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# Physiological regulation of the heat shock response by glutamine: implications for chronic low-grade inflammatory diseases in age-related conditions

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## Abstract

Aging is an intricate process modulated by different molecular and cellular events, such as genome instability, epigenetic and transcriptional changes, molecular damage, cell death and senescence, inflammation, and metabolic dysfunction. Particularly, protein quality control (chaperone systems) tends to be negatively affected by aging, thus leading to cellular senescence in metabolic tissues and, as a consequence, to the increasing dissemination of inflammation throughout the body. The heat shock (HS) response and its associated expression of the 70 kDa family of heat shock proteins (HSP70), which are anti-inflammatory molecular chaperones, are found to be markedly decreased during muscle inactivity and aging, while evidence supports the loss of HSP70 as a key mechanism which may drive muscle atrophy, contractile dysfunction, and reduced regenerative capacity. In addition, abnormal stress response is linked with higher incidence of neurodegenerative diseases as well as low-grade inflammatory diseases that are associated with physical inactivity and obesity. Therefore, strategies to increase or, at least, to maintain the levels of HSP70, and its accompanying HS response to stress, are key to reduce biological cell dysfunctions that occur in aging. In this sense, physical exercise is of note as it is the most powerful inducer of the HS response, comparable only to heat stress and fever-like conditions. On the other hand, the amino acid L-glutamine, whose production within the skeletal muscle and liberation into the blood stream is dependent on muscle activity, is a potentializer of HSP70 expression and HS response, particularly via its entering in hexosamine biosynthetic pathway (HBP). Herein, we discuss the collaborative role of glutamine (and its donors/precursors) and physical exercise (mostly responsible for glutamine release into the circulation) as potential tools to increase HSP70 expression and the HS response in the elderly.

**Keywords:** Aging, Heat shock response, HSP70, Stress response, Inflammation, Exercise, Glutamine, Age-related condition, Hexosamine biosynthetic pathway (HBP)

## Background

With the worldwide increase in longevity, the incidence of a series of chronic degenerative diseases has been rising at the same pace. Particularly in developing countries, where public health systems cannot cope with the desired preventive actions, the situation is dramatic. The concept of healthy aging has been expanding with the rapid growth of the elderly population in developing countries [1]. As a consequence, the economic burden associated with possible loss of independence in elderly people has brought attention to nutritional and exercise interventions as an effective way of delaying the negative effects of aging over cognitive function, fitness status, and metabolic and cardiovascular parameters [1, 2].

In a panoramic view, aging comprises a set of interconnected processes which are modulated by different molecular and cellular events, such as genome instability, epigenetic and transcriptional changes, molecular damage, cell death and senescence, inflammation, and metabolic

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dysfunction [3]. As life expectancy continues to rise, healthspan is not keeping pace because current disease treatment often decreases mortality without preventing or reversing the decline in overall health. Elderly people are sick longer, often coping with multiple chronic diseases simultaneously. Therefore, it is imperative to better understand healthy aging necessary to extend healthspan [4].

During the entire process of aging that may commence during the second decade of life in humans, there is an overall decline of biological functions, which includes deterioration of the cardiovascular, gastrointestinal, urinary, respiratory, endocrine, skeletal muscle, and nervous functions, paralleled by unfavorable changes in body composition (e.g., visceral obesity) that predispose the individual to chronic inflammatory diseases of low grade [5, 6]. Specifically, adipose tissue expansion is linked to a state of chronic unresolved inflammation [7] that, when associated with aging, is often called "inflammaging". This term has been coined to highlight the increased levels of circulating pro-inflammatory molecules that is observed in older people [8] and is a common feature not only in aging but also in obesity and diabetes [9, 10].

Inflammaging is a highly significant risk factor for both morbidity and mortality among elderly people, as most if not all age-related diseases share an inflammatory pathogenesis [11]. Some of highly pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNFα) and interleukin-1β (IL-1β), create an intracellular state of oxidative stress, alongside they induce, per se, the inactivation of the insulin receptor and associated downstream molecules in metabolic tissues, especially in the muscle and adipose tissue [12]. The consequence of chronic receptor inactivation in aging is insulin resistance (IR) [13] and skeletal muscle atrophy, leading to loss of muscle function and, finally, to sarcopenia in many cases [10].

Aging is still an inevitable process observed all around the animal kingdom that involves the accumulation of increased DNA repair malfunctions and enhanced exposure to environmental noxious substances that lead to tissue oxidative stress and cellular dysfunctions. Particularly, in humans, this is aggravated by changes in lifestyle, mainly low physical exercise in coexistence with positive energy balance which determine (avoidable) epigenetic alterations that can transmit age-related metabolic diseases transgenerationally [14]. Such a scenario slowly evolves to accumulated tissue dysfunctions that finally become chronic degenerative diseases. As age-related chronic degenerative diseases are characterized by the progressive loss of protein quality control that leads to chronic inflammation, we shall focus on the gradual decline of the heat shock (HS) response that is observed in such conditions because HS response is crucial to ensure against protein denaturation at the same time it is anti-inflammatory.

## Age-related inflammatory diseases of high prevalence

For the purpose of the present work, we shall discuss the most prevalent and emblematic examples of age-related diseases that share in common the establishment of a chronic state of unresolved inflammation. As the inflammatory stimuli are not withdrawn, such conditions eventually evolve to neurodegenerative diseases, metabolic diseases (e.g., obesities and diabetes mellitus) that complicate into cardiovascular diseases (CVD, including atherosclerosis- and hypertension-based ones), as well as neuromuscular degeneration (e.g., sarcopenia) and systemic inflammatory diseases, such as rheumatoid arthritis (RA) and inflammatory bowel disease (IBD). Additionally, an increasing body of evidence suggests that some of the 116 million US adults who suffer from chronic pain (fibromyalgia, i.e., an intractable widespread pain disorder that is most frequently diagnosed in women [15]) are also at an increased risk for developing age-related diseases prematurely, suffering earlier cognitive and physical decline and experiencing earlier mortality [16].

Neurodegenerative diseases disproportionately affect older individuals and, therefore, disease-related morbidity has increased along with the general increase in longevity [17]. Among these age-related diseases, Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), polyglutamine diseases, amyotrophic lateral sclerosis (ALS), and cerebrovascular disease have drawn a lot of attention due to their irreversibility, lack of effective treatment, and accompanied social and economical burdens [17, 18]. Unfortunately, however, currently available therapies for adult onset neurodegenerative diseases provide symptomatic relief but do not modify disease progression. In common, all the above illnesses have a progressive disturb of protein quality control leading to the accumulation of unfolded proteins and protein aggregates that trigger inflammation in brain tissues [19].

Also mainly due to increasing life expectancy, the population of elderly individuals with rheumatoid arthritis (RA) is expanding [20]. This is noteworthy because people with RA die at a younger age than people without the disease, whereas age exerts an exponentially increasing effect on CVD risk in RA patients [21]. RA is characterized by a sequence of age-dependent degenerative conditions that usually starts with an acute inflammatory reaction, followed by a continuous pro-inflammatory overburden that induces endocrinosenescence, neurosenescence, and senescence of the muscular system [7, 22]. Age-related premature atherosclerosis has also been recognized as an important factor in the morbidity and mortality of patients with systemic lupus erythematosus (SLE), this being attributed to vasculitis and corticosteroid use by these patients [22]. SLE is an autoimmune multi-system disease frequently accompanied by arthritis, fever, serositis, Raynaud's syndrome, lung disease, and neuropsychiatric

symptoms that are very common among elderly patients [23]. SLE, in turn, is frequently observed in aged patients along with psoriasis, another form of immunoimbalance that many times is followed by a rheumatoid component, the psoriasis arthropathy [24].

Contributing significantly to decreased physical activity in the elderly is a debilitating and progressive loss of skeletal muscle function and mass known as sarcopenia [25]. Although its underlying mechanisms are far from being completely settled, studies in model organisms indicate that sarcopenia is driven by a combination of muscle tissue extrinsic and intrinsic factors and that it fundamentally differs from the rapid atrophy of muscles observed following disuse and fasting [25]. Furthermore, decreased ability of muscles to respond to anabolic stimuli is part of the causal mechanisms for muscle loss with aging [26]. Sarcopenia has also origins in intestinal absorption of dietary protein amino acids. Aging per se does not inevitably reduce the anabolic response to a high-quality protein meal, as ingestion of approximately 25–30 g of protein per meal has been found to stimulate muscle protein synthesis in both young and older individuals. However, muscle protein synthesis is blunted in elderly when protein and carbohydrate are co-ingested or when the quantity of protein is less than approximately 20 g per meal [27]. Moreover, although there is a recognizably increased splanchnic first-pass extraction of amino acids in the elderly, muscle protein anabolism has proven to be stimulated by oral amino acids in the elderly as well as in the young [28]. Independent of directly causal factors, the establishment of sarcopenia is closely related to inflammatory processes and is aggravated by the concomitant age-related changes in cytoprotective mechanisms (particularly those involving protein quality control) [29]. In any way, all the above conditions surrounding sarcopenia tend to limit physical activity which, in turn, predisposes the elderly to chronic inflammatory diseases, including obesities and type 2 diabetes mellitus (T2DM) [13, 30].

Chronic inflammatory bowel disease (IBD), in its own, is associated with unresolved inflammation at the level of gut mucosa, being commonly found in the elderly [31]. Dysbiosis and dysregulation of the immune system have been found to play a major role in IBD, leading to enhanced permeability to bacterial components and loss of physiological transport systems. IBD includes Crohn's disease, ulcerative colitis, and the consequent intolerance to certain components of diet (e.g., gluten and lactose). If, on the one hand, protein quality control is not, at present, definitely ascribed as causal or result of chronic IBD, both physiological and pharmacological maneuvers leading to the overexpression of protein chaperones, which avoid the formation of protein aggregates and consequent chronic inflammation, have been found to prevent the development of inflammatory process in the large intestinal mucosa provoked by various damaging factors [32]. In this regard, diet is an extremely important factor, since gut microbiota strongly influences the physiology of the gastrointestinal tract, being dramatically affected by what one chronically eats [33]. Despite multiple effective medical and surgical treatment strategies for adults with Crohn's disease and ulcerative colitis, efficacy studies typically have excluded older subjects. A rapidly aging population and increasing rates of Crohn's and ulcerative colitis make the paucity of data in older adults with IBD an increasingly important clinical issue [31].

In total, aging is associated with impaired resolution of inflammation that perpetuates a series of degenerative diseases in many organs and physiological systems. Such inflammatory diseases have underlying basis on chronic overburden of protein quality control, which leads to the formation of protein aggregates that triggers more inflammatory signals; this eternalizes inflammation that spreads throughout the body [7]. Hence, understanding of intracellular protein quality control system (the chaperone machinery and the HS response) is crucial for adequately treating age-related chronic degenerative diseases.

#### Anti-misfolding protein quality control systems

Aging is associated with increased cellular dysfunctions. Nonetheless, nature evolved a variety of cell defensive strategies aimed to combat these imbalances. Among such cell defenses is the expression of heat shock proteins (HSPs), which are a principal focus of the present article. HSPs have attracted significant attention due to its versatility and range of functions in and out of the cells [34]. The genes encoding HSPs are highly conserved and many of them, as well as their protein products, can be assigned to families on the basis of typical molecular weight [35]. In eukaryotes, different HSP families comprise multiple members that differ in inducibility, intracellular localization, and function [36]. In the context of the present discussion, a review of the diverse HSP types, location, function, and sensitivity to exercise is highly recommended [35, 37, 38]. In the present paper, the 70 kDa family of HSPs (HSP70) will be contemplated.

HSP70 is a cytoprotective and anti-inflammatory molecular chaperone primarily devoted to avoid protein misfolding and to correct unfolded proteins, thus allowing for the proteins homeostasis (i.e., proteostasis) within the cellular compartments [7, 34]. Since proteostasis-threatening situations rapidly evoke a strong expression of HSP70, intracellularly located HSP70 (iHSP70) is, as a consequence, a universal marker of stress. As further discussed, iHSP70 expression is induced by different cell stressors and signals of imminent dangerous situations, such as heat, metabolite deprivation, redox imbalances and, particularly, during (and after) physical exercise, due to

sympathetic nervous system activation, intracellular calcium mobilization, and exercise-induced changes in intracellular pH; all of the nominated situations being powerful inducers of iHSP70 gene expression.

The activation of iHSP70 is critical for the promotion of tissue repair, since the expression of this chaperone, by virtue of avoiding misfolded protein aggregates, confers cytoprotection and also exerts anti-inflammatory effects [39]. Hence, since aging is associated with chronic low-grade inflammation and impaired skeletal muscle repair, the activation of HSP70 expression and its effective response against cellular stress play a key role against cell dysfunction observed in aging. Consequently, any tiny impairment in the ability of cells to respond to stress via iHSP70 expression (i.e., the HS response) may have profound consequences to cell viability, tissue repair and, as a corollary, to organism longevity. Unfortunately, however, aging and age-related chronic inflammatory diseases are marked up by a conspicuous depression of stress-elicited HS response [7]. Additionally, iHSP70 expression is decreased during muscle inactivity and aging, and evidence supports the loss of iHSP70 as a key mechanism which may drive muscle atrophy, contractile dysfunction, and reduced regenerative capacity associated with these conditions. Conversely, several interventions have shown that normal and overexpression of HSP70 are associated with improvements in skeletal muscle atrophic conditions [40]. In fact, upregulation of HSP70 contributes to the maintenance of muscle fiber integrity and facilitates muscle regeneration and recovery [40].

In addition to the HS response, cells further evolved autophagy, which is a cellular strategy to sequester and deliver for degradation to lysosomes, large protein aggregates and whole damaged organelles inaccessible to smaller proteolytic systems in the cell. Therefore, the HS response and macroautophagy represent two ends of the spectrum of cellular protein quality control, with the former being ubiquitous in all living organisms, whereas the latter is restricted to eukaryotic cells [41]. During stress, increased levels of autophagy permit cells to adapt to changing nutritional and energy demands through protein catabolism [42]. Such a self-digestion not only provides nutrients to maintain vital cellular functions during fasting but also can make the cell free of superfluous or damaged organelles, misfolded proteins, and invading microorganisms [43]. Autophagy, a process that is potently triggered by fasting, is now emerging as a central biological pathway that functions to promote health and longevity [43]. Moreover, in animal models, autophagy protects against diseases such as cancer, neurodegenerative disorders, infections, inflammatory diseases, insulin resistance, and aging [43–45].

As a whole, strategies to increase or, at least, to maintain "appropriate" levels of iHSP70 and its accompanying HS response (and autophagy) to stress are key to reduce the biological cell dysfunctions that occur in aging. Following that, physical exercise, which is the most powerful physiological inducer of iHSP70 expression, compared only to heat stress and fever-related conditions [34, 46–48], is considered, at the same time, the best solution to unfasten this perceived Gordian knot (an intractable problem that may be solved by "thinking outside the box") of senescence-associated chronic inflammatory diseases, as recently suggested [7]. In addition to that, several studies have reported that the amino acid L-glutamine (thereafter referred to as glutamine) strongly enhances the HS response by acting as a potentializer of iHSP70 expression [49, 50], mainly via the hexosamine biosynthetic pathway (HBP) [51–56]. Glutamine is important for protein quality control also by stimulating autophagy, so also avoiding the formation of undesirable protein aggregates [57]. Inasmuch as glutamine is liberated into the blood by active skeletal muscle, it follows that physical exercise may warrant a healthy HS response also via glutamine metabolism. Ergo, we shall discuss herein the possible collaborative role of glutamine (and its donors/precursors) and physical exercise as potential tools to increase HSP70 expression in the elderly, thus reverting age-associated degenerative diseases of inflammatory nature.

## Heat shock proteins and the heat shock response

Mammalians developed a range of adaptations to survive in the presence of acutely and chronically non-lethal stressful situations [58]. Among these adaptations, the HS response (a type of stress response) is striking because it is probably the most highly conserved genetic system ever known, existing in every organism in which it has been sought, from archaebacteria to eubacteria, from plants to animals [59, 60]. The HS response evolved to adapt organisms appropriately against several stressful insults, whether from heat, cold, oxidation, free radicals, toxins, hypoxia, or metabolic stress [61]. And, however impressive as it may seem, the HS response is also recruited from other branches of metabolism very far from proteostasis, at least a priori. This is the case of inflammation, energy preservation, and immune responses [7]. Impaired HS response, however, is a common feature in several age-related conditions associated with inflammation, such as T1DM and T2DM, aging, and obesity [61–64].

Members of the 70 kDa family of heat shock proteins (HSP70) mediate cytoprotective stress responses [63]. Within the HSP70 family, the constitutive heat shock cognate, HSC70 (or HSP73, encoded by the *HSPA8* gene in humans), and its inducible form (HSP72, encoded by *HSPA1A*) have received more attention for their ubiquity and high level of expression. Although iHSP70 had been serendipitously discovered in heat-shocked *Drosophila*

*busckii* cells by Prof. Ferruccio Ritossa in 1962 [65], HSP70 expression is associated with a variety of homeostatically stressful situations, not only heat [66]. It is noteworthy that the inducible expression of HSP72 is impressively and highly conserved in nature from bacteria to humans: in order to manage on chaperone and cytoprotective intracellular functions, at least 13 genes were identified in humans that are responsible for HSP70 family coding [35, 38]. HSP72 (*HSPA1A* gene) is inducible during cell stress and represents the most abundant of all HSPs, accounting for 1–2 % (!) of intracellular proteins [37], including in skeletal muscle [67]. As a molecular chaperone, the intracellular HSP72 protein (referred thereafter simply as iHSP70) can interact with other proteins (either unfolded, in non-native state or in stress-denatured conformations) avoiding inappropriate interactions, impeding formation of protein aggregates, and leading to the degradation of damaged proteins, as well as helping the correct refolding of proteins [67]. Other functions include protein translocation [68], anti-apoptosis, [69] and anti-inflammatory responses, the latter via HS response-dependent blockade of NF-κB transcription factor downstream pathways [7, 70]. More recently, HSP roles have been expanded to include control of cell signaling [71], modulation of immune responses [72–74], in chronic diseases such as diabetes, obesity, and insulin resistance [12, 63]. Figure 1 depicts the principal known functions of HSP70.

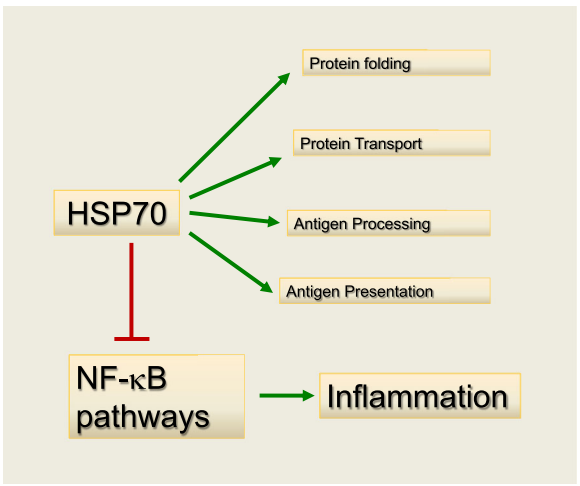


**Fig. 1** General heat shock protein functions. Heat shock proteins (HSPs), particularly those from the 70 kDa family (HSP70) are molecular chaperones whose principal function is to assist in protein folding and to correct misfolded proteins to avoid intracellular inflammatory signals elicited by protein aggregates. As chaperones, HSP70s attach to and help in protein transport from intracellular compartments to others, which is also observed when HSP70s facilitate antigen processing and presentation by antigen-presenting cells. On the other hand, HSP70s "protect" also the inhibitor of kappaB (IκB) proteins thus preventing its phosphorylation by IκB kinases (IKK). Consequently, HSP70s blunt nuclear factor κB (NF-κB)-dependent inflammatory pathways, so that the activation of HSP70 is anti-inflammatory as a corollary

The synthesis of iHSP70 in mammalian cells is mainly controlled by the heat shock transcription factor-1 (HSF1), while the activation of HSF1, necessary for full cytoprotective HS response, involves a multistep mechanism that comprises its phosphorylation, trimerization, nuclear translocation, and DNA binding to the heat shock elements (HSE) located at the promoter regions of targeted heat shock genes [63, 74, 75]. At rest, HSF1 is inactive in a monomeric state bound to iHSP70 molecules, located in the cytosol. Under stress conditions (i.e., upon any shift from cellular homeostasis), particularly in the presence of denatured proteins or protein threatening conditions (e.g., heat, heavy metals), iHSP70 releases HSF1 and subsequently binds to denatured proteins, acting as a molecular chaperone (aiding protein refolding), eventually releasing HSF which is then able to trigger the synthesis of more iHSP70 molecules. Serine-phosphorylation and trimerization of HSF1 induces enhanced HSF1 DNA-binding affinity for the *cis*-acting regulatory domains (the HSE described above) in target genes, inducing the expression of more iHSP70 (HSP72, indeed) molecules which, in turn, enhances cellular stress responses, and defense capacity [37]. As HSF1 is the primary regulator of the anti-inflammatory HS response, low expression of HSF1 is associated with a number of human pathologies of inflammatory nature, including T2DM [47], obesity-related fatty liver disease [76], and neurodegenerative diseases [63].

## The heat shock response in inflammation and its resolution

Age-related chronic inflammatory diseases, such as systemic inflammatory diseases (e.g., RA, IBD), obesities and their associated co-morbidities, T2DM, and CVD, as well as neurodegenerative and neuromuscular diseases, share in common a state of unresolved inflammation throughout body tissues. This points to the question as to why inflammatory responses do not achieve an expected physiological resolution phase in aging and/or in age-related degenerative diseases. However, inflammation evolved to be an acute response, as physiological mechanisms to cope with ad infinitum inflammatory responses were not predicted (naturally selected).

### Anti-inflammatory role of intracellular HSP70

During the activation of an inflammatory response, the production of pro-inflammatory arachidonic acid-derived prostaglandins (PGs), as well as other lipid mediators and vasoactive compounds, take place. This increases vascular permeability, allowing the arrival and activation of inflammatory cells and tissue repair [77]. In fact, maximal cyclooxygenase-2 (COX-2)-dependent prostaglandin $E_2$

($PGE_2$) production occurs at 2 h after challenge, whereas COX-2 expression is much higher at 48 h, but pro-inflammatory $PGE_2$ production is much lower [78]. This strongly suggests the existence of some metabolic deviation of arachidonic acid metabolites toward another mediator. Additionally, and perhaps unexpectedly, both selective COX-2 inhibitors (COXIBs) and dual COX-1/COX-2 (classic NSAID) blockers inhibit early phase but, at the same time, remarkably exacerbate inflammation at mononuclear late stage (48 h), which prevents the resolution phase of inflammation [78]. Therefore, the so-called "bad COX", responsible for the production of pro-inflammatory eicosanoids and cytokines, is not that "bad" since it is crucial for the resolution of inflammation [79].

During the entire inflammatory response (including its resolution phase), there is a finely orchestrated expression of inducible proteins centered at nuclear transcription factors from the kappa light chain enhancer of activated B cells (κB) family (NF-κB), [80], which propel inflammation during the challenging phase but, simultaneously, prepares its resolution. At the beginning of an inflammatory response and under the control of activated NF-κB transcription factors, inducible enzymes (including COX-2) drive the synthesis of $PGE_2$, which induces fever by changing bodily thermoneutral range upwardly. As a consequence of elevation in core temperature, the highly evolutionarily conserved HS response initiates the activation of a transcriptional program based on the activation of the heat shock transcription factor HSF1 [81]. The chief impact of HSF1 activation is the elevated production of HSPs whose major representative is HSP70. Small heat shock proteins induced by fever, such as HSP27, also contribute to cytoprotection [82, 83].

Since heat stress faced during fever episodes stimulates HSF1-induced HSP70 expression, cells become protected against proteotoxic stress that could emerge from heat-induced protein denaturation. Therefore, HS response supports proteostasis (protein homeostasis) and cytoprotection [75]. Additionally, hyperthermia enhances toll-like receptor-4 (TLR4) expression and downstream signaling in vivo [84], whereas activation of TLR2, TLR3, and TLR4 acts synergistically with fever-associated hyperthermia to induce HSP70 expression and release to the extracellular space both in vivo and in vitro [85]. This means that, under microbial pathogenous attack, fever is even more protective because bacterial lipopolysaccharides (LPS) may signal to phagocytes via TLRs more efficiently, thus enhancing their microbicidal capacity.

Aside being a molecular chaperone which works to reduce the formation of protein aggregates and reverse protein denaturation, iHSP70 is able to associate with the complex formed by NF-κB with its inhibitor (IκB) thus impeding NF-κB translocation to the nucleus [86]. Therefore, the HS response is anti-inflammatory in its very nature. Moreover, $PGE_2$ and other PGs produced during the onset of inflammation may be converted into their respective electrophilic dehydration products, such as $PGA_2$ and J-family PGs, which are α,β-unsaturated cyclopentenone prostaglandins (cyPGs) possessing strong anti-inflammatory activities in vitro as well as in vivo [87]. As demonstrated in the classic studies by Prof. M. Gabriella Santoro's group in Italy, this is partially dependent on cyPG-dependent inhibition of NF-κB activation, because cyPGs are the strongest physiological inducers of HSP70 comparable only to HS itself and exercise. In other words, cyPG anti-inflammatory action is maximal only if HSP70 expression is elevated [88]. Finally, HS-activated HSF1 directly controls COX-2 transcription, thus allowing for high-throughput $PGE_2$ production during inflammation [89], whether to exacerbate ($PGE_2$ itself) or resolve inflammation ($PGE_2$ conversion into $PGA_2$, a cyPG).

Inasmuch as cyPGs are strong electrophiles, they promptly conjugate with reactive thiols present in cysteine moieties of proteins and peptides (e.g., glutathione, GSH) via Michael addition reactions [90]. Because of this, cyPGs are inflammation-derived anti-inflammatory compounds by virtue of directly inhibiting, at Cys179, IκB kinase-β (IKKβ), which, in turn, phosphorylates IκB leading to NF-κB activation during inflammation [91]. These eicosanoids block NF-κB activity also directly after Michael addition reaction at Cys62 of p50 and Cys38 of p65 subunits of NF-κB [87]. At the same time, the increase in cyPG intracellular contents during inflammation momentarily creates a state of redox imbalance because cyPGs briefly reduce intracellular GSH contents in every cell type and tissue so far tested [92–95] and react with Nrf2-transcription factor repressor Keap1 [96], thus triggering the expression of a number of redox-protective genes, such as γ-glutamylcysteine synthetase (γ-GCS), glutathione *S*-transferases (GST), glutathione disulfide (GSSG) reductase, glutamine synthetase, glucose-6-phosphate dehydrogenase (G6PDH), and superoxide dismutase [87, 97, 98]. Therefore, besides inducing HSP70 expression, cyPG, at physiological concentrations, are cytoprotective by activating redox-sensitive gene expression. Please, see Fig. 2, which summarizes HS response during inflammation and its physiological resolution.

HSP70 blocks NF-κB activation at different levels. For instance, HSP70 impedes the phosphorylation of IκBs, while heat-induced HSP70 protein molecules are able to directly bind to IκB kinase gamma (IKKγ) thus inhibiting TNFα-induced apoptosis [99]. The perception that HSP70 might act intracellularly as a suppressor of NF-κB pathways has been raised after a number of seminal discoveries in which HSP70 was intentionally induced, such as the inhibition of TNFα-induced activation of phospholipase $A_2$ in murine fibrosarcoma cells [100],

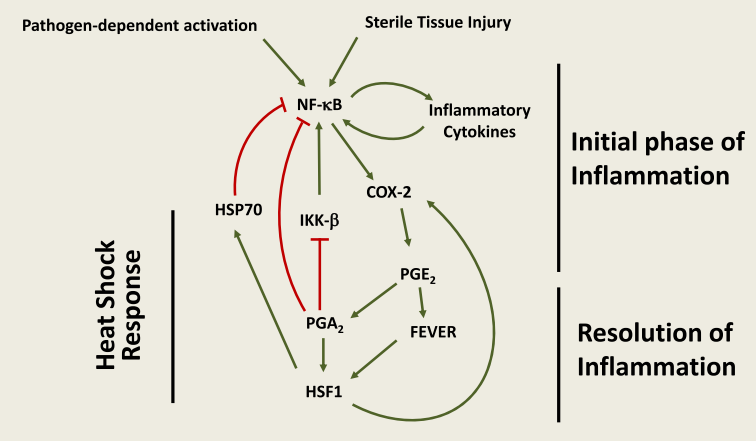


**Fig. 2** Physiology of the heat shock response during inflammation and its resolution. Injury- and pathogen-initiated acute inflammatory processes trigger a variety of signals that lead to the activation of the nuclear factor NF-κB, the master regulator of inducible production of cytokines and inflammatory enzymes, such as cyclooxygenase-2 (COX-2). At the same time, such noxious signals stimulate the liberation of arachidonic acid from cellular stores toward the cytosol where it is converted into inflammatory prostaglandins (PGs), among them is $PGE_2$, which induces hyperthermia. Fever, in turn, activates heat shock factor-1 (HSF1), leading to the expression of anti-inflammatory and cytoprotective 70 kDa heat shock proteins (HSP70) that turn off NF-κB downstream pathways. At the same time, fever-activated HSF1 induces the expression of more COX-2 molecules, which in turn exacerbate $PGE_2$ production. As the inflammation progresses over 24 to 48 h, $PGE_2$ and other prostanoids may be converted into cyclopentenone PGs (cyPGs), such as $PGA_2$. CyPGs are the strongest inducers of HSF1 activation along with heat shock, so that inflammation can be resolved within its own. *Arrows* indicate stimulation of the indicated pathways while *broken lines* represent inhibition. This illustration was redesigned and adapted from [7]

the suppression of astroglial inducible nitric oxide synthase (iNOS, encoded by the NF-κB-inducible *NOS2* gene) expression, paralleled by decreased NF-κB activation [101], and the protection of rat hepatocytes from TNFα-induced apoptosis by treating cells with the nitric oxide (NO)-donor SNAP, which reacts with intracellular GSH molecules generating *S*-nitrosoglutathione (SNOG) that induces HSP70, and, consequently, HSP70 expression [102]. iHSP70 confers also protection against sepsis-related circulatory fatality via the inhibition of iNOS (*NOS2*) gene expression in the rostral ventrolateral medulla through the prevention of NF-κB activation, inhibition of IκB kinase activation and consequent inhibition of IκB degradation [103]. This is corroborated by the finding that iHSP70 assembles with liver NF-κB/IκB complex in the cytosol thus impeding further transcription of NF-κB-depending TNFα and NOS-2 genes that worsen sepsis in rats [86]. This may also be unequivocally demonstrated by treating cells or tissues with HSP70 antisense oligonucleotides that completely reverses the beneficial NF-κB-inhibiting effect of heat shock and inducible HSP70 expression (see, for instance, ref. [102, 103]). Hence, HSP70 is anti-inflammatory per se, when intracellularly located.

Another striking effect of HSP70 is the inhibition of apoptosis. Caspases form an apoptotic cascade by an intrinsic pathway characterized by the release of mitochondrial pro-apoptotic factors into the cytosol, while stimulation of cell surface receptors triggers the extrinsic pathway by external signaling factors that may induce the apoptotic process. The inhibitory potential of iHSP70 over apoptosis occurs via many intracellular downstream pathways (e.g., JNK, NF-κB, and Akt), which are both directly and indirectly blocked by iHSP70 either, besides the inhibition of Bcl-2 release from mitochondria. Together, these mechanisms are responsible for iHSP70 anti-apoptotic function in cells under stress conditions [104]. Therefore, iHSP is both cytoprotective and anti-inflammatory by avoiding protein denaturation and excessive NF-κB activation which may be damaging to the cells [105]. Figure 3 highlights the steps where HS response obliterates NF-κB-elicited downstream inflammatory signals.

Finally, it is noteworthy that HSP70 expression and regulation of the HS response are both modulated by another key player, the nicotinamide adenine dinucleotide ($NAD^+$)-dependent protein deacetylase of class III family sirtuin-1 (SIRT1). Multiple studies that have imputed a role for SIRT1 to the activation of HSF1 and, consequently, the enhanced synthesis of molecular chaperones, including iHSP70, in order to regulate the stability and function of intracellular proteins. It has been shown that activation of SIRT1 prolongs HSF1 binding to the promoter (HSE) regions of heat shock genes by maintaining HSF1 in a deacetylated and DNA-binding competent state [106], so enhancing the transcription of molecular chaperones such as HSP72 and HSP25 [106, 107]. The importance of SIRT1 for the chaperone machinery is clearly demonstrated by SIRT1 knockdown, which attenuates heat shock

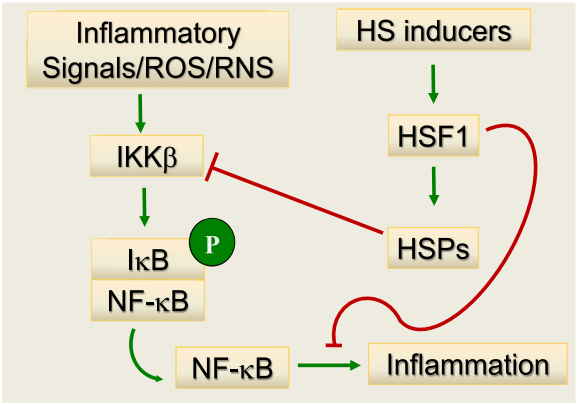


**Fig. 3** Anti-inflammatory profile of the heat shock response. If, on the one hand, inflammatory signals and their consequent (and sometime causal) formation of reactive oxygen and nitrogen species (ROS/RNS) activate NF-κB downstream inflammatory pathways, on the other hand, heat shock (HS) response inducers (e.g., fever, hyperthermia, exercise) block inflammation. Accordingly, the above inflammatory signals activate IKKβ which phosphorylate IκB proteins leading to NF-κB-dependent production of inflammatory cytokines and related proteins. However, HS response can completely revert NF-κB-elicited pathways, as heat shock factor-1 (HSF1) impedes transcription of NF-κB-dependent genes whereas HSP70 may block IKKβ activity

response [108]. Conversely, it has recently been demonstrated that whole-body heat shock treatment of high-fat diet (HFD)-fed rats reverses insulin resistance-induced vascular defects while increasing SIRT1 expression/activity in parallel [109]. Additionally and strikingly, SIRT1 physically interacts with the RelA/p65 subunit of NF-κB and inhibits transcription of inflammatory genes by deacetylating RelA/p65 at Lys310 [110], whereas SIRT1 has recently found to directly inhibit NLRP3 inflammasome activation [111].

### Extracellular HSP70 and the role of HSP70 balance between intra- and extracellular space in inflammation

After both acute and chronic stressful situations, HSPs can also be found in the extracellular milieu (eHSP70). This happens following a finely concerted secretion, mainly from lymphocytes and tissues from the hepatosplanchnic territories [34, 112]. In general, eHSP70 acts as an alert signal to physiological systems for the presence of homeostatically threatening situations [105]. eHSP70 is associated with the activation of the immune system and inflammation [113]. For example, eHSP70 has been reported to stimulate neutrophil microbicidal capacity [114] and chemotaxis [115] and recruitment of natural killer (NK) cells [116], as well as cytokine production by immune cells [73, 117]. In addition, eHSP70 has been recently hypothesized to be involved in motor neuron cell protection under stress conditions and neurodegenerative diseases [63, 118]. However, contrarily to that which occurs when HSP70 is within the intracellular space (iHSP70), when exported to the extracellular space (eHSP70), it functions as a stress signaling and pro-inflammatory molecule possibly by acting via TLR2 and TLR4 (see, for instance, ref. [85]). eHSP70 has been reported to be negatively correlated with intramuscular HSP70 content in obesity and diabetes [47]. Indeed, elevated levels of eHSP70 are positively associated with insulin resistance in elderly volunteers and induce TLR-dependent β cell failure [62]. Because of this, detection of plasma eHSP70 when not linked to any acute stress (e.g., exercise, α-adrenergic stimulation) is reputed as a marker of inflammation-associated chronic stress [34, 47, 112].

Secretion of eHSP70 by non-canonical mechanisms (exosomes) has been documented in lymphocytes, macrophages, epithelial cells, dendritic cells, neuronal cells, and hepatocytes [119]. Once secreted, eHSP70 can bind to TLR2 and TLR4 in a variety of cells, leading to the activation of pro-inflammatory pathways via MyD88 and TIRAP that signal downstream to NF-κB via IRAK4, TRAF6, and IKK, and inducing JNK activation via MEKK4/7 [120, 121]. High-affinity binding of eHSP70 to other surface receptors, including LRP/CD91, CD40, scavenger receptors, and c-type lectins, has also been described [72].

The signal triggered by eHSP70 promotes typical immunoinflammatory responses directed to the combat of infections and bacterial infiltration through the production and release of nitric oxide (NO) and pro-inflammatory cytokines, such as TNFα and IL-1β [24]. Furthermore, eHSP70 responses are positively associated with classical inflammatory parameters such as C-reactive protein (CRP), fibrinogen, and monocyte counts [122], being commonly found in clinical situations in which danger signaling to immune system must be required [119]. Indeed, increased serum eHSP70 has been reported in chronic and age-related diseases [123–125]. In addition, serum eHSP70 levels were found to be higher in long-term (>5 years) T2DM patients as compared to newly diagnosed ones [126]. Interestingly, during conditions in which individuals are chronically exposed to elevated eHSP70 levels (e.g., obesity, T2DM), a marked reduction in HSF1 and iHSP70 contents in skeletal muscle and adipose tissue is observed [12, 47, 76, 127, 128].

Ser307 phosphorylation of insulin receptor substrate-1 (IRS-1) is a physiological feedback mechanism to block insulin/IGF1 signaling pathways [128] that can be triggered by inflammatory cytokines via IKKs. This process is inhibitable by cyPGs [129], which, as discussed above, are powerful anti-inflammatory autacoids possessing iHSP70-inducing capacity [87, 91]. eHSP70-elicited TLR4 expression and signaling is increased in obese and T2DM subjects, an effect that may explain the high basal rate of MAPK phosphorylation and NF-κB activation found in these patients [130–133]. On the other hand, the above findings also help to explain why inhibition (or absence)

of TLR4 confers protection against insulin resistance in skeletal muscle [134], adipose tissue, and liver [135, 136]. Moreover, eHSP70 is positively correlated with insulin resistance and inflammation in elderly people, being ascribed as a key player in the impairment of insulin signaling in the skeletal muscle that occurs with advanced age and in T2DM [62]. In addition, chronic exposure of β cells and islets to increased concentrations of eHSP70 results in β cell death and altered cell bioenergetics, a phenomenon that, apparently, is mediated through TLR-2 and 4 activation [62]. Since, in T1DM, there is a dramatic increase in plasma eHSP70 and, in T2DM and aging, there is a slow chronic increase in the concentration of this protein in the plasma, we have deduced that chronic exposure of pancreatic β cells to eHSP70 may lead to β cell failure and loss of functional integrity in vivo [34].

Based on the above discussion, it is sensible to state that, while iHSP70 is clearly protective, anti-apoptotic, anti-inflammatory, and associated with normal insulin sensitivity, eHSP70 is related to a pro-inflammatory response, decreased expression of the anti-inflammatory iHSP70, and reduced insulin sensitivity. Because of this, we have suggested that the ratio of compartmental distributions of HSP70 between extra and intracellular locations may determine the outcome of inflammation in chronic degenerative diseases. In a recent study, our group observed that the ratio between plasma eHSP70 and cellular iHSP70 in lymphocytes from rats submitted to different loads of acute exercise (an acute stressful situation) can indicate the inflammatory status [34]. Indeed, extracellular to intracellular HSP70 ratio index (H-index) measured in peripheral blood mononuclear cells (PBMC) in relation to serum values has been recently assumed as novel and overall index of immunoinflammatory status of an individual [34, 39, 105, 112]. The rationale for this is that the higher eHSP70 amounts, the more inflammatory signals are coming into play because eHSP70 is pro-inflammatory in nature. On the other hand, for any specific situation, the more the cells are able to respond to stressful stimuli by enhancing iHSP70, the more such cells are in a state of anti-inflammation and cytoprotection. Therefore, if one takes $R_c = [eHSP70]_c/[iHSP70]_c$ as the HSP70 ratio in a control situation, whatever the techniques used to assess each eHSP70 and iHSP70, then H-index can be calculated as the quotient of any $R_j = [eHSP70]_j/[iHSP70]_j$ by $R_c$, which will be therefore considered as the unity ($R_c = 1$), normalizing all the remaining results in this situation "j". Hence, H-index = $R_j/R_c$ may allow for the comparisons between any stressful situation "j" and the situation assumed as the control one.

H-index can be applied to estimate immunoinflammatory status in many different situations, such as immune responses, CVD, neurodegenerative diseases, diabetes, and immunological impacts of exercise. For example, as previously argued [34], assuming H-index for the controls (resting, unstimulated) as the unity, exercise produces a shift in H-indices to up to ca. 5, which is paralleled by an elevation in inflammatory markers and stimulation of cell proliferation. H values higher than 5 denote an exacerbated pro-inflammatory response. Conversely, H-indices between 0 and 1 indicate a predominantly anti-inflammatory status. Thus, changes in H-index emerge as a potentially new biomarker for inflammation and as a very sensitive indicator of inflammatory status.

## Suppression of the heat shock response in age-related degenerative conditions associated with chronic inflammation

Several studies have shown that HSP synthesis and the HS response may be negatively affected by aging [137, 138]. This can be clinically assessed with ease by examining HSP70 expression in heat-treated PBMC after an appropriate time [34]. For example, Njemini and colleagues have demonstrated, in human monocytes and lymphocytes, that basal (37 °C) and heat-induced (42 °C) HSP70 expression is reduced with advanced age [137], a behavior that is inversely correlated with higher pro-inflammatory cytokine levels. Later, the same group has demonstrated the age-related increase in basal (unstimulated) levels of iHSP70, iHSP32, and iHSP90 in PBMC from healthy human subjects [138]. In addition, low-grade inflamed patients have higher basal levels of iHSP70, iHSP32, and iHSP90 in PBMC that positively correlate with serum concentrations of inflammatory mediators (CRP and IL-6) [138]. However, while basal levels of iHSP70 may increase due to the effects of pro-inflammatory cytokines and the associated oxidative stress, the essential machinery which should rapidly respond to cellular stress inducing HSP70 expression (i.e., an adequate HS response) is reduced, and the stress response become compromised.

Apparently, basal levels of iHSP70 in metabolic tissues (e.g., skeletal muscle) do not reduce with aging as long as insulin sensitivity is normal; however, if aging is associated with long-term insulin resistance, then basal iHSP70 levels in the muscle tend to reduce [139]. This is supposed to be related to the fact that insulin resistance, per se, is a consequence of decreased HS response [7]. In any way, compromised HS response is observed in tissues of aged subjects, thus allowing for the establishment of an unresolved inflammatory state. In addition, during aging, cellular ROS levels can increase due to a limited capacity of antioxidant systems and repair mechanisms. Then, excessive ROS generation associated with impaired resistance to cell stress has been proposed to play an important role in accelerating aging process [140]. However, it is difficult to determine whether ROS-induced oxidative stress is the cause or just a consequence of aging. Moreover, it is important to highlight that in neutrophils, for example, ROS

are essential for pathogen destruction through phagocytosis and for robust inflammatory responses [141]. Interestingly, in the elderly (60–89 years), a positive correlation has been found between iHSP70 and spontaneous ROS production by neutrophils, but the same correlation has not been confirmed in nonagenarians (>90 years) [142]. The lack of a positive correlation between neutrophil HSP70 levels and ROS in the latter group might be associated with the longer lifespan of these specific people. In general, however, strong evidence suggests that higher iHSP70 contents represent a more protective profile against ROS effects in aging [143].

It is a unanimity, among the studies on the underlying mechanisms of age-related chronic inflammation, that HS response capacity (not necessarily iHSP70 basal levels) is seriously defective in metabolic tissues of individuals bearing age-related chronic diseases, especially when associated with obesity and physical inactivity. This scenario leads to a myriad of inflammatory disorders associated with aging. As stated above, age-associated RA is characterized by a sequence of age-dependent degenerative conditions which starts with an acute inflammatory reaction that is perpetuated into endocrinosenescence, neurosenescence, and senescence of the muscular system [22]. Beside of this, age exponentially increases CVD risk in RA patients [21]. On the other hand, HSF1 and iHSP70 play a role in protecting against both irritant-induced gastric lesions and IBD-related colitis. This is corroborated by the fact that irritant-induced gastric lesions is aggravated in HSF1-null mice due to their inability to up-regulate HSP70, i.e., to arm a healthy and sufficient HS response. Conversely, the protective role of iHSP70 against colitis is associated to its suppressive effect on the expression of pro-inflammatory cytokines [144]. In addition, overexpression of HSP70 was found to prevent the development of inflammatory processes in the large intestinal mucosa provoked by various damaging factors [32].

As preliminarily stated above, sarcopenia is a geriatric syndrome in which there is a decrease of muscle mass and strength with aging and constitute a fundamental cause of frailty, functional decline, and disability. Although its etiology is not completely understood, sarcopenia is also closely related to inflammatory processes and aggravated by the concomitant age-related changes in cytoprotective mechanisms, particularly those involving protein quality control and HS response [29]. In line with an inflammatory nature of sarcopenic disturbs is the observation that aging contributes to enhanced extracellular eHSP70 [123], which, as discussed above, works as a pro-inflammatory cytokine worsening the picture. Hence, elevated plasma eHSP70 is linked to sarcopenia being a potential biomarker and predictor of the illness [123]. Known primary causes of sarcopenia include also a sedentary lifestyle and malnutrition [145]. While resistance training could be a promising intervention [145], elderly individuals normally fail to adequately respond to exercise stimuli. The decrement in regenerative capacity may also be due to a dramatic reduction in postprandial anabolism as well as an increase in generation (or decrease in removal) of reactive oxygen species (ROS) [146]. Indeed, ROS production by normal metabolism and its overproduction in inflamed states are direct causes of aging and many aging-related degenerative complications [147]. This may be because levels of ROS during aging can increase due to a limited capacity of antioxidant systems and repair mechanisms [148]. Thus, excessive ROS production and the impaired resistance against oxidative stress, as well as a defective HS response capacity (which could alleviate ROS consequences) have been proposed to play a major role to accelerate aging process [140]. In aged (20–24 months old) female C57BL/6 mice chronically (8 weeks) treated with either geranylgeranylacetone (100 $mg.kg^{-1}$ $day^{-1}$, a pharmacological inducer of iHSP70) or heat therapy (twice a week) was found to increase muscular endurance, although muscle power, contractile force, capillary perfusion, and innervation were not different [149]. Both treatments resulted in the expected improvement in peripheral insulin response and glycemic status. Moreover, mitochondrial protein carbonylation (an indicative of oxidative stress) increases moderately with age, whereas this increase may impact upon skeletal muscle function, though it is not a hallmark of sarcopenia per se [150]. In these studies, HSP70 basal expression is not altered in sarcopenia, but nothing is known about the capability of HS response in such condition.

Skeletal muscle is a key reservoir of amino acids that sustain protein synthesis in other tissues, and limited muscle mass often associates with impaired responses to both stress and critical illness [151]. Nevertheless, loss of muscle mass is not that simple. In both sarcopenia and cancer cachexia (another muscle degenerative condition frequently observed in the elderly), type IIb (glycolytic, fast twitch) muscle fibers are smaller and are preferentially lost, while loss of oxidative type I (low twitch) fibers is a common feature observed in obese individuals. Myofiber loss can be accompanied by inflammation, the infiltration of adipose tissue, fibrosis, and decreased capillarization [25]. Although both muscle mass and strength are needed for optimal performance, loss of muscle strength is a better predictor of mortality (related to any cause) during aging [152], suggesting that muscle function is a more important health parameter than muscle mass per se [25]. Extrinsic changes in innervation, stem cell function, and endocrine regulation of muscle homeostasis contribute to muscle aging. In addition, organelle dysfunction and compromised protein homeostasis are among the primary intrinsic causes. Some of these age-related changes can, in turn, contribute to the induction of compensatory

stress responses that have a protective role during muscle aging [25]. Progression of sarcopenia depends also on the intestinal absorption of dietary protein amino acids. However, it has been shown that muscle protein synthesis is blunted in elderly when protein and carbohydrate are co-ingested or when the quantity of protein is less than approximately 20 g per meal [27]. However, despite directly causal factors, the establishment of sarcopenia is closely related to inflammatory processes and aggravated by the concomitant age-related changes in cytoprotective mechanisms (particularly those involving protein quality control) [29]. In any way, all the above conditions surrounding sarcopenia tend to limit physical activity which, in turn, predisposes the elderly to chronic inflammatory diseases, including obesities and T2DM [13, 148].

Another crucial issue in aging is the development of neurodegenerative diseases [143]. Aging and age-related neurodegenerative disorders are tightly associated with chronic oxidative stress and impaired protein quality control systems (HS response and autophagy), which are the primary pathogenic mechanisms contributing to neuronal dysfunction, degeneration, death, and cognitive decline in both humans and experimental animals [153]. Aging leads to an accumulation of disabilities and diseases that limit normal body functions and is a major risk factor for neurodegenerative diseases [143]. In fact, recent evidence has shown that HSPs are critically involved in the progression of neurodegeneration [154, 155]. Reduced expression of many iHSPs has been observed in the brain tissue of aged humans and animal models of aging, as well as in tissues from elderly patients with neurodegeneration. This strongly suggests their involvement in the pathophysiology of age-related neurodegenerative disorders [153]. Additionally, as observed in relation to sarcopenia, plasma levels of the pro-inflammatory eHSP70 are correlated with neurodegeneration [154].

The bulk of currently available information converges upon the observation that chaperone-directed protein quality control and HS response are markedly hindered in neurodegenerative diseases in general. The totality of major neurodegenerative illnesses is associated with the accumulation of unfolded proteins and the formation of toxic protein aggregates. This is the case of the aggregates of polyglutamine androgen receptor in spinal and bulbar muscular atrophy, huntingtin in Huntington's disease (HD), α-synuclein in Parkinson's disease (PD), and *tau* protein in Alzheimer's disease (AD). All of them are client proteins of iHSP90, and their turnover is regulated by the protein quality control function of the iHSP90/iHSP70-based chaperone machinery [19]. Interestingly, iHSP90 and iHSP70 have opposing effects on client protein stability in protein quality control: iHSP90 stabilizes the clients and inhibits their ubiquitination, whereas iHSP70 promotes ubiquitination-dependent and proteasomal degradation [19]. iHSP70, working as a chaperone over the above client proteins, protects neurons from protein aggregation and its consequent cytotoxicity in PD, AD, polyglutamine diseases, and amyotrophic lateral sclerosis (ALS), thus avoiding the establishment of an inflammatory status resulting from chronically non-removed protein aggregates [17]. Inasmuch as protein aggregates are not withdrawn from the brain tissue, a state of endoplasmic reticulum (ER) stress is achieved that, becoming chronic, triggers inflammation invariably [7]. As a consequence, neurodegenerative diseases are characterized by an out-of-control situation of oxidative stress and inflammatory markers. An example is the pro-inflammatory eHSP70, whose plasma concentrations are correlated with cognitive decline in language and executive functions in elderly people [154, 156].

It has long been recognized that all aggregative neurodegenerative disorders have in their very heart an altered capacity of cells to produce molecular chaperones (particularly HSP70) at levels compatible with protein synthesis demands [157]. AD is the most common neurodegenerative disease causing dementia and having no treatment or cure as yet [158]. Although the exact physiopathology of AD is still unsettled, it is clear that brain dysfunctions and atrophy (due to neuronal loss) that accompany AD are correlated with the accumulation of unfolded proteins that tend to form neurotoxic protein aggregates, such as extracellular deposition of amyloid plaques, accumulation of intracellular neurofibrillary tangles (NFTs), inflammation, and oxidative stress [159–161]. Abundant extraneuronal deposits of amyloid-beta (Aβ) are the major pathological hallmark of AD and play an early pathologic role in the development of the disease [162]. Aβ is a 40 or 42 amino acid polypeptide derived from amyloid precursor protein (APP) after its sequential cleavage by β- and γ-secretases. Its physiological role is likely related to the modulation of synaptic activity, although still controversial. In AD, Aβ accumulates forming intermediate soluble oligomers that are synaptotoxic as well as insoluble β-sheet pleated amyloid fibrils that are the main constituents of dense-core plaques (mainly Aβ42) and cerebral amyloid angiopathy (primarily Aβ40) [159]. In fact, Aβ protein dimers are directly associated with impairment of synaptic plasticity and memory [162].

If depressed HSP70 may be at the core of AD, in vitro and in vivo studies have shown that rising iHSP70 contents is able to prevent protein aggregates and the formation of Aβ in brain cells, thus suppressing AD conditions [163, 164]. In primary neuron cultures, adenovirus-induced HSP70 has been shown to be neuroprotective against intracellular Aβ accumulation and Aβ-mediated cytotoxicity in AD [163]. Furthermore, transgenic mice expressing HSP70 also displayed lower levels of Aβ, Aβ plaque deposition, and neuronal and synaptic loss than control mice [164].

Another type of misfolded polypeptides found in AD are the neurofibrillary tangles (NFT), which are composed by aggregates of hyperphosphorylated forms of the *tau* protein that become extraneuronal ("ghost" tangles) when tangle-bearing neurons die. NFTs have a stereotypical spatiotemporal progression that correlates with the severity of the cognitive decline, while topographic staging of NFTs (from stages I to VI) is used for the pathological diagnosis of AD [161]. Under physiological conditions, *tau* is a soluble microtubule-associated protein located to the axon, where it physiologically facilitates the axonal transport by binding and stabilizing the microtubules [159, 165]. However, in AD, *tau* translocates to the somatodendritic compartment and dissociates from microtubules undergoing hyperphosphorylation, misfolding, and aggregation due to self-associations to form both fibrillar and prefibrillar oligomeric clumps [166]. These aggregates give rise to NFT and neuropil threads [159]. Not surprisingly, therefore, iHSPs inhibits *tau* aggregation by a mechanism that seems to involve preferential associations with soluble, monomeric, and prefibrillar oligomeric *tau* species [158].

Stimulation of the HS response has conspicuously shown to block progression of virtually all neurodegenerative diseases studied [167]. iHSP70 prevents protein aggregation by binding to the exposed hydrophobic residues of *tau* [168]. Thence, at least in vitro, iHSP70 interaction with soluble *tau* is supposed to inhibit self-association of *tau* into aggregates. In addition, iHSP70 has also been found to interact with pre-existing *tau* aggregates, having a preferential selectivity for oligomeric versus filamentous *tau* tangles. Fibromyalgia, which is a disseminated pain disorder mainly diagnosed in middle-aged women, has traditionally been classified as either a musculoskeletal disease or a psychological disorder. However, accumulating evidence now suggests that fibromyalgia may be associated with CNS dysfunction with loss of gray matter [15], similarly to that described for classical neurodegenerative diseases of aggregative nature. It is of note, indeed, that fibromyalgia is associated with abnormal protein ubiquitination and HS response pathways [169], at the same time, fibromyalgia predisposes the patient to an increased risk for developing age-related diseases prematurely, suffering earlier cognitive and physical decline and experiencing earlier mortality [16]. On the other hand, long-term intranasal administration of recombinant HSP70 (in order for HSP70 to reach different cerebral structures intracellularly, so to enhance iHSP70) to middle-aged and old mice has convincingly demonstrated that iHSP70 enhances animal lifespan, improves learn, memory, and locomotor and exploratory activities in old mice [118]. This suggests that pharmacological administration of tissue-directed iHSP70 may be of value in reverting aging-associated disorders in humans. Therefore, HSPs may be envisaged as potential therapeutic tools to prevent neurodegeneration by avoiding protein aggregation processes, thus reducing the toxicity of such oligomers [170]. However, more studies are required to identify the specific signaling pathways and routes of administration of HSP70 to avoid possible harmful effects because, if HSP70 is not accurately introduced inside brain cells, it could remain within the extracellular space, where eHSP70s is a pro-inflammatory by virtue of what the binding to TLR2 and TLR4, at least in other cells, may exert [62, 112]. Still in support of a major role of HS response for normal brain function, numerous studies have shown that the plant polyphenol resveratrol (3,5,4′-trihydroxystilbene) may extend the lifespan of several species, preventing age-related diseases beside possessing anti-inflammatory action. The beneficial effects of resveratrol are believed to be associated with the activation of SIRT1 [18], which, as discussed above, enhances the HS response. Unfortunately, however, the accumulation of protein aggregates in many elderly people was found to surpass the ability of neuronal tissue to cope with appropriate HS response so that the end of story is a consequent chronic inflammatory response and tissue degeneration.

Although not completely understood, the exact mechanisms by which inflammation is chronically attained in neurodegenerative as well as in other prevalent age-associated diseases, cellular senescence in metabolic tissues may shed light on the whys of persistent unresolved inflammation that lead to tissue dysfunctions. In aged mammals, it seems that while insulin resistance is not chronically sustained or not so severe, cells are still able to compensate increasing their HSP70 levels [139, 171]. After long-term insulin resistance, notwithstanding, stress response (i.e., HSP70 machinery) is blunted by the senescent effect of obesity [7, 30] and the levels of HSP70 fall [139]. Whether this scenario is also attained in other age-related chronic degenerative diseases is a matter of current dispute.

## Cellular senescence as the underlying mechanism of chronically depressed HS response and the consequent unresolved inflammation in age-related conditions

As discussed above, elders are sick longer, often coping with multiple chronic diseases simultaneously [4]. Senescent cells accumulate in many tissues during aging and start to present a unique senescence-associated secretory profile (SASP) that includes many pro-inflammatory cytokines [4, 7]. On the other hand, HS response, which is critical to promote the resolution of inflammation, is severely impaired in metabolic tissues during chronic inflammation. For instance, with respect to insulin resistance and T2DM, it has been found that the expression of messenger RNA (mRNA) coding for the inducible form of HSP70

(*HSPA1A* gene) was dramatically reduced (90 % decrease) in skeletal muscle biopsies of T2DM patients as compared to healthy volunteers [127]. Similar observations have been reported in obese and non-obese T2DM patients, in which a marked reduction in the protein expression of HSP70 has been noticed in comparison with obese controls [47]. Moreover, T2DM patients show decreased intramuscular expression of both HSP70 and heme-oxygenase [172], so that HS response-associated anti-inflammatory and antioxidant defenses are impaired leading to an inflammatory state, high *NOS2*-dependent NO production and impaired insulin receptor downstream signaling pathways function by *S*-nitrosation [173]. We have recently observed that HSF1-HSP70 axis is progressively suppressed in adipose tissue and liver of insulin resistant obese patients, as nonalcoholic fatty liver disease (NAFLD) evolves from steatosis, toward more inflammatory forms of the disease, e.g., steatohepatitis accompanied by fibrosis [76]. Moreover, such suppression was found to be strongly correlated with the degree of enhancement of JNK1 and JNK2 expression in adipose tissue, which was followed by similar rises in the amounts of Thr183/Tyr185-diphosphorylated activated p-JNK1 and p-JNK2 in the same tissue. Hence, adipose tissue of insulin-resistant patients is embraced in a suppressed HS response, as observed in the age-related chronic degenerative diseases discussed in the previous section. This is a complex situation because stress-induced iHSP70 should inhibit JNK-dependent signal transduction [174, 175] under physiological conditions.

The association between NF-κB-centered unresolved inflammation and chronic diseases involves the unfolded protein response (UPR) (a cellular reaction to overnutrition) and ER stress, as observed in obesity, atherosclerosis, insulin resistance, and T2DM [176–181]. In all these cases, unremitted low-grade inflammation, which follows chronic ER stress, is a consequence of impaired resolution of inflammation [182]. Age-related chronic inflammation and HS response pathways also intercross at gene regulatory level. Accordingly, the promoter region of TNFα gene contains an HSF1 binding site that represses TNFα transcription, and thus loss of this repressor results in sustained expression of TNFα [183], which possibly explains why HSF1 knockout is associated with a chronic increase in TNFα levels and increased susceptibility to endotoxin challenge [174, 184, 185]. Regulation of this network in the opposite direction also occurs: TNFα may transiently repress HSF1 activation [186]. Moreover, JNK1 phosphorylates HSF1 in its regulatory domain causing suppression of HSF1 transcribing activity [187] while iHSP70 prevents apoptosis by inhibiting the JNK/Bim pathway [185, 188]. However, if inflammation evolved to present both an initiation and a resolution phase (Fig. 2), why does inflammation not resolve in age-related diseases? The answer to this question is linked to cellular senescence and SASP.

Cellular senescence and its associated SASP is an alternative mechanism to UPR, in order for the cell to avoid apoptotic death, which would be an expected result after an inoperative anti-inflammatory HS response. In fact, a senescent-like state can emerge in fat cells from obese individuals (even young obese subjects), this being an adaptation to fat cell overutilization which resembles cellular aging [189]. High-fat diet (HFD)-induced obesity also leads to vascular senescence in a process involving long-term activation of Akt1 and mTOR [190]. On the other hand, fibroblasts from adult segmental progerioid Werner syndrome, in which the cells undergo premature senescence, are associated with a strong positive feedback system in which over-activation of the p38-NF-κB pathway leads to SASP that then attenuates the expression of the mRNA-binding protein HuR, a critical factor for full activity of the $NAD^+$-dependent protein deacetylase SIRT1 [191–193]. HuR enhances the stability of several target mRNAs, including that encoding for SIRT1, via HuR association to the 3′-untranslated region of SIRT1 mRNA which promotes its stability and thus a rise in SIRT1 protein expression levels [191]. Conversely, $H_2O_2$-induced oxidative stress disrupts HuR-SIRT1 mRNA interaction lowering cell survival in a cycle checkpoint kinase-2 (Chk2)-dependent manner [191]. SIRT1, in turn, enhances HSF1 expression [192] and prolongs HSF1 binding to the promoters of HS genes by maintaining HSF1 in a deacetylated, DNA-binding competent state [106], while HS itself increases cellular $NAD^+$/NADH ratio and augments the recruitment of SIRT1 to the HSP70 promoter [194]. SIRT1 knockdown, on the other hand, attenuates HS response [108] whereas SIRT1 modulators were found to also modulate HSF1 activity and HS response in HeLa cells [194].

Following a cellular insult (e.g., genotoxic stress), HuR associates with SIRT1 mRNA, triggering an anti-apoptotic and pro-survival gene expression program [195]. However, HuR participation in cellular homeostasis goes beyond that, as HuR is involved in the differentiation of pre-adipocytes, including translation and stability of glucose transporter GLUT1 mRNA. Therefore, experimental data support a role for HuR in muscle and adipose tissue differentiation processes [196]. Contrarily, reduced HuR levels are associated with enhanced cellular senescence and because of these observations, HuR is considered a factor implicated in the maintenance of a "young cell" [197]. Interestingly, HS and calorie restriction (which enhances SIRT1 deacetylase activity) seem to act synergistically with respect to the HS response [198]. SIRT1 attenuates saturated fatty acid-induced ER stress and insulin resistance in hepatocyte-like cells [199]. Moreover, resveratrol, an inducer of SIRT1 metabolic action, increases insulin

sensitivity, 5′-AMP-activated protein kinase (AMPK), and peroxisome proliferator-activated receptor-γ (PPARγ) coactivator-1α (PGC-1α), leading to increased mitochondrial number and oxidative metabolism [200]. AMPK, in turn, inhibits glycogen synthase kinase-3β (GSK-3β), an enzyme that constitutively inhibits HSF1 activity [55], so that energy sensing (AMPK) is linked to anti-inflammation (HSP70) via AMPK and SIRT1-dependent AMPK activity (Fig. 4).

Alongside other metabolic effects, SIRT1 activates PGC-1α by deacetylation [201], while PGC-1α stimulates the production and secretion of a novel myokine (IRISIN) which acts in white adipose cells, both in vitro and in vivo, driving a brown-fat-like phenotype via stimulation of uncoupling protein-1 (UCP1) expression [202]. This links calorie restriction and physical exercise to protective energy-consuming oxidative metabolism. As could be inferred from the above statements, since HuR-SIRT1 duet controls the expression and transcribing activity of HSF1, any decrease in the flux through HuR-SIRT1 pathways, suppress the HS response. Unfortunately, however, this is exactly what happens during the establishment of age-associated chronic inflammatory diseases.

SASP-related production of inflammatory cytokines (e.g., IL-1β, IL-6, IL-8, IL-18) is connected to persistent DNA damage-like response. During caspase-mediated apoptosis, HuR switches its function from pro-survival to pro-apoptotic [203]. Caspases can mediate cleavage of HuR under different situations [204]. In parallel is the observation that HFD induces, in white adipose tissue, the cleavage of SIRT1 by caspase-1 which is, in turn, activated by the diet-induced inflammatory response in adipocytes [205]. Moreover, HFD feeding or systemic inflammation leads to the activation NLRP3 inflammasome, which is actually the key event that will lead to production and activation of caspase-1 [205]. Inflammasomes are large multimeric danger-sensing platforms that promote autocatalytic activation of caspase-1 and mediate the cleavage of inactive pro-interleukins, among other proteins, into their active forms. Inflammasomes of NLR [nucleotide-binding oligomerization domain (NOD)-leucine-rich repeat and pyrin-domain (LRP) containing protein] family are the best studied; particularly the NLRP3 inflammasome that mediates a series of metabolic diseases, including atherosclerosis and insulin resistance in adipocytes [206]. Remarkably, injuring stimuli that cause senescence, such as UVB irradiation, also induce NLRP3 activation while inflammasome activation seems to work as a "danger sensor," as observed in the metabolic stress induced by high extracellular glucose that activates NLRP3 inflammasome [207].

Although not exclusive of aging, obesity and adipose tissue disturbances are highly prevalent among elderly people. Present-day human beings were metabolically selected during the last glaciation (*ca.* 19,000-10,000 years ago) for possessing high energy saving capacity in times of famine. Therefore, our present life style in a genetic background favoring energy conservation led us to the obesity epidemic [7]. This scenario is aggravated because changing feeding habits from a paleolithic diet (lean meat, fruits, vegetables and nuts, but not cereal grains, dairy or legumes) to the "fast food" style, dramatically affected gut microbiota, favoring the harboring of bacteria that stimulate inflammatory responses at gut mucosa that spread out toward other tissues [33]. Metabolic overutilization of adipose tissue, in the face of energy surplus, overwhelms adipocyte endoplasmic reticulum leading to ER stress and unfolded protein response (UPR). As the positive energy balance is not reversed, UPR becomes inflammatory leading to chronic activation of NLRP3 inflammasome. Therefore, genome instability, excess energy imbalance and epigenomic alterations observed in aging lead to the persistent activation of NLRP3 inflammasomes in metabolic tissues so that an uninterrupted supply of ILs and other inflammatory cytokines (SASP) disseminate inflammation throughout the body. At the same time, NLRP3-dependent caspase-1 cleaves HuR, leading to depression of HSF1 expression, thus resulting in a marked failure to resolve inflammation via the HS response, as illustrated in Fig. 4.

## Glutamine metabolism and its importance for the heat shock response

Glutamine is recognized as a crucial amino acid for cell survival and growth, playing an important role in intermediate metabolism (for more information on the historical aspects of glutamine research and general view of metabolic regulation in metabolism, please, consult [208]). Compared with all other amino acids in the body, glutamine is present at the highest extracellular concentration being the most abundant free amino acid in the blood. Because the organism can synthesize and release glutamine from many tissues, the amino acid is classified as nutritionally non-essential. However, in some catabolic conditions, such as sepsis, recovery from burns or surgery, as well as after high-intensity exercise, glutamine stores (particularly in the skeletal muscle and liver) may fall sharply [208–211]. This effect is due to increased bodily requirement for glutamine under such conditions, especially by the muscle itself (in the case of high-intensity exercise) and the rapidly dividing cells of the immune system (in the remaining above cases) [50, 212]. Moreover, glutamine is released in significant quantities from skeletal muscle stores following stress and injury [213].

Glutamine can be used as fuel for the essential production of ATP, NADPH, and $CO_2$ and donates 2 amide nitrogen atoms during the synthesis of macromolecules, including purines, pyrimidines, and amino sugars [214].

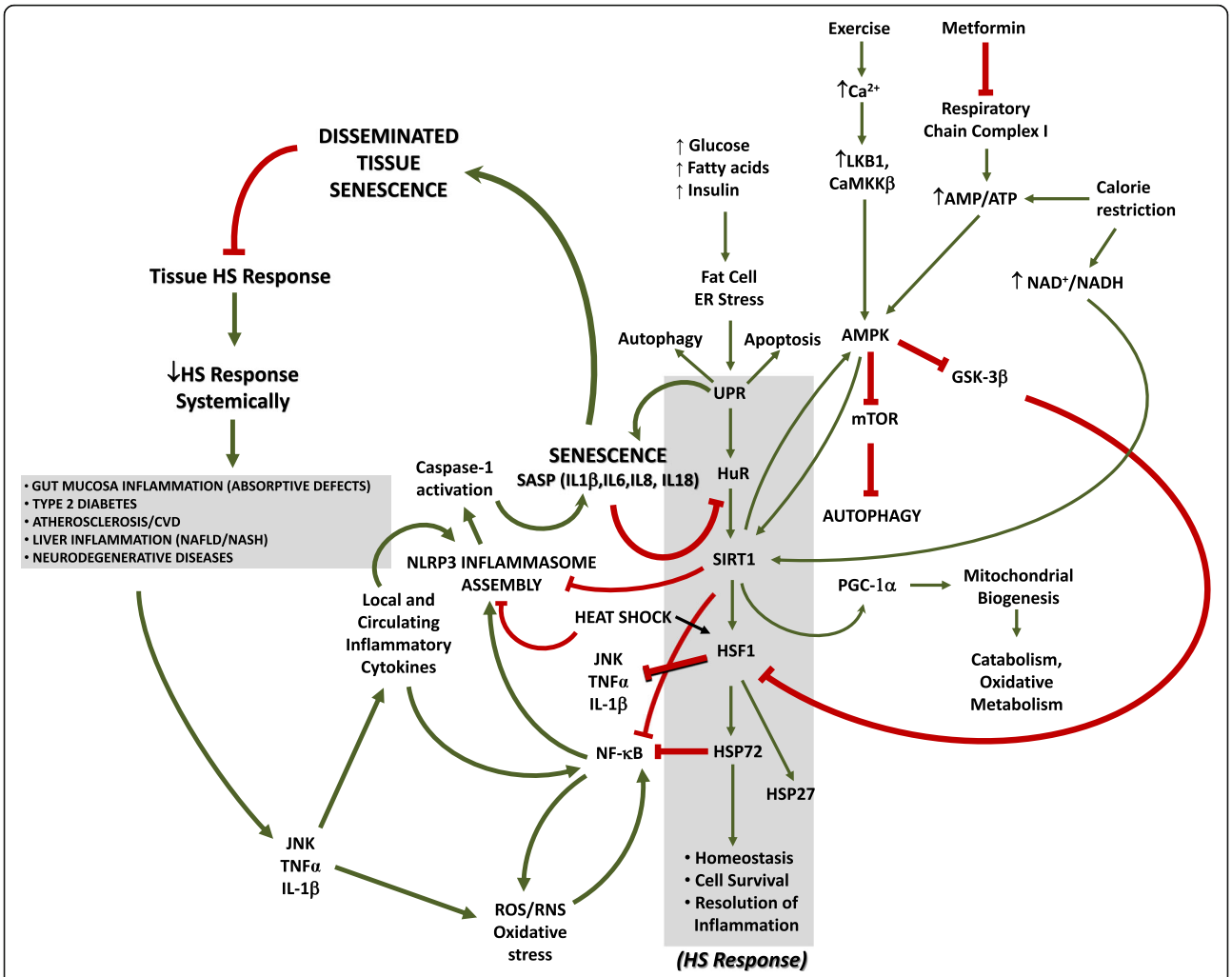


**Fig. 4** Heat shock response failure in chronic inflammatory diseases: role of cellular senescence. Under normal nutrient supply (i.e., equivalent to energy expenditure, physical activity), glucose and fatty acids are utilized in adipose tissue upon physiological amounts of insulin. Any excess of demand is counteracted by enhanced heat shock (HS) response in order supply the correct furnishing of chaperones thus avoiding or correcting endoplasmic reticulum (ER) stress and the resulting unfolded protein response (UPR). When circulating glucose and fatty acids (especially saturated) overcome energy expenditure and high amounts of surplus energetic metabolites should be stored in adipose tissue under a higher insulin command, ER stress develops. Should energy expenditure be still and chronically lower than energy intake, ER stress is followed by the UPR, a cellular strategy evolved in order to evaluate the capacity of the cell to arrange a physiological HS response (which conveys cells to protein/metabolite homeostasis). In the case of irremediable HS response, cells may undergo apoptosis and irreversible cell death. On the other hand, if proteostasis is not attained but cells still have conditions to avoid apoptosis, an alternative metabolic pathway may be taken in which cells do not dye but activate senescence, assuming a senescence-associated secretory phenotype (SASP). This is accomplished because adipocytes chronically challenged by excess fatty acids, cholesterol, high-fat diet, and hyperglycemia prepare an inflammatory response, which becomes chronic. Under the persistence of risk factors, the cells develop an UPR that is diverted to the inflammatory branch since continuous inflammatory stimuli do not cease to activate NLRP3 inflammasome, leading to the activation of caspase-1. Activated caspase-1 determines, in adipocytes, a state of frank cellular senescence which culminates in SASP that can spread out to other tissues and cell types, including adipose tissue infiltrating macrophages, skeletal muscle cells, pancreatic β cells, hepatocytes, vascular cells, and brain structures. In all these cell types, including adipocytes, SASP leads to cleavage of HuR, an mRNA-binding protein responsible for enhancing SIRT1 expression. As a consequence, HSF1 expression and transcribing activity becomes depressed, because SIRT1 enhances both. Therefore, HS response is hindered accordingly and a state of enhanced inflammation is noted because HS response is of crucial importance for the resolution of inflammation. As a healthy HS response cannot resume, resolution of inflammation is more and more impaired thus impeding autophagy and an efficient resolution of UPR via HS response. Beside of this, several studies indicate that senescent cells are resistant to undergo apoptosis (which should be an alternative to break this vicious cycle), so that chronically inflamed cells are likely to persist in tissues. This illustration was redesigned and adapted from [7]

In rapidly proliferating cells (e.g., lymphocytes, enterocytes, tumor cells), glutamine plays a unique role in intermediary metabolism that differs in much from that of other amino acids. Glutamine acts as a precursor of lipids after running through the left-hand side of the Krebs cycle until the formation of citrate. Accordingly, due to the high demand for lipid synthesis in quickly dividing cells, glutamine-derived citrate is exported toward the cytosol (pyruvate/malate shuttle) being converted into cytosolic acetyl-CoA and, eventually into lipids, thus assisting in membrane and lipid mediator synthesis during cell proliferation. Glutamine also influences the expression of a number of genes related to cell protection and survival [215]. Importantly, glutamine is the immediate precursor of the glutamate moiety for glutathione (GSH = γ-glutamyl-cysteinyl-glycine), the main soluble antioxidant species within the cell, this being demonstrated in a number of cell types and tissues [208, 212–219]. Inasmuch as redox imbalances are characteristic of degenerative disorders [140, 148] and aggregative diseases [141–143, 153, 154], glutamine status becomes of importance in dictating a healthy condition.

At the same time, glutamine is cytoprotective by promoting redox protection (GSH), it has anti-inflammatory effects by preventing the activation of NF-κB and stress kinase pathways (p38/MAPK, ERK, and MKP-1), leading to attenuation of inflammatory cytokine release and prevention of acute respiratory distress syndrome (ARDS) following sepsis [220]. In ARDS, glutamine-elicited suppression of NF-κB blocks NOS2 expression and, therefore, excess NO production [220]. These protective effects of glutamine, however, have long been recognized to be associated with glutamine-mediated potentiation of the HS response. In fact, glutamine attenuates endotoxin-induced lung metabolic dysfunction [221] and reduces lung injury after sepsis, thus improving survival, by enhancing HSP70 expression [222, 223]. Moreover, glutamine protects against renal ischemia-reperfusion injury [224]. This is because glutamine protects against cellular injury by increasing HSF1 function [225]. Glutamine not only enhances transactivation of HSF1 over heat shock genes through increased HSF1 trimerization (both spontaneous and heat-induced) but also induces HSF1 expression by activating its transcription in a CCAAT enhancer-binding protein-β (C/EBPβ)-dependent manner [56]. This was also confirmed in in vivo studies with trained rats [49]. Glutamine increases HSF1 nuclear localization and DNA binding, which is accompanied by augmented relative abundance of activating phosphorylation at Ser230 of HSF1 [56]. Apart from its role in facilitating HSF1 trimerization (a necessary step for its nuclear migration and activity), glutamine induces HSP expression via *N*-acetyl-*O*-glycosylation (O-GlcNAcylation) and phosphorylation of HSF1 and Sp1 transcription factors [53]. In turn, glucosamine 6-phosphate needed for O-GlcNAc-dependent post-translational modifications is furnished by a special and nutrient-sensing pathway, the hexosamine biosynthetic pathway (HBP), which is a metabolic shunt from glycolysis, normally representing 1–3 % of incoming glucose in cells [226].

HBP is a metabolic pathway that leads to the eventual synthesis of uridine diphosphate (UDP)-*N*-acetylglucosamine and (UDP)-*N*-acetylgalactosamine (UDP-GlcNAc and UDP-GalNAc, respectively) after processing through glutamine:fructose-6-phosphate amidotransferase (GFAT, the first and rate-limiting step of HBP). UDP-GlcNAc and UDP-GalNAc, in turn, may be attached to serine or threonine hydroxyl moieties in nuclear and cytoplasmic proteins by the enzymic action of *O*-linked-*N*-acetylglucosaminyl (O-GlcNAc) transferase (a.k.a. OGT) [227]. The main donors for UDP-GlcNAc are glucose, glutamine and uridine triphosphate (UTP) from the HBP. Similar to the widespread cascades of phosphorylation that work as switchers and/or fine-tuners of intermediate metabolism, the O-GlcNAcylation occurs in many post-translational modifications in response to internal or environmental changes [228]. O-GlcNAcylation is often competitive with phosphorylation at the same sites (or at proximal sites) on proteins. Indeed, O-GlcNAc signaling and its crosstalk with phosphorylation reactions affects the post-translational state of hundreds of proteins in response to nutrients and stress, and is also altered in several metabolic diseases and inflammatory processes [229]. For instance, glutamine stimulates the expression of the argininosuccinate synthetase (ASS) gene (involved in the regulation of NO production via NOS in many cells [230]) via O-GlcNAcylation of Sp1 [54, 231], a key transcription factor required for full HS response [54, 105, 231]. Glutamine availability has also been identified as a limiting step for the activation of the mammalian target of rapamycin (mTOR) [232]. This is of note because many initiation transcription factor complexes (e.g., eIF2, eIF4F), which are assembled from multiple subunits, are sensitive to the activation by the mTOR cascade [233], thus resulting in coordinated protein synthesis and degradation [148]. Key intracellular proteins and transcriptional factors, such as Sp1 [54, 231], are known to be O-GlcNAcylated via HBP during stress, injury, or illness, while phosphorylation of the eIF2 promotes the activation of HSF1 [41], leading to the expression of HSPs under stress conditions [56].

Increasing evidence suggest that glutamine may also act on HBP via p38/MAPK, which participates in its downstream actions [234]. Indeed, p38/MAP kinase, but not phosphoinositol-3 kinase (PI3K), signals downstream of glutamine-mediated fibronectin-integrin pathway after intestinal injury, and this dramatically enhances HS response [235]. In this case, glutamine has been shown to play a cytoprotective role by dephosphorylating p38/

MAPK downstream of glutamine-mediated fibronectin-integrin osmosignaling after HS [235]. In rat kidney proximal tubular cells, high glucose stimulates angiotensinogen gene expression and cell hypertrophy via activation of HBP, in a process partially mediated by p38/MAPK [236]. This is similar to that found in neutrophils obtained from exercised rats, in which glutamine supplementation prevents exercise-induced neutrophil apoptosis in parallel with a reduction in p38/MAPK and JNK phosphorylation besides p53 and caspase 3 expression [237].

There is another important point where glutamine metabolism intercepts HS response via HBP. Accordingly, OGT-mediated UDP-GlcNAc addition reaction regulates HS response by blocking GSK-3β, an enzyme that constitutively inhibits HSF1 activation by phosphorylating the transcription factor at Ser303 [55]. Thence, glutamine-mediated increased fluxes through HBP may, on the one hand, block GSK-3β, thus liberating glycogen synthesis by glycogen synthase and, on the other, may liberate HSF1 thus allowing enhanced expression of HSP70 [55, 238]. HBP is a nutrient-sensing pathway [226] that presents multiple connections with energy metabolism, not only with glycogen synthesis [219]. AMPK, which occupies a central position in metabolic regulation in order to avoid inflammatory dysregulation, phosphorylates and inhibits GFAT1, the flux-generating step of HBP, thus allowing for the downregulation of such a shunt from the glycolysis under low glucose situations [239]. Conversely, chronic hexosamine flux stimulates fatty acid oxidation by activating AMPK [240]. The metabolic flux through HBP is dependent on glucose availability [51]. Therefore, in high-glucose states, HBP may act adversely as GFAT1 gene expression is enhanced by hyperglycemia contributing to an exaggerated flux through HBP that can be deleterious [52]. Indeed, exacerbated HBP activity does contribute to insulin resistance rather than being cytoprotective [219]. In fact, overenhanced flux through HBP is an inducer of ER stress, while being associated with insulin resistance [52, 241], obesity [242], and abnormal glucose disposal rate in T1DM [243] and T2DM itself [241].

Physiologically, glutamine-elicited increase in the flux through HBP leads to a momentary redox imbalance by depleting pentose phosphate shunt (cf. Fig. 5a, b). Therefore, at the same time that glutamine is cytoprotective by enhancing HS response, it may induce a small redox imbalance that suffices to increase the expression of redox-protecting genes, including those involved in GSH biosynthesis [244]. De novo GSH synthesis is primarily induced by transcriptional regulation [245–247], via a cascade of signaling events leading to nuclear factor-erythroid 2 p45-related factor 2 (Nrf2) binding to promoter regions of antioxidant response elements (ARE) in the nucleus [247–250]. Similarly to that observed for NF-κB, under basal conditions, cytoplasmic Nrf2 is bound to the Kelch-like ECH-associated protein 1 (Keap1). However, when cells are exposed to oxidative stress, the oxidation of critical cysteines present in Keap1 protein liberates Nrf2 to dissociate and traverse to the nucleus (see, for instance, ref. [87]), triggering the expression of a number of redox-protective genes, such as γ-GCS, glutathione *S*-transferases (GST), GSSG reductase, glutamine synthetase (GS), glucose-6-phosphate dehydrogenase (G6PDH), and superoxide dismutase [97, 98]. The same is observed when cells are treated with anti-inflammatory and HS response inducers cyPGs, which are able to undergo Michael addition reactions directed to Keap1 [87]. This passingly redox challenge is believed to be produced by glutamine-evoked depletion of glucose-6-phosphate (G-6P), which is necessary for the synthesis of NADPH. In turn, NADPH is used to regenerate GSH from GSSG via a GSSG reductase-catalyzed reaction [49]. Therefore, we believe that glutamine diverts G-6P from the hexose-monophosphate shunt toward glucose-6-phosphate isomerase to form fructose-6-phosphate (F-6P) which can be further metabolized through the HBP. As depicted in Fig. 5, glutamine may divert muscle glycolysis and glycogenesis favoring the formation of UDP-*N*-acetylglucosamine, the end-product of HBP [55, 239]. When performing a flux balance analysis, based on metabolite concentrations of the rat muscle [49], it is predicted that the flux through G6PDH should be around 700 μmol $min^{-1}$ g $tissue^{-1}$ (corresponding to 3.3 % of incoming glucose after hexokinase reaction), whereas the flux through GFAT, 80 μmol/min/g tissue, responds to 4.3 % of fructose-6-phsophate utilized by the muscle (or 0.9 % of total incoming glucose). Under a high-glutamine environment, however, the flux through GFAT may enhance up to approx 450 μmol $min^{-1}$ g $tissue^{-1}$, which corresponds to 20 % of incoming fructose-6-phoshate (or 4.3 % of total incoming glucose through hexokinase), substantially reducing (by roughly 50 %) the amount of G-6P available to enter pentose phosphate shunt, via G6PDH. Hence, high intracellular glutamine concentrations are expected to reduce the formation of NADPH leading to a transient decrease in GSH which rapidly triggers the transcription of Nrf2-dependent genes, such as G6PDH, γ-GCS, and GS (Fig. 5b). Beside of this, *N*-acetylglucosamine produced by the HBP regulates the activity of HSF1 and GSK-3β, leading to increased HS response [55]. In fact, chronic glutamine supplementation to trained rats has shown to increase muscle GSH contents and GS at the same time that it has enhanced HS response [49]. Corroborating this assumption is the fact that the activation of GFAT depletes intracellular glucose stores [239]. Oppositely, GSH depletion, per se, increases OGT gene expression leading to O-GlcNAcylation of different regulatory proteins [251], thus strongly suggesting that HBP has a short-loop positive feedback system

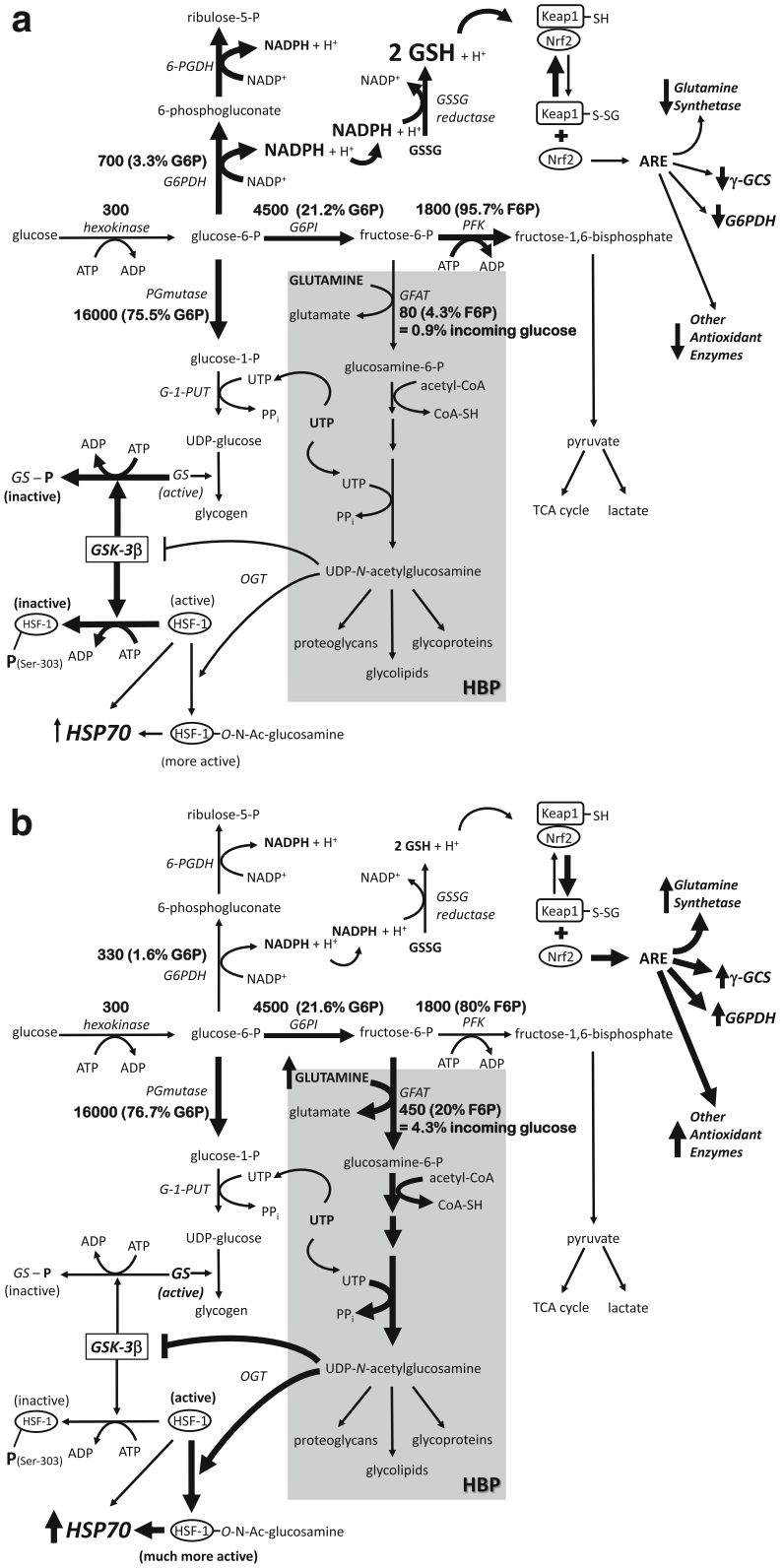


**Fig. 5** (See legend on next page.)

(See figure on previous page.)
**Fig. 5** Heat shock response interplay with glutamine metabolism via hexosamine biosynthetic pathway (HBP). Depicted are the major routes of glucose utilization after its entry in cells. Soon after passing hexokinase (HK) bottleneck, phosphorylated glucose may be diverted to glycolysis, glycogen synthesis, or pentose phosphate shunt (hexose-monophosphate shunt), in a proportion that depends on the cell type and physiological conditions. The present artwork is a graphic illustration of experimental values obtained from soleus and gastrocnemius muscles of 8-week trained (treadmill) rats treated or not with L-glutamine supplementations during the last 21 days [49]. Hence, this is an example of a very metabolically active skeletal muscle. *Thicknesses of arrows* indicate the approximate proportion of each metabolite entering each given sub-pathway. It is noteworthy that, under normal conditions, the muscle preferentially (~75.5 %) utilizes massive amounts of G6P to build up glycogen, at the same time one fifth of entering G6P flows toward glycolysis. In this case, only a minor proportion (~3.3 %) is diverted to hexose-monophosphate shunt in order to feed NADPH generation and redox protection (please, confront **a** and **b**). Under L-glutamine supplementations, excess intramuscular L-glutamine supply enforces fructose 6-phosphate (F6P) to divert from glycolysis and enter hexosamine biosynthetic pathway (HBP, *shaded box* in the center) after its conversion to glucosamine 6-phosphate by glutamine 6-phosphate amidotransferase (GFAT, a.k.a. glutamine-fructose-6-phosphate transaminase). UDP-*N*-acetylglucosamine (UDP-GlcNAc), the final HBP metabolite, operates to enhance the heat shock response by acting at two different points: (1) by blocking glycogen synthase kinase-3β (which phosphorylates and inactivates HSF1, under basal conditions) and (2) by covalent modification of HSF1, which becomes *O*-linked-*N*-acetylglucosaminylated, having more DNA-binding and transcribing activities onto heat shock genes. Moreover, at the estimated L-glutamine concentrations for the soleus (17–18 mM, [49]) and gastrocnemius (ca. 8.5 mM, [49]) muscles, a possibility does exist in that substrate flux through glutamine 6-phosphate amidotransferase (GFAT), whose basal capacity is low in comparison with those of the main concurrent pathway (PFK, **a**), should be conspicuously faster toward the formation of UDP-GlcNAc than in the direction of glycolysis, hexose-monophosphate shunt, or glycogenesis, causing enhanced generation of glucosamine 6-phosphate at the expense of much more glucose 6-phosphate (**b**). This empties hexose-monophosphate shunt leading to momentary deficit of NADPH which triggers Nrf2 transcription factor-dependent gene transcription that accounts for the enhanced expression glutamine synthetase (EC 6.3.1.2 a.k.a. glutamate-ammonia ligase), γ-glutamylcysteine synthetase (EC 6.3.2.2, a.k.a. glutamate-cysteine ligase) and more G6PDH in order to counteract this redox imbalance. HSF1, whose activity must be enhanced following high-intensity exercise training, is potentiated by *O*-linked *N*-acetylglucosamine modification [55] thus increasing cytoprotection through the production of more HSP72 protein chaperone molecules needed during the recovery phase. The fluxes through the biochemical pathways showed here were calculated by using Michaelis-Menten function, intracellular muscle L-glutamine, and L-glutamate estimated from [49] and the following data for the rat muscle: hexokinase (HK, EC 2.7.1.1, $K_m = 0.035$ mM for D-glucose, $V_{max} = 20$ μmol min$^{-1}$ mg protein$^{-1}$; [342]) and glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49, $K_m = 0.002$–$0.070$ mM for glucose-6-phosphate, $V_{max} = 54$ μmol min$^{-1}$ mg protein$^{-1}$ [343, 344]); glucose-6-phosphate isomerase (G6PI, a.k.a. phosphoglucoisomerase, EC 5.3.1.9, $K_m = 0.25$ mM for glucose-6-phosphate, $V_{max} = 520$ μmol min$^{-1}$ mg protein$^{-1}$ [345], assumed to be a near-equilibrium step; 6-phosphofructokinase (PFK, EC 2.7.1.11; $K_m = 0.086$ for fructose-6-phosphate, $V_{max} = 265$ μmol min$^{-1}$ mg protein$^{-1}$ [346, 347]); phosphoglucomutase (PGmutase, EC 5.4.2.2, $K_m \cong 0.07$ mM for glucose-6-P and glucose-1-P, $V_{max} \cong 1300$ μmol min$^{-1}$ mg protein$^{-1}$ [348]; glutamine 6-phosphate amidotransferase (GFAT, EC 2.6.1.16, $K_m \cong 0.4$ mM for fructose-6-phosphate and $K_m = 0.8$–$1.6$ mM for L-glutamine; $V_{max} \cong 35$ μmol min$^{-1}$ mg protein$^{-1}$ [349–351])

devoted to assist in cytoprotection via HS response soon as some redox threatening situation is ongoing.

Finally, it is import to distinguish that glutamine remarkably enhances HS response but glutamine is not a HS inducer itself. Although glutamine is able to slightly increase HSF1 trimerization in non-stressful situations [49, 56], glutamine acts physiologically as an enhancer of the HS response, which means that a pre-existent (e.g., exercise, heat stress, elevated sympathetic nervous system tonus) stress-induced HSF1 activation must be present for glutamine to fully enhance HSF1 activation and transcribing activity. In addition, iHSP70 per se participates in glutamine-induced HS response [252].

As a whole, glutamine synthesis and availability is indispensable for full and accurate cytoprotective stress responses. Therefore, conditions likely to induce glutamine depletion in the blood stream, reducing its availability to other cells, may result in cellular dysfunction.

## Glutamine depletion states impair the heat shock response

In response to several forms of stress, cells can rapidly increase uptake and utilization of glucose and glutamine, mainly for the maintenance of basal metabolism and cell defense response purposes [212–214, 216, 218, 253]. Consequently, glutamine depletion does impair cellular stress response in human leucocytes [254]. In fact, it has long been recognized that the HS response of eukaryotic cells depends on glutamine and/or some glutamine metabolite [255]. Now, it is clear that, besides its classic metabolic roles, glutamine is key for survival and cytoprotection via HBP. Studies suggest that inhibition of both glycolysis and HBP results in decreased cell survival [256]. Due to its crucial position in regulating the HS response, HBP-emanated O-GlcNAc post-translational modifications mediate cell function and survival in the cardiovascular [257–259] and neuromuscular [260] systems, while its defective function is associated with cancer [261]. Therefore, it is not surprising that in vitro attenuation of HBP remarkably reduce maximal HS response [54], whereas genetic mutations in GFAT gene, in which HBP activity is reduced, lead to impairment of neuromuscular junction development and function [260].

Reductions of bodily glutamine concentration may contribute to cell death due to a reduced stress response capacity [209, 262]. Interestingly, upon depletion of intracellular glutamine, the uptake of some amino acids, such as leucine, declines and mTORC1 becomes

inactivated. However, mTORC1-S6K1 signal is critical for the regulation of cell cycle progression, cell size, and cell survival [232, 263], so that reduction in intracellular glutamine stores may hamper cell survival. Recent studies have demonstrated that glutamine-assisted HS response may modulate autophagy, by regulating mTOR/Akt pathway and by blocking signaling pathways associated with protein degradation [41]. In cell culture, glutamine induces autophagy under basal and stressed conditions and prevents apoptosis under heat stress through its regulation of the mTOR and p38/MAP kinase pathways [57]. This is of note because, besides chaperone-based, eukaryotes lay hold on autophagy as a part of protein quality control, as discussed in previous sections. Hence, by facilitating autophagy via modulation of the HS response, glutamine becomes of importance in chronic degenerative diseases of aggregative nature. Indeed, as glutamine may enhance HS response over an initiating proteostasis defect, glutamine can even avoid the triggering of autophagy in some circumstances, such as acute exercise (which is capable of inducing toxic imbalances that lead to autophagy) [264, 265].

Glutamine deprivation reduces proliferation of lymphocytes, influences expression of surface activation markers on lymphocytes and monocytes, affects the production of cytokines, and stimulates apoptosis. Moreover, glutamine administration seems to have a positive effect on glucose metabolism in the state of insulin resistance [266]. This protective effect of glutamine is related to glutamine-induced stabilization of mRNA encoding HSP70 [266], possibly via HBP.

As discussed above, fever is a protective acute-phase response to infection. However, in critically ill patients, the harmful effects of fever seem to be predominant. Critical illness is frequently (but not necessarily) associated with reduced plasma glutamine levels, which contribute to the immune suppression in these patients due to impaired monocyte function [267]. In vitro studies with glutamine-depleted monocytes (obtained from PBMC of health human donors) have shown that glutamine deprivation dramatically reduces PBMC thermoresistance and suggests that elevated body temperature may damage monocytes in critically ill patients with reduced plasma glutamine levels, possibly via inhibition of the cytoprotective HS response [267]. Age-related intestinal dysfunctions may also contribute to deficient passage of amino acids to the circulation, as inflamed gut mucosa utilizes glutamine in large quantities [213]. Studies in animal models of inflammatory bowel disease (IBD) suggest that supplementation of total parenteral nutrition with glutamine may increase glutamine plasma concentrations, reducing intestinal damage, improving nitrogen balance and the course of the disease. However, human data supporting this assumption are either missing or contradictory [268]. Nevertheless, glutamine supplementation has convincingly been demonstrated to prevent exercise-induced intestinal permeability, possibly through HSF1 activation [269, 270].

In conclusion, it is evident that the overall metabolism of glutamine in aging and age-associated degenerative diseases of inflammatory nature may be partially compromised and this may negatively impact the HS response in the elderly. Diminished bodily synthetic capacity that occur in aging due to muscular disuse (and/or sarcopenia) and defects in glutamine absorption by the intestine and/or in their utilization by the gut may also be at the center of glutamine depletion observed in some age-related conditions. Hence, interventions devoted to improve glutamine turnover and metabolism are predicted to be of value in assisting the improvement of the HS response in the elderly.

## Physical exercise, glutamine, and HS response in aging

Physical exercise is one of the most powerful physiological inducers of the HS response, comparable only to fever and anti-inflammatory cyPGs. Since exercise is a homeostatically threatening situation that evoke a series of physiological adjustments, it has long been thought that exercise should mandatorily induce the HS response. Some of the conditions known to elicit cellular stress response are similar to those experienced by cells in response to physical exercise. They include hyperthermia, transient ischemia, exercise-elicited generation of ROS/RNS and oxidative stress, cytokine production, muscular stretch-stress, intramuscular glucose and glycogen reduction, and alterations in intracellular calcium and pH values. All of them are potent inducers of HSP expression in different cell types and tissues [271, 272].

Locke and co-workers [273] were the first to demonstrate that vigorous physical activity is associated with the induction of HSP70 in rodents. Subsequently, increased expression of HSPs in humans following exercise was confirmed [274]. Now, it is peaceful that HSPs, such as HSP72 (iHSP70), are induced or activated upon acute exercise bouts and after chronic exercise training regimens, whereas HSP induction and HS response are key components of exercise adaptation that could contribute to improvements in athletic performance [275].

Although exercise has been being increasingly prescribed to elderly people in order to combat chronic inflammatory diseases [276, 277], only a few studies have addressed exercise impacts on HS response in aging [278–280]. Exercise-induced transient increases of iHSP70 inhibit the generation of inflammatory mediators and vascular inflammation, metabolic disorders (e.g., obesity and T2DM), and atherosclerotic CVD. In all these conditions, benefits of exercise on inflammation and metabolism

depend on the type, intensity, and duration of physical activity [274]. Exercise has also been shown to produce favorable effects against neurodegenerative diseases, by both preventing and avoiding the progress of age-related AD and PD [281]. This is linked to the fact that exercise enhances hippocampal neurogenesis, thus improving learning and memory in aged people [282].

The expression pattern of HSPs in skeletal muscles has been demonstrated to decrease in old rats compared with young ones. Interestingly, however, exercise training significantly increases HSPs in aged rats [283]. Hence, if acute exercise-induced HS response is severely blunted in the muscle of elderly individuals [284], exercise training regimens are emerging as more appropriate approaches for the elderly. Although there are some discrepancies in relation to HSP70 expression in response to exercise training in young people [285], expression of HSPs in skeletal muscles of aged individuals depends on the frequency and duration of exercise training [283]. Exercise induces autophagy in multiple organs involved in metabolic regulation, such as skeletal muscle, liver, pancreatic β cells, adipose tissue, and brain [41, 264, 265], so that exercise influences protein quality control via HS response per se and through HS response-dependent autophagy. Indeed, exercise stress and molecular control of proteostasis provides evidence that the HS response and autophagy coordinate and undergo sequential activation and downregulation and that this is essential for proper proteostasis in eukaryotic systems [41]. A systematic analysis carried out by Dokladny and colleagues [41] on the association between exercise-induced HS response (assessed by iHSP70 expression) and autophagy in humans (231 humans; 22 studies, including data from their own group) supports the notion that autophagy is upregulated during the early degradation phase of exercise while iHSP70 expression tends to increase during the later building and protein synthesis phase. Hence, HSP70 expression appears to be the main controller of protein synthesis and degradation, whereas autophagy remains under inhibitory control of the HS response. Therefore, exercise-induced autophagy represents, up to a certain point, a desirable response to avoid the formation of misfolded protein aggregates and defective organelles, provided not in excess.

Pharmaceuticals that activate HSPs and produce a "training-like" HS response are now under clinical trials. This is the case of hydroxylamine derivatives, such as the compound BGP-15, a small molecule that has been demonstrated to activate HSP70 in skeletal muscle, inhibiting the early-phase acetylation of HSF1 and prolonging the duration of its binding to HSEs [275]. In mouse models of muscular dystrophy, the HS response potentializer BGP-15 decreases kyphosis, improves the dystrophic pathophysiology in limb and diaphragm muscles, and extends lifespan [286]. It has been demonstrated that iHSP70 interacts with sarcoplasmic/endoplasmic reticulum $Ca^{2+}$-ATPase (SERCA) to preserve its function under conditions of stress, ultimately contributing to the decreased muscle degeneration observed after HSP70 upregulation [286]. The HS response inducer BGP-15 has proven to efficiently reverse the noxious effects of HFD-induced obesity in the muscle and adipose tissue, by increasing the HS response [12]. BGP-15 improves cardiac function and reduces arrhythmic episodes in different mouse models that progressively develop heart failure and atrial fibrillation and can provide cytoprotection and normalization of cell signaling, which are often defective in the aged and diseased heart [287]. Therefore, if BGP-15 was developed to simulate the beneficial effects of exercise, it is plausible to suppose that exercise itself should present better (or the best) accomplishment.

As a powerful exercise-related enhancer of the HS response, glutamine is a potential target for intervention in age-related conditions. Several studies with different cell types, including muscle, intestinal mucosa, immune cells, specific neurons of the central nervous system, hepatocytes, and pancreatic β cells, just to cite a few examples, have irrefragably demonstrated that glutamine is required in incubation/culture media for normal growth and function [214, 216, 218, 288]. In catabolic (e.g., sepsis, recovery from burns and surgery, exhaustive exercise) and inflammation-related (age-associated chronic diseases) situations, glutamine requirements increase dramatically. However, at the same time that the body increases its demand for glutamine, several organs reduce their ability to produce the amino acid (e.g., liver, kidneys), which leads to an overall deficit of glutamine in the body. Therefore, catabolic processes, which increase amino acid utilization in order to generate other necessary compounds (including glucose and acute phase proteins), contribute to the diminution of glutamine stores, a situation that is aggravated in high-throughput inflammatory and oxidative stress responses, as activated immune cells dramatically increment their glutamine utilization [208, 214, 216, 218, 289]. Although there is no single explanation for the glutamine deficit found in the above situations [210, 211], this effect is clearly observed in both humans [290] and experimental animal models [291]. Hence, under various conditions, glutamine can become a conditionally essential amino acid [292].

Exercise is a homeostasis-challenging situation that tends to induce, at the beginning of the session, an accelerated glutamine and alanine release from activated skeletal muscles, thus enhancing plasma concentrations of both amino acids [208]. This transient increase is due to the high production of $NH_4^+$ following muscle transaminations that occur upon the increased energy demand for ATP during muscle contraction. Additionally, alanine released by the working muscle is taken up by the liver in

order to furnish glucose to the circulation (glucose-alanine cycle) via gluconeogenesis. Still in the liver, $NH_4^+$ produced during transaminations must be diverted to urea (a bicarbonate-consuming reaction) or glutamine (a bicarbonate-saving pathway). Since excess intracellular $NH_4^+$ could not be poured into the circulation because $NH_4^+$ is cytotoxic (and, particularly neurotoxic) when at high concentrations, glutamine was settled during the evolution of metabolism as a safe circulating "carrier" of $NH_4^+$ moieties [208]. Moreover, sparing bicarbonate (the main physiological alkaline defense of the body) is of extreme importance during exercise, in view of acid-base imbalances imposed by the contracting skeletal muscle and exercise-related metabolic adjustments that tend to increase acid production.

In different catabolic conditions, including overtraining, the overall glutamine stores may be threatened. The availability of glutamine is thought to be a major factor during critical illnesses, such as sepsis, extensive burns, pancreatitis, trauma, and surgery [209, 293, 294]. Moreover, the concentration of glutamine in patients diagnosed with T2DM has shown a significant reduction (20 %), when compared with healthy individuals [295]. In colonic cancer patients undergoing surgery, plasma glutamine levels drop by 30 %, independently of the previous glutamine depletion [296]. In severe pancreatitis [297] and trauma patients [298], plasma glutamine levels decrease to less than 50 % of its basal levels as compared to control values. Similar effects have also been seen in experimental animal models of sepsis, followed by severe adaptive immune system suppression (low T and B lymphocyte responses) [50, 209, 299]. In such conditions, plasma glutamine depletion is an independent outcome factor in critically ill patients [300]. But even in non-ill conditions, such as during and after exhaustive exercise, glutamine metabolism may be affected in tissues, thus undoubtedly impairing the immune system, in spite of the absence of any observed change in plasma glutamine levels [301]. Hence, numerous studies in animal models of catabolic and critical illness indicate that total parenteral nutrition (TPN) supplemented with glutamine may enhance protein anabolism, gut-associated barrier functions, systemic immunity, and gut mucosal repair. This is apparently due to the potential of glutamine as an important fuel substrate for the gut itself because glutamine upregulates cytoprotective pathways [302]. However, in a recent study with intensive care unit (ICU) patients, it has been encountered that TPN supplemented with glutamine dipeptide is safe, but does not alter clinical outcomes among the patients [302], while clinical trials have not demonstrated prolonged advantages, such as reductions in mortality or risk of infections in adults [303]. In a recent meta-analysis of randomized clinical trials [304], no difference has been found to allow the recommendation of glutamine supplementation to generic population of critically ills. Therefore, the efficacy of glutamine supplementation is still under debate.

Aged people tend to present an array of intestinal dysfunctions, including those associated with gut mucosa transport and dysbiosis [31–33]. Since glutamine is of absolute requirement as a fuel substrate for the enterocyte, intestinal utilization of glutamine is important for maintaining the integrity of the intestinal barrier, with subsequent prevention of bacterial translocation and, through stimulation of the gut-associated immune system, prevention of gut barrier atrophy. It is assumed that a derangement of the gut mucosal barrier function, which occurs during aging and critical illnesses, results in an amplification of the general inflammatory response predisposing patients to multiple organ failure [305]. In fact, chronic glutamine supplementation reduces exercise-induced intestinal permeability while inhibiting NF-κB pro-inflammatory pathways in human PBMC, in a mechanism associated with the activation of HSP70 expression [269]. The same was confirmed in physically active subjects acutely treated with oral doses of glutamine prior to exercise [270]. Increased permeability may be a factor in the pathogenesis of Crohn's disease, which is a chronic inflammatory disease, frequently observed in the elderly, characterized by low-HS response that perpetuates the inflammatory state [306]. In fact, approximately 50 % of patients with Crohn's disease have an increased small intestinal permeability [307], whereas oral glutamine supplementation initiated before advanced age in rats increases gut mass and improves the villus height of mucosa, thereby preventing the gut atrophy encountered in advanced age [303]. Even though, a 4-week treatment with oral glutamine supplementations had shown no effect on impaired intestinal permeability in Crohn's disease patients [307].

There have been raised several theories to explain the persistent, relapsing inflammation observed in patients with Crohn's disease or ulcerative colitis. However, all of them converge to the fact that gut mucosa cannot overcome the persistent inflammatory milieu resolving inflammation [308] that becomes chronic. Crohn's disease progression is dependent on persistent inflammatory status based on NF-κB- and p38/MAPK-elicited signaling [309]. In any way, induction of the HS response (employing heat treatment, for example) has been shown to protect against TNFα-induced inflammatory shock and this is associated with HSP70 expression in many organs, including small and large intestines [310]. Additionally, intestinal malabsorption of glutamine has been referred to as causal to the ineffectiveness of oral glutamine treatment of obese insulin-resistant mice [311]. If, on the one hand, HSP70 expression is not definitely ascribed as causal or result of chronic inflammatory bowel diseases, overexpression of

HSP70 was found to prevent the development of inflammatory process in the large intestinal mucosa provoked by various damaging factors [32]. Corroborating this viewpoint is the finding that combined glutamine and arginine administration is able to decrease pro-inflammatory cytokine production by biopsies from Crohn's patients, this being associated with suppression of NF-κB- and p38/MAPK-based inflammatory pathways [312]. Although intestinal obstruction is not exactly a chronic inflammatory disease, glutamine supplementation decreases intestinal permeability and preserves gut mucosa integrity in experimental mouse model of intestinal obstruction [313].

As stated in the "Background" section, aging process may affect the HS response leading to conformational neurodegenerative diseases of inflammatory nature, this being affected by bodily glutamine status. In fact, the association with aging is one of the most distinctive characteristics of protein conformational diseases. This connection is particularly striking in neurodegeneration associated with deficient protein quality control (e.g., HS response, autophagy) where, for each specific disease, age is the strongest predictor of disease onset, even for the familial variants. On the other hand, age appears to have a modifying, rather than causative, influence on disease onset, as each disease has its characteristic age of onset, with AD and PD being late onset, ALS occurring most frequently in early to mid-life, and HD exhibiting a strong positive correlation between age of onset and polyglutamine length polymorphism [167]. In vitro studies suggest that healthy neuronal cells require both intracellular and extracellular glutamine and that the neuroprotective effects of glutamine supplementation may prove beneficial in the treatment of AD. In fact, glutamine acts as a neuroprotectant against DNA damage and Aβ- and $H_2O_2$-induced stress [314]. However, no clinical trial is currently being carried out as to assess the possible beneficial effects of glutamine supplementation, or combined glutamine plus exercise schedules, over HS response in neurodegenerative diseases in aging.

Although clinical studies with aged people remain scarce, experimental studies reported that glutamine supplementations may protect cells, tissues, and whole organisms from stress and injury by blocking NF-κB downstream inflammatory signals, thus promoting a balance between pro- and anti-inflammatory cytokines, by improving intestinal integrity and immune cell function, and, finally, by enhancing HS response, so that parenteral glutamine (> 0.50 g $kg^{-1}$ $day^{-1}$) may be of potential benefit to elderly individuals [305]. When (and preferably) orally given, glutamine may be administered as free amino acid or in its dipeptide forms. However, even though a priori safe, caution should be taken in supplementing middle-aged and elderly individuals with glutamine at the above dosage, as increases in serum urea and creatinine, paralleled by decreased in estimated glomerular filtration rate, have been reported in this specific population after glutamine supplementations [315]. Alternatively, glutamine can be administered as a part of TPN (0.3–0.5 g/kg body weight) and, as such, can reduce the dramatic decrease in glutaminemia and tissue glutamine in glutamine depletion states [294]. Glutamine, typically furnished to the patient as the dipeptide L-alanyl-L-glutamine, can be commercially found at a concentration of 200 g/L sterile solution, with an osmolality <900 mOsm/kg $H_2O$. These parenteral solutions are more effective than oral or enteral solutions, when the maintenance of glutamine concentration in the body is desired [294]. Nevertheless, TPN is very invasive and may expose the patient to increased risk of infections, so that, as far as possible, enteral alternatives should be chosen. Moreover, enteral routes are much more physiological and provide the physiological generation of other amino acid derivatives (e.g., citrulline and arginine), which can only be accounted for if glutamine is given enterally [64, 230].

Since intestinal dysfunctions are commonly found in the elderly that hamper the ability of aged people to maintain a good nutritional state [31–33], more efficient ways to deliver glutamine into the circulation have been permanently being investigated. In this regard, the excellent effects of glutamine dipeptides on glutamine availability have been attributed to the fact that enterocytes have a more efficient transport mechanism for the absorption of dipeptides and tripeptides than for the absorption of free amino acids [316]. The glycopeptide transport protein (PepT-1), which is located in the luminal membrane of the jejunum and the ileum, has a broad substrate specificity and actively transports dipeptides and tripeptides from diet into the enterocytes of humans and animals [317, 318]. Research utilizing radioactively labeled glutamine dipeptides has shown that nearly 90 % of the radioactivity accumulates intact in the cytosol. Through this route, it can be avoided intracellular hydrolysis of glutamine and its subsequent metabolism by enterocytes, proceeding directly into systemic circulation [291, 319]. Intestinal glutamine uptake is regulated by the high-affinity glutamine transporter solute carrier family 1 member 5 (SLC1A5) and its inhibition blocks glutamine entry in enterocytes leading to the inhibition of mTORC1 signaling and consequent dysregulation of autophagy [208]. In addition, glutamine can be carried by SLC7A5, which is a bidirectional transporter that regulates the simultaneous efflux of glutamine out of cells and the transport of other amino acids into cells. This directional control allows for excess glutamine to signal into cell growth promoting pathways, while suppressing catabolism in different tissues and cells [232, 320].

Studies in experimental animal models with acute oral glutamine supplementation, in its free form or as a

dipeptide, have demonstrated an increase in plasma glutamine concentrations between 30 to 120 min after supplementation [321]. Nevertheless, the concentration and the area under the curve of the dipeptide acutely supplemented group has been 26 % superior to that of free L-glutamine supplemented group, 30 min after the supplementation [321]. In another study, animals submitted to exhausting physical exercise and chronic supplementation with L-alanyl-L-glutamine have demonstrated that the nutritional intervention may attenuate the reduction of glutamine concentration in the soleus and gastrocnemius muscles immediately and 1 h after the exercise session [319]. Combined exercise training and glutamine supplementation has also been shown to increase hepatic and muscular concentrations of glutamine and glutamate [291, 322].

HSP70 expression is decreased during muscle inactivity and aging, and evidence supports the loss of HSP70 and its accompanying HS response as a key mechanism that may drive muscle atrophy, contractile dysfunction, and reduced regenerative capacity associated with these conditions [40]. Besides neurodegenerative diseases discussed above, aging also predisposes individuals to a progressive loss of muscle mass and function (sarcopenia). Primary causes of sarcopenia include a sedentary lifestyle and malnutrition. And, although the causes for this loss in skeletal muscle mass are multifactorial, sarcopenia is known to be associated with a state of chronic low-grade inflammation, being correlated with insulin resistance that culminates in an imbalance between protein synthesis and degradation rates. Considering that the skeletal muscle is an essential producer of glutamine, reducing muscle mass may limit the availability of glutamine for the rest of cells (e.g., immune cells) [323, 324]. In addition, aging generally increases protein and amino acid needs above those required by younger adults [325]. However, glutamine supplementation seems not to be able to combat the muscle wasting associated with disease or age-related sarcopenia [303]. In any case, a combination of resistance exercise plus nutritional interventions has been raised as promising in treating sarcopenia [26, 148]. This is reinforced by the fact that whey protein hydrolysate (WPH), a notable source of glutamine dipeptide, was found to enhance exercise-induced HSP70 response in rats [326].

While resistance training appears to be the most adequate intervention [26], older individuals fail to adequately respond to exercise stimuli. Decrement in regenerative capacity may also be due to a dramatic reduction in postprandial anabolism as well as an increase in generation (or decrease in removal) of ROS. On the other hand, recent work has suggested that increasing HSP expression through the manipulation of duration and frequency of exercise can lead to protection and training-induced adaptation against aging-induced structural weakness in skeletal muscles. Accordingly, at least in aged (20 months old) male Sprague-Dawley rats, muscular HSP70 expression (alongside HSP27, HSP60, and HSP90) has recently found to be seriously reduced, as compared to young animals [238]. This is particularly true for oxidative slow twitch fibers (soleus). And, as observed in other models, different schedules of exercise and exercise training were able to revert this profile to near-young patterns, which was followed by enhanced antioxidant enzyme (SOD) expression and decreased pro-inflammatory (p38/JNK) pathways. In total, it is reasonable to believe that the restoration of glutamine availability in aging (i.e., by supplementation, particularly *p.o.*) may lead to improved mTOR downstream pathway activation/sensitivity that would allow for normal HSF1/HSP70 activation (HS response), thus improving muscle integrity, regeneration, and recovery. Interestingly, lower plasma glutamine availability and reduced basal and induced levels of HSP70 are also characteristics of obese, insulin-resistant, and T2DM people. Similarly to the elderly, these people also present reduced muscle mass (sarcopenic obesity) [327] and low-grade inflammation [7, 112].

Besides its influence on intestinal absorptive capacity and inflammatory status, gut microbiota may modulate aging-related changes in innate immunity, sarcopenia, and cognitive function, all of which are elements of frailty [328]; strikingly, gut microbiota may be shaped by exercise, as discussed below. Changes occurring in the microbiota during aging can have an unfavorable impact on host health, particularly due to microbiota-induced intestinal inflammation and impaired absorption of vital nutrients. The most noticeable feature in the microbiota of elderly individuals is an alteration in the relative proportions of the Firmicutes and the Bacteroidetes phyla, with the elderly having a higher proportion of Bacteroidetes while young adults have higher proportions of Firmicutes [329]. A polemic paper published in *Diabetes* in 2007 [330] was the very first work approaching the influence of gut microbiota ecosystem on low-grade inflammation and its associated chronic inflammatory diseases [33]. Accordingly, bacterial lipopolysaccharide (LPS) was identified as a triggering factor for such diseases, whereas mice feeding on a high-fat diet (HFD) were found to increase the proportion of LPS-containing microbiota in their intestines. These observations were further confirmed in many other subsequent studies [331–333]. In fact, a huge body of evidence indicates that gut microbiota participates in whole-body metabolism by affecting energy balance, glucose metabolism, and low-grade inflammation associated with obesity and related metabolic disorders, particularly because gut microbiota controls gut barrier function and the onset of metabolic endotoxemia [331]. Many studies that have examined changes in the gut microbiota in obesity almost unanimously demonstrate that obese individuals

have altered levels of certain bacterial groups with a loss of biodiversity (with the predominance of Firmicutes genera) being the consistent outcome [334]. However, only a few studies addressed microbiota in aging [328, 329], thus suggesting that a lot of further efforts must be endeavored to shed light on novel perspectives for therapeutics and changes in our lifestyle [33].

That exercise is definitely the best and cheapest way of reverting chronic inflammatory diseases of whatever underlying nature is peaceful. What is novel is that exercise influences host-gut microbiota axis [335]. In fact, low-calorie and increased physical activity program during 10 weeks has shown a positive impact on gut microbiota composition, decreasing the amount of Firmicutes genera and increasing Bacterioidetes ones [335]. Fairly more recently, the triad diet-metabolism-exercise has become neat, particularly after carefully controlled studies on elite athletes (see, for instance, ref. [336] for review). In this regard, whey protein (WP) supplementations occupy a highlighted place. WP and whey protein hydrolysates (WPH), which are considerable sources of glutamine dipeptide and known for their postexercise recovery effects and muscle hypertrophy results, may contribute to gut microbiota composition and potentially to lipid metabolism, as judged by metagenomic analysis in samples from these subjects [336]. Furthermore, WPH are now referred to as novel antidiabetic agents that affect glycemia in animals and humans. WPH has proven to ameliorate blood glucose clearance, reducing hyperinsulinemia and restoring pancreatic islet capacity to secrete insulin in response to glucose in ob/ob mice [337]. Therefore, besides its well-known effects on cardiovascular and endocrine systems, exercise alone can also influence gut microbiota composition, although the impact of exercise on gut motility and transit time must also be considered in further studies [334].

Unequivocally, exercise improves health gains in diabetes, CVD, cellular senescence, and age-related degenerative diseases [13, 148]. The question is: what type and frequency should be prescribed to elderly subjects? Although there is as yet no clinical study on the joint contribution of glutamine supplementations and exercise to improve the HS response in the elderly, resistance training emerges as the best intervention in sarcopenia [26], whereas resistance exercise plus nutritional interventions (glutamine dipeptide) have also been raised as promising in combating sarcopenia in aging [148]. Physical inactivity or a decreased physical activity level is a part of the underlying mechanisms of sarcopenia and, therefore, physical activity can be seen as an important factor to reverse or modify the development of this condition. Exercise represents the most important approach to prevent and treat sarcopenia. Moreover, exercising before protein intake allows a greater use of dietary protein-derived amino acids for the de novo muscle protein synthesis in elderly men [338]. Although some clinical trials of exercise interventions demonstrate positive effects of exercise on cognitive performance, other trials show minimal to no effect. Physical exercise interventions aimed at improving brain health through neuroprotective mechanisms show promise for preserving cognitive performance. Exercise programs that are structured, individualized, higher intensity, longer duration, and multicomponent show potential for preserving cognitive performance in older adults [339]. Increased risk for cognitive impairment has been linked to CVD risk factors such as hypertension, dyslipidemia, metabolic syndrome, uncontrolled diabetes, hyperinsulinemia, and high levels of inflammatory markers, all of which are modifiable by increasing exercise levels [340]. Besides compelling evidence from animal models, people who are more active in mid-life and late life have lower risk for global cognitive decline and incident dementia. Aerobic exercise randomized controlled trials in older adults have demonstrated positive effects on cognitive performance in conjunction with changes in regional brain volume, neurotrophin levels, and brain activation patterns. Additionally, although few studies have examined the effects of resistance training on cognitive function, there is some evidence that resistance-only training has a positive effect [339]. Aerobic exercise has long been linked to improvements in cardiorespiratory fitness and avoidance/reversion of chronic inflammatory diseases, but progressive resistance exercise is also of value, particularly in preventing sarcopenia and other age-related degenerative diseases. In summary, four categories of specific exercises are recommended for elderly people: aerobic exercise, progressive resistance exercise, flexibility, and balance [341].

Inducers of the HS response, such as physical exercise, heat treatment itself (e.g., hot tub, sauna), and calorie restriction may efficiently interrupt the vicious cycle of age-related chronic degenerative diseases of inflammatory nature in elderly, being envisaged as the best and most economical treatments for such chronic diseases [7, 39]. Extracellular to intracellular HSP70 ratio index (H-index), measured in PCMC in relation to serum values, has also been recently described as novel and overall index of immunoinflammatory status of an individual and could be invaluable in estimating immunoinflammatory status in as many different situations as immune responses, obesities, T2DM, CVD, and immunological impacts of exercise in aging [34].

Although, to the best of our knowledge, there is as yet no one published work, a question emerges as to whether exercise combined with glutamine supplementation would be able to maintain HSP levels and an adequate HS response in the elderly. Since exercise increases both glutamine production and HS response in the muscle, alongside the fact that glutamine is a potentializer of

the HS response, it is not unreasonable that an association between exercise training and glutamine supplementation should be invaluable in preventing age-related chronic degenerative diseases of inflammatory nature. Equally, how exercise and glutamine interact with gut microbiota, under the viewpoint of local and systemic HS response, is another interrogation. These testable possibilities need to be urgently addressed.

## Conclusions

Glutamine is essential for the maintenance of normal neuronal physiology and skeletal muscle size and function due to its capability of controlling the HS response. Changes in physiological systems (e.g., cardiovascular, endocrine, muscular, nervous) that occurs with aging, along with simultaneous unfavorable changes in body composition (i.e., sarcopenia and visceral/abdominal obesity) may lead to, respectively, lower availability of glutamine and chronic low-grade inflammation. Under these conditions, bodily levels of glutamine may reduce, thus affecting its physiological roles. As glutamine is essential for normal HSF1/HSP70 axis activation, the stress response is likely to be reduced in many elderly people. Exercise is a powerful and low-cost physiological inducer of the HS response, being capable of reverting age-associated low-HS response states. Therefore, exercise training associated with glutamine supplementation and heat treatment itself are envisaged as important therapeutic tools able to restore the stress response in the elderly, allowing normal HSP70 synthesis and the maintenance of muscle integrity, size, regeneration, and rapid recovery from injury. In addition, the re-establishment of the HS response by glutamine supplements, under specific and controlled conditions, may also reduce the incidence of neurodegenerative diseases thus increasing longevity with health.

> "Quæ medicamenta non sanat, æ ferrum sanat. Quæ ferrum non sanat, æ ignis sanat. Quæ vero ignis non sanat, æ insanabilia existimare oportet.
> *That which drugs fail to cure, the scalpel can cure. That which the scalpel fails to cure, heat can cure. If heat cannot cure, it must be determined to be incurable.*"
> (Aphorisms of Hippocrates, by Elias Marks, from the Latin version of Verhoofd, Collins & Co., New York, 1817)

#### Abbreviations

ACTH: Adrenocorticotropic hormone; AD: Alzheimer's disease; ALS: Amyotrophic lateral sclerosis; ARDS: Acute respiratory distress syndrome; ARE: Antioxidant response elements; ASS: Argininosuccinate asynthetase; Aβ: Amyloid-beta; CVD: Cardiovascular disease; eHSP: Extracellularly located HSP; ER: Endoplasmic reticulum; GFAT: Glutamine:fructose-6-phosphate amidotransferase (a.k.a. glutamine:fructose-6-phosphate transaminase); GLP-1: Glucagon-like peptide 1; HBP: Hexosamine biosynthetic pathway; HD: Huntington's disease; HFD: High-fat diet; HS response: Heat shock response; HS: Heat shock; HSE: Heat shock element; HSF: Heat shock factor; HSP: Heat shock protein; HSP70: the 70 kDa family of heat shock proteins; IBD: Inflammatory bowel disease; ICU: Intensive care unit; iHSP: Intracellularly located HSP70; IL: Interleukin; IR: Insulin resistance; Keap1: Kelch-like ECH-associated protein-1; LPS: Lipopolysaccharide; mTOR: Mammalian target of rapamycin; NF-κB: Nuclear transcription factors from the kappa light chain enhancer of activated B cells (κB) family; NFT: Neurofibrillary tangle; Nrf2: Nuclear factor-erythroid 2 p45-related factor 2; OGT: O-linked-N-acetylglucosaminyl (O-GlcNAc) transferase; PBMC: Peripheral blood mononuclear cells; PD: Parkinson's disease; PDG: Phosphate-dependent glutaminase; RA: Rheumatoid arthritis; ROS: Reactive oxygen species; SIRT1: Nicotinamide adenine dinucleotide ($NAD^+$)-dependent protein deacetylase of class III family sirtuin-1; SLE: Systemic lupus erythematosus; T1DM: Type 1 diabetes mellitus; T2DM: Type 2 diabetes mellitus; TLR: Toll-like receptor; TNFα: Tumor necrosis factor alpha; TPN: Total parenteral nutrition; UDP-GalNAc: Uridine diphosphate (UDP)-*N*-acetylgalactosamine; UDP-GlcNAc: Uridine diphosphate (UDP)-*N*-acetylglucosamine

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#### Authors' contributions

All the authors were involved in co-writing the manuscript. PIHBJ, prepared the figures, supervised the elaboration of the manuscript, and wrote its revised final version. All authors read and approved the final manuscript.

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#### Ethics approval and consent to participate

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