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REVIEW ARTICLE

The role of Hsp70 in oxi-inflamm-aging and its use as a potential biomarker of lifespan

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Abstract The heat-shock protein 70 (HSPA1A or Hsp70) acts as a cellular defense mechanism its expression being induced under stressful conditions. Aging has been related to an impairment in this induction. However, an extended longevity has been associated with its increased expression. According to the oxidation-inflammation theory of aging, chronic oxidative stress and inflammatory stress situations (with higher levels of oxidant and inflammatory compounds and lower antioxidant and anti-inflammatory defenses) are the basis of the age-related alterations of body cells. Since oxidation and inflammation are interlinked processes, and Hsp70 has been shown to confer protection against the harmful effects of oxidative stress as well as modulating the inflammatory status, it could play a role as a regulator of the rate of aging. This role may be different in mitotic and post-mitotic tissues due to the differences in their age-related mechanisms of response, such as apoptosis. Mechanisms affected by Hsp70 that can interfere with the deleterious effects of excessive oxidative stress and chronic low-grade inflammation and that are closely related to the aging process have been detailed. In addition, the potential use of the basal levels (with their differences in post-mitotic and mitotic tissues), the inducible levels, as well as the extracellular levels of Hsp70 as possible biomarkers of the rate of aging and lifespan, have also been discussed.

Keywords Hsp70 · Aging · Longevity · Biomarker · Oxidation · Inflammation

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Introduction

The oxidative-inflammatory theory of aging proposes a chronic oxidative and inflammatory stress state as a cause of the age-related changes, especially those affecting the homeodynamic systems, i.e. the nervous, endocrine and immune systems. The subsequent impairment of these systems, as well as the communication between them, could explain the altered homeostasis/homeodynamics and the increase of morbidity and mortality (De La Fuente and Miquel 2009). At the molecular level, to cope with conditions of stress such as oxidative and inflammatory stress, organisms have developed a wide range of sophisticated stress response mechanisms. These act at the cellular or organelle-specific level, initiating a series of events for maintenance, repair, adaptation, remodeling and survival (Demirovic and Rattan 2013).

Mammalian cellular stress responses can be categorized into seven main distinct pathways (Demirovic et al. 2013). Of all of them, the most evolutionary

conserved among species, is the heat-shock response (Verbeke et al. 2001). The main effector of this response, which is induced by HSF-1 activation, is the chaperone Hsp70, the product of the HSPA1A gene. Although this chaperone is also called Hsp70-1, Hsp72, HspA1, Hsp70-1a, Hsp70i (Tavaria et al. 1996; Kampinga et al. 2009), and recently the term proposed has been HSPA1A (Kampinga et al. 2009), in the present review, this heat shock protein will be referred to as Hsp70, which is the term most frequently used in the literature.

In addition to the role of Hsp70 in the protein quality control system, which is of vital relevance in counteracting the aging process, novel intracellular functions of this chaperone have emerged. Interestingly, these are also closely related to the oxi-inflamm-aging process. Thus, anti-apoptotic properties as well as NF-kB repression capacity of Hsp70 have been observed (Guzhova et al. 1997; Buzzard et al. 1998; Asea et al. 2002). Moreover, it has been demonstrated that extracellular Hsp70 can exert a wide range of actions, triggering pro-inflammatory cascade signalling or, in the cases where there is an over-activation of the immune system, blocking it (Asea et al. 2002; Lehner et al. 2004; Van Eden et al. 2005). Thus, in the context of oxi-inflamm-aging, Hsp70 can be proposed as one of the possible regulators of this process. Moreover, this chaperone could be a good candidate as a biomarker of the rate of aging and lifespan. These aspects will be discussed in the present review.

Oxidative-inflammatory theory of aging

The aging process is characterized by an oxidative stress state (Harman 1956; Barja 2004) and by a chronic low-grade of inflammation, so-called "inflamm-aging (Franceschi et al. 2000). The free radical theory of aging (Harman 1956), proposed that aging is the consequence of the accumulation of damage by deleterious oxidation in biomolecules caused by the reactivity of free radicals of oxygen when a chronic oxidative stress occurs as consequence of higher amount of oxidant compounds than antioxidant defenses. In addition, the term inflamm-aging denotes an upregulation of the inflammatory response that occurs with age, resulting in a low-grade chronic systemic proinflammatory state. It is characterized by raised levels of proinflammatory cytokines (interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), etc.) and other inflammatory compounds, all of which have been shown to rise with age and be involved in the pathogenesis of most age-associated diseases (Vasto et al. 2007). Regarding these two very interrelated (Vida et al., 2014) and important aging characteristics, the term oxi-inflamm-aging was proposed to better define what occurs during the aging process (De La Fuente and Miquel 2009). The theory of oxidation and inflammation of aging links the chronic oxidative and inflammatory stress with a persistent activation of the transcription factor NF-kB, and suggests the involvement of the immune system in this activation and in the aging process (De la Fuente and Miquel, 2009). The age-related changes in the immune system, called immunosenescence, which is characterized by the impairment of many functions but also by the overactivation of others, could at the same time, contribute to the increase of the oxidative-inflammatory stress situation, and thus, could be responsible for the acceleration of aging (De La Fuente 2014). Thus, successful aging and longevity will depend on the maintenance of efficient anti-inflammatory and antioxidant mechanisms.

Heat-shock response, chaperones and Hsp70

Heat shock proteins (Hsp), also termed stress proteins, are highly conserved molecules which have been observed in every organism, from eubacteria to archebacteria, and from mice to soybeans (Richter et al. 2010). Under physiological conditions, heat shock proteins (Hsps) are expressed at low levels. However, a wide variety of pathological and physiological stressful stimuli can induce a marked increase in the synthesis of intracellular Hsp, a process known as the heat-shock response (Verbeke et al. 2001). The heat shock factor 1 (HSF-1) is responsible for the induction of most chaperones acting as a "sensor" of the stress status of the cell. Under normal conditions, Hsp90 and Hsc70 are bound to HSF-1 forming an inhibitory complex, which maintains HSF-1 inactive in the cytoplasm. However, when the cytosolic level of damaged or misfolded proteins increases, the transcription factor HSF-1 is released from its inhibitory chaperone complex and translocates into the nucleus where it induces the synthesis of chaperone proteins,

which are the effectors of the heat-shock response (Shamovsky and Gershon 2004).

Chaperones are involved in various aspects of proteome maintenance, including assistance in macromolecular complex assembly, protein transport and degradation, aggregate dissociation and refolding of stress-denatured proteins (Liberek et al. 2008). Chaperones were first classified into different families according to their molecular weight: Hsp40s, Hsp60s, Hsp70s, Hsp90s, Hsp100s, and the small Hsps. Recently another terminology has been proposed based on the HUGO Gene Nomenclature Committee (Kampinga et al. 2009). Many, but not all of them, are stress proteins or heat shock proteins (Hsps), since their synthesis is induced under several conditions of stress (e.g., heat shock, oxidative stress, cellular energy depletion, toxins, heavy metals, antibiotics, infections, ethanol, inflammation, etc.), which structurally destabilize a subset of cellular proteins. A common aspect of these stressors is that they result in proteins having non-native conformations, which is consistent with the function of Hsps as molecular chaperones. The Hsp70 family encompasses at least 11 genes which encode a group of highly related proteins; from all of them the gene HSPA1A is the one that encodes the inducible Hsp70, also called Hsp70-1, Hsp72, HspA1, Hsp70-1a and Hsp70i (Tavaria et al. 1996; Kampinga et al. 2009). In the present review we will refer to this protein as Hsp70 being consistent with the large body of literature. Out of all the chaperones that are induced when the heat shock response is triggered, the product of the HSPA1A gene, Hsp70 is the one that is induced by HSF-1 in the highest amount and it is the main effector of this response. Caution should be taken given that the term Hsp70 used in the literature includes two different proteins, the inducible one, also called Hsp70 or Hsp72 (HSPA1A) and its heat-shock cognate protein, which is constitutively expressed, called Hsc70 or Hsp73 (HSPA8). However, this distinction is ambiguous because on one hand, in non-stressed cells measurable concentrations of the so-called inducible Hsp70 have been reported and on the other hand increased synthesis of some cognate proteins due to stress has also been found.

Hsp70 in addition to being the heat shock protein that is induced in the highest amount under stress conditions and acting as the main effector of the heat-shock response, is also the most highly conserved of the Hsps. This is the reason why it has aroused the greatest interest and it has been the most widely studied. The human Hsp70 protein is 73 % identical to the *Drosophila* protein and 50 % identical to the *E. coli* dnaK product. Therefore, it can be assumed from the universality and conservation of Hsp70 through species, that it must play an essential role in cell survival. Moreover, a strong correlation of Hsp70 levels with longevity in mammalian and avian species has been found, suggesting that one of the mechanisms underlying the evolution of longevity and lifespan is improved protein homeodynamics via increased constitutive expression of Hsp70 (Salway et al. 2011). Its universality and evolutionary preservation across species, together with its known implication in the aging process, makes Hsp70 a promising biomarker of this process.

Role of Hsp70 in oxidation

The accumulation of oxidized proteins is a hallmark of the aging process and of many age-related diseases. Oxidation impairs protein function as the proteins are unfolded leading to an increase of protein hydrophobicity and often results in the formation of toxic aggregates, which interfere with normal cell functioning. At the molecular level, and to ensure survival, cells have developed several defense mechanisms to counteract this oxidative damage accumulation. One of the first lines of defense is the action of the antioxidants, which try to counteract and ameliorate the effects of free radicals and reactive oxygen species (ROS). Once the free radicals have already caused damage, molecular chaperones act to counteract the toxicity of the damaged proteins, RNA and DNA. These molecular mechanisms, despite acting "downstream" are of great importance given that their correct functioning can also prevent the damage caused by oxidative stress (Broome et al. 2006).

The molecular chaperone Hsp70 plays a pivotal role in the protein quality control system, ensuring the correct folding of proteins, the re-folding of misfolded proteins and controlling the targeting of proteins for subsequent degradation. Thus, Hsp70 counteracts the oxidative stress damage of proteins. In fact, it has been demonstrated that overexpression of Hsp70 in the muscle of mice, abrogates the age-related increase in post-translational modifications of proteins such as their carbonylation, oxidation and formation of

disulfide bonds (Broome et al. 2006). In addition, cell membranes, DNA, RNA and proteins have all been suggested as being protected by Hsp70 due to its folding activities. Moreover, it has also been suggested that Hsp70 may increase the expression or the activity of endogenous scavengers of ROS, such as catalase and superoxide dismutase (Polla et al. 1995) and it has also been postulated that Hsp70 protects cells from oxidative stress injury, maintaining mitochondrial membrane potential (Polla et al. 1996). Figure 1 schematically shows the proposed molecular targets through which, Hsp70 could mediate protection against oxidative stress.

Role of Hsp70 in inflammation

Intracellular Hsp70

Intracellular Hsp70 has a wide range of anti-inflammatory actions. It can prevent responses to inflammatory cytokines such as tumor necrosis factor α (TNF α) and interleukin 1 (IL-1). Mice subjected to heat shock have been shown to be protected from normally lethal inflammatory shock after systemic administration of high doses of TNF-α, whereas mice missing the Hsp70.1 (HSPA1A) gene are no longer protected (Van Molle et al. 2002). In addition to modulating the response to inflammatory cytokines, Hsp70 also down-regulates their production. Thus, overexpression of Hsp70 in human macrophages blocks lipopolysaccharide-induced increases in the production of TNF, IL-1, IL-10, and IL- 12 (Ding et al. 2001). In the setting of focal cerebral ischemia, overexpression of Hsp70 has also been associated with the decreased production of TNF-α and IL-1β (Zheng et al. 2008).

Many, if not most, of the modulatory effects of intracellular Hsp70 on inflammation can be attributed to the regulation of the NF-kB pathway. Transcription factors of the NF-kB family are key players in the initiation of the inflammatory response (Gilmore 2006)

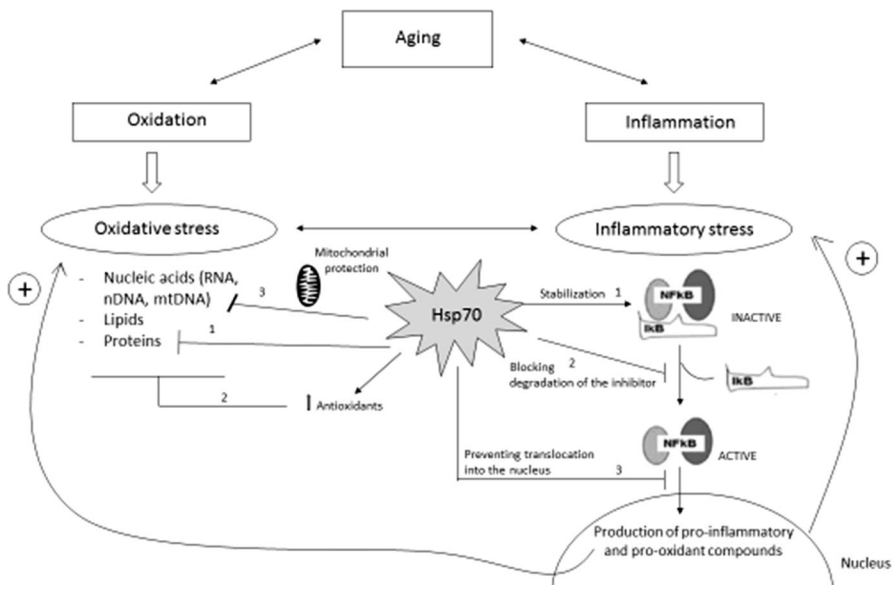

Fig. 1 Role of intracellular Hsp70 in oxi-inflamm-aging. The oxidation-inflammation theory of aging proposes a chronic oxidative and inflammatory stress state as the base of the age-related alterations of body cells. A chronic oxidative stress causes oxidative damage to nucleic acids, lipids and proteins and together with a chronic inflammatory stress, lead, by the persistent activation of NF-kB, to the production of pro-inflammatory and pro-oxidant compounds. These could cause a positive feedback loop in both oxidation and inflammation damage in a vicious circle. In this context, the intracellular Hsp70 acts as a defense mechanism counteracting oxidative damage by *1* refolding or targeting, for degradation, misfolded proteins, *2* increasing the expression or activity of endogenous scavengers of ROS, such as catalase or superoxide dismutase and *3*) maintaining mitochondrial membrane potential. In addition, Hsp70 may counteract the inflammatory stress by blocking the activation of the transcription factor NF-kB by: *1* stabilization of the inactive Nf-kB–IkB complex, *2* blocking the degradation of the inhibitor IkB or *3* preventing translocation of the active NF-kB complex

given that they induce the expression of several genes involved in inflammatory and immune responses, including those of TNF- α, IL-1, iNOS, and matrix metalloproteinase 9 (Matthews and Hay 1995; Gilmore 2006). Since intracellular Hsp70 blocks the activation of NF-kB, the induction of Hsp70 inhibits the nuclear translocation of NF-kB in response to inflammatory cytokines and other stimuli (Guzhova et al. 1997). Thus, mice overexpressing Hsp70 showed decreased NF-kB activation after a stroke (Zheng et al. 2008). These effects may be carried out through the direct interaction of Hsp70 with NF-kB proteins or by interactions with other proteins in the NF-kB regulatory pathway. Some authors found a direct interaction with NF-kB after heat-shock, since they observed co-immune-precipitation of Hsp70 with members of the NF-kB family: p65, p50 and rel-c (Guzhova et al. 1997). Other authors found that Hsp70 blocks the degradation of the inhibitor of NF-kB: IkB, thereby preventing activation of NF-kB (Wong et al. 1997). Another proposed mechanism has been the Hsp70-mediated stabilization of the IkB–NF-kB complex (Zheng et al. 2008). In Fig. 1 the above mentioned molecular mechanisms through which Hsp70 blocks NF-kB activation are shown schematically.

In summary, it has been demonstrated that intracellular Hsp70 can operate at many levels of the NF-kB pathway to inhibit or dampen its activation and consequently, it could be a promising target to block the establishment of age-related chronic inflammation.

Extracellular Hsp70

It has been documented that a variety of cell types, including neural cells, (Guzhova et al. 2001; Taylor et al. 2007), epithelial cells (Broquet et al. 2003), embryo cells (Hightower and Guidon 1989), B lymphocytes and dendritic cells (Théry et al. 1999; Clayton et al. 2005), maturing erythrocytes (Mathew et al. 1995) and tumor cells (Gastpar et al. 2005) can release Hsp70. Moreover, Hsp70 and anti-Hsp70 antibodies have been identified in human serum (Pockley et al. 1998). Numerous recent studies have examined the levels of extracellular Hsp70 in relation to diseases and pathologic states, as well as in relation to aging, though in some instances both Hsp70 and the non-inducible (cognate) form of the 70 kDa heat shock protein (Hsc70) were not distinguishable. Current thinking suggests that Hsp70 is released both by functioning as well as dying cells, and can act on a variety of receptors (Calderwood et al. 2007; Schmitt et al. 2007).

Different secretion mechanisms have been postulated. Since transport of other proteins across lipid membranes is one of the tasks of Hsp70, it is assumed that cytosolic Hsp70 could be transported to the plasma membrane in concert with other proteins possessing transmembrane domains that fulfil shuttle functions (Mambula et al. 2007). However, the molecular nature of these associated proteins has not yet been identified. Other authors reported an active nonclassical secretory pathway that could not be affected by inhibitors that antagonize transport through the ER-Golgi system (Hunter-Lavin et al. 2004). It has also been reported that after stress, Hsp70 and Hsc70 display an interaction with phosphatidylserine moieties on membranes, followed by rapid incorporation into the lipid bilayer. Finally, it has also been demonstrated that once Hsp70 and Hsc70 are anchored in the membrane via their C-terminus sequences, they can be released via exosome formations that activate macrophages (Vega et al. 2008).

If several physiological mechanisms have been proposed for the release of Hsp70, there should also be physiological functions for these extracellular proteins. Although Hsp70 release from dying cells can serve as a danger signal, release from living cells can signal a successful stress response (Ganter et al. 2006) and suggests a modulatory or signalling role. In fact, some authors have shown that extracellular Hsp70 acts as a chaperokyne. Several reports have shown that extracellular Hsp70 can induce the release of cytokines, including TNFα, IL-6, and IL-1β from monocytes (Asea et al. 2002; Vabulas et al. 2002; Lehner et al. 2004; Svensson et al. 2006). Extracellular Hsp70-induced cytokine release was found to be mediated through Toll-like receptor 2 (TLR2), TLR4, and downstream activation of NF-kB (Asea et al. 2002). It is known that these pro-inflammatory cytokines can stimulate the HPA axis, an effect that results in the increased release of glucocorticoids and other adrenal steroids that are powerful anti-inflammatory agents. Thus, Hsp70 could be involved in the triggering of a feedback anti-inflammatory mechanism. Glucocorticoids, at certain levels, not only inhibit pro-inflammatory cytokine synthesis and release, but also increase IL-10 production, a prototypic anti-inflammatory cytokine. Thus, Hsp70–glucocorticoid–IL-10

interactions appear to be relevant as an anti-inflammatory mechanism (Ortega et al. 2012).

Moreover, in the cases where there is an overactivation of the immune system, such as in some chronic inflammatory diseases (rheumatoid arthritis and diabetes), it has been described that extracellular Hsp70 can prevent or arrest inflammatory damage and promote production of anti-inflammatory cytokines such as IL-10 (Van Eden et al. 2005).

Therefore, several different functions have already been described for extracellular Hsp70, including enhancement of the immune system response as well as dampening, in the cases where there is a harmful overactivation of the immune system, suggesting that a fine-tuned regulation of this extracellular Hsp70 is needed to keep inflamm-aging under control (Fig. 2).

Hsp70 and the immune system

The immune system plays a pivotal role in the establishment of the oxidative-inflammatory condition of aging, through NF-kB modulation, as previously mentioned (De La Fuente and Miquel 2009; Arranz et al. 2010; De La Fuente 2014). The dysregulation and overall impairment in host immunity that occur with aging are evidenced by the higher risk and severity of infections and the increased susceptibility to cancer among aged individuals, exerting a great influence on age-related morbidity and mortality (Vida et al. 2014).

As explained in detail above, the double control that Hsp70 exerts on inflammation, through inhibition, when intracellular, or activation when extracellular, is

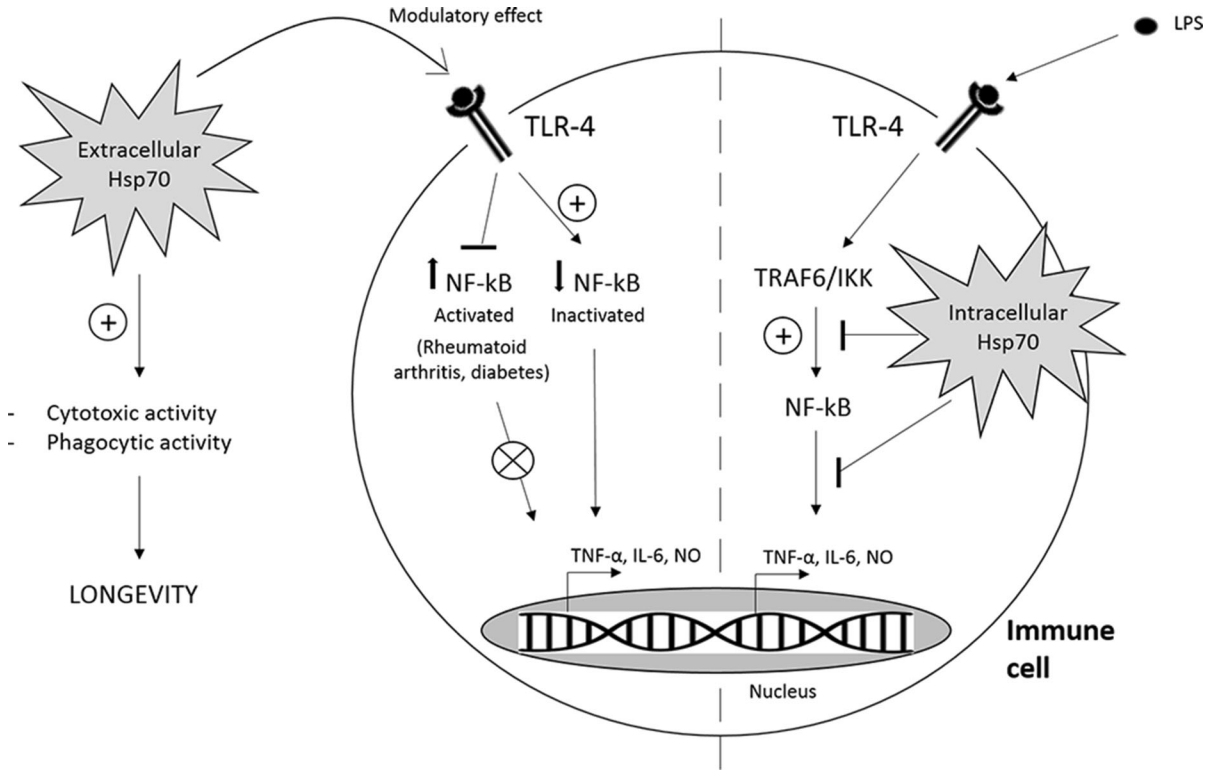

Fig. 2 Modulation of immune response by Hsp70. Immune cells are under tight control of both extracellular and intracellular Hsp70. The extracellular Hsp70 exerts a modulatory effect, through Toll receptor 4 (TLR4), depending on the activation state of the immune cells. Thus, it can act as a chaperokyne promoting the activation of NF-kB when the immune cells are not activated. Nevertheless, in some chronic inflammatory diseases such as rheumatoid arthritis and diabetes, extracellular Hsp70 can prevent or arrest inflammatory damage by inhibiting NF-kB. Moreover, extracellular Hsp70 plays an important role in enhancing the immune response, triggering the cytotoxic activity of natural killer cells and the function of phagocytic cells, which are immune responses enfeebled during aging, and as a result it contributes to longevity. The intracellular Hsp70 exerts an anti-inflammatory action impairing the activation of NF-kB by direct interaction with NF-kB proteins or with other proteins in the NF-kB regulatory pathway. (See "Role of Hsp70 in age-related apoptosis" for more detail)

carried out involving the NF-kB pathway. But Hsp70 can also directly affect the activity of immune cells. In fact, extracellular Hsp70 has been shown to play an important role in enhancing immune cell response. It has been shown to be important in triggering the activity of natural killer cells (Multhoff et al. 2001), which is thought to be mediated through the C-type lectin receptor CD94 and the adhesion molecule CD56 (Gross et al. 2003). It has also been shown to stimulate neutrophil functions (chemotaxis, phagocytosis and microbicidal activity) (Giraldo et al. 2013), effects that are mediated through adrenoreceptors (Hinchado et al. 2012). Interestingly, administration of Hsp70 in vivo promotes wound healing by stimulating macrophage phagocytic activity (Kovalchin et al. 2006). The capacity of extracellular Hsp70 to affect and to be affected directly or indirectly by the immune network indicates that this heat shock protein is part of the signaling system that can modulate the inflammatory status influencing the aging process.

Thus, in the context of aging, extracellular Hsp70 could ameliorate the age-related enfeeblement of the immune cell functions and at the same time could modulate the inflammatory status, preventing the inflammatory damage caused by the age-related chronic inflammation (Fig. 2).

Role of Hsp70 in age-related apoptosis

The relationship between aging and apoptosis has been long discussed, the most prevalent thought being that aging is associated with an up-regulation of apoptosis. The common view was that apoptosis would have primarily a negative impact on aging by destroying essential and often irreplaceable cells. That view has now changed to one that acknowledges that there are two general ways in which apoptosis can play a role in aging: the first one, by the elimination of damaged and presumably dysfunctional mitotic cells (e.g., fibroblasts, hepatocytes), which can then be replaced by cell proliferation, thereby maintaining the homeodynamics. The second could be the elimination of essential post-mitotic cells (e.g., neurons, cardiac myocytes), which cannot be replaced, thereby leading to deterioration. Thus, the proposed involvement of Hsp70 in the process of apoptosis is very relevant. In fact, the cytoprotective role of Hsp70 has been related with the inhibition of apoptotic pathways (Buzzard et al. 1998). Moreover, Hsp70 has been demonstrated to be involved in abolishing the apoptosis cascade at different key points, both upstream and downstream, affecting both the intrinsic and extrinsic pathways of apoptosis. This regulatory role of Hsp70 on apoptosis is carried out at the following levels: (1) At a pre-mitochondrial level by inhibiting the stress inducing signalling cascade (Guo et al. 2005) or by stabilizing survival kinases, such as PKC or Akt (Gao and Newton 2002); (2) At the mitochondrial level, by preventing mitochondrial membrane permeabilization through the inhibition of BID activation, a pro-apoptotic protein (Gabai et al. 2002) and blocking BAX translocation. This prevention of mitochondrial outer membrane permeabilization, avoids DNA fragmentation; (3) At the post-mitochondrial level by inhibiting caspase activation and DNA fragmentation (Beere et al. 2000).

Promising use of Hsp70 as a biomarker of biological age

The ability of Hsp70 to ameliorate the toxicity of proteins and regulate key aging pathways such as inflammation, immunosenescence and apoptosis, indicates that Hsp70 could be a promising biomarker of lifespan and biological age.

Intracellular Hsp70

In the context of intracellular Hsp70, the different actions of the inducible and basal levels of this chaperone must be considered.

Inducible levels

There is a large number of reports demonstrating that the induction of various chaperones is impaired in aged organisms (Deguchi et al. 1988; Fargnoli et al. 1990; Heydari et al. 1994; Nitta et al. 1994; Liu et al. 1996; Verbeke et al. 2001). Nevertheless, the level of response depends on the type of stimulus. Thus, in aged rats while heat-induced synthesis of Hsp70 is impaired, exercise in the same animal is able to induce a significant amount of Hsp70 (Kregel and Moseley 1996). These differences are also shown when comparing other aged animal species, both experimental animals and human subjects (Söti and Csermely 2000). This indicates there is not a general age-related

impairment in the transcriptional process of molecular chaperones. Indeed, the levels of HSF-1, which is the transcription factor responsible for the induction of most chaperones, are practically unchanged during aging. However, the levels of activation and binding of this HSF-1 to the heat shock element and its DNA-binding site in the promoter region of molecular chaperones, are decreased in aged animals (Heydari et al. 1994; Pahlavani et al. 1995; Locke and Tanguay 1996). Interestingly long-lived animals as well as human centenarians do not experience this impairment at the level of Hsp70 induction since they synthesize it in response to a given stressor as if they were young (Ambra et al. 2004). Moreover, Hsp70 induction has been found to be impaired in some prematurely aging mice models. Thus, Hsp70 induction was lower in the liver of aged mice prone to accelerated senescence than in mice that were resistant to accelerated senescence (Nakanishi and Yasumoto 1997).

In addition, the relative amount of Hsp expression after a given stressor has been found to be predictive of remaining life span in *C. elegans* as described by (Rea et al. 2005). These authors have found that the relative amount of expression of an sHsp transgene (Hsp16.2-GFP) in response to brief heat stress in a young worm is partially predictive of that individual's remaining life span. Young worms with the most robust expression of this gene had the longest life spans and were most resistant to subsequent toxic heat stresses.

Furthermore, genetic studies carried out on Hsp70 genes in humans have demonstrated that specific polymorphisms are significantly associated with human longevity and survival, these polymorphisms being related to a differential induction of the heat shock response (Singh et al. 2007). In this way, these authors have shown that isolated peripheral blood cells from those genotypes, which are negatively associated with human longevity, also have less ability to respond to heat shock (Singh et al. 2006).

Given that the Hsp70 induction levels after a given stressor are high in adult and long-lived individuals but low in old and prematurely aged subjects, these inducible levels can be proposed as a biomarker of biological age.

Basal levels

Despite the unifying results demonstrating the age-related impaired induction of Hsp70 to a given stressor, the age-related changes of Hsp70 basal levels is still controversial due to conflicting results. Cells in vitro of aged human beings and other species (*Drosophila*, *C. elegans*,..) develop a constitutively increased level of Hsp70 (Wheeler et al. 1995; Fonager et al. 2002). The accumulation of misfolded proteins in aged cells would require an increased amount of chaperones to prevent protein aggregation and to assist in refolding, or degradation. So this increase in the Hsp70 basal synthesis can be explained as an attempt by the cells, to try to counteract the increase in damaged proteins or as an attempt to try to compensate for the decreased functionality of Hsp70 in aged cells. Indeed, the term "sick chaperone" arose to explain this increased basal synthesis of Hsp70. However, several reports have shown that Hsp70 basal levels decrease with age. Together with an impaired induction, these decreased basal levels could make the damage accumulation even worse. Interestingly, most of the reports demonstrating an age-related decrease in Hsp70 basal levels were performed in post-mitotic tissues such as: retina, heart, muscle or brain (Colotti et al. 2005; Senf 2013; Gleixner et al. 2014). As previously mentioned, according to the oxidative-inflammatory theory of aging, post-mitotic cells and tissues are the ones that age at the fastest rate (De La Fuente and Miquel 2009). Thus, it can be the reason why they show a different pattern in the variation of Hsp70 basal levels during aging, being no longer able to constitutively express Hsp70, due to damage accumulation. Furthermore, this differential accumulation of Hsp70 between mitotic and post-mitotic tissues could contribute to the age-related damage accumulation and tissue deterioration, taking into account the anti-apoptotic properties of Hsp70. Thus, on one hand, the age-related accumulation of Hsp70 observed in mitotic tissues could impair the elimination of damaged mitotic cells, contributing to damage accumulation. On the other hand, the age-related decrease of Hsp70 observed in post-mitotic tissues could allow the elimination of essential post-mitotic cells, leading to tissue deterioration (Fig. 3).

In order to clarify the role of Hsp70 in mitotic and post-mitotic cells, we performed a comprehensive analysis of Hsp70 basal levels in several tissues (mitotic and post-mitotic) from ICR-CD1 and Balb/C female mice at different ages (adult, mature, old), including mice showing high longevity. Table 1 illustrates the changes observed when comparing old versus adult and long-lived versus old mice.

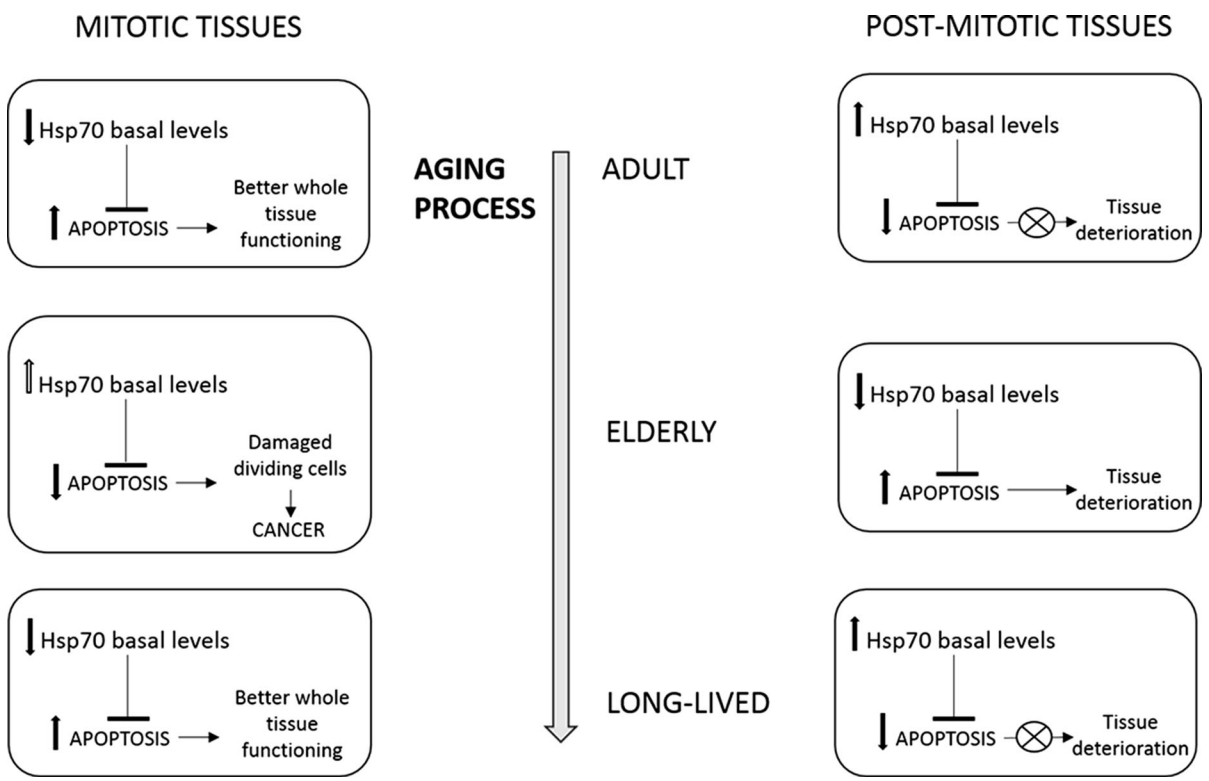

Fig. 3 Involvement of Hsp70 basal levels in age-related apoptosis. In adults, mitotic tissues have low Hsp70 basal levels so apoptosis is not inhibited. Thus, the homeodynamics is maintained by the elimination of damaged and presumably dysfunctional mitotic cells, which can then be replaced by cell proliferation. In the elderly, mitotic tissues have high Hsp70 basal levels, which block apoptosis, and consequently, the further division of damaged cells can contribute to cancer. Interestingly, in long-lived individuals, mitotic tissues have low Hsp70 basal levels similar to adults, allowing apoptosis to occur improving whole tissue function. In post-mitotic tissues there is a decline in Hsp70 basal levels in the elderly that allows the elimination of essential post-mitotic cells, which can not be replaced, leading to tissue deterioration. Nevertheless, in long-lived individuals, post-mitotic tissues have high Hsp70 basal levels, similar to those of adults, which could account for the avoidance of tissue deterioration

Table 1 Variation of Hsp70 basal levels in mitotic and post-mitotic tissues comparing old vs adult and long-lived versus old ICR-CD1 and Balb/C female mice

		Old versus adult	Long-lived versus old
Mitotic tissues	Liver	↑	↓
	Renal medulla	↑	↓
Post-mitotic tissues	Heart	↓	↑
	Renal cortex	↓	↑
	Skeletal muscle	↓	↑
	Cerebral cortex	↓	↑

In mitotic tissues, such as liver and kidney medulla, old mice have increased Hsp70 basal levels compared to adult mice, whereas naturally long-lived mice have lower levels than the old group of mice and comparable to the levels from adults. Nevertheless, post-mitotic tissues of old mice have decreased Hsp70 basal levels compared to adults, whereas naturally long-lived mice have higher levels than old mice and being again similar to those in adults. These results could explain the different rate of aging of both cellular types: mitotic and post-mitotic.

In post-mitotic cells, the age-related decrease of Hsp70 basal levels in the old group can be explained on the basis that due to the accumulation of damage, these cells are no longer able to synthesize Hsp70. This phenotype increases the toxicity of protein aggregates driving the cell to enter apoptosis. Interestingly, post-mitotic cells from long-lived mice show higher levels than the ones found for the old group.

This fact suggests that only the long-lived mice maintain the ability to synthesize Hsp70 in post-mitotic cells. These cells show an adaptive mechanism in maintaining high constitutive Hsp70 levels, which helps them to control the protein homeodynamics and protects them from entering apoptosis preventing tissue degeneration.

In mitotic cells, given that they can divide their misfolded proteins during cell cycle division, the ability to synthesize Hsp70 is not ameliorated during aging. That is the reason why mitotic cells from old mice show higher Hsp70 basal levels, reflecting the age-related increase of damaged proteins. Interestingly, mitotic cells from long-lived mice show decreased Hsp70 basal levels, which may reflect the maintenance of the homeodynamics control, which is a characteristic of long-lived individuals. Another possible explanation could be that the maintenance of lower levels does not block apoptosis pathways making non-functional mitotic cells enter apoptosis for a better whole tissue function.

Since the Hsp70 basal levels in post-mitotic tissues are high in adult and long-lived individuals but low in old individuals and in mitotic tissues low in adult and long-lived but high in old individuals, these basal levels could be proposed as a tissue biomarker of biological age.

Extracellular Hsp70

Circulating Hsp70 was first identified in groups of normal individuals (Pockley et al. 1998) and it was believed to be the result of dying cells. It was not until a few years later, once the immunomodulatory properties of Hsp70 commented above were described, when serum Hsp70 levels were studied in relation to aging. Thus, an age-related decline in serum Hsp70 levels was found in a study performed on 60 individuals aged between 20 and 96 years (Rea et al. 2001). Several reasons could explain why serum Hsp70 levels are lower at very old age. It is possible that cells in these individuals are exposed to similar levels of stress but have a diminished response in terms of Hsp70 synthesis. Another reason could be that cells from very old individuals synthesize the same amount of intracellular Hsp70 as adults, but the active release mechanism by which Hsp70 enters the circulation is defective. This is an intriguing possibility since recent findings demonstrate that disruption of lipid rafts on cell membrane abrogates the release of Hsp70 from living cells (Hunter-Lavin et al. 2004; Bausero et al. 2005; Gastpar et al. 2005; Lancaster and Febbraio 2005). Interestingly, in a study performed in human centenarians and their offspring compared to an old group and its respective offspring, significantly lower mean serum Hsp70 levels were shown in centenarians and centenarian offspring than in unrelated controls. Thus, the authors proposed low Hsp70 serum level as a predictor of longevity (Terry et al. 2004). Given that the centenarian offspring also had lower levels than the controls, it is plausible that the reason why they live to very old age is due to the preservation of low Hsp70 extracellular levels during their whole lifetime. Given the pro-inflammatory effects of extracellular Hsp70, having lifelong low extracellular Hsp70 levels may result in decreased exposure to inflammation. In fact, a study (Njemini et al. 2004) using 65 patients aged between 67 and 97 years of age, and classified as having inflammation or not, showed an age-related increase in serum Hsp70 levels and these levels were positively correlated with the level of inflammation.

Conclusion

Our understanding of the molecular pathways that affect aging and lifespan is advancing rapidly and increasingly implicates the Hsps as relevant mediators in the aging process. Specifically, the protective roles of Hsp70 in key cellular aging processes such as proteotoxicity, oxidation, inflammation, immunosenescence and apoptosis, make it unique as an important component in the regulation of aging and longevity. Thus, Hsp70 could be a promising target for future interventions in aging and in age-related diseases.

However given the wide spectrum of functions described for Hsp70, further research is needed in order to elucidate what determines the specific role that Hsp70 performs in each case. Another important aspect for future study is the measurement of the changes in Hsp70 activity (by the refolding of damaged proteins or by the ability to activate immune cells) in relation to aging.

Although there can be many potential biomarkers that change with aging, not all comply with the conditions required to be used as biomarkers of biological age. For this, the biomarker has to fulfil at least two more requirements: the first being that if an

adult individual shows values characteristic of a chronologically old individual he/she would die prematurely. The second is that a long-lived individual, known to have experienced healthy aging, must have a value of this biomarker similar to that of an adult. The Hsp70 levels show great promise as the long-sought-after biomarkers of biological age, given that both inducible as well as basal levels have been shown to be preserved in long-lived individuals. Moreover, the maintenance of low extracellular Hsp70 levels has been proposed as a biomarker of longevity. Nevertheless, to validate this suggestion, longitudinal studies should be carried out to check if levels of Hsp70 (inducible, basal and extracellular) may be predictive of remaining lifespan.

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