Agradecimentos

Ao Doutor Paulo Pereira, pela supervisão, apoio, confiança e, não menos, pelo stress.

Ao pessoal do laboratório: João (companheiro de gabinete, companheiro de boa e má música), Carla Bento (tutora espectacular), Steve (benurron e estupidezas), Hugo (não companheiro de gabinete, mas quase), Vanda, Rosa, Carla Marques, Henrique e Murat. Às cervejas, ao tropical, ao Go Between convosco.

Ao David, à Xu e ao Pedro Rosmaninho, por aturarem o meu mau humor (nem sempre com um sorriso no rosto mas quase sempre com grande paciência) e por, ainda assim, me disponibilizarem um banquinho em casa para jantar e trabalhar.

Às meninas (e menino) de Coimbra, Ana Teresa, Sandra, Dina, Liliana, Milka (e João Rafael), pelos últimos 11 anos.

Ao pessoal do curso e mais que isso, David, Xu, Ki, Pedro da Ki, Fernando, Steve, Vasco, Tiago, Tunning, Luísa, Silvino, Juliana, Vinagre, Vidigueira, Atmosfera, pelas cartas, festas, saídas e momentos de grande proximidade.

Acima de tudo, ao meu pai e à minha mãe.

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ABBREVIATIONS

3-DG	3-deoxyglucosone
AGE	Advanced glycation end-product
AMD	Age-related macular degeneration
Ang-1	Angiopoietin-1
Ang-2	Angiopoietin-2
Arg	Arginine
ATP	Adenosine-5'-triphosphate
CEL	N^{ε}-(1-carboxyethyl)-lysine
CHIP	Carboxyl terminus of the Hsc70-interacting protein
CMA	Chaperone-mediated autophagy
CML	N^{ε}-carboxymethyl-lysine
Cys	Cysteine
DBD	DNA-binding domain
DM	*Diabetes mellitus*
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin-ligase
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
Glo1	Glyoxalase I
Glo2	Glyoxalase II
GO	Glyoxal
GSH	Thiol-containing tripeptide glutathione
DNA	Deoxyribonucleic acid
Hsc70	Heat-shock cognate protein 70
HSE	Heat-shock element
Hsf	Heat-shock transcription factor
Hsp	Heat-shock protein
Hspbp1	Heat-shock factor binding protein 1

Lys	Lysine
MAPK	Mitogen-activated protein kinase
MGO	Methylglyoxal
MGO-H	MGO-derived hydroimidazolone
MGO-H1	N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)ornithine
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-Kb	Nuclear factor-κB
PCV	Packed cell volume
PDGF	Platelet-derived growth factor
PEDF	Pigment epithelium-derived factor
POS	Photoreceptor outer segments
RAGE	AGE-specific cell-surface receptor
ROS	Reactive oxygen species
RPE	Retinal pigment epithelium
sHsp	Small heat-shock protein
Ser	Serine
SOD	Superoxide dismutase
STUB1	STIP1 homology and U-box-containing protein1
Trh	Threonine
TRP	Tetracopeptide repeat
UPP	Ubiquitin-proteasome pathway
VEGF	Vascular endothelial growth factor

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SUMÁRIO

O metilglioxal (MGO) é um metabolito dicarbonil altamente reactivo que se forma principalmente a partir da glicólise. Este composto constitui um dos principais precursores dos produtos finais avançados de glicação (AGEs – sigla inglesa), uma vez que reage com proteínas, nucleótidos e lípidos, alterando a sua estrutura e função. O papel do MGO, assim como dos AGEs seus derivados, nas alterações deletérias sofridas por células e tecidos, tem sido extensivamente abordado nas últimas décadas. A acumulação de ambos os produtos contribui não só para o envelhecimento, mas também para uma série de doenças que incluem a *diabetes mellitus* (DM). No entanto, pouco se sabe acerca do efeito do MGO nas vias de controlo de qualidade proteica, bem como acerca da forma como as células respondem ao insulto tóxico resultante do MGO.

Este estudo focou-se no efeito do MGO na resposta *heat-shock*. Esta via de resposta ao stress é mediada por chaperonas moleculares designadas *heat-shock proteins* (Hsps), que são capazes de reconhecer proteínas desnaturadas/modificadas e de promover o seu enrolamento. Alternativamente, as Hsps podem enviar as proteínas irreversivelmente modificadas para degradação no proteasoma, numa forma dependente da ligase de ubiquitina CHIP.

Com este trabalho, mostramos que o tratamento de células ARPE-19 com MGO resulta na activação do factor de transcrição *heat-shock factor* (Hsf-1), o principal regulador da resposta *heat-shock*. Além disso, demonstramos que o stress induzido por MGO conduz a um decréscimo de algumas Hsps, seguido por um aumento da expressão destas chaperonas, e que este decréscimo inicial é prevenido pela sobrexpressão do Hsf-1. Esta resposta *heat-shock* induzida pelo MGO coincide com um aumento da viabilidade celular e com uma diminuição dos níveis totais de proteínas ubiquitinadas.

Os dados obtidos sugerem que o MGO compromete o controlo de qualidade proteica dependente de chaperonas moleculares, levando a uma acumulação de substratos ubiquitinados e a um decréscimo da viabilidade celular. Contudo, estas modificações produzidas pelo MGO desencadeiam uma resposta mediada pelo Hsf-1, essencial ao restabelecimento da homeostase celular.

ABSTRACT

Methylglyoxal (MGO) is a highly reactive dicarbonyl metabolite that forms mainly during glycolysis. MGO constitutes a major precursor of advanced glycation-end products (AGEs) since it reacts with proteins, nucleotides and lipids, disrupting their structure and function. Both MGO and MGO-induced AGEs have important toxic effects in cells and tissues. Indeed, these products are known to accumulate and contribute to ageing, as well as to a number of diseases, including *diabetes mellitus* (DM). Nonetheless, very little is known on the effect of MGO on cell protein quality control system and on cell response to MGO. In this study we focus on the effect of MGO on the heat-shock response. This stress-pathway is mediated by stress-inducible molecular chaperones called heat-shock proteins (Hsps), which bind to denatured/modified proteins and assist their proper folding/refolding. Alternatively, Hsps can deliver fatally damaged proteins to the proteasome in a process that requires binding to the carboxyl terminus of Hsc70-interacting protein (CHIP).

Herein, we show that MGO treatment in ARPE-19 results in the activation of heat-shock factor 1 (Hsf-1), the master regulator of heat-shock response, by increasing oligomerization, decreasing interaction with Hsp90 and increasing translocation to the nucleus. We further show that MGO-induced stress leads to a decrease in levels of several Hsps, which is followed by an increase in the expression of these chaperones. Moreover, we show that the initial decrease in Hsps levels can be counteracted by overexpressing Hsf-1. We further show that the delayed heat-shock response induced by MGO is concomitant with an increase in cell viability and a decrease in the total levels of ubiquitin conjugates.

The data presented in this work suggests that MGO impairs the protein quality control by a mechanism that is dependent on molecular chaperones and leads to accumula-

tion of ubiquitinated substrates and to a decrease in cell viability. However, these MGO-induced changes appear to elicit a response mediated by Hsf-1, which is crucial to help cells to cope with stress conditions and to re-establish homeostasis.

Key-words: heat-shock factor-1, Hsf-1; heat shock protein, Hsp; methylglyoxal, MGO; carboxyl terminus of Hsc70-interacting protein, CHIP; ubiquitin-proteasome pathway, UPP.

Chapter 1

INTRODUCTION AND OVERVIEW

Chapter 1

INTRODUCTION AND OVERVIEW

1.1. Methylglyoxal (MGO) in physiological systems

1.1.1. Brief summary on MGO metabolism

Aldehydes are highly reactive biologically significant compounds that result from parametabolic reactions (Dimitriev and Titov, 2009). Among them are the products of lipid peroxidation – malonic dialdehyde, MDA and 4-hydroxynonenal, 4-HNE – and anomalous glucose metabolites – glyoxal, GO, methylglyoxal, MGO and 3-deoxyglucosone, 3-DG (figure 1). The latest ones have been largely implicated as glycotoxic mediators of carbonyl stress (Dimitriev and Titov, 2009; Turk, 2010).

MGO is a highly reactive α-oxaldehyde that is formed intracellularly from several metabolic pathways, mainly from enzymatic and non-enzymatic β-elimination of the phosphate group from triose phosphate intermediates of glycolysis, dihydroxyacetone phosphate and glyceraldehydes-3-phosphate, respectively, but also from degradation of Amadori products, lipid peroxidation, threonine catabolism and ketone body metabolism (Ramasamy *et al*., 2005; Gomes *et al*., 2006; Thornalley, 2008; Price and Knight, 2009; Turk, 2010). MGO can cross membranes, probably by passive diffusion, and thus can be found in all tissues and body fluids (Gomes *et al*., 2006). However, MGO is kept at low concentrations in normal physiological conditions in order to protect nucleic acids and proteins (Thornalley, 2003; Turk, 2010).

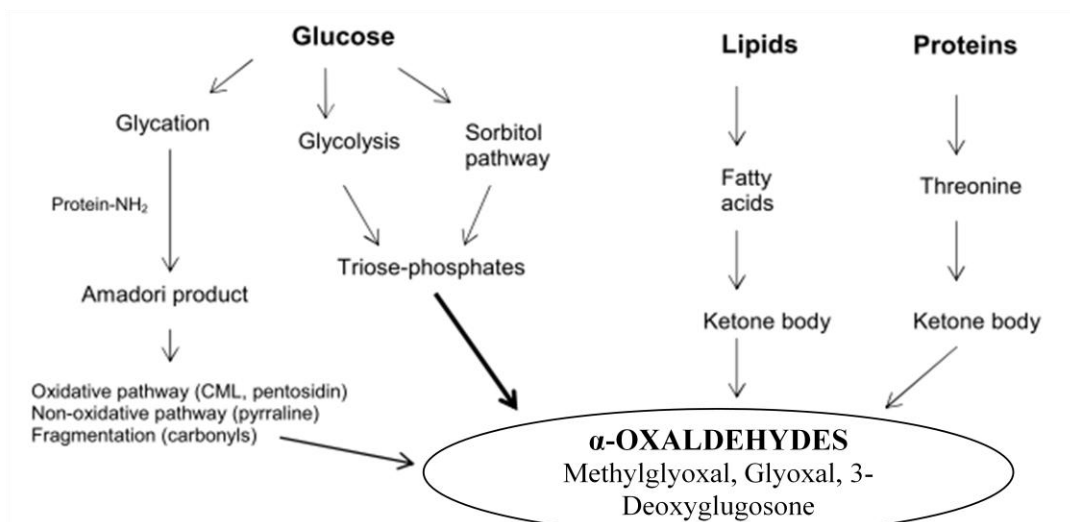

Figure 1.

Reaction scheme for reactive carbonyl compounds. Dicarbonyls are formed as glycolytic intermediates in metabolic conversion of glucose, *via* Maillard reaction by degradation of glycated proteins, and during lipid peroxidation and threonine catabolism (adapted from Turk *et al*., 2010).

1.1.1.1. MGO-induced formation of AGEs

Glucose, as well as other glycolytic intermediates such as the phosphorylated monocarbonyl compounds (*e.g.* glucose-6-phosphate, dihydroxyacetone phosphate and fructose-6-phosphate) and α-dicarbonyl compounds (*e.g.* MGO, GO and 3-DG), form stable end-stage adducts called advanced glycation end-products (AGEs) on free amine and thiol groups within aminoacids, proteins, nucleotides, lipoproteins and basic phospholipids (Thornalley, 2008; Price and Knight, 2009; Tessier, 2009). These glycation processes contribute to cell and tissue damage *in vivo* (Thornalley, 2003). The non-enzymatic reaction of glycine with glucose, also known as browning reaction, was first described in 1912 by Louis Camille Maillard (Tessier, 2009; Price and Knight, 2009). It was only in the early 1980s that the *in vivo* reaction between proteins amino groups and glucose, without the aid of enzymes, was named "non-enzymatic glycosylation". A few

years later, the name of this reaction was changed to "glycation" in order to differentiate it from enzymatic glycosylation (Tessier, 2009). Simultaneously, Monnier and Cerami linked the "non-enzymatic glycosylation" to the browning reaction discovered by Maillard and comprehensively described by Hodge in 1953 (Hodge, 1953; Monnier and Cerami, 1981; Tessier, 2009).

Glycation of proteins is a complex series of parallel and sequential non-enzymatic reactions by aldehyde groups on sugars and α-dicarbonyls (figure 2), collectively called the Maillard reaction (Thornalley, 2008; Glenn and Stitt, 2009). In biological systems, this reaction is subdivided into early and advanced steps. The initial step begins with the formation of an imine intermediate, also called Shiff base, between a reducing sugar, such as glucose, and ε-amino groups in a protein or free amino acid. This unstable imine can generate reactive α-dicarbonyl compounds, also called α-oxaldehydes, through oxidative reaction (Namiki and Hayashi, 1983), or rearrange itself into the relatively stable Amadori adducts (Turk, 2010). In the advanced step, the Amadori product under-

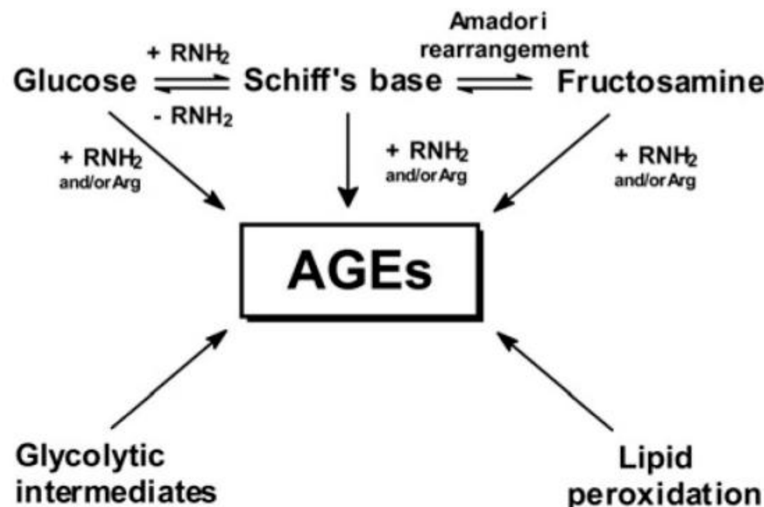

Figure 2.

Major pathways for the formation of AGEs in physiological systems. Formation of early and advanced glycation adducts from glucose and glycolytic intermidiates and products of lipid peroxidation (from Rabbani and Thornalley, 2008).

goes further oxidation and dehydration, complex rearrangements, cleavage and covalent binding reactions, which give rise to a group of heterogeneous, stable adducts and cross-links of proteins, AGEs (Tessier, 2009; Turk, 2010).

It has become increasingly appreciated that glucose is one of the least reactive ketoses. In fact, under physiological conditions, 99% of glucose exists in a ring structure, which is highly stable and less prone to nucleophilic attacks while comparing with other sugars (Bunn and Higgins, 1981; Tessier, 2009). Glucose *per se* is also much less reactive than low molecular weight carbonyls such as GO, MGO and 3-DG, which are up to 200-500,000-fold as reactive as glucose (Glenn and Stitt, 2009; Tessier, 2009; Turk, 2010). Thus, despite the low concentration of these glycation agents, they are extremely reactive and account for an important part of glycation inside cells (Tessier, 2009).

Oxaldehydes can directly and relatively rapidly induce modifications in the chemi-

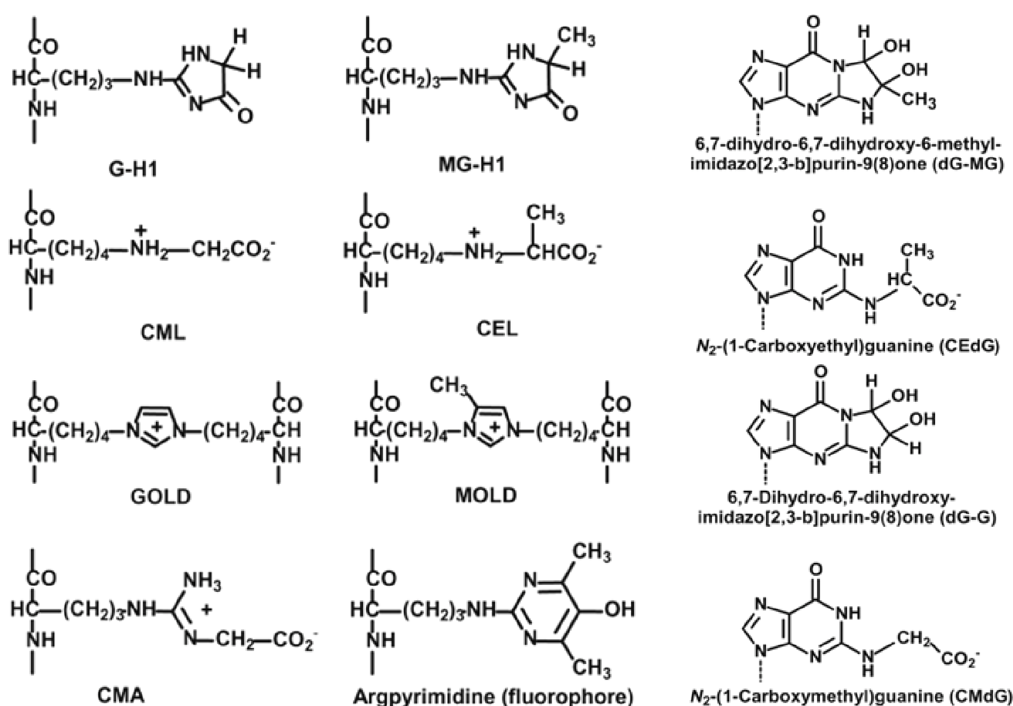

Figure 3.

Schematic representation of AGEs formed from the reaction of MGO and GO on lysine and arginine residues and on nucleotides (adapted from Thornalley, 2008).

cal structure of some amino acid residues in proteins, constituting an important source of intra- and extracellular AGEs (Thornalley, 2008; Tessier, 2009; Glenn and Stitt, 2009). MGO is thought to be the most abundant of the α-dicarbonyls and a potent AGE precursor, forming adducts on arginine (Arg), lysine (Lys) and cysteine (Cys) residues (Price and Knight, 2009). The most prevalent MGO adduct in proteins identified *in vivo* is MGO-derived hydroimidazolone (MGO-H), which has been found in highest concentrations in lens protein of elderly human subjects (Thornalley, 2008; Glenn and Stitt, 2009). Nδ-(5-hydro-5-methyl-4-imidazolon-2-yl)ornithine (MGO-H1), Nδ-(4-carboxy-4,6-dimethyl-5,6-dihydroxy-1,4,5,6-tetrahy-dropyrimidin-2-yl)ornithine (argpyrimidine), Nε-(1-carboxyethyl) lysine (CEL) and the cross-link MGO-derived lysine dimer 1,3-di(Nε-lysino)-4-methyl-imidazolium salt (MOLD) are also formed (figure 3). Similar adducts are formed by GO (*e.g.* N^{ε}-carboxymethyl-lysine, CML and glyoxal-derived lysine dimer, 1,3-di(N^{ε}-lysino-imidazolium salt) (GOLD) and probably by other α-dicarbonyl compounds (Thornalley, 2008; Price and Knight, 2009).

Deoxyribonucleic acid (DNA) suffers as well from continuous damage induced by glycation *in vivo* (Thornalley, 2008). The major AGE adducts found in DNA (nucleotide advanced glycation end-products) are MGO-H and CEL equivalents on deoxyguanosine (dG) residues (dG-MGO and CEdG), the most reactive nucleotide under physiological conditions, depicted in figure 3 (Thornalley, 2008; Price and Knight, 2009). Finally, AGEs can also be formed from lipid peroxidation (*e.g.* oxidative products of polyunsaturated fatty acids, PUFAs) that leads to accumulation of a class of Maillard products named advanced lipoxidation end-products (ALEs) (Tessier, 2009; Glenn and Stitt, 2009).

Protein damage by glycation is implicated in protein misfolding, functional impairment (*e.g.* enzyme inactivation) and resistance to proteolysis due to covalent cross-

linking, as well as in reduced protein-protein interaction and protein-DNA interaction (for transcription factors) (Thornalley, 2008). DNA adducts, on the other hand, are associated with increased mutation frequency, the most prominent being multibase deletions and base-pair substitutions, DNA strand breaks, decreased DNA replication, cross-linking between DNA and proteins, cytotoxicity and, ultimately, apoptosis (Thornalley, 2008; Price and Knight, 2009). Finally, excessive lipid glycation is associated with lipid bilayer disruption (Thornalley, 2008).

AGEs may also result in the cross-linking of long-living proteins (*e.g.* collagen, fibronectin and laminin) and of structural and basement membranes, thereby modifying key cellular structures that impact permeability and cellular motility (Sell *et al.*, 1992; Gawlowski *et al.*, 2009). In addition, AGEs may transduce signals and thereby exert their effects *via* interaction with AGE-specific cell-surface receptors (RAGE), which have been identified on a variety of cells and that act as signaling receptors for at least two distinct AGE adducts, CML and hydroimidazolone adducts, as well as by internalization and degradation *via* monocyte/macrophage RAGEs (Ramasamy, 2006; Goldin, 2010; Turk, 2010).

There are several factors that affect the accumulation of glycation products *in vivo* and that can act either in synergy or in opposition (Tessier, 2009). Among the biological parameters that are known to affect glycation and that continue to be widely studied, are glucose levels in cells and in the extracellular matrix, the concentration and reactivity of monosaccharides and other carbonyl compounds, the accessibility and reactivity of the amino groups on proteins, the longevity of the modified molecules, oxidative processes, renal clearance of free AGEs and reactive precursors, along with glycation adducts absorbed from food, and the chemical or enzymatic de-glycation and degradation of Mail-

lard products and reactive AGE precursors (Thornalley, 2008; Tessier, 2009). The latter parameter will be described in further detail in the next section.

1.1.1.2. Avoiding or reducing AGEs formation: anti-glycation defences

All of the above described damages to biological macromolecules by glycation agents occur in normal physiological states, but are maintained at low levels by a series of anti-glycation defences that either suppress or remove glycated residues (Thornalley, 2003; Thornalley 2008; Tessier, 2009).

The turnover of glycation adducts is an important defence against their accumulation. Protein glycation adducts are removed by proteasomal and lysosomal proteolysis, phospholipid glycation adducts are removed by lipid turnover, and nucleotide glycation adducts are cleared by nucleotide excision repair. The rapid turnover of intracellular proteins and other molecules might indirectly protect them from large accumulation of chemical damage that would, otherwise, impair or disrupt their function and, ultimately, induce citotoxicity (Brownlee, 2000; Tessier, 2009).

In order to suppress AGE-forming dicarbonyls, cells have endogenous protection against their reactivity, which is provided by several “detoxifying” enzymes, namely by aldo-keto reductases (AKRs), aldehyde dehydrogenase, 2-oxaldehyde dehydrogenase and by the specialized glyoxalase system (Nemet *et al*., 2006; Glenn and Stitt, 2009; Price and Knight, 2009; Baba *et al*., 2009). The later system plays a central role in eliminating MGO and has been widely studied in both prokaryotic and eukaryotic organisms.

1.1.1.2.1. The glyoxalase system

The glyoxalase system represented in figure 4 is present in the cytosol of all mammalian cells and comprises two enzymes: the glutathione (GSH)-dependent enzyme glyoxalase I (Glo1) and glyoxalase II (Glo2). Glo1 catalyzes the isomerization of the hemithioacetals, formed spontaneously from reactive, acyclic α-oxoaldehydes (RCO-CHO) and GSH, into S-2-hydroxyacylglutathione derivatives (Thornalley, 2003). In turn, Glo2 catalyzes de conversion of these molecules into α-hydroxyacids (*e.g.* GO into glycolate and MGO into D-lactate), and reforms GSH consumed by the first reaction catalyzed by Glo1 (Thornalley, 2003; Thornalley, 2008).

Human Glo1 is a dimer and is present in all human tissues (Thornalley, 2008). The major physiological substrate for this enzyme is MGO since it accumulates markedly,

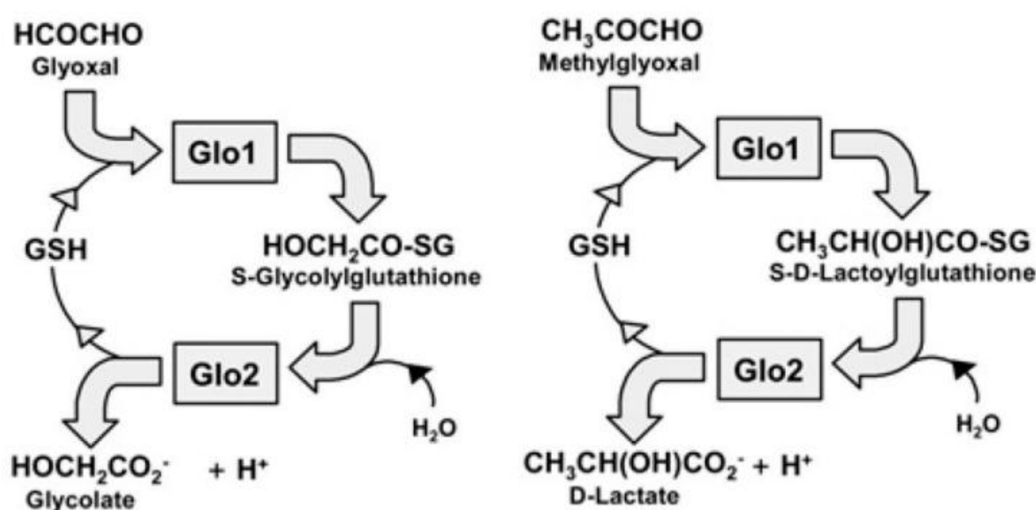

Figure 4.

Metabolism of GO and MGO by the glyoxalase system. The biotransformation of GO and MGO involves two separate enzymes, glyoxalase I (Glo1) and glyoxalase II (Glo2), which bring about two consecutive reactions involving the thiol-containing tripeptide glutathione (GSH) as a cofactor. GO and MGO are converted by Glo1 into S-Glycolyl-glutathione and S-D-lactoyl-glutathione in the first reaction, respectively. Subsequently, Glo2 catalyzes the hydrolysis of these thiolesters into Glycolate and D-lactic acid, respectively, as well as into free GSH (from Rabbani and Thornalley, 2008).

along with glycated molecules, when Glo1 is inhibited and/or by depletion of GSH (Abordo *et al*., 1996; Thornalley *et al*., 1996; Thornalley, 2008). Moreover, cells that overexpress Glo1 show less accumulation of MGO-derived AGEs (Shinohara *et al*., 1998), as well as extend lifespan (Morcos *et al*., 2008; Glenn and Stitt, 2009). By preventing the accumulation of these reactive α-oxoaldehydes, Glo1 supresses α-oxoaldehyde-mediated glycation reactions and associated cytotoxicity, that should be avoided under normal physiological conditions (Thornalley, 2003). Thereby, Glo1 is considered a key enzyme in the anti-glycation defence (Thornalley, 2003).

1.1.2. MGO and metabolic dysfunction in ageing and disease

Over the past 30 years, scientists have been investigating the multiple and diverse effects that the Maillard reaction exert in living systems (Ramasamy *et al*., 2006; Tessier, 2009). Glycation products significantly accumulate on long-lived macromolecules with age and contribute to morbidity and mortality of people suffering from diseases of major social impact such as diabetes, heart disease, chronic and acute renal failure, age-related macular degeneration (AMD) and Alzheimer's disease, as well as to tumourigeneses and multidrug resistance in cancer chemotherapy (Thornalley, 2008; Tessier, 2009). These products might also play a role in pathologic anxiety, autism, obesity and other disorders (Thornalley, 2008).

The direct role of these glycation products, both in clinical manifestations of ageing (*e.g.* stiffening of tissues that are rich in extracellular matrices and long-lived proteins such as skeletal muscle, tendons, joints, bone, heart, arteries, lung, skin and lens), and in other diseases, remains an open question and much has to be done to elucidate the physiological and pathological roles of AGEs (Tessier, 2009). In the next sections, the most

important effects of these bioactive molecules in certain biological contexts will be briefly described.

1.1.2.1. Ageing

Ageing can be defined as a complex multifactorial process characterized by accumulation of deleterious changes in cells and tissues, progressive deterioration of structural integrity and physiological function across multiple organ systems, and increased risk of death (Glenn and Stitt, 2009; Semba *et al.*, 2010). In summary, the ageing response is inevitable, ultimately leading to decreased viability of the organism (Glenn and Stitt, 2009). More than 300 theories on ageing mechanisms have been proposed and no one can tell which of these theories is best able to explain the phenomenon of ageing (Tessier, 2009). However, it has been observed that various post-synthetic chemical modifications of proteins accumulate during the ageing process (Tessier, 2009). In the early 1980s, Monnier and Cerami postulated that the Maillard reaction of proteins could have a causative role in ageing and related pathologies since the formation of AGEs exceeds that predicted by first order kinetics in both cases (Monnier and Cerami, 1981; Tessier, 2009). This “glycation hypothesis of ageing” was at the origin of the growing interest in the field of the Maillard reaction *in vivo* (Tessier, 2009).

Recent epidemiological studies demonstrate that late-stage Maillard processes contribute to the multisystem decline that occurs with ageing and are associated with increased risk of developing many chronic diseases that disproportionally affect older individuals (Semba *et al.*, 2010). Several studies have consistently observed that old age is associated with progressive impairment of mitochondrial function, increased oxidative stress and immune activation (Semba *et al.*, 2010). These ageing features, which are also common to diabetic complications and age-related diseases (figure 5), correlate and

may partially result from increased MGO concentration and consequent accelerated AGEs accumulation in cells and tissues (*e.g.* hydroimidazolone glycation end-products accumulation with age in human lens proteins) (Ayoub *et al.*, 1993; Haik *et al.*, 1994; Thornalley, 2008; Glenn and Stitt, 2009; Turk, 2010). Accordingly, Glo1 enzymatic activity was recently shown to become progressively impaired with ageing, as well as in diabetic tissues (Morcos *et al.*, 2008), contributing to the decreased clearance of AGEs precursors.

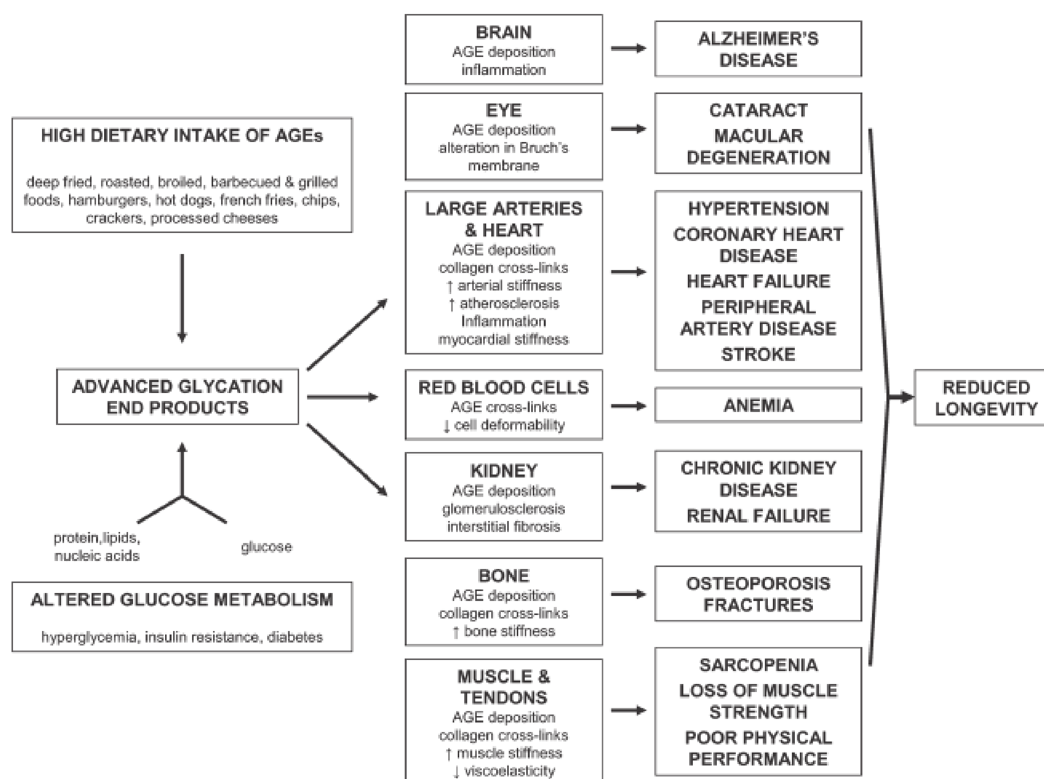

Figure 5.
Conceptual model f the effects of AGEs in multiple organ systems during ageing (from Semba *et al.*, 2010).

1.1.2.2. Diabetes

The most widely known glycated protein is a modification of hemoglobin (HbA), HbA1c, first discovered by Kunkel and Wallenius in 1955 (Kunkel and Wallenius,

1955; Tessier, 2009; Glenn and Stitt, 2009). This modified hemoglobin is glycated in the N-terminal valine of the b chain and is found to be elevated in red blood cells of

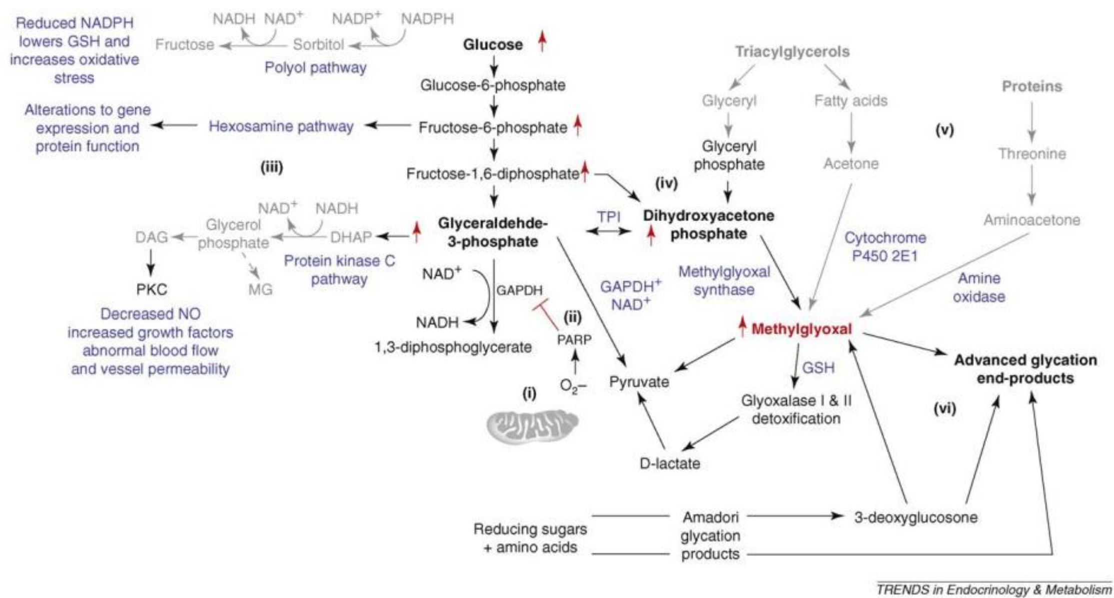

Figure 6.

MGO metabolism in diabetes. (i) Excess glucose metabolism within mitochondria leads to overproduction of reactive oxygen species (ROS) and leakage of superoxide anions (O_2^-) from the electron transport chain. (ii) This oxidative stress upregulates the nuclear enzyme poly(ADP-ribose) polymerase-1 (PARP), which is an inhibitor of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH inhibition slows conversion of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate and causes increased concentrations of glycolytic intermediates. (iii) Excess glycolytic intermediates are driven down their respective metabolic pathways: the polyol, hexosamine and protein kinase C (PKC) pathways, causing oxidative or leading to the production of MGO. (v) MG can also be formed from fatty acid and threonine metabolism. (vi) Both MGO and reducing sugars, such as glucose, form adducts on free amine groups of proteins, advanced glycation end-products (AGEs). MGO is detoxified largely by the glyoxalase system. When it becomes overwhelmed, *e.g.*, in diabetes, MGO levels rise. TPI, triose-phosphate isomerase; GSH, glutathione; NAD + /NADH, oxidised/reduced forms of nicotinamide adenine dinucleotide; NADP + / NADPH, oxidised/reduced forms of nicotinamide adenine dinucleotide phosphate; DHAP, dihydroxyacetone phosphate; DAG, diacylglycerol (from Price and Knight, 2009).

diabetic patients (Tessier, 2009). This was incidentally the first observation of the high concentration of a glycated protein in patients with *diabetes mellitus* (DM) (Tessier, 2009).

In patients with type 2 diabetes, weak function of pancreatic β-cells, low sensitivity of insulin receptors in peripheral tissues and/or weak amplification of the hormone signal hinder the buffering of plasma glucose concentration (Dimitriev and Titov, 2009). Therefore, the result of DM is inevitably hyperglycaemia, which has been shown to coincide with increases in the concentrations of MGO, D-lactate and AGEs in the plasma and tissue of diabetic patients (Thornalley *et al*., 2001; Thornalley, 2003; Ramasamy *et al*., 2006; Dimitriev and Titov, 2009; Price and Knight, 2009). These molecules have been implicated in the diabetes-related complications such as heart failure, atherosclerosis, microcirculation disorders, susceptibility to common infections, diabetic nephropathy, diabetic retinopathy, diabetic neuropathy, among others, due to maladaptive responses in multiple tissues (Cooper, 2004; Gawlowski *et al*., 2009; Price and Knight, 2009; Turk, 2010).

About 80% of patients with DM suffers from cardiovascular diseases due to arising dysfunctions in cardiomyocytes and morphological changes in large conduit arteries, coronary arteries and microvasculature. An increasing body of evidence implicates MGO in these complications (Gawlowski *et al*., 2009; Dimitriev and Titov, 2009). For instance, increased formation of AGEs has been directly linked to alterations of the myocardial calcium transient and hence in cardiac contractility, constituting a possible mechanism by which DM affects cardiac muscle structure and function (Gawlowski *et al*., 2009). Other studies report the involvement of MGO in ATP (adenosine-5'-triphosphate) exhaustion in cardiomyocytes (Scheuermann-Freestone *et al*., 2003). This is due the ability of creatine to scavenge MGO in cardiomyocytes. Creatine is also used

as a substrate of phosphorylation, enabling energy conservation in the form of phospho-creatine. By scavenging MGO, this protein loses the later function and the energy reserves decrease. In addition to these findings, increased RAGEs expression was observed in endothelial cells, vascular smooth muscle and cardiac myocytes of diabetic patients (Schmidt *et al.*, 1999). Furthermore, Cipollone *et al.* observed up-regulated RAGE expression in diabetic atherosclerotic plaques retrieved from human subjects (Cipollone *et al.*, 2003). These receptors contribute to the diffuse accumulation of AGEs in the subendothelial space, initiates vascular permeability, increased migration of macrophages and T-lymphocytes into the intima and impaired endothelium dependent arterial relaxation (Schmidt *et al.*, 1994). Interaction of AGEs with endothelial surface RAGEs also induces the generation of reactive oxygen species (ROS) (Yan *et al.*, 1994) and induction of adhesion molecules and pro-inflammatory cytokines (Basta *et al.*, 2004; Ramasamy *et al.*, 2006; Semba *et al.*, 2010).

Several studies also implicate MGO in the apoptosis of pancreatic β-cells (Sheader *et al.*, 2001) and impaired insulin signaling in diabetes, probably as a consequence of insulin receptor substrate-1 (IRS1) impairment resulting from the binding of MGO (Riboulet-Chavey *et al.*, 2006). Other reports made by Yao and colleagues showed that MGO induces increased expression of angiopoietin-2 (Ang-2) in Müller glia cells of the rat retina by modifying mSin3A, the corepressor protein of a glucose-responsive GC box in the *ang-2* gene promoter (Yao *et al.*, 2006; Ramasamy *et al.*, 2006). Increased levels of this proangiogenic factor contribute to proliferative changes that characterize the advanced stages of diabetic retinopathy (Ramasamy *et al.*, 2006). These findings suggest a new role for MGO and glycation in the modulation of gene expression in tissues that are susceptible to complications of diabetes and in other contexts, such as cancer, renal failure, ageing, infection and inflammation (Ramasamy *et al.*, 2006).

The generation of ROS is intertwined with MGO metabolism since MGO production, as well as degradation, yields hydrogen peroxide (H_2O_2) and other radical species (*e.g.* superoxide anion, O_2^-) as byproducts (Kalapos, 2008; Thornalley, 2008; Price and Knight, 2009). Furthermore, the modification of thiol-containing tripeptide glutathione (GSH) and superoxide dismutase (SOD) by MGO might hinder antioxidant defences, thereby contributing to oxidative stress (Kalapos, 2008; Ramasamy *et al.*, 2006; Price and Knight, 2009). Interaction of AGEs with RAGEs itself results in oxidative stress *via* activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and mitochondrial pathways (Ramasamy *et al.*, 2005). This interaction may also be responsible for decreased expression levels of Glo1. This is supported by studies reporting that the induction of diabetes in wild type mice decreases the expression of Glo1, whereas induction of diabetes in RAGE $^{(-/-)}$ mice does not (Thornalley, 2008). The MGO-derived depletion of GSH and NADPH also decreases the *in situ* activities of Glo1 and thereby increases the concentrations of GO and MGO, along with associated glycation reactions (Abordo *et al.*, 1996; Thornalley, 2008; Glenn and Stitt, 2009). In addition, by propagating oxidative damage through ROS production and by lowering antioxidant defence, MGO alters or inhibits DNA replication *via* oxidative damage to DNA (Price and Knight, 2009). Taking this into account, MGO-induced oxidative stress may be implicated in tissue damage in diabetic as well as in aged tissues (Nishikawa *et al.*, 2000; Ramasamy *et al.*, 2006).

Another feature of both ageing and DM is the lower resistance to common infections. In fact, hyperglycaemia affects different facets of the immune function, leading to increased risk of pneumonia, asymptomatic urinary tract infection, skin infection and inflammation of the heart lining, among others (Price and Knight, 2009). This might be due to the disruption of immune responses by MGO. For instance, through the interac-

tion with RAGEs, AGEs may render altered protein production and mRNA expression of a host of proinflammatory molecules such as interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α) and interleukin-10 (IL-10) in myeloid and T cells, as well as disrupt the expression of adhesion factors (*e.g.* intercellular-adhesion molecules, ICAM and vascular-adhesion molecules, VCAM) and antigen presentation markers in dendritic cells and monocytes (Price *et al.*, 2009; Price and Knight, 2009). Price *et al.* further suggest that glycation may promote dendritic cell development but impair the ability of these cells to stimulate primary T cells responses (Price *et al.*, 2004; Ramasamy, 2006).

MGO can also modify individual components of the immune system. For example, Cantero *et al*. showed that MGO can disrupt platelet-derived growth factor (PDGF) signaling by reacting with PDGF tyrosine kinase receptor β and reducing its tyrosine kinase activity (Cantero *et al.*, 2007). PDGF signaling is highly important in regulating cell proliferation, development, differentiation and growth. Its disruption is responsible for reduced lymphocyte proliferation in diabetes and could conceivably impact differentiation of T_H cells (Cantero et al., 2007; Ramasamy *et al.*, 2006). MGO is also responsible for the modification of a Cys residue in the DNA binding loop of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) p65 subunit (Laga *et al.*, 2007). NF-κB is a protein complex that acts as a transcription factor for a large number of genes (including those controlling programmed cell death, cell adhesion, inflammation – cytokines –, tissue remodeling and angiogenesis), and that is sequestered in the cytosol by the inhibitor I-kB, in the absence of a stimuli (Wu *et al.*, 2009; Price and Knight, 2009). Upon an activation signal, I-kB is degraded and NF-κB is translocated to the nucleus. MGO-induced NF-κB p65 subunit modification impairs these events and thereby affects inflammatory responses to pathogens, innate mechanisms of infection clearance, T cell activation and cytokine-driven cell differentiation, among other func-

tions (Price and Knight, 2009). Another example is MGO-induced modification of Cys residues in the active site of cathepsins, a family of lysosomal proteases essential to general protein turnover and antigen processing for presentation (Zeng *et al*., 2006; Price and Knight, 2009). Cathepsin adducts show impaired activity, which might influence diabetes complications in both vascular protein turnover and immune response (Price and Knight, 2009).

In summary, MGO might underlie dysfunctions in inflammation and infection clearance in diabetes and subsequently contribute to the development of accelerated atherosclerosis, diabetes neuronal complications and microvascular complications such as diabetic nephropathy (Ramasamy *et al*., 2005; Ramasamy *et al*., 2006; Price *et al*., 2004; Price and Knight, 2009).

1.1.2.3. Tumours

Cancer cells exhibit a glycolysis rate similar to that of diabetic organs and tissues since their proliferation depends on this abnormally high carbohydrate metabolism (Dimitriev and Titov, 2009). However, the expression and activity of both Glo1 and Glo2 are higher in several cell lines (Ayoub *et al*., 1993; Thornalley, 2008; Talukdar *et al*., 2009). This is likely to be linked to the high anaerobic glycolytic activity inherent to cancer cells, which leads invariably to a high flux of triosephosphates and associated MGO formation (Thornalley, 2008). Thus, both high expression and activity of Glo1 confer protection against proteome and genome functional impairment and mutation induced by Glo1 substrates, respectively, as mentioned above. Despite the beneficial role of this enzyme in certain biological contexts, potentiation of MGO effects appears to be of great value when it comes to tumour therapy. In fact, evidences suggest that MGO production and accumulation induced by inhibition of Glo1 activity can promote

apoptosis of tumour cells (Thornalley *et al*., 1996), probably by inducing modifications in DNA and proteins involved in activation of apoptosis. These evidences support the hypothesis that Glo1 activity might be responsible for multidrug resistance in cancer chemotherapy (Thornalley, 2003).

On the contrary, several authors reckon that the consequences of glycation in a tumour milieu may be exacerbated by MGO modification of proteins such as transcription factors involved in oncogenesis, more specifically in metastasis and angiogenesis (Ramasamy *et al*., 2006). An example is MGO-induced modification of the c-terminal Arg residue in heat-shock protein (Hsp) 27, a major target of this α-oxaldehyde. This modification leads to suppression of cytochrome c-mediated caspase activation and therefore inhibits apoptosis of tumour cells (Sakamoto *et al*., 2002; Oya-Ito *et al*., 2006).

1.2. Intracellular protein quality control and housekeeping

1.2.1. How do cells respond to/cope with stress?

Proteins are particularly fragile macromolecules due to their structural and functional complexity and, therefore, have the generic tendency to misfold (Ellis, 2007; Hinault and Goloubinoff, 2007). This poses a universal problem for all types of cells given the high macromolecular crowding in the cytosol, with little or no "free" water, which provides a friendly environment to interactions between exposed interactive surfaces (Fink, 1999; Ellis, 2007). In principle, the primary sequence of all proteins should contain all the information needed to their spontaneous self-assembly into natively functional structures, even in the absence of an energy source or other macromolecules (Anfisen, 1973; Ellis, 2007; Hinault and Goloubinoff, 2007). However, when nascent polypeptides exit the ribosome in the unfolded state, or when labile mature proteins are partially unfolded, they may achieve misfolded conformations that abnormally expose hy-

drophobic surfaces (Hinault and Goloubinoff, 2007). Seeking stabilization, newly exposed hydrophobic surfaces tend to spontaneously associate to form stable protein aggregates and leave solution, or to combine with cellular membranes, thereby disturbing vital membranal functions (Goldberg, 2003; Hinault and Goloubinoff, 2007).

Organisms across the kingdoms are constantly being challenged by events that cause acute and chronic stresses, resulting in deleterious effects on cellular infrastructure and disruption of cellular homeostasis (Gupta *et al.*, 2010). The intracellular milieu itself is a highly hazardous environment, where ROS are continually generated, wherein sugars can glycate proteins, enzymes modify or destroy proteins, fatty acids function as detergents and partially folded nascent chains can act as nuclei for aggregation (Goldberg, 2003; Martinez-Vicente *et al.*, 2005). In order to avoid these harmful events, organisms have developed the capacity to initiate a number of adaptive cellular stress response pathways that attempt to reduce damage to a minimum and maintain or reestablish cellular homeostasis (Gupta *et al.*, 2010).

The central role in monitoring mature proteins for post-synthetic denaturation or chemical damage is played by several highly conserved pathways among prokaryotes and eukaryotes. These pathways constitute the protein quality control system and are also primordial in responding to environmental insults (Goldberg, 2003; Gupta *et al.*, 2010; Koga *et al.*, 2010). Amid these stress-response pathways, we can find the proteolytic response (performed by the lysosomal and proteasomal systems) and the heat-shock response, responsible for degradation and chaperone-mediated protein protection/refolding, respectively (Martinez-Vicente *et al.*, 2005). The coordinated interplay and partitioning of proteins between these surveillance systems, as well as their relevance in specific contexts, in particular the one in which this work is based, will be discussed in the next sections.

1.2.1.1. The autophagic/lysosomal system

Proteins within cells are continuously being degraded to amino acids and replaced by newly synthesized proteins (Goldberg, 2003). Actually, a large fraction of newly synthesized proteins (30%) is rapidly degraded in eukaryotes after release from the ribosome, probably because of the inherent inefficiency of protein folding or multimer assembly (Schubert *et al*., 2000; Goldberg, 2003). This process is highly selective and precisely regulated, serving as a protein quality control mechanism that rapidly eliminates misfolded or damaged proteins, whose accumulation would interfere with normal cell function and survival, without the non-specific destruction of essential cell constituents (Goldberg, 2003). This balance between protein synthesis and degradation further allows cells to rapidly modify intracellular levels of proteins in order to adapt to changes in the extracellular environment (Semba *et al*., 2010).

The term autophagy refers to any process resulting in the degradation of intracellular components inside lysosomes or vacuoles, the equivalent to lysosomes in yeast (Martinez-Vicente *et al*., 2005). Lysosomes consist of single membrane acidic organelles with an internal pH of 5.1-5.5, fully devoted to the degradation of intra- and extracellular components. These organelles contain a large assortment of hydrolases with acidic pH optima, capable of degrading most naturally-occurring macromolecules (Majeski and Dice, 2004; Martinez-Vicente *et al*., 2005; Koga *et al*., 2010). The best characterized autophagic pathways described in the mammalian cells are macroautophagy, microautophagy and chaperone-mediated autophagy (CMA), which differ in the way they deliver substrates into the lysosomal lumen, in the types of substrates carried for degradation and in their activity/regulation (Figure 7) (Kaarniranta *et al*., 2009).

Microautophagy involves the invagination or tubulation of the lysosomal membrane to engulf and degrade whole regions of the cytosol, including both soluble proteins and organelles such as peroxisomes, and even portions of the nucleus, without requiring the formation of an intermediate autophagic vacuole (Martinez-Vicente *et al.*, 2005; Koga *et al.*, 2010). Although poorly understood in mammals, microautophagy has been traditionally considered as the form of autophagy constitutively active to guarantee proper turnover of long-lived proteins under basal conditions (Martinez-Vicente *et al.*, 2005).

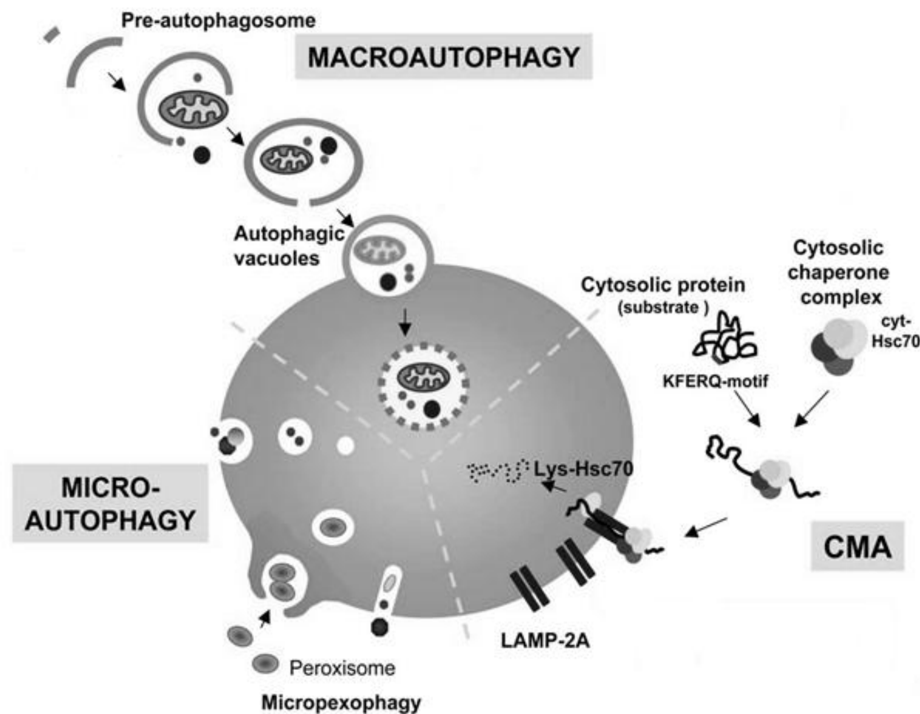

Figure 7.

Schematic model of the main forms of intracellular autophagic pathways in mammalian cells. Three different mechanisms contribute to the delivery of cytosolic cargo to the lysosomes: macroautophagy, microautophagy and chaperone-mediated autophagy (CMA). Abbreviations: Hsc70, heat-shock cognate protein of 70 kDa; LAMP-2A, lysosome associate membrane protein type 2A (adapted from Martinez-Vicente *et al.*, 2005).

Macroautophagy is an inducible form of autophagy responsible for the degradationn of both long-lived soluble proteins and complete organelles under stress conditions (Martinez-Vicente *et al*., 2005; Kaarniranta *et al*., 2009). It combines the action of two different ubiquitin-conjugation cascades (a protein to protein and a protein to lipid conjugation) and by two phosphorylation complexes, that induce the formation of a *de novo* formed membrane (limiting membrane), which elongates and seals to form a double-membrane organelle called autophagic vacuole or autophagosome (Majeski and Dice, 2004; Koga *et al*., 2010). Fusion of lysosomes with these autophagic vacuoles provides them with the enzymes required for the degradation of the sequestered material (Martinez-Vicente *et al*., 2005). Although basal macroautophagy activity occurs in different tissues, macroautophagy is often a stress-induced pathway essential for the maintenance of cellular homeostasis and energetic balance, for the defence against exogenous and endogenous aggressors, as well as in circumstances where extensive cellular remodeling is required (Koga *et al*., 2010).

The third form of autophagy, CMA, can degrade single soluble cytosolic proteins, which are directly translocated across the lysosomal membrane without involving vesicular traffic (Majeski and Dice, 2004; Kon and Cuervo, 2010). All CMA substrates contain a peptide sequence related to KFERQ, which is selectively recognized by the cytosolic heat-shock cognate chaperone of 70 kDa (Hsc70). The substrate/chaperone complex is then targeted to the lysosomal surface where it interacts with a receptor protein, the lysosome-associated membrane protein type 2A (LAMP-2A). Once unfolded, the substrate translocates across the membrane assisted by a luminal chaperone, lys-Hsc70, and is rapidly degraded (Martinez-Vicente *et al*., 2005; Koga *et al*., 2010).

Although basal CMA can be detected in almost all cell types, CMA is maximally activated in response to cell stress (starvation, oxidative stress and in conditions that

cause protein damage). Under these conditions, the selectivity of CMA allows the removal of altered proteins without affecting neighboring functional ones. CMA has been described only in mammalian cells (Koga *et al.*, 2010).

The lysosomal system undergoes striking changes as cells age (*e.g.* increase in lysosome volume, changes in some hydrolases activites, intralysosomal accumulation of indigestible materials – lipofuscin – and impaired regulation of lysosomal pH). These changes render cells an inefficient turnover of intracellular components and the inability to properly adapt to changes in the intracellular environment (Martinez-Vicente *et al.*, 2005). In fact, the decreased efficiency of the autophagic system has been linked to the pathogenesis of different age-related disorders such as neurodegenerative diseases, cancer, diabetes, retinopathies, among others.

1.2.1.2. The ubiquitin-proteasome pathway (UPP)

The ubiquitin-proteasome pathway (UPP) is the major non-lysosomal proteolytic pathway critical in eukaryotic cell cycle and pro-survival pathways (Zang *et al.* 2008). It comprises two main stages: the ubiquitin-tagging of substrates and their proteolytic degradation (Shang and Taylor, 2004). In this pathway, mutant, misfolded, or damaged proteins are flagged by conjugation with an ubiquitin chain in order to be recognized and degraded by the 26S proteasome, a large multicatalytic enzyme complex expressed in the nucleus and cytoplasm of all eukaryotic cells (Adams, 2003). The proteasome complex consists of the proteolytic core 20S particle and two 19S regulatory complexes that cap the entry to the cylindrical catalytic core (Figure 8). The 19S cap recognizes and recruits ubiquitin conjugates to the proteasome, cleaves ubiquitin moieties from the substrate, unfolds and linearizes the polypeptide and translocates it through the catalytic

cavity of the 20S particle (Martinez-Vicente *et al*., 2005; Shang *et al*., 2005; Fernandes *et al*., 2006).

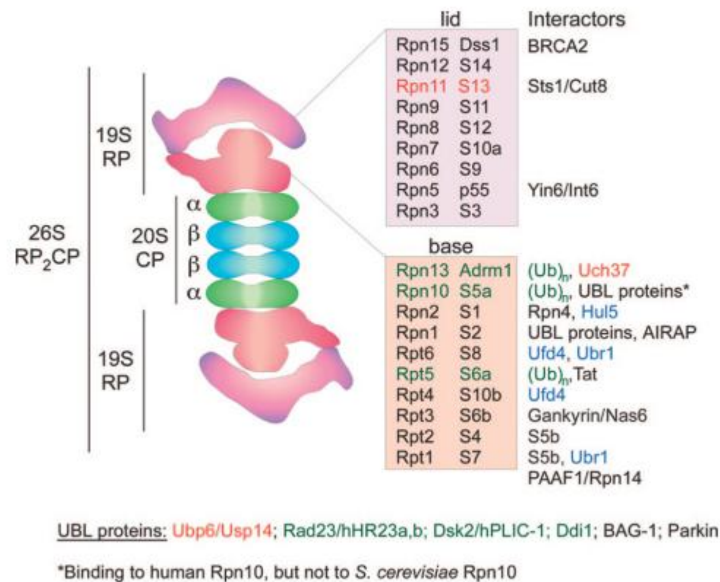

Figure 8.

Schematic representation of the 26S proteasome and its interactions with other proteins. The 26S proteasome is composed of a 20S core particle (CP) and two 19S regulatory particles (RP). The catalytic core is composed of four stacked rings of seven different α_{1-7} or β_{1-7} subunits, some of which bear the proteolytic active sites. Different components of the 19S RP participate in substrate recognition, untagging, and folding. The subunit composition (systematic and alternative names) of the 19S RP subcomplexes lid and base, as well as important interactors, are shown on the right. Abbreviations: Rpt, regulatory particle ATPase; Rpn, regulatory particle non-ATPase (from Marques *et al*., 2009).

Ubiquitin conjugation or ubiquitination is a highly ordered ATP-dependent process that consists of the addition of one or more monomers of ubiquitin, a small (76 aminoacids), highly-conserved, heat-stable, regulatory protein ubiquitously expressed in eukaryotic cells, to the protein substrates (Liang and Godley, 2003). This conjugation process (Figure 9) requires the sequential activities of three groups of enzymes: an ubiquitin-

activating enzyme (E1), an ubiquitin-conjugating enzyme (E2) and an ubiquitin-ligase (E3). In the first place, an E1 activates and transfers ubiquitin to an E2. This carrier protein presents ubiquitin to an E3, which is specific for different protein substrates and that binds to the target protein, acting in concert with E2 to covalently attach ubiquitin to a lysine residue on the substrate (Goldberg, 2003; Adams, 2003). Formation of the ubiquitin-substrate conjugates is usually followed by additional rounds of conjugation of more ubiquitins to the initial adduct. A chain of at least four Lys48-linked ubiquitins, but often more, functions as a targeting signal for substrate recognition and degradation by the 26S proteasome complex (Liang and Godley, 2003). Prior or coordinated with

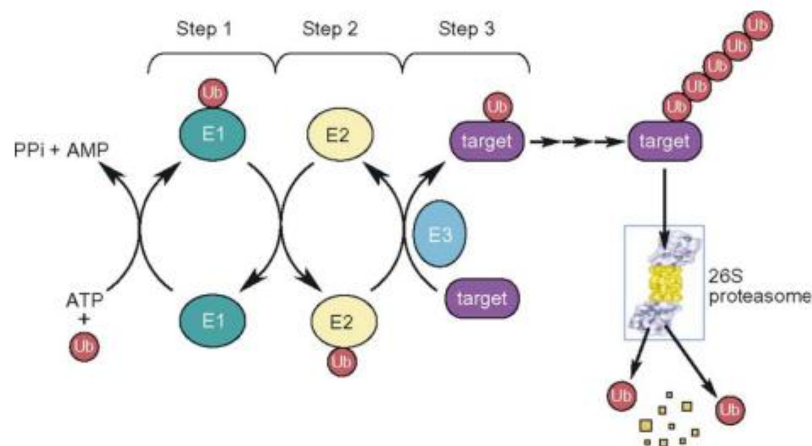

Figure 9.
Ubiquitination of proteins targeted to the proteasome. An ubiquitin-activating enzyme (E1) binds ubiquitin, which is transferred to an ubiquitin-conjugating enzyme (E2). An ubiquitin–protein ligase (E3) then transfers ubiquitin from the E2 to the target substrate. Multiple ubiquitin molecules are attached to proteins before recognition and degradation by the 26S proteasome (from Adams, 2003).

degradation, the ubiquitin chains are disassembled and the ubiquitin is recycled (Liu *et al.*, 2006).

UPP is an important protein quality control system that plays a critical role in a myriad of cellular functions, including regulation of the cell cycle, organogenesis, signal transduction, development, differentiation, stress response, DNA repair, transcriptional regulation, receptor down-regulation, synaptic plasticity and inflammation (Shang and Taylor, 2004; Shang *et al.*, 2005; Fernandes *et al.*, 2008). Many of these functions are mediated by the conditional turnover of regulatory and abnormal proteins or by UPP non-proteolytic activity when polyubiquitin chains are linked to target proteins through lysine residues other than Lys48. The UPP proteolytic activity is of special importance to cell withstanding and recovery from various environmental stresses (Shang *et al.*, 2005; Wu *et al.*, 2009). In fact, certain E2s (*e.g.* Ubc4 and Ubc5) are induced as part of the heat-shock response (Goldberg, 2003). Still, the UPP itself is also a target of such stresses and its abnormal regulation or inactivation has been implicated in many age-related diseases such as Alzheimer's disease, Parkinson's disease, diabetic retinopathy and cataract (Fernandes *et al.*, 2008; Zhang *et al.*, 2008).

1.2.1.3. Heat-shock proteins and stress response

As mentioned above, it was thought that proteins could spontaneously self-assembly into natively functional structures in the absence of an energy source or other macromolecules. Nowadays, it is known that the self-assembly of newly synthesized, stress-unfolded, or folded proteins in near-native conformations requires assistance by chaperones (Ellis, 2007). The molecular chaperones are expressed in the cytoplasm at low concentrations, which are sufficient to carry their physiological and housekeeping functions and to remove sporadically-forming misfolded protein species (Hinault and Goloubinoff, 2007). In contrast, during a stress such as heat-shock, many molecular chaperones are massively synthesized by the cell (Hinault and Goloubinoff, 2007). The

stress-inducible nature of many molecular chaperones has led to their early classification among the heat-shock proteins (Hsps) (Hinault and Goloubinoff, 2007).

The heat-shock response was first reported by Ritossa in 1962, who observed activation of a set of puffs in polytene chromosomes in the salivary glands of *Drosophila melanogaster* larvae exposed to sudden elevated temperature or to chemical agents that induced oxidative stress (Ritossa, 1962; Gupta *et al*., 2010). The massive synthesis of a common set of new proteins (later called heat-shock proteins or stress proteins) upon heat shock in *D. melanogaster* was only observed in 1974 (Tissières *et al*., 1974). Since then, the induction of this set of proteins has been observed in cells of a broad spectrum of eukaryotes and in response to other stresses such as light, oxidation, dehydration, or pathogen attacks (Ellis, 2007; Gupta *et al*., 2010).

The major chaperone function can be defined as the prevention and reversal of incorrect interactions that may occur between potentially interactive surfaces exposed to the crowded intracellular environment (Ellis, 2007). Chaperones assist the correct folding of newly synthesized or stress-accumulated misfolded proteins, prevent protein aggregation, or promote selective degradation of misfolded proteins (Schlesinger, 1990; Gupta *et al*., 2010). They also participate in the unfolding of proteins during transport across membranes, in the association of monomers into oligomers, and in macromolecular disassembly processes, such as the remodeling of chromatin during fertilization and transcription, and the resolubilisation of insoluble microaggregates that have escaped the attention of other chaperones (Goldberg, 2003; Ellis, 2007). Furthermore, chaperones translocate to the cell nucleus after an environmental damage and protect it by direct protection and repair of damaged proteins, as well as by changing intranuclear traffic and organization (Michels *et al*., 1997; Csermely and Blatch, 2007). Additionally, molecular chaperones regulate both the activation and the disassembly of numerous

transcriptional complexes (Guo *et al*., 2001; Ahn *et al*., 2005; Csermely *et al*., 2007) and likewise affect cell survival by interacting with various components of the programmed-cell death machinery, both upstream and downstream of the mitochondrial events (Garrido *et al*. 2001; Gupta *et al*., 2010).

The major classes of molecular chaperones are the small Hsps (sHsps), Hsp40, Hsp60, Hsp70 (comprises the constitutive cytosolic member heat-shock cognate protein 70, Hsc70, the stress-induced cytosolic member Hsp70, the endoplasmic reticulum (ER) form BiP and the mitochondrial form mHsp70), Hsp90 and Hsp100 proteins, which include heat-shock/stress proteins. These different chaperones display mutually non-exclusive properties. Hsp60s (GroEL in *E. coli*) are large oligomeric ring-shaped proteins known as chaperonins, which facilitate protein folding and assembly by providing a folding chamber with the co-chaperone Hsp10 (GroES in *E. coli*). Hsp60-Hsp10-containing structures engulf their clients and allow the folding of single partially unfolded chains in the absence of others (Fink, 1999; Ellis, 2007; Calderwood *et al*., 2009). On the other hand, the molecular chaperones Hsp100, Hsp90 and Hsp70 bind to unfolded sequences in polypeptide substrates free in the cytoplasm, showing preference for hydrophobic regions (Calderwood *et al*., 2009). These chaperones form large complexes containing accessory proteins such as the co-chaperones Hsp40 and Hsp20, that bind the primary chaperones in order to mediate client selection, ATP hydrolysis and cycles of association with and dissociation from the client (Mayer and Bukau, 2005; Calderwood *et al*., 2009). Hsp100, Hsp90 and Hsp70 bind transiently to short hydrophobic sequences on partially denatured polypeptides or oligomerization subunits, reducing the time that these potentially interactive surfaces are exposed by cycling on and off them until they are buried by folding. This cyclic process requires ATP hydrolyses (Mayer and Bukau, 2005; Hinault and Goloubinoff, 2007). sHsps, such as Hsp27 and α-

crystallins, found in the eye lens, consist of 12 to 43-kDa proteins which operate differently from other chaperones by constituting high-molecular-weight structures that mediate client holding and folding in an ATP-independent manner (Fink, 1999; Calderwood *et al*., 2009).

The folding of newly synthesized proteins or misfolded polypeptides to their proper conformations involves the optimal cooperation between multiple molecular chaperones (Hartl and Mayer-Hartl, 2002; Goldberg, 2003; Hinault and Goloubinoff, 2007). For instance, it has been proposed that the complexes formed by sHsps may provide a reservoir for Hsp70 and other molecular chaperones machinery to renature the bound protein (Fink, 1999). Though this process can take many minutes or even longer, and often be unsuccessful, this solution is apparently less costly in terms of ATP-consumption than the alternative one of having to degrade and resynthesize indiscriminately all the misfolded proteins that occur during the lifetime of a cell (Goldberg, 2003; Hinault and Goloubinoff, 2007). Nonetheless, cells have placed the repairing system by the chaperones in competition with more radical systems carried by proteases, such as the 26S proteasome or the lysosomal/autophagic system, leading to the elimination and recycling of irreversibly damaged and likely toxic polypeptides that remain misfolded too long, despite the recurrent unfolding efforts by chaperones (Hinault and Goloubinoff, 2007). Recently, the E3 carboxyl terminus of Hsc70-interacting protein (CHIP), also termed STIP1 homology and U-box-containing protein1 (STUB1), was found to selectively ubiquitinate certain mutant proteins in a process requiring the abundant chaperones Hsp70 or Hsp90, which bind unfolded or hydrophobic domains and facilitate substrate recognition by CHIP, and to direct them to degradation in the proteasome (Murata *et al*., 2001; Goldberg, 2003; Dai *et al*., 2003). Prolonged chaperone binding can help in the identification of fatally damaged proteins (Murata *et al*., 2001), but the chaperones

might also function as cofactors in maintaining the substrate in a soluble, unfolded state that facilitates proteolytic attack (Wickner *et al.*, 1999). An attractive model is that failure of the chaperones to perform protein folding or the refolding of damaged proteins leads to recruitment of ubiquitination enzymes to eliminate the potentially dangerous polypeptides (Wickner *et al.*, 1999; Murata *et al.*, 2001; Goldberg, 2003).

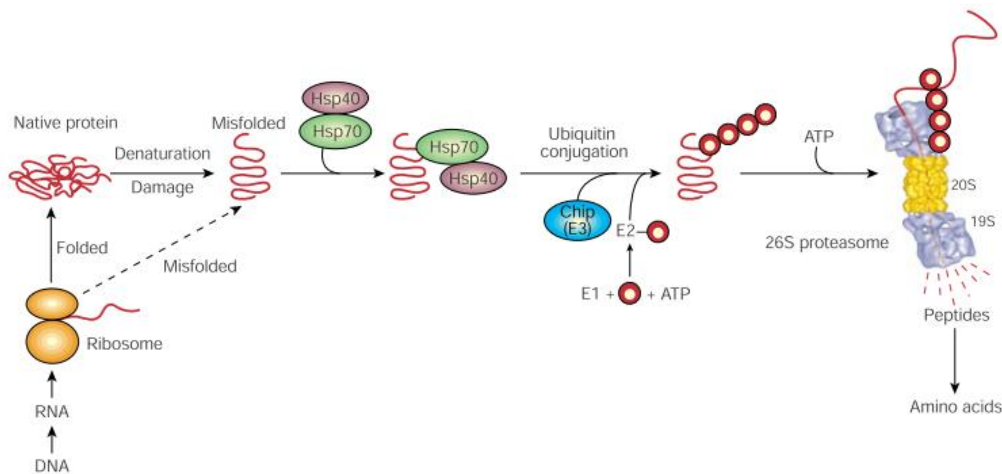

Figure 10.
The ubiquitin-proteasome pathway. Molecular chaperones may function in protein folding and in the degradation of misfolded species. By associating hydrophobic domains, chaperones Hsp70/40 promote the folding of newly synthesized proteins and favour their refolding. Alternatively, they can facilitate the recognition of abnormal proteins, leading to their ubiquitylation by CHIP and degradation by the 26S proteasome (from Golberg, 2003).

1.2.2. Heat-shock factors and the heat-shock response

The heat-shock response is one of the most ancient and conserved cellular stress responses that allow cells to avoid increases in the levels of unfolded proteins, disruption of the cytoskeleton and loss of mitochondrial function (Ahn *et al.*, 2005; Nadeau and Landry, 2007). As stated above, this response was originally identified upon observation of a set of chromosomal puffs in the salivary glands of *Drosophila melanogaster* larvae exposed to sudden elevated temperature or to chemical agents that result in un-

coupling of oxidative phosphorylation (Ahn and Thiele, 2003). Subsequent analysis of the heat-induced puffs confirmed that the loci were transcriptionally active regions, where genes encoding for Hsps were located (Ritossa, 1962; Ahn and Thiele, 2003; Anckar and Sistonen, 2007; Nadeau and Landry, 2007). The transcriptional response to hyperthermia and other protein-damaging stresses has later been shown to be a highly regulated process in which the transcription of most non-stress genes is repressed, but that of heat-shock genes rapidly upregulated in order to provide resistance to cells (Lindquist, 1986; Anckar and Sistonen, 2007; Nadeau and Landry, 2007).

The transcriptional response to heat and other proteotoxic stress is regulated by a family of heat-shock transcription factors (Hsfs), remarkably conserved from yeast to humans (Ahn *et al.*, 2005; Anckar and Sistonen, 2007; Voellmy and Boellman, 2007). In non-vertebrate organisms (*e.g.* nematode, fruit fly, yeast), the transcriptional activation of the heat-shock response is regulated by a single Hsf, which is also responsible for growth and viability under non-stressful conditions (Pirkkala *et al.*, 2001). Vertebrates, in turn, have evolved a family of Hsf homologues, Hsf-1, Hsf-2, Hsf-3 and Hsf-4 (Figure 11), of which Hsf-3 is specific for avian species and Hsf-4 for mammals (Nakai, 1999; Anckar and Sistonen, 2007). The Hsfs cross-species conservation reflects their importance as regulators of the responses to various forms of physiological and environmental stimuli, suggesting that different members of the Hsf family could share a subset of target genes (Leppä and Sistonen, 1997; Pirkkala *et al.*, 2001; Anckar and Sistonen, 2007). Nevertheless, since the *hsf1*, *hsf2*, *hsf3* and *hsf4* knockout mice have clearly distinct phenotypes, the Hsfs might be restricted to different fields of action (Xiao *et al*, 1999; Wang *et al.*, 2003; Fujimoto *et al.*, 2004; Anckar and Sistonen, 2007).

Among the different vertebrate Hsfs, Hsf-1 is the predominant isoform required for stress regulation of Hsp expression. Hsf-1 cannot be substituted by other Hsf members

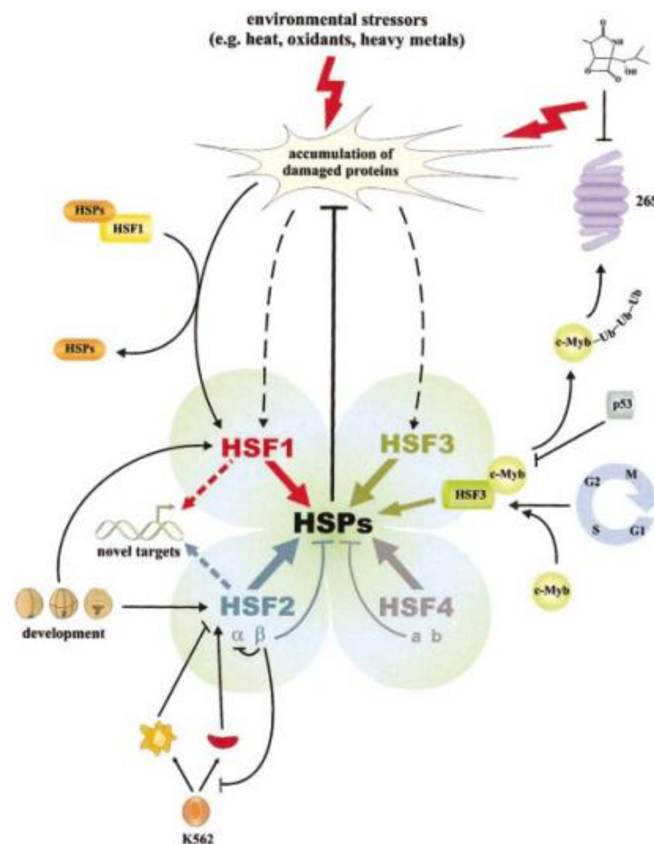

Figure 11.

Model of differential Hsf functions. Environmental stressors and dysfunctions in the ubiquitin-proteasome pathway induce accumulation of aberrant and short-lived proteins, which in turn leads to dissociation of Hsf-1/Hsp complexes and activation of Hsf-1. Activation of the heat-shock response ultimately leads to synthesis of Hsps, thereby forming a negative feedback loop for attenuation of the stress response. Beyond the heat-shock response, Hsf-3 cooperates with c-Myb to ensure an adequate supply for Hsps, an enhanced need for which is observed during the cell cycle progression. Hsf-2 is activated under certain developmental conditions and upon erythroid differentiation of K562 cells, leading to transcriptional activation of its target genes, *i.e.* the heat-shock genes. Both Hsf-1 and Hsf-2 are likely to have additional target genes. The stimuli responsible for activation of Hsf-4 are uncharacterized, but the *in vitro* evidence points to opposite roles for the two Hsf-4 isoforms in regulation of the heat-shock genes (from Pirkkala *et al.*, 2001).

since the heat-shock response is obliterated in its absence, as previously demonstrated in fibroblasts and mice lacking *hsf1*, which are unable to induce expression of Hsp70 in

stressful conditions (McMillan *et al*., 1998; Xiao *et al*., 1999; Kallio *et al*., 2002; Ahn *et al*., 2005; Zhang *et al*., 2002). In contrast, Hsf-2 and Hsf-4 are known to play important roles in differentiation and development, but are incapable of responding to classical stress stimuli. For instance, *hsf2* knockout mice have an intact heat-shock response, but disrupted spermatogenesis and neuronal development (Kallio *et al*., 2002; Mcmillan *et al*., 2002; Wang *et al*., 2003). Hsf-4, on the other hand, is expressed in only a few tissues and required for proper eye development (Fujimoto *et al*., 2004; Anckar and Sistonen, 2007).

1.2.2.1. Hsf-1: functional domains and activation mechanism

Hsf-1 binds to DNA as a homotrimer, in which each individual DNA-binding domain recognizes a pentameric sequence nGAAn in the DNA double helix. The minimal functional upstream regulatory elements recognized by Hsf-1 contain at least three nGAAn repeats and are called heat-shock elements (HSEs) (Wu, 1955; Trinklein *et al*., 2004; Anckar and Sistonen, 2007; Voellmy and Boellman, 2007). The promoters of *hsp* genes contain more than one HSE, allowing for multiple Hsf-1 trimers to bind simultaneously in a cooperative manner, in which one DNA-bound trimer facilitates the binding of the next trimer (Kroeger and Morimoto, 1994; Ahn and Thiele, 2003; Anckar and Sistonen, 2007). Such cooperation may be involved in regulating the magnitude of Hsf-1-mediated transcription, although some HSEs are not required for downstream gene expression (Wu, 1955; Anckar and Sistonen, 2007). Furthermore, HSEs precise architecture itself influences the conformations adopted by Hsf-1, as well as their displayed distinct requirements for temperature and posttranslational modifications (Santoro *et al*., 1998, Anckar and Sistonen, 2007).

There are several functional modules that compose Hsf-1 (Figure 12) and that are involved in regulation of the factor trimerization at both intra- and intermolecular levels. The DNA-binding domain (DBD), capable of interacting specifically with HSE sequences in the promoters of *hsp* genes, is the most conserved functional domain in Hsfs and is located near the N-terminus (Damberger *et al.*, 1994; Pirkkala *et al.*, 2001; Voellmy and Boellman, 2007). The oligomerization domain consists of a long, interrupted, hydrophobic repeat sequence (HR-A/B) and is found adjacent to the DNA-

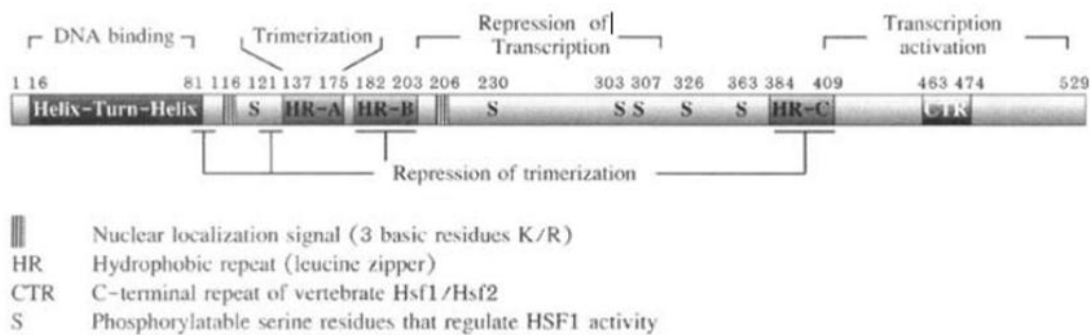

Figure 12.
Linear map of human Hsf-1. Functions of individual domains/regions are indicated below or above the map (from Voellmy and Boellman, 2007).

binding domain (Sorger and Nelson, 1989; Anckar and Sistonen, 2007). In the trimeric Hsf-1, these heptad repeats are proposed to form an unusual triple-stranded coiled-coil configuration through the interactions of the hydrophobic residues (Peteranderl and Nelson, 1992). In fact, it has been shown that the deletion of HR-A/B abolishes Hsf-1 trimerization (Rabindran *et al.*, 1993; Voellmy and Boellman, 2007). In addition, most members of the Hsf family, including Hsf-1, contain an additional hydrophobic repeat sequence (HR-C) in the carboxyl terminus of their sequences, whose absence – *e.g.* Hsf-4 (Nakai *et al.*, 1997) –, or deletion – *e.g.* Hsf-1 (Rabindran *et al.*, 1993) –, generates

constitutive trimerization of Hsfs, suggesting that HR-C supresses Hsf-1 as well as other Hsfs trimerization (Pirkkala *et al.*, 2001; Anckar and Sistonen, 2007).

This data have impelled a model in which the HR-C represses Hsf-1 trimerization by folding back and directly interacting with HR-A/B (Figure 13). The hydrophobic

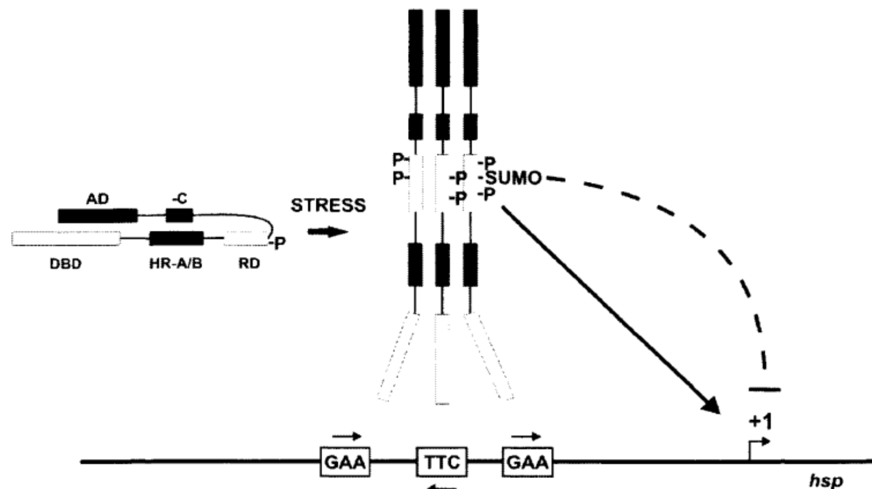

Figure 13.
Multistep activation mechanism of Hsf-1. When exposed to stress, Hsf-1 acquires DNA-binding activity through a monomer-to-trimer transition and subsequent nuclear accumulation. In the nucleus, Hsf-1 binds to HSEs in the promoters of target genes such as Hsps. During activation, Hsf-1 is hyper-phosphorylated (P) at several serine residues, mostly in the regulatory domain (RD). Abbreviations: HR-A/B/C, leucin-zipper-like heptad-repeat domains; AD, activation domain; DBD, DNA-binding domain (from Anckar and Sistonen, 2007).

contacts between HR-A/B and HR-C are thought to restrain the HR-A/B, forming an intramolecular coiled-coil structure and thereby maintaining the monomeric Hsf-1 in a closed inactive state in the absence of a stress (Wu, 1955; Anckar and Sistonen, 2007). The transactivation domain, which is rich in hydrophobic and acidic amino acids, was found near the carboxyl end of the mammalian Hsf-1 and other Hsfs (Newton *et al.*, 1996; Voellmy and Boellman, 2007). This domain interacts with several regulatory pro-

teins as well as with components of the transcription pre-initiation complex (Green *et al*., 1995). Under non-stressful conditions, the transactivation domain is kept inactive by a regulatory domain located between the HR-A/B and HR-C domains (Green *et al*., 1995; Newton *et al*., 1996). This regulatory domain can work as a gatekeeper, on which stress stimuli act to unleash the function of Hsf-1 (Anckar and Sistonen, 2007).

1.2.2.2. Hsf-1 regulation

In unstressed cells, Hsf-1 is continuously present and largely localizes in the cytoplasm in a non-homooligomeric state (Pirkkala *et al*., 2001; Guettouche *et al*., 2005; Nadeau and Landry, 2007). In response to a cellular stress, Hsf-1 undergoes the transition from the monomeric to the homotrimeric form and re-localizes to the nucleus, where it acquires DNA-binding and transactivation activities (Pirkkala *et al*., 2001; Ahn *et al*., 2005). The rapidity of Hsf-1 activation in response to stress *in vivo* (Boehm *et al*., 2003), along with the finding that the regulation of Hsf-1 activity does not depend on new protein synthesis or degradation, suggest that the stress signal is transmitted rapidly to Hsf-1 and not *via* a multistep signal transduction cascade (Cotto *et al*., 1996; Ahn and Thiele, 2003; Voellmy and Boellmann, 2007). An increasing body of evidence indicates that the inactive state of Hsf-1 in the absence of a stress is maintained through both intra- and intermolecular interactions, as well as by posttranslational modifications (Anckar and Sistonen, 2007). Besides, the transcriptional activity of the homotrimeric Hsf-1 itself requires additional layers of regulation. This is supported by data showing that the transcriptional activity of overexpressed homotrimeric Hsf-1 is negligible compared to that of the stress-activated factor, and that certain compounds (*e.g.* salicylates, menadione and H_2O_2) induce Hsf-1 DNA-binding activity but not expression of inducible *hsp* genes (Bruce *et al*., 1993; Voellmy and Boellmann, 2007).

Several hypothesis and findings on Hsf-1 regulation mechanisms will be described in the next sections.

1.2.2.2.1. Feedback regulation of Hsf-1 by molecular chaperones

It is unclear how the multitude of stresses that are greatly different in nature, including heat-shock, H_2O_2, low pH, infection, inflammation, nonsteroidal anti-inflammatory drugs, heavy metals, developmental cues and others, exhibit equal capacity to trigger Hsf-1 homotrimerizarion (Ahn and Thiele, 2003; Nadeau and Landry, 2007; Anckar and Sistonen, 2007). Nonetheless, these diverse stresses lead to the generation and accumulation of non-native proteins resulting from chemical or physical denaturation, a common denominator that might underlie Hsf-1 regulation (Nadeau and Landry, 2007; Voellmy and Boellmann, 2007; Anckar and Sistonen, 2007). Several studies observed that, under conditions of moderate heat stress, the Hsp response was self-regulated, meaning that Hsp expression increased after initiation of a heat treatment, continued for some time, and then decreased to a low rate approximately corresponding to a pre-stress rate, a phenomenon called attenuation (Lindiquist, 1980; Dioimenico *et al*., 1982; Abravaya *et al*., 1991; Voellmy and Boellmann, 2007). The reduction of heat-induced transcription or translation rates resulted in the extension of the period of elevated *hsp* gene transcription during stress recovery, suggesting that one or more synthesized Hsps feed-back regulated the Hsp response (Voellmy and Boellmann, 2007). This has led to a widely accepted model in which the elevated level of misfolded protein intermediates liberates Hsps from Hsf-1, thereby allowing Hsf-1 to be converted into an active homotrimeric state (Anckar and Sistonen, 2007). Nowadays, it is known that Hsps and co-chaperones that are assembled into multichaperone complexes regulate Hsf-1 activity at different levels, providing a mean of autoregulation of this pathway.

Several studies initially focused on Hsp70 as a molecular chaperone involved in Hsf-1 deactivation. Indeed, the direct interaction between monomeric Hsf-1 and Hsp70 was shown to occur in unstressed cells and to attenuate heat-shock response, in part by preventing recruitment of the general transcriptional machinery and by stabilizing Hsf-1 monomeric conformation as well (Rabindran *et al*., 1994; Shi *et al*., 1998; Pirkkala *et al*., 2001). However, several eukaryotic cells express low basal levels of Hsp70 and are still perfectly capable of inhibiting Hsf-1 trimerization (Velazquez *et al*., 1983; Voellmy and Boellmann, 2007).

An increasing body of evidence points to Hsp90 as an important chaperone involved in the inhibition of Hsf-1 oligomerization in the absence of a stress, whether alone or in concert with other factors, and both *in vitro* and *in vivo* (Zuo *et al*., 1995; Ali *et al*., 1998; Knowlton and Sun, 2001). Actually, the available information suggests that a mature Hsp90-containing multichaperone complex, which appears to be the end result of several intermediate assembly steps involving Hsp70 and Hsp40, as well as adaptor proteins such as Hop, interacts with Hsf-1 and prevents its oligomerization (Duina *et al*., 1998; Bharadwaj *et al*., 1999; Voellmy and Boellmann, 2007). Because Hsp90-containing complexes interact with denatured proteins in response to cellular stress, these non-native protein substrates compete with Hsf-1 for binding to Hsp90, leading to Hsp90-Hsf-1 complex interruption and, subsequently, to an increase in Hsf-1 DNA-binding competence (Zuo *et al*., 1998; Nadeau and Landry, 2007; Ahn *et al*., 2005). Wang *et al*. corroborated these results by reporting that the phosphorylation of a specific serine on human Hsf-1 might suppress its activation by stabilizing the interaction between non-homotrimeric factor and Hsp90-containing complexes (Wang *et al*., 2006). Thus, this mechanism may serve to prevent inadvertently formed Hsf-1 trimers from

becoming active, as well as to ensure that Hsf-1 activity varies proportionally to the level of stress (Voellmy and Boellmann, 2007).

Another mechanism for suppression of Hsf-1 transcriptional competence, particularly during the recovery from a stress, consists of binding of Hsp70 and Hsp40 to the transcriptional activation domain region of Hsf-1 (Shi *et al.*, 1998). Other studies reported that Hsf-1 and Hsc70 associate in a high-molecular-mass complex in the cytoplasm of unstressed cells (Nunes and Calderwood, 1995). Ahn and colleagues further contributed to this work by showing that the C-terminal region of Hsf-1, which includes its transactivation domain, interacts with Hsc70, and that this complex translocates into the nucleus upon heat-shock treatment (Ahn *et al.*, 2005). Moreover, they showed that this interaction is required for the Hsf-1 trimerization/transcriptional competence. Although both Hsc70 and Hsp70 interact tightly with Hsf-1, the function of their respective interactions is probably different due to their distinct expression patterns and considerable differences in their C-terminal domains, among other things (Ahn *et al.*, 2005).

1.2.2.2.2. Hsf-1 regulation by CHIP and other interacting factors

CHIP has also been implicated in Hsf-1 regulation. CHIP is the only known higher eukaryotes protein able to target proteins that are unable to achieve tertiary structures and that remain chaperone substrates, for proteasomal degradation by facilitating their ubiquitination (Ballinger *et al.*, 1999; Voellmy and Boellmann, 2007; Nadeau and Landry, 2007). This co-chaperone contains a N-terminal tetracopeptide repeat (TRP) domain responsible for binding the conserved C-terminus of several chaperones such as Hsc70, Hsp70 and Hsp90, and for attenuating the rate of the folding pathway by competing with co-chaperones such as Hop, Bag-1, Bag-2 and heat-shock factor binding protein 1 (Hspbp1), among others (Dice, 2007). CHIP also contains a modified RING

finger-like domain called a U-box, that has E3 ubiquitin ligase activity and that allows CHIP to attach polyubiquitin side chains to chaperone-bound non-native proteins (Murata *et al.*, 2001; Kim *et al.*, 2005; McDonough and Patterson, 2003; Dai *et al.*, 2003; Ballinger *et al.*, 1999). Therefore, CHIP seems to determine whether a protein is assisted by chaperones in achieving its final conformation or whether it becomes a proteasome substrate following CHIP-mediated polyubiquitination.

Experimental results suggest both direct and indirect mechanisms for the functional interaction between Hsf-1 and CHIP, which seems to regulate Hsf-1 activation prior and during a stress (Voellmy and Boellmann, 2007). By being able to bind chaperones *via* its TRP domain, CHIP interferes with the assembly of Hsp90-containing chaperones complexes, forcing them to disassembly, and hence allowing Hsf-1 activation (Figure 14) (Dai *et al.*, 2003). In parallel, other results suggest a direct physical interaction between the N-terminal region of Hsf-1 and CHIP with an intact TRP domain, only upon heat stress (Dai *et al.*, 2003; Kim *et al.*, 2005). These results point to the necessity for Hsf-1 to undergo an overall structure modification so that this interaction may occur (Kim *et al.*, 2005).

Hsf-1 is exposed to several other regulatory influences. For instance, Hspbp1 was found to interact with the oligomerization domain of an active Hsf-1 together with Hsp70, thereby negatively affecting Hsf-1 DNA binding activity (Satyal *et al*, 1998; Pirkkala *et al.*, 2001).

1.2.2.2.3. Hsf-1 regulation by phosphorylation

Beyond the interaction with other proteins, phosphorylation of Hsf-1 regulatory domain serines (Ser) and threonines (Thr) emerged as a major post-translational mechanism for a rapid change in this transcription factor activity, independently of its oli-

gomeric status. Heat shock activates in minutes an array of signal-transducing kinases, which comprises three mitogen-activated protein kinase (MAPK) pathways, namely the extracellular signal-regulated kinase (ERK) pathway, the c-jun N-terminal kinase (JNK) pathway, and the p38 pathway (Nadeau and Landry, 2007). In fact, Hsf-1 was found to be extensively phosphorylated on at least 12 sites, most of which are located in the regulatory domain (Guettouche *et al.*, 2005). Therefore, stress-induced phosphorylation of the regulatory domain likely acts as a switch by relieving the inhibitory effect of this domain, rendering Hsf-1 transactivation-competent (Anckar and Sistonen, 2007). Nonetheless, only a few studies have been able to link phosphorylation to Hsf-1 transcriptional activation, but not DNA-binding activity, upon heat-shock stress – *e.g.* phosphorylation of Ser230 (Holmberg *et al.*, 2001) and Ser326 (Kline and Morimoto, 1997; Guettouche *et al.*, 2005) –, whereas most phosphorylation events were shown to repress the transcriptional activity of Hsf-1, in particular phosphorylation of Ser303, Ser307 and Ser363 located in the regulatory domain of human Hsf-1 (Kline and Morimoto, 1997; Anckar and Sistonen, 2007). The phosphorylation of these residues leads to Hsf-1 sequestration in the cytoplasm by the scaffolding protein 14-3-3ε under non-stress conditions (Wang *et al.*, 2004). Thus, Hsf-1 is kept in a transcriptionally inactive state by this inhibitory effect, which can be overridden by heat shock (Holmberg *et al.*, 2001; Knauf *et al.*, 1996).

1.2.2.3. Hsf-1 activation upon different stimuli

Summarizing, the common signal generated by various stress stimuli that underlies Hsf-1 activation is likely to be protein damage. However, it could possible that distinct stimuli differentially modulate Hsf-1-dependent transcriptional processes (Voellmy and Boellmann, 2007; Pirkkala and Sistonen, 2007). For instance, Hsf-1 activated by oxida-

tive stress shows a phosphorylation pattern distinct from when it's activated by heat (Liu and Thiele, 1996; Anckar and Sistonen, 2007). Although phosphorylation is involved in a stress-specific activation mechanism of Hsf-1, it may also specify the subset of target genes that are to be transcribed in response to a particular stress (Anckar and Sistonen, 2007). This is further emphasized by the fact that Hsf-1 DNA-binding and transactivation capacities are uncoupled and can presumably proceed in a stimulus-specific manner (Pirkkala *et al*., 2001; Anckar and Sistonen, 2007). This suggests that, depending on the stress stimuli, the various events associated with Hsf-1 activation are differentially affected.

1.2.3. Decline in the protein quality control pathways upon ageing and disease

The amount of damaged or newly folded proteins and the available chaperone capacity are two sides of a carefully balanced system in our cells (Csermely and Blatch, 2007). There are extreme situations where the chaperone system, as well as the proteolytic systems, cannot keep up with the rate of production of unfolded proteins and, thus, become overloaded. This might occur when there is an excess of chaperone substrates production or insufficient content in molecular chaperones and proteases in cells (Goldberg, 2003; Csermely and Blatch, 2007; Hinault and Goloubinoff, 2007). Chaperone overload becomes especially large in aged subjects, where damage to proteins occurs due to the abundance of oxidative stress, glycation and addition of sugar residues. Non-dividing cells are particularly susceptible to accumulation of these modified molecules (Csermely and Blatch, 2007; Calderwood *et al*., 2009).

Recent studies propose that increased protein damage in ageing cells may be a consequence of a time-dependent decline of protein quality control (Figure 14), both in non-mammal and mammal species (Kurapati et al., 2000; Steinkraus *et al*., 2008; Gray

et al., 2008; Winklhofer *et al.*, 2008). This might be due to a reduced capacity to activate the transcriptional pathways that lead to Hsps synthesis and thereby to a decline in the heat-shock response, resulting in stress-mediated damage and cell death (Sherman and Goldberg, 2001; Garrido *et al.*, 2006; Csemerly and Blatch, 2007; Calderwood *et al.*, 2009). This is a general effect observed in neuronal tissues (Sherman and Goldberg, 2001), skeletal and cardiac muscle (Kayani *et al.*, 2008), and liver (Gagliano *et al.*, 2007).

The decline in protein quality control has been reported as the probable cause of neurological diseases that involve aggregation-prone proteins and formation of inclu-

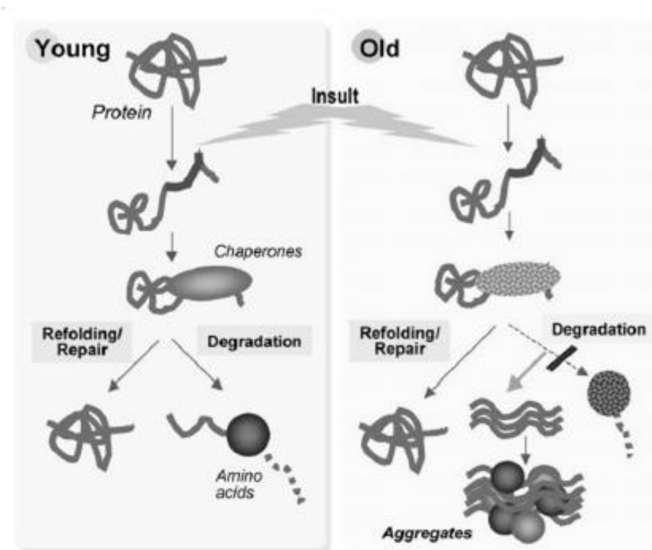

Figure 14.
Intracellular fates of modified proteins. Damaged proteins are recognized by molecular chaperones that attempt to repair (refold) them. If refolding is not possible, chaperones target the damaged protein for degradation by the intracellular proteolytic systems. As cells age, the activity of the major proteolytic systems becomes defective, leading to chaperone "overload" and to the intracellular accumulation of damaged/unfolded protein products, which often aggregate, sequestering chaperones, proteases and other neighboring proteins. Protein aggregates slowly accumulate in all cells through the life, but their formation can be precipitate under particular cellular conditions or in certain pathologies (from Martinez-Vicente *et al.*, 2005).

sion bodies, whose occurrence is linked to pathology. These diseases include Alzheimer's disease, Parkinson's disease and Huntington's disease, whose pathological symptoms are linked to accumulation of amyloid peptide and cytoskeletal protein tau, α-synuclein and huntingtin, and androgen receptors containing areas of polyglutamine expansions aggregate, respectively (Winklhofer *et al*., 2008; Hands *et al*., 2008). The striking feature of these neuronal disorders is that the disease does not become established immediately but is instead observed as the mature individual ages (Sherman and Goldberg, 2001), which points to the decline in protein quality control as a trigger or promoter of these pathologies. In addition, chaperone-overload in folding diseases is probably aggravated by the sequestration of most chaperones by aggregation-prone proteins (Csermely and Blatch, 2007).

Age-dependent decline in the cytoprotective heat-shock response is also observed in muscle tissues, which loose mass and suffer a decline in force generation upon ageing (Kayani *et al*., 2008). The heat-shock response is also essential to the protection of cells against the toxic effects of alcohols, heavy metals, xenobiotics and oxidants, presumably being particularly significant in liver function. Indeed, Gagliano and colleagues recently showed a decline in the heat-shock response in aged livers (Gagliano *et al*., 2007).

The molecular chaperone system is just one of the strategies of the protein quality control. A second strategy consists of the degradation of damaged proteins by the UPP, which is tightly linked to the molecular chaperone pathway. As mentioned above, the bridge between both pathways is provided by CHIP (Marques *et al*., 2006). This protein binds to Hsp70, Hsc70 and Hsp90, and catalyzes the ubiquitin tagging of these chaperones substrates, thereby selecting them for degradation through the proteasome. Thus, CHIP plays a major role in longevity, which is corroborated by results showing that

CHIP inactivation in mice leads to a marked reduction in lifespan, along with accelerated age-related pathophysiological phenotypes (Min *et al*., 2008; Calderwood *et al*., 2009). Furthermore, Jana *et al*. observed that CHIP associates and is sequestered by protein bearing expanded polyglutamine repeats that mediate aggregation during ageing, which might repress Hsf-1 activation during ageing (Jana *et al*., 2005). Deregulation of Hsp-CHIP interactions may therefore underlie some of the changes in Hsp levels that accompany ageing (Min *et al*., 2008).

Finally, damaged proteins can also be processed by autophagy, a pathway of autodigestion that comprises several forms, as mentioned above, two of which undergo age-dependent decline: macroauthophagy and CMA (Dice, 2007). CMA resembles CHIP-mediated proteasomal degradation since damaged proteins form complexes containing Hsc70, Hspbp1, Hsp40, Hop, Hsp90 and Bag-1, that mediate CMA-substrate recognition and unfolding prior to lysosomal delivery, insertion and acceptance in the lumen by an intralysosomal population of Hsc70 molecules (Dice, 2007; Calderwood *et al*., 2009). Decline in CMA is also observed in Parkinson's disease (Dice, 2007).

Taking into account the abovementioned findings that point to the central role played by Hsps in protein quality control, one could say that Hsf-1 activation and increased Hsps levels are coupled to the mechanisms that ensure longevity in non-mammalian and mammalian species, whereas the opposite associates with degeneration and death.

1.3. Retinal pigment epithelium (RPE): a good cell model

1.3.1. The eye and the retina

The perception of light begins in the retina, a highly differentiated neuroectodermal light-sensitive tissue at the back of the eye, which is composed of multiple cell-types

within defined layers and is responsible for capturing high-resolution visual information (Rakoczy *et al*., 2006; Rodieck, 2008; Glenn and Stitt, 2009; Swaroop *et al*., 2009; Xu *et al*., 2009). Retinal anatomists traditionally sub-divide the layers into inner and outer retina. The inner retina is composed of ganglion cells, amacrine cells, bipolar cells glia and astrocytes, while the outer retina consists of horizontal cells and photoreceptors. Other retinal cells are the Müller glia cells, that span the thickness of the inner and most of the outer retina, and the non-neuronal retinal cells that constitute the retinal pigment epithelium (RPE) (Glenn and Stitt, 2009). Beneath the retina lies the dense capillary bed in the choroid, which supplies the outer retina with oxygen and nutrients, thereby playing a critical role in maintaining photoreceptor health and integrity (Figure 15) (Campochiaro and Hackett, 2003; Fernandes *et al*., 2006; Ehrlich *et al*., 2008; Snodderly, 2008; Glenn and Stitt, 2009). Retinal circulation nourishes the inner half of the retina (Snodderly, 2008; Campochiaro and Hackett, 2003).

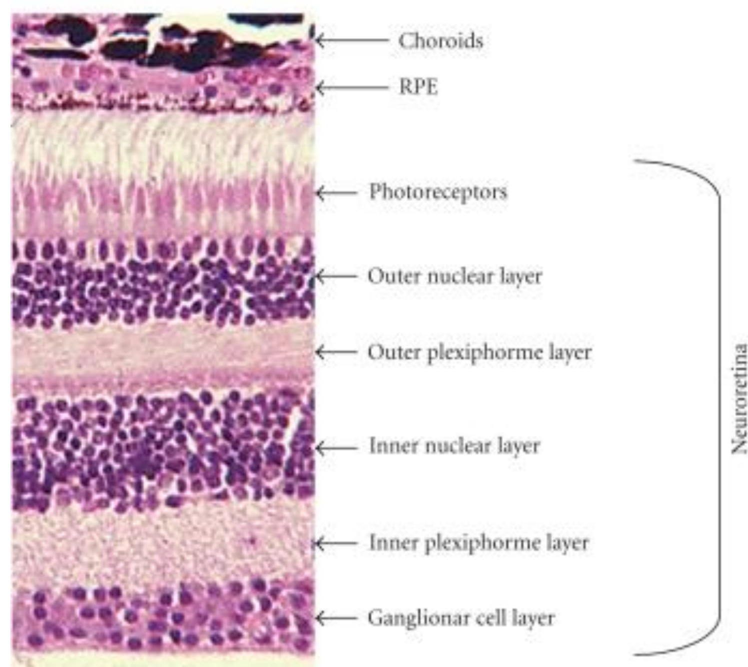

Figure 15.

Retinal section of the retina showing the location of the retinal pigment epithelium (RPE) (adapted from Simó *et al*., 2010).

Photoreceptors are primary neurons in the visual pathway that respond to photons through changes in their membrane potential, process through which chemical stimuli (light energy) is converted into image-forming signals that are transmitted through the axons of the optic nerve to the brain (Rodieck, 2008; Glenn and Stitt, 2009). There are two types of photoreceptors: rods and cones. Both rods and cones contain specialized visual phototransduction proteins that are concentrated in membranous disk-like structures, the outer segments. These disks have high metabolic demands and undergo continuous renewal in a process controlled by light and circadian rhythms (Young, 1978; Swaroop *et al.*, 2009). During each day, the concentration of light-induced toxic substances increases inside the photoreceptors (*e.g.* photo-damaged proteins and lipids, photo-oxidative radicals). Therefore, to maintain the excitability of photoreceptors, the photoreceptor outer segments (POS) or outer-segment disks undergo a constant renewal process (Strauss, 2005). The shed old disks are phagocytosed by the RPE (Ehrlich *et al.*, 2008), a monolayer of post-mitotic pigmented cells that also participate in the visual cycle (*e.g.* uptake, processing, transport and release of vitamin A derivatives) (Nowak, 2006; Swaropp *et al.*, 2009), transport of nutrients and waste products into and out of the retina, and nurture of photoreceptors and choriocapillaris by secreting various growth factors and cytokines such as pigment epithelium-derived factor (PEDF), vascular endothelial growth factor (VEGF), angiopoietin-1 (Ang-1), angiopoietin-2 (Ang-2) and monocyte chemoattractant protein-1 (MCP-1) (Strauss, 2005; Swaroop *et al.*, 2009). The apical membrane of the RPE faces the photoreceptor outer segments, whereas its basolateral membrane faces Bruch's membrane, a thin elastin-rich semi-permeable exchange lamina that separates the RPE from the choroid blood vessels (Strauss, 2005; Swaroop *et al.*, 2009). Together with Bruch's membrane, RPE forms the outer blood-retinal barrier (oBRB) to the small fenestrated capillaries of the choroidal

blood vessels (choriocapillaries) due to its tight junctions and specialized transport systems (Swaroop *et al*., 2009; Glenn and Stitt, 2009).

1.3.2. RPE and AGEs

Retina cells have little regenerative capacity after insult, often becoming dysfunctional during ageing. The cellular manifestations of this process include increased chemical damage to proteins, accumulation of intra- and/or extracellular deposits, and decreased efficiency of antioxidant defences (Glenn and Stitt, 2009). These manifestations may be directly related to free radical generation by blue light or light in the visible spectrum. This is probably best appreciated in the outer retina, at the level of RPE-Bruch's-choroid complex that sustain the neurosensory retina (Figure 15) (Glenn and Stitt, 2009).

The RPE is central when discussing the ageing process. This susceptibility is related to photoxidative stress, POS shedding, lipid peroxidation, high metabolic requirements and high oxygen demands (Tian *et al*., 2005; Liang and Godley, 2003). To an extent, and in contrast to other retinal cells, RPE is capable of some regeneration following damage, but these largely pos-mitotic cells are constantly subjected to damaging processes (Glenn and Stitt, 2009). In fact, there is a net reduction in RPE with ageing and the surviving RPE show decreased melanin content, mitochondria dysfunction, altered lysosomal degradative capacity and proteasome dysfunction (Ambati *et al*., 2003; Liang and Godley, 2003; Glenn and Stitt, 2009). The retinal cycle itself is also responsible for the generation of considerable metabolic waste products such as the cytoplasmic, non-degradable, autofluorescent material called lipofuscin, which continues to accumulate throughout life, and extracellular deposits referred to as drusen, which are

found between the RPE and Bruch's membrane and that result from the phagocytosis of POS (Maeda *et al*., 2005; Glenn and Stitt, 2009).

As mentioned in the previous sections, AGEs accumulate within ageing and diabetic tissues. In particular, these molecules were shown to accumulate in the inner retina. This is of special relevance in diabetic patients, where they contribute to retinal microvasculature and neuroglial abnormalities that characterize diabetic retinopathy (Stitt *et al*., 1997; Glenn and Stitt, 2009). The outer retina is also highly prone to AGEs accumulation given the fact that it constitutes a highly oxygenated and glucose-enriched tissue (Glenn and Stitt, 2009). Indeed, AGEs accumulate in RPE, where they can be found as free adducts or as AGE-modified proteins in lipofuscin granules (Schutt *et al*., 2003). RPE growing on an AGE-modified substrate, such as RPE cells nearby drusen deposits, show enhanced accumulation of lipofuscin, which is related to a suppression of lysosomal enzymatic activity (Glenn *et al*., 2009). In addition to accumulation of AGE adducts, RAGE is highly expressed in the outer retina by RPE, especially on cells adjacent to drusen (Yamada, 2006). Several studies show that AGEs can induce apoptosis following prolonged exposure (Howes *et al*., 2004), as well as various abnormal responses in the RPE such as an imbalance of the VEGF/Ang-2 ratio secreted by these cells, a recently published work from our lab (Bento *et al*., 2010).

Chapter 2

OBJECTIVES

Chapter 2

OBJECTIVES

Recent data from our lab suggests that MGO might exert an effect upon components of the protein quality control (Bento *et al*., submitted). The main results described in this work show that MGO impairs the 20S proteasome activity and that it also affects the chaperone-mediated protein quality control system. It also shows that, in spite of the negative effects of MGO upon the later, this compound can also induce activation of Hsf-1 through increased oligomerization, DNA-binding and transactivation activities. The present work was based in these findings and aimed to:

… determine whether activation of Hsf-1 through increased homotrimerization upon MGO treatment impacts cellular viability;

… assess the importance of the interaction of several Hsps, previously shown to either negatively or positively regulate Hsf-1, in the activation of this transcription factor;

… assess the effect of MGO-induced Hsf-1 activation on the expression of several target genes, namely Hsp90, Hsc70, Hsp70, Hsp40 and Hsp27.

… determine whether the studied Hsps preserve their folding activity upon MGO treatment;

… determine the effects of MGO on ARPE-19 cells overexpressing wild type Hsf-1, a constitutively active Hsf-1 construct, or a dominant negative Hsf-1 construct.

Chapter 3

METHODS

Chapter 3

METHODS

3.1. Cell culture and treatments

The human retinal pigment epithelium cell line ARPE-19 (LGC Promochem, Teddington, UK) were cultivated in Dulbecco's modified Eagle's medium/Ham's F12 (DMEM:F12) (1:1), supplemented with 10% fetal bovine serum (FBS), 1x antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin and 250 ng/ml amphotericin B) and 1x GlutaMax. ARPE-19 cells were grown at 37 ºC in a incubator with a humidified atmosphere containing of 5% CO_2 in air. Medium, GlutaMax and antibiotics were purchased from Invitrogen (Carlsbad, CA, USA). Cells were treated with 1 mM methylglyoxal (MGO; Sigma-Aldrich, St. Louis, MO, USA) for up to 10 hours.

3.2. Western blot analysis

After treatment, cells seeded onto 6-well plates were washed twice in cold 1x phosphate-buffered saline (PBS) solution, denatured with 2x Laemmli buffer and collected to microfuge tubes with a cell scraper. They were then boiled at 100ºC for 5 minutes and sonicated (four times for 5 seconds) in order to break down nuclear DNA. Total cell lysates were resolved in SDS-7.5%, 10%, or 12% reducing polyacrylamide electrophoresis gels and electrophoretically transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5% nonfat milk in TBS-T (20 mM Tris, 150 mM NaCl,0.2% Tween 20, pH 7.6) for 1 hour. Next, they were incubated with gentle agitation for 1 hour at room temperature or, otherwise, overnight at 4 ºC with specific primary antibodies against Hsf-1, c-myc, Hsp90, Hsc70, Hsp70, Hsp40, Hsp27, CHIP and β-actin, listed in Table 1. The blots were then washed with 1x TBS-T for several times and incubated for 1 hour at room temperature with

appropriate horseradish peroxidise (HRP)-conjugated secondary antibodies. The secondary antibodies used in this work are also listed in Table 1. The membranes were washed with 1x TBS-T for several times and the immunoreactive bands were visualized with an ECL (enhanced chemioluminescence) system (GE Healthcare Bio-Sciences, Uppsala, Sweden). To quantify the proteins, films were scanned and the optical density of the bands was measured with Gel-Pro Analyzer 4.0 software (Media Cybernetics, Silver Spring, MD).

3.3. Preparation of nuclear and cytosolic extracts

After treatments, cells seeded in 60 x 15 mm plates were washed twice in cold 1x PBS, collected in 500µl of cold 1x PBS and transferred to previously cooled microfuge tubes. Cells were pelleted by centrifugation at 5,000 g for 10 minutes at 4 ºC. After removing the supernatant, cells were lysed with 4 packed cell volumes (PCVs) of Buffer A (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.4% NP-40, 1 mM DTT, 2 mM PMSF and 1x protease inhibitor cocktail) and incubated on ice for 30 minutes. This was followed by centrifugation at 4,000 g for 5 minutes at 4 ºC. The supernatants containing the cytosolic proteins were transferred to previously cooled microfuge tubes and stored on ice during the remaining isolation steps. Nuclear pellets were resuspended in 3.5 PCVs of Buffer B (20 mM HEPES, 0.4 M NaCl, 1 mM EDTA, 10% Glycerol, 1 mM DTT, 2 mM PMSF and 1x protease inhibitor cocktail) and briefly vortexed. After extraction on ice for 1 hour, the samples were briefly sonicated (two times for 1 second) and centrifuged at 16,000 g for 5 minutes at 4 ºC. Supernatants containing the nuclear proteins were transferred to previously cooled microfuge tubes. Protein concentrations of both nuclear and cytosolic extracts were determined using the BCA method (Pierce-Thermo Scientific, Waltham, MA, USA). Nuclear and cytosolic extracts were mixed

with Laemmli buffer, boiled at 100 ºC for 5 minutes and sonicated. Proteins were resolved in SDS-7.5% or 12% polyacrylamide gels, transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA) and immunobloted against Hsf-1, Hsp90, Hsc70, Hsp70, Hsp40, Hsp27, CHIP, β-actin, LAMP-2A and Lamin B, using specific primary and horseradish peroxidise (HRP)-conjugated secondary antibodies listed in Table 1.

3.4. Immunoprecipitation

After treatment, ARPE-19 cells cultured in 60 x 15 mm plates were washed twice with ice-cold 1x PBS, scraped off the dishes and collected in ice-cold 1x PBS. Pellets were resuspended in 100 µl of lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 10 nM IOD, 2mM PMSF, 20 mM Na_3MoO_4, 0.25% NP-40 and complete-mini protease inhibitor cocktail; Roche Applied Science, Indianapolis, IN, USA), incubated for 30 minutes on ice and briefly sonicated. Following centrifugation at 16,000 g for 10 minutes at 4 ºC, supernatants were transferred to new tubes. Part of the supernatants was denatured with 2x Laemmli buffer, boiled at 100 ºC for 5 minutes and sonicated (input). 2.5 µg of anti-c-myc antibody were added to the remaining supernatants. Subsequently, the samples were incubated overnight at 4 ºC, with gentle agitation. Thereafter, 50 µg of protein G-Sepharose (GE Healthcare Bio-Sciences, Uppsala, Sweden) were added and the incubation proceeded at 4 ºC for 2 hours. The immune complexes were collected after centrifugation and washed 3 times with lysis buffer. The immunoprecipitated proteins were eluted with 2x Laemmli buffer, boiled at 100ºC for 5 minutes and sonicated. The samples were analyzed by western blot, as previously described, using specific primary antibodies against c-myc and Hsp90. The primary and secondary antibodies used for this work are listed in Table 1.

Table 1 – List of primary and secondary antibodies used for Western Blot.

Antibody	Host	Clone/Cat.#	≈ M.W. (kDa)	Dilution	Company Supplier
Anti-Actin	Mouse	C4	43	1:100,000	Abcam, Cambridge, UK
Anti-Lamin B	Mouse	101-B7	68	1:500	Calbiochem, San Diego, CA, USA
Anti-Ubiquitin	Mouse	P4D1	*	1:1,000	Covance, Princeton, NJ, USA
Anti-Hsp90	Mouse	16F1	90	1:1,000	Stressgen, Farmingdale, NY, USA
Anti-Hsp70	Mouse	C92F3A-5	70	1:300	Stressgen, Farmingdale, NY, USA
Anti-Hsp40	Rabbit	SPA-400 #	40	1:1,000	Stressgen, Farmingdale, NY, USA
Anti-Hsp27	Rabbit	SPA-803 #	27	1:100,000	Stressgen, Farmingdale, NY, USA
Anti-Hsf-1	Rat	10H8	85	1:1,000	Stressgen, Farmingdale, NY, USA
Anti-Hsc70	Rat	1B5	73	1:5,000	Stressgen, Farmingdale, NY, USA
Anti-STUB1/CHIP	Goat	ab2482 #	35	1:500	Abcam, Cambridge, UK
Anti-Mouse-HRP	Goat	626520 #	-	1:10,000	Bio-Rad, Hercules, CA, USA
Anti-Rabbit-HRP	Goat	656120 #	-	1:5,000	Bio-Rad, Hercules, CA, USA
Anti-Rat-HRP	Goat	#81-9520	-	1:7,500	Zymed-Invitrogen, Carlsbad, CA, USA
Anti-Goat-HRP	Rabbit	611620 #	-	1:7,500	Bio-Rad, Hercules, CA, USA

* P4D1 antibody – recognizes free ubiquitin (8 kDa), polyubiquitin and ubiquitin-conjugated proteins.

Clone/Cat. # – Clone designation or catalog number of the antibodies.

3.5. Transient transfection

One day prior to the transfection, the cells were seeded onto 6-well plates in 2 ml of fully supplemented DMEM:F12 medium (for western blotting), or onto 24-well plates in 1 ml of DMEM:F12 medium. The cells were incubated overnight at 37 ºC so that they were 80-90% confluent at the time of the transfection. For each transfection sample, 2 µg/0.8 µg (6-well plates and 24-well plates, respectively) of the plasmid pcDNA3.1 hHsf-1 c-myc (provided by Dr. Lea Sistonen from de University of Turku,

Turku, Finland) were diluted in 100 µl of Opti-MEM I Reduced Serum Medium (Invitrogen, Carlsbad, CA, USA). Subsequently, 10 µl /2 µl (6-well plates and 24-well plates, respectively) of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) transfection reagent were diluted in 100 µl of Opti-MEM I Medium, according to manufacturer's specifications, and incubated for 5 minutes at room temperature. Diluted DNA and diluted Lipofectamine 2000 were then combined and incubated together for 20 minutes at room temperature. Meanwhile, the culture medium of each well was removed, cells were washed twice with 1x PBS and cultured in 2 ml /1 ml (6-well plates and 24-well plates, respectively) of Opti-MEM I Medium. After the incubation, the DNA/Lipofectamine complexes (total volume 200 µl) were added to each well. The wells were mixed gently and cells were incubated at 37 ºC in a CO2 incubator. Opti-MEM I Medium was replaced by fully supplemented DMEM:F12 medium after 6 hours of DNA/Lipofectamine incubation. 24-30 hours after transfection, the transfected cultures were treated with 1 mM MGO for up to 9 hours and used for Western blot analysis (cells seeded onto 6-well plates) or to determine cellular viability (cells seeded onto 24-well plates).

3.6. Cell viability assay (MTT assay)

MGO cytotoxicity in ARPE-19 cells seeded onto 24-well plates was assayed using the MTT cytotoxicity assay, which is based in the cleavage of a tetrazolium salt by mitochondrial dehydrogenase in viable cells. Following treatments, cells were washed twice with 1x PBS and incubated with 0.5 mg/ml MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Invitrogen, Carlsbad, CA, USA] dissolved in Krebs buffer (130 mM NaCl, 4 mM KCl, 1.5 mM MgCl2, 1 mM CaCl2, 6 mM glucose, 10 mM HEPES, pH 7.4) for 2 hours at 37°C, in a humidified incubator with 5% CO_2. Subsequently, supernatants were removed and the formazan precipitate was solubilized in

300 µl 0.04 M HCl (in isopropanol) and resuspended with a P200. 200 µl of each well were transferred to a 96-well plate. The optical densities of the well were then quantified at a wavelength of 570 nm, with reference wavelength at 620 nm, using a Biotek Synergy HT spectrophotometer (Biotek, Winooski, VT, USA). By referring to the negative control, the percentage of viable cells was assessed. All experiments were done in triplicate.

3.7. Statistics

Data are reported as the mean ± standard deviation (SD) of at least three independent experiments, except when it is stated otherwisey. Comparisons between multiple groups were performed by one-way analysis of variance test (ANOVA) with the Tukey's multiple comparison test [GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA, USA)]. In all cases, $p < 0.05$ was considered significant.

Chapter 4

RESULTS

Chapter 4

Results

4.1. MGO-induced stress decreases cellular viability

Increased production and accumulation of MGO has been associated with cell and tissue dysfunction in ageing and diabetes, both characterized by decreased proteasome activity (Vernace *et al*., 2007; Merforth *et al*., 2003). Recently, we and others showed that MGO significantly impairs the 20S proteasome activity (Queisser *et al*., 2010; Bento *et al*., submitted), leading to accumulation of ubiquitin conjugates and to depletion of free ubiquitin. Data from our lab further shows that MGO decreases the levels of the molecular chaperones Hsc70 and Hsp90 and leads to accumulation of CHIP-, Hsp40-, and ubiquitin-containing aggregates, presumably as a consequence of binding to MGO-induced modified proteins and to molecular chaperones (Bento *et al*., submitted).

The impairment of protein quality control and protein modifications induced by MGO are likely to lead to the accumulation of denatured and/or aggregated proteins that become obsolete or even toxic and that might affect cellular viability, as observed for sudden increases in temperature (Riezman, 2004). In order to determine if this is the case, we used the spontaneously immortalized human RPE cell line ARPE-19, which retains both morphological and biochemical features characteristic of RPE (Dunn *et al*., 1996; Dunn *et al*., 1998). The viability of ARPE-19 cells treated with 1mM MGO for 1, 5, 8 and 10 hours was assessed by the well-described colorimetric tetrazolium salt (MTT) assay, which monitors the metabolic activity of cultured cells (Figure 16). As expected, a decrease in cellular viability can be observed for the 8-hour treatment. However, the cellular viability is restored following 10 hours of incubation with MGO. A possible explanation could be that, at this point, cells might be activating or upregulating components of mechanisms that allow them to cope with MGO-induced stress.

However, one could not exclude the possibility that MGO has lost its effectiveness or that it has been completely consumed after 10 hours of treatment.

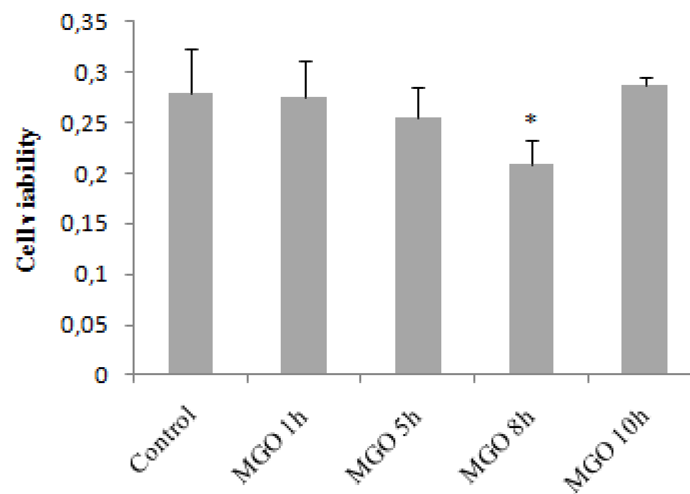

Figure 16.

MGO-induced stress decreases cell viability. ARPE-19 cells were treated with 1mM MGO for 1, 5, 8, and 10 hours and used to assess viability through the MTT colorimetric assay. The results represent the mean ± SD of at least three independent experiments. * $p < 0.05$, significantly different from the control; one-way ANOVA (Tukey *post hoc* test).

4.2. MGO-induced stress results in Hsf-1 oligomerization and redistribution to the nucleus

Stimuli that induce cell stress and protein damage normally activate sensing mechanisms and various forms of cell response to stress in order to reestablish homeostasis. This often includes a dramatic change in the pattern of gene expression and increased synthesis of Hsps, whose expression generally results in folding, repair or elimination of altered proteins, and that has been shown to enhance the survival of cells exposed to numerous types of stimuli that induce stress and apoptosis (Pirkkala *et al.*, 2001; Koga *et al.*, 2010).

In mammalian cells, the inducible expression of Hsps in response to environmental stresses is regulated by Hsf-1 (Pirkkala *et al*., 2001). Activation of mammalian *hsp* gene expression involves the reversible stress-inducible conversion of Hsf-1 from the inactive monomeric form to the DNA-binding competent homotrimer (Ahn and Thiele, 2003). To assess whether activation of Hsf-1 is part of the cell response to the noxious effects induced by MGO, we used non-transfected ARPE-19 cells and ARPE-19 cells transfected with a plasmid containing the wild-type form of Hsf-1 tagged with c-myc. The transfected cells were allowed to overexpress the protein for ≈16 hours and then both transfected and non-transfected ARPE-19 cells were treated with 1mM MGO for 1, 5 and 9 hours. Following treatment, the whole cell extracts were harvested with Laemmli buffer (1-2% β-mercaptoethanol) and separated in a 7.5% SDS-PAGE electrophoresis gel. C-myc (Figure 17A) and endogenous Hsf-1 (Figure 17B) were detected by Western blot.

Figure 17A and 17B show that Hsf-1 exists mostly in the monomeric/inactive form in unstressed conditions in ARPE-19 cells, and that MGO induces dimerization and trimerization of both endogenous and overexpressed Hsf-1. This suggests that Hsf-1 is activated upon MGO-induced stress, confirming results previously obtained in our lab (Bento *et al*., submitted). Hsf-1 multimerization correlates with disulfide bond formation (Ahn and Thiele, 2003). Therefore, it should be noted that the multimerization pattern of Hsf-1 is, although probably not fully, preserved during the SDS-PAGE procedure due to the low content of β-mercaptoethanol (1-2%) on the denaturation buffer.

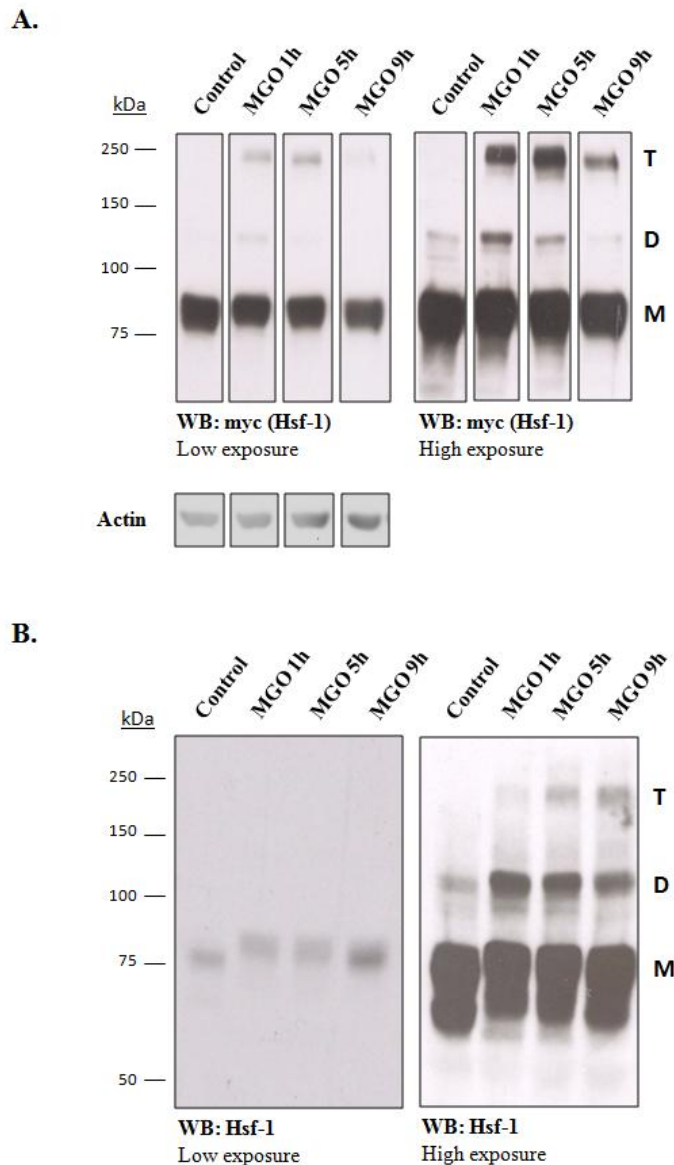

Figure 17.

MGO induces activation of Hsf-1 through increased oligomerization. (A) ARPE-19 cells were transfected with c-myc-Hsf-1 wild type and treated with 1 mM MGO for 1,5, or 9 hours. Control cells were left untreated. The cells were harvested in 2x Laemmli buffer (1% SDS) and the whole extracts were boiled at 100 ºC for 5 minutes and then sonicated. Samples were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were probed with specific antibodies against c-myc (Hsf-1) and β-actin. β-actin was used as a loading control. (B) ARPE-19 cells were treated with 1mM MGO for 1, 5, or 9 hours. Control cells were left untreated. Total lysates were analyzed by western blotting, as described above, using a specific antibody against Hsf-1. M, monomeric Hsf-1; D, dimeric Hsf-1; T, trimeric Hsf-1.

We further examined the subcellular localization of endogenous Hsf-1 before and after exposure to MGO. It is known that, in unstressed cells, Hsf-1 largely localizes in the cytoplasm as an inactive monomer. In response to a cellular stress, Hsf-1 undergoes the transition from a monomer to a homotrimer and localizes to the nucleus, where it acquires DNA-binding and transactivation activity (Wu, 1995). To assess the distribution of Hsf-1, the cellular extracts harvested in PBS were fractioned and the nuclear and

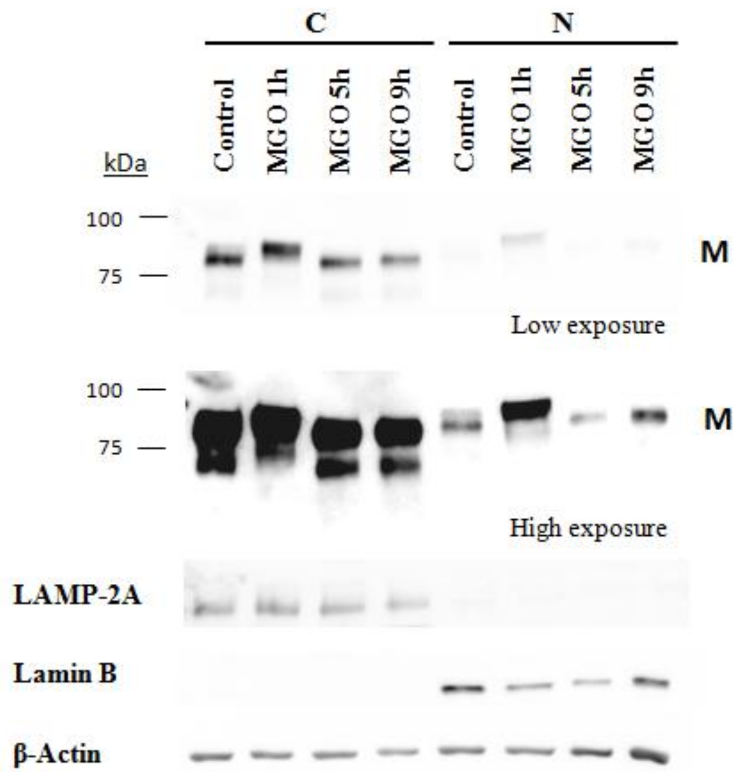

Figure 18.

MGO induces Hsf-1 translocation from the cytoplasm to the nucleus. ARPE-19 cells were treated with 1mM MGO for 1, 5, or 9 hours. Control cells were left untreated. The cells were harvested in PBS and the nuclear and cytosolic fractions were prepared. The samples were then analyzed by Western blot, as described in figure 17, and the membrane was immunoblotted against Hsf-1, Lamin B, LAMP-2A and β-actin. LAMP-2A was used as a cytosolic fraction loading control, Lamin B was used as nuclear fraction loading control. β-actin was used as a loading control. C, cytosolic fraction; N, nuclear fraction; M, monomeric Hsf-1.

cytoplasmic samples were prepared with Laemmli Buffer (1-2% β-mercaptoethanol), separated in a 7.5% SDS-PAGE electrophoresis gel and analyzed by Western blot (Figure 18).

As expected, under non-stressed conditions, Hsf-1 was predominantly localized in the cytoplasm of ARPE-19 cells, whereas upon MGO treatment Hsf-1 redistributed to the nucleus. Immunoblotting using the same extracts assured that LAMP-2A localized in the cytoplasm, whereas Lamin B localized in the nucleus. We used LAMP-2A as a cytosolic loading control due to problems with the antibody against β-Tubulin. We were not able to observe the formation of Hsf-1 trimers in neither fractions, presumably because the cell fractioning was performed in the presence of ditiothreitol (DTT), which is a powerful reductant capable of reverse multimerization. Other possible explanations could be the difficulty in transferring higher molecular weight protein to nitrocellulose membranes, as well and the use of small amounts of protein for the Western blot analyzes.

The activation of Hsf-1 by MGO-induced stress was further evaluated in our lab through a HSE-luciferase gene reporter assay, which confirmed the increased ability of Hsf-1 to bind HSEs upon MGO treatment (Bento *et al*. submitted).

4.3. MGO induces increased expression of several Hsps

In summary, we observed some of the steps of the multistep process that involves the activation of Hsf-1: conversion from the inactive monomer to the homotrimer, nuclear accumulation and DNA-binding activation. We next assessed the expression of known Hsf-1-dependent target genes. The mRNA levels of HspB1 (heat-shock 27 kDa protein 1, also known as Hsp27), HspB2 (heat-shock 27 kDa protein 2), Hsp70, Hsc70 and Hsp90, as well as the protein levels of Hsc70 and Hsp90, were quantified (Bento *et*

al., submitted). MGO appears to have a biphasic effect on the regulation of molecular chaperones. In an early stage, MGO decreases the mRNA levels of these molecular chaperones, as well as the proteins levels of Hsc70 and Hsp90 (up to 5/7 hours of incubation). However, this response is reverted for longer periods of incubation with MGO, suggesting the mounting of a cell response to stress, which is likely to be triggered by Hsf-1.

To further support the previously obtained data pointing to the mounting of a stress response to MGO, we treated ARPE-19 cells with 1mM MGO for 1, 3, 5, 7 and 9 hours. The cells were harvested in Laemmli buffer and the levels of Hsp90, Hsc70, Hsp70, Hsp40 and Hsp27 were analyzed by Western blot, following a SDS-12% PAGE. The relative levels of the Hsps were determined by quantification of the bands (Figure 19). The results confirmed the decrease in the protein levels of Hsp90 and Hsc70 in the first 7 hours of treatment with MGO, followed by an increase that puts these molecular chaperones levels closer to the control ones. We observed a similar behavioral pattern for Hsp70 protein levels. This is coincident with the increase in cell viability observed for the 9-hour treatment with MGO (Figure 16). The protein levels of Hsp27 and Hsp40 behave somehow differently. We can observe a slight non-significant decreasing tendency of Hsp40 protein levels upon MGO treatment for up to 7 hours and an increase to significantly higher levels than the control ones for the 9-hour treatment with MGO.

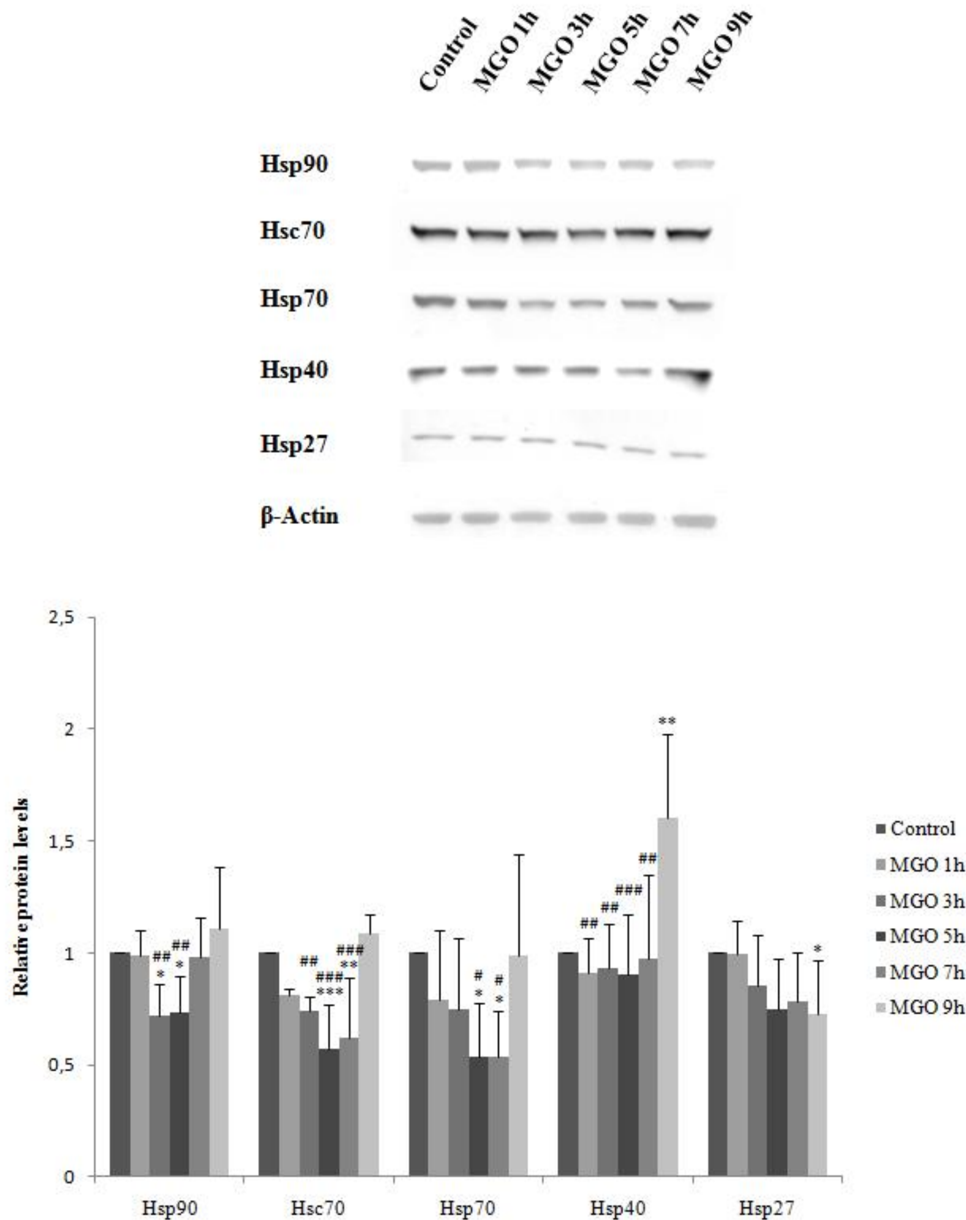

Figure 19.

MGO-induced stress induces increased expression of Hsp90, Hsc70, Hsp70 and Hsp40, but not Hsp27. ARPE-19 cells were treated with 1mM MGO for 1, 3, 5, 7, or 9 hours. Control cells were left untreated. The samples were analyzed by western blotting, as described in figure 17, and the membranes were probed against Hsp90, Hsc70, Hsp70, Hsp40, Hsp27 and β-actin. β-actin was used as a loading control. The quantified results represent the mean ± SD of at least four independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ significantly different from the control; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, significantly different from MGO 9 hours; one-way ANOVA (Tukey *post hoc* test).

For Hsp27, we can observe a decrease in the protein levels upon MGO treatment, but we were not able to detect increased levels of this protein, whose expression is also under control of Hsf-1.

This data further confirms that there is a reversion in the cell response for longer periods of MGO incubation, most likely to be triggered by Hsf-1, but also that this response is somewhat specific, considering the different MGO effects upon distinct Hsps, which were shown to be upregulated in response to other stress stimuli such as increased temperatures.

4.4. MGO-induced stress leads to rapid upregulation of several Hsps in Hsf-1-overexpressing cells

In order to evaluate the importance of Hsf-1 in the upregulation of Hsp90, Hsc70, Hsp70 and Hsp40 observed for the 9-hour treatment with MGO, ARPE-19 cells were transfected with a plasmid containing the wild type form of Hsf-1 tagged with c-myc and were allowed to express the construct for ≈ 16 hours. The cells were then treated with 1mM MGO for 1, 5 and 9 hours, except for those used to generate an appropriate control. After incubating with MGO, the cells were harvested in Laemmli buffer and the samples were analyzed by Western blot following a SDS-12% PAGE. The relative levels of the Hsps were determined by quantification of the bands (Figure 20A).

We observed an increase in Hsp90, Hsc70 and Hsp70 levels upon 1 hour of MGO treatment, followed by a return to levels similar to the control ones. It is interesting to observe that the Hsp40 and Hsp27 levels remain similar to the control ones, even upon overexpression of Hsf-1. Although important to have a significant number of experiments, this supports the results from Figure 19, which seem to point to a specific regulation of Hsps by Hsf-1.

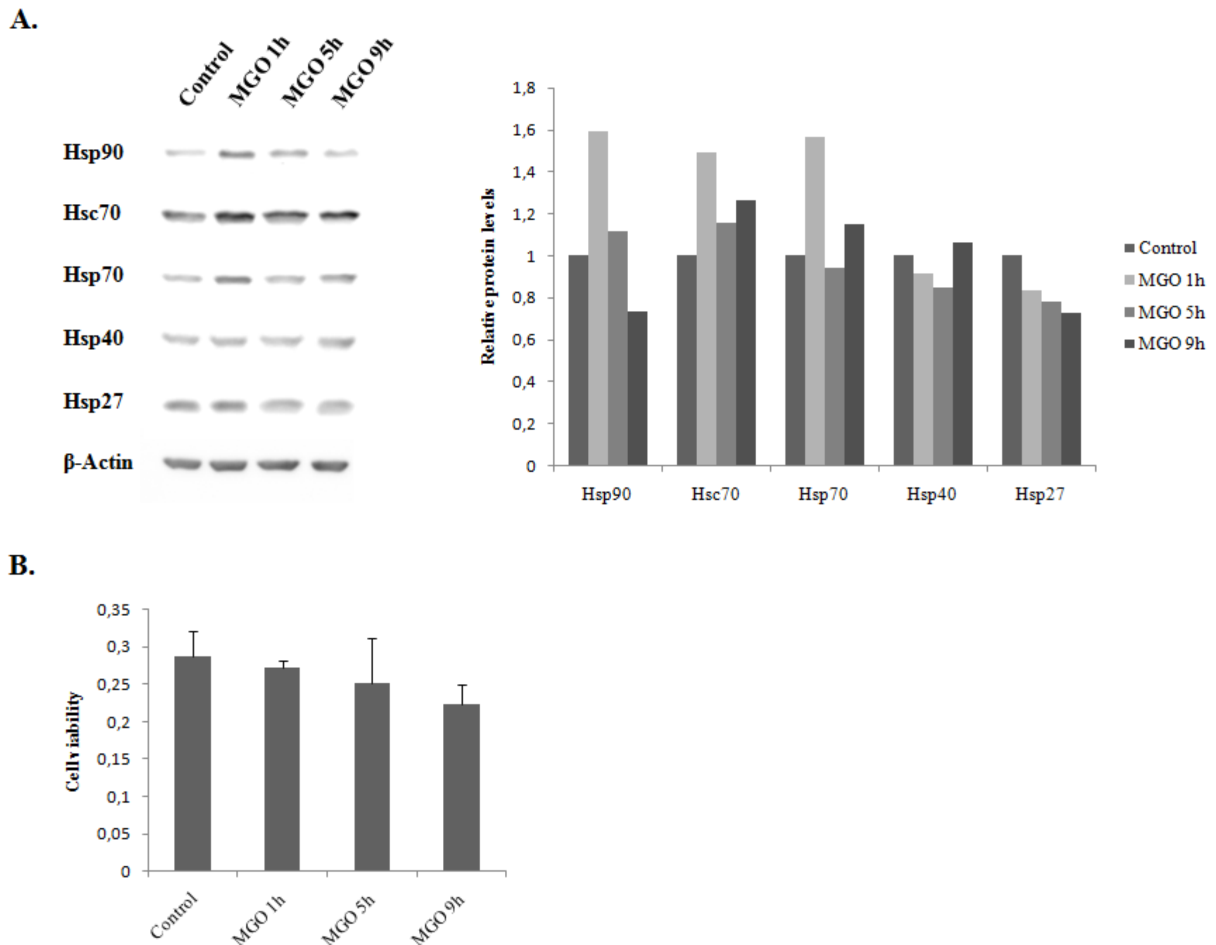

Figure 20.

Overexpression of Hsf-1 leads to a rapid upregulation of Hsp90, Hsc70 and Hsp70, but not Hsp40 and Hsp27, upon MGO-treatment. (A) ARPE-19 cells were transfected with c-myc-Hsf-1 wild type and treated with 1mM MGO for 1, 5, or 9 hours. Control transfected cells were left untreated. A western blot was performed as described in figure 13 and the membranes were probed against Hsp90, Hsc70, Hsp70, Hsp40, Hsp27 and β-actin. β-actin was used as a loading control. The graph data represents quantification of relative band intensities of one experiment. (B) ARPE-19 cells were transfected with c-myc-Hsf-1 wild type and treated with 1mM MGO for 1, 5, or 9 hours. The MTT colorimetric assay was used to assess cell viability. The results represent the mean ± SD of three independent experiments; one-way ANOVA (Tukey *post hoc* test).

The MGO treatment of ARPE-19 overexpressing Hsf-1 did not altered significantly their viability (Figure 20B). However, a slight tendency towards the decrease of cellular viability with increasing hours of treatment can be observed. This might be due to the fact that the period between the end of the transfection and the beginning of the treatments wasn't respected, rendering the cells more susceptible to MGO treatments.

4.5. MGO-induced stress decreases interaction between Hsp90 and Hsf-1

In addition to putative intramolecular interactions, the activation of Hsf-1 from the monomeric to the homotrimeric form is known to be modulated *via* direct interactions with Hsps and other factors (Ahn and Thiele, 2003). The Hsp90-containing multichaperone complexes, and possibly Hsp90 alone, appear to be the most relevant repressors of Hsf-1 activity by dynamically interacting with the factor in unstressed conditions (Voellmy and Boellman, 2007). Upon exposure to a proteotoxic stress, the increase in total concentration of molecular chaperone client proteins leads to a decrease in the concentration of chaperone-bound Hsf-1. Consequently, unbound Hsf-1 homotrimerizes, acquires transcriptional activity and transactivates *hsp* genes (Voellmy and Boellman, 2007).

As previously mentioned, MGO induces aggregation and structural modifications in proteins. Hence, the MGO-induced increase in molecular chaperone client proteins might underlie Hsf-1 activation by leading to a decrease in the concentration of chaperone-bound Hsf-1. In order to evaluate the importance of these events in Hsf-1 activation upon MGO-treatment, we transfected ARPE-19 cells with a plasmid containing wild-type Hsf-1 tagged with c-myc and treated the cells for 1, 3, 5 and 8 hours with 1mM MGO. We then immunoprecipitated the overexpressed Hsf-1 and the immunoprecipitates were probed against c-myc (Hsf-1) and Hsp90 (Figure 21).

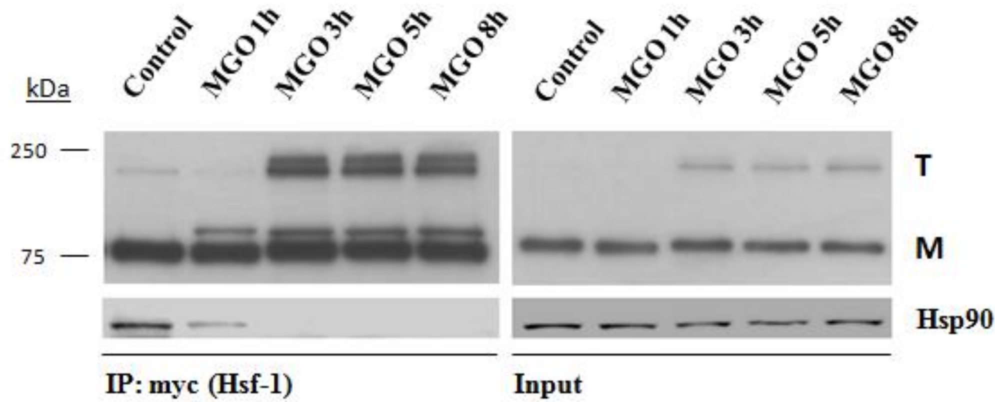

Figure 21.

MGO-induced stress decreases interaction between Hsf-1 and Hsp90. ARPE-19 cells were transfected with c-myc-Hsf-1 wild type and treated with 1mM MGO for 1, 3, 5 and 8 hours. Control cells were left untreated. C-myc (Hsf-1) was immunoprecipitated and the immunoprecipitates were probed against c-myc (Hsf-1) and Hsp90. M, monomeric Hsf-1; T, trimeric Hsf-1.

As previously observed (Figure 17), MGO treatment induced the trimerization of Hsf-1. Furthermore, in agreement with findings suggesting that Hsp90-containing complexes repress Hsf-1 oligomerization in the absence of a stress (Bharadwaj *et al.*, 1999), we observed a decrease in the association between Hsf-1 and Hsp90 upon MGO treatment, which seems to be independent from the decrease in Hsp90 protein levels observed for shorter periods of incubation with MGO (input; Figure 19).

4.6. MGO-induced stress results in increased ubiquitination, which decreases upon longer periods of incubation of MGO

Hsp40 and CHIP are crucial regulators of the protein quality control. Hsp40 is a co-chaperone that facilitates individual reactions between primary chaperones and unfolded clients and that also facilitates chaperone ATPase activity necessary for peptide binding and folding (Ballinger *et al.*, 1999; Li *et al.*, 2009; Calderwood *et al.*, 2009). Hsp40

functions in cooperation with CHIP, which was originally identified as a co-chaperone of Hsc70 but that also exhibits ubiquitin-ligase activity. CHIP binds *via* a TPR domain to Hsc70, Hsp70 and Hsp90, and diminishes their substrate affinity by negatively regulating Hsp40-ATPase and refolding activities of these Hsps (Ballinger *et al*., 1999; Pratt *et al*., 2006). Subsequently, CHIP polyubiquitinates damaged proteins that fail to achieve stable tertiary structures and remain chaperone substrates, targeting them to proteasomal degradation (Murata *et al*., 2003).

Data from our lab shows that MGO strongly increases, in a time- and dose-dependent manner, the accumulation of large aggregates containing ubiquitinated proteins, presumably those modified by MGO, chaperone and co-chaperone complexes, including Hsp40 and CHIP (Bento *et al*., submitted). This might suggest a cooperative action of chaperones and UPP in rescuing or degrading misfolded and/or post-translational modified substrates.

To address this question, we assessed total ubiquitin protein levels for 1, 5, and 9 hours of treatment with 1mM MGO (Figure 22) and observed that, following an increase in ubiquitinated proteins upon MGO treatment, there seems to be a decrease in the ubiquitinated substrates after incubation with MGO for 9 hours. The ubiquitin antibody used in this work recognizes both monoubiquitinated and polyubiquitinated substrates. Therefore, we cannot say for sure that the increase in the ubiquitination targets the substrates for degradation. However, it is possible to infer a correlation between the increased levels of ubiquitinated proteins and the decreased levels of several Hsps for the 5-hour treatment with MGO (Figure 19), and between the reversion of both observed at 9 hours of incubation. Thus, one could argue that the Hsps themselves might be initially ubiquitinated and targeted for degradation and that the decreased levels of ubiquitination observed for longer periods of incubation with MGO might account for

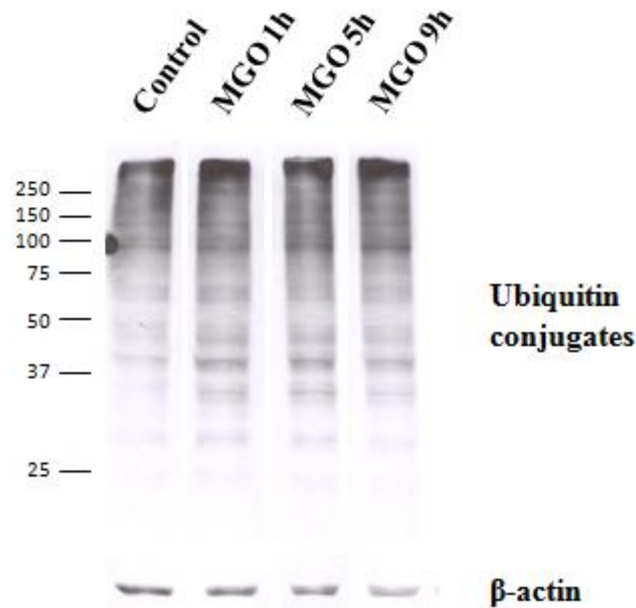

Figure 22.

Total protein ubiquitination decreases for longer periods of incubation with MGO. ARPE-19 cells were treated with 1mM MGO for 1, 5, or 9 hours. Control cells were left untreated. The samples were analyzed by western blotting, as described in figure 17, and the membranes were probed against ubiquitin (mono and polyubiquitin chains) and β-actin. β-actin was used as a loading control.

the function of upregulated Hsps. Furthermore, we can also establish a relationship between decreased total protein ubiquitination and increased cellular viability observed for the same period (Figure 16).

4.7. MGO-induced stress doesn't affect protein levels of CHIP but alters CHIP subcellular localization

Several studies show that CHIP levels are regulated upon proteasome inhibition (Dai *et al*., 2003). As mentioned above, we and others showed that MGO decreases both chymotrypsin-like and caspase-like proteasome activities (Queisser *et al*., 2010; Bento *et al*., submitted). To ascribe whether the protein levels of CHIP increase upon MGO-treatment, ARPE-19 cells were treated with 1mM MGO for 1, 3, 5, 7 and 9 hours. The

samples were separated in a 12% SDS-PAGE electrophoresis gel and immunoblotted against STUB-1/CHIP (Figure 23A). We didn't observe significant differences between the protein levels of CHIP in control cells and in cells exposed to MGO, although there seems to be a slight tendency to a decrease. On the other hand, overexpression of Hsf-1

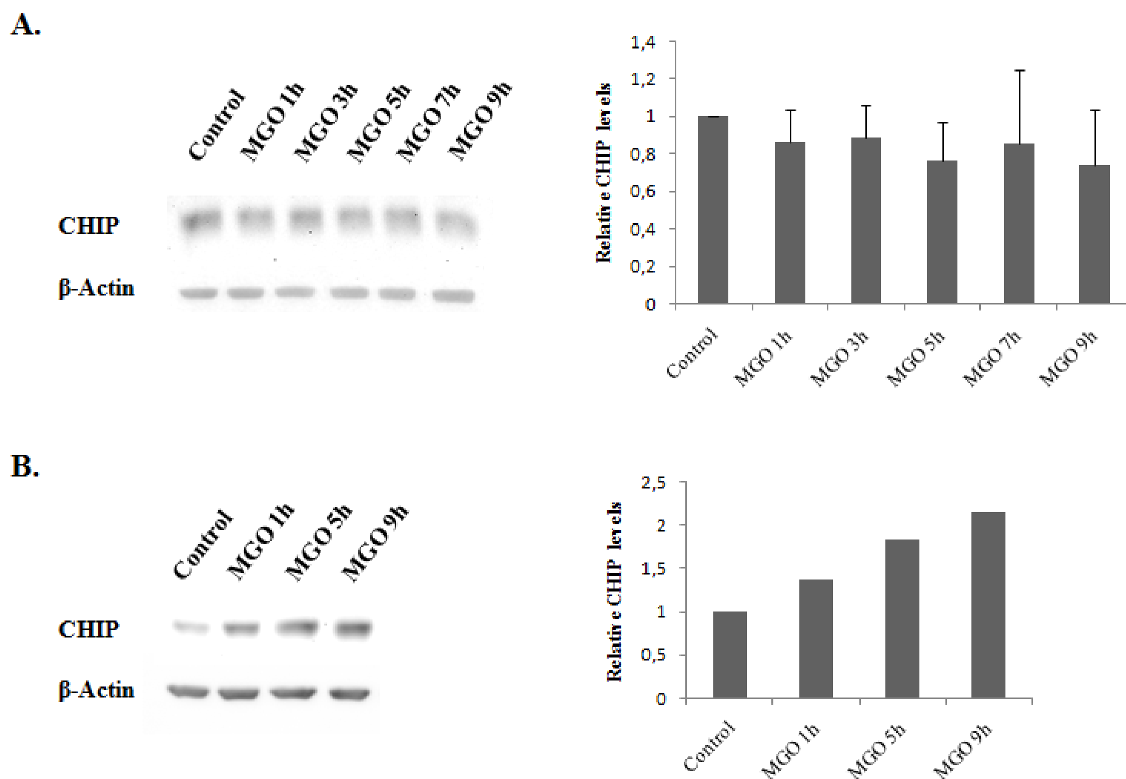

Figure 23.

MGO-induced stress increases CHIP protein levels in Hsf-1-overexpressing cells (A) ARPE-19 cells were treated with 1mM MGO for 1, 3, 5, 7, or 9 hours. Control cells were left untreated. The samples were analyzed by western blotting, as described in figure 17, and the membranes were probed against STUB-1/CHIP and β-actin. β-actin was used as a loading control. The quantified results represent the mean ± SD of at least four independent experiments; one-way ANOVA (Tukey *post hoc* test). (B) ARPE-19 cells were transfected with c-myc-Hsf-1 wild type and treated with 1 mM MGO for 1, 5, or 9 hours. Control cells were left untreated. The samples were analyzed by western blotting, as described in figure 13, using specific antibodies against STUB-1/CHIP and β-actin. β-actin was used as a loading control. The graph data represents quantification of relative band intensities of one experiment.

leads to time-dependent increase in the levels of CHIP upon MGO treatment (Figure 23B).

Furthermore, in order to balance protection and degradation of chaperone substrates, CHIP can activate Hsf-1 to regulate protein quality control. Several studies showed that CHIP interacts with Hsf-1 upon heat-shock treatment through Hsf-1s N-terminal region and that this interaction depends on a conformational change suffered by Hsf-1 upon heat shock (Dai *et al.*, 2003; Kim *et al.*, 2005). They further observed that, in unstressed conditions, CHIP largely localizes in the cytosol, whereas after heat shock there is an inversion in this protein cytoplasmic/nuclear ratio, in a similar way to Hsf-1 behaviour upon the same stress (Dai *et al.*, 2003). According to the authors, CHIP might regulate the chaperone response to stress through increased trimerization and transcriptional activation of Hsf-1.

In order to determine if CHIP's subcellular localization alters upon MGO-induced stress, we treated ARPE-19 cells with 1mM MGO for 1, 5 and 9 hours and performed a subcellular fractioning. The cytosolic and nuclear proteins were prepared with Laemmli buffer, separated in a 12% SDS-PAGE electrophoresis gel, and analyzed by Western blot (Figure 24). Immunoblotting using the same extracts assured that LAMP-2A localized in the cytoplasm, whereas Lamin B localized in the nucleus.

We observed an increase in CHIP levels in the cytoplasm upon MGO 1 and 5 hours, when comparing with the cytosolic levels of CHIP in untreated cells, which is accompanied by a decrease in the CHIP levels found in the nucleus for the same treatment times. For the 9-hour treatment, however, we can observe the translocation of CHIP from the cytosol to the nucleus, which coincides with the upregulation of several chaperones such as Hsp90, Hsp70, Hsc70 and Hsp40 (Figure 19), as well as with decreased protein ubiquitination (Figure 22) and increased cellular viability (Figure 16).

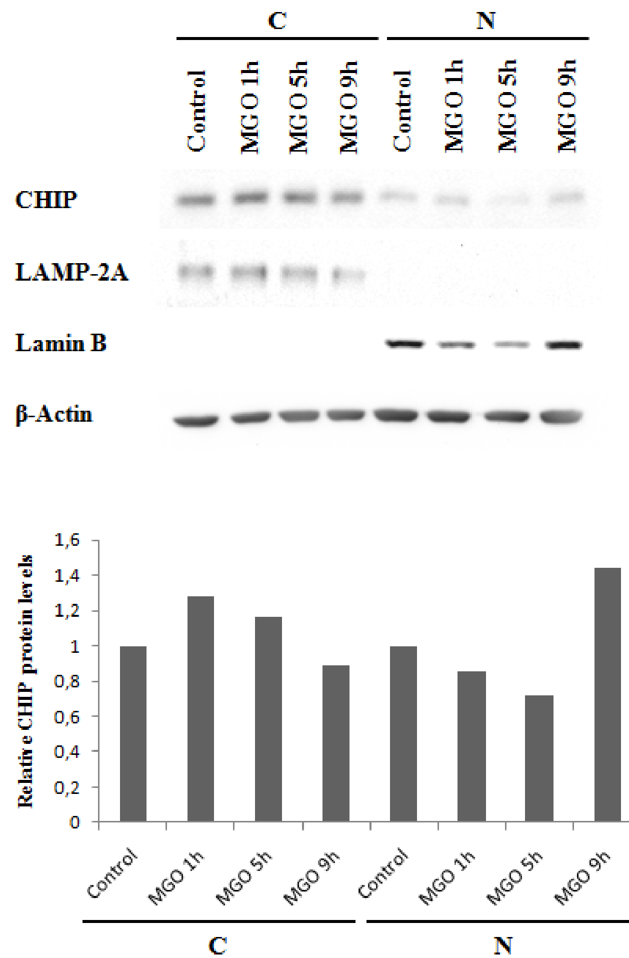

Figure 24.

MGO treatment induces translocation of CHIP to the nucleus for longer periods of incubation. ARPE-19 cells were treated with 1mM MGO for 1, 5, or 9 hours. Control cells were left untreated. The cells were harvested in PBS and the nuclear and cytosolic fractions were prepared. The samples were then denatured in Laemmli buffer, boiled at 100 ºC and sonicated. The proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The cytosolic and nuclear fractions were analyzed using antibodies against STUB-1/CHIP, LAMP-2A, Lamin B and β-actin. LAMP-2A was used as a cytosolic fraction loading control, Lamin B was used as nuclear fraction loading control. β-actin was used as a loading control. C, cytosolic fraction; N, nuclear fraction. The graph data represents quantification of relative band intensities of one experiment.

Chapter 5

DISCUSSION

Chapter 5

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5.1. Overview

MGO is an endogenous highly reactive dicarbonyl compound formed primarily from the triose phosphate intermediates of glucose metabolism and that, under normal physiological conditions, is controlled by the glyoxalase system present in the cytosol (Thornalley, 2003; Price and Knight, 2009). Increased production and/or reduced degradation resulting in accumulation of MGO are hallmarks of ageing and a number of pathophysiological conditions such as diabetes, neurodegenerative diseases and connective tissue disorders (Thornalley, 2008; Desai *et al*., 2010).

MGO readily binds and modifies free ε-NH_2 groups of Lys, Arg and Cys amino acid residues, leading to cross-linking and formation of a variety of AGEs and thereby altering the structure and function of target proteins (Turk, 2010). The accumulation of MGO-modified proteins can be toxic to cells and have many deleterious effects on several critical mechanisms. For instance, MGO-induced AGEs on mitochondrial proteins has been shown to affect the mitochondrial electron transport chain and the energetic status of the cell, increasing the formation of intracellular ROS and thus contributing to increased oxidation of proteins (de Arriba *et al*., 2007; Kalapos, 2008; Desai *et al*., 2010; Turk, 2010).

MGO-induced post-translational modifications by glycoxidation, a type of damage that arises from a combination of protein glycation and oxidation, result in the exposure of hydrophobic surfaces of the substrate proteins and, in many cases, in loss of secondary and tertiary structure (Kumar *et al*., 2004; Lamark and Johansen, 2010). Molecular chaperones recognize the exposed hydrophobic domains of modified proteins and assist their folding, either in an ATP-dependent or independent manner, preventing aggrega-

tion (Pratt *et al.*, 2010). Furthermore, molecular chaperones exhibit other functions distinct from their chaperone activity that include delivery of fatally damaged proteins to the UPP and/or autophagic pathways, and modulation of the apoptotic response (Dai *et al.*, 2003; McDonough and Patterson, 2003). Considering the extensive role of molecular chaperones in conferring a protected environment within cells upon different stress stimuli, our purpose with this study was to investigate the effects of MGO on the protein quality control mechanism played by molecular chaperones, as well as its role in cell coping with MGO-induced stress.

5.2. MGO induces Hsf-1 activation through increased olimerization, decreased interaction with Hsp90 and translocation to the nucleus

In this study we show that MGO leads to the activation of Hsf-1, the main transcription factor involved in the regulation of Hsps, not only through increased oligomerization (Figure 17), as previously observed in our lab (Bento *et al.*, submitted), but also through increased translocation to the nucleus (Figure 18) and decreased interaction with Hsp90 (Figure 21). Activation of Hsf-1 is an essential aspect of the heat-shock protein response since it regulates the abundance of cytosolic and nuclear chaperones (Voellmy and Boellmann, 2007). As previously described, Hsf-1 activation requires its ability to oligomerize, translocate to the nucleus and bind DNA in response to environmental stresses. Ahn and Thiele suggested that the redox state of Hsf-1 changes *in vivo* in response to heat-shock and H_2O_2 stress and that it might regulate Hsf-1 homotrimerization (Ahn and Thiele, 2003). Taking this into account, the slight decrease in the electrophoretic mobility of monomeric Hsf-1 upon MGO treatment that can be observed in Figures 17 and 18 might result from stress-dependent changes in the redox state of Hsf-1. In fact, MGO-induced stress is tightly linked to changes in cellular redox state since

MGO has an inhibitory effect on the electron respiratory chain (Rosca *et al.*, 2002; Rosca *et al.*, 2005). Ahn and Thiele further show that heat-shock- and H2O2-induced Hsf-1 homotrimerization requires two Cys residues, within or adjacent to the Hsf-1 DNA-binding domain, which are engaged in redox-sensitive disulfide bonds (Ahn and Thiele, 2003). This evidence may explain the higher preservation of Hsf-1 homodimers and homotrimers when samples are prepared using low amounts of β-mercaptoethanol, since MGO is known to induce the formation of disulfide cross-links and modify the SH-group of Cys residues. The slower migration through SDS-PAGE might also be due to hyperphosphorylation of the factor, which has been described to accompany its activation (Guettouche *et al.*, 2005).

Several studies suggest that the regulation of Hsf-1 activity is done post-translationally at multiple levels and involves not only intra- but also intermolecular interactions. These include interaction with regulatory factors DAXX and 14-3-3ε, other transcription factors such as STAT-1 and NF-IL6, splicing factors, chromatin-remodeling factors, components of the transcriptional machinery, Hspbp1, and chaperones and/or chaperone complexes (Satyal *et al.*, 1998; Pirkkala *et al.*, 2001; Voellmy and Boellmann, 2007). In fact, the latter, with some exceptions, feedback regulate the heat-shock protein response by binding Hsf-1 and keeping it in a non-homotrimeric and non-transcriptionally competent state (Voellmy and Boellmann, 2007). Mature multi-chaperone complexes containing Hsp90, p23 and an immunophilin were shown to assembly with both monomeric and trimeric forms of Hsf-1, repressing either its oligomerization or transcriptional competence (Duina *et al.*, 1998; Bharadwaj *et al.*, 1999; Guo *et al.*, 2001). Guo *et al.* further reported that, in different experimental situations that caused concentration of chaperone substrates to rise, the concentration of Hsp90-

containing complexes found in association with Hsf-1 declined and the transcriptional competence of Hsf-1 increased (Guo *et al*., 2001).

MGO-induced stress results in aggregation and structural modifications in proteins, which are common features of other stressful conditions such as elevated temperature. In other words, MGO induces an increase in molecular chaperone client proteins, including Hsp90 client proteins, possibly contributing to Hsf-1 derepression by leading to a decrease in the concentration of chaperone-bound Hsf-1, whether in the monomeric or in the homotrimeric form. In accordance with the abovementioned data, we show that MGO-treatment disrupts Hsf-1-Hsp90 interaction and that it coincides with increased Hsf-1 oligomerization (Figure 21), suggesting that this mechanism of regulation is common to a number of stresses in which we might include MGO-induced stress.

Other studies further emphasize the role of molecular chaperones other than Hsp90 in Hsf-1 regulation. Among these chaperones we find Hsp70 and Hsp40, which were shown to interact with monomeric Hsf-1 in unstressed cells, as well as to attenuate heat-shock response (Rabindran *et al*., 1994; Shi *et al*., 1998; Pirkkala *et al*., 2001), and Hsc70, which is apparently required for Hsf-1 trimerization and transcriptional competence (Ahn *et al*., 2005). Others report that CHIP regulates activation of stress-chaperone response either indirectly, by interfering with the assembly of molecular chaperone-containing complexes on Hsf-1, or by physically interacting with this transcription factor, thereby inducing trimerization and transcriptional activation of Hsf-1 (Dai *et al*., 2003; Kim *et al*., 2005). In fact, Dai *et al*. showed that CHIP largely localizes in the cytoplasm in unstressed conditions, which is confirmed by our results in ARPE-19 cells (Figure 24), whereas upon thermal challenge it translocates to the nucleus (Dai *et al*., 2003). Our data show that, upon MGO-induced stress, there is a tendency to a decrease in the nuclear-localized CHIP, at least until the 5-hour treatment (Figure 24). This de-

crease might account for the recruitment of CHIP to the cytosol in order to regulate the destiny of MGO-modified proteins since CHIP regulates of Hsc/Hsp70 and Hsp90 function by determining whether chaperone client proteins enter the productive folding-refolding pathway or the proteasome-dependent degradation pathway (McDonough and Patterson, 2003; Kundrat and Regan, 2010a; Pratt *et al*., 2010). Following 9 hours of treatment, it is possible to observe a strong accumulation of CHIP in the nucleus. This inversion in CHIP cytosolic/nuclear ratio seems to coincide with the increase in the mRNA (Bento *et al*., submitted) and protein levels of Hsp90, Hsc70, Hsp70 and Hsp40 observed in the same period (Figure 19). This might suggest a direct role of CHIP in Hsf-1 activation upon MGO-induced stress. The observed increase also correlates with increased cellular viability (Figure 16), which might be related with suppression of the p53 apoptotic pathway, which is induced by MGO (Hsieh and Chan, 2009), by CHIP-DAXX interaction in the nucleus, as reported for heat-shocked mouse embryonic fibroblasts (McDonough *et al*., 2009).

5.3 MGO-induced stress results in the decrease of several Hsps but Hsf-1 activation precedes induction of their expression

Upon activation, Hsf-1 binds to *hsps* promoter elements called HSEs and induces the expression of several Hsps that help limite the damage caused by stress and facilitate cell recovery in concert with other cellular responses (Voellmy and Boellmann, 2007). In this study we show that MGO leads to a decrease in the levels of some molecular chaperones such as Hsp90, Hsc70, Hsp70, followed by an upregulation upon longer periods of exposition to MGO (Figure 19). The significant decrease in Hsp27 levels observed for the 9-hour treatment with MGO is in agreement with previous studies showing that downregulation of Hsp27 is induced by high glucose levels (Gawlowski *et*

al., 2009). Moreover, Hsp27 was shown to be a major MGO-modified protein in cells, which may account for the observed decrease in this molecular chaperone levels (Sakamoto *et al*., 2002). Hsc70 was also found to be a potential intracellular target for AGE modification in human fibroblasts (Unterluggauer *et al*., 2009). Thus, MGO-induced glycoxidative modifications of other chaperones might underlie the initial decrease in Hsp90, Hsp70 and Hsc70 levels. In fact, as observed for α-crystallin (Kumar *et al*., 2004), the enhanced degradation of MGO-modified proteins may increase their susceptibility to proteolytic cleavage by proteases such as trypsin and chymotrypsin, as a consequence of the exposure of proteolytic cleavage sites. Nonetheless, the decrease observed for Hsp27 protein levels, as well as the slight decrease initially observed for Hsp40 levels, are not comparable with other chaperones', which may result from their trapping in large aggregates containing proteins in non-native conformation.

In addition, Hsp70 and Hsp90 turnover was shown to rely on CHIP-dependent ubiquitination, which adds polyubiquitin chains to these chaperones through K11, K48 and K63 linkages, even when they are bound to client proteins (Kundrat and Regan, 2010a; Kundrat and Regan, 2010b). CHIP is known to be involved in substrate delivery to the proteasome (McDonough and Patterson, 2003). However, we showed that MGO impairs proteasome activity (Bento *et al*., submitted) and, in parallel, Queisser *et al*. showed that hyperglycaemia-induced formation of MGO covalently modifies 20S proteasome, decreasing its activity in cultured endothelial cells and extracts of diabetic kidneys (Queisser *et al*., 2010). In agreement with this data, the function (Friguet *et al*., 2000) and distribution (Li *et al*., 2008) of the proteasome have been reported to be impaired and changed, respectively, upon ageing. This may account for the inefficient removal of damaged proteins, rendering the cells more susceptible to their cytotoxic effect. Nonetheless, ubiquitination was recently shown to target substrates for degradation

via both USP and macroautophagy. In fact, several key proteins involved in selection of misfolded proteins for autophagic degradation are ubiquitin-binding proteins and K63-linked have been associated with autophagic degradation (Lamark and Johansen, 2010; Korolchuk *et al.*, 2010). Moreover, proteasome inhibiton was shown to induce macroautophagy, probably as a compensatory mechanism (Lamark and Johansen, 2010). Taking this into account, we can reason that the observed downregulation of Hsps for the 5- and 7-hour treatment might be related with increased autophagy activity.

It is also interesting to emphasize the fact that the marked decrease in the levels of molecular chaperones induced by MGO-treatment is only observed for the ATP-dependent Hsp90, Hsc70 and Hsp70 chaperones, but not for the ATP-independent chaperones Hsp40 and Hsp27. Several studies noted that the presence of ATP seems to protect αβ-crystallin against degradation by serine proteases (Kumar *et al.*, 2004). Moreover, MGO-modified α-crystallin was shown to have reduced ATP binding sites and increased susceptibility to proteolytic degradation (Kumar *et al.*, 2004). Thus, we may also suggest that downregulation of Hsp90, Hsc70 and Hsp70, whose activity is ATP-dependent, might be related to a decrease in the protection against proteolytic degradation conferred by ATP, whether due to a reduction in the ATP-binding sites resulting from conformational changes, or due to the decreased energy production resulting from MGO-induced impairment of mitochondrial function.

The elicited expression of Hsp90, Hsc70, Hsp70 and Hsp40 coincides with a decrease in the total protein ubiquitination (Figure 22). This expression also coincides with an increase in cellular viability (Figure 16). In fact, the studied chaperones are known not only to provide important defence against the dire consequences of protein misfolding and aberrant protein interactions, but also to promote cell survival by interfering with several signaling pathways that include the apoptotic pathways (Koga *et al.*,

2010). Stressed and damaged cells trigger apoptotic cell death through several pathways. However, several Hsps such as Hsp90, Hsp70 and Hsp27, have been shown to modulate apoptotic cascades mostly by inhibiting or blocking several steps of these pathways, including translocation of Bax into mitochondria, release of cytochrome c from mitochondria, and activation of initiation caspases, among other things (Arya *et al*., 2007). Kim *et al*. showed that the intracellular damages caused by MGO lead to apoptosis in human lens epithelial cells (Kim *et al*., 2010). Other studies show that the modification of Hsp27 by MGO is essential to Hsp27 repressing activity for cytochrome-c mediated caspase activation (Sakamoto *et al*., 2002; Oya-Ito *et al*., 2006). Taking this into account, we can suggest that the induced expression of several Hsps might promote ARPE-19 cells survival not only by assisting misfolded proteins to regain their native states or to be degraded, which might explain the decreased levels of total ubiquitinated proteins, but also by interacting with members of the apoptotic cascades to inhibit cell death.

5.4. Overexpression of Hsf-1 induces rapid expression of several (but not all) Hsps and CHIP

In this study, we further observed that the initial downregulation of several Hsps can be countered and, most importantly, their expression can be rapidly induced upon MGO-treatment by overexpressing Hsf-1 (Figure 20). This can be explained by the existence of higher levels of free Hsf-1 not associated with chaperone/chaperone complexes, which might rapidly homotrimerize, translocate to the nucleus and induce *hsp* genes. The decrease in Hsp90, Hsc70 and Hsp70 levels at the 5-hour treatment with MGO might account for a decrease in *hsps* genes transcription due to the Hsf-1 negative feedback regulation by chaperones or chaperone-complexes or presumably by changes

in Hsf-1 DNA-binding or transactivation activities as a result of the induction of different post-translational modifications. Another interesting observation is that neither Hsp40 nor Hsp27, whose levels increase upon other stressful conditions such as heat shock, are upregulated in these MGO-treated ARPE-19 cells overexpressing Hsf-1. This data suggest that the regulation of Hsps by Hsf-1 might be somehow stress-specific. For instance, Hsf-1 was shown to be differentially phosphorylated and to induce distinct cell responses in response to distinct activating stimuli (Liu and Thiele, 1996). Hsf-1 activation upon different stress stimuli might also be cell-specific and/or presumably involve other factors also capable of regulating Hsps expression. In fact, several reports have suggested that both Hsf-1 and Hsf-1 are affected during downregulation of UPP (Pirkkala *et al.*, 2000).

On the other hand, the levels of CHIP are not upregulated upon MGO-induced stress in normal ARPE-19 cells (Figure 23A), as would be expected since the proteasome is inhibited by MGO. On the contrary, in Hsf-1-overexpressing cells they are rapidly upregulated (Figure 23B). A possible explanation could be that the overexpressed and abundant Hsf-1 might be interacting with factors involved in *chip* gene transcription. Consistent with the increase in CHIP levels in Hsf-1 overexpressing cells, the levels of Hsp90, Hsc70 and Hsp70 decrease for the 5-hour and 9-hour treatments with MGO. In fact, it has been observed that overexpression of CHIP leads to a decrease in Hsp70 levels, whereas its absence leads to an increase (Kundrat and Regan, 2010a). This might explain the observed relation. However, we cannot exclude the possibility that the transfection itself might be interfering with CHIP and Hsps levels upon MGO-treatment. Thus, in order to confirm this result we need to generate a proper control consisting of ARPE-19 cells transfected with an empty vector.

5.5. Concluding remarks

As a major conclusion, the data obtained in this work suggests that accumulation of MGO, which occurs in a variety of situations such as diabetes and ageing, initially impairs the protein quality control dependent on molecular chaperones, leading to decreased cellular viability and accumulation of ubiquitinated proteins. However, for longer periods of exposure to MGO, the changes induced by this highly reactive α-oxaldehyde seem to elicit a response mediated by Hsf-1. The resulting upregulation of several Hsps, which might be exerting their effect not only by mediating protein folding/degradation, but also by modulating apoptotic pathways, might be crucial to cellular coping with MGO-induced stress.

Even though, many questions arose while doing this work: are the studied molecular chaperones modified by MGO and does this account for their degradation? Do they keep their protein refolding ability or exhibit anti-apoptotic activity in the presence of MGO? Are decreased ubiquitination and increased cellular viability related to the delayed upregulation of Hsps upon MGO-induced stress? Is Hsf-1 the only transcription factor involved in the *hsp* genes induction in these experimental settings? How is it regulated and is this regulation specific of MGO-induced stress? Future work will have to be done in order to address these and other questions, as well as to confirm and further support our hypotheses. Nonetheless, our data emphasizes the important role of the heat-shock response in the re-establishment of cellular homeostasis upon exposure to different stress stimuli. Furthermore, since MGO is known to be intimately linked to ageing and many diseases such as diabetes, this work might shed a new light on another possible mechanism through which MGO exerts their effects.

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