CHAPTER 21

# OXIDATIVE STRESS IN DIABETES

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**Abstract:** Oxidative stress and diabetes, both Type 1 and Type 2 as well as their related conditions have been extensively studied. As diabetes, obesity and metabolic syndrome have reached at epidemic levels, there is a huge need and effort to understand the detailed molecular mechanisms of the possible redox imbalance, underlying the cause of pathology and progression of the disease. These studies provide new insights at cellular and subcellular levels to design effective clinical interventions. This chapter is intended to emphasize the latest knowledge and current evidence on the role of oxidative stress in diabetes as well as to discuss some key questions that are currently under discussion.

## INTRODUCTION

Diabetes mellitus (DM) is still a major health concern, and on the rise worldwide, with the estimation of reaching approximately 300 million patients very soon.[1] Most of them (around 90%) are Type 2 diabetic, often linked with long years of metabolic syndrome, with no signs of hyperglycemia or overt conditions, combined with obesity and insulin resistance. Even with careful glycemic control, complications including nephropathy, retinopathy, vascular and cardiac conditions and neuropathy still affect many suffering from diabetes with often devastating consequences. Infarction and cardiac problems are leading causes of death among diabetic patients.[1] Microvascular complications can lead to blindness, cataract, numbing, painful neurological conditions and renal failure.[2,3] In the past decades, free radicals and oxidative stress-related mechanisms have been implicated in the pathology and progress of various diseases, with a huge body of literature focusing on DM and its complications.[4-6]

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First we consider only deleterious effects: The free radicals also participate in important signaling mechanisms and cell homeostasis. If the finely tuned redox balance is impaired in the cell, free radical mediated mechanisms and oxidative stress can lead to the pathology of certain disease. The potential role of these molecules and mechanisms has been investigated by several groups, helping to solve important questions about the involvement of oxidative stress in diabetes and its complications. While some of these studies sought the relationship between free radicals and islet damage, the majority has been focusing generally on hyperglycemia as a characteristic feature of the disease[7] and hyperglycemia-induced processes culminating in oxidative stress and diabetic tissue damage in various organs.[8-10] The studies then expanded to metabolic syndrome, obesity and Type 2 diabetes mellitus (T2DM), where a long and constant, chronic proinflammatory condition as well as lipid metabolites can trigger oxidative stress mechanisms. These approaches aimed to solve important questions about the role of oxidative stress in diabetes, metabolic syndrome and related conditions, therefore offering a possible intervention to treat them.

This chapter makes an attempt to give a good, diverse and close-to-complete as possible overview, summarizing the major oxidative stress pathways in diabetes and its complications, lining up the most important, interesting and significant results, achievements and discussion exploring mechanisms or attempting to modulate a redox imbalance. We also briefly highlight the most common direct and indirect approaches and techniques to detect reactive oxygen species (ROS) in diabetic animal models, tissues and biological fluids.

## HYPERGLYCEMIA AND OXIDATIVE STRESS

Hyperglycemia and glucotoxicity are generally considered as primary driving sources in diabetes-related oxidative stress.[7,11] Two major trials noted that hyperglycemia has a key role in clinically relevant tissue damage at the sites of complications in the disease therefore these oxidative stress pathways can represent an important link between high glucose levels and cell/tissue damage.[12,13] First, several different biochemical pathways were suggested to be involved in the pathogenesis, which were seemingly unconnected. The initial investigations go back as early as the 1960s-70s. The well defined mechanisms are: the increased flux of the polyol pathway,[14,15] the activation of protein kinase C (PKC),[16,17] the increased intracellular production of advanced glycation end products (AGEs)[18-20] and the increased activity of the hexosamine pathway.[21-23]

In case of the polyol pathway, the increased glucose flux due to hyperglycemia forces the enzyme aldose reductase to convert the excess glucose to sorbitol. For this conversion, the enzyme consumes NADPH.[24] This creates a problem as NADPH is also an important source to regenerate glutathione (GSH) from oxidized glutathione (GSSG). This process subsequently contributes to oxidative stress due to the depletion of the GSH pool, which is one of the cell's main intracellular defense mechanisms.

Hyperglycemia also activates protein kinase Cs (PKCs) as it increases the levels of diacyl-glycerol.[17] This pathway then has several undesirable effects, including increased TGF-β levels which can culminate into increased fibrosis in the kidney, liver, etc.,[25,26] increased NF-κB activity and therefore proinflammation; most importantly PKC activation leads to the increased activity of NOXes (NADPH oxidases)[27] which again contribute to ROS production and oxidative stress.

The problem with the increased formation and accumulation of AGEs through enzymatic and non-enzymatic glycation is that through these reactions, important proteins can be glycated, including those involved in the regulation of gene transcription, leading to the loss of their function. AGEs also contribute to crosslink formation in the extracellular matrix.[28] From the oxidative stress viewpoint, modified proteins can bind to AGE receptors (RAGE), which in turn activate NF-κB, cytokine formation and proinflammatory pathways.[29,30] Lastly, hyperglycemia increases the undesired flux through the hexosamine pathway, where fructose-6-phosphate gets diverted from normal glycolysis. Here, the end product is UDP-N-acetyl glucosamine, which in turn can modify functionally important proteins on their serine and threonine residues, resulting in aberrant changes in gene expression.[31]

Although several in vitro and in vivo studies offered promising results, inhibiting various pathways in cell cultures and animal models, and showed the beneficiary effects of aldose reductase inhibitors, clinical trials were not encouraging for pharmacologic inhibition of AGEs or PKCs at various sites of diabetic tissues. Later, Brownlee hypothesized that there has to be a common hyperglycemia induced pathway, a unifying mechanism that could offer a solution linking all the above mentioned mechanisms together.[32] Their initial observation was that hyperglycemia increased oxidative stress and ROS production at the sites of diabetic tissue/cell damage.[33] This unifying mechanism was proven to be excess superoxide ($O_2^{-\cdot}$) production from the mitochondria, which ultimately inactivates GAPDH, consequently linking hyperglycemia and oxidative stress as well as the four hyperglycemia-related mechanisms described above.[34,35] The first observation in this mechanism was that in a diabetic cell, more glucose is getting metabolized and oxidized through the tricarboxylic acid (TCA) cycle, due to hyperglycemia.[32] This process consumes a lot more NADH and $FADH_2$ than in a normal cell, which eventually leads to a back up of excess electrons in the mitochondrial electron transport chain at the level of co-enzyme Q.[36] This "reverse electron flow" then passes the electrons to oxygen, resulting in excess $O_2^{-\cdot}$ formation, which leaks mostly at Complex I. If this is true, then a mitochondrial defense enzyme such as manganese superoxide dismutase (MnSOD), or collapsing the mitochondrial membrane potential and proton gradient should remove this excess superoxide. Indeed, in MnSOD transgenic mice it has been shown that hyperglycemia induced oxidative stress is abolished by endothelial cells overexpressing MnSOD or the mitochondrial uncoupling protein UCP-1.[34] More interestingly, the above discussed four pathological pathways are attenuated as well. The same group of researchers later concluded that this superoxide generating process controls all of the four pathways by inhibiting the activity of an important enzyme in glycolysis, glyceraldehyde-3-phosphate dehydrogenase (GAPDH);[33] also MnSOD or UCP-1 overexpression prevented this damage. Because in vitro experiments suggested that the amount of $O_2^{-\cdot}$, necessary for the inhibition, was far higher that can be produced in hyperglycemia, this inhibition was thought to be indirect. As it was shown later, the mechanism acts through poly-ADP-ribose polymerase (PARP) pathway, which modifies GAPDH with these polymers, MnSOD or UCP-1 prevented the process.[35] PARP is probably activated in hyperglycemia when it "senses" single strand breaks in the DNA caused by oxidative stress.[37,38] At the end, reduced activity of GAPDH, contributes to the switch to polyol pathway, activates PKC, increases AGEs formation in the cells and increases the hexosamine pathway. As hyperglycemia is thought to be one of the major factors in microvascular diseases,[12,13] this mechanism provides an elegant link between hyperglycemia-related oxidative stress and the progression of diabetes and its complications (Fig. 1).

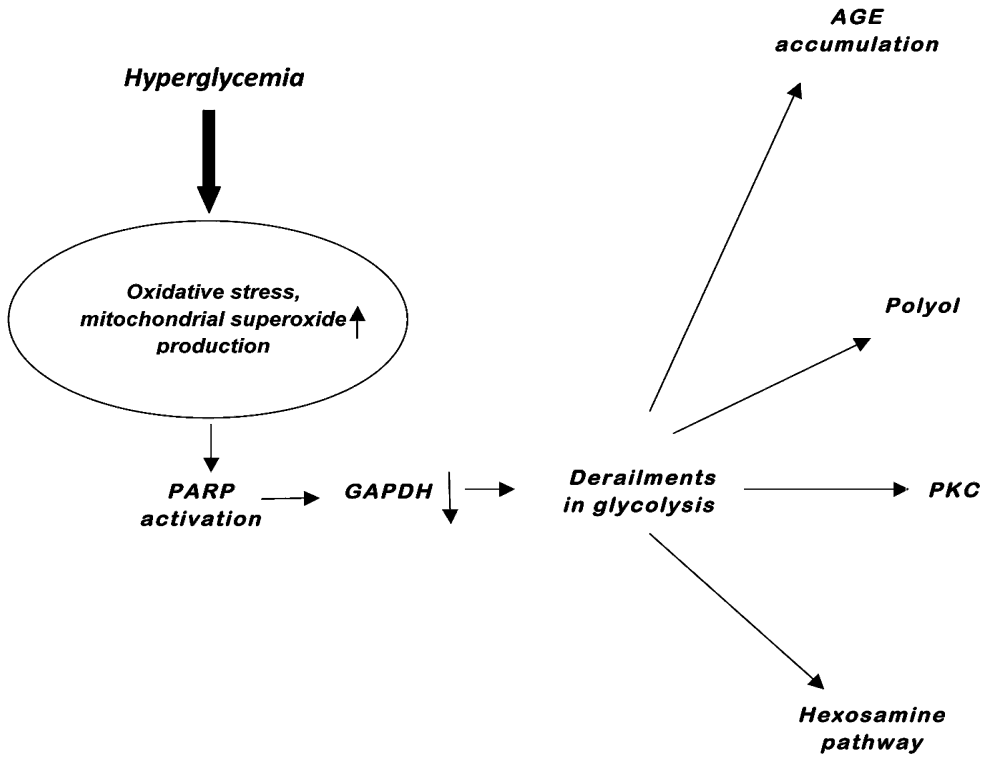


**Figure 1.** Relationship between hyperglycemia, oxidative stress and a central mechanism connecting different pathways together.

A substantial body of work deals with one or more of these individual pathways and their relation to diabetic complications, linking oxidative stress and the potential beneficial applications of novel compounds or inhibitors in these complications. Diabetic nephropathy, retinopathy or peripheral neuropathy are progressively painful conditions associated with oxidative stress. They also correlate one or more of the pathways detailed above such as increased activity of PARP, aldose reductase in the polyol pathway, accumulation of 3-nitrotyrosine, possibly with the contribution of peroxynitrite or increased protein carbonylation.[2,3,39-48] The approaches focus on novel and specific PARP inhibitors, aldose reductase inhibitors to counteract the polyol pathway or peroxynitrite decomposition catalysts as well as inhibitors of the inducible nitric oxide synthase (iNOS).[49-55] Peroxynitrite is a potent biological oxidant and exerts deleterious effects mostly through mediating other free radical reactions due to its decomposition.[56-58] This includes the activation of PARP as well.[59] These studies indicate the possibility of novel treatments to minimize oxidative stress and influence the outcome of a diabetic condition or complication. They also provide evidence that modulating oxidative stress through these pathways attenuates the progression of late complications in almost every organ that is considered the target, such as the peripheral nerves, retina or the kidney. Pathophysiological markers in nephropathy (albuminuria, albumin/creatinine ratio), indicators of neuropathy (tactal allodynia, slow nerve conductivity, sensory neuropathy)

or retinopathy (ischemia/reperfusion injury, permeability, aneurism) could be improved by interfering with the above mentioned pathways.[46,60-62]

As β-cell dysfunction is mainly characteristic of Type 1 diabetes mellitus (T1DM), a relationship between oxidative stress and the pancreas is important to include. The pancreas is different from many organs in that it lacks most of the major antioxidant and defense systems against oxidative stress.[63,64] This of course leaves the organ including the β cells vulnerable to ROS mediated attacks in a redox imbalance, including lipid peroxidation, protein oxidation, DNA damage and modification of signaling pathways. These processes can contribute to the death and dysfunction of the β cells in T1DM and to a certain degree of impairments of various functions in T2DM. In T1DM the process is essentially a cytokine-mediated apoptotic cell death induced by oxidative stress. The major cytokines from the infiltrating immune cells include interleukin 1β (IL-1β) and tumor necrosis factor a (TNFα).[65] In addition to their toxicity, these cytokines are strong activators of the inducible nitric oxide synthase (iNOS).[65] Nitric oxide (NO) produced from iNOS is believed to potentiate the cytokine effect. The activation of this oxidative stress related enzyme can accelerate the toxicity process as the β cells have little or no defense mechanisms and hence cannot survive much longer. In the early phase of development of metabolic syndrome and T2DM, the loss of β cells is slow but becomes significant in a late phase. The role of oxidative stress has been linked with increased levels of fatty acids and glucolipotoxicity as the latter is thought to be an important factor contributing to the development of the disease.[66-68] A novel body of work increasingly focuses on the role of the inflammasome complex and IL-1β and its relation to oxidative stress and T2DM, including pancreatic β-cell dysfunction.[69,70]

## METABOLIC SYNDROME, TYPE 2 DIABETES, INSULIN RESISTANCE AND OXIDATIVE STRESS

In obesity-related metabolic syndrome, with prolonged impaired glucose tolerance and insulin resistance eventually leading to T2DM; hyperglycemia is not necessarily a major factor in relation to oxidative stress or the developing complications. Importantly, this type of disease is often associated with increased risk of cardiovascular diseases, ischemic heart disease, atherosclerosis and hypertension.[71,72] These patients, and especially most of the animal models used in research do not develop overt hyperglycemia for a long time, but are insulin resistant, glucose intolerant, obese and show markers of oxidative stress as well.[73]

The focus of research and the debate is, whether insulin resistance and mitochondrial dysfunction are derived from the same cause, oxidative stress, and whether cellular and mitochondrial oxidative stress is somewhat a contributory factor in the development of insulin resistance in insulin sensitive tissues or it is just a consequence. Research on high fat diet-fed animals, *db/db* mice and Zucker rats, has shown that nutrients in excess and the increased flux of substrates lead to lipid accumulation and glucolipotoxicity.[74,75] This lipid accumulation, moreover, has commonly been associated with insulin resistance and decreased insulin sensitivity in the skeletal muscle.[74] The intracellular lipid metabolites, for example diacylglycerol, ceramides, and fatty acid metabolites are known to inhibit insulin action.[76,77] On the contrary, novel views suggest that this nutrient excess leads to rather excessive β-oxidation and lipid induced mitochondrial stress in the skeletal muscle.[78] How can they play role in oxidative stress? "Overfueling" of the mitochondria and oxidative

stress, as a partial consequence, seems as an attractive factor. In the past years, a popular theory suggested that either inherited or acquired mitochondrial dysfunction may be the underlying cause in lipid accumulation and insulin resistance in the peripherals.[79] It was further suggested that this dysfunction has an impact on the fat oxidizing capacity in the mitochondria, hence contributing to the accumulation of lipids in nonlipid storage tissues. Novel research suggests that, particularly in the skeletal muscle, partially oxidized fatty acid products accumulate on high fat diet.[78] This suggests an oversupply of lipids and overfueling the β-oxidation and TCA cycle, where the outcome is then partially oxidized otherwise non-existing products. These products eventually shift the redox balance and use up the ubiquinone pool, creating a reverse electron flow in the mitochondria, leading to excess $H_2O_2$ emission.

Recently, it was shown in both humans and rodents that high dietary fat intake will lead to this $H_2O_2$ release in the mitochondria of skeletal muscle fibers, indicating a redox shift.[80] These latter phenomena are more compatible with the patterns of mitochondrial bioenergetics and suggest a direct mechanism by which a high lipid supply exceeding metabolic demand leads to increased mitochondrial oxidant emission in obesity and metabolic syndrome (Fig. 2). Related data also suggest that insulin resistance appears a lot earlier than the structural and functional defects of mitochondria.[81,82] One month feeding of high fat high sucrose diet to mice is sufficient to induce glucose intolerance but these animals do not show any significant alterations in mitochondrial structure or dysfunction.[81] An extended diet such as four months of feeding leads to mitochondrial changes in biogenesis and other abnormalities and ROS production. These observations also imply that overt mitochondrial dysfunction is rather a consequence rather than a cause of the altered metabolism.

High fat diet fed rodents with mitochondrially targeted antioxidants or genetically engineered manipulations such as mitochondrially targeted catalase completely preserve insulin sensitivity.[80,83] Similar experiments have been reported by various independent investigators.[80,83,84] In addition numerous groups have found a relationship between oxidative stress and insulin resistance with indirect approaches or general antioxidants.[81,85] As previous results with nonspecific antioxidants had been usually disappointing or contradictory, efforts to develop mitochondria targeted specific compounds to counteract ROS can have the potential to successfully treat metabolic syndrome and related diseases. As Neufer et al suggest, revealing detailed mechanisms in mitochondrial bioenergetics, oxidant production and insulin resistance can provide the basis for the most effective therapies, or simply just can lead to rethinking the strategies for achieving the best metabolic balance by calorie restriction and a less sedentary lifestyle.[80] Note that careful consideration should be given to any mitochondrial approach as it will attenuate the subcellular oxidative processes, it may also change certain hormetic responses or could undesirably affect other pathways related to cellular nutrition and accumulation of glycation products etc.[84]

Several lines of evidence indicates that toxic lipid metabolite accumulation and its shifting to a more oxidized state leads to a number of processes that disrupt insulin signaling, where oxidative stress plays a role (Fig. 2). Most importantly, numerous studies indicate the role of the NF-κB/IKKβ/iNOS pathway linking to oxidative stress, chronic low grade proinflammatory state and lipotoxicity to insulin resistance.[86-88] The first step in these studies was the discovery that targeted disruption of the NF-κB pathway or pharmacological approaches to block it, reverses and prevents insulin resistance and obesity.[89] Further work characterized the details of redox processes and identified iNOS as another key player and often a mediator of inflammatory processes. First attributed to

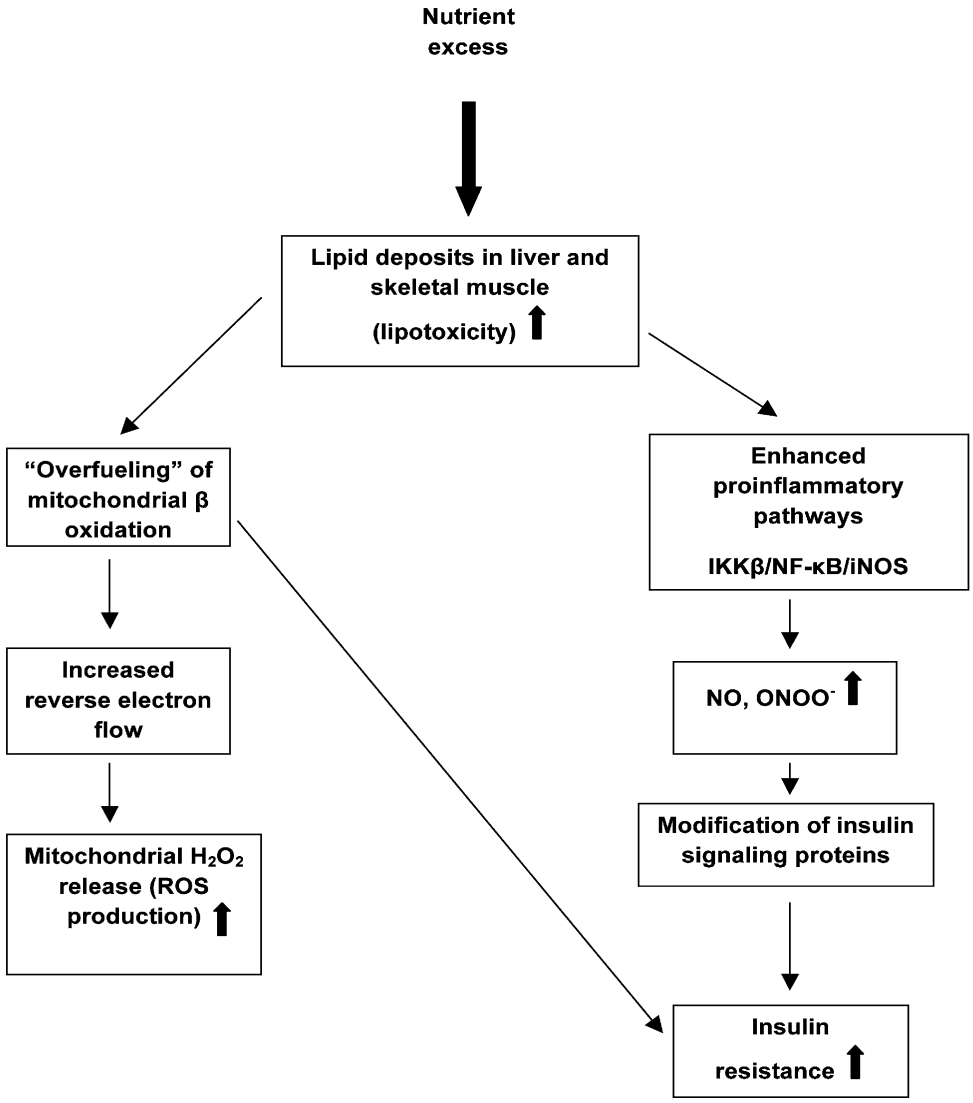


**Figure 2.** Nutrient excess, obesity and metabolic syndrome-related oxidative stress and its relation to insulin resistance as currently viewed.

macrophages, iNOS was shown to be expressed in insulin sensitive tissues such as the adipose tissue or liver and skeletal muscle in rodents and humans. Expression of iNOS is upregulated by several inducers of insulin resistance such as hyperglycemia,[90] free fatty acids[91] or cytokines.[92] Its activity can contribute to increase 3-nitrotyrosine formation possibly through the formation and decomposition or various free radical reactions of the potent oxidant peroxynitrite. If iNOS is uncoupled, it can simultaneously produce NO and $O_2^{\cdot-}$, which may indeed favor the formation of peroxynitrite.[93] The consequence is that such reactions can modify proteins (carbonylation, nitration), altering, enhancing or suppressing

their activity and therefore function.[94-96] Accumulation of toxic lipid products such as 4-hydroxynonenal and lipid radicals can also interfere and initiate such reactions.[97] Due to the altered lipid metabolism this could be an additive and relevant concern in obesity and metabolic syndrome. These modifications may happen on insulin signaling proteins, interfering with their important function such as disrupting the phosphorylation cascade.

Recent work provided some important insights on the role of iNOS and oxidative modifications on insulin signaling proteins. First, *iNOS* –/– mice are resistant to diet induced obesity.[98] Then they improve their whole body insulin sensitivity as well as glucose tolerance when fed on a high fat diet. Furthermore, these knockout obese mice had normal PI3K/Akt phosphorylation signaling in the skeletal muscle. Similar studies have been shown parallel beneficial effects of targeting iNOS in the liver as well.[99] A question therefore arise, if the redox reactions driven by iNOS can directly act on the insulin signaling cascade and if this is the case, what is the nature of these modifications? Circulating free fatty acids can induce iNOS and also trigger hepatic and skeletal insulin resistance as well as a proinflammatory response.

The insulin resistance is characterized by the disruption of the tyrosin/serine phosphorylation cascade involving the insulin receptor β, IRS-1/2, p85 subunit of PI3K and Akt. Therefore, this scenario provides a good model to study the molecular details and the deleterious redox actions of iNOS on insulin signaling. Evidence exists that lipid infusion leads to iNOS overexpression and consistently nitration of the insulin signaling proteins.[100] As this reaction happens on the tyrosine residues, which are also involved in a healthy phosphorylation signal, iNOS is a key player to the lipid induced redox modifications of insulin signaling proteins. Furthermore, iNOS –/– mice, similar to previous studies, show lack of such reaction.[100] Though could be formed in several reactions, nitration of proteins is considered a good indication of oxidative stress in pathological processes related to iNOS activation. It is difficult to explain, if in vivo this nitration happens through peroxynitrite formation, given the compartmentalization and the rather complex nature of the biological systems. On the other hand, in vitro generated peroxynitrite indeed modifies IRS-1 and Akt. In biologically relevant systems, various reactions of peroxynitrite are considered a very efficient and potent way of nitrating protein residues.[101-103] In summary, these results provide pharmacologic as well as genetic evidence for the role of iNOS in oxidative stress associated with metabolic syndrome and other allied diseases.

Achievements in this field give further insights into the complex nature and relation of altered lipid metabolism, the "overfueling" concept, nutrient excess, accumulation of toxic metabolites and resulting cellular and subcellular (mitochondrial) oxidative stress to insulin resistance and obesity.

## DIRECT AND INDIRECT DETECTION OF FREE RADICALS IN DIABETES: BIOMARKER OR MECHANISM?

Proper detection and characterization of free radicals in vivo in tissues or biological fluids in disease models like diabetes has traditionally been challenging. This is due to the nature of these ROS with a very short half life and their rapid reaction with the surrounding molecules such as lipids, proteins, or with each other (annihilation). The options are either to successfully compete with these reactions to capture at least a fraction of the radicals to represent the real in vivo situation or to detect their "fingerprints"—products that are

formed from biomolecules during their reactions with free radicals. Both approaches can and has provided useful information about the nature of free radical reactions and oxidative stress in diabetes.

In the first case, electron spin (paramagnetic) resonance spectroscopy (ESR or EPR) has been the gold standard method of free radical detection, usually combined with spin trapping. In spin trapping, free radicals react with a diamagnetic compound to form paramagnetic adducts which are usually more stable and less reactive and therefore can then be detected with EPR. This method requires certain amount of chemical and biophysical knowledge and experience and expertise to be properly done, including the interpretation of the spectra, the selection of spin trapping compounds and their application, and the avoidance of the formation of possible impostor radicals and artifacts. Interpretation of a complex EPR spectra is often difficult and requires thorough computer simulation. Because of these considerations, biological EPRs have never gained real widespread popularity, although an EPR spectrum is exact in terms of no diamagnetic compounds or side products will ever interfere with the detection.

The first in vivo application dates back to 1979[104] and methods have greatly developed since.[105,106] In vivo applications in diabetes require the spin trap to be nontoxic up to a desirable dose. To compete with fast radical-radical reactions, a huge excess of spin trap is given to the animal model in the study. The greatest limitation of any in vivo trapping is that the rate of radical formation relies on the animal. This is usually a lot lower than any chemical system therefore, state-of-the art detection and sensitivity are the key. As the whole diabetic animal is rarely placed inside the EPR cavity (which is possible, but requires a special low-band EPR spectroscope due to the high dielectric constant of the water in the tissues),[107] the approaches are usually considered as ex vivo detection methods. Ex vivo detection can be carried out by a Folch-type lipid extraction of tissues with chloroform/methanol to confirm lipid radical formation,[104] and hence lipid peroxidation in diabetes, or in biological fluids such as the bile where spin traps can be excreted into and adducts are easily detectable,[106] reflecting mainly the redox state of the liver. The extract or the biological fluid can be placed in a small quartz flat cell in the machine to carry out a sensitive detection. In each case, proper ex vivo controls are necessary to exclude any ex vivo lipid peroxidation or Fenton type reaction in the collection tube. These methods have been successfully used to detect nitric oxide formation in streptozotocin-induced diabetes with the spin trap DETC and iron directly in frozen tissues,[108] an iNOS-mediated lipid free radical production in the same model, detected by the spin trap POBN,[93] or lipid free radical production in ketosis.[109]

To gain more precise details about the mechanism(s) contributing to the free radical production, various inhibitors can be given to diabetic animals to modulate the detectable free radical adduct formation or knockout animals can be used to modulate redox enzyme levels. This way, the role of these enzymes such as NADPH oxidase, xanthine oxidase, NOS isoforms etc., can be studied. Spin trapping has also been successfully carried out in a Type 2 diabetic model, where we detected lipid radical formation, which was attenuated by iNOS inhibition. A ten day rosiglitazone treatment also attenuated the signal, indicating the beneficial role of the drug on an iNOS-mediated lipid free radical production (Stadler et al, data currently under revision).

With a sensitive EPR spectrometer in hand, and with proper biophysical planning and approach, this method can provide vital and novel information about the nature of oxidative stress in diabetic models. To overcome the previously mentioned limitations of this technique, Mason et al introduced immuno-spin trapping as a revolutionary idea to the

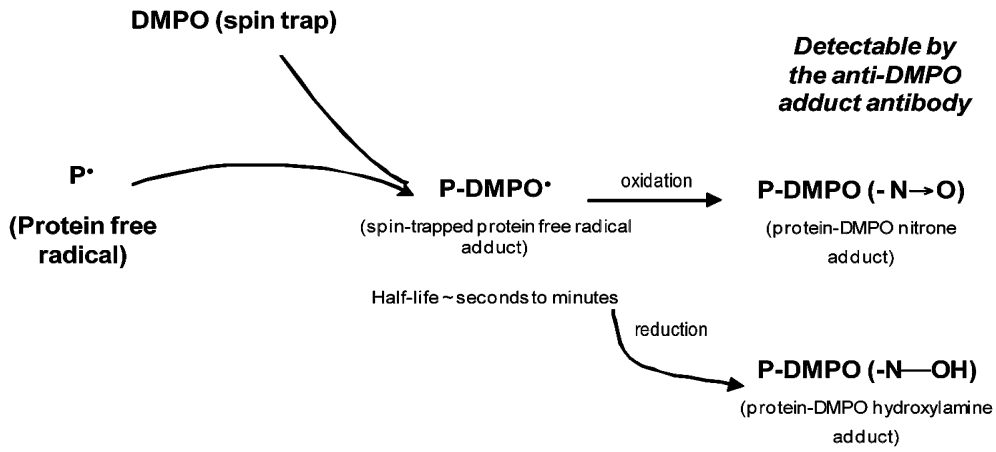


**Figure 3.** Schematic representation of the immuno-spin trapping technique. The approach has been validated in vivo including diabetic models.

field.[110] This method combines the specificity of spin trapping with the sensitivity threshold of the immunological detection. The backbone of the method was the development of the anti-DMPO antibody in rabbits. DMPO is a nitrone spin trap originally designed to detect oxygen-centered radicals mainly in vitro. Due to the low half-life of the adducts, the EPR detection gets complicated in vivo, as these adducts decay rapidly to an EPR silent form. The new method takes this into account and the antibody was designed to recognize the EPR silent DMPO-protein radical adducts (Fig. 3). This way, a more simple ELISA or Western blot run can give beneficial information about the nature of the trapped radicals.[111] The method has been validated in vivo by us and others,[109,112-115] in cells and in tissues, including diabetes or obesity models. DMPO was nontoxic up to a considerably high level which is enough to successfully outcompete other reactions of radicals for example the intracellular GSH levels (10-100 mM DMPO in vitro, 1-2 g/kg DMPO in vivo).[111] For the first time, it has become possible to detect and visualize radicals in space and time, in tissues and cells of these models, and gain valuable information about protein oxidation and protein radicals in the liver after ketosis[109] or in the kidney after 12 and 16 weeks of high fat diet feeding and diet-induced obesity.[116] This tool has tremendous possibilities of future applications in the field of diabetes-related oxidative stress studies, with the antibody being available for research use. An immunoprecipitation study with anti-DMPO antibody combined with powerful mass spectrometry or Western blots can identify specific proteins in the damage process of diabetes, or modification of insulin signaling proteins due to oxidative stress in obesity and T2DM. This can shed light on molecular mechanistic details, providing basis for perhaps new therapeutic approaches.

The traditional and more widespread indirect "fingerprint" methods have also been valuable to contribute to the understanding of various mechanisms underlying the pathology of the disease, or related symptoms and conditions. In this case, the secondary products of a certain free radical pathway are detected, which are more or less specific for the primary reaction. Numerous studies identified the accumulation of these end products in Type 1 and Type 2 diabetic animal models in various tissues and even in human studies.[117-119] These products are usually stable and if specific enough, can be

considered as oxidative stress related hallmarks of a pathological process in diabetes. In the light of the current literature, the biomarkers that have been validated and represent the most consistent fingerprints are:

- 4-hydroxynonenal (4-HNE) adducts indicating lipid peroxidation processes, protein carbonylation and modification of proteins by 4-HNE,
- Isoprostanes for lipid peroxidation,
- 3-nitrotyrosine accumulation indicating protein oxidation and nitration.

Careful consideration should always be given to find if the observed change on a protein or enzyme is a functional alteration and therefore a mechanistic contribution to the pathology or it is only the biomarker of the oxidative stress process in diabetes. For example, 3-nitrotyrosine is not exclusively a result of peroxynitrite formation in diabetes or other diseases, and it can lead to gain or loss of function or even no change in function of enzymes.[96,120] The yield of these reactions in biology is mostly low or site specific. It should therefore always be evaluated whether nitration, carbonylation or other changes play a role in the progression of diabetes or its complications or they are simply correlated with the disease and serve as indicative and reliable markers of oxidative stress. In addition, the use of fluorescent probes and dyes gained tremendous popularity in diabetes related oxidative stress research. The sole use of these probes including 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA), dihydroethidium, rhodamin 123 etc. received increased debate and scrutiny in the literature lately mostly due to their rather complex in vivo chemistry and that most of them require a catalyst or more importantly, they can generate the radical they are originally purported to measure.[121-124] Only with proper controls and coupled with an HPLC method to identify the products, they can provide additional information about radical species in diabetes, if the above mentioned chemical concerns are adequately addressed.

Nevertheless, direct and indirect approaches provide mounting evidence that oxidative stress and free radical species not only correlate with the pathology and damage in diabetic organs but they contribute to significant signaling pathways and mechanisms. A direct EPR or immuno-spin trapping detection and an indirect measurement of damaged biomolecules can complement each other to gain a better picture of the relationship between oxidative stress and the disease process itself. Sophisticated and diverse detection approaches contribute to a better and deeper understanding of these mechanisms, some of which can be a novel target to alleviate oxidative stress related alterations in diabetes.

## CONCLUSION

Innovative research on oxidative stress and diabetes has led to the understanding of the major pathways that are correlated with the pathology and progress of the disease. This warrants further investigation to answer and further clarify the most pressing questions. Which mechanisms contribute as a cause and which are only a consequence of the disease? What are the similarities and differences in each organ affected by oxidative stress? How does mitochondrial dysfunction contribute to the overall picture of the complications, and whether it has any involvement in insulin resistance at all. How oxidative stress can modify important signaling proteins in the canonical insulin signaling cascade? Can we develop effective therapies to target multiple pathways in relation to redox imbalance

to restore a healthy homeostasis? Current and further research aimed to understand the precise redox pathways will help to resolve these questions and contribute to new avenues of treatment to tackle obesity, diabetes and its devastating long term symptoms.

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