content (nmol/mg of tissue) decreased significantly to values similar to non-loaded controls when subjected to dynamic loading of 0.25 MPa at 0.5 Hz for 1 h. With minimal strain possibility, very low oxidative stress and ATP synthesis were observed in tissues subjected to 1 MPa of hydrostatic pressure supporting the requirement of mechanically induced oxidants to drive ATP synthesis in articular cartilage

[a] and [b] denote significant difference with $p < 0.001$

17.4.3 Relevance of Oxidant Conditioning in Cartilage by Mechanical Loading

Joint loading plays a significant role by maintaining cartilage health and function by modulating composition, histomorphology, and structural properties. During development, the mechanical environment at the joint modulates tissue biology and growth to establish the surface geometry and tissue topology [49]. In other words, growth and development increases mechanical demands on the joint, and cartilage adapts to its new state of equilibrium. This is achieved by chondrocytes partly by increasing metabolism and matrix synthesis. The process of ROS regulation in response to mechanical cues for functional adaptation is complex and not completely understood. Previous evidence indicates that mechanically induced ROS are critical to support glycolysis in cartilage. Although a threshold effect is apparent, it is reasonable to predict that sublethal levels of ROS can be generated by certain mechanical stimuli to induce enhanced biosynthetic activity, a phenomenon of mechanically induced "oxidant conditioning." Along with improved matrix synthesis, current understanding supports the theory that oxidant conditioning exerts influence on numerous cell-signaling pathways that will enhance cartilage's ability in response to increasing mechanical demands.

With the existing scientific philosophy that boasts the application of antioxidants as the definitive treatment for a myriad of oxidant damage-related diseases, it becomes imperative to prove unequivocally that oxidants are as necessary as their reducing agents. One of our earlier works laid the foundation to support the theory of oxidant conditioning in cartilage and its potential benefits in injury tolerance. Inspiration for our work was derived from in vivo effects of mechanical loading on cartilage and the phenomenon of hormetic oxidant conditioning. We hypothesized that mechanical loading is the hormetic agent, when exposed in low doses induces ROS that would deliver beneficial effects in cartilage. As a biochemical equivalent of joint loading, exogenous ROS can mimic effects of physical stimuli regulating

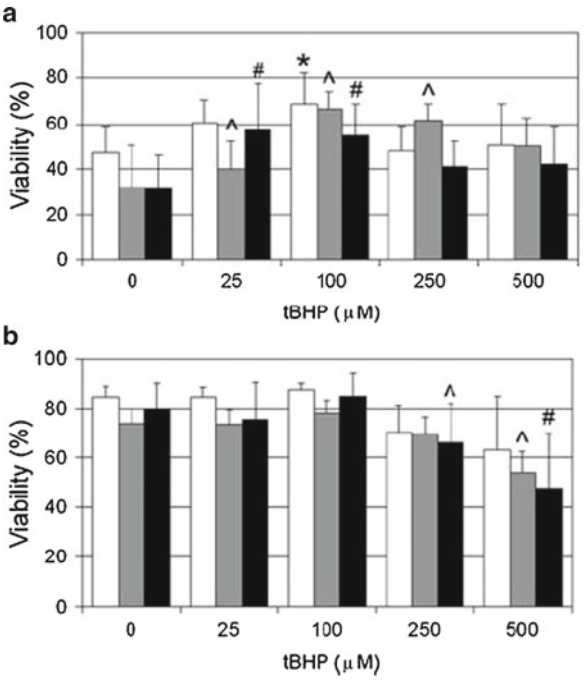

Fig. 17.3 Effects of oxidant preconditioning on chondrocyte viability. Viability was measured in compressed explants (**a**) and uncompressed explants (**b**) after treatment with the indicated doses of tBHP. Explants were treated with tBHP once (*white columns*), twice (*gray columns*), or four times (*black columns*) prior to compression. *Columns* and *bars* show means and standard deviations based on six explants. *Symbols* above the *columns* indicate significant differences vs. 0 tBHP control for 1-day treatment (*), 2 days of treatment (^), and 4 days of treatment (#) (copyright (2010) Wiley, used with permission from Prem Ramakrishnan. Oxidant conditioning protects cartilage from mechanically induced damage. Journal of Orthopaedic Research, John Wiley and Sons)

cartilage health and augment stress response. We found that preconditioning of cartilage with low doses of H_2O_2 mitigated the harmful effects of injurious mechanical compression [97]. In this study, porcine cartilage explants were treated with nonlethal concentrations (25, 100, 250, and 500 μM) tert-butyl hydrogen peroxide (tBHP) every 48 h for varying number of times (one, two, or four) prior to exposing them to unconfined axial compression (5 MPa, 1 Hz, 1,800 cycles). Our results showed that pretreatment with 100 μM tBHP mitigated compression-induced chondrocyte death (Fig. 17.3), increased glycolysis, and decreased proteoglycan losses. Gene expression analysis revealed elevations of HIF-1α, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and catalase along with suppression of matrix metalloproteinase (MMP-3) with increasing number of treatments (Fig. 17.4). These findings demonstrated that exogenous oxidants stimulate pertinent cellular signaling pathways that enhance chondrocytes' resistance to mechanical injury by boosting metabolism, antioxidant defenses, and suppression of pro-inflammatory signals. The demonstrated analogy between mechanical forces and oxidants has generated

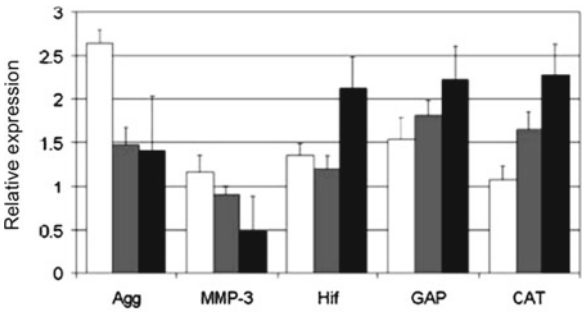

Fig. 17.4 Effects of oxidant preconditioning on gene expression. Real-time PCR analysis of gene expression is shown for explants treated with 100 μM tBHP once, twice, or four times. Expression levels for the indicated genes were normalized to untreated controls (relative expression). *Columns* and *error bars* show means and standard deviations based on four explants (copyright (2010) Wiley, used with permission from Prem Ramakrishnan. Oxidant conditioning protects cartilage from mechanically induced damage. Journal of Orthopaedic Research, John Wiley and Sons)

enthusiasm in the OA research community, and current efforts are geared towards leveraging the effect of oxidants on cartilage functional adaptation for development of therapeutic modalities.

17.5 Potential Pathways Involved in Oxidant Conditioning

Based on evidences of our study and review of current literature, we have identified prospective pathways that may have the most significant role in chondrocyte oxidant conditioning and functional adaptation. Current literature on redox balance and cell signaling is quite expansive, and literature reviews are available for more comprehensive understanding on this subject [3, 98, 99].

17.5.1 Nuclear Factor-κB Signaling

Transcription factor NF-κB is among the most important transcription factors shown to respond directly to oxidative stress [100–102]. NF-κB was originally recognized in regulating gene expression in B cell lymphocytes [103]. Activation and regulation of the NF-κB/Rel transcription family requires nuclear translocation of complexes, and this process plays the pivotal role in regulation of genes involved in encoding pro-inflammatory cytokines and other inflammatory mediators [104, 105]. In chondrocytes, NF-κB has been shown to play a central role in cartilage degeneration and arthritis development [106]. Treatment with antioxidants inhibits the activation of NF-κB downstream signaling by ROS [107, 108] possibly by scavenging oxidants and stabilizing the association between IκB and NF-κB heterodimeric

complex. Because NF-κB can be rapidly induced in a variety of cell types by a diverse set of seemingly unrelated agents, it has been proposed that agents activating this transcription factor do so by increasing a minimum intracellular effective oxidative stress threshold [101, 107]. In smooth muscle cells, ROS upregulated A1 adenosine receptor (A1AR) via an NF-κB pathway, leading to a cytoprotective role [100]. Preconditioning with xanthine oxidase and $FeSO_4$ induced neuroprotection via NF-κB [109]. Increase in the protein expression of superoxide dismutase (SOD-2) after xanthine oxidase preconditioning but not by $FeSO_4$ suggests that superoxides and peroxides may have a role in NF-κB activation and subsequent transcription of SOD-2. Protective effects of NF-κB activation have not been clearly defined yet in cartilage physiology, although oxidant preconditioning seems to, at least in part, suppress catabolic effects of some cytokines via the NF-κB pathway[110, 111].

17.5.2 HIF-1α Signaling

HIF-1α is an important transcription factor that is a crucial regulatory element in sensing hypoxic conditions and response by modulating gene expression of oxygen-sensitive enzymes and their cofactors [25, 112]. HIF-1α is constitutively expressed in cells but is rapidly degraded by the ubiquitin–proteasome system which is regulated by the intracellular redox state and is activated by elevated levels of ROS. In general, degradation of HIF-1α prevails under normoxia, whereas hypoxia inhibits the ubiquitin pathway, resulting in upregulation of HIF-1α expression [113]. Although unclear on a mechanistic standpoint, it is widely believed that activation of HIF-1α relies on the availability of oxidants. This was observed when HIF-1α stabilization and nuclear translocation were achieved in a non-hypoxic but cytokine-mediated process [114–118]. ROS-induced HIF-1α stabilization and activation was observed to be dependent on tumor necrosis factor (TNF-α) expression in normoxic conditions. Antioxidants and inhibitors of free radical sources such as NADPH oxidase and mitochondrial complex I attenuated TNF-α-induced HIF-1α activation. Posttranslational stability, nuclear translocation, and gene expression of HIF-1alpha are regulated by peroxide generation via an NADPH oxidase-type activity [119]. Expression of NO synthase could cause HIF-1α accumulation, thus underscoring the role of NO as an intracellular activator of this transcription factor [120]. HIF-1α has direct influence on genes that encode erythropoietin (EPO), vascular endothelial growth factor (VEGF), glycolytic enzymes, and glucose transporters (GLUT), as well as cytokines and other inflammatory mediators [119]. In chondrocytes, HIF-1α has been found to be necessary for chondrocyte viability, metabolism, and matrix production [121–123]. Stabilization and activation of HIF-1α in hypoxia is essential for metabolic homeostasis in chondrocytes [124]. As in many other cell types, non-hypoxic stresses such as mechanical stimuli, oxidants, and cytokines are known modulators of HIF-1α [123, 125, 126]. A major process that chondrocytes potentially benefit from oxidant conditioning would be through expression and activation

of glucose transporters and glycolytic enzymes via the HIF-1α. In support of this claim, HIF-1α enhances GLUT-mediated dehydroascorbate transport in chondrocytes that may increase intracellular ascorbate levels, support matrix production, and supplement intracellular antioxidant defenses [127]. ROS-mediated VEGF [128] and EPO[129–131] expressions are a few notable mechanisms of interest, but their potential roles in oxidant conditioning in cartilage pathology need further elucidation.

17.5.3 Transcription of Antioxidant Defenses

Elevation of intracellular antioxidant capacity by upregulation of antioxidant genes is critical for adaptation to oxidative stress and protection against oxidative damage. One interesting transcription factor called redox factor 1 (Ref-1) was shown to be upregulated by genotoxic agents and oxidants, such as bleomycin and H_2O_2, and as a result protected cells from DNA and oxidative damage [132]. Nuclear translocation of cytoplasmic Ref-1 is observed under oxidative stress conditions [132, 133]. In addition, Ref-1 modulates co-transcriptional activity by modulating genes expression of NF-κB, p53, and HIF-1α [134], eliciting its oxidant-induced cellular defense function. Another transcription factor called antioxidant responsive element (ARE) activates a variety of antioxidant genes in response to ROS via an NF-κB independent pathway [135–137]. In chondrocytes, oxidative stress was observed to regulate the transcription of ARE, providing some basis for its influence on antioxidant defense and anti-apoptosis regulation [138]. Genetic knockout of an ARE-related factor resulted in severe arthritis in an antibody-induced arthritis model, indicating its protective role in joint disease conditions [139]. Antioxidant levels are regulated by ROS through multiple initiators such as growth factors/cytokines [140], mechanical forces [97, 141], and other exogenous sources [136, 142]. The regulation of Ref-1 and ARE may be significant in mediating adaptive responses in cartilage's effort against elevated oxidative stress by promoting both DNA repair and redox activation of key transcription factors.

17.5.4 Other Potential Signaling Mechanisms

Phosphoinositide 3-kinase (PI3K) pathway plays a key role in cell survival and proliferation in response to stimulation by growth factors, hormones, and cytokines. Stimulation of PI3K was shown to elicit protective effects by abrogating oxidative-stress-induced premature senescence [143] and apoptosis [144] in chondrocytes. Mitogen-activated protein kinases (MAPK) contribute significantly in various cellular processes and are activated by numerous signals such as growth factors, hormones, and cytokines, in addition to genotoxic and oxidative stressors [145]. Apoptosis signal-regulated kinase 1 (ASK1), a member of the MAPK family, regu-

lates the JNK and p38 MAPK pathways leading to apoptosis [146] under oxidative stress but can activate survival pathways in certain conditions [147]. Mechanical injury and damage-associated fragments caused chondrocyte death and proteoglycan loss in cartilage that were mediated by MAPK signaling [148]. Considered as an apoptosis initiator under high levels of oxidative stress, P53 transcription under low levels of NO preconditions chondrocytes' defense mechanism against fatal doses of ROS by elevating hemo-oxygenase-1 and NF-κB expression [149]. NO-induced heat-shock proteins (especially HSP70) are demonstrated as cytoprotective in chondrocytes and are modulated by NO expression [150, 151].

17.6 Summary and Conclusions

Functional adaptation is observed in every organism throughout its lifetime. This phenomenon is observed as a response not only to normal growth and development of an organism but also to various environmental stressors. This essential feature is critical and will impart resistance to a variety of internal and external stresses that would otherwise be quite detrimental for the organism's survival. While universal acceptance prevails that oxidative stress is a causative agent of the process of aging and several degenerative diseases, their role as an essential regulator of homeostasis, growth, and remodeling is quite conclusive, and mechanisms is becoming quite apparent. Currently, there is growing support to the theory that certain levels of oxidative stress increase resistance to future stresses. Oxidants are important initiators and intermediaries to multiple signaling pathways critical for cartilage function. Although the pervading mechanical environment seems to be the primary regulator of ROS, the mechanism by which regulation is induced is unclear and requires further investigation.

Cartilage is a tissue that exhibits considerable structural, biological, and mechanical inhomogeneities. At a cellular level, these regional differences provide a very unique environment for resident chondrocytes, depending on their relative location within the articular cartilage. In other words, a chondrocyte at the superficial zone does not experience a similar environment as a chondrocyte in the middle or the deep zone. Hence, it is undeniable that chondrocyte response to stimuli would also be dictated by its local environments. ROS production in response to depth-dependent response in chondrocytes can occur, and this cannot be ruled out based on the findings discussed here. It is still not clear whether cells at the different regions would elicit the same response when provided with similar stimulus. Currently, the definition of oxidant conditioning is speculative, but great strides are being made in closing the gap in our knowledge of oxidants and their beneficial roles in joint pathology.

Antioxidants are part of an ensemble of potential therapeutic interventions for numerous disease pathologies including OA. Antioxidants are also considered as the elixir of youth, touted to potentially slow or even reverse the process of aging. While their benefits are undeniable in some cases, the potential long-term effects of abrogation and continued suppression of oxidative stress remain unanswered.

Oxidant conditioning is a relevant and demonstrable phenomenon in cartilage physiology as in a variety of other tissues in the human body. We discussed the role of ROS as regulators of discrete pathways that are potentially responsible for cartilage's stress responsiveness and adaptation to increased mechanical demands. Evidences supporting the contribution of ROS in aggravating cartilage pathology are also apparent, fuelling the current excitement for novel therapies with the focus of counteracting the deleterious effects of oxidative damage. With all evidences taken together, it is reasonable to conclude that our current understanding of oxidants in physiology is far from complete. Although the "duality" exhibited by ROS seems to be primarily dose dependent, it is quite possible that their mechanisms of action are the product of a myriad of factors. Better understanding of mechanisms that regulate redox balance in cartilage is warranted so that clinical applications targeting oxidative stress could be employed with the least probability of undesired outcomes. As such, oxidants are essential for cartilage adaptation, but the jury is still out on the question of "how much is too much."

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Chapter 18
The Role of Sirtuins in Arthritis

Diego Kyburz and Fabienne Niederer

Abstract Sirtuins (SIRTs) are a family of histone deacetylases, targeting a multitude of histone but also nonhistone proteins. Via a nicotinamide adenine dinucleotide-dependent deacetylation reaction, sirtuins regulate the activity of transcription factors important for cell proliferation, apoptosis, and cell metabolism. In particular, the finding that SIRT1 mediates the life-prolonging effects of calorie restriction has highlighted the crucial role of sirtuins in cellular homeostasis. In addition, sirtuins have been shown to affect the immune system by targeting transcription factors such as nuclear factor-κB (NFκB). Effects on inflammation have been demonstrated in SIRT1-deficient mice which show increased transcription of proinflammatory cytokines and display a systemic lupus erythematosus-like phenotype. Data on the role of sirtuins in arthritis are only beginning to emerge. In this review, we summarize recent findings on the expression and function of sirtuins in rheumatoid arthritis and osteoarthritis.

Abbreviations

AP-1	Activator protein 1
BMDM	Bone marrow-derived macrophages
cAMP	Cyclic adenosine monophosphate
ChIP	Chromosome immunoprecipitation
eNOS	Endothelial nitric oxide synthase
FOXO	Forkhead box type O
HDAC	Histone deacetylases
HDMEC	Human dermal microvascular endothelial cells

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HIF-1α	Hypoxia-inducible factor 1 α
HSF-1	Heat shock factor protein 1
IGFR	Insulin-like growth factor receptor
IL	Interleukin
LPS	Lipopolysaccharide
MCP	Monocyte chemoattractant protein
MMP	Matrix metalloproteinases
NaBy	Sodium butyrate
NAD+	Nicotinamide adenine dinucleotide
NAM	Nicotinamide
NFκB	Nuclear factor "kappa-light-chain-enhancer" of activated B cells
OA	Osteoarthritis
OSM	Oncostatin M
PARP-1	Poly(ADP-ribose)-polymerase 1
PGC-1α	PPARγ coactivator 1 α
PPARγ	Peroxisome proliferator-activated receptor γ
PTP1B	Protein tyrosine phosphatase 1B
RA	Rheumatoid arthritis
Rantes	Regulated on activation normal T cell expressed and secreted
SIRT	Sirtuin
TNF-α	Tumor necrosis factor α
TSA	Trichostatin A

18.1 Introduction

Research on aging in the last years has led to the discovery of genes and pathways that regulate longevity. Mutations in the yeast sir2 gene were found to be associated with the replicative life span of yeast cells [1, 2]. Subsequently, orthologues of yeast Sir2 were described in other species including mammals [3]. The finding that sirtuins slowed aging in mice sparked the interest in this gene family as targets for the development of antiaging drugs [4]. Seven different sirtuin genes have been identified in mammals, SIRT1 to SIRT7, of which SIRT1 is the best characterized. In particular, SIRT1 has been reported to mediate the life-prolonging effect of calorie restriction by the polyphenol resveratrol [5]. Sirtuins are nicotinamide adenine dinucleotide (NAD+)-dependent histone deacetylases (HDAC). HDAC are enzymes inhibiting gene expression by reversing acetylation of histone proteins. Within the HDAC family, the sirtuins constitute an own class, the class III HDAC. In contrast to the class I and II HDAC, which use zinc as cofactor and are inhibited by trichostatin A (TSA), the sirtuins are not TSA sensitive and catalyze protein deacetylation in a reaction that uses NAD+. SIRT1–3 and SIRT5 catalyze a deacetylation reaction of lysine residues whereby NAD+ is used as a cofactor and nicotinamide (NAM) is released with 2′-*O*-acetyl-ADP-ribose (Fig. 18.1). In contrast, SIRTs 4 and 6 catalyze an ADP-ribosylation reaction,

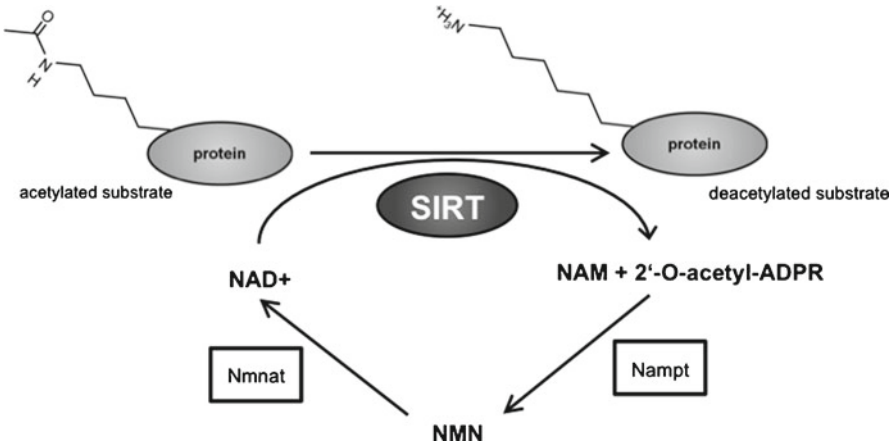

Fig. 18.1 Deacetylase reaction catalyzed by sirtuins. An acetyl group from a target protein is transferred to nicotinamide adenine dinucleotide (NAD+). Nicotinamide (NAM) and a 2′-*O*-acetyl-ADP-ribose (ADPR) are formed. In two steps, requiring nicotinamide phosphoribosyltransferase (Nampt) and nicotinamide nucleotide adenylyltransferase (Nmnat), NAD+ is resynthesized

whereas the substrate for SIRT7 is unknown [6, 7]. However, sirtuins not only target histones but also nonhistone proteins, thereby modulating key cellular functions in metabolism and stress response.

In addition to their effects on cell proliferation, survival, and metabolism, sirtuins also affect central signaling pathways of the immune system by targeting nuclear factor-κB (NFκB) and activator protein 1 (AP-1) [8–10]. SIRT1-deficient mice were found to develop signs of autoimmunity reminiscent of systemic lupus erythematosus [11, 12], suggesting that sirtuins may be involved in the pathogenesis of autoimmune diseases. So far, the data on the role of sirtuins in the development of arthritis is still limited. The observation of a beneficial effect of the sirtuin-activator resveratrol in a rabbit model of inflammatory arthritis has raised the interest in the sirtuin family as modulators of joint inflammation and possible therapeutic targets [13]. In this review, the emerging data on the role of sirtuins in rheumatoid arthritis and osteoarthritis is summarized.

18.2 Sirtuin Expression and Signal Transduction

18.2.1 Expression of Sirtuins

Although SIRTs are all different from each other, the seven mammalian sirtuins share a common characteristic as they require NAD+ as an essential cofactor [14]. Thus, they all carry a highly conserved NAD+-binding domain. However, the individual family members have distinct flanking N- and C-terminal extensions. These

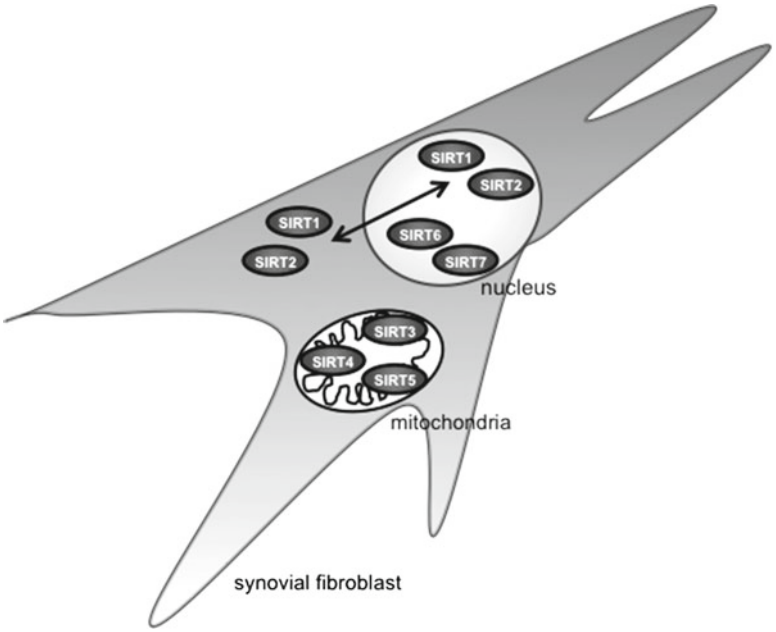

Fig. 18.2 Subcellular location of individual SIRTs in mammalian cells. SIRT1, SIRT6, and SIRT7 are mainly found in the nucleus, while SIRT2 is located to the cytoplasm of mammalian cells, though SIRT1 and 2 have been reported to shuttle between these two compartments. The mitochondria contain the sirtuins 3, 4, and 5

divergent N- and C-termini are responsible for their variation in binding partners, substrates, and subcellular localization. Thus, SIRT1 and SIRT6 are found in the nucleus, SIRT2 is located in the cytoplasm, SIRT3–5 in the mitochondria, and SIRT7 in the nucleolus. However, recent data suggest trafficking of individual SIRTs between different subcellular compartments. SIRT1 carries two nuclear localization signals and two nuclear export signals. It has been shown that the nucleus-to-cytoplasm transfer is a regulatory mechanism of the activity of SIRT1. Similarly, SIRT2 was found to shuttle between the cytoplasm and the nucleus [15] (Fig. 18.2).

In addition to the differences in their subcellular localization, the sirtuins are also expressed in varying amounts in different tissues. SIRT1 is highly expressed in several brain regions and has also been found in heart, kidney, liver, pancreas, skeletal muscle, spleen, and white adipose tissue. SIRT2 is reported to be the most abundant sirtuin in adipose tissue but is also found in the brain and the nervous system. The mitochondrial SIRT3 is highly expressed in metabolically active tissues such as brown adipose tissue, muscle, liver, kidney, heart, and brain [14]. SIRT4 was reported to be expressed in islets of Langerhans where it colocalizes with insulin-expressing β-cells [16]. Both SIRT5 and SIRT6 were identified to be ubiquitously expressed in various tissues. Finally, high expression levels of SIRT7 were found in adipocytes and cardiomyocytes [17].

18.2.2 Signal Transduction of Sirtuins

Sirtuins act primarily by the hydrolytic removal of an acetyl group from a lysine residue within proteins in the presence of NAD+. The acetyl group is then transferred to the ADP-ribose component of NAD+ to generate 2′-*O*-acetyl-ADP-ribose and nicotinamide (NAM). Removal of a lysine acetyl group confers a positive charge to this amino acid, resulting in a tight binding to the negatively charged phosphate backbone of the DNA. Such dense chromatin structure provides only little space for transcription factors. Thus, the protein biosynthesis rate is ultimately reduced upon deacetylation. As a consequence, sirtuins mainly silence gene expression. However, although sirtuins belong to the HDAC family, these molecules do not exclusively target histones. An increasing number of nonhistone substrates of SIRTs have been identified. Most of the substrates consist of acetylated protein except for SIRT4 and SIRT6, which exert ADP-ribosyltransferase activity instead of catalyzing a deacetylation reaction [14].

18.2.3 SIRT1 Buffers Stress-Induced Damage in Different Tissues

SIRT1 is emerging as a master regulator of metabolic and stress responses with several critical downstream targets. Many factors controlling cell proliferation and apoptosis as well as metabolism have been identified as sirtuin substrates (Table 18.1). Among them are the tumor suppressor protein p53 [18–20], the forkhead box type O transcription factors (FOXO) family [21–23], and the peroxisome proliferator-activated receptor γ (PPARγ) coactivator 1α (PGC-1α), a master regulator of glucose metabolism [24, 25]. The relevance of the sirtuins for control of metabolism has been underlined by the finding that overexpression of SIRT1 in transgenic mice may prevent diabetes induced by high-fat diet or even in the course of normal aging [26, 27]. SIRT1 also has protective properties on cardiovascular function. SIRT1 inhibits senescence of endothelial cells [28], activates endothelial nitric oxide synthase (eNOS) [29], and inhibits macrophage foam cell formation [30]. Transgenic mice overexpressing SIRT1 in myocytes are protected against cardiac hypertrophy [31]. Besides cardiovascular diseases, sirtuins are of importance for the development of neurodegenerative diseases, which are increasingly common as humans are living longer. The generation of β-amyloid peptide by cleavage of amyloid precursor protein was reduced in the brains of mice overexpressing SIRT1 [32]. β-amyloid peptide is the main component of the plaques found in the brain of Alzheimer's disease patients. Remarkably, the number of plaques in mice overexpressing β-amyloid was significantly reduced when they overexpressed SIRT1 at the same time. Moreover, SIRT1 was shown to be able to reduce the formation of tangles of tau protein in neurons of mice, another characteristic finding in Alzheimer's disease [33].

Table 18.1 Selected pathways regulated by SIRT1

Target of SIRT1	Tissue	Related disease
p53	Ubiquitous	Cancer
FOXO	Ubiquitous, adipose tissue	Metabolic diseases
PGC-1α	Ubiquitous, adipose tissue	Diabetes
eNOS	Heart, liver	Cardiovascular diseases
Alpha-secretase	Brain	Alzheimer's disease
Ku70, PARP1	Ubiquitous	Cancer
NFκB	Ubiquitous	Inflammatory diseases
AP-1	Ubiquitous	Inflammatory diseases

Furthermore, SIRT1 is also involved in the regulation of DNA repair mechanisms via regulation of Ku70 [34] and poly(ADP-ribose)-polymerase 1 (PARP1), the circadian clock modulation via BMAL1 and PER2 [35, 36], and general stress responses via targeting hypoxia-inducible factor 1α (HIF-1α), heat shock factor protein 1 (HSF-1), and p53 [37]. Of note, through the suppression of p53 function, SIRT1 may act as an oncogene. Increased levels of SIRT1 are found in several human tumors and are followed by a decrease in the active p53, leading to genome instability and resistance to apoptosis [19, 20]. Similarly, deacetylation of the DNA repair factor Ku70 by SIRT1 leads to inhibition of apoptotic pathways. Contrary to these findings, SIRT1 deletion was shown to be associated with decreased TNF-α-induced apoptosis of bone marrow-derived macrophages (BMDM) *in vitro*. The antiapoptotic effects of myeloid SIRT1 deletion were proposed to be due to the NFκB hyperactivity, as it had been previously reported that NFκB has antiapoptotic effects [38]. Similarly, in HEK293 epithelial cells, SIRT1 augmented apoptosis in response to tumor necrosis factor (TNF)-α [8], highlighting that SIRT1 might have different biological outcomes depending on the apoptotic stimuli and on the different cell types.

In addition to the effects on cell proliferation, metabolism, and apoptosis, SIRT1 was shown to physically interact with NFκB, a crucial transcription factor involved in the regulation of inflammatory responses [8]. Upon deacetylation of the RelA/p65 subunit, the interaction of NFκB with promoter regions of target genes was inhibited. Further, SIRT1 has been demonstrated to directly interact with c-Fos and c-Jun, the major components of AP-1, thus inhibiting the transcriptional activity of AP-1 [9, 10]. It is of no surprise that impaired SIRT1 function was found to result in an increased proinflammatory activity. SIRT1-deficient mice show a lupus-like phenotype with antinuclear antibodies in the serum and deposition of IgM and IgG immune complexes in liver and kidney [11, 12]. Macrophages from myeloid SIRT1 knockout mice displayed increased NFκB-mediated inflammation in response to environmental stress, indicating an anti-inflammatory activity of SIRT1 [39]. Interestingly, when these mice with myeloid-specific SIRT1 deletion were fed a high-fat diet, serum levels of the proinflammatory cytokines interleukin (IL)-6, TNF-α, IL-12, and monocyte chemoattractant protein (CCL)-1 were increased, suggesting a chronic inflammatory state.

In agreement, Pfluger et al. found that mice moderately overexpressing SIRT1 under its natural promoter showed a better glucose tolerance under a high-fat diet and were protected from the development of liver steatosis. This protective effect of SIRT1 was accompanied by lower levels of IL-6 and TNF-α in the liver [27]. Embryonic fibroblasts of SIRT1 transgenic mice showed a decreased NFκB activation after stimulation with TNF-α, suggesting an inhibitory effect of SIRT1 on NFκB. However, published data on the effects of SIRT1 on inflammation are controversial. Inhibition of SIRT1 was shown to attenuate antigen-induced airway inflammation and hyperresponsiveness [40]. Similarly, in human dermal microvascular endothelial cells, SIRT1 inhibition decreased the inflammatory response to stimulation with TNF-α, documenting that depending on the cellular systems, SIRT1 may also have proinflammatory effects [41].

18.2.4 Downstream Targets of Other SIRTs

Not so extensive research has been conducted on sirtuins other than SIRT1. Therefore, it still remains much to be learnt about the proteins targeted by those HDACs.

SIRT2 has been mainly implicated in cytoskeletal organization by targeting structural proteins like α-tubulin [42]. Furthermore, SIRT2 is considered to be involved in cell cycle regulation under stress conditions. Whereas under normal conditions SIRT2 did not affect cell cycle progression, overexpression of SIRT2 delayed mitotic exit in response to stress [43].

SIRT2 appears to target similar molecules to those under SIRT1 control. Like SIRT1, SIRT2 inhibits adipogenesis through the deacetylation of FOXO transcription factors [44]. In addition, SIRT2 was reported to interact and regulate the tumor suppressor gene p53, the p300 deacetylase, and the PPARγ [17].

The mitochondrial sirtuins SIRT3, 4, and 5 appear to regulate metabolic functions such as the synthesis of ATP or the urea cycle [45–47].

In the absence of SIRT6, mice displayed severe developmental defects and suffered from premature aging [48]. In addition, SIRT6 was found to regulate the production of TNF-α [49], indicating that SIRT6 might influence similar pathways as SIRT1. In the nucleolus, SIRT7 interacts with RNA polymerase I and, thus, is involved in the regulation of cell growth and metabolism by driving ribosome biogenesis [50].

18.3 Sirtuins in Rheumatoid Arthritis

No information on the expression and function of sirtuins in rheumatoid arthritis (RA) was available until very recently. Studies in animal models of arthritis have shown beneficial effects of HDAC inhibitors; however, the inhibitors used were not targeting the sirtuin family members [51–54]. A remarkable publication by Grabiec et al.

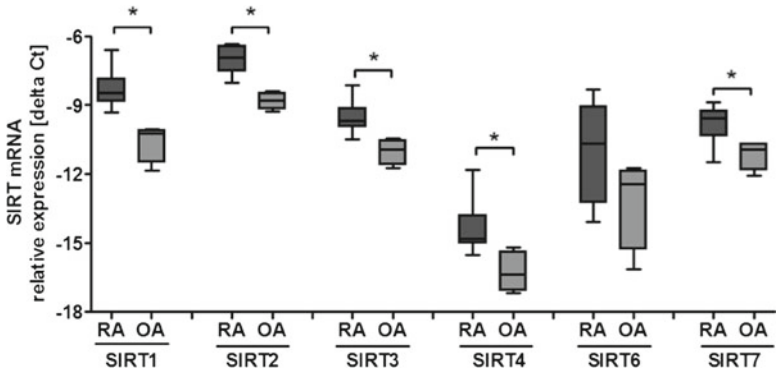

Fig. 18.3 Expression of SIRT mRNA in synovial fibroblasts from patients with RA and OA. RT-PCR showing relative expression of SIRT mRNA in synovial fibroblasts from patients with RA and OA. Results are presented as the difference in threshold cycle (delta Ct), relative to the endogenous control 18S rRNA. *$p < 0.05$, unpaired *t*-test

assessed the effects of various HDAC inhibitors in monocyte-derived macrophages and synovial explants of patients with RA [55]. Among other HDAC inhibitors, they used NAM, a nonspecific inhibitor of sirtuins. Addition of NAM to cultured macrophages of healthy individuals reduced LPS- and TNF-α-induced production of IL-6 and LPS-induced TNF-α production. The same results were found with synovial fluid macrophages from patients with RA. Nicotinamide dose-dependently reduced the TNF-α-induced production of IL-6 and IL-8 as well as the LPS-stimulated IL-6 production. The baseline expression levels of IL-6, IL-8, and TNF-α in unstimulated cells were however not affected by NAM treatment. Consistent with the data obtained in isolated synovial fluid macrophages, treatment of whole tissue synovial explants from RA patients with NAM resulted in decreased production of a variety of cytokines, including IL-6 and IL-8 as well as several chemokines. Interestingly, the effects of NAM were not restricted to proinflammatory cytokines, as IL-10 was also found to be reduced in the explants. The findings of Grabiec et al. were surprising, as the data obtained with SIRT1-deficient or SIRT1 transgenic mice rather suggested that SIRT1 has antiinflammatory effects by inhibiting NFκB activation. Since NAM, the molecule used in these studies, is a nonspecific inhibitor, the findings could be due to effects on the other members of the sirtuin family.

A recent study from our own laboratory has analyzed the specific impact of SIRT1 on the inflammatory response of cultured synovial cells of patients with RA [56]. To analyze the expression in RA synovial cells, we performed an expression profile of sirtuins in synovial fibroblasts from patients with RA and noninflammatory osteoarthritis (OA) (Fig. 18.3). Interestingly, SIRT1–4 and SIRT7 were found overexpressed in RA synovial tissue as compared to OA tissue. Synovial fibroblasts as well as macrophages expressed the SIRT1 protein as shown by immunohistochemistry double stainings. TNF-α was shown to induce SIRT1 expression in cultured synovial fibroblasts as well as in monocytes. Consistent with previous reports in

other cellular systems [57, 58], SIRT1 had an antiapoptotic effect on synovial fibroblasts. Knockdown of SIRT1 with specific siRNA resulted in a significantly increased rate of spontaneous apoptosis. In contrast, transfection of synovial fibroblasts with a vector encoding SIRT1 led to a further decrease of spontaneous apoptosis. Surprisingly, SIRT1 inhibition by siRNA reduced the LPS-induced production of the proinflammatory cytokines IL-6 and IL-8 by synovial fibroblasts. Similarly, LPS-induced TNF-α production was lower in monocytes transfected with SIRT1 siRNA. The same effect was found when synovial fibroblasts were treated with the small molecular SIRT1-specific inhibitor EX-527. The inhibition of LPS-induced IL-6 production was also seen with the pan-sirtuin inhibitor sirtinol. These result suggested that the overall effect of sirtuins is proinflammatory in synovial cells and argues against other members of the sirtuin family that may counteract SIRT1. The molecular mechanisms involved in these proinflammatory effects of SIRT1 in RA synovial fibroblasts and monocytes are not clearly delineated. It was however shown that the increase in LPS-induced TNF-α by overexpression of SIRT1 was abrogated by NFκB inhibition, arguing for an NFκB-dependent mechanism. Collectively, the data in synovial cells of RA patients show that SIRT1 promotes the production of proinflammatory cytokines directly and additionally maintains inflammation by an antiapoptotic effect on synovial cells.

Additional data have very recently been published supporting a proinflammatory role for SIRT1. Fernandes et al. have used J774 macrophages to study the effect of the sirtuin inhibitors sirtinol and cambinol. They found significantly decreased LPS-induced expression of IL-6, TNF-α, and the chemokine Rantes [59]. Interestingly, they observed decreased IκBα phosphorylation with increasing concentrations of cambinol, suggesting that sirtuin inhibitors act via inhibiting NFκB activation.

Further support for a proinflammatory role of SIRT1 has been provided by a study of primary human dermal microvascular endothelial cells (HDMEC). Treatment of these cells *in vitro* with the sirtuin inhibitor sirtinol led to a decrease in the expression of adhesion molecules as well as to a reduction in the production of the chemokines CXCL10 and CCL2. Consequently, the adhesion of monocytes to HDMEC was significantly reduced. By the use of SIRT1- and SIRT2-specific inhibitors, it was shown that the modulation of adhesion molecule expression was predominantly controlled by SIRT1. The authors concluded that SIRT1 contributed to skin inflammation and proposed SIRT1 as a therapeutic target in skin diseases such as psoriasis [41].

As the polyphenol resveratrol is mediating its effects partly via SIRT1, studies of resveratrol in synovial cells may be of relevance for understanding the role of SIRTs in RA. Byun et al. have shown that treatment of synovial fibroblasts derived from patients with RA with resveratrol induces apoptosis. This apoptosis induction was independent of p53 but required activation of caspase 8 [60]. Another group has confirmed the apoptosis-inducing effect of resveratrol in a human RA synovial cell line. In this cell line, apoptosis induction by resveratrol was shown to be dependent on SIRT1 as cell viability remained high in presence of the sirtuin inhibitor sirtinol. However, apoptosis was also inhibited by TSA, an inhibitor of class I and II HDAC, suggesting that other mechanisms could be involved. In contrast to the

previous study with primary synovial fibroblasts, resveratrol did not activate caspase 8 but caspases 9 and 3 in a SIRT1-dependent manner [61]. These results suggest that SIRT1 has a proapoptotic effect, which contrasts with the inhibitory effects of SIRT1 overexpression on spontaneous apoptosis of synovial fibroblasts reported earlier [56]. Resveratrol-induced apoptosis may however be distinct from spontaneous or TNF-α-induced apoptosis of synovial cells. In addition, it was recently shown that resveratrol does not directly target SIRT1 but rather enhances SIRT1 activity indirectly by inhibiting cAMP phosphodiesterases and increasing NAD+ [62]. Therefore, the effects of resveratrol on apoptosis may only partially reflect SIRT1 activity.

There are so far no data available regarding the function of SIRT2–7 in RA.

18.4 Sirtuins in Osteoarthritis

Osteoarthritis is characterized by a disturbed cartilage homeostasis. The expression of cartilage-specific genes is important for the maintenance of a normal chondrocyte phenotype. The HDAC inhibitors control the expression of cartilage-specific genes. The HDAC inhibitors TSA and sodium butyrate (NaBy) were shown to decrease cartilage resorption induced by IL-1β and oncostatin M (OSM). Cultured bovine nasal explant cultures treated with IL-1β and OSM released lower amounts of proteoglycans and collagens when exposed to TSA or NaBy. Both HDAC inhibitors reduced the activity of collagenases. In cultured primary human articular chondrocytes, TSA repressed the induction of the matrix metalloproteinases MMP-1 and MMP-13 by IL-1β and OSM. Since MMP are key enzymes involved in cartilage degradation, these studies suggested an important regulatory role for HDAC in osteoarthritis.

SIRT1 was shown to be expressed in primary human chondrocytes. Immunofluorescence staining revealed a nuclear localization. The expression levels were reduced under nutritional, metabolic, and catabolic stress [58]. In line with these findings, SIRT1 expression was found to be reduced in OA chondrocytes as compared to chondrocytes from normal individuals. The first study on the role of sirtuins in OA was published in 2008 by Dvir-Ginzberg et al. The authors reported that, in contrast to class I HDAC, SIRT1 did not repress cartilage-specific gene expression but rather increased it. Interestingly, transfection of primary chondrocytes with a dominant negative mutant form of SIRT1 decreased the expression of cartilage-specific genes, suggesting that constitutive expression was enhanced by SIRT1. Chromosome immunoprecipitation (ChIP) analysis revealed that SIRT1 associates with the transcription factor Sox9. SIRT1 deacetylates Sox9 in a NAD+-dependent fashion, thereby enhancing transactivation of the collagen II (a1) promoter. Targeting of SIRT1 to the promoter and enhancer of collagen II (a1) led to recruitment of the coactivators GCN5, PGC-1α, and p300, resulting in an increased gene transcription [63].

In the same fashion of other cell types, SIRT1 was shown to have antiapoptotic properties in chondrocytes. Treatment of primary human articular chondrocytes with

the sirtuin inhibitors sirtinol and nicotinamide increased spontaneous apoptosis, and knockdown of SIRT1 with siRNA significantly increased nitric oxide induced apoptosis. Levels of Bax were found increased, whereas Bcl-2 was decreased, in siSIRT1-treated chondrocytes [58]. Evidence has been presented that SIRT1 activates the insulin-like growth factor receptor (IGFR) pathway. SIRT1 thereby activates Akt, leading to phosphorylation of MDM2, a protein that binds to and inhibits the proapoptotic protein p53. In addition, it was shown that SIRT1 represses protein tyrosine phosphatase 1B (PTP1B), a proapoptotic protein. Overexpression of PTP1B, but not of an enzymatically inactive mutant, in human chondrocytes resulted in a drastic increase of apoptosis, while levels of phosphorylated IGFR were reduced. These data confirmed PTP1B as a proapoptotic protein targeting the IGFR pathway in chondrocytes. Interestingly, SIRT1 and PTP1B showed an inversely correlated expression pattern in normal and OA articular cartilage. Considering the fact that chondrocyte death is increased in OA, overexpression of PTP1B may contribute to the pathogenesis of OA [64].

As regards the reduced levels of SIRT1 in OA cartilage, it has recently been reported that TNF-α leads to a decreased SIRT1 activity in chondrocytes. In contrast, SIRT1 mRNA was at the same time increased. Immunoblot analysis of TNF-α-stimulated chondrocytes revealed that SIRT1 was present in two forms: the full-length 110-kDa SIRT1 and a short 75-kDa protein. It was shown that its cleavage was dependent on cathepsin B. SIRT1 activity following TNF-α stimulation of cultured chondrocytes could be restored by adding a cathepsin B antagonist [65]. This study suggests that the proinflammatory cytokine TNF-α reduces SIRT1 activity by enzymatic cleavage and thereby inhibits the expression of cartilage-specific genes.

For a long time, it has been known that cathepsin B levels are increased in OA as compared to normal cartilage [66]. In a follow-up study, the same authors have shown that the addition of OA synovial fluid to *in vitro* cultured chondrocytes led to the formation of 75-kDa SIRT1, suggesting that the synovial fluid contained proinflammatory cytokines inducing cathepsin B. It was found that the inactive 75-kDa SIRT1 was resistant to further degradation and accumulated in the cytoplasm, colocalizing with the mitochondrial membrane [67]. Inhibition of the nuclear export of 75-kDa SIRT1 led to markedly increased apoptosis rates in TNF-α-treated chondrocytes. As 75-kDa SIRT1 associates with cytochrome c, they proposed that 75-kDa SIRT1 may protect cells from TNF-induced apoptosis by binding cytochrome c, thereby blocking apoptosome formation [68]. Such an effect could add to the well-documented antiapoptotic activity of full-length SIRT1.

Studies of cartilage in mice revealed that SIRT1 expression decreases over time with no detectable SIRT1 at 9 months of age, while the inactive form 75-kDa SIRT1 was well detectable at this age. In contrast, SIRT1 heterozygous mice had decreased levels of 75-kDa SIRT1 and no detectable full-length SIRT1. In correlation with these results, chondrocyte death as assessed by staining for cleaved caspase 3 was markedly increased in heterozygous mice as compared to wild-type mice. Cartilage staining for aggrecan and collagen 2a1 was decreased in heterozygous mice and OA scores higher than in wild-type mice. These results suggested

that with age, and possibly under the influence of inflammatory cytokines, SIRT1 is cleaved to a short 75-kDa SIRT1, which may have a protective effect on chondrocyte apoptosis but may not protect from cartilage degradation caused by decreased production of extracellular matrix proteins, being necessary for this function the presence of full-length SIRT1 [69].

18.5 Summary and Conclusions

Sirtuins have gained a lot of interest recently due to their antiaging effects. As deacetylases, they target a multitude of histone as well as nonhistone proteins explaining the broad range of effects of this family of HDAC. In addition to their role in cell proliferation and metabolism, effects on the pathways of immunity and inflammation have been found. SIRT1-deficient mice display systemic lupus erythematosus-like manifestations, suggesting that SIRT1 may be involved in autoimmune processes. SIRT1 targets two major proinflammatory pathways, the NFκB and AP-1 pathways, both of which are implicated in the pathogenesis of chronic arthritis. Therefore, SIRT1 may play a role in the pathogenesis of RA and other forms of arthritis. Research on SIRT1 in arthritis is still at an early stage. The published data have confirmed that SIRT1 is overexpressed in RA and is involved in the inflammatory process in the RA synovium. However, pro- as well as anti-inflammatory properties of SIRT1 have been described. Synovial fibroblasts of patients with RA overexpressing SIRT1 produce higher amounts of proinflammatory cytokines, and silencing of SIRT1 leads to the downregulation of TNF-α and IL-6 production. This proinflammatory effect of SIRT1 was enhanced by the inhibition of spontaneous apoptosis mediated by SIRT1, resulting in a prolonged life span of the activated synovial fibroblasts. Thus, in cultured RA synovial fibroblasts, SIRT1 has an overall proinflammatory effect [56]. These data are in agreement with a previous report of anti-inflammatory effects of the nonspecific sirtuin inhibitor NAM on synovial macrophages and synovial explants of patients with RA [55]. Similarly, in a macrophage cell line, the sirtuin inhibitor sirtinol inhibited LPS-induced TNF-α production in a NFκB-dependent fashion [59]. On the other hand, embryonic fibroblasts of SIRT1 transgenic mice showed decreased NFκB activation after TNF-α stimulation [27], whereas SIRT1-deficient macrophages showed hyperactive NFκB [39]. These discrepancies suggest that the effects of SIRT1 on NFκB and other transcription factors involved in chronic inflammation may depend on the cell type as well as on the cellular milieu, determining whether the overall outcome is pro- or anti-inflammatory.

In osteoarthritis, it seems that SIRT1 expression in the cartilage decreases over time paralleling the progression of cartilage damage [69]. Interestingly, SIRT1 was found to exist in two forms, the full-length SIRT1 and a short 75-kDa SIRT1. The formation of the 75-kDa SIRT1 was induced by TNF-α and dependent on cathepsin B, an enzyme that has previously been shown to be induced in osteoarthritic cartilage [65, 66]. As only the full-length SIRT1 induced the production of cartilage-specific

genes, the data suggested that under the influence of TNF-α, the cleavage of SIRT1 leads to a loss of complete SIRT1 and consequently degradation of cartilage due to a lack of extracellular matrix proteins [69]. Thus, SIRT1 was demonstrated to have cartilage protective properties.

In summary, sirtuins are important modulators of inflammation and immunity. In particular, SIRT1 is involved in the development of chronic joint inflammation in rheumatoid arthritis, with proinflammatory effects on synovial cells. In addition, anti-apoptotic and cartilage protective effects were shown for SIRT1. Further studies are needed to delineate the role of SIRT1 in chronic joint inflammation and to establish the critical parameters deciding on whether a pro- or antiinflammatory outcome results. Moreover, research needs to address the other members of the sirtuin family which may synergize with or oppose the activity of SIRT1. Increased understanding of the impact of sirtuins on immune pathways is needed to be able to design therapeutic approaches targeting sirtuins.

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Chapter 19
MicroRNA Molecules, Master Regulators, Biomarkers, and Potential Therapies

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Abstract A group of small noncoding RNAs-known as microRNAs-are master posttranscriptional regulators of cellular processes during specification and also preserve tissue homeostasis in adults. Most of the microRNA molecules (miRNAs) are tissue and stage specific, so that patterns of miRNA expression can be useful biomarkers in neoplastic, degenerative, and inflammatory diseases. Environmental factors have a great impact on the pathogenesis of rheumatic diseases, due to their ability to induce epigenetic modifications in gene expression patterns. A common vehicle of exogenously induced epigenetic modifications is oxidative stress. A number of miRNA are induced by redox-sensitive transcription factors and/or by epigenetic modifications. These miRNAs participate in the cellular response to oxidative stress either by protecting cells or promoting damage. In this sense, high-throughput miRNA expression studies can identify miRNA signatures that will help assess the risk of oxidative stress-dependent damage in defined cell populations. Importantly, a single miRNA can regulate an entire molecular pathway through the binding and translational inhibition of several end products. Furthermore, some miRNAs control the activity of transcriptional regulators. This ability makes miRNA attractive as therapeutic agents, an emerging field that is starting to be explored.

Abbreviations

Ago	Argonaute
DGCR	DiGeorge critical region
DNMT	DNA methyltransferase

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DUSP	Domain in ubiquitin-specific proteases
ERK	Extracellular signal-regulated kinase
FGF	Fibroblastic growth factor(s)
Fox	Forkhead box transcription factor
GSH	Reduced glutathione
GW	Glycin-tryptophan dipeptide-rich protein
HDAC	Histone deacetylase(s)
HIF	Hypoxia-inducible factor
HO	Heme oxygenase
ICOS	Inducible T cell costimulator
IFN	Interferon
IL	Interleukin
IRAK	Interleukin-1 receptor-associated kinase
IRF	Interferon regulatory factor
LDL	Low-density lipoproteins
MMP	Metalloproteinase
miRNA	MicroRNA molecule(s)
NF	Nuclear factor
NO	Nitric oxide
NOS	Nitric oxide synthase
Nrf2	Erythroid 2-related factor
P bodies	Processing bodies
PBMC	Peripheral blood mononuclear cells
PDCD	Programmed cell death
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PTPN	Protein tyrosine phosphatase nonreceptor type
RISC	RNA-induced silencing complex
ROS	Reactive oxygen species
RUNX	Runt-related transcription factor
SOD	Superoxide dismutase
SPARC	Secreted protein acidic and rich in cysteine
SP	Specificity protein
TCR	T cell receptor
TLR	Toll-like receptor(s)
TNF	Tumor necrosis factor
TRAF	Tumor necrosis factor receptor-associated factor
TRBP	TAR RNA-binding protein
Txnrd	Thioredoxin reductase
UTR	Untranslated region
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor

19.1 Introduction

The ability of plants to silence selective genes has been known for many years. In 1990, a system of inhibitory RNA, or RNA interference, was shown to account for selective depigmentation of petunia flowers [1]. It was found that a second copy of a gene inhibited the expression of both the gene and the transgene. The characterization of this regulatory system of gene expression in worms and the ability of synthetic RNA duplexes to reproduce gene expression interference in mammals were later on awarded the Nobel Prize [2, 3]. Over the last decade, the powerful control that this system exerts on the expression of human genes has been unveiled. An increasing number of RNA-regulated cell processes are being recognized, and alterations of this regulatory network have been associated with disease. In this chapter, we discuss the relevance of microRNA, the genome- encoded inhibitory RNA system, to the understanding and treatment of oxidation and rheumatic conditions.

19.2 The Biogenesis of MicroRNA

19.2.1 Small Noncoding RNA Orchestrate Cell Processes

MicroRNAs (miRNAs) are a family of noncoding small RNA molecules that control cell behavior under physiologic and pathologic conditions.

Mature miRNAs are single-stranded RNA of around 21–23 nucleotides in length that bind specific mRNA, marking these molecules for degradation and, in this way, leading to their translational inhibition. Over 1,000 different miRNAs have already been identified in humans, and they are thought to regulate the expression of at least one-third of our genes [2].

The expression and the activity of miRNA are coordinated by sophisticated molecular machinery (Fig. 19.1). Most miRNAs are synthesized by the action of RNA polymerases II and III as longer primary transcripts (pri-miRNA) in the form of hairpins followed by a sequential digestion by endonucleases to acquire their mature form. The first cleavage takes place inside the nucleus by the Drosha-DiGeorge critical region (DGCR)-8 complex and results in a 5′ and 3′ shorter molecule known as pre-miRNA. Exportin 5 is a carrier protein that binds pre-miRNA and transports them to the cytoplasm, where the molecules are put into contact with Dicer, another endonuclease, and with HIV TAR RNA-binding protein (TRBP) to be cleaved into a double-stranded RNA molecule with 2 base 3′ overhangs.

One of the strands, known as the guide, is then released from the complementary one (the passenger) and together with Dicer is assembled in the RNA-induced silencing complex (RISC). The RISC is a multimolecular complex in which different mRNA molecules assemble, so that miRNA can interact with them [4].

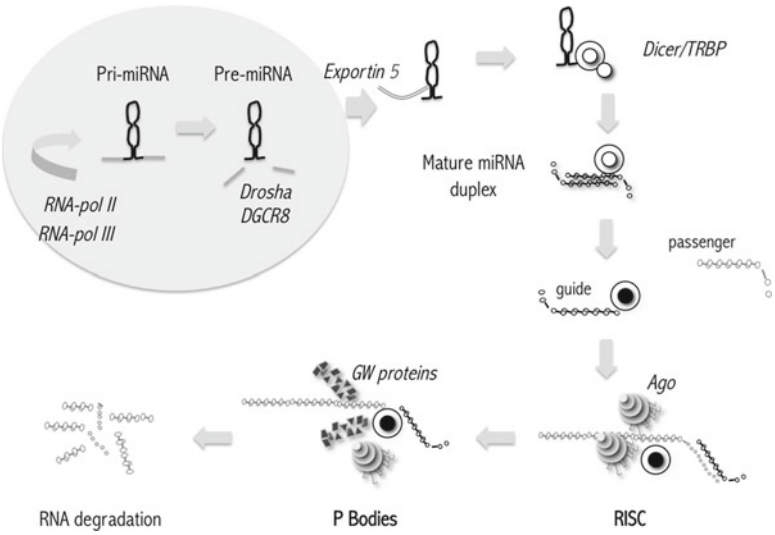

Fig. 19.1 Biogenesis of the microRNA. The molecules are synthesized as precursors by the action of RNA polymerases II and III and follow a serial cleavage to produce the mature molecule, which is inserted into the microRNA-induced silencing complex to interact with potential complementary mRNA. Once these transcripts are recognized by microRNA molecules, additional RNA-binding proteins proceed to their degradation

Once a miRNA molecule binds its target, the latter is marked for degradation. The RISC contains a cluster of molecules which play specific activities in the transport, cleavage, and recycling of RNA. There are additional structures, the processing bodies (P bodies or GW bodies), growing in the proximity of RISC, where the degradation of the mRNA molecules is executed [5].

19.2.2 RISC-Dependent mRNA Neutralization

The binding of mRNA by miRNA is based on complementarity between nucleotides 2–8 of the miRNA—the seed—and consecutive nucleotides at the 3′-untranslated region (3′-UTR) of its target. The simplicity of the binding of this relative short sequence has facilitated the in silico prediction of roles and targets of the different miRNA molecules [6].

As a result of the binding, translation into protein of the targeted mRNA is intercepted by different mechanisms, including inhibition of translation, blockade of translation initiation, and destabilization [7]. Usually, complementarity between the seed and the mRNA tag is not perfect. Incomplete matches are thought to render weaker interactions and partial degradation.

There are two prominent families of proteins participating in RISC and P bodies. These are the argonaute (Ago) and the GW182 (for glycine–tryptophan

dipeptide-rich protein of 182 kDa) proteins. Ago1 to Ago4 interact with miRNA and repress protein translation when artificially coupled to reporter mRNAs [8]. The three components of GW182 act downstream of Ago2 and appear to block translational initiation and 80S complex assembly, as well as post-initiation steps. Also they could interact with the poly(A)-binding protein and recruit a deadenylase complex to promote mRNA decay [8].

Interestingly, molecules participating in a biological process usually share binding motifs for miRNA at their 3′-UTR. Therefore, a single miRNA can shut down a whole metabolic pathway. Also frequently, miRNA are encoded at introns inside the genomic DNA of their targets. This type of miRNA is called mirtrons and is coregulated with the embedding gene, so that when the expression of this gene is triggered, its natural regulator is ready to deactivate it [9].

In summary, a single molecule of microRNA can neutralize a cluster of different mRNAs, having a great impact on the copy number of proteins in a tissue and developmental-specific (i.e., temporal and spatial) fashion [10].

19.2.3 Participation of MicroRNA in Disease

Because of its role in diverse homeostatic processes, microRNA activity is necessary for the integrity of individuals. In particular, the microRNA system is a natural defense against viral infections and can block the mutagenic activity of transposable elements, conferring genomic stability [11, 12]. A breach in this surveillance mechanism can lead to the development of tumors, and mutations in some miRNA have already been associated to different neoplasms [13].

Some miRNAs are considered master conductors of specification, due to their ability to suppress the expression of molecules not associated to cell lineage. Along with this role in differentiation and development, distinct miRNA molecules orchestrate metabolic pathways in the adult, in a cell-type-specific fashion, such as insulin secretion, cholesterol biosynthesis, and metabolism of triglycerides, carbohydrates, and lipids [14]. The importance of this regulation is illustrated by the development of diabetes after the selective deletion of Dicer1 in pancreatic β cells [15].

The discovery of this sophisticated mechanism to regulate the molecular profiles of the cells provides new avenues for understanding complex diseases [16]. Inflammatory disorders are driven by inducible molecules, which are generally under mirtron control. It can be easily envisioned what complex effects might derive from a single polymorphism at a mirtron seed. Moreover, the microRNA also targets transcriptional regulators, with dramatic consequences in activating or regulating whole networks [17].

Modifications in the sequences of particular miRNA molecules are not the only mechanism through which miRNAs can cause organ malfunction. Even more dramatic are the consequences of a failure in the microRNA biogenesis. Deficiencies in the proteases involved in miRNA maturation, such as Dicer or Drosha, are lethal at early embryonic stage. Alterations in these molecules have a great impact

on normal development and on the maturation of the immune system, as shown by selective deletion models in mice [18]. Dicer deficiency in immature T cells leads to a fall in mature T cell counts, and its absence in double positive T cells results in a preferential Th1 differentiation related to the inability of cells to repress IFNγ. These animals show organomegaly, intestinal lymphadenopathy, colitis, and low counts of Treg cells [19]. Moreover, when both Dicer and Drosha are suppressed in double positive T cell populations, the animals develop a fatal autoimmune syndrome comparable to the forkhead transcription factor FoxP3 deletion [20]. On the other hand, deletion of Dicer in B cell precursors is associated with their premature apoptosis and with impairment of pro-B to pre-B transition. Finally, conditional deletion of Ago2 in hematopoietic cells blocks development of erythrocytes and B cells [21].

While severe mutations in the microRNA network are not found in human adults, the activity of Dicer and related proteases can be locally exceeded and result in functional alterations. This situation is typical of virus-infected cells. During high viral replication, a variety of miRNA molecules from the host cell target viral RNAs, so that the RNA-engaging proteins and the degradation machinery jam with unexpected activity [21]. This can hamper their work on endogenous molecules and eventually put the cell function into danger. In this way, viral infections may indirectly induce cytotoxicity because of the saturation of the endogenous microRNA pathway, an issue that needs also be considered in the design of miRNA-based therapies. As mentioned above, a major consequence of the microRNA network jam is loss of tolerance. Precisely, autoimmunity in the setting of viral infections could partially derive from microRNA dysfunction [22].

19.2.4 MiRNA Molecules as Biomarkers

MiRNA expression signatures are useful for diagnosis and therapy of several human diseases, including cancer, viral infections, and inflammatory conditions. Tumors can be classified according to the levels of around 200 miRNA molecules that are not only consistently deregulated in most patients but also display a pathogenic role in tumorigenesis [23]. This role can be predicted based on the specificity of the binding of target mRNAs.

Interestingly, miRNAs are secreted into organic fluids, such as peripheral blood, where they can travel in circulating exosomes. Their small size and their protection from digestion by various cellular factors allow their detection at low levels. Moreover, since many of the miRNAs found in the bloodstream are tissue specific, their expression in the periphery can be easily associated to their source [24]. It should be underlined that even very small changes in miRNA expression levels might be biologically significant. There are many ongoing efforts searching for miRNA signatures in different patient cohorts, and some miRNA-based diagnostic tests are already available showing high capacity to discriminate between clinically related conditions, such as pancreatic cancer and pancreatitis [25].

19.3 Prediction of miRNA Involved in Oxidative Stress in Rheumatic Conditions

In spite of the identification of multiple susceptibility genes associated to distinct rheumatic conditions, there is, in general, a low concordance rate in the phenotype of monozygotic twins [26]. Over the years, experts have come to regard the incidence of environmental factors as decisive in their development. There are recognized triggering agents in systemic lupus and similar diseases, including drugs, UV radiation, or infections. Through different pathways, all these potential inductors contribute to alter the immune threshold. Lately, epigenetic modifications have been suggested as common pathogenic pathways following the incidence of environmental factors [27]. Interestingly, oxidation is a general mediator of cell damage associated to infections, UV radiation, and toxics. An excessive oxidative load acts on redox-sensitive transcriptional regulators and is able to modify patterns of gene expression, through stable epigenetic modifications [28].

The relevance of oxidation to the development and progression of rheumatic diseases is unquestionable and is comprehensively discussed in other chapters of this book. Oxidation, like other critical cell processes, is controlled by master molecules, including miRNA. In this regard, the detection of particular miRNA might provide information about the cell oxidation status and could help assess specific risk in order to modify a therapeutic strategy accordingly. To illustrate this concept, a recent experiment showed activation of mmu-miR-466h-5p in mouse cells exposed to glucose deprivation. Reactive oxygen species (ROS) accumulated in glucose-deprived cells exhausting reduced glutathione (GSH) reserves and inhibiting histone deacetylases (HDAC) activity. This resulted in the release of miR-466 expression, which indicates that this proapoptotic miRNA is under epigenetic control and mediates oxidation-dependent damage [29].

In a similar way, during radiation-mediated oxidative stress, DNA modifications are associated to an enhancement of p53 expression. This oncogene is able to downregulate the constitutive miRNA, Let7. Additionally, p53 directly trans-activates miR-34a, a commonly deleted molecule in human cancers that targets transcripts involved in cell cycle/apoptosis, as well as in DNA repair and angiogenesis [30].

Most of the available data concerning miRNA roles come from in silico prediction of targets, followed by tissue expression studies and by confirmation of functional changes in a selective target following forced expression of its regulating miRNA [31].

Along with the Watson–Crick complementarity between the seed and an oligosequence at the 3′UTR of transcripts, also the mRNA nucleotide at position 8 with regard to the miRNA (m8 match) is highly conserved, and the nucleotide at position 9 (t9 A-anchor) is usually an adenosine. This particular structure allows the prediction of sets of genes that could be regulated by a given miRNA molecule. The prediction is further clarified by two essential paradigms. Firstly, molecules associated in a role or pathway are usually regulated by the same miRNA, and, secondly, a limitation for the interaction between miRNA and target mRNA is obviously derived from their synchronic availability.

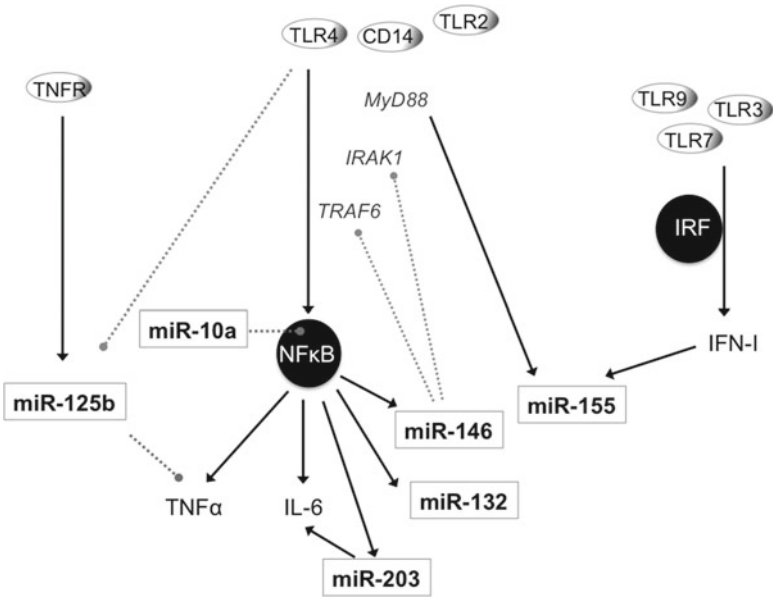

Fig. 19.2 The graph shows the regulation of innate immune activation pathways by different miRNA molecules. The binding of innate receptors, such as the members of the Toll-like family (TLR), leads to the activation of transcriptor factors NF-κB and interferon regulatory factors (IRF), which in turn trigger the gene expression of cytokines as well as their regulatory miRNA

19.3.1 Innate Responses

The role of innate immune responses in inflammatory joint diseases is supported by the high production of proinflammatory cytokines, such as IL-1β, IL-6, IL-17, and TNFα in the target tissues [32–34]. These molecules not only participate in the development of the disease but correlate with activity and severity markers as well [35, 36]. Elegant studies have shown that the normally tight regulation of their gene expression is lost in rheumatic conditions [35, 37].

A few miRNAs have been identified as regulators of the innate pathways, both in experimental models and in humans (Fig. 19.2). Interestingly, some of them are differentially upregulated in patients with inflammatory joint diseases and thus can be regarded as biomarkers. In 2008, we among others showed an increase in miR-146a in joints as well as in circulating monocytes from patients with rheumatoid arthritis [38–40]. Levels of this miRNA were shown to correlate with disease activity. Interestingly, miR-146a, miR-155, and miR-132—which are all increased in rheumatoid arthritis—are upregulated in monocytes in response to Toll-like receptor (TLR) ligands and proinflammatory cytokines. The transcriptional activity of NF-κB triggers the expression of miR-146a, and the miRNA in turn targets two upstream molecules in the NF-κB activation pathway—interleukin-1 receptor-associated kinase (IRAK)1

and tumor necrosis factor receptor-associated factor (TRAF)6—thereby helping to shut down the proinflammatory circuit [41]. On the other hand, miR-125b is downregulated in response to lipopolysaccharide. Since this miRNA targets TNFα, the induction of the cytokine is further facilitated by this loop upon TLR activation [42].

In contrast, miR-155 is induced both in response to TLR ligands and to IFN-Iβ, and its activity shifts T differentiation toward Th1 and Th17 patterns, in this way promoting autoimmunity [43]. Interestingly, miR-155-deficient mice are protected against collagen-induced arthritis, while in the K/BxN serum transfer arthritis model, absence of this miRNA results in a decrease of auto-reactive B cells, lower levels of IL-6 and IL-17, and milder bone damage.

More recently, another miRNA, miR-203, came out as an intriguing mediator of inflammatory injury. It was found to be increased in synovial cells from rheumatoid arthritis patients, compared to patients with osteoarthritis, and it was upregulated following incubation of cells with the demethylating agent, 5-azacytidine [44]. Overexpression of this miRNA by pre-miR-203 transfection resulted in an increased production of IL-6 and metalloproteinase (MMP)1, both of which are markers of severity in patients with rheumatoid arthritis. Also of interest was the fact that miR-203 activity could be abrogated with an NF-κB inhibitor. Altogether, these findings suggest that miR-203 might be triggered by oxidative stress through DNA demethylation and promote tissue damage. However, miR-203 is possibly the ugly but not the bad guy in this adventure. There is evidence indicating that miR-203 acts as tumor suppressor and could participate in mechanisms of cell repair against stress [45].

19.3.2 Distant Effects of Inflammation

Systemic inflammation is associated to the development of premature atherosclerosis, being the latter one of the major causes of reduced life span in patients with rheumatic conditions [46]. Oxidative stress has a prominent participation in vascular injury and in inflammation-mediated plaque instability. The arterial wall is infiltrated with activated inflammatory cells that produce an excessive amount of ROS, which in turn oxidize lipoproteins.

Different studies as well as epidemiological data have shown that the vascular risk of patients with rheumatoid arthritis or with lupus can be reduced with disease modifying drugs, even though some of these agents have a well-established negative incidence in traditional proatherogenic markers [47]. There seems to exist, therefore, a collateral vascular benefit from achieving remission of the inflammatory condition [48]. However, the number of drugs added to antirheumatic regimes focused on the prevention of vascular risk is increasing. Currently, selection of candidates that would clearly benefit from such regimes is strongly desirable.

As has been recently suggested, oxidative stress in the vessel wall and in adipose tissues contributes to the pathogenesis of inflammation-dependent atherogenesis. Microvesicles containing bioactive molecules, including miRNA, can be found in

peripheral blood [24]. The ability of the microRNA to travel inside vesicles facilitates the contact between adipose tissue and/or local inflamed cells with distant endothelial cells and may help to propagate insulting mechanisms to the blood vessel wall. On the other hand, the traveling habits of miRNA are convenient both for diagnostic purposes and for delivery of miRNA-based therapies [49].

There is in vitro evidence suggesting that microRNA regulates redox signaling in endothelial cells [50]. In human microvascular endothelial cells, Dicer selective deficiency was found to increase the expression of vascular endothelial growth factor (VEGF), while it reduced migration of cells and Matrigel tube formation—an indicator of impaired angiogenesis. Interestingly, Dicer-deficient cells showed decreased production of ROS upon cytokine stimulation, and the angiogenic response could be rescued with H_2O_2.

Two important miRNAs for endothelial cell migration, proliferation, and angiogenesis are miR-221 and miR-222, which are thought to target both VEGF and nitric oxide synthase (NOS) pathways and in this way increase hypoxia [51]. These molecules are upregulated by TNFα, and their levels have been shown to inversely correlate with those of adiponectin. The adipogenesis-associated miRNAs, miR-103 and miR-143, have been found decreased in the presence of this cytokine, an effect that could explain inflammatory cachexia [52].

Different miRNAs are involved in the production of proinflammatory adipocytokines and chemokines, including miR-155, miR-183, and miR-872, which repress anti-inflammatory and antioxidative actions of heme oxygenase-1 (HO-1). The principal source of these miRNAs seems to be macrophages infiltrating adipose tissues [53].

Regulatory miRNAs in the vascular environment have also been identified, including miR-126, which mediates the angiogenic effects of VEGF and fibroblastic growth factors (FGF), at the same time targeting vascular cell adhesion molecule (VCAM)-1. A decrease in endogenous endothelial cell miR-126 levels was shown to promote leukocyte adherence to endothelial walls, a fact which further supports the anti-inflammatory role of this miRNA [54]. Another regulatory miRNA is miR-21, which is triggered by mechanic forces and protects endothelial cells by increasing eNOS and NO production. MiR-21 is induced by IL-6 and targets the programmed cell death (PDCD) 4 gene, in this way rescuing vascular cells from ROS-mediated apoptosis.

One of the major pathogenic pathways shared by atherogenesis and inflammation is transcriptional activity of NF-κB. In this regard, miRNA targeting this circuit might have a protective role on inflammation-induced vascular injury. MiR-132 is NF-κB sensitive and displays an anti-inflammatory role, while miR-10a has been found downregulated at athero-susceptible sites in vivo, and its deletion leads to NF-κB activation [55].

The former miRNA molecules could be helpful to assess vascular risk, and miR-126 mimetics could even be employed to prevent intravascular leukocyte activation. But if a single molecule has to be chosen as global predictive factor for cardiovascular risk in rheumatic conditions as well as in metabolic disorders, miR-27a is probably the best candidate and will perhaps substitute traditional markers

in the next future. The reason is that this master miRNA targets key transcriptional regulators, such as Runx1, FoxO1, and SP1, all of which participate in adipogenesis, inflammation-dependent oxidative stress, and angiogenesis [56]. MiR-27a is upregulated in obesity and its expression is favored by hypoxia.

The production of hydrogen peroxide in macrophages causes oxidative damage to lipoproteins, such as phospholipids in LDL. This is associated to an upregulation of miR-9, miR-125a, miR-146a, miR-146b, and miR-155 in monocytes. In turn, some of these molecules inhibit lipid uptake, through the targeting of scavenger receptors [57]. On the other hand, cholesterol efflux is also tightly regulated and blocked by the production of miR-33, which interferes with ATP-binding cassette transporters.

19.3.3 Systemic Autoimmune Diseases

From selective deletion models, we have learned that the integrity of the microRNA machinery is needed for the normal maturation of the immune system. Additionally, there are approximately 100 miRNA molecules, or immunomirs, involved in immune cell responses, and some of them have been found to be altered in the context of autoimmune diseases.

The miR-17-92 cluster is one of the principal systems regulating cell proliferation and death mechanisms. It is composed of six different miRNAs synthesized together, and they target different proteases involved in cell cycle progression, such as Rb:E2F and p21, as well as the apoptosis mediators Pten and Bim [58]. Alterations in their activity can hamper progression in the differentiation of immune precursors.

MiR-181 is the principal regulator of the immune threshold in T cells. This miRNA is highly expressed at the immature stage. It suppresses the proteases PTPN22, DUSP5, and DUSP6, which in turn are inhibitors of both cytoplasmic and nuclear activation of ERK1/2. In consequence, immature T cells depict a high activation status of ERK1/2, and they are characterized by positive selection of TCR binding peptides. As differentiation progresses, levels of miR-181 drop, the activity of its targets increases, and ERK1/2 stays in a latent form. In this way, auto-reactivity of mature T cells is prevented [59].

In a similar fashion, the maturation of B cells is controlled by miR-150, whose expression increases along the differentiation process. Thus, immature precursors are characterized by suppression of miR-150 and high levels of c-Myb, which is a target of this miRNA. This molecular footprint is necessary for pro-B to pre-B transition and is thereafter progressively inverted [58]. In mature B cells, another miRNA, miR-155, is upregulated and drives heavy chain switch of immunoglobulins.

Interestingly, components of the microRNA machinery are targeted by autoantibodies associated to systemic autoimmune diseases. In fact, GW182 was cloned as a novel protein recognized by an autoimmune serum from a patient with motor and sensory neuropathy. Later on, Ago2 was shown to be responsible for the

reactivity of Su antibodies, which can be found in lupus patients along with various autoimmune and infectious diseases [60]. Although the relevance of the loss of tolerance to these molecules is yet to be defined, there are increasing studies showing selective expression profiles of miRNA in relation to autoimmune conditions.

The first studies addressing miRNA patterns in patients with systemic lupus erythematosus revealed that miR-146a, a biomarker of rheumatoid arthritis, was selectively suppressed in PBMC from the patients [61]. Furthermore, levels of miR-146a negatively correlated with disease activity and with the IFN signature found in more than one-half of patients, altogether indicating antagonistic pathogenic pathways in rheumatoid arthritis and systemic lupus [62].

Although several miRNAs have been later on associated to specific features of lupus, such as a cluster of molecules upregulated during nephritis, miR-126 overexpression is probably the most decisive in terms of loss of tolerance in this disease. This miRNA regulates levels of DNA methyltransferase (DNMT)1 [63]. Genomic hypomethylation has been observed in T cells from patients with lupus and correlated with a lack of repression of activation markers. This suggests that restoring miR-126 to normal levels might provide a way to attenuate T auto-reactivity.

Different works have also addressed patterns of miRNA associated to other connective tissue diseases. In this regard, the miR-17-92 cluster is downregulated in minor salivary glands from patients with Sjögren's syndrome, a fact that has been associated to maturation arrest and accumulation of pro-B cells [64]. Also miR-574-3p and miR-768-3p have been found to be overexpressed in the salivary glands from patients with Sjögren's syndrome, while in PBMC, miR-146a shows a similar profile as in rheumatoid arthritis.

As for systemic sclerosis, some studies have been focused on the in silico prediction of miRNA molecules targeting pathogenic cytokines and matrix components. In this regard, the miR-29 family has shown up as a master regulator of collagen deposition in this disease [65]. In particular, miR-29a—clustered together with miR-29b1 and located in chromosome 7—was predicted to target PDGF-B, PDGFR β, the transcriptional regulator SP-1, thrombospondin, and SPARC, along with coll III.

19.3.4 Bone and Cartilage Damage

FoxO1 is a key transcriptional regulator that protects cells from the effect of ROS, inhibiting apoptosis and promoting cell differentiation. It is also the principal component of the FoxO family in bone, and a loss of function with age has been suggested to participate in bone loss. MiR-182 targets FoxO1 and shows a negative effect on bone proliferation [66].

Along with inflammatory arthritides, miR-146a has been found to be upregulated in osteoarthritic chondrocytes, in an experimental model of joint instability. Although it is difficult to assess miR-146a actual role in this pathological situation,

its widespread distribution in the target tissue clearly indicates that the miRNA participates in cartilage homeostasis. In silico analysis has identified Smad4 as a putative target of miR-146a, and functional studies have suggested that this pro-anabolic molecule could be blocked in osteoarthritis due to a microRNA unbalance. In turn, Smad4 decreased expression correlated with the upregulation of VEGF, angiogenesis, development of hypertrophic chondrocytes, and increased cell death [67]. The upregulation of miR-146a in chondrocytes is the result of the activation of NF-κB in response to the local release of proinflammatory cytokines and in particular to IL-1β, as suggested by in vitro studies.

Interesting to explore in joint and bone degeneration are miRNAs associated to redox-sensitive transcription factors, such as the erythroid 2-related factor (Nrf2), a master regulator of antioxidative responses coupled to the CO-producing enzyme HO-1. This system acts on mechanisms of proliferation/apoptosis and angiogenesis, and it attenuates inflammation. Some key myomirs, including miR-1, miR-133, miR-206, and miR-146a, are downregulated by this pathway. MiR-210 is induced in response to hypoxia-inducible factor (HIF)1, and its activation promotes angiogenesis [68].

Another subject of interest is the identification of the miRNA involved in the regulation of mitochondrial enzymes. Positively, alterations in the mitochondrial antioxidative buffer capacity have been associated to the development of joint degeneration (discussed in depth in Chaps. 4 and 5 of this book). In this regard, miR-335 and miR-34a have been recently shown to inhibit superoxide dismutase (SOD)2 and thioredoxin reductase (Txnrd)2 and to promote senescence in renal cells [69].

19.4 The MicroRNA as a Therapeutic Tool

19.4.1 Transduction of Cells with miRNA Works Safely

Both the participation of miRNA in regulatory networks and the alterations found in some miRNA in association to disease make them attractive as therapeutic tools. Defective miRNA can be restored with the administration of miRNA mimetics, and the expression of disease-associated miRNA, such as the oncomirs, can be shut down with antisense oligonucleotides or antagomirs.

There are some widely expressed miRNAs participating in critical processes, such as cell growth and survival. Modulating these miRNAs may have generalized off-target effects. They are known as systemic miRNA, and an example of them is the miR-17-92 cluster. In contrast, others are highly specific, so that their administration would lead to targeted effects. Indeed, the appeal of using miRNA in therapeutics relays in their selective activity. So far, miRNA-based therapeutic approaches in animal studies have been promising, since no toxic effects have been observed [70].

One of the difficulties that needs to be solved in miRNA-based therapies is to optimize delivery both to avoid toxicity and to efficiently reach the target tissue. In this regard, some rheumatic conditions offer the opportunity of intra-articular injections as an interesting approach to deliver miRNA molecules. This possibility has been elegantly explored in mice with autoantibody-mediated arthritis. Double-stranded miR-15a, a proapoptotic miRNA that targets Bcl-2, was injected in the knee joints and was found to efficiently enter synovial cells [71].

Vascular processes, including thrombophilias and atherogenesis, might be directly approached with intravascular delivery of miRNA [72].

The first application to the treatment of human diseases is miravirsen, an antisense molecule against miR-122, which is a liver-specific miRNA that acts as survival factor for hepatitis C virus [73].

19.4.2 Do Not Kill the Messenger

There are enough data pointing to specific miRNA as biomarkers of disease activity and progression in rheumatic conditions. Some of them act as master regulators of pathogenic processes. However, in many circumstances, their upregulation reflects an increased transcriptional activity of their targets instead of a malfunction of the microRNA system. It is therefore important to read the whole miRNA message in order to accurately select a therapeutic option.

This is the case of miR-146a, which acts as a biomarker in rheumatoid arthritis and other inflammatory diseases. While the molecule is upregulated via NF-κB, it helps shutting down the circuit through the repression of IRAK1 and TRAF6. Its upregulation reflects the magnitude of the activation but should be regarded as protective. The miR-146a-deficient mouse further illustrates the effects that the suppression of this miRNA would draw. This mouse is characterized by a generalized loss of immunological tolerance, leading to fatal IFNγ-dependent immune-mediated lesions in different organs [74]. This phenotype is not very different from the clinical picture of active lupus in which a downregulation of miR-146a can be found. Should we then enhance miR-146a beyond its physiological levels? This approach could be useful to treat particular phenotypes with constitutive activation of NF-κB. Nevertheless, an excessive inhibition of TLR signaling is dangerous, since these receptors are the first barrier against invasion and help produce an efficient defensive response [75]. Additionally, as shown by the specific actions of miR-146a in mature T cells, it is not clear whether its upregulation would induce tolerance. Although its expression leads to the suppression of IL-2 upon TCR engagement, miR-146a also acts as an antiapoptotic factor for these cells [76].

The miR-146a example illustrates the extensive characterization that needs to be carried out in order to select miRNA for therapeutics. Potential candidates to combat the effects of oxidative stress associated to rheumatic conditions are summarized in Table 19.1.

Table 19.1 Putative microRNA targets in oxidative-dependent injury associated to rheumatic diseases

Up	Down
Beneficial effects	*Pathogenic*
miR-10a	miR-27
miR-21	miR-33
miR-125b	miR-34a
miR-132	miR-155
miR-146a	miR-182
miR-210	miR-183
	miR-203
	miR-221
	miR-222
	miR-335
	miR-466
	miR-872

19.4.3 What Alters MicroRNA Patterns of Expression?

From a different perspective, it would be useful to find out whether activation profiles of miRNA are static or can be exogenously modified in the long run. There is already some information suggesting that modification of miRNA patterns of expression with drugs or with dietary supplements could represent a therapeutic opportunity. It has been shown that dietary factors, including micronutrients and nonnutrient components of diets, have an impact on the expression of miRNA.

The effect of dietary polyphenols on miRNA signatures is an indirect proof of the ability of oxidative stress to induce microRNA malfunction. Polyphenols are abundant in plants and many of them in food. Proanthocyanidins, the commonest polyphenols in the human diet, are contained in fruits, beans, nuts, cocoa, tea, and wine. They appear to bear free radical-scavenging properties and have shown anti-inflammatory, antimicrobial, cardioprotective, hypolipidemic, and antidiabetic activities. One of the suggested targets of these molecules is miR-30b*, which was found to be downregulated in hepatocarcinoma cells exposed to different dietary polyphenols [77].

Also soy isoflavones and green tea have been shown to modulate patterns of microRNA, an effect possibly associated to their antineoplastic activity.

Finally, high doses of conjugated linoleic acid (CLA), a dietary supplement recommended for weight loss, were associated to decreased levels of miR-107 and miR-143.

19.5 Conclusions

With the introduction of molecular targeting therapies, the so-called biologics, our approach to treat human diseases has experienced a considerable change. There is now a tendency toward using more natural weapons, both to prevent collateral effects and to try and restore the tissue homeostasis. Under this general principle, miRNA-based therapies appear as sophisticated strategies that will substitute less selective treatments [49]. It is still early to forecast how will these therapies improve the outcome of rheumatic conditions, but we are already starting to measure their applicability in the characterization of these diseases.

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Chapter 20
Estrogen-Dependent Transcriptional Activity: A Protection Against ROS in Osteoarthritis

Laura Lugo and Gabriel Herrero-Beaumont

Abstract Osteoarthritis (OA) is a chronic degenerative joint disorder characterized by morphological, biochemical, molecular, and biomechanical changes in all joint tissues. Despite the heterogenic pathogenesis, it has been described that the imbalance between production of reactive oxygen species and cellular scavenging mechanisms—or oxidative stress—is critical for disease progression. Thus, the production of ROS not only destroys cartilage but also amplifies the inflammatory process that helps to perpetuate disease. On the other hand, since estrogens play an important role in preserving homeostasis of articular tissues, they could also act protecting the joint tissues against oxidative stress. In agreement, it has been shown that estrogen deficiency results in an increased oxidation load eventually leading to DNA damage. Moreover, the total antioxidant capacity could be restored with the administration of 17β-estradiol, indicating that estrogens might buffer the impact of oxidative stress. Estrogens represent an interesting approach for the treatment of OA, with favorable direct effects on the chondrocyte metabolism and antioxidative properties.

Abbreviations

Akt Protein kinase B
AP Activating protein

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AP-1	Activating protein-1
CAT	Catalase
Cat D	Cathepsin D
COMP	Cartilage oligomeric matrix protein
COX-2	Cyclooxygenase-2
CREB	Cyclic adenosine monophosphate response element-binding
CTX	Carboxy telopeptide of type I collagen
Cu^{2+}/Zn^{2+}	Copper and zinc oxides
E2	17β-estradiol
EC-SOD	Extracellular superoxide dismutase
ER	Estrogen receptor
ERE	Estrogen response element
ERK	Extracellular signal-regulated kinase
FGF	Fibroblast growth factor
GAG	Glycosaminoglycan
GPX	Glutathione peroxidase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
H_2O_2	Hydrogen peroxide
HIF	Hypoxia-inducible factor
HOCl	Hypochlorous acid
HRT	Hormone replacement therapy
IGF-I	Insulin-like growth factor I
IL	Interleukin
iNOS	Inducible NO synthase
LDL	Low-density lipoprotein
L-NMMA	*N*-monomethyl-L-arginine
MDA	Malondialdehyde
MMP	Matrix metalloproteinases
MnSOD	Manganese superoxide dismutase
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
NF	Nuclear factor-κB
$^{\bullet}NO$	Nitric oxide
$^{\bullet}NO_2$	Nitrosyl radical
NOS	NO synthase
$^{\bullet}O_2^-$	Superoxide anion
OA	Osteoarthritis
OB	Osteoblast
$ONOO^-$	Peroxynitrite
OP	Osteoporosis
OPG	Osteoprotegerin
OVX	Bilateral ovariectomy
PI3K	Phosphatidylinositol-3 kinase
PRDX	Peroxiredoxins

PTH	Parathyroid hormone
RANKL	Receptor activator of NF-κB ligand
ROS	Reactive oxygen species
SA-beta-gal	Senescence-associated beta-galactosidase
SERM	Selective estrogen receptor modulator
SIN-1	3-Morpholinosydnonimine hydrochloride
SNP	Sodium nitroprusside
SOD	Superoxide dismutase
Sp	Specificity protein
TGF	Transforming growth factor
TNF-α	Tumor necrosis factor-α
TRAF	TNF receptor-associated factor
VDR	Vitamin D receptor
VEGF	Vascular endothelial growth factor

20.1 Defining Osteoarthritis

Osteoarthritis (OA) is a chronic degenerative joint disorder manifested by morphological, biochemical, molecular, and biomechanical changes produced in all joint tissues. These alterations cause softening, fibrillation, ulceration, and loss of articular cartilage due to the disruption in the normal balance between anabolic and catabolic mechanisms that maintain extracellular matrix homeostasis in articular cartilage. Moreover, pathologic changes in non-cartilaginous tissues include subchondral bone sclerosis, eburnation, and synovial inflammation and are also determinant factors both in the initiation and the progression of OA. Clinical manifestations of late stage OA are well characterized and include joint pain, tenderness, limitation of movement, crepitus, occasional effusion, and variable degrees of inflammation without systemic effects; however, it is difficult to identify or to characterize clinically this disease at onset.

Perhaps due to its heterogeneity, the duration of the early stages as well as the initial tissue lesions are not well established. What is apparent is that events involved in OA initiation are not necessarily the same as those taking place during disease progression. In some cases, its initiation may result from the existence of a steep stiffness gradient at the underlying subchondral bone. On the other hand, changes in the architecture and turnover of osteoarthritic subchondral bone alter its stiffness and its quality, thus contributing to disease progression, as has been recently shown [1]. However, whether the changes in the bone precede cartilage degeneration remains controversial, as some investigators consider that OA is initiated in the cartilage leading to subchondral bone sclerosis, but others suggest that OA is primarily a bone disease, where the thickening or subchondral bone density changes are crucial for the initiation and progression of OA [2–5].

There is also some controversy about the relevance of synovial membrane inflammation to OA pathogenesis, although the involvement of this tissue in OA progression seems clear. In this regard, a high percentage of patients with advanced OA of the knee depict persistent synovitis, a feature that directly correlates with the degree of joint destruction. Additionally, the synovial membrane is a main source of proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-1α or β, not only at advanced stages of OA but also in patients with early disease. The leakage of these mediators into the synovial fluid accelerates cartilage degradation, thereby contributing to the pathogenesis of OA [6, 7].

20.2 Epidemiology

According to the WHO, 10% of the world population over 60 years of age, which represents 70.85 million people, suffers from symptomatic osteoarthritis. Therefore, OA is the most frequent articular disease, mostly affecting elderly people. Taking into consideration that the population over 60 is expected to increase by 1.25 billion between 2005 and 2050, there could be an additional 125 million patients with symptomatic OA in the next decades. These figures indicate that OA not only constitutes a worldwide public healthcare challenge but a significant burden to global economy as well.

Radiologic criteria have been considered the goal standard in OA diagnosis in epidemiological studies, but due to their elevated costs, more recent studies are based solely on clinical symptoms. Few studies have examined the incidence of OA. Oliveria et al. reported findings from a large-scale study set in Massachusetts, in which the incidence of OA was defined according to radiographic findings as well as to clinical symptoms. Taking together all patients with OA, the knee was affected in 40% of the cases, with hand and hip showing an incidence of 18% each [8]. Considering all subtypes of OA, males were affected more often than females below the age of 45, while females over 60 had an 8% higher incidence than males [9, 10].

Although OA has been traditionally classified as primary or secondary, the division is somewhat artificial. The difference between both types is the existence of an identifiable etiological factor. Thus, secondary OA is caused by well-known risk factors such as congenital disorders of joints, diabetes, inflammatory diseases, injury to joints or ligaments, and obesity. In turn, primary OA is considered a "wear and tear" condition and is broadly defined as an idiopathic condition occurring in previously intact joints in the absence of an apparent triggering event. People tend to develop this type of osteoarthritis around age 55–60. In turn, secondary osteoarthritis tends to strike at an earlier age, around 45–50 years.

As more underlying causes of osteoarthritis are identifies, the term primary or idiopathic osteoarthritis may become obsolete. Herrero-Beaumont et al.

propose that primary OA—associated with an unknown etiologic factor—might be categorized in a new classification based on the relative incidence of etiologic, pathologic, or molecular mechanisms. According to this classification of the disease, genetic, hormone-dependent, and aging-related factors, all of them relevant for the development of OA, could define three distinct subsets of primary OA, instead of being associated with secondary OA. These three domains act in concert with additional risk factors, such as bone mineral density, acute injury, obesity, ethnicity, nutrition, as well as biomechanical aspects, like overload, joint deformity, muscle weakness, and atrophy, to shape the expression of the disease in a given individual [11, 12].

20.3 Estrogen Deficiency Is Associated with Osteoarthritis

Substantial efforts have been made to understand the potential role of estrogens in the biology of joint tissues, as well as in the development and progression of OA. Several studies have demonstrated that sex hormones, in particular estrogens, play an important role in preserving the homeostasis of articular tissues. Indeed, they orchestrate the activity and expression of crucial signaling molecules through the regulation of pleiotropic networks. These include the canonical estrogen receptor (ER) signaling pathway, non-estrogen response element-mediated genomic ER signaling, non-genomic ER signaling pathways, and ligand-independent signaling pathways [13].

As mentioned above, estrogens exert important functions on the joint tissues. For instance, estrogen's actions regulate the growth and remodeling of the subchondral bone, the osteoblast (OB) development and function, as well as the matrix production and mineralization of this bone [13].

Indeed, different experimental studies have shown the ability of 17β-estradiol (E2) to target the expression of matrix components by cartilage cells. In isolated chondrocytes from rabbit joints, E2 increased the synthesis of glycosaminoglycan, while in bovine cartilage explants subjected to catabolic cytokines, addition of E2 prevented cartilage degradation, as indicated by a decrease in the secretion of type II collagen C-telopeptide. An additional role in the protection of cartilage from oxidative stress was suggested in bovine articular chondrocytes, in which E2 was found to inhibit the expression of cyclooxygenase-2 (COX-2) [14–16].

From a clinical point of view, there is enough data to support a subtype of OA related to estrogen deficiency. Indeed, the results from a meta-analysis carried out to identify sex differences in OA showed that women not only had higher prevalence but also increased rates of incident OA in the knee. Moreover, women over 55 years of age, who have low estrogens levels, tend to depict a more severe disease compared to males, thus putting females generally at higher risk when it comes to prevalence and incidence of OA [17–19].

In turn, several experimental models illustrate how an abrupt estrogen deficiency can aggravate the erosion of the cartilage surface and lead to an increased subchondral bone turnover. In fact, induction of osteoporosis (OP) by bilateral ovariectomy (OVX) and systemic corticosteroid administration during 4 weeks increases the severity of cartilage damage in experimental OA in mature female rabbits and was correlated with the bone loss observed at the lumbar spine or subchondral bone. Additionally, Sprague Dawley OVX rats were used to evaluate whether ovariectomy (estrogen deficit) could affect OA, in order to validate an experimental model of postmenopausal OA. In this model, the levels of CTX-I and CTX-II were increased and correlated strongly with the severity of cartilage surface erosion. Moreover, treating the OVX rats with estrogen or with a selective estrogen receptor modulator inhibited the OVX-induced acceleration of cartilage and bone turnover and significantly suppressed cartilage degradation and erosion.

Similarly, in feral adult female OVX cynomolgus macaques, the cartilage OA-type lesions were significantly less severe in the animals that received hormone replacement therapy (HRT) with conjugated equine estrogens for 3 years compared with those without any treatment. In addition, the bone turnover parameters in subchondral bone or epiphyseal/metaphyseal trabecular bone were decreased as previously described in monkeys and humans. Together, these results demonstrate that long-term HRT significantly reduces the severity of OA lesions, by reducing bone turnover in postmenopausal OA [20–23].

20.4 Oxidative Stress in OA

Among other factors, the imbalance between the production of reactive oxygen species and cellular scavenging mechanisms defined as oxidative stress is involved in the pathogenesis of OA. Different sources of oxidative stress work during OA development, including exogenous factors like overload, trauma, and synovial inflammation. The production of reactive oxygen species (ROS) not only stimulates cartilage destruction but is also involved in the inflammatory process that promotes disease progression in OA. In particular, ROS contribute to posttranslational modification of proteins that is in turn associated to an impaired biological activity. The major modifications induced by oxidative stress are nitrosylation, nitration, oxidation, and chlorination of specific amino acids, leading to changes in protein structure. A subsequent side effect of inflammation-induced proteins is their accumulation in the target tissue, because it is not unusual that they become difficult to degrade after oxidative stress-dependent posttranslational modifications. In this situation, the tissue is subjected to an additional biological overload affecting various cell processes, such as intracellular signaling, proliferation, senescence and death, and synthesis or degradation of the extracellular matrix [24] (Fig. 20.1).

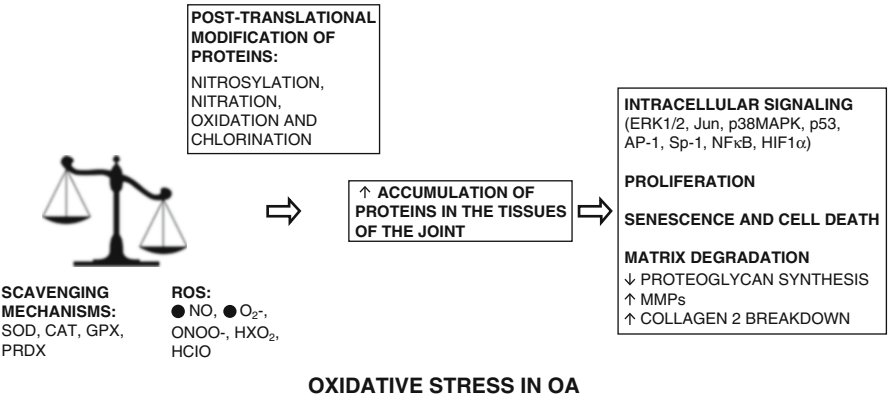

Fig. 20.1 Oxidative processes happening during OA. The disproportion between the ROS production and cellular antioxidant mechanisms produce oxidative stress during OA, fact that stimulates cartilage destruction by the accumulation in the joint tissue of posttranslational modified (nitrosylation, nitration, oxidation, and chlorination) proteins that is in turn associated to an impaired biological activity in the joint cells. The normal intracellular signaling pathways are affected, driving to senescence and cell death and to the degradation of the extracellular matrix

20.4.1 Major Mediators of Oxidative Stress in the Joint

Under the influence of mechanical stress, oxygen pressure variations, and immunomodulatory or inflammatory mediators, chondrocytes produce high levels of ROS. The principal ROS generated by chondrocytes are nitric oxide ($^{\bullet}NO$) and superoxide anion ($^{\bullet}O_2^-$), subsequently transformed into derivative radicals, including peroxynitrite ($ONOO^-$) and hydrogen peroxide (H_2O_2). These species can be generated by different enzymes such as the NO synthase (NOS) enzymes, drawing the production of $NO^{\bullet-}$; the NADPH enzyme complex that produces superoxide anion radicals through the reduction of molecular oxygen; and myeloperoxidase that can be involved in the production of nitrosyl radical ($^{\bullet}NO_2$) as well as hypochlorous acid (HOCl) [25].

Chondrocytes express both endothelial NOS and inducible NOS. The inducible isoform is regulated at the gene level by shear stress, compression, and soluble mediators, such as growth factors, cytokines, and endotoxins. In this regard, IL-1β appears to be an important mediator of inflammatory oxidation. Its ability to induce $^{\bullet}NO$ production in chondrocytes has been described [25]. On the other hand, there could be also some regulatory cytokines working to prevent an oxidative overflow. Although further evidence is needed, this could be the case for transforming growth factor (TGF)-β, which has been shown to inhibit $NO^{\bullet}$ production in rheumatoid arthritic synoviocytes [26–28].

20.4.2 Effects of ROS on Joint Chondrocyte Intracellular Pathways

Reactive oxygen species may directly regulate the activation of different signaling cascades involved in cell growth, proliferation, differentiation, death, and senescence. Intracellular ROS levels become elevated after the binding of growth factors and cytokines to different receptors, all of them present in joint cells. There are several kinase cascades that can be then activated in response to the intracellular ROS increase: in particular, extracellular signal-regulated kinase (ERK)1/2, Jun-NH2-terminal kinase, and the p38 MAPK. Interestingly, some of the principal transcription factors involved in inflammation, including nuclear factor (NF)-κB, activating protein (AP)-1, specificity protein (Sp)-1, c-Myc, p53, and hypoxia-inducible factor (HIF)-1α, have been shown to be redox-sensitive. It is therefore believed that ROS may regulate the activity of these transcription factors, through oxidative modifications [29].

Interestingly, it appears that elevated levels of ROS during chondrogenesis inhibit proliferation and initiate chondrocyte hypertrophy. This process could depend on the activation of ERK and p38 MAPK, a pathway needed for chondrocyte hypertrophic transformation. In this regard, a raise of ROS in chondrocytes during differentiation has been reported to activate these two kinases [30]. Activating protein-1 is a heterodimeric complex of Fos and Jun proteins required for the activation of matrix metalloproteinases, as well as for cell proliferation; hence, AP-1 represents the link between oxidative stress and tissue degradation. As mentioned above, AP-1 is sensitive to oxidative modifications; thus, its DNA-binding activity can be regulated by redox reactions. Conserved cysteine residues located in the DNA-binding domains of Fos and Jun and flanked by basic amino acids are oxidized and modulate AP-1 DNA binding [31, 32]. Similar redox regulation has also been showed in lymphocytes involving the small GTP-binding protein Ras. In this case, the activity of Ras is modulated by ·NO, by nitrosylation of a critical cysteine residue [33].

Reactive oxygen species, acting as signaling intermediates for TNF-α and IL-1β, were found to upregulate c-Fos, increasing collagenase gene expression [34, 35].

Some other studies showed that intracellular ROS generated by Nox2 and Nox4 (two types of NADPH oxidases) are required for chondrogenic differentiation of the cell line ATDC5 (a mouse embryonic carcinoma-derived cell line that exhibits the multistep chondrogenic differentiation observed during endochondral bone formation) and primary chondrocytes. When these cells where treated with siRNA for Nox2 or Nox4, the levels of ROS decreased and the chondrogenic differentiation was suppressed [36].

Hypoxia-inducible factor-1 (HIF-1) is a transcription factor that regulates the expression of genes involved in the protection of chondrocytes during osteoarthritis. Its activity is modulated by the ubiquitin-dependent protein degradation pathway, which in turn is activated by ROS. However, when the cell redox status is altered and there are high levels of ROS, the degradation of HIF-1 is increased [37].

Chondrocyte death may represent an important component in the pathogenesis of osteoarthritis possibly related to an increased production of ROS. The effect of NO on chondrocyte death remains controversial, as IL-1β does not induce apoptosis in primary human chondrocytes despite the generation of high levels of NO. To explain this effect, some authors suggest that the proapoptotic effect of NO could be blocked by other ROS, while others sustain that NO is not toxic to cells by itself but requires the participation of additional ROS in order to mediate cell death. Interestingly, NO has also been considered as antiapoptotic for chondrocytes, because it was able to reduce H_2O_2-induced apoptosis in bovine-cultured chondrocytes [38–40].

Overall, it appears that the role of ROS in chondrocyte death depends on the global ROS composition in the cell. Thus, HOCl negative effects on chondrocyte viability are modified when NO_2^- is simultaneously present at a physiological concentration, presumably because the final product of the reaction is NO_2Cl, which is less damaging for cells than HOCl [41].

Recently, the role for O_2^-, H_2O_2, and $ONOO^-$ in chondrocyte death has been underscored by different studies. These ROS can be released mechanically or after static compression of articular cartilage and drive chondrocyte death. Removing O_2^- and H_2O_2 by preincubation of the cells with a cell-permeable superoxide dismutase (SOD) mimetic can prevent mechanically induced cell death [42].

Chondrocytes become senescent as a result of aging or disease. They depict phenotypic changes contributing to the development or progression of OA. In a postmitotic tissue-like articular cartilage, some cellular events such as oxidative damage to DNA or the accumulation of damaged proteins and lipids might trigger stress-induced senescence [43]. In a study carried out in articular cartilage from OA, senescent cells were identified using the senescence-associated beta-galactosidase (SA-beta-gal) marker. No SA-beta-gal staining was observed in control cartilage regardless of patient age. However, SA-beta-gal staining was observed in damaged OA cartilage adjacent to the lesion. Moreover, a greater percentage of SA-beta-gal positive cells were detected in cultured chondrocytes obtained from lesion-adjacent sites. Mean telomere length was shorter in cells located at these areas. In addition, the expression of collagenases was altered in OA cartilage [44].

20.4.3 Effects of ROS on Cartilage Matrix

Cartilage is a flexible connective tissue composed of specialized cells called chondrocytes and large areas of extracellular matrix, which is mainly composed of type II collagen, proteoglycans, and elastin. Reactive oxygen species contribute to cartilage degradation by different mechanisms including inhibition of matrix synthesis, affecting the cell migration and chondrocyte sensitivity to growth factors, directly by matrix breakdown, and by activating the expression of matrix-degrading enzymes.

The direct exposure of chondrocytes to •NO or H_2O_2 or $ONOO^-$ inhibits proteoglycan synthesis. Furthermore, the treatment of chondrocytes with the •NOS inhibitor *N*-monomethyl-L-arginine (L-NMMA) restores IL-1 suppressed proteoglycan synthesis, thus indicating that IL-1 induces •NO, which in turn mediates the inhibition of proteoglycan synthesis [45–47]. Additionally, when chondrocytes are transduced with iNOS to release endogenous •NO, matrix synthesis is also decreased.

Regarding type II collagen, extracts of cells treated with IL-1 or SNAP (the •NO donor *S*-nitroso-*N*-acetylpenicillamine) had lower prolyl-hydroxylase activity, and L-NMMA was partially able to revert the effects of IL-1, indicating that prolyl-hydroxylase, an important enzyme in the posttranslational processing of collagen, can be sensitive to •NO [48].

In vitro and in vivo studies have confirmed the role of growth factors in the regulation of cartilage matrix. Insulin-like growth factor I (IGF-I) stimulates the expression and synthesis of collagen type II and proteoglycans. A role for NO in chondrocyte insensitivity to IGF-I is suggested by recent studies showing that cartilage from mice unable to produce NO maintained the capacity to increase proteoglycan synthesis in response to IGF-I. This suggests that the increase in NO is important in the development of chondrocyte insensitivity to the actions of IGF-I [49, 50]. NO not only has a direct catabolic effect on chondrocyte metabolism but could indirectly contribute to an anabolic imbalance as well, inducing insensitivity to the actions of several growth factors in chondrocytes.

The migration of chondrogenic precursors is believed to play a role in cartilage growth and repair. The exposure to NO has been described to impair cartilage repair through inhibition of migration and attachment of chondrocytes to fibronectin, associated to a disrupted assembly of actin filaments [51].

ROS may also cause matrix breakdown due to a direct attack of proteoglycan and collagen molecules by free radicals. It was shown that collagen solutions exposed to superoxide radical failed to form gels normally. So, altering collagen biochemistry could negatively affect the structural or functional integrity of cartilage. In addition, the microfibrillar form of acid-soluble collagen incubated with superoxide ion was degraded by a nonenzymatic reaction. Additionally, HOCl has been described to be able to degrade type I collagen producing smaller fragments with different amino acid composition to that of alpha-chains, but not the synovial fluid hyaluronic acid [52–55].

Furthermore, free radicals can start collagen cleavage, making it more sensitive to proteolytic enzymes [54]. For example, acting like signaling mediators in the chondrocyte α5β1 integrin pathway in response to fibronectin, ROS levels led to the activation of NF-κB and subsequently the production of MMP-13 [56].

Recently, it was suggested that lipid peroxides could play a key role in the structural destabilization of cartilage matrix. The increase of lipid peroxidation activity was observed in a cartilage collagen degradation model comprised by primary rabbit chondrocytes treated with calcium ionophore, leading to the oxidation and loss of collagen matrix. Moreover, this effect could be blocked by vitamin E suggesting that the mechanism of matrix degradation and release is related to lipid peroxidation [57, 58].

20.4.4 ROS and the Synovial Membrane

The synovial membrane in OA is characterized by synovial inflammation, irregular enlargement, and eventually death of synovial cells. The synovial membrane can be affected by degradation products resulting from cartilage breakdown, which contribute to worsen inflammation. In addition, this tissue is a major source of molecules, which mediate cartilage degradation, such as the proinflammatory cytokines IL-1α, β, and TNF-α, matrix-degrading proteases including collagenases and aggrecanases, arachidonic acid derivates like prostaglandin E2 and leukotriene, and NO.

Immunohistochemical analysis as well as in situ hybridization studies demonstrated that iNOS was predominantly expressed in synovial lining cells and, to a lesser extent, in infiltrating mononuclear cells and synovial fibroblasts. Moreover, in vitro stimulation with IL-1, TNF-α, and LPS significantly increased the production of NO in freshly isolated synovial fibroblasts. Thus, synovial cells are able to express high levels of inducible NO synthase (iNOS) but also to produce high amounts of NO during OA [59, 60].

When the amount of ROS in the synovial fluid rise beyond physiological levels, several effects can be observed in the synovial membrane, including depolymerization and degradation of glycosaminoglycan (GAG) and hyaluronan, which results in a loss of the rheologic properties of the joint fluid [61]. Concerning inflammation, it has been shown that nuclear factor (NF)-κB, a master transcriptional regulator of the inflammatory response, can be activated through a process involving reactive oxygen intermediates. In contrast, experimental models have suggested that NOS could exert a protective effect limiting the extent of inflammation. Indeed, in a mouse model of arthritis, genetic disruption of NOS2 exacerbated the severity of the inflammatory process [62].

In other studies, NO has been found to induce synoviocyte death, acting through p53 and caspase 3 [63]. Furthermore, SNP (a NO donor sodium nitroprusside) was found to reduce the survival of synovial fibroblasts and downregulated the expression of the antiapoptotic proteins mcl-1 and bcl-2 [64].

20.4.5 ROS and Subchondral Bone

Subchondral bone lesions appear at early stages of OA and are associated to a decrease in bone volume, indicating that bone resorption might trigger the degradation of the overlying cartilage in OA [65]. Indeed, osteoclasts, bone-specific multinucleated cells, contribute to bone remodeling by breaking down bone matrix, not only under physiologic conditions but also in OA. It has been shown that osteoblastic cells regulate osteoclast formation through the receptor activator of NF-κB (RANK) ligand (RANKL), osteoprotegerin (OPG)/RANK pathway. Signaling through RANKL/RANK enhances the formation, activation, and survival of osteoclasts, whereas OPG prevents RANK engagement by RANKL, thereby protecting bone from excessive resorption. Therefore, the relative concentrations of RANKL and OPG in bone are major determinants of bone mass and strength.

It has been found that ROS act as intracellular signal mediators in the processes of osteoclast differentiation and activation, a fact that abounds on the osteoclast susceptibility to oxidative stress, pointing to a potential therapeutic target [66]. Pretreatment of osteoclasts with antioxidants reduced RANKL-induced activation of protein kinase B (Akt), NF-κB, and extracellular signal-regulated kinase (ERK) [67]. Additionally, the stimulation with RANKL of bone marrow osteoclast precursors of the monocyte–macrophage lineage transiently increased the intracellular level of ROS through a signaling cascade involving TNF receptor-associated factor (TRAF)-6, Rac1, and NADPH oxidase [68]. Again, treatment with antioxidants made these cells insensitive to RANKL.

On the other hand, the effect of bone-acting drugs has been tested in different experimental models of OA, globally showing how modifications in the bone metabolism have an impact on the joint lesion. In brief, OA could be prevented with agents inhibiting bone resorption, like alendronate, and also by restoring bone formation with parathyroid hormone (PTH) [2, 69]. The administration of OPG to mice with experimental OA was found to decrease bone resorption and importantly also cartilage degradation [70].

20.5 Cartilage Antioxidants in Osteoarthritis

Osteoarthritis is often associated with a disruption of the oxidative equilibrium in joint tissues. To counteract ROS toxicity, chondrocytes have a well-coordinated enzymatic antioxidant system formed principally by superoxide dismutases (SODs), catalase (CAT) and glutathione peroxidase (GPX), glutathione reductase, and peroxiredoxins (PRDX).

In a study carried out in 34 osteoarthritic patients who underwent hip or knee arthroplasty, the pre- and postoperative activities of the antioxidant enzymes SOD, CAT, and GPX activity in erythrocytes were assessed. Enzymatic activities were significantly lower in the OA group than in the healthy control group. Moreover, the activity increased after arthroplasty [71].

In a Dunkin–Hartley guinea pig model, which develops spontaneous age-related osteoarthritis (OA) of the knee and other joints, type 2 SOD (SOD2) was associated with the earliest stages of OA. SOD2 expression was decreased in cartilage before and after the development of OA-like lesions [72]. Similarly, in an experimental model in which OA is induced by the surgical transection of the anterior cruciate ligament, serum levels of malondialdehyde (MDA), a marker of lipid peroxidation, significantly correlated with the amounts in serum of C2C neoepitope, which is a degradation product of collagen type II degradation. This finding suggests that chondrocyte lipid peroxidation might mediate collagen degradation during the development of OA [73].

Glutathione reductase regenerates reduced glutathione (GSH) from oxidized glutathione (GSSG), contributing to defend the cell against oxidative stress. Moreover it has been described that a reduction in the antioxidant capacity of

the glutathione system had detrimental effects on the extracellular matrix of articular cartilage. In fact, chondrocytes were more susceptible to cell death when they were incubated with 3-morpholinosydnonimine hydrochloride (SIN-1) to induce oxidative stress in vitro and when depleted of intracellular glutathione [74–76].

The PRDXs directly remove and neutralize H_2O_2, as well as other oxidizing chemicals. The characterization of the crystal structure of human type 5 PRDX (PRDX5) suggested that it might have a broader activity against ROS, as compared to other isoforms. Moreover, human cartilage constitutively expresses the PRDX5, being its transcript considerably upregulated in OA cartilage, probably due to the local high levels of inflammatory cytokines, such as IL-1 and TNF-α, which can act as inductors [77–79].

Interestingly, some authors found that IL-1β enhanced manganese superoxide dismutase (MnSOD) and GPX gene expressions, but decreased Cu^{2+}/Zn^{2+} SOD, extracellular superoxide dismutase (EC-SOD), and CAT gene expressions in bovine chondrocytes. Induction of SOD and MnSOD enzymatic activity and mRNA expression respectively were inhibited by NF-κB inhibitors but not by MAPK inhibitors. IL-1β dysregulates enzymatic antioxidant defenses in chondrocytes, a fact that might lead to an accumulation of H_2O_2 [80].

Growth factors are important in the development, maintenance, and repair of cartilage. It has been found that IGF-I, but not fibroblast growth factor (FGF) or TGF-β1, significantly reduced ROS levels and enhanced the activity of GPX in rat mature cartilage explants and human chondrocytes, suggesting that IGF-I might protect cells against ROS possibly by increasing the activity of GPX [81].

20.5.1 Antioxidants Intake

Nowadays it seems difficult to stop the degenerative process in OA, once there is surface structural damage. As mentioned above, the cartilage is degraded by the activation of several complex pathways that can contribute to the worsening or progression of the OA, like oxidative stress. In this regard, detecting potentially protective factors that could counteract the oxidation pathways involved is a challenge. Dietary supplements might interfere with the pathogenic process, through their ability to block oxidative damage, enhance synthesis of collagen and proteoglycans, and/or increasing the threshold to ROS-dependent cartilage injury.

Tocopherols are a series of organic compounds consisting of various methylated phenols that have vitamin E activity. These compounds are effective antioxidants in blood, breaking free-radical chain reactions of lipid peroxidation [82]. Interestingly, it has been suggested that tocopherols may act in the OA process and help subdue inflammation. In a recent study, it was suggested that tocopherols can inhibit cyclooxygenase-2, as well as iNOS, and subsequently reduce levels of ROS [83]. In addition, levels of α-tocopherol were significantly lower in the synovial fluid of

knees than in serum in patients with inflammatory disease, which suggests that α-tocopherol might be locally consumed at the site of increased oxidation because of the inflammatory activity [84].

It is difficult to analyze what is the actual impact of these components on the incidence and progression of human OA. In general, epidemiologic studies have been carried out in small cohorts and/or at short-term follow-up, therefore showing important limitations. Besides, some methodological problems of these studies include the existence of confounding variables such as sex, ethnicity, and the intake of other antioxidants (vitamin C or selenium) [85–87].

In male STR/1N mice, a mice that develop OA easily because of a varus deformity-induced mechanical overload of the medial tibial plateau, a 12-month feeding with a vitamin E-, C-, A-, B6-, and B2- and selenium-supplemented diet led to an increase in the activity of serum GPX and other antioxidative enzymes. Additionally, the expression of both GPX and Cu^{2+}/Zn^{2+} SOD were upregulated in the articular cartilage of the mice fed with supplemented diet [88].

20.5.2 Estrogens as Antioxidants

20.5.2.1 The Oxidative Stress Is Estrogen Dependent

As mentioned above, some pathophysiologic mechanisms involved in OA are related to estrogen depletion. Indeed, postmenopausal women have a higher incidence rate of OA than age-matched males. An estrogen replacement therapy (ERT) was able to decrease the development of radiographic knee and hip OA, suggesting a protective effect of estrogens in OA [89]. It has been shown that adult joint tissues express estrogen receptors that can be activated, thereby playing a relevant role in maintaining the homeostasis of articular tissues [90].

Since oxidative stress plays an essential role in the pathogenesis of OA, we could speculate that estrogens could help balance the antioxidant reserves of joint cells. In this line, estrogen deficiency induced in rats by surgical removal of the ovaries was associated with increased oxidative stress and DNA damage. Moreover, the serum total antioxidant capacity in OVX rats was restored after the administration of 17β-estradiol, indicating that estrogens have a protective effect on oxidative stress [91].

Similarly, the beneficial effect of estrogens against oxidative stress has been studied in a recent work, in which postmenopausal women taking HRT had lower levels of oxidation markers, such as 8-hydroxy-2-deoxy guanosine, thiobarbituric acid reactive substances, and protein carbonyl. They also showed a higher total antioxidant status compared to women taking no HRT, which suggests that estrogens are able to decrease oxidative damage to both DNA and lipids [92].

Oxidative stress leads to joint aging, characterized by the loss of viable cells due to apoptosis or similar cell mechanisms. Cartilage matrix aging is associated to an increase in water content and the degradation of the cartilage's proteins. This situation

makes the cartilage more prone to degeneration and may trigger osteoarthritis. According to this, it has been showed that mitochondrial oxidative stress is higher in males than in females; hence, the higher levels of estrogens in females protect them against aging, by activating the expression of antioxidant enzymes or by upregulating the expression of antioxidant, longevity-related genes.

Taking into consideration the interaction between antioxidant enzymes (AE) and sexual steroid hormones, some authors monitored the activity of relevant antioxidant enzymes such as SODs, CAT, GPX, glutathione-S-transferase, and glutathione reductase in the brain of female and male rats that were estradiol and progesterone dependent. Their experimental results revealed that the enzyme activity showed a certain dependence on the concentration of both hormones [93].

Taking into account the previously mentioned studies, it seems clear that estrogens bear an antioxidant effect. But, what molecular mechanisms could be involved in this process?

20.5.2.2 Potential Mechanisms of Estrogens in the Regulation of Gene Expression

Estrogens are a group of active molecules that regulate critical cellular signaling pathways participating in cell proliferation, differentiation, and homeostasis. To carry out these functions, estrogens orchestrate the expression of a wide range of genes. Most of these mechanisms follow the activation of the estrogen receptor (ER), which is a ligand-activated enhancer protein that works both as signal transducer and transcription factor. Alternatively, ERs may be activated independently of a hormonal ligand. Both non-genomic ER signaling pathways and genomic actions mediate the stimulation of target gene expression in response to estrogens [13].

The non-genomic responses associated with estrogen signaling are responsible for more rapid effects and trigger the activation of intracellular kinase cascades, such as the extracellular signal-regulated kinase 1/2 (ERK1/2), p38, and phosphatidylinositol-3 kinase (PI3K)/Akt, leading to phosphorylation of ERs and thereby permitting their translocation to the nucleus and then participate as transcriptional regulators.

The genomic action can be estrogen response element (ERE) dependent/independent. In the first case, activated ERs dimerize and bind to specific sequences of DNA in the regulatory region of target genes, which are known as ERE. Subsequently, there is an interaction with coactivator proteins and components of the RNA polymerase II transcription initiation complex to modulate the transcription of target genes. In some of the cases, it has been described that even when EREs sequence differ by one to three nucleotides from the consensus sequence expected to bind ERs, certain genes can be regulated by an estrogenic ERE-dependent action. This is the case of cathepsin D and vascular endothelial growth factor (VEGF). In other situations, the ERE-related sequences display synergic action or overlap with other regulatory elements within the promoter region of the gen as occurs with c-Fos and TGF-α [94, 95].

In contrast, in ERE-independent genomic actions ERs do not directly bind consensus sequences in DNA. Instead, the activated ERs interact with a DNA-bound transcription factor and so form a more stable complex by favoring the recruitment of coactivators. Some examples of this kind of activity are the upregulation of the transcription factors AP-1 and Sp-1.

The AP-1 transcription factor consists of dimers of Fos (Fos, Fra1, Fra2, and FosB) and Jun (Jun, JunB, and JunD) that bind to the AP-1 site of DNA to mediate the production of matrix metalloproteinases and chondrocyte proliferation. To complete the coactivator complex that confers estrogen responsiveness to genes encoding c-Fos, the active ERs need to interact with CREB-binding protein and the glucocorticoid receptor-interacting protein to finally trigger the binding of the activator complex to the AP-1 site of DNA. Similar reactions lead to the transcriptional regulation of collagenases and IGF [94].

The activation of AP-1 is required for the overexpression of matrix metalloproteinases and leads to an uncontrolled cell proliferation, thus contributing to cartilage degradation. It has been shown that ROS production can stimulate c-Fos and c-Jun expression and subsequently enhance AP-1 binding activity in chondrocytes. Proinflammatory cytokines such as IL-1 and TNF-α increase ROS production and induce the expression of c-Fos, effects that were attenuated in presence of antioxidants. Taken together, this information suggests that decreasing ROS levels by antioxidants suppresses cytokine induction of c-Fos expression in bovine and rabbit chondrocytes [34, 96].

Of note, estrogens can exert opposite effects on AP-1 complex depending on the presence or not of certain cytokines such as IL-1. Indeed, estradiol alone supports the formation of the activator complex and consequently favors the binding of the transcription factor to the AP-1 site, driving the production of metalloproteinases. But when it incubated with IL-1, estradiol triggers Fra-1, which is in turn involved in the negative regulation of AP-1-dependent transactivation [97].

In a murine macrophage cell line that produces osteoclasts in response to RANKL, a selective estrogen receptor modulator (SERM) was able to suppress indirectly the transcription factor AP-1 by inhibiting the RANKL-induced ROS generation and thereby suppressing the activation of MAP kinases ERK and JNK [98].

Sp-1 is a member of a large family of zinc finger proteins that is expressed in chondrocytes. The activation of the Sp-1 specially stimulates type II collagen and cartilage oligomeric matrix protein (COMP) synthesis due to the presence of several SP-1 binding sites in the promoter regions of these genes. In OA, levels of proinflammatory cytokines and ROS are high, triggering mechanisms that support the final matrix degradation as well as induce hypertrophic differentiation of chondrocytes. Indeed H_2O_2 downregulates type II collagen and aggrecan expression and increases that of MMP-1 and MMP-3 [99]. Additionally it has been found that oxidized LDL induces a hypertrophic chondrocyte-like phenotype through oxidative stress, indicating that Ox-LDL plays a role in the degeneration of cartilage [100].

Estrogens can activate Sp-1 sites through the MAPK pathways (ERK, JNK, and p38) as well as by the binding of ERs to SP-1, which modulate the transcription of promoter containing Sp-1 sites. In fact, E2 can upregulate vitamin D receptor (VDR)

transcription by regulating the SP-1 transcription factor in intestinal HT29 cells. In this experiment, there was an increase in Sp-1 binding to the respective consensus site in the VDR promoter after the incubation with E2. Thus, chondrocytes treated with E2 could activate the synthesis of type II collagen or aggrecan, since they have Sp-1 consensus elements in their promoter.

Cathepsin D (Cat D) is a lysosomal aspartic proteinase that plays an important role in the degradation of proteins and in apoptotic processes induced by oxidative stress, cytokines, and aging. In OA, Cat D was identified in chondrocytes and synoviocytes localized predominantly in those areas where there was formation of a fibrous connective tissue, pannus, supporting a role for Cat D in extracellular matrix degradation [101, 102]. The inhibition of Cat D in endothelial cells by pepstatin A or genetic knockdown preserved the cells against the H_2O_2-induced apoptosis. Moreover, it was also described that Cat D by modifying the levels of ROS scavenging proteins can promote the formation of ROS and trigger the apoptotic process in these cells [103].

Cat D is an estrogen-responsive enzyme, whose levels are increased following estrogen treatment [104, 105]. Therefore, estrogens could mediate degradation or apoptotic processes induced by oxidative stress in the cartilage by increasing Cat D levels in cartilage or in the synovial membrane. However, estrogens are also known to increase the expression of scavenging proteins that could counterbalance the increase of estrogen-induced Cat D expression.

The transcriptional regulator NF-κB control genes involved in inflammation, proliferation, and apoptosis. In this regard, a sustained activation of NF-κB is associated with pathological conditions. Additionally, ROS can also function as signaling messengers to activate NF-κB transcription factor [106]. Moreover, it has been described that E2 can trigger the NF-κB pathway through non-genomic actions but also inhibit its activation by genomic E2 alternative mechanisms as well as by inducing the expression of protective proteins that can block NF-κB activation [107].

20.6 Conclusions

In order to assess the interaction between antioxidant enzymes (AE) and sexual steroid hormones, some authors monitored the activity of relevant antioxidant enzymes such as SODs, CAT, GPX, glutathione-S-transferase, and glutathione reductase, globally finding that they were estradiol and progesterone dependent.

In addition, estrogens regulate critical cellular signaling pathways participating in cell proliferation, differentiation, and homeostasis in the joint. They act as transcriptional regulators of a variety of genes as well as interact with other transcription factors such as AP-1, Sp-1, and NF-κB. In the particular case of cartilage, ROS trigger the activation of c-Fos and c-Jun and subsequently enhance AP-1 binding activity in chondrocytes. This action drives the increase of matrix metalloproteinases and has also been associated to an uncontrolled cell proliferation that finally contributes to cartilage degradation.

Estradiol triggers Fra-1, which blocks AP-1-dependent transactivation, and could in this way counteract the negative effects of the ROS.

Estradiol has also been found to inhibit the activation of NF-κB, which is in turn activated by ROS.

On the whole, estrogens represent an interesting approach for the treatment of OA, not only because of their favorable direct effects on the chondrocyte metabolism but also for the antioxidative properties found in different experimental system in the last few years.

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ERRATUM

Chapter 17
Oxidative Conditioning and Treatment for Osteoarthritis

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