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ACTIN CYTOSKELETON CARBONYLATION IN HUMAN T-LYMPHOCYTES:
A ROLE IN CELLULAR SENESCENCE

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"I have not failed, I have just found 10000 ways that won´t work"

Thomas Edison

"An expert is a person who has made all the mistakes that can be made in a narrow field"

Niels Bohr

PREFÁCIO

É com agrado e entusiasmo que escrevo este prefácio….Traduzir um caminho de 6 anos, 2 de Mestrado e 4 de Doutoramento em apenas algumas linhas é bem mais complicado do que Biologia Molecular, isso posso garantir. Este sentimento é apenas sentido por quem partilha, de igual forma o meu gosto pela ciência e pelo poder de descobrir coisas novas todos os dias….bem…não será bem todos os dias! Claro que este gosto esteve sempre presente, mas foi sendo cultivado e refinado ao longo do tempo, teve como responsáveis dois extraordinários cientistas de quem eu tive a sorte e privilégio de me terem aceite como aluno.

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Em obediência ao disposto no Decreto-Lei 388/70, Artigo 8º, parágrafo 2, declaro que efectuei o planeamento e execução do trabalho experimental, observação do material e análise dos resultados e redigi as publicações que fazem parte integrante desta dissertação.

Publicações:

I - Castro, JP, Ott, C, Jung, T, Grune, T; Almeida, H. 2012. **Carbonylation of the cytoskeletal protein actin leads to aggregate formation**. *Free Radical Biology & Medicine.* Aug 15;53(4):916-25. Epub 2012 Jun 15.

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ABSTRACT

Oxidative stress has been used to explain phenomena such as cancer progression, neurodegeneration and even ageing itself. Its implication in dysfunction and disease has been widely described. At a molecular level, oxidative stress means that the levels of oxidants are higher than the antioxidants, disrupting cellular signaling and provoking biomolecules damage, which are aged cells common features. Proteins are sensitive to oxidation, a cause of their dysfunction and accumulation as protein aggregates. Moreover, this insoluble material can alter cellular functionality. From previous studies I could verify in oxidative stress challenged human lymphocytes that cytoplasmatic actin becomes easily and heavily carbonylated, a non-enzymatic, irreversible type of protein oxidation. Therefore as a first aim I set to verify whether actin carbonylation leads to cellular functional impairment by assessing proliferation rates and proteasome activity, the main site for oxidized proteins degradation.

I could verify that carbonylated actin forms aggregates with direct effect on impairing proliferation and diminishing proteasome enzymatic action. Due to the underlying proteostasis disturbance, I decided, for the second aim, to address Hsp90. As a molecular chaperone, it is involved in keeping a functional proteome; having actin as client protein, Hsp90 assists oxidized proteins degradation and protects the 20S proteasome from oxidative inactivation. Aiming the role of Hsp90 in actin aggregates formation, I found a likely mechanism for protein aggregates formation. This takes part mainly due to Hsp90 cleavage at a very highly conserved N-terminal amino acid motif, generating a non-functional N-terminal lacking fragment. I confirmed that cleavage antedates oxidized actin accumulation, and also that Hsp90 enhances oxidized actin degradation. The data favor the point that Hsp90 cleavage is an important precursor for the chain of events that goes from oxidized protein accumulation to proteasome inactivation. This consequence appears to be very important for autophagy upregulation that follows upon proteasome inhibition and protein aggregates formation. Further studies should follow to understand what happens later time points that could allow to have a larger view of the problem. Knowing this, procedures to prevent Hsp90 cleavage would perhaps allow cells escape oxidative stress harmful effects and retard their entry in senescence like state.

RESUMO

O stress oxidativo tem sido utilizado para explicar fenómenos como o cancro, neurodegeneração, e até o envelhecimento. As suas implicações na disfunção e doença têm sido largamente descritas. Ao nível molecular, stress oxidativo significa que os níveis de oxidantes são superiores aos níveis de antioxidantes, desregulando a sinalização celular e provocando danos nas biomoléculas, características estas que são típicas de células envelhecidas. As proteínas são sensíveis à oxidação, podendo levar à sua disfunção e acumulação como agregados proteicos. Estes agregados tendem a ser insolúveis, podendo alterar a funcionalidade celular. Em estudos prévios, consegui verificar que em linfócitos humanos sujeitos a acção oxidativa, a proteína citoplasmática actina sofre facil e abundantemente carbonilação, um processo irreversível e não enzimático que pode levar à perda de função e em último caso, à formação de agregados proteicos. Sendo assim, o meu primeiro objectivo proposto, foi verificar se a carbonilação da actina leva a uma diminuição de função celular, através da avaliação da taxa proliferativa celular e actividade do proteossoma. Fui capaz de verificar um efeito directo de agregados de actina oxidada na diminuição da proliferação e na diminuição da actividade enzimática do proteossoma. Devido ao facto de nestas condições oxidativas haver um distúrbio na proteostasia, tais como acumulação de agregados de proteínas oxidadas (especialmente de actina) e diminuição da actividade do proteossoma, decidi, como segundo objectivo, estudar a chaperona molecular Hsp90. A Hsp90 está envolvida na manutenção da proteostasia, tem a actina como proteína cliente, tem um papel na degradação de proteínas oxidadas, e protege o proteossoma de inativação oxidativa; como tal, propus-me a perceber o papel da Hsp90 na formação de agregados de actina. Descobri, um possível mecanismo pelo qual os agregados de proteínas oxidadas se poderão formar. Em parte, isto pode dever-se ao facto de haver menos Hsp90 funcional em condições de stress oxidativo, devido a clivagem proteolítica, acontece no N-terminal da proteína, gerando um fragmento parcialmente funcional. A clivagem foi confirmada, e que de facto, antecede a acumulação insolúvel de actina. Foi também confirmado o papel da Hsp90 no aumento da degradação de actina oxidada. Estou convencido que a clivagem da Hsp90 será um importante percursor numa cadeia de eventos que vai desde a acumulação de proteínas oxidadas, passando pela inactivação do proteossoma, e culmina na activação de autofagia, um processo verificado aquando da inibição do proteossoma. Contudo, mais estudos serão necessários para entender o que se passa mais à frente no modelo, possibilitando-nos uma visão mais alargada do problema. Percebendo isto, procedimentos experimentais poderão ser desenhados de forma a prevenir a clivagem da Hsp90, o que levaria a um melhoramento celular e ao seu retardar na entrada de estadios de senescência ou semelhantes a senescência.

INTRODUCTION

INTRODUCTION – PART I

Along Earth´s geological time, evolution tailored living organisms according to environmental conditions.

Around 2 billion years ago oxygen levels had risen up to 18% of our time era. However, only 1,5 billion years after this critical event, around 500 million years ago, earth witnessed a vast expansion of multicellular life known as the "Cambrian explosion". With it, a major achievement was the expansion of the use of oxygen in aerobic metabolism with the inherent increase in the efficiency of cellular energy production. As a consequence, more Adenosine TriPhosphate (ATP) was synthesized, to become the most important source of energy in living organisms.

In mammals, the main site for ATP synthesis is the mitochondria, especially its inner membrane, which houses enzyme complexes that act as electron carriers. In the final processes of nutrient oxidation breakdown that take place in the mitochondrial matrix, a large number of electrons are conveyed to reduce NAD (or FAD). These electrons are then transported to and released from successive electron carriers, that harness the energy they contain and combine with molecular oxygen to produce water. At some points of the chain, H+ is moved out of the matrix to the intermembrane space, which creates a transmembrane electrochemical gradient; in turn, it promotes proton return to the matrix in a way that activates ATP synthase rotation and generates ATP.

1.1 Reactive oxygen species

The essential role of oxygen is thus to be reduced into a water molecule. Because in its ground state, the oxygen outer shell contains two unpaired electrons and both have the same spin, oxygen reacts successively with one electron at a time, which precludes the production of very reactive molecules. It is estimated however that 1-2% of oxygen does not get fully reduced into water; this results in the formation of intermediary byproducts, such as superoxide anion (O2-.), with one unpaired electron, which makes of it a powerful oxidant, categorized as a reactive oxygen species (ROS).

This occurs because during aerobic metabolism, electron transfer at the respiratory chain is not fully efficient due to leakage at complex I (NADH dehydrogenase) and Complex III (cytochrome bc1 complex); the efficiency depends on specific organs or cells, and also on the lifespan of the considered species [1].

ROS can be categorized as radicals and non-radicals; while radicals have at least one unpaired electron, non-radicals are oxidants whose electronic grounds are completed

[2]. ROS are unstable, explaining why their activities have short half-lives, that range from nanoseconds, as the case of hydroxyl, to minutes, as hydrogen peroxide [3].

Cells may be affected by endogenous or exogenous ROS (Table 1). The major source of endogenous ROS is the mitochondria [4]; however, other sources should be referred as they can also produce relevant amounts, or lead to their production, as occurs in nitric oxidase reaction, Fenton reaction, arginine metabolism, microsomal cytochrome P450 detoxification, peroxisomal B-oxidation, respiratory burst (in phagocytic cells) and prostaglandin synthesis, among others [5, 6] (Figure 1). Exogenous sources, are exemplified by UV radiation X-Rays, gamma-rays, nanoparticles, ultrasounds, pesticides/herbicides, metals (Cu, Fe), ozone and xenobiotics [5, 6] (Figure 1).

In addition, cells can use ROS to participate as second messengers of transductive pathways that regulate processes as cytokine secretion, growth, differentiation, gene expression, and as active players in immune responses against invading pathogens [7]. Moreover, when produced in small amounts, ROS are able to elicit a wide spectrum of responses/signaling-pathways to include the JAK/STAT, PI3K/AKT, p38, JNK/SAPK and Ras/MEK/ERK1/2 pathways [8].

1.2 Anti-oxidants defenses

ROS intrinsic high reactivity is of major concern as it may result in cell damage.

To cope with that risk, cells evolved anti-oxidant mechanisms that counter-balance ROS action and accumulation. One of them is dismutation, performed by superoxide dismutases (SOD) including Mn-SOD, present in mitochondria, and Cu-Zn-SOD that can be either cytoplasmatic or extracellular.

SODs promote additional reduction of oxygen to yield hydrogen peroxide, H_2O_2, a non-radical. Its reactivity comes from its open conformation, and leads to biomolecule oxidation, as will be discussed ahead. While superoxide instability and inability to cross membranes limits its effects exerted on cells, hydrogen peroxide diffuses easily across membranes [9], a property that is commonly used in cell culture. Additional enzymatic resources as catalase or peroxidase are employed to detoxify H_2O_2 (Table 1). Apart from these and other enzymatic processes, cells developed non-enzymatic anti-oxidants, some of which are dietary supplements as ascorbic acid (Vitamin C), tocopherol (Vitamin E), Zinc, Selenium and carotenoids [5] (Table 1).

ROS - Oxidants	Anti-oxidants
Superoxide anion	Superoxide dismutase (Enz)
Hydrogen peroxide	Catalase (Enz)
Hydroxyl	Glutatione Peroxidase (Enz)
Hydroperoxyl	Tocopherol (Non-Enz)
Peroxyl	Glutathione (Non-Enz)
Hypochlorous acid	Zinc, Selenium, Carotenoids (Non-Enz)

Table 1. The most common Reactive Oxygen Species and Antioxidants. Cells are exposed to a multitude of ROS with different origins, reactivity and stability. To counterbalance oxidants, cells have evolved antioxidant defenses. They can be divided in enzymatic (Enz) or non-enzymatic (Non-Enz). Some of the most common examples are shown.

1.3 Oxidative Stress

In spite of the detoxifying mechanisms, H_2O_2 can still be converted into hydroxyl anion by transition metal catalysed reactions, as is the case when Fe^{2+} or Cu^{+} ions are oxidized (Fenton reaction). It appears that these reactions are a major source of damage [10] because hydroxyl is an highly reactive species and a major contributor to oxidative stress. This condition is usually referred as an unbalance between anti-oxidants and oxidants, that favors the oxidation state. Sies defined it as "*a disturbance in the pro-oxidant / antioxidant balance in favor of the former, leading to potential damage*" [11].

Such damage results from the oxidation of biomolecules as sugars, lipids, DNA and proteins. The large array of products formed from their oxidation is strongly dependent on the nature of the biomolecule damaged, the oxidant and the biological environment. Briefly, oxidation of polysaccharides results in their depolymerization or fragmentation [12] but continued oxidation of glycated proteins promotes advanced-glycated end products (AGEs) formation and ability to interact with the receptor for AGEs and further enhance the oxidative burden upon nearby molecules [5]. Also worth of

consideration is the effect of oxidants onto the sugars of nucleotides that lead to their carbonylation, ability to bind iron and intensify the Fenton reaction. In addition, ROS target nucleotide purines and pyrimidines that result in base deamination, ring openings, adduct formation and whole nitrogen base loss, which, together with the failure of repairing mechanisms allows the establishment of mutations. Lipids are another class of molecules attacked by ROS, where the carbon-carbon double bonds of unsaturated fatty acids are an important target. The effects may be relatively limited, or may progress in the presence of oxygen to the formation of new peroxyl radicals that may continue new rounds of oxidative damage on lipids and produce even more oxidized lipid hydroperoxides. Proteins are also markedly affected when oxidized as will be explained in the following chapter.

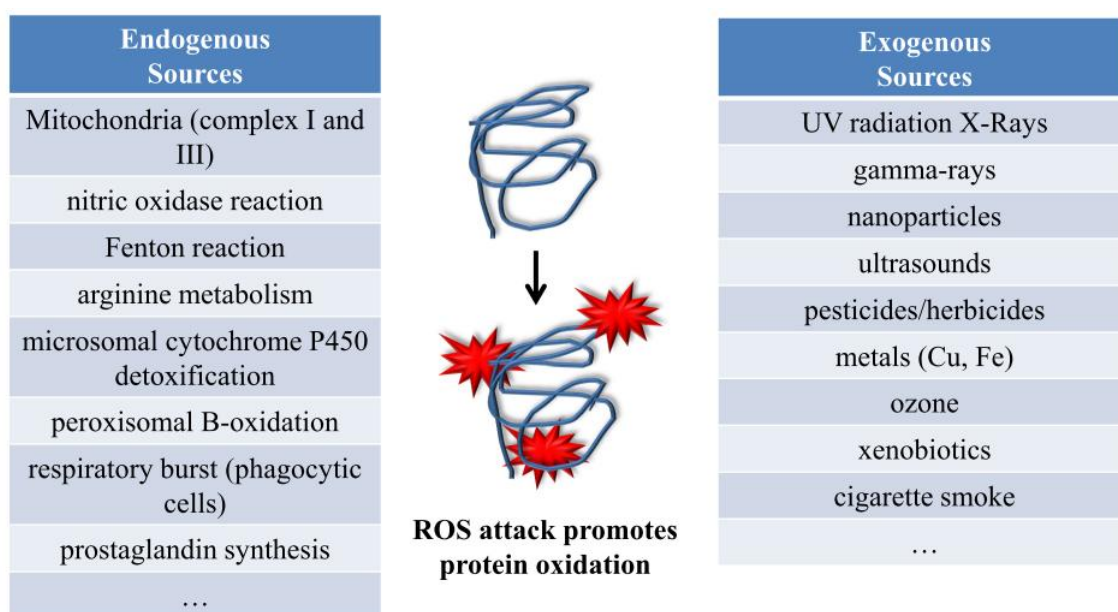

Figure 1. Reactive Oxygen Species sources. ROS are an inevitable consequence of daily life. ROS are continuously generated by enzymatic or non-enzymatic reactions. Their source can either be endogenous, from metabolism and energy-transferring processes, or be exogenous, resulting from exposure to environmental contact. Either sources can oxidize proteins, leading to their functional impairment or/and accumulation.

A large body of evidence has been generated over the years, showing the correlation of oxidative stress consequences and pathologies like cancer [13], neurodegenerative disorders, and ageing [11]. In fact, one of the most experimentally tested theories that attempt to explain ageing is the Free Radical Theory of Ageing, proposed by Denham

Harman in 1956. Since then, the theory has been updated, but its fundamental principle has been retained. It postulates that free radical / oxidative stress leads to biomolecule error accumulation over time, being a major contributor to the age-related functional involution of cells, tissues and organisms [14] (Figure 2).

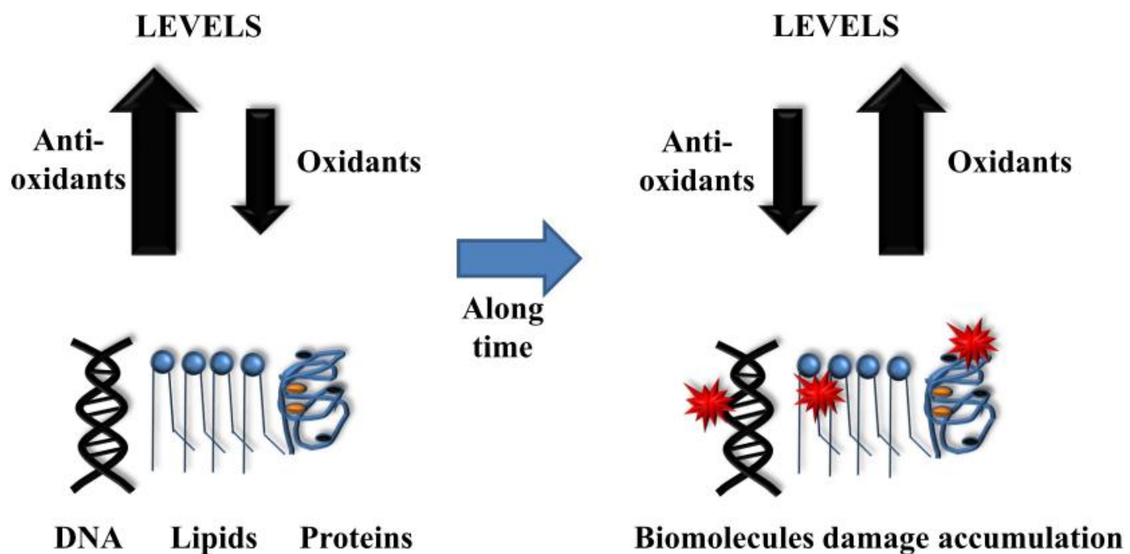

Figure 2. How oxidative stress occurs. Across different organisms, young cells have higher levels of antioxidants comparing to oxidants, promoting a reduced cellular environment. However, in old cells, redox state changes towards oxidation, due to less efficient antioxidant defenses and less efficient mitochondria, generating the so called oxidative stress state. As the condition persists, biomolecules such as proteins become oxidized which leads to functional impairment and contributes to cell dysfunction. Oxidative stress has been linked with disease and ageing progression due to the error accumulation nature of the process.

The interplay between oxidative stress, lifespan extension and ageing has been documented. In fact, overexpression studies of antioxidant enzymes such as SOD, lead to life extension through oxidative stress reduction in mice and *Drosophila* [15, 16]. The accumulation of errors, due to oxidative stress especially in proteins is thought to play an important role in the ageing process [14]. The following chapter is focused on protein oxidation.

2. The special case of oxidative stress: Protein oxidation

Proteins are an important set of biomolecules affected by ROS. Why should protein oxidation be considered so important? Because every cellular process requires the involvement of proteins and protein is the main intracellular category of biomolecules, making them an easy and reachable target for ROS.

When the cellular redox state goes beyond physiological levels, protein oxidation is likely to occur. The specific features of oxidation depend on the type of ROS, its severity (concentration and duration) and the affected amino acid residues [5]. As protein structure is maintained by amino acid interactions, their oxidation, thus leads to protein structure loss and consequent functional impairment. For example, enzymes, as lysozyme and ribonuclease, have been shown to be inactivated by radiation originated ROS, particulary when the most effective $HO^{\cdot}$ is involved [17, 18].

The affected amino acid residues can be an important markers of extensive oxidation damage. Oxidation may be reversible when it involves amino acids such as cysteine and methionine, which are highly sensitive to slight increases in ROS levels and originate methionine sulfoxide and cysteinyl derivatives respectively. The former can be restored back to its reduced form by methionine sulfoxide reductase [19], and the latter can be repaired by enzymes such as glutaredoxin or thioredoxin [20] thus reverting oxidative conditions. This ability to oxidize and to revert is thought to underlie redox cellular signaling [21-23].

2.1 Protein carbonylation – a case of irreversible oxidation

In contrast to methionine and cysteine oxidation, there is no enzymatic mechanism able to revert some types of protein oxidation. Irreversible events occur when ROS oxidizes a variety of amino acid residues having aromatic or long side chains, as histidine, tryptophan, tyrosine or phenylalanine [24]. Oxidation of other amino acid residues as proline, arginine, lysine and threonine results in irreversible carbonyl formation, a consequence of major importance due to their quantity and irreversibility [25-29]. Therefore its occurrence should be taken as serious in a cellular context. But how exactly is protein carbonylation generated?

Carbonyls can be formed directly by metal catalysed oxidative attack on the amino acid side chains of proline, arginine, lysine, and threonine or secondary to lysine, cysteine or histidine by reactive carbonyls on carbohydrates (glycoxidation products), lipids, and advanced glycation/lipoxidation end products. The more abundant products from

carbonylation reaction are glutamic semialdehyde from proline, and aminoadipic semialdehyde from lysine.
Protein carbonylation is an indicator of severe or permanent oxidative damage [30] as happens in many disorders that affect cell functioning and survival [10] and following exposure to oxidants in experimental conditions [9, 31]. It is not surprising that protein oxidation has been related to oxidative stress, disease and aging [10, 32]. In the turn of the century Levine and Stadtman gathered data from studies that included tissues from organisms at different ages and even whole organisms; they showed that in the last third of their lives there was a dramatic increase in carbonylated protein content, thus indicating an extensive accumulation that lead the authors to point out that the increase in *"oxidized proteins rises to a level likely to cause substantial disruption of cellular function"*[32]. Interestingly, mouse mitochondria carbonylated proteins were decreased upon subjecting animals to calorie restriction diet [33], a condition that has been proved to prolong certain organism lifespan by activating some intracellular pathways. These findings received additional support from studies with old *C. elegans* [34]. Therefore, the correlation of increased levels of carbonylated proteins and aging progression suggests an important connection.
Along the years, our knowledge regarding the correlation of aging and carbonylated proteins has increased due to the development of carbonyl detecting techniques.
Its assessment is currently simplified by the development of techniques as the derivatization of carbonyls with 2,4-dinitrophenylhydrazine (DNPH), followed by immunodetection with an anti-DNP antibody in a western blot or ELISA assay, or the derivatization with biotinhydrazide and detection with fluorescent avidin probes. Frequently these procedures are based on 2D gels which amplifies protein resolution and allows further analysis as the quantification of protein oxidation level, in relation to protein overall content, or its identity employing mass spectrometry, for detailed review of methods, see [35].

2.3 Among the most susceptible proteins to carbonylation is cytoplasmatic actin

The concept of oxidative stress has evolved. While, in early days, it used to describe random damage to the nearby biomolecules, current thinking favors selective damage as there is strong evidence for carbonylation specificity in the sense that some proteins are more susceptible to be carbonylated compared to others [36]. In fact, susceptibility

variation has been seen in different models as plant *Arabidopsis thaliana,* bacteria *E. coli*, yeast *Saccharomyces cerevisiae* and even human samples [37-44]. The cause for these different carbonylation susceptibilities remain elusive, but explanations have been proposed, including ROS and anti-oxidants concentration, ROS proximity, protein structure and location, metal ions binding and protein abundance in cells [45-47].

Cytoskeletal protein actin (reviewed elsewhere [9, 48-50]) has been recognized as a most susceptible proteins to oxidation. This finding has gathered increased consistence and interest in recent years, from *in vitro* cell free studies, through cells and organisms.

In vitro, purified actin is oxidized in a way that is dependent on the concentration of the oxidant and the time of exposure [51]. Metazoan cysteine 374, at the carboxyl extremity of the α actin (or Cys272 in β and γ actin) [52] and the yeast actin [53] are very sensitive to oxidation due to their localization at or close to the surface of the actin molecule, followed by methionine 44, 47 and 355 [50]. Actin oxidation may be elicited by a variety of different compounds as chloramines [50], acrolein [54], hypochlorite [51], hydrogen peroxide [31, 55] and others [56-59].

Although some of these studies were addressing actin changes at the outset, others were unexpected observations in different contexts and fields. In fact, it was shown that the exposure of different cells or biological samples to oxidative conditions affects a large number of proteins, of which actin was found to be a rather sensitive one as evidenced by carbonylation assays.

2.4 Settings

Diet composition, for example, may lead to a variation in actin carbonylation and nitrotyrosination [60], suggesting the possibility for its prevention; this point is supported by a study on the beneficial effects of including oats in the diet [61]. In addition, antioxidants have been studied intensely due to their hampering effect on proliferation, albeit the underlying mechanism may differ according to the compound. For instance, the use of hops antioxidants as proanthocyanidins resulted in HT29 colon cell line proliferation arrest [62], an effect attributed to substantial actin carbonylation and cytoskeleton functional impairment. Similar consequence was noted in the epithelial Caco-2 cells [63], yeasts upon hydrogen peroxide administration [53] and a neuronal cell line after inflammatory cytokine challenge [64].

Animal studies on the effects of other stressors and environmental agents on carbonylation were also examined in a variety of conditions that emphasized the

relevance of cytoskeletal protein oxidation. For example, α-actin from cod fish muscle is rather sensitive to ferrous catalyzed oxidation [65] and ferric nitrilotriacetate administration to rats results in increased oxidized proteins in gastrocnemius muscle [57], whereas the *in vivo* contact of mussels with environmental pollutants was followed by carbonylation enhancement of β-actin in their muscular cells [66]. In a different setting, quite unexpected was the detection of carbonylated actin in the serum of pigs housed at high density [67], a finding considered a potential stress biomarker.

2.5 Consequences and biological importance

The progressive modifications on actin molecule have functional consequences. While mild oxidation of surface sulfur containing residues has limited effect on actin polymerization ability, additional oxidation strongly inhibits it and even enhances its fragmentation [51].

Moreover, when the intense oxidative insult is continued, actin aggregate formation ensues. This was shown in muscle [48] and non-muscle [31].

The reason for actin susceptibility is not known, as mentioned earlier when protein oxidation susceptibility was discussed in a general context. As hypothesized before, one reason could be the intracellular content. Actin amounts to 10% of the whole protein in normal and leukemic lymphocytes, c. 8% and 5% respectively [68], myeloid leukemia cells (5%, [69]), adrenocortical cells (10-12%, [70]) and fibroblasts (7-14%, [71]). Another reason is the main localization: it is present throughout the cytoplasm, off the lumina of membranous compartments and so easily accessible to diffusible oxidative compounds. Finally, actin may have specific structural features that make it more prone to oxidation, similarly to other proteins. For example, upon submission of yeasts to H_2O_2, only 8% of the whole yeast protein becomes oxidized, but it amounts to 80% when ribosomal protein is considered [72]. In contrast, in *E. coli* cultures, H_2O_2 oxidation targets the protein synthesis factor EF-G [44] and when the cells are grown in a medium with a methionine substitute and stressed, the small increase in total carbonyls is outweighed by remarkable carbonylation of EF-Tu, the most plentiful protein in that bacteria [73]. Similarly, oxidation of arthropod mitochondria results in isolated aconitase carbonylation [74].

Actin has additional relevance in the context of ageing. In a recent study [75], while verifying carbonylation along ageing, the most susceptible proteins were categorized according to their involvement in cell functions, they included: glucose metabolism,

pyruvate dehydrogenase and tricarboxylic acid cycle, electron transport chain and ATP metabolism, plant metabolism, amino acid and protein metabolism, lipid metabolism, antioxidant defenses, heat shock proteins/chaperones, receptors and cell signaling, membrane transport, plasma proteins and related, and cytoskeleton. Actin was highlighted as a sensitive one in yeast, mouse, rat or even human [75]. The recognition of actin sensivity to carbonylate, is supported by several lines of evidence, ranging from *in vitro* cell free models through cells and organisms [31, 50-57, 63, 66, 67].

Why should this matter? Apart from the enhanced academic interest in recent years new areas with notorious medical relevance are being unraveled. Actin participates in a number of different and fundamental cell and tissue functions [76] that range from the mechanical support for cell shape and muscular contraction, to endocytosis, organelle movement, neuronal and immunological synapses and cell movement towards chemoattractants [9, 31]. Beyond actin involvement in a large number of cellular processes, its study is also important due to the fact that actin has been correlated with neurodegenerative diseases, shown by abnormal F-actin accumulation in neuronal death conditions [77] and cancer [78].

Due to its decisive role in cellular functionality, it is important to understand how actin is oxidized and how is degraded.

3. Actin and other oxidized proteins fate – proteasome degradation or aggregate formation?

3.1 Proteasome degradation

Non-oxidized proteins have different degradation mechanisms. While some proteins have their turnover mechanism well documented others remain obscure. This is the case of non-oxidized actin.

The fate of actin inside cells under physiological non-oxidizing conditions is still debatable. For example, what happens to G-actin after F-actin depolymerization and dismantlement? To our current knowledge the mechanism whereby F-actin is degraded is currently unkown, apart from the involvement in the cycle of F-actin polymerization or depolymerization by actin binding protein as gelsolin [79].

How G-actin is degraded is currently unsettled too. However, there is evidence that three different mechanisms may be operating. The first line of evidence favors caspase-2 role in G-actin degradation by proteolytic cleavage; in fact, in chick embryonic

cardiomyocytes, following lovastatin mediated F-actin disassembly, it was shown that a caspase-2 inhibition resulted in actin degradation prevention [80]. Secondly, there is evidence from cultured neonatal rat heart cells, demonstrating that when muscle contraction was pharmacologically inhibited, α-actin was partially degraded by the lysosome [81]. In addtion, actin degradation has been linked to an E3 ubiquitin ligase motif-containing 32 (TRIM32) which binds myosin in skeletal muscle and ubiquitinates α-actin in vitro. Overexpression of TRIM32 in human embryonic kidney cells lead to a decrease in cytoplasmatic actin supporting the involvement of TRIM32 in actin degradation [82]. Thus, non-oxidized actin employs different degradation mechanisms likely to be preferentially activated in some tissues and according to particular functional features. It is a matter that awaits further investigation. In contrast, oxidized proteins appear to be an attractive target to the proteasome, the main cellular non-lysosomal degradation system, following its degradation pathway [83, 84]. The proteasome is active in two main forms: one called the 26S, which requires ubiquitin tagging and ATP hydrolysis, and the 20S that does not need ubiquitin or either ATP to complete the process.

The 20S structure is a barrel shaped, composed of α and β rings organized in a αββα manner; each ring has seven subunits (as α1-α7; β1-β7) (Figure 3). The inner β rings responsible for specific proteolytic activity, contain three subunits: β1 for peptidyl-glutamyl-peptide-hydrolyzing or caspase like activity, β2 for trypsin like activity, and β5 for chymotrypsin like activity. The outer α-rings are responsible for substrate detection and binding of regulatory complexes such as 11S and 19S which determines the activity and the specificity of the proteasome [85]. The combination of 20S with 11S or 19S results in the 26S proteasome form. It is involved in the so called Ubiquitin-Proteasome-System (UPS), and localized in the cytosol and nucleus.

The ubiquitin part of the system, consists of three types of enzymes: E1 ubiquitin activating enzymes, E2 ubiquitin conjugating enzymes and E3 ubiquitin ligases. Ubiquitination is a highly ordered and specific mechanism, and due to the diversity of existing enzymes is able to form countless possibilities of arrangements. The process starts when the E1 enzymes bind one ubiquitin molecule to its C-terminal glycine; is bound to ubiquitin is then transferred to E2 enzymes and, in the last step, ubiquitin is bound to E3 enzymes. Depending on E3 enzyme type, ubiquitin is either tagged directly to lysine residues in the substrate or indirectly in a two-step mechanism [85]. This form

of the proteasome is usually responsible for the degradation of short-lived, not required or even misfolded proteins.

Oxidized proteins seem to be degraded exclusively by the 20S proteasome, the main site for oxidized proteins degradation [85-91]. There are three important reasons for such preeminent involvement; first, the 20S proteasome itself is relatively more resistant to oxidative stress than the 26S [92]; second, oxidized proteins expose hydrophobic residues that can act as a trigger signal for degradation independently of ubiquitin tagging, not wasting ATP in a cellular stress situation [84, 93]; third, as lysine residues are preferential targets of oxidative modification, ubiquitin lysines would be less able to bind to substrates and thus, could impair the whole process [45, 85, 86, 94]. It has been shown that proteasome inhibition conversely increases the steady state amount of carbonylated proteins; this, in fact in post-ischemic rat heart, actin was found to be oxidized [95] without actin ubiquitin adducts formation, thus suggesting a 20S proteasome-mediated proteolysis [96]. In support, Grune and Davies groups demonstrated that the disruption, of the ubiquitin tag system by compromising ubiquitin conjugating activity, did not impair the degradation of oxidatively modified proteins [94].

Despite of 20S proteasome turnover, carbonylated proteins can accumulate during time, possibly due to an efficiency decrease or even to oxidized proteins increased rate formation. When this happens, protein aggregates can form as it will be described in the following chapter.

3.2 Aggregate formation

In spite of the large number of studies favoring the 20S proteasome role in degrading mildly oxidized proteins [84-86] the rate of oxidized proteins formation may be higher than the proteasome capacity to promote their turnover. In that case, because proteasome may become less functional during a stressful period of time, oxidized proteins may accumulate into insoluble protein aggregates, which further inhibit the proteasome [31, 97, 98] (Figure 3).

Changes in protein structure often result in exposure of the usually concealed protein core. It is often constituted by hydrophobic residues that upon exposure, enhance binding to other oxidized proteins due to its sticky nature; this results in cross-link promotion, culminating in insoluble high molecular protein aggregates [25, 30, 98-100]. The generation of a Schiff base by the reaction of a carbonyl group from one protein

with an amino group from another, adds further to aggregate accumulation and points to the interesting aspect of aggregate enlargement without any further oxidation [101]. Interestingly, the view that protein aggregates are inert materials has changed. Recent data show that they interfere with cell gene expression dynamics, as they can alter cellular metabolism at a transcriptional level and even lead to cell death [102, 103]. Moreover, protein aggregates are a common feature of aged cells [98]. They have been deeply correlated with aging progression across different models such as bacteria [39, 104], yeast [72], *C. elegans* [34] and mammalian cells [105].

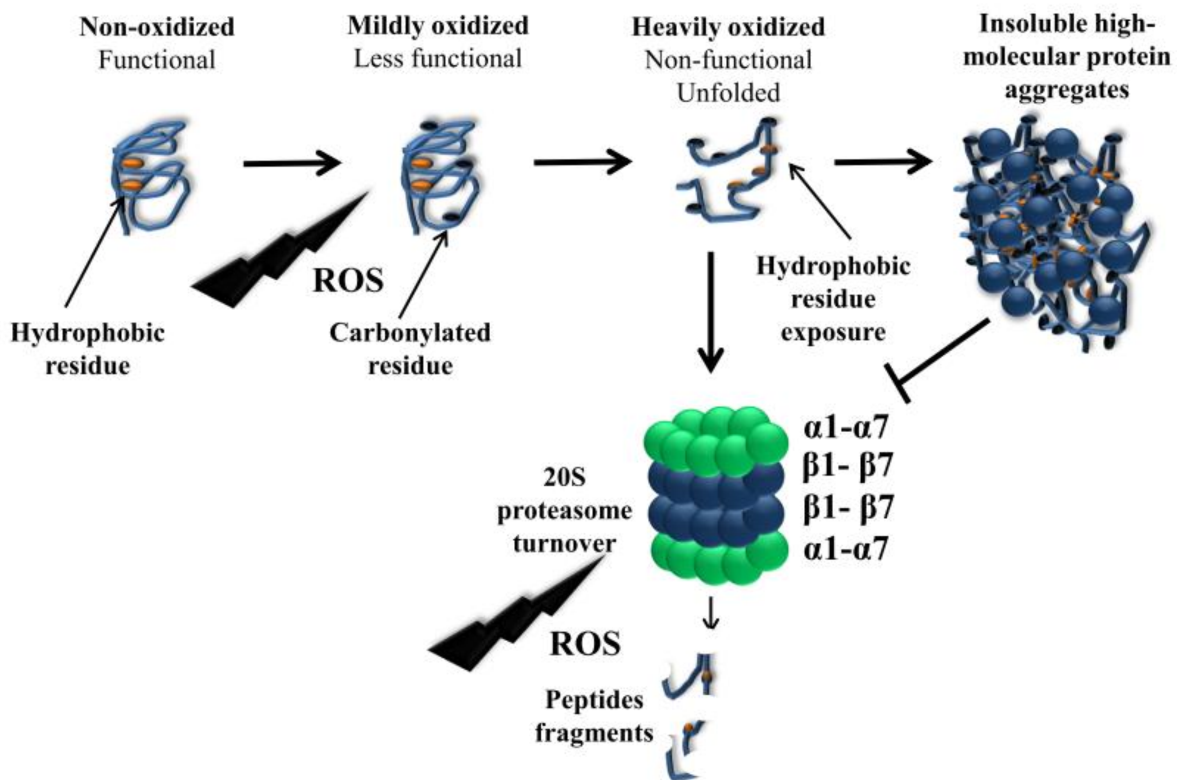

Figure 3. Actin carbonylation and aggregation fate: proteasome 20S or protein aggregates? Native actin gets moderately oxidized when cells are challenged with hydrogen peroxide to promote oxidative stress. Some of the oxidized actin is degraded by the 20S proteasome; however, because the 20S proteasome is also functionally affected, oxidized proteins tend to accumulate. As the stress endures, actin becomes heavily oxidized, exposes hydrophobic residues and starts to aggregate. These bind to the proteasome, inhibit its proteolytic activity and further disturb the proteasome and cell proliferation.

THE ESTABLISHMENT OF HYPOTHESIS AND AIMS

The data supporting the effect of ageing on tissue carbonylation is varied as evidenced in Levine and Stadtman evaluation [10, 32], suggesting that a similar consequence is found in tissues of aged humans. To verify this point, collection of tissue from aged individuals would be required. However, such samples are hard to obtain due to the invasive character of the procedure and early assessments were likely to result in tissue loss. As alternative, primary T-cells were isolated from buffy coats obtained during the preparation of blood units for transfusion and then submitted to expected oxidative conditions employing H_2O_2. By performing immunoblots after DNPH derivatization, the procedure demonstrated a regular pattern of protein carbonylation; a most intense band was studied by *MALDI-TOF* and identified as actin. The results were the core of my Master thesis completed in 2008.

The conditions for actin oxidation and lymphocyte dysfunctional consequences thereof became one of the aims in my PhD project. Because of the variability of primary cells, the project derived employing a Jurkat T-cell line.

In **Publication I**, the establishment of cell culture protocols and the effects following H_2O_2 administration were the central subject. Hydrogen peroxide mediated oxidative stress resulted in cell proliferation decrease, an effect accompanied by an enhancement in carbonylated actin (also confirmed in cell free extracts). Actin aggregates fed to cells were shown to promote their growth arrest too. A decrement in proteasome activity and morphological evidence of aggregates was indicative that actin accumulation in oxidative conditions results in features of cellular ageing. This was correlated with a time-dependent increase in carbonylated actin, but a direct effect was also observed when we produced carbonylated actin aggregates in vitro and fed them to Jurkat cells. Under these conditions, cells stopped dividing and had their proteasome activity levels decreased, which corresponded to what had been observed in hydrogen peroxide challenged cells. Therefore, it was concluded, that oxidized actin aggregates are formed under oxidative stress, which leads to cell proliferation inhibition and reduced proteasome activity.

The remarkable susceptibility and time-related feature of actin oxidation (Publication I) lead us to reason that it might be involved in other oxidative prone conditions due to its abundance and cytosolic localization. After a thorough search, we concluded that actin carbonylation had been verified in a number of conditions, from cell free systems to cell culture, whole organisms study and even apparently distant biotechnological matters as

food storage (**Publication II**); nevertheless, it was our conviction that such extension of findings had not generated equivalent interest.

We reasoned though that, in daily cell life, such actin susceptibility to oxidation and to produce harmful accumulation would be balanced by appropriate regulatory mechanisms. One of them was the involvement of the proteasome (Publication I)**,** but it was an important matter to understand how oxidized actin would be conveyed to it and what might happen should the proteasome functional ability become exhausted.

PUBLICATION I

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Original Contribution

Carbonylation of the cytoskeletal protein actin leads to aggregate formation

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Protein carbonylation is a common feature in cells exposed to oxidants, leading to protein dysfunction and protein aggregates. Actin, which is involved in manifold cellular processes, is a sensitive target protein to this oxidative modification. T-cell proteins have been widely described to be sensitive targets to oxidative modifications. The aim of this work was to test whether the formation of protein aggregates contributes to the impaired proliferation of Jurkat cells after oxidative stress and to test whether actin as a major oxidation-prone cytoskeletal protein is an integral part of such protein aggregates. We used Jurkat cells, an established T-cell model, showing the formation of actin aggregates along with the decrease of proteasome activity. The presence of these protein aggregates inhibits Jurkat proliferation even under conditions not influencing viability. As a conclusion, we propose that an oxidative environment leads to actin aggregates contributing to T-cell cellular functional impairment.
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Introduction

Several theories have been proposed to elucidate the reduced functionality of the immune system during aging but none fully explain the biology of the process [1]. Although presenescent T cells are still metabolically active, they have a reduced capacity to divide after activation [2,3]. Excessive previous proliferation on chronic stimulation and the consequences of free radicals are among the most discussed mechanisms explaining this phenomenon [4,5].

Among the effects caused by free radical attack protein carbonylation is a frequent event [6]. Protein carbonylation is a nonenzymatic process, leading to the formation of an irreversible product and to protein dysfunction both by changing its correct folding and by promoting the formation of nonsoluble protein aggregates [7]. These protein aggregates can in turn modify the cellular metabolism and influence cellular functionality [8,9]. Possibly, as a result of a continuous oxidation, protein dysfunction underlies the age-related involution and, in fact, an enhanced content in carbonylated protein was observed in the course of ageing in a number of species [10].

Although, it is assumed that any protein is susceptible to carbonylation, some may be more susceptible compared to others [7]. It was suggested, that one of these is actin which, *in vitro*, exhibits elevated susceptibility to carbonylation and can form covalent cross-linking as well as noncovalent aggregates [11]. Such actin susceptibility was also observed in rat spinal cord cells [12] and mussel muscular cells [13] and additional actin filament disruption and polymerization failure were also noted in the Caco-2 colonic cell line [14] and yeast [15] when submitted to oxidative challenge. The data support the point that actin is thus a protein with a major likelihood to oxidative modifications. Due to its ubiquitous presence and variety of actions inside the cells [16], this would indicate that its oxidation and functional disturbance might be a common occurrence. However, the diversity of cells so far studied is rather limited, which does not allow a generalization regarding such effect.

To maintain a homeostatic proteome, cells degrade not required or damaged proteins with the help of the proteasomal system [17], which is a multicomplex proteolytic machinery existing mainly in two forms, the ATP-ubiquitin dependent (26S) and the ATP-ubiquitin independent (20S). Oxidative conditions, such as those leading to protein carbonylation, are known to result in a decline of ubiquitin-dependent 26S preoteasome activity, in contrast with 20S proteasome, whose activity is practically maintained [18]. Interestingly, these effects do not hinder the cellular ability to degrade carbonylated protein generated under oxidative conditions. Indeed, all existing data point to the fact that oxidatively damaged proteins become partially unfolded due to primary oxidative modification, followed by an exposure of hydrophobic patches which in turn act as a trigger signal for 20S proteasomal degradation, excluding the need for ubiquitin tagging [19–21]. In fact carbonylated proteins also follow this pattern of events [22]. Moreover, actin, after being

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carbonylated, has been described to be degraded by the 20S proteasome in cardiomyocytes [23]. An unbalance in the proteome homeostasis can occur when the formation of oxidized proteins outreaches the degrading capacity of the proteasome and heavily modified proteins are formed. Under these conditions cross-linked proteins may be formed and lead to insoluble protein aggregates. These have been described to act as a proteasome inhibitor, partially due to the direct binding of the proteasome to aggregate surface domains resulting in loss of proteasomal activity [9]. Aggregates appear to induce senescence in some cell types, resulting in a loss of cell dividing capacity [8,24].

Senescent cells are known to increase in organisms with time, and immune cells do not seem to be an exception to this phenomenon. In humans, such changes include reduction of naïve T-cell production, either CD4+ and CD8+, and clonal expansion of specific CD8 T-lymphocyte subsets as the CD8+CD28– [25]. These CD8+ cells are particularly prone to oxidation [26] and when grown *in vitro*, they obtained a senescent like phenotype including growth arrest, resistance to mitogenic stimuli, altered cytokine secretion, diminished telomere length, and the loss of CD28 expression [27].

Here we test the hypothesis that the formation of protein aggregates and the resulting inhibition of proteasome also take place in immune cells, therefore contributing to the reduced proliferation ability of T cells. We were able to demonstrate the formation of protein aggregates already at low doses of oxidative stress, the inhibitory effect on the proteasome, and, furthermore, we were able to show that carbonylated actin is a major component of these aggregates.

Materials and methods

Cell viability/proliferation

Jurkat cells (6×10^5 cells/ml) were allowed to adapt in 75-mm culture flasks containing RPMI 1640 (Biochrome), with 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin at 37 °C, 5% CO_2 in a Nuairetm US Autoflow CO_2 water-jacketed for 3 days. They were then divided and 6×10^5 cells/ml were cultured in the same medium under seven different conditions: controls, 6.25, 12.5, 25, 50, and 100 μM H_2O_2 or 1 μM lactacystin (Invitrogen) for a variable time depending on the experiments described below. Cell suspensions from each flask were mixed with trypan blue (1:1) and evaluated in an automated cell counter (Invitrogen) for cell viability and proliferation (cell number).

Immunoblotting

Cells cultured in the RPMI 1640 medium, as above, were submitted to oxidation by adding 6.25, 12.5, 25, 50, and 100 μM H_2O_2 for 3 or 24 h, and for another set of experiments to 1 μM lactacystin, 100 μM H_2O_2, and both combined. They were counted, and an equal number (8×10^5 of cells) were lysed directly in a lysis buffer (glycerol 12.5%, 6.25 mM Tris, pH 6.8, SDS 5%, bromophenol blue 0.08%), briefly sonicated, and boiled at 65 °C for 20 min. The same volume (20 μl) from each sample was loaded and resolved in a 10% SDS-PAGE gel. Proteins were blotted onto Hybond membranes (Amersham Biosciences Europe) (equal concentration of protein in each lane was confirmed by staining the membrane with Ponceau solution). When the experiment was performed to detect protein carbonylation, the membranes were directly derivatized with 2,4-dinitrophenylhydrazine (DNPH, Sigma Aldrich). Briefly, after the transfer, the membranes were equilibrated in TBS (Tris buffer saline)/20% methanol, washed for 5 min in 10% trifluoroacetic acid (TFA, VWR), incubated for 10 min with 5 mM DNPH/TFA (10%) in the dark, washed with TFA (10%) to remove the excess of DNPH, and finally washed again five times (5 min each) with 50% methanol. Following this, membranes were blocked with 5% BSA/TBST (Tris buffer saline with Tween 20, 0.1%) for 1 h.

After blocking, the membranes were probed with rabbit IgG anti-DNP (Sigma) at a 1:10,000 dilution as the primary antibody, goat IgG anti-actin (Abcam) at a 1:750 dilution, and rabbit anti-β5 proteasome subunit at 1:1000 (Abcam). For the secondary antibodies, IRDye 680LT goat anti-rabbit IgG, IRDye 800CW goat anti-rabbit IgG, and IRDye 800CW donkey anti-goat IgG from Li-Cor were used. The detection was performed by infrared, using the Li-Cor equipment from Odyssey. Band intensities were quantified by densitometry using Odyssey software. Graphics were made using GraphPad Prism 5 software.

Immunocytochemistry

Cells were grown on 6-well plates for 24 h, and after washing in PBS, they were placed on poly-L-Lysine slides (Jurkat are suspension cells) and allowed to dry in the incubator described above (see Cell viability/proliferation). Immediately after, they were fixed in 4% paraformaldehyde, washed with PBS, PBST (phosphate buffer saline with Tween 20 0.1%), and PBSTx (phosphate buffer saline with Triton X 0.1%) 0.1%, and incubated for 1 h in PBST/BSA 2%, to lower unspecific background signal; however, for the experiments detecting carbonylated proteins, before the blocking step, cells were incubated in with 5 mM DNPH for 10 min, and to neutralize the reaction 2M Tris was used. Afterward, cells were incubated overnight at 4 °C with a solution containing the primary antibodies, 1:250 of goat anti-β-actin from Santa Cruz (USA) and/or 1:1000 of rabbit anti-DNP from Sigma, 1:1000 of mouse anti-CFTR from R&D Systems (USA), 1:100 of rabbit anti-β5 proteasome subunit from Abcam. After washing twice in PBST, cells were incubated with the secondary antibodies conjugated with Alexa fluorochromes from Molecular Probes, donkey anti-goat secondary antibody conjugated with Alexa 488 and 568 (1:1000 prepared in PBST with 2% BSA) and with anti-rabbit secondary antibody conjugated with Alexa 488 and 568 (1:1000 prepared in PBST with 2% BSA) and donkey anti-mouse secondary antibody conjugated with Alexa 568 (Molecular Probes) 1:750 prepared in PBST with 2% BSA). Fluorescence samples were analyzed on a Zeiss AxioImager Z1 fluorescence microscope in Porto, Portugal, and a Zeiss Axiovert in Jena, Germany. For phalloidin staining experiments, actin-stain 488 fluorescent phalloidin was purchased from Cytoskeleton Inc. The same protocol as above was used until the blocking step. Cells were incubated with 100 nM phalloidin for 30 min in the dark, washed three times in PBS, and analyzed in a Zeiss LSM 510 confocal microscope, also in Jena, Germany, with help from Dr. S. Monajembaschi, Fritz Lippman Institute, Jena.

Proteasome activity

Cells were counted, and the same number of cells was washed twice with PBS in each tube. Cells suspensions were centrifuged for 5 min at 30*g* and lysis buffer (250 mM sucrose, 25 mM Hepes, 10 mM magnesium chloride, 1 mM EDTA, and 1.7 mM dithiothreitol (DTT)) was added to the pellet. Cells were lysed using a 20-gauge syringe, followed by repeated freeze–thaw cycles. Afterward, the cell lysates were centrifuged at 3500*g* for 30 min and the supernatants were used for determination of proteasomal activity. Samples of the supernatants were incubated in 225 mM Tris buffer (pH 7.8), 45 mM potassium chloride, 7.5 mM magnesium acetate, 7.5 mM magnesium chloride, and 1 mM DTT. To measure chymotrypsin-like activity of the proteasome the

fluorogenic peptide Suc-LLVY-MCA was used as a substrate at a final concentration of 200 μM. Samples were incubated for 30 min at 37 °C. The proteolytic MCA liberation was measured using a fluorescence reader (Reader EL 340; Bio Tek Instruments, Bad Friedrichshall, Germany) at 360 nm excitation and 460 nm emission. Free MCA was used as the standard for quantification.

Actin aggregate formation in vitro

Purified, nonmuscle, 232.56 μM actin (Cytoskeleton, Inc.) was suspended in distilled water. A volume of 10 μl totaling 50 μg of actin was mixed with 10 μl of general actin buffer (GAB, Cytoskeleton, Inc.) supplemented with 5% sucrose, 1% dextran and 0.2 mM ATP, purposed to stabilize protein and prevent unwanted aggregation. Samples were incubated on ice for 1 h to depolymerize actin oligomers that may have formed during storage or resuspension. After incubation for 1 h, 5 μl of samples was mixed with GAB + H_2O_2 (1:1) to obtain final concentrations of 1, 0.5, and 0.1 mM of hydrogen peroxide. Next, samples were mixed for 30 min at 300 rpm at room temperature. Twofold gel loading buffer was added 1:1 (composition described in Immunoblotting), and samples were heated to 65 °C for 20 min. Sample aliquots of 5 μl were loaded and analyzed in a 10% SDS-PAGE gel, followed by silver staining.

Silver staining

At the end of the electrophoresis, gels were fixed in 30% ethanol (Merck)/10% acetic acid (Merck) for no less than 2 h. Next, gels were washed in 20% ethanol and distilled water for 10 min each. For sensitization, sodium thiosulfate 0.2 g/L was used for 1 min followed by two quick washes in distilled water. Gels were incubated in a silver nitrate solution (2 g/L, Sigma) for 10 min in the dark room. To develop protein bands, 700 μl 37% formaldehyde (Merck), 30 g sodium carbonate (Merck), and 5 ml sodium thiosulfate were mixed to a final volume of 1 L. The development was stopped using Tris (Sigma) 50 g/L and 2.5% acetic acid. Silver-stained gel images were captured using ChemiDoc equipment.

Actin aggregates incubation with Jurkat cells

Actin aggregates were produced as described above in Actin aggregates formation *in vitro*. Using 96-well plates, Jurkat cells (6×10^5 cells/ml) were placed in contact with purified aggregates (1–2%) for 24 h. Proliferation and viability indexes were obtained using the same method described above (Cell viability/proliferation).

Peptide mass fingerprinting

Silver-stained gel bands of protein aggregates were cut out from the gel, destained by a first wash of 50 mM ammonium bicarbonate (ABC)/5% acetonitrile (ACN) and two additional washes of 50 mM ABC/50% ACN. Afterward gel pieces were dried with 100% ACN, and then rehydrated with a 10 mg/ml trypsin solution (Promega, Madison, WI, USA, sequencing grade) overnight at 37 °C. Peptide was extracted by a 60% ACN/0.1% trifluoroacetic acid (TFA) solution (twice). Protein digests were desalted and concentrated using ZipTips (Millipore, Bedford, MA, USA) following the manufacturer instruction. Samples were applied onto a stainless-steel 192-well MALDI plate using the dried droplet method. For the matrix, a solution of 5–10 mg/ml cyano-4-hydroxycinnamic acid in 50% ACN/0.1% TFA was used. Samples were analyzed at the Proteomics Unit of IPATIMUP (Porto, Portugal) using the 4700 proteomics analyzer MALDI-TOF/TOF (Applied Biosystems, Foster City, CA, USA). Peptide mass fingerprint data were collected in positive MS reflector mode in the range of 700–4000 mass-to-charge ratio (*m/z*) using 1000–3000 laser shots for each sample and were calibrated internally using trypsin autolysis peaks. Several of the highest intensity nontrypsin peaks were selected for tandem MS (MS/MS) analysis. Air, at 9×10^{-7}, was used as the collision gas for MS/MS and the collision energy was set at 1 kV. Data from 3000 to 5000 laser shots were collected. The spectra were analyzed using GPS Explorer (Version 3.6; Applied Biosystems), which acts as an interface between the Oracle database containing raw spectra and a local copy of the Mascot search engine (Version 2.1.04). The MS and MS/MS data were searched together against a locally stored copy of the NCBInr and SwissProt using the Mascot search engine. The search included peaks with a signal-to-noise ratio greater than 10 and allowed for up to two missed trypsin cleavage sites. To be considered a match, a confidence interval (CI), calculated by Applied Biosystems GPS Explorer (GPS), of at least 95% was required. The closer a confidence interval is to 100%, the higher the probability that the identification is correct.

Results

Proliferation Is impaired under oxidative stress conditions but not viability

To evaluate the viability and proliferation rate, Jurkat T cells were submitted to oxidative conditions using hydrogen peroxide at various concentrations and analyzed up to 72 h (see Materials and methods for details). Viabilities were high for all groups (above 90%) and did not show significant differences between control and treated cells, suggesting that a noncytotoxic concentration of hydrogen peroxide was used (see supplementary material Fig. S1A).

Actin carbonylation and aggregation in Jurkat cells

In order to test whether protein carbonylation already takes place at the used oxidant concentrations, we measured protein carbonylation by immunoblot. Interestingly, we observed a major DNPH-positive band at 42 kDa. Due to the known susceptibility of actin to carbonylation [11, Castro and Almeida, unpublished data] and the molecular weight of the major carbonylated band, we assumed this to be actin and performed an identification using an anti-actin antibody. By using the double channel infrared Li-Cor instrument we could verify an absolute match of the protein bands. Surprisingly, the carbonylation intensity of actin declined, rather than increased, with increasing concentrations of H_2O_2 (Fig. 1A). However, the amount of actin was also decreased dramatically, indicating a clear concentration-dependent loss of the protein after 24 h (Fig. 1A).

Possible explanations for this result could be either the degradation of actin by the proteasome or the formation of high molecular aggregates. Both processes are taking place within several hours after oxidative stress, in most tested cells in a time frame from 3 to 24 h [22,28–30]. Therefore, we tested whether the actin loss is taking place already in an early time frame. As demonstrated in Fig. 1B no loss of actin could be observed within 3 h after starting the oxidative challenge. Under moderate oxidizing conditions also no dramatic increase of actin carbonylation was observed (Fig. 1B). As already noted one explanation for the later reduction of the actin content would be that the stress-related action carbonylation leads to the formation of high molecular weight protein aggregates, thereby reducing the actin levels at the molecular weight range of 42 kDa. In order to test

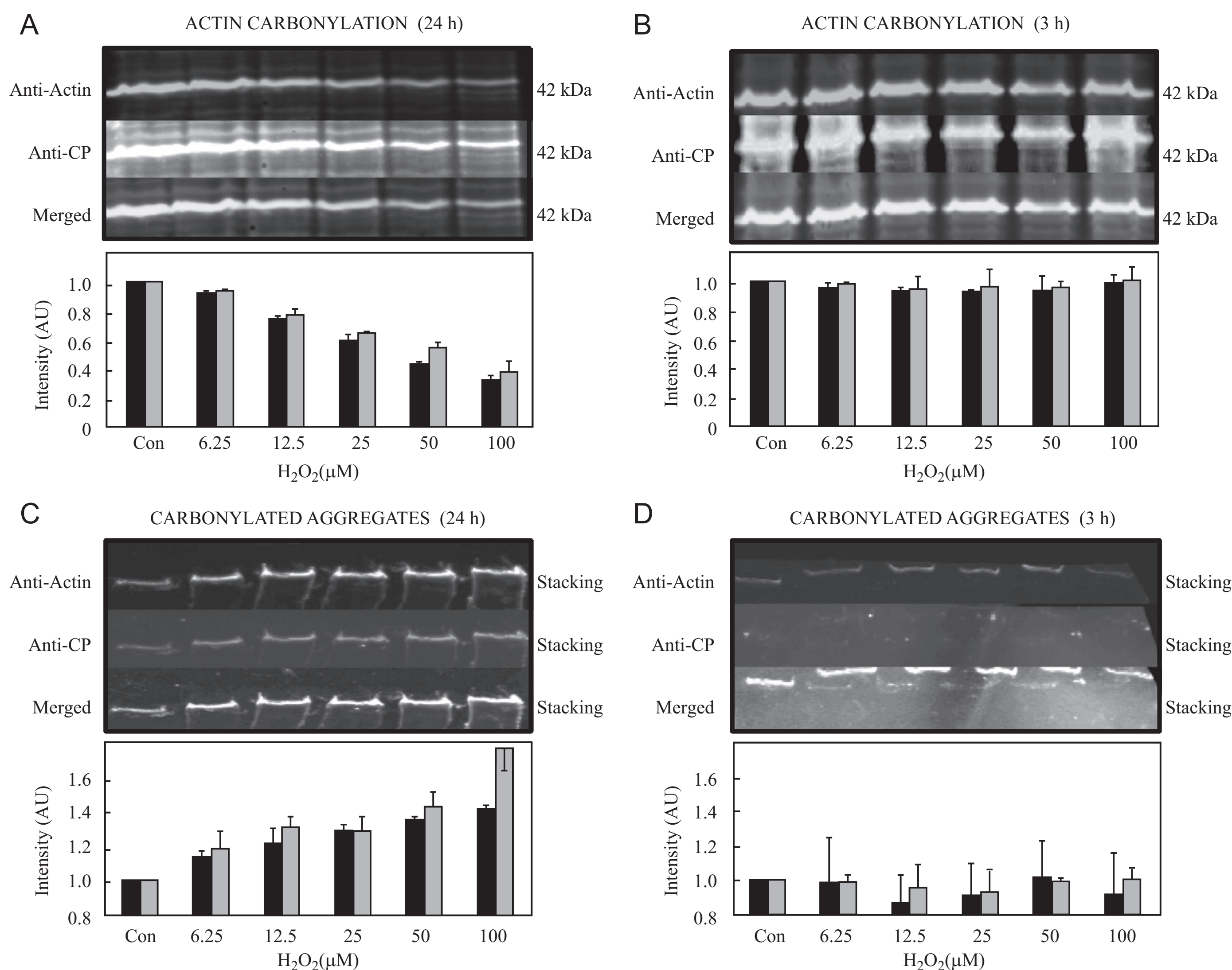

Fig. 1. Carbonylated actin aggregates formation is time and concentration dependent. Actin carbonylation following exposure of Jurkat cells to hydrogen peroxide for 24 and 3 h. Lanes represent controls (normal conditions) and hydrogen peroxide concentrations of 6.25, 12.5, 25, 50, and 100 μM H_2O_2. The graphs depict band quantification of actin (black columns) and the carbonylated bands (gray columns) from three independent experiments (mean ± SD are shown). Membranes were probed against DNP and β-actin (see Materials and methods). (A–D) Immunoblots from top to bottom: immunoblots for actin, carbonylated proteins (CP), and the respective merged blot; this is repeated throughout the figure, for each time point and zone. The 42-kDa band is shown at a time point of 24 h (A) and 3 h (B). The stacking zones of the immunoblots, as the potential localization of protein aggregates, are shown again at 24 h (C) and 3 h (D).

this we analyzed in a series of experiments the high molecular weight fraction in the stacking zone of the immunoblot. As demonstrated in Fig. 1C we could clearly identify a signal of protein carbonyls and actin in the stacking gel. This signal increased with increasing concentrations of hydrogen peroxide 24 h after the stress, indicating a shift of actin toward the high molecular weight zone. Interestingly, such protein aggregates cannot be observed 3 h after exposure (Fig. 1D), which is in accordance with the remaining band intensity of actin at 42 kDa at this time point.

Considering these results, we speculate that actin aggregation is obviously a major factor in the loss of functional actin. However, since the proteasome is able to degrade preferentially proteins which are oxidized, we decided to test the role of the proteasome in the degradation of oxidized actin.

Proteasome inhibition favors actin aggregate formation

Since we observed a most dramatic decrease in actin and actin carbonylation levels 24 h after treatment with 100 μM H_2O_2 accompanied by an increase in actin aggregates, our next step was to confirm that actin was actually mainly aggregating and not being degraded. To achieve this we inhibited the proteasome with lactacystin, and after viability indexes and proliferation measurements (supplementary material Fig. S2) we decided to employ the proteasome inhibitor lactacystin (LC) during hydrogen peroxide treatment. Since high concentrations of lactacystin under our conditions are toxic to Jurkat cells we used a concentration of 1 μM lactacystin. As demonstrated in Fig. 2B such a low concentration of lactacystin already decreases significantly the activity of the proteasome by some 25%, without affecting the amount of the proteasome. In order to test whether lactacystin addition is influencing the loss of the actin band, we performed an actin immunoblot with and without hydrogen peroxide and lactacystin. As demonstrated in Fig. 2A (42-kDa band) no significant changes due to lactacystin levels in the controls or hydrogen peroxide-treated cells were observed. However, as already demonstrated in Fig. 1A, a loss of actin occurs due to hydrogen peroxide treatment. With this new set of experiments, we noted two major zones of actin localization in the high molecular range: one in the stacking

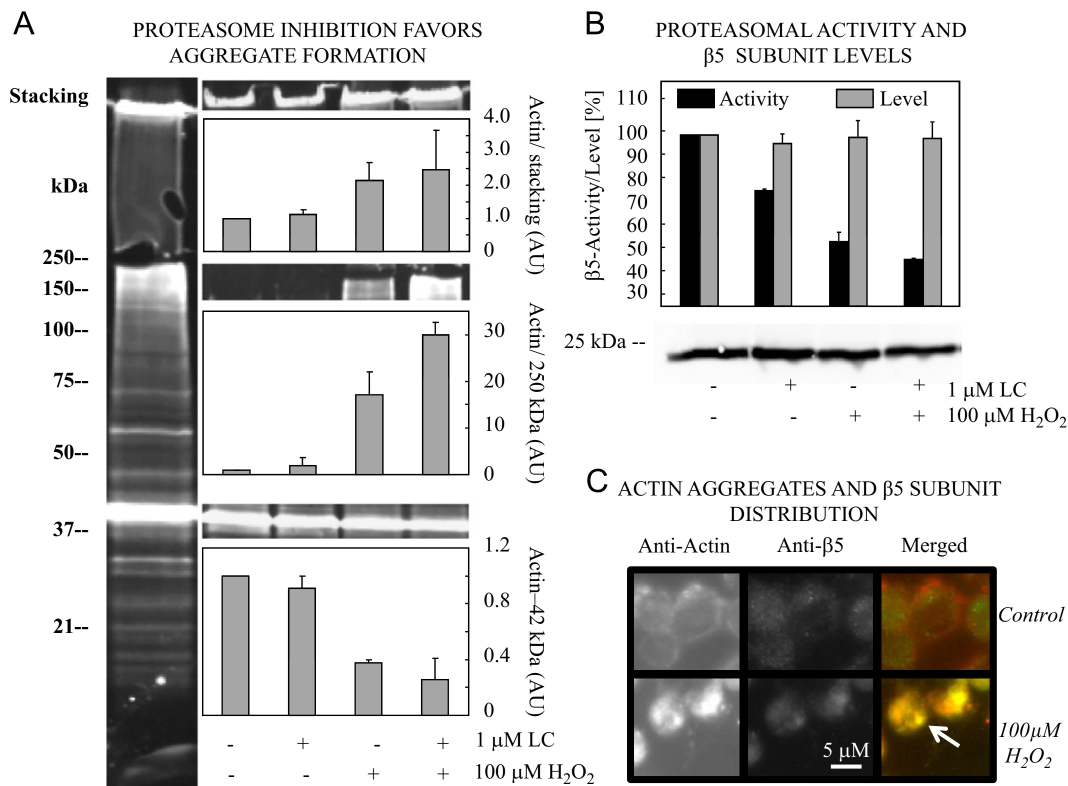

Fig. 2. Actin aggregation and proteasome inhibition. Jurkat cells were incubated as described above with the exception that the proteasome inhibitor lactacystin was present in some of the experiments. (A) The four different tested conditions were control, control + lactacystin, 100 μM hydrogen peroxide, and 100 μM hydrogen peroxide + lactacystin. Analysis was performed after 24 h. After probing with an anti-actin antibody the 42-kDa band of lactacystin and the two regions of aggregate formation, 150–250 kDa and the stacking gel zone, were quantified by analyzing three independent experiments (mean ± SD are shown). (B) At 24 h after hydrogen peroxide treatment the proteasomal activity was measured as described under Materials and methods (black columns). Three independent experiments were performed (mean ± SD are shown). Conditions are the same as in described in Panel A. A representative immunoblot for the β5-proteasomal subunit is shown below the activity panel. Three blots were performed and one representative is shown. Immunoblot quantification of the three blots is shown by gray columns. (C) Actin distribution in Jurkat cells with and without treatment with hydrogen peroxide is shown in the left column images. The cellular localization of proteasome β5 subunit is shown in the middle row, whereas on the right, the figures demonstrate the overlay of both images. These were captured with a fluorescence microscope (see Materials and methods).

zone, already noted in Fig. 1C and D, and one in the high molecular weight of 150–250 kDa exhibiting colocalization with protein carbonyls. It is clearly visible that the quantification of both zones revealed that they are increasing dramatically due to hydrogen peroxide treatment, independently of proteasome inhibition.

Since hydrogen peroxide treatment increases protein aggregation with little or no effect of proteasome inhibitor lactacystin, we decided to test for proteasomal activity. As already noted lactacystin reduced the proteasomal activity by some 25%. However, as demonstrated in Fig. 2B hydrogen peroxide treatment reduced the proteasomal activity by some 50% after 24 h. Next we decided to test whether the reduced proteasomal activity is due to a reduced expression. As demonstrated in Fig. 2B the expression of the β5 subunit is not changed due to lactacystin or hydrogen peroxide treatment. Since it is known that the 20S proteasome is largely resistant to oxidative stress, at least compared to the 26S [18], we proposed that protein aggregates are a major factor in reducing the proteasomal activity, as shown earlier [8,9]. Nevertheless, to make sure that the aggregates were held responsible for this decrease, we checked for proteasome activity at time points where no relevant aggregation took place. So we tested the proteasomal activity at the 3 h time point, where we could observe no significant aggregate formation (see Fig. 1). At this time point the proteasomal inhibition by lactacystin (80% inhibition) exaggerates clearly the inhibition by hydrogen peroxide (35% inhibition), whereas this relation turns vice versa at 24 h (see Fig. 2B). Since there is no aggregation at the 3 h time point, we attribute the ongoing proteasome inhibition at later time points (24 h) to protein (actin) aggregation. These data provide evidence that proteasome activity inhibition, not its protein level, is the mechanism that allows the accumulation of carbonylated actin and makes it prone to form aggregates in these cells.

Actin aggregates colocalize to the proteasome

In order to test whether the decrease in the proteasomal activity could be related to interaction of actin aggregates with the proteasome, we used an immunocytochemical assay with antibodies against actin and the proteasomal β5 subunit in control and 100 μM H_2O_2-treated cells. Treated cells displayed one or more dense spots with intensive actin labeling which, most interestingly, colocalized with the proteasome (Fig. 2C). This suggests a binding between actin aggregates and the proteasome. This was not seen in control cells, which exhibited a delicate homogeneous distribution of actin and a uniform proteasome labeling (Fig. 2C). Taking these results into account, it seems that the proteasome does lose its activity by binding to actin aggregates.

Actin aggregates as a result of oxidative challenge

After noting that oxidative stress induced actin aggregation in Jurkat cells, we decided to analyze these aggregates further by immunocytochemistry. First, we could clearly demonstrate that, as expected, the main amount of carbonylated proteins is found in the cytosol (Fig. 3A), as already reported earlier [31,32]. Interestingly, the distribution is not even as reported for other cell lines,

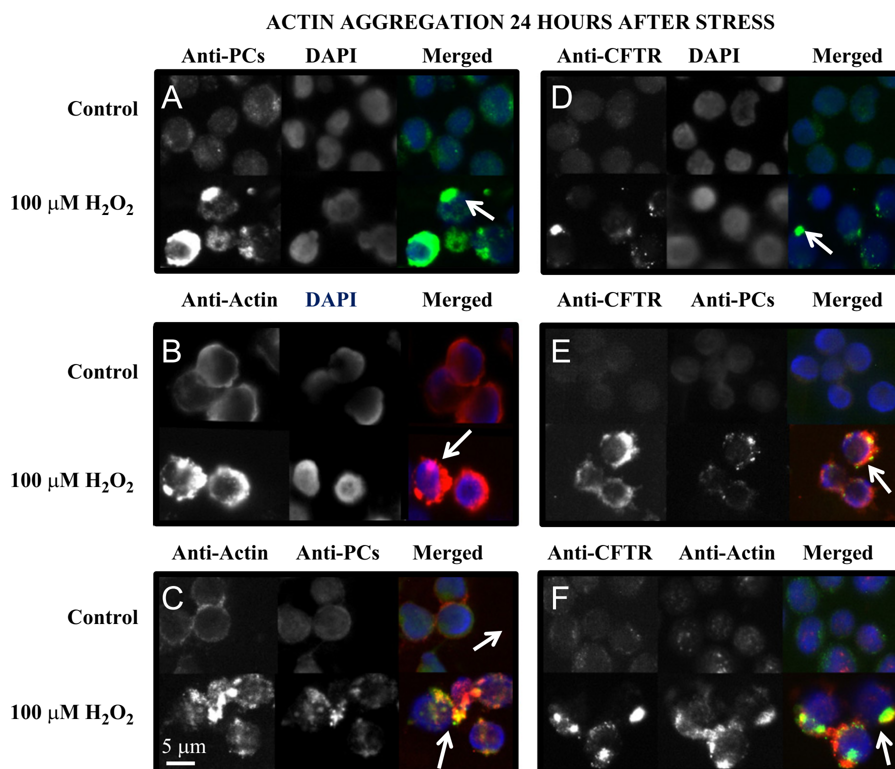

Fig. 3. Actin aggregates in aggresomes. For each panel upper and bottom images are from control and 100 μM H_2O_2, respectively, and are taken after 24 h of exposure to hydrogen peroxide. Localization of protein carbonyls and DNA (A); actin and DNA (B) and actin and protein carbonyls (C) are shown on the left panels. The right panels show the colocalization of CFTR, an aggresome marker protein, with DNA (D), protein carbonyls (E), and actin (F). Arrows indicate the presence of protein aggregates/ aggresomes. Images captured with a fluorescence microscope (see Materials and methods).

but isolated spots are visible. In a next series of experiments we could clearly show that actin shows the same uneven distribution pattern after treatment with hydrogen peroxide (Fig. 3B) and in colocalization studies a clear colocalization of actin and the protein carbonyls was found (Fig. 3C). This confirms the results of our electrophoretic studies (Fig. 1 and Fig. 2A) where an aggregation of carbonylated actin was suggested. For several years it is known that damaged proteins might accumulate in structures called aggresomes [33] and that CFTR (cystic fibrosis transmembrane conductance regulator) is a marker of aggresomes [33,34] we decided to test the colocalization of protein carbonyls and actin with CFTR (Fig. 3D–F). A clear colocalization can be found in hydrogen peroxide-treated cells between CFTR and carbonylated actin aggregates. Taking these results into account, there seems to be sufficient evidence in favor of carbonylated actin aggregating in aggresomes and binding to the proteasome, therefore reducing the functional capacity of the proteasomal system.

Oxidative stress leads to F-actin rearrangements

Following actin aggregate mass verification in Fig. 3, we wanted to check in which way the cytoskeleton arrangement would be affected. To achieve that we stained cells with phalloidin, a high affinity drug for F-actin. Using confocal microscopy and Z-stack settings, which seemed the best approach for structural findings, we can see by visualizing (Fig. 4) that there are notorious differences between control and hydrogen peroxide-treated cells. Control cells exhibit normal actin display occupying almost the entire periphery of the cell. This was not seen in treated cells that showed not only major F-actin aggregates, but cellular zones devoid of F-actin. These results confirm the earlier results of actin aggregate formation and showed that actin condensation leads to cytoskeleton rearrangements.

Oxidative stress leads to an irreversible growth arrest state

Our initial attempt was to test whether low dose oxidative stress might impair the cellular function of Jurkat cells. As seen in Fig. 5, under the tested conditions the cells are viable, but due to malfunction of the proteasomal system are forming aggresomes with F-actin as a major component (Figs. 2–4). In order to test whether the presence of protein aggregates also under nontoxic conditions influences the dividing ability of the Jurkat cells, we performed proliferation experiments. In Fig. 5A it is clearly visible that hydrogen peroxide-treated cells are losing their dividing ability in a concentration-dependent manner. Also 72 h after the start of the oxidative challenge the proliferating ability had not recovered. However, since the loss of proliferation might be due to the loss of some essential (perhaps reducing) components in the cells or the surrounding media, we decided to perform an additional set of experiments and changed the cell culture media after 72 h and monitored cellular proliferation for some 72 h. As shown clearly in Fig. 5B the oxidatively challenged Jurkat cells did not regain proliferation, even under optimal new growth conditions. This permanent inhibition in proliferation we attributed to the persistent presence of protein aggregates in Jurkat cells.

In vitro-formed actin aggregates inhibit the proteasome and block Jurkat proliferation

To reveal some mechanistic evidence to demonstrate the leading role of actin aggregates in inhibiting the proteasome and interfering with Jurkat proliferation, we decided to produce actin aggregates *in vitro*, employing a purified nonmuscle actin.

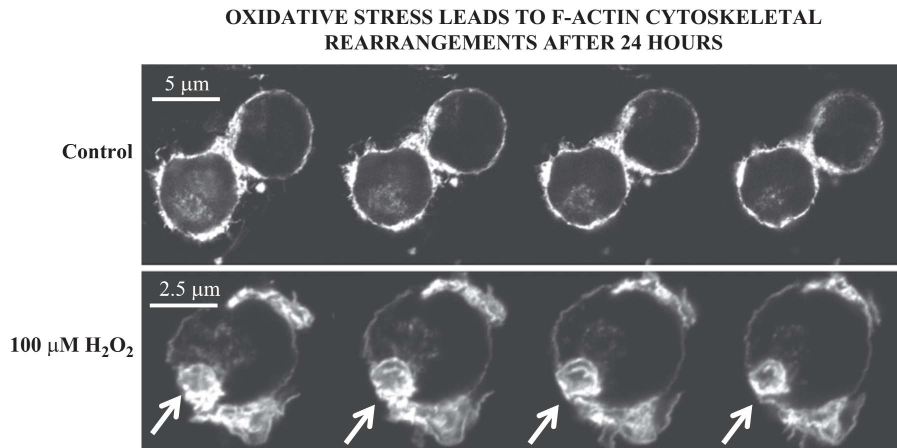

Fig. 4. F-actin redistribution after hydrogen peroxide treatment. Jurkat cells were cultured as described above under control conditions or with 100 μM H_2O_2 treatment. Using the specific F-actin staining phalloidin, images show uniform F-actin display in control cells, but not in cells treated with hydrogen peroxide. Actin aggregates can be visualized (shown by the arrow); also worthy of note are cellular zones apparently lacking actin, which seems to be condensed in aggregates. Z-stack images captured from a confocal microscope (see Materials and methods). Cells were analyzed in 16 Z-stacks each 0.55 μm wide. Stacks 6 to 9 are shown. Arrows indicate the presence of actin aggregates/aggresomes.

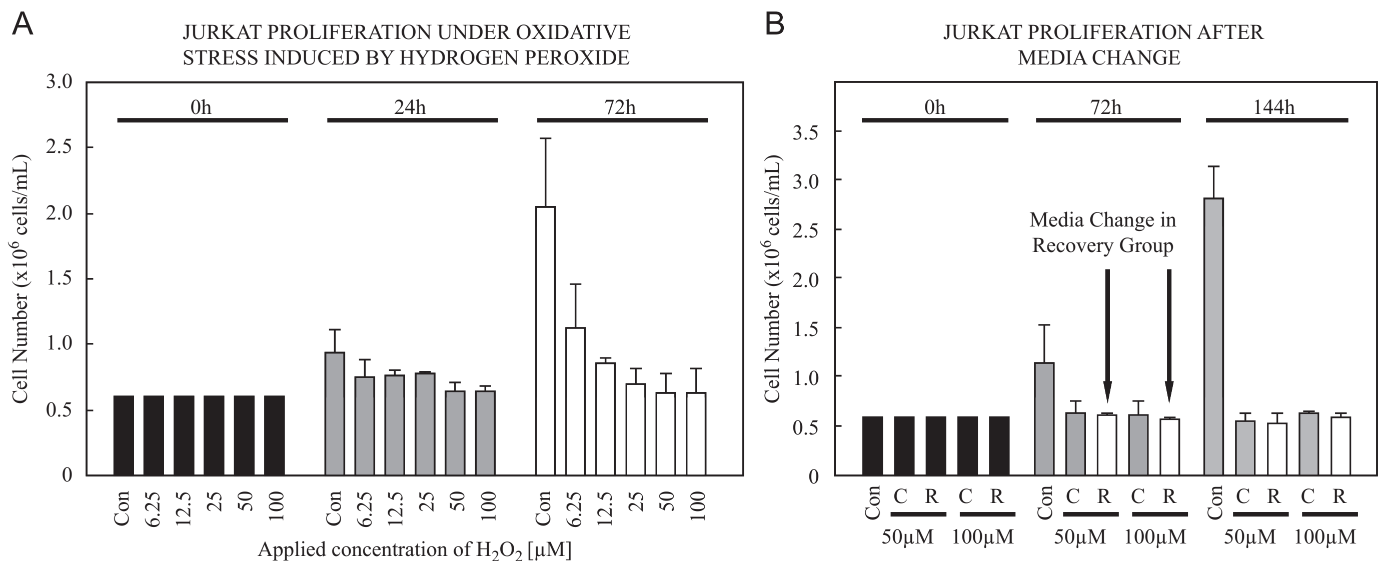

Fig. 5. Proliferation of Jurkat cells is impaired by hydrogen peroxide treatment and protein aggregates. Jurkat cells were treated with the indicated concentrations of hydrogen peroxide. (A) Cells were cultivated and after the indicated time points cells were harvested and counted. In panel B the same experiment was performed, with the exception that after 72 h of cultivation in some culture flasks the media were changed once (R = recovery after stress media), whereas in the control (C) the media were not changed. All measurements were performed using the trypan blue method (see Materials and methods). The mean ± SD of three independent experiments is shown.

As shown in Fig. 6A, exposure to increasing amounts of hydrogen peroxide results in a 42-kDa normal actin band intensity decrease. Conversely, several bands of protein appear above 75 kDa. Peptide mass fingerprint provided clear evidence that they were composed of actin (data not shown). In contrast to the study with Jurkat cell extracts, aggregates were absent from the stacking zone of the gel in this experiment, suggesting a further processing of initial actin aggregates in cells.

Next we tested whether these *in vitro*-formed aggregates are taken up by Jurkat cells, as reported for other cell types [8]. After incubation of Jurkat cells with actin aggregates for 24 h, we performed an immunocytochemistry staining technique against actin. As shown in Fig. 6B, control cells exhibited a typical uniform distribution throughout the cell, already seen in Fig. 4, and cells incubated with nonoxidized actin showed a similar distribution. However, cells incubated with oxidized actin/actin aggregates showed clearly the accumulation of actin aggregates as seen previously in 100 μM H_2O_2-treated cells (Fig. 4) and indicating cellular actin aggregate uptake.

The uptake of actin did not influence the cell viability (Fig. S3). However, in these treated cells we could clearly demonstrate a decline of the proteasomal activity with some remaining 70% of the chymotrypsin-like activity after 24 h incubation of Jurkat cells with oxidized actin. This decline was not accompanied by changes in the amount of the β5-proteasomal subunit (data non shown).

Next we tested, whether the accumulation of actin aggregates—without exposing the cells to hydrogen peroxide—is influencing the cellular proliferation of Jurkat cells. Cells treated with oxidized actin/actin aggregates for 24 h lost proliferating ability as well as hydrogen peroxide-treated cells (Fig. 6C). None of the controls (GAB buffer or nonoxidized actin) are able to influence cell proliferation. This indicates that actin aggregates mimic the ability

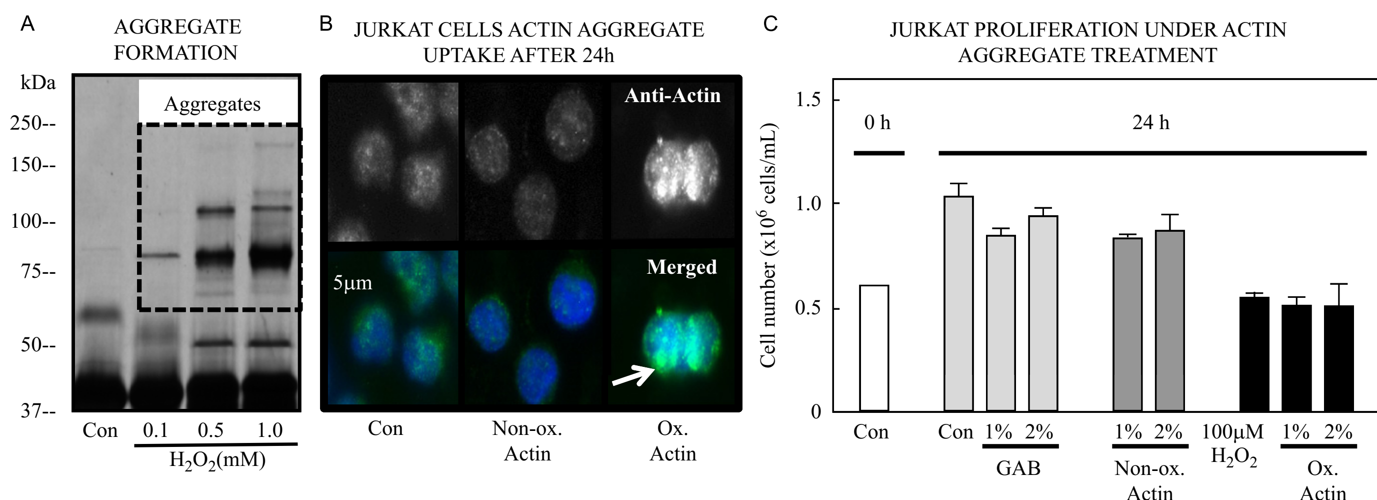

Fig. 6. Actin aggregates are taken up by Jurkat cells and block proliferation. An *in vitro* exposure of actin to hydrogen peroxide was performed as described under Materials and methods. Panel A shows a representative silver-stained gel of untreated actin (control) and actin treated with hydrogen peroxide (0.1, 0.5, and 1 mM). The formed actin aggregates are highlighted by the dashed line. (B) The uptake of nonoxidized (non-ox.) and oxidized (ox.) actin was monitored 24 h after exposure of Jurkat cells. An anti-actin antibody was used (upper row) and a merged figure with the nuclear DAPI stain is shown in the lower row. (C) Jurkat cells were incubated with the indicated amounts of nonoxidized or oxidized actin or the general actin buffer (GAB) for 24 h. All measurements were performed using the trypan blue method (see Materials and methods). The mean $\pm$ SD of three independent experiments is shown.

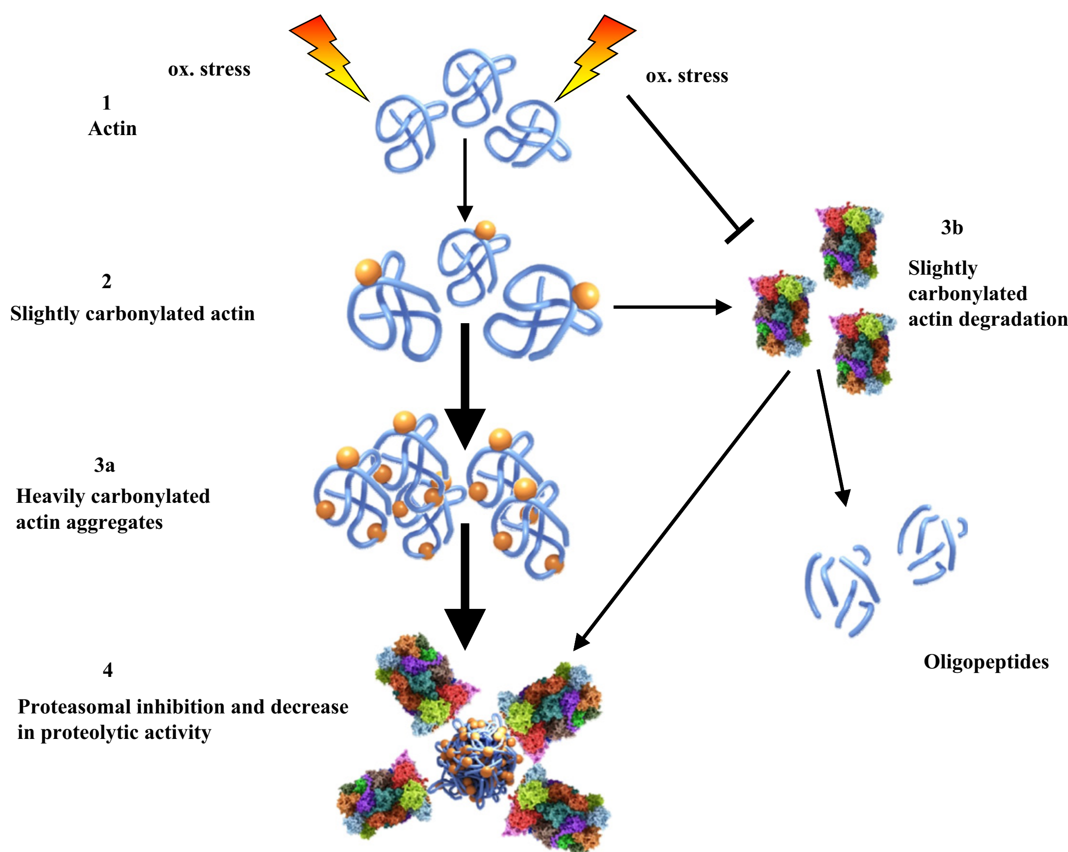

Fig. 7. Proposed events for actin aggregate formation and proteasome inhibition. Native actin (1) is moderately oxidized if cells are challenged with hydrogen peroxide (2). At the same time oxidative stress slightly impairs the proteasome; however, some of this oxidized actin is degraded (3b), but as the stress persists, and because the proteasome was partially inhibited, parts of it are heavily oxidized and starts to form actin aggregates (3a). Actin aggregates bind to the proteasome inhibiting its proteolytic activity (4) and influence, as accumulated aggregates, the cell metabolism.

of H_2O_2 to cause growth arrest (Fig. 6C). Taking these results into account, we can clearly conclude that artificial actin aggregates can directly affect normal cellular processes such as proliferation and proteasome activity

Discussion

Within the damage provoked by exposing biomolecules to free radicals, proteins account for a major target, resulting often in

protein carbonylation. There seems to be different protein susceptibilities to carbonylation, but one protein that has been widely described as a sensitive one is actin [11]. Its ubiquity and cellular abundance not only in human cells but in several other organisms, and its involvement in manifold cellular processes, such as motility [35], adherence [36], gene expression [37], immunological synapse [38], cell secretion [39], and cell division [40], make actin a relevant target of study. In fact, alterations in the structure and dynamics of the cytoskeleton have been reported in many age-related diseases such as cancer [41] and neurodegenerative disorders [42].

In this work with a T-cell model, we have shown the susceptibility of actin to aggregation. Together with the known effects of protein aggregates on cellular processes, we hypothesize a role of actin aggregation in the diminishment of T-cell functionality, namely proliferation. Our data suggest a two-way event for actin aggregates formation, following the general mechanism proposed by Jung et al. [32]: the oxidative attack leads first to a slight protein oxidation, causing misfolding in the native protein state; if the stress persists, further oxidation aims at the hydrophobic residues that are normally buried inside proteins and these become heavily oxidized, which favors the formation of protein aggregates.

To cope with and prevent the accumulation of oxidized proteins, which can lead to an imbalance of the proteome, they are normally degraded by the proteasome. However, this process may be impaired when the rate of oxidized protein formation exceeds proteasomal capacity or when the proteasome itself has a decreased activity. In fact, Das et al. [43] showed a loss of catalytic activity of the proteasome, following exposure to prooxidants, resulting in a decline in activation-induced proliferation and an increase in the accumulation of carbonylated proteins. These data perfectly fit with our results evidencing decreased proteasome activity and cell proliferation in oxidatively challenged cells. In addition, we observed carbonylated actin aggregates and, furthermore, we were able to make an immunocytochemical colocalization of the aggregates with the proteasome, an additional support to the view that they contribute to proteasome inhibition and the decline of its proteolytic activity. To support our view, we were even able to show a direct effect of artificial actin aggregates in proliferation and proteasome activity. In Fig. 7 we summarized our working scheme on the role of oxidative stress in the formation of actin aggregates. If native actin is exposed to oxidants it becomes moderately oxidized. At the same time, oxidative stress impairs the proteasome [18]; however, some of the oxidized actin is degraded. But as the stress persists, and because the proteasome was momentarily inhibited, it allows heavily oxidized actin to start aggregation. The formed aggregates interact with the proteasome, inhibit its activity, and lead to a loss of Jurkat functionality, e.g., proliferation.

In fact, a decreased proteasome-mediated degradation has been shown in T cells from elderly people and was thus proposed to play a role in immunosenescence [44]. In addition, studies by Chondrogianni et al. pointed out that the inhibition of the proteasome promotes the induction of a senescent state, while the stable expression of some proteasome subunits results in increased proteasome activity and cell survival following oxidants exposure [45].

So, as a conclusion, we propose that actin aggregates contribute to T-cell functional impairment, in part, by inhibiting the proteasome activity. We also hypothesize that actin's ability to form high molecular aggregates is extended to other cells, such as brain and skeletal and heart muscle, with similar functional outcome.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2012.06.005.

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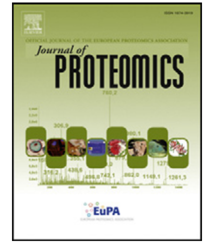

Review

Actin carbonylation: From cell dysfunction to organism disorder☆

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ABSTRACT

Protein carbonylation is an important event in the context of proteostasis because of its frequency, non-enzymatic nature and irreversible effects. The carbonylation of proteins disturbs their function and leads to protein aggregates, which may precede cellular senescence and cell death.
Actin, an evolutionarily conserved cytoskeletal protein that is involved in important cellular processes, is one of the proteins most susceptible to carbonylation. Conditions resulting in oxidative stress are likely to lead to its carbonylation, loss of function and aggregate formation. In this review, we summarise actin susceptibility to carbonylation, as verified in cell free extracts, cell lines and animal models, and review its fate through the activation of cell mechanisms aimed at removing damaged proteins. Their insufficient activity may underlie age-related diseases and the ageing process.
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Introduction

During aerobic metabolism, the transfer of electrons in the respiratory chain is not entirely efficient because of electron leakage at Complex I (NADH dehydrogenase) and Complex III (cytochrome bc1 complex), possibly at variable proportions, according to specific organs or cells [1] and to the life span of the considered species [2].

It is estimated that 1–2% of the oxygen used by mitochondria *in vitro* [1] is converted into superoxide in this way. Although this production in mitochondria seems to be the most important source of oxidising species, other locations in the cell provide additional contributions. That is the case of the endoplasmic reticulum membrane, where cytochrome P450 catalyses the oxidation of substrates, such as prostaglandins, steroid hormones, drugs and environmental xenobiotics [3]. Furthermore, other oxygen-metabolising enzymes are known to contribute to the release of oxidising species, such as xanthine oxidase, NADPH oxidases, cycloxygenases, lipoxygenases and others [4]. An additional oxidative burden results from the enzymatic production of nitric oxide (NO) radicals by at least three different types of NO synthase enzymes [5], autoxidation processes and the possibility of rapid reactions between all these species. Thus, although the superoxide anion (O_2^-) is likely the most permanently produced primary reactive oxygen species (ROS), it is also the precursor of other oxidising molecules that contribute to the whole level of oxidative compounds. When not buffered by antioxidants, they lead to oxidative stress and disturbing effects on biomolecules.

1. Effects of oxidising species on biomolecules

The effects of the superoxide anion in different cell compartments are restricted by the anion's limited ability to diffuse across membranes. Moreover, this anion is rapidly scavenged and dismutated by superoxide dismutases into hydrogen peroxide, a non-radical oxidant, which, in contrast with superoxide, rapidly diffuses within the cell. This is a useful property that is explored when cells in culture are experimentally challenged with hydrogen peroxide added to the medium. It is detoxified by catalases or by peroxidases from a variety of cell compartments but may also be converted into the highly reactive hydroxyl anion upon its cleavage by transition metal catalysed reactions, as is the case when Fe^{++} or Cu^{+} ions are oxidised. It appears that under normal conditions, metal catalysed oxidation is a major source of oxidative damage [6] that is permanently exerted on such molecules as sugars, nucleic acids, lipids and proteins.

The large array of products formed from the oxidation of biomolecules is dependent on the nature of the damaged biomolecule, the oxidant and the biological environment. Briefly, the oxidation of polysaccharides results in their depolymerisation or fragmentation [7], whereas continued oxidation of glycated proteins promotes the formation of advanced-glycated end products (AGEs) and the ability to interact with the receptor for AGEs and enhance the oxidative burden upon nearby molecules [8]. Also worth consideration is the effect of oxidants on the sugars of nucleotides that lead to their carbonylation, ability to bind iron and to intensify the Fenton reaction. In addition, ROS target nucleotide purines and pyrimidines that result in base deamination, ring openings, adduct formation and whole nitrogen base loss, which, together with the failure of repairing mechanisms, allow the establishment of mutations. Lipids are another class of molecules attacked by ROS, where the carbon–carbon double bonds of unsaturated fatty acids are an important target. The effects may be limited, but in the presence of oxygen, they may progress to the formation of new peroxyl radicals that continue oxidative damage on new lipids and produce more oxidised lipid hydroperoxides.

An important category of biomolecules that are subjected to the oxidation process are proteins. The effect of free radicals on them is diverse and relates to the intensity of the challenge, the actual oxidised amino acids and the type of protein involved [9]. The oxidation may result from the direct interaction with free radicals or may be indirect, secondary to the action of other previously oxidised proteins, lipids or sugars [10–12]. The effects may be mild and reversible when the oxidation is limited to a few amino acids, for example, when sulphur-containing residues of cysteine or methionine are oxidised to cysteinyl derivatives or methionine sulphoxide and benefit from the repairing ability of specific reductive systems [13]. By contrast, the effects may be irreversible, for example, when the attack addresses a variety of amino acid residues with aromatic or long side chains, such as histidine, tryptophan, tyrosine or phenylalanine [9]; moreover, the oxidation of other amino acid residues as proline, arginine, lysine and threonine, results in irreversible carbonyl formation, a consequence of major importance because of their quantity [12,14–17].

Protein carbonylation is an indicator of severe or permanent oxidative damage [18], as it occurs in many disorders that affect cell functioning and survival [6] and following exposure to oxidants in experimental conditions. In this case, the assessment of protein oxidation is performed primarily using common methods of carbonylation assay, which is one reason why carbonylation and oxidation are frequently used synonymously, as we will do in the rest of this manuscript. Protein carbonylation assessment is currently simplified by the development of such techniques as the derivatisation of carbonyls with 2,4-dinitrophenylhydrazine (DNPH) followed by immunodetection with an anti-DNP antibody in a Western blot or ELISA assay or the derivatisation with biotinhydrazide and detection with fluorescent avidin probes. Frequently, these procedures are based on 2D gels, which amplify protein resolution and allow further analysis, such as the quantification of the oxidation level in relation to the overall protein content, or the protein identity *via* mass spectrometry. In specific cases of protein identification, previous protein enrichment procedures are necessary. Recently, the 2-iminobiotinylation of carbonylated peptides or derivatisation with Girard P reagent were proposed before further protein quantitation or identification (for a detailed review of methods, see [19]).

It is generally accepted that the damage of proteins leads to their abnormal conformation, loss of functional ability, unfolding and exposure of hydrophobic domains, which mark them for degradation by the proteasome [20,21]; alternatively, such domains are the targets for ROS attack and further protein derangement. Of equal importance, the exposure of hydrophobic, sticky domains, usually concealed inside the protein structure,

enhances unspecific binding with other oxidised proteins and promotes aggregation, which, *via* cross-linking, leads to the establishment of high-molecular-weight aggregates [11,12,18, 22,23]. Additionally, the generation of a Schiff base by the reaction of a carbonyl group of one protein with an amino group from another, *e.g.*, lysine residues, also contributes to the formation of aggregates and indicates that they are able to grow without further oxidation reactions [24].

Notably, such aggregates are hard to remove from the cellular environment and are stored. Independent of their formation, the majority of these oxidised protein aggregates are compiled in the lysosomes and are taken up *via* macrophagy [25]. However, there is another storage form – the aggresomal structure – which is aimed at specific proteins (perhaps cytoskeletal proteins) or is an intermediate storage form [26–28].

The historical view that those stored proteins are in an inert final deposit, where they cannot influence the cellular metabolism, has long been overcome. The aggregates are able to affect the cellular metabolism and lead to cell death [27,28]. In addition, beyond exhibiting resistance to proteasome degradation, they disturb the cellular degradative ability of other proteins [6,29–31] by inhibiting the proteasome [31,32]. Moreover, it is known that because of the presence of various metals in accumulated protein aggregates [33], these aggregates can produce oxidants in a senescent cell [34]. Furthermore, by various mechanisms described, such protein aggregates are able to influence gene expression on a transcriptional level [35,36].

2. Actin: a cell-wide dynamic polymer with active involvement in cell functioning

The discovery of actin was reported by Brunó Straub et al. in 1942 upon its extraction from skeletal muscle cells, where it occupies a large proportion; later, actin's presence was verified in many other cells, albeit exhibiting a different isoform [37]. The protein is not restricted to eukaryotic cytoplasm because it takes part in nuclear structure and functioning. In addition, the presence of structurally similar proteins in bacteria [38] indicates that actin is an ancient cell protein and an evolutionarily successful one. Thus, it is not surprising that actin participates in many different and fundamental cell and tissue functions [39] that range from mechanical support for cell shape and muscular contraction to endocytosis, organelle movement, neuronal and immunological synapses and cell movement toward chemical attractants.

The actin monomer, actin G, is the fundamental actin structure, whichever isoform is considered. Actin is a 375-amino-acid protein composed of two main domains separated by a cleft, where an ATP complexed with Mg^{++} is bound and ATPase activity is located. Monomers polymerise after slight rotational movement of the incoming monomer that eventually results in an apparent double stranded helical conformation. An important prerequisite of this process appears to be a hydrophobic loop of actin G monomer that interacts with a hydrophobic pocket located between other actin monomers and stabilises the filament thereof.

The human genome has 6 actin genes that encode 4 muscle (α) and 2 non-muscle (β and γ) actin isoforms; however, despite this diversity, the protein amino acid sequence is rather similar among all isoforms, except the amino terminal [40]. A noteworthy example of the conservation of actin among eukaryotes is the 88% identity between vertebrate striated muscle actin and yeast single gene coded actin [41].

3. From cells to organisms — actin carbonylation as a result of stressful conditions

From *in vitro* cell-free studies, through cells and organisms, the verification of actin susceptibility to oxidation has gathered increased interest in recent years.

In vitro, purified actin is oxidised in a way that is dependent on the concentration of the oxidant and on the time of exposure [42]. Cysteine 374, at the carboxyl extremity of the α actin (or Cys272 in β and γ actin) [43] and the yeast actin [44] are notably sensitive to oxidation because of their localisation at or close to the surface of the actin molecule; these reactions are followed by oxidation of methionines 44, 47 and 355 [45]. Actin carbonylation imparts the enhanced exposure of its hydrophobic regions and narrowing of the cleft separating the 2 main domains, which leads to the reduced ability to complex with ATP [45] and to polymerise. Actin oxidation may be elicited by a variety of different compounds, such as chloramines [45], acrolein [46], hypochlorite [42], hydrogen peroxide [47,48] and others [49–52].

Although several of these studies were addressing actin changes, others were unexpected observations in different contexts and fields. In fact, it was shown that the exposure of different cells or biological samples to oxidative conditions affects many proteins, of which actin was found to be notably sensitive, as evidenced by carbonylation assays.

Diet composition, for example, may lead to a variation in actin carbonylation and nitrotyrosination [53], suggesting the possibility for its prevention; a point supported by the beneficial effects of adding oats to the diet [54]. Antioxidants have been studied intensely because of their hampering effect on proliferation, although the underlying mechanism may differ according to the compound. For instance, the use of hops antioxidants, such as proanthocyanidins, resulted in HT29 colon cell line proliferation arrest [55], an effect attributed to substantial actin carbonylation and cytoskeleton functional impairment. A similar consequence was noted in epithelial Caco-2 cells [56], yeast cells upon hydrogen peroxide administration [44] and a neuronal cell line after inflammatory cytokine challenge [57].

Animal studies on the effects of other stressors and environmental agents on carbonylation were also examined in conditions that emphasised the relevance of cytoskeletal protein oxidation. For example, α-actin from cod fish muscle is rather sensitive to ferrous catalysed oxidation [58] and ferric nitrilotriacetate administration to rats results in increased oxidised proteins in gastrocnemius muscle [50], whereas the *in vivo* contact of mussels with environmental pollutants was followed by carbonylation enhancement of β-actin in their muscle cells [59]. In a different setting, the unexpected detection of carbonylated actin in the serum of pigs housed at high densities [60] was considered a potential stress biomarker.

The progressive modifications of actin molecule have functional consequences. For example, whereas mild oxidation of surface sulphur-containing residues has a limited effect on actin polymerisation ability, additional oxidation strongly inhibits it and even enhances its fragmentation [45].

In addition, when oxidative insult is intense and continued, actin aggregate formation ensues. This was shown in muscle [42] and non-muscle [48] actin-rich cell-free fractions. Furthermore, in a cell line model of hydrogen peroxide oxidative stress [48], such aggregates were noticed at 24 h of cell culture, but not at a shorter, 3 hour stress time, further indicating that beyond its susceptibility to carbonylate, actin's ability to form aggregates is time-related.

All these data converge with the view that actin is among the most preeminent proteins oxidised upon cell exposure to oxidants [43], but the reason for this susceptibility is not known. One possible reason is the intracellular actin content: actin constitutes 10% of the whole protein in normal and leukaemic lymphocytes, approximately 8% and 5%, respectively [61], myeloid leukaemia cells (5%, [62]), adrenocortical cells (10–12%, [63]) and fibroblasts (7–14%, [64]). Another possible reason is the actin localisation: it is present throughout the cytoplasm, off the lumina of membranous compartments, therefore, it is easily accessible to diffusible oxidative compounds. Additionally, similar to other proteins, actin may have specific structural features that make it more prone to oxidation. For example, upon submission of yeasts to H_2O_2, only 8% of the whole yeast protein becomes oxidised, but this oxidation amounts to 80% when ribosomal protein is considered [65]. By contrast, in *Escherichia coli* cultures, H_2O_2 oxidation targets the protein synthesis factor EF-G [66], and when the cells are grown in a medium with a methionine substitute and stressed, the small increase in total carbonyls is outweighed by remarkable carbonylation of EF-Tu, the most plentiful protein in that bacteria [67]. Similarly, oxidation of arthropod mitochondria results in isolated aconitase carbonylation [68].

We would add that muscle actin extracted and processed for scientific purposes may spontaneously oxidise [69], and its storage at usual freezing temperatures may not be sufficient to prevent its oxidation after some time [70], further emphasising its susceptibility and the need to employ carefully controlled conditions.

Actin carbonylation/aggregation and its biological significance will be discussed further in the next sections.

4. The fate of carbonylated actin: turnover or aggregation?

The fate of actin inside cells under physiological non-oxidising conditions remains debatable. For example, what occurs to G-actin after F-actin depolymerisation and dismantlement? To the best of our current knowledge, there is no mechanism that promotes F-actin degradation, only depolymerisation by actin binding proteins, such as gelsolin [71]. However, evidence from cultured chick embryonic cardiomyocytes favours a role for caspase-2 in G-actin degradation by proteolytic cleavage. It was shown that lovastatin, a drug used to induce the loss of F-actin, operates in a caspase-2 dependent manner; when a caspase-2 inhibitor was employed, no actin degradation was found in this cell type [72], suggesting an important role for caspase-2 in non-oxidised actin turnover. There is also evidence from cultured neonatal rat heart cells that, when muscle contraction was inhibited by pharmacological drugs, α-actin was partially degraded by the lysosome, pointing to a different mechanism of degradation [73]. Recently, actin degradation has been linked to an E3 ubiquitin ligase motif-containing 32 (TRIM32), which binds myosin in skeletal muscle and ubiquitinates α-actin *in vitro*. The overexpression of TRIM32 in human embryonic kidney cells leads to a decrease in cytoplasmic actin, supporting the involvement of TRIM32 [74]. Thus, non-oxidised actin seems to be degraded by different mechanisms, therefore maintaining the debate of how it is actually degraded — an open question that needs further investigation.

Under non-physiological oxidative environments, cells have to cope with damaged proteins and prevent their accumulation; for that purpose, they use degrading systems, such as the proteasome. Although mildly oxidised proteins are an attractive substrate for proteasomal degradation [10,21,75,76], in conditions of ROS burst from environmental or intracellular sources, proteasome function is compromised or insufficient [77], and proteins tend to accumulate as non-degradable, insoluble aggregates that additionally contribute to proteasome inhibition [31,32]. In time, cells may attain the growth arrest condition and even die by apoptosis [22,34,78], with consequent tissue damage and dysfunction.

The proteasome, the most important non-lysosomal mechanism for protein turnover in cells [79], is a complex catalytic feature that exhibits two predominant forms, the 20S catalytic core, which is ATP and ubiquitin tagging independent, and the 26S form that combines the 20S core and at least one 19S regulatory cap, involved in the recognition of ubiquitinated substrates, which is, therefore, ATP and ubiquitin dependent. Extensive and detailed description of the structure and regulation of the proteasome is reviewed elsewhere [79].

The 20S proteasome form has been shown to be the main site for oxidised protein degradation [10,75,76,79–82], and there are at least three reasons for its preeminent involvement. First, the 20S proteasome itself is relatively resistant to oxidative stress [83]; second, oxidised proteins expose hydrophobic residues that under stressful conditions can act as a trigger for degradation, independently of ATP and ubiquitin tagging [20,21]; third, because lysine residues are preferential targets of oxidative modification, ubiquitin lysines would be less able to bind to substrates, therefore, could impair the whole process [10,79,84,85]. Thus, despite the large number of studies favouring the 20S proteasome role in degrading mildly oxidised proteins [10,21,79], when oxidation is too extensive, protein aggregates can form and even inhibit the proteasome [14,31,32].

The 26S proteasome generally requires the preceding action of the ubiquitin system, consisting of three types of enzymes: E1 ubiquitin activating enzymes, E2 ubiquitin conjugating enzymes and E3 ubiquitin ligases. Ubiquitination is a highly ordered and specific mechanism, and because of the diversity of existing enzymes, ubiquitination can form countless possibilities of arrangements. The process starts when the E1 enzymes bind one ubiquitin molecule to its C-terminal glycine, then ubiquitin is transferred to E2 enzymes. The last step of the chain is

composed of the ubiquitin binding from E2 to E3 enzymes and finally, depending on E3 enzyme type, ubiquitin is either tagged directly to lysine residues in the substrate or indirectly in a two-step mechanism (for detailed process description, see [79]).

Conversely, it was shown that proteasome inhibition increases the steady state amount of carbonylated proteins. In the post-ischaemic rat heart, oxidised actin, but not actin ubiquitin adducts, were found, suggesting a 20S proteasome-mediated proteolysis [86,87]. In support, we and the Davies group demonstrated that the disruption of the ubiquitin tag system by compromising ubiquitin conjugating activity did not impair the degradation of oxidised proteins [84]. Early on, cysteine and methionine were described as prone to oxidative stress, therefore, it is noteworthy mentioning that these modifications, particularly the Cys^{374} residue of actin, contribute to a decrease in actin susceptibility to α-chymotrypsin and subtilisin proteolysis, by changing the structure of the subdomain 2 in the actin molecule [88,89]. This could allow the formation of insoluble high molecular weight actin carbonylated aggregates. In fact, in hydrogen peroxide treated cells, we showed that carbonylated actin is partially degraded by the proteasome [48]. After inhibiting the proteasome with lactacystin in stress conditions, an increase of carbonylated actin and high molecular aggregate formation and accumulation were detected, thus pointing to the proteasome's ability to degrade moderate actin carbonylation; however, progressive impairment in a continuous oxidative environment and after the formation of actin aggregates was also observed. The binding of aggregates to the proteasome was shown by immunofluorescence co-localization studies of actin aggregates and the β5 proteasome subunit. These studies favour two possible fates of oxidised actin, depending on the duration of oxidative stress and proteasome functionality. Aggregate formation is, therefore, a likely scenario that can alter cellular metabolism and contribute to cell and tissue dysfunction (Fig. 1).

5. Actin carbonylation involvement in cytoskeleton disturbance, cell dysfunction and age-related diseases

External conditions, such as ionising radiation and atmospheric chemicals, may exert acute or chronic oxidative stress on cells or organisms that results in functional abnormalities. Similar consequences are observed in the persistent effects of internal disorders that include inflammatory reactions, chronic degenerative diseases or the simple living process throughout the organism's lifespan.

Ageing, particularly the last third of life, is associated with an enhancement of protein carbonyl content [90–94]; indeed, the continued effect of ROS on cells and tissues was proposed as a cause of the ageing process itself [95]. Because ROS are produced as long as cells and organisms are alive, the consequences are continuous and progressive. Not unexpectedly, the outcome is the reduced functional ability of cells or tissues, ill adaptation to stressful conditions and clinical findings of overt disease. In a variety of human disorders, a 2–3-fold increase in oxidised protein content was estimated, resulting in a level of up to one carbonylated protein in every 3 present [6].

A target protein that is part of this generalised age-related carbonylation is actin. Among others, actin carbonylation was found to be enhanced in the aged brain of mice [96], which was prevented by lipid peroxide scavengers in specific experimental conditions [97]. Despite the scarcity of data in this respect, such observations suggest that the carbonylation of proteins, such as actin, is included in the ageing process, and its prevention could be an approach to relieving the ageing phenotype.

Notably, the carbonyl content increases in a variety of tissues or cells, and its targeting of actin was shown in human disorders with cytoskeleton functional impairment, such as Alzheimer disease (AD). In necropsies of selected brain areas of AD patients, extracellular deposition of Aβ amyloid protein, intracellular fibrillary tangle accumulation and neuronal cell loss were described [98]. The tangle stem from intracellular aggregates of tau protein that normally promote microtubule stabilisation; in AD however, tau hyperphosphorylates, loses microtubule interaction and aggregates in abnormal helical polymers [98]. Furthermore, there is mechanistic evidence that Aβ and tau protein aggregates interfere with cellular protein turnover [32,99–101] and interact with actin, resulting in actin aggregation, which is hypothesised as the feature underlying cell loss in AD [102].

There is considerable evidence in favour of oxidative stress intervention in the pathogenesis of AD and other human degenerative disorders [103]. Indeed, the increase in protein oxidation is an important finding in the hippocampus and inferior parietal lobule of AD patients [104]; in addition, there is compelling evidence for increased carbonylated β-actin in AD-affected brain regions [105]. Moreover, protein oxidation increase was also verified in post-mortem exams of the brains from humans that were classified as having mild cognitive impairment [106], which provided additional support for actin carbonylation involvement in the pathogenesis of AD.

Similarly, enhanced actin carbonylation was found in human heart failure [107,108], in a fashion that correlated with organ dysfunction.

In the experimental conditions of an ischaemia–reperfusion injury model, the reperfusion of a previously ischaemic tissue is accompanied by a burst of inflammatory mediators and ROS production. Notably, the microglia of the hippocampus from reperfused monkey brains exhibited selective actin and HSP70 protein carbonylation [109], as did ischaemic rat heart muscle reperfused *ex vivo* upon previous ischaemia, in an apparent time-dependent fashion [47,86]. There is evidence that these processes result in strong actin carbonylation of up to 7-fold [47], and several findings also suggest that this might be suppressed by antioxidants [110].

Similar selective effects on actin were noticed in rat diaphragm muscles, following a systemic inflammatory state mimicked by LPS injection [111], and in the spinal cord of an experimental model of autoimmune encephalomyelitis [112]. Moreover, a model to study inflammation, employing macrophage cultures in excess of oxygen, also evidenced enhanced actin carbonylation together with actin depolymerisation in the cell periphery and its polymerisation deep in the cytoplasm [113].

Carbonylation of actin also occurs in malignant transformations, as was recently observed in human cervical cells from dysplastic lesions caused by the human papilloma virus infection

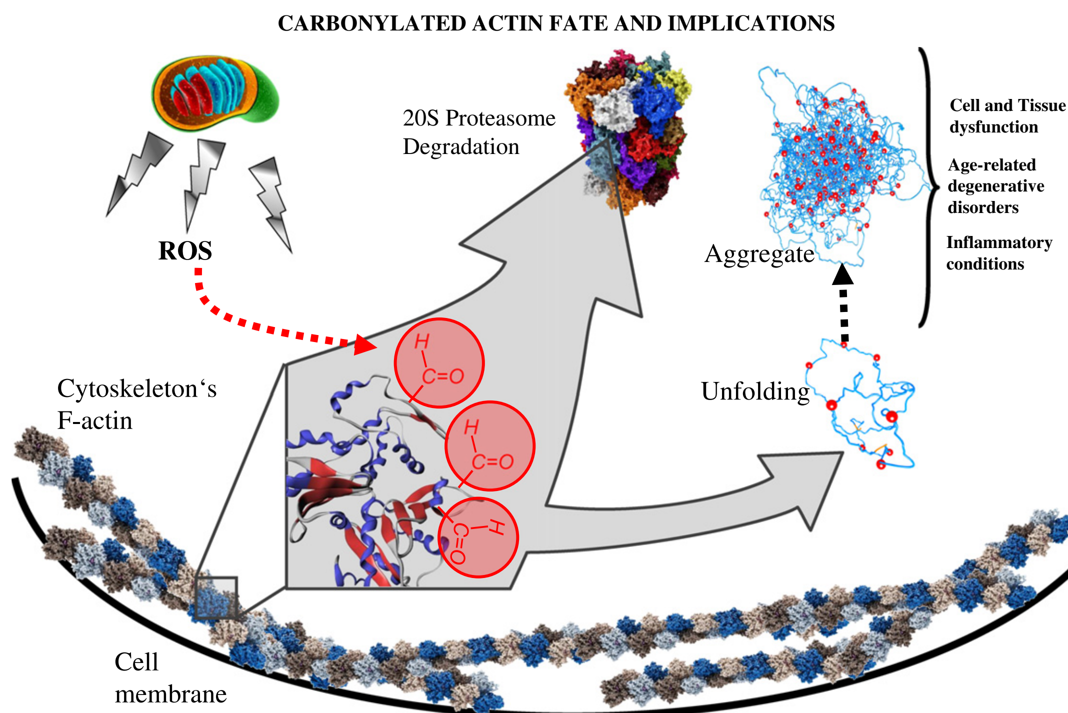

Fig. 1 – Fate of carbonylated actin and biological implications. When the cellular internal redox state changes because of enhanced ROS production, mainly by mitochondria, proteins become carbonylated. Actin (in blue), has a high susceptibility to this oxidative modification and undergoes functional impairment along three main proposed stages that range from mild protein oxidation to aggregation. In the first stage, actin is carbonylated (carbonyl groups in red), though it partially maintains its function; as stress persists, it becomes more oxidised, unfolds, exposes hydrophobic domains (in orange) and loses its function. At the third stage, upon further oxidation and/or proteasome inhibition, hydrophobic domains favour binding and aggregation with other carbonylated proteins. Although abundant in cells and engaged in many important activities, actin susceptibility to carbonylation and to form aggregates relates to cellular dysfunction. In fact, that was noticed in many conditions that include age-related degenerative disorders and chronic inflammation.

[114]. Carbonylation may also appear during carcinogenesis and cancer treatment. For example, acrolein, an oncogenic agent and a metabolite of cyclophosphamide was found to cause the aggregation of F-actin in rat Sertoli cells [46]. In addition, photodynamic therapy, a technique that is receiving greater attention and wider application in the field of superficial cancer in recent times [115–117], was found to cause actin carbonylation when experimentally applied to a cell line of bladder cancer [118].

6. Conclusions and prospects

From an evolutionary perspective, actin became an indispensable protein to cells and organisms because of its involvement in important biological activities.

Although the actin network is robust, it is also sensitive to derangements caused by daily living processes. One of these processes is the continuous production of reactive oxygen species that takes place mostly in mitochondria during aerobic metabolism. A major effect of ROS production is protein oxidation, which may range from simple reversible effects to irreversible consequences that include protein unfolding and carbonylation. This process, the best characterised protein oxidative modification, is non-enzymatic and irreversible and, depending on the type of stress, duration, and sort of protein involved, leads to protein loss of function, additional severe oxidation and the formation of high molecular weight oxidised aggregates. *Per se*, these may induce changes in cellular metabolism, such as inhibition of protein turnover by the proteasome, cell cycle arrest and even cell death by apoptosis.

Actin is organised in such a way that some of its amino acids are rather sensitive targets to oxidation. By contrast to the irreversibility of carbonylation, one may speculate that under milder oxidation, the localisation and susceptibility of some cysteine and methionine residues sense, buffer and revert the oxidation; in this case, the cell skeleton would work in a way that parallels the body skeleton when it buffers organism acidosis in the long term. However, actin intervention likely goes beyond that process. In fact, actin appears to promote mitochondrial integrity by preventing ROS release [119], and, during cytoskeleton organisation, it is glutathionylated at Cys374, in a process where ROS act as second messengers [120]; notably, actin was found as a largely glutathionylated protein under oxidative conditions [52].

Therefore, the mainly structural roles of actin may extend into sensor and regulatory post-translational properties. The refinement of methods employed in the assessment of actin molecule structural features may still uncover unexpected abilities.

Apart from its importance for the understanding of fine-tuned biological processes, actin is now an attractive molecule for the comprehension of human degenerative disorders and health as a whole, including the ageing process itself, the quality of food, heart disease, Alzheimer disease, sustained inflammatory conditions, carcinogenesis and cancer management. The recently enhanced interest for this widely present and evolutionarily conserved protein envisages exciting new knowledge in the near future.

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INTRODUCTION –PART II

In order to approach the mechanism whereby oxidized actin was conveyed to the proteasome, it was hypothesized that a transporter with the ability to bind actin and the 20S proteasome was the likely molecule. For this purpose, the proteostasis assuring Hsp90 chaperone appeared to adapt well.

1. Hsp90 – A role in assisting oxidized proteins degradation?

Protein structure is determined by its amino acid composition and the laws of thermodynamics; however, the macromolecular overcrowded cytosol can promote protein misfolding and aggregation in low stability proteins [106]. To cope with unfavorable consequences, cells evolved to synthesize proteins whose role is to assist and stabilize other proteins in a constitutively manner. They are usually referred as molecular chaperones [107] and most of them belong to heat shock proteins (Hsp) family. Under heat or oxidative stress, they are induced [108, 109] to quickly establish proteostasis.

Molecular chaperones as Hsp90 are ATP-dependent and are co-regulated by co-chaperones that define its final function [106, 110, 111]. Co-chaperones can regulate Hsp90 activity and cycle by stimulating or inhibiting ATP hydrolysis, by recruiting specific client proteins, by coordinating the interplay with other chaperones as Hsp70 or even by their enzymatic activities [106].

Hsp90 is highly abundant [106] and has been considered to have a major role in stabilizing a diversity of client proteins as kinases, nuclear hormones, receptors, cell surface receptors, or transcription factors in the cytosol [112]. Actin has also been show to be a client protein of Hsp90 [113-118].

The Hsp90 chaperone is a dynamic homodimer. Each monomer can be divided in three main domains, the N-terminal, the middle and the C-terminal domains. The C-terminal is responsible for dimerization and co-chaperone binding, the middle domain is responsible for substrate binding, whereas the N-terminal role is to hydrolyse ATP [106]. In a rest state, N-terminal is bond to ADP instead of ATP (Figure 4).

Hsp90 has a role in stabilizing proteins, but in oxidatively challenged cells, it appears to exert additional actions. It can protect the proteasome from oxidative inactivation [119, 120] and enhance oxidized proteins degradation [121]. Thus, a likely scenario, if Hsp90 contribution is lost during oxidative stress, degradation of oxidized proteins would diminished and accumulate in the form of insoluble protein aggregates. In turn, protein aggregates would contribute to further proteasome inhibition.

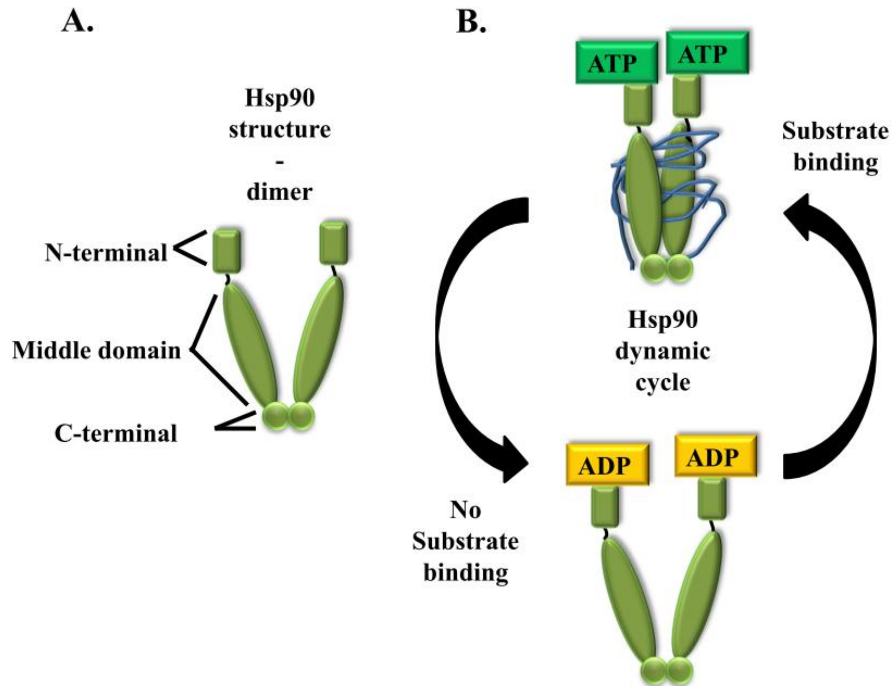

Figure 4. The molecular chaperone Heat Shock Protein 90. (A) The Hsp90 monomer is composed of three main domains; N-terminal for ATP hydrolysis, Middle domain for substrate binding and C-terminal for dimerization and co-chaperone binding. **(B)** Hsp90 cycle. The open conformation (bottom) refers to Hsp90 dimer rest state with no substrate but with ADP to the N-terminal. When Hsp90 binds a substrate, it adopts a closed conformation in order to refold, stabilize or send forward its client protein (top). At this point of the cycle, ADP is replaced by ATP, which is later hydrolyzed, which complete the cycle.

2. Crosstalk between proteasome and lysosomal autophagy

Besides the proteasome, the other main site for protein and organelle turnover is the lysosome. Proposed by Christian de Duve [122], the lysosome has an important role in cellular homeostasis. Due to lysosomal richness in hydrolases, it destroys and clears extraneous material and cellular short lived proteins, damaged organelles or even larger aggregates. In the past, this process, autophagy, was thought to be unselective but it is now recognized to be specific. Autophagy is divided in three types, chaperone-mediated autophagy, a type of protein transport due to recognition by molecular chaperone Hsp70 which fuses with LAMP-2, a lysosomal membrane protein, in the lysosome [123, 124]; microautophagy, described as a primary engulfment of small amounts of cytoplasm into lysosomes [125]; and macroautophagy, which is responsible for clearance of damaged organelles and protein aggregates through the formation and extension of a membrane that envelops the substrate and later fuses with the lysosomes [126]. Because protein aggregates are related to macroautophagy special emphasis will be given to it.

Macroautophagy, hereafter referred as autophagy, starts with a double membrane feature that expands and engulfs target organelles or protein aggregates before making them available for lysosome digestion. The characterization of the original membrane has received a considerable attention lately. It may result from *de novo* synthesis, but may also be originated from the ER, the mitochondria or the plasma membrane [127-129]; the exact source remains elusive. Current knowledge indicates that the process is regulated by about 30 genes, collectively designated as ATG (AuTophaGy) related genes. The ultimate aim of the process is to fuse with lysosomes, taking advantage on their enzyme rich content, and when fusion is achieved, the features are now called autolysosomes, to promote content degradation.

Briefly, in mammalian cells, when the signaling protein TOR (target of rapamycin) is inhibited due to starvation or oxidative stress. The beclin 1-class III PI3K (phosphoinositide 3-kinase) protein is required to form the pre-autophagosomal structure which is the putative site for autophagosome formation. Afterwards, Atg13, an essential autophagy factor, is dephosphosporylated, which allows its association with and activation of Atg1 homologues (ULK; uncoordinated family member-51-like-kinase). This two-protein complex is recruited by FIP200 (Focal adhesion family-interacting of 200 kDa) to form a new ULK-Atg13-FIP200 complex that will promote membrane elongation and complete the autophagosome structure and the encircling of the target. At the same time, the membrane is enriched in LC3-II (Light chain), the lipidated form of LC3-I. This is an ubiquitin like-protein that undergoes post-translational modification by ubiquitin-like reactions that culminate in the addition of a phosphatidylethanolamine (PE) to LC3-I. This conjugation process is critical in the autophagosome expansion process.

But how are protein aggregates or damaged organelles recognized directed to phagosomes in the first place? It is thought to involve ubiquitin and a p62/SQSTM1 adaptor protein. However, instead of ubiquitinating the protein at lysine 48, as occurs before directing substrates to the proteasome, it is made through lysine 63; in addition, it recruits p62 and the complex is forwarded to LC3-II enriched autophagosome membrane. Later, the autophagosome is completed in a process mediated by Rab7, a small GTPase, and LAMP-2. The structure then fuses with lysosomes providing direct contact with hydrolytic enzymes and with the substrate for destruction [6]. Together with proteasome mediated degradation, macroautophagy is a decisive turnover mechanism for clearing protein aggregates or damaged organelles. Not unexpectedly, in

recent years a cross-talk between the proteasome and lysosomal autophagy has been described, that conduces to autophagy upregulation when the proteasome gets inhibited.

PUBLICATION III

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HSP90 CLEAVAGE ASSOCIATES WITH OXIDIZED PROTEINS ACCUMULATION AFTER OXIDATIVE STRESS

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ABSTRACT

To remove mildly oxidized proteins, cells employ the 20S form of the proteasome. However, if the rate of protein clearance is inferior to its formation, as occurs under oxidative stress (OS) additional oxidation results in non-enzymatic, irreversible, protein carbonylation and functional loss, and the promotion of protein aggregates formation and accumulation. In Jurkat cells, upon stressful conditions, there is oxidized actin aggregates accumulation, impaired proliferation and reduced proteasome activity, conditions frequently observed in cell senescence. As Hsp90 is a molecular chaperone that assists oxidized proteins degradation and protects the proteasome from oxidative inactivation, it was hypothesized that oxidation would prevent its role as actin chaperone.

The study shows that Hsp90 is cleaved into a 73KDa form, the Hsp90c, by a non-enzymatic, time-related, iron and stress intensity dependent process. It reveals that cleavage appears early, progressively increases in the insoluble cytoplasmic fraction and later co-localizes with the actin aggregates. In addition, it demonstrates that autophagocytosis is activated along the time, as evidenced by LC3-II protein increase in cellular extracts.

These data thus indicate that Hsp90 has an actin chaperoning role and probably directs its oxidized form for destruction in the proteasome. In addition, the study provides evidence for a dual intervention of cell clearing processes, possibly compensatory, because when proteasome activity is reduced, phagocytosis is activated likely as a cell survival mechanism.

INTRODUCTION

The functional disability associated to ageing has been attributed to random, molecular dysfunction and errors, that accumulate in cells and tissues [1, 2]. While the underlying cause is debated, there is evidence favoring the processes of continued biological oxidation that, through the generation of by-products as reactive oxygen species (ROS), impinge on biomolecules, including proteins.

An important consequence is protein carbonylation, a non-enzymatic chemical change that is frequently observed in aged cells and tissues [3] and results in an irreversible protein structural modification and functional loss [4].

Mildly carbonylated soluble proteins are recycled at the proteasome. This multicatalytic feature assembles mainly in two forms, the ATP and ubiquitin independent 20S proteasome and the ATP and ubiquitin dependent 26S proteasome. Both have in common a cylinder shaped structure resulting from the apposition of αββα protein rings containing specific subunits; as the catalytic activity is exerted by some of the β subunits, the side positioning of α subunits limits the catalytic area exposure to hazards. While both forms of the proteasome are able to destroy and recycle proteins, it is the 20S proteasome that has been shown to be the main destination of oxidized or otherwise damaged proteins [5-11]; in turn, the 26S proteasome is able to degrade naturally folded and even functional proteins, when they are ubiquitin tagged [12].

As long as the proteasome clears oxidized proteins from the cytoplasm, their accumulation is prevented; eventually, as oxidation continues, proteasome capacity is exceeded, protein destruction decreases and further oxidation is fostered. This event promotes protein unfolding, hydrophobic residues exposure and the establishment of oxidized protein cross-links that result in insoluble, high molecular weight aggregates that directly impair the ubiquitin-proteasome system [13], disturb cell functioning, and may cause cell senescence or apoptosis [14-16]. Protein aggregate accumulation thus reflects cellular proteostasis impairment and proteasome dysfunction; ultimately, it accompanies the ageing process and conduces to organism disorder [17].

The mechanisms whereby oxidized proteins are delivered to the proteasome have remained elusive, but its elucidation would be of utmost importance for the understanding of cell functioning under stresses. It is reasonable to admit that there is a transporter having a strong interaction with the oxidized protein and the 20S proteasome, their major destruction site. Such a process was described for the cytosolic protein calmodulin that, when oxidized by hydrogen peroxide, is delivered for destruction at the 20S proteasome in a selective interaction with the heat shock protein 90 (Hsp90) chaperone [18].

Hsp90 functions as a homodimer, whose monomers are composed of three distinct domains. The N-terminal domain promotes ATP hydrolysis required for chaperone substrate remodeling, the middle domain has a role in substrate binding and the C-terminal interacts with the other Hsp90 monomer and binds co-chaperones by the use of a TRP binding segment [19].

As a chaperone, Hsp90 assists a large number of native (client) proteins, mostly transcription factors and kinases, to fold properly into their functional conformation [19]. In addition, Hsp90 interacts with pre-folded, configured proteins to assist them in stabilization of ligand binding regions, which frequently exibit hydrophilic charged surfaces, next to hydrophobic clefts [20]. Not surprisingly, Hsp90 inhibition is followed by client protein unfolding and ubiquitin tagging for destruction [19].

Interestingly, Hsp90 also interacts with the 20S proteasome, as reflected in the unusual difficulty in separating them [21]. In particular, Hsp90 binds to the proteasome alfa subunits [22] that, in the setting of oxidation, assist client protein degradation by the proteasome [18]. Moreover, such alfa subunit interaction appears to afford protection to the deeply located beta type, catalytic subunits; in fact, Hsp90 silencing in conditions of metal catalyzed oxidation, results in proteasome marked functional decrement and even inactivation [23, 24].

In a previous study, it was verified that submitting a T cell line to hydrogen peroxide mediated oxidative stress resulted in proteasome inhibition, oxidized protein aggregates formation and cell proliferation arrest [25], characteristic features of aged and senescent T cells in vivo [26, 27] and in vitro [28]. Furthermore, at the same conditions, actin was found as most susceptible to oxidation and prone to aggregate formation [25]. However,

while actin containing aggregates were shown at 24h upon stress, they were not demonstrated at an earlier time, when a smaller decrement in proteasome functioning was already evidenced.

Those findings led us to hypothesize that, similarly to calmodulin, Hsp90 interacted with oxidized actin. Indeed, in a lymphocyte cell line [29] and skeletal muscle extracts [30], Hsp90 was found to bind actin, to establish crosslinks with its filaments [29] and to promote bundle formation [19]. In contrast, HSP90 inhibition was found to reduce actin polymerization [31] further emphasizing HSP90 importance in actin role. As actin has been considered a client protein of Hsp90 chaperone [29-35], it is possible that a peculiar hydrophobic cleft with relevance for actin filament growth [36] is the structural feature that provides support for HSP90 interaction.

However, in the setting of oxidation, as proteasome impairment appears early and aggregates are evidenced later, this shift in cells under a proliferative arrest suggests that additional mechanisms would be involved. In fact, to cope with redox imbalance and promote proteostasis, it has been proposed that challenged cells make use of mechanisms that lead to protein fold, hold or degradation [37]; when degradation is activated, the first line is proteasome intervention but if protein changes are too marked, as when they carbonylate or aggregate or the proteasome is inhibited, it is the autophagy-lysosome system that is called to compensate [37, 38].

So, on account of the time-related proteasome inhibition and actin aggregate formation, Hsp90 involvement in actin fate following oxidative stress challenge was further explored, hypothesizing that in time, 20S proteasome inhibition might give way to autophagocytosis.

MATERIAL AND METHODS

Cell culture conditions

Jurkat cells were cultured in a medium containing RPMI 1640, with 10% FBS, 1% L-glutamine, 1% Penicillin/Streptomycin (Biochrom) at 37°C, 5% CO2 in a NUAIRE™ US Autoflow CO_2 water-jacketed were submitted to oxidation by adding 100 µM hydrogen peroxide (H_2O_2, Sigma) for 3h, 6h, 9h and 24h and comparing to 0h time-point. When employed, 500µM Desferoxamine (*DFO*, Novartis) was used 18h before exposure.

Immunoblotting

For each experiment or time point, Jurkat cells were lysed in 0,1%

TritonX-100, 20mM NaCl, 30mM Tris pH 7,4, 1mM EDTA and 100mM DTT (Lysis Buffer, LB), collected, briefly sonicated and protein quantified by the Bradford method [39]. A total of 10 µg of protein was heated at 65°C for 30 minutes and samples were loaded and resolved in a 10-12% SDS-PAGE gel. Proteins were blotted onto nitrocellulose membranes (BioRad) and stained with Ponceau S for protein loading control. Next, membranes were blocked with 5% BSA/TBST (Tris Buffer Saline with Tween 20, 0.1%) for 1h. When the experiment was designed to detect protein carbonylation, membranes were directly derivatized with 2,4-dinitrophenylhydrazine (DNPH, Sigma Aldrich). Briefly, after the transfer, membranes were equilibrated in TBS (Tris Buffer Saline)/20% Methanol, washed for 5 min in 10% Trifluoroacetic acid (TFA, VWR), incubated for 10 min with 5 mM DNPH/TFA (10%) in the dark, washed with TFA (10%) to remove DNPH excess and washed again five times (5 min each) with 50% methanol. They were then blocked with 5% BSA/TBST (Tris Buffer Saline with Tween 20, 0.1%) for 1h.

After blocking, the membranes were probed with rabbit anti-Hsp90 full length (Abcam), and goat anti-actin (Santa Cruz), rabbit IgG anti-DNP (Sigma) at dilutions of 1:7500, at a 1:10000, 1:5000 as the primary antibodies respectively, rabbit anti-LC3 (Novus Biological) at a 1:2500 dilution and rabbit anti-LAMP-1 at 1:2500 (Abcam), mouse anti-Hsp90 N-terminal at 1:1000 (Abcam). For secondary antibodies, IRDye 800CW or IRDye 680LT were used at 1:15000 dilution, depending on primary antibody. They were purchased from Li-Cor Odyssey. The detection was performed at infrared, using the Li-Cor equipment from Odyssey. Band intensities were quantified by densitometry using Odyssey software. Graphics were made using GraphPad Prism 5 software.

Immunocytochemistry

Cells were grown on 6-well plates for 24h, and after washing in PBS, they were placed in poly-L-Lysine slides (Jurkat are suspension cells) and allowed to dry in the incubator described above (see *Cell culture conditions*). Immediately after, they were fixed in 4% PFA (Para-formaldehyde), washed with PBS, PBST (Phosphate Buffer Saline with Tween 20 0.1%) and PBSTx (Phosphate Buffer Saline with Triton X 0,1%) 0,1% and incubated for one hour in PBST/BSA 2%, to lower unspecific

background signal. Then, they were incubated overnight at 4°C in a solution containing the diluted primary antibodies: 1:250 of goat anti-β-actin (Santa Cruz, USA), 1:200 for Rabbit anti-LC3 (Novus Biologicals), 1:200 of rabbit anti-Hsp90 (full length) and 1:100 of Rabbit ant-LAMP-1 from Abcam. After washed twice in PBST, cells were incubated with the secondary antibodies conjugated with Alexa fluorochromes from Molecular Probes, donkey anti-goat secondary antibody conjugated with Alexa 488 and 568 (1:1000 prepared in PBST with 2% BSA) and with anti-rabbit secondary antibody conjugated with Alexa 488 and 568 (1:1000 prepared in PBST with 2% BSA) and donkey anti-mouse secondary antibody conjugated with Alexa 568 (Molecular Probes) 1:1000 prepared in PBST with 2% BSA). For aggregate detection and co-localization, a marker dye (Aggresome Detection Kit, Enzo Life Sciences) was prepared according to the manufacturer protocol. Fluorescence samples were analysed on a fluorescence microscope Zeiss AxioImager Z1 in Porto, Portugal and, in a Confocal Microscope Zeiss LSM 510 with help from Dr. S. Monajembaschi, Fritz Lippman Institute, Jena.

Cellular fractioning

After several time-points control cells and 100 μM H_2O_2 treated cells were lysed in LB, and centrifuged at 21,100g for 35min 4ºC to separate soluble fraction (SF) from insoluble fraction (IF). Supernatants (SF) were collected, and pellets (IF) were resuspended in resolubilization buffer (7M Urea, 2M thiourea, 4% CHAPS and 100mM DTT, RB), adapting a previously described procedure [40].

***In vitro* Hps90 cleavage**

In a tube, 0,6 μg recombinant human Hsp90β (Enzo Life Sciences, ADI-SPP-777) was subjected to 0,2mM H_2O_2, 0,2mMADP and 0,5mM $FeCl_3$ for 1h at 25ºC, with mild agitation. The reaction was stopped adding Catalase. DTT was added to the tube, and the sample was loaded for SDS-PAGE, followed by immunoblot detection against Hsp90, as described above.

Proteasomal degradation of oxidized actin

Non-muscle actin (Cytoskeleton Inc.) was diluted 1:2 with General Actin Buffer (GAB) (Cytoskeleton Inc.), supplemented with 5 % Sucrose, 1 % Dextrose and 0.2 mM ATP, and incubated for 1 h at 4°C. Actin oxidation was accomplished incubating

20μg actin in 0.1 mM H_2O_2 for 2 h at 25°C. Controls (non-oxidized Actin and Hsp90β) were incubated solely with GAB. The reaction was stopped using 40 μg/ml catalase and 50 mM DTT. To the mixture, 1 μg of isolated 20S proteasome [41], and 2 μg of recombinant human Hsp90β (Enzo Life Sciences, ADI-SPP-777) were added, incubated for 2 h at 37°C and later centrifuged for 10 min at 3000 g. To 125 μl of supernatant and 625 μl of 1 M HEPES, fluorescamine (Sigma Aldrich, 47614) solution was added for a final concentration of 0.03 % and incubated for 5 min. Fluorescamine reacts with primary amines, leading to the formation of a fluorescent product measured at 390 nm excitation/475 nm emission wavelength. A series of dilutions of glycine (from 0.05 to 10mM) were used as standard. Proteolysis rate was calculated as the difference between proteasome and blank samples (without addition of isolated 20S proteasome).

RESULTS

Hsp90 accumulates in protein aggregates upon oxidative stress and associates with actin

In Jurkat cells submitted to a 24h hydrogen peroxide mediated oxidative stress, a decrement in proteasome activity and the accumulation of cytoplasmatic aggregates containing carbonylated actin was reported [25]. As these findings commonly accompany the increase in protein unfolding or misfolding and proteostasis impairment [42-44], it was reasoned that oxidized actin accumulation was consequent to reduced proteasome ability and abnormal actin interaction with Hsp90. Therefore, it was decided to verify whether this chaperone was somehow involved in actin aggregates formation and fate.

At 24h upon stress, when protein aggregates are well established [25], Hsp90 full-length antibody detecting protein and the aggregate dye co-localize at coarse areas in the cytoplasm, in contrast to 0h (Fig 1A). In addition, Hsp90 co-localizes with actin (Figure 1B); in fact, while at 0h stress a uniform cytoplasmic display of Hsp90 and peripheral concentration of actin was noticed as previously [25], after 24h of stress, Hsp90 labeling was evidenced as condensed masses, with a distribution that co-localized to actin aggregates (Figure 1B, bottom).

Hsp90 is cleaved after oxidative stress into a 73kDa fragment in an iron dependent process

Because we were interested in the interaction between Hsp90 and oxidized actin, that would promote actin degradation, it was expected that if Hsp90 became less functional, oxidized actin degradation would be impaired, contributing to protein aggregates formation. It is known that oxidative stressful conditions promote Hsp90 cleavage. In fact, there was a notorious decrease in Hsp90 level at 24h of oxidative stress, compared to 0h (Fig 2A); however, instead of a single Hsp90 band, a second band ~70kDa was evidenced. While a dramatic expression of an Hsp90 inducible form was unlikely, as it would be heavier, an Hsp90 proteolytic cleavage was most probable, similarly to previous reports showing the appearance of a 73kDa band upon Hsp90c cleavage at its N-terminal extremity [45] [46].

To confirm, a Hsp90 N-terminal detecting antibody was employed, anticipating that no 73 kDa band would be evidenced at 24h. So, 100μM H_2O_2 treated cells extracts were reacted with antibodies against HSP90 full length and N-terminal segment. Interestingly, after fluorochrome probed secondary antibodies detection, the full length antibody evidenced a double band at 84 and 73 KDa (Fig 2B, left panel) but only the heavier 84 KDa band was labeled when the antibody against the N-terminal was employed instead (Fig 2B, middle panel),. Merging both channels (right panel) overlay was evidenced only in the heavier, upper band, as had been shown before [46].

In addition, as iron was considered a co-factor for HSP90 cleavage [46], DFO was used to deplete iron from cells, before submitting them to oxidative stress. As expected, in cells not subjected to DFO, there was HSP90 cleavage as evidenced by the double band (Figure 2C, left lane); in contrast, in DFO treated cells, there was a single 84 KDa band showing that Hsp90c had not been cleaved (Fig. 2C, right lane), thus confirming that the process is iron dependent. Moreover, employing a cell-free system with purified recombinant Hsp90, it was shown that Hsp90 cleavage is non enzymatic (Fig.2D) and hydrogen peroxide concentration dependent.

Therefore, these data indicate that oxidative stress promoting conditions cause Hsp90 cleavage into a 73 kDa protein, by a non-enzymatic, iron-dependent process.

Cleaved Hsp90 accumulates as insoluble protein aggregates

To further verify Hsp90 binding to the aggregates, two experiments were

made. In the first, advantage was taken from a previous (unpublished) study showing that DTT (Dithiothreitol) addition breaks down oxidized aggregates – in fact, extracts from H_2O_2 treated cells grown in DTT free medium exhibited a strong band at the stacking zone of the gel that upon DDT use disappeared from that zone and appeared at the resolving gel, suggesting they had migrated there. In the current study, DTT-free cell extracts exhibited a 84 KDa band and an additional band at the stacking zone, which disappeared when DTT was used (Figure 3A, right lane). Moreover, Hsp90c (73 KDa) band increased remarkably, indicating that it was bound to the aggregates previously noticed at the stacking zone.

The second approach departed from the recognition that proteins become insoluble upon aggregation. Extracts from cells studied at time point 0h and 24h of stress were separated into two fractions, soluble (SF) and insoluble (IF), and cleaved Hsp90 was assessed. Quantification in the IF at 24h of stress revealed a 3 fold increase compared to 0h (Fig 3B, lower chart), thus providing strong evidence that upon oxidative stress Hsp90c accumulates together with the aggregates as insoluble material. Interestingly, actin levels increased in the IF at 24h of stress but not before, further supporting the view that Hsp90c and actin accumulate in aggregates when they are fully established.

Hsp90 cleavage antedates actin accumulation

As Hsp90 plays an important role in oxidized proteins degradation and in 20S proteasome protection (Whittier et al., 2004), we admitted that a cleaved Hsp90 does not prevent oxidized proteins, as cytoplasmic actin, to accumulate.

To test that, a temporal assessment was made at 0h, 3h, 6h and 9h to verify actin and Hsp90 abundance in soluble (SF) and insoluble (IF) fractions. As depicted in Fig 4A, at each time point, actin is more abundant in SFs but accumulation in the IF starts at 6h time-point and continues afterwards (Fig. 4A).

As to Hsp90c, its presence at 0h is probably due to the high ROS production by cancer cells [47-49]; however, later at 3h time-point, there is cleavage enhancement (Fig. 4B) and, from 6h onwards, HSP90c shifts from SF to IFs (Fig. 4A), confirmed by densitometry quantification (Fig. 4C), showing that it accumulates as insoluble material. Why Hsp90c insolubilization starts earlier than actin is unknown but

might related to its own susceptibility to oxidation or its binding to other, unknown, susceptible proteins.

While at 24h of stress, actin aggregates accumulate and the proteasome function is impaired, at 3h such changes are slight, a difference that is probably related to the amount of oxidative burden imposed. In fact, a mild oxidation of actin and particularly its association with Hsp90 is useful for proteasome functioning, suggesting that the association is beneficial for protein degradation at the proteasome (Figure 4D).

LC3-I converts into LC3-II and enhances concentration at the insoluble fraction

In recent years, a crosstalk between two major degradation systems, proteasome and lysosomal autophagy has been proposed, indicating that when the proteasome is inhibited, autophagy becomes up-regulated [50-52]. Such change may be studied by the conversion ratio of LC3-I protein into LC3-II [53]. In the process, LC3-I acquires a lipidated, SDS PAGE faster flowing form that is incorporated in the autophagosome. Due to the reduction of the proteasome activity at 3h and accumulation of oxidized protein aggregates at 24h [25], macroautophagy was tested using LC3 protein conversion as marker and the experimental conditions as referred for Fig. 4A.

The western blots of soluble and insoluble fractions along different time-points are displayed in Fig 5, on the left. The charts on the right depict LC3-II/LC3-I ratios in soluble (Fig. 5A, left chart) and insoluble (Fig. 5A, right chart) fractions. There is an initial ratio increase in the soluble fraction (left chart), antedating its phagophore binding as previously suggested [54], and a decline after 3h time-point, possibly due to the lipidation enhancement and exhaustion of available soluble LC3 protein; this is in agreement with the Figure 5A, right chart, which shows a progressive LC3-II/LC3-I ratio increase in the insoluble fractions, especially between 9h and 24h (around 8-fold increase), by the time the aggregates are formed.

Thus, progressive conversion from soluble to insoluble form suggests that, when proteasome inhibition establishes, insoluble protein aggregates accumulation leads to autophagosome formation.

To ascertain this point, an immunocytochemical study was made, employing antibodies against LC3 and LAMP-1, a lysosome marker [55], and a

protein aggregate-binding dye in cells submitted to oxidative stress at 0h (initial experiment time-point) and 24h. In the experiments employing LC3 antibody and the aggregate dye, at 0h no aggregates were found and the cytoplasmic LC3 distribution was uniform (Fig. 5, B1); however, at 24h, LC3 labeling appeared as dense spots that co-stained with the aggregate marker (right panel); interestingly, in other experiments (Fig. 5 B2), at 0h, lysosomes exhibited a fine labeling in the cytoplasm, similarly to the aggregate marker, that became intense and co-localized with protein aggregates at 24h.

Taken together, the results point to autophagosome assembly, following protein aggregate formation and proteasome functional disability.

Hsp90 cleavage prevention blunts aggregate formation and autophagy up-regulation

It had been noticed that HSP90 cleavage is dependent on iron (Figure 2D and [46]). In order to test whether Hsp90 cleavage contributes to compensatory autophagy up-regulation, it was blocked employing desferoxamine (DFO) as iron chelator. Cells were incubated 18h with 500µM DFO prior to hydrogen peroxide stress and extracts were prepared for western blotting against Hsp90. As expected (Fig. 6A, left), DFO treated cells did not exhibit Hsp90 cleavage when compared to non DFO treated cells and the LC3-I conversion into LC3-II was blunted. In addition, an important role in aggregate formation and accumulation was shown as blocking Hsp90 cleavage, resulted in marked reduction in irreversible protein oxidation (Fig 6B). Moreover, in an immunocytochemical study, fluorescent labeled protein aggregates, evidenced as coarse intracellular areas upon H_2O_2 challenging, were reduced or absent when treated with DFO beforehand (Fig 6C). The relative contribution of each factor, iron and Hsp90, for protein oxidation and autophagy, although uncertain is relevant. In fact, only 3h after stress, oxidized proteins were increased ~1,5-fold and continued for 24h. However, when DFO was used, the increase was blunted and the levels were similar to 0h.

DISCUSSION

In the course of cell metabolism, oxygen use generates a variety of by-products that include the reactive oxygen species whose continued production subjects biomolecules to harmful, oxidant effect [56]. The

appearance of irreversibly affected molecules has been considered a mark of oxidative stress and their accumulation in the last third of organism´s life [3] is evidence in favour of the importance of oxidation for ageing.

In cells, adding to functional impairment, oxidation results in protein structural abnormalities as unfolding. Consequently, usually buried hydrophobic domains become exposed and promote the establishment of cross-links with other oxidized proteins that antedate the formation of insoluble protein aggregates [57]. These findings, apart from being consequences of a continued or intense oxidative environment, are features of aged cells, able to interfere with their metabolism, cause changes in gene expression and lead to their senescence or death. Aggregates have been correlated with aging progression across different models such as bacteria [58, 59], yeast [60], *C. elegans* [61] and mammalian cells [62].

The appearance of abnormal or no longer necessary proteins triggers a disposable mechanism that directs them for destruction at the 26S proteasome upon tagging by ubiquitin molecules. Yet, in the case that proteins are mildly oxidized, they may be directed through an additional, different process that targets them to the 20S proteasome, also aiming at cell homeostasis maintenance and cross-linked protein accumulation prevention [8, 63, 64]. In fact, the 20S proteasome was considered the main system involved in the degradation of oxidized proteins [24]. In the case of calmodulin [18, 41] and actin (current study), there is evidence that oxidized proteins, compared to native proteins, are degraded by the proteasome at an higher rate. The nature of chemical changes undergone by both molecules emphasizes further similarity as it is the sulphur containing methionine residues in calmodulin [18] and specific cysteine and methionine residues of actin [65, 66] that are sensitive to the early oxidative modification.

Oxidized proteins tagged for destruction require the interaction with chaperones before they are transported to the proteasome. In this setting, the Hsp90 chaperone protein is an important cellular tool when proteostasis is challenged due to its cytoplasmic abundance and ability to interact with a vast number of proteins [19]. In particular, Hsp90 is known to bind strongly to actin [29, 30] and calmodulin [18]. In addition, the Hsp90 ability to enhance their degradation at the proteasome as shown before [18]

and in the current study, favors its involvement in their transportation. Hsp90, indeed, was recognized as an active intracellular chaperone when cells are affected by carbonylation [67].

When the oxidative insult is intense, actin aggregates are formed [25, 66] (and the current data). Although they associate with the Hsp90 molecule, it is a 73KDa fragment, the Hsp90c, that becomes an important component of the aggregate, as shown in the present study; such cleavage is not unexpected as oxidative stress impinges on amino acid residues and may cause polypeptide backbone fragmentation [68]. In the case of Hsp90, the cleavage is detectable in intact cells, their lysates and purified recombinant proteins and is consequent to a non-enzymatic process requiring iron; this is thought to be chelated to adenine nucleotides and cause HSP90 break at an highly conserved N-terminal amino acid motif following a local Fenton reaction [46]. The consequences are loss of ATP binding domain and impairment of Hsp90 chaperoning function [46].

Moreover, the oxidative stress related HSP90 functional loss is likely to contribute to the observed proteasome derangement. In fact, in the current model of oxidative stress on Jurkat cells, proteasome activity reduction was reported as early as 3h and had intensified at 24h [25]. In another report, Hsp90 silencing was found to account to reduced proteasome trypsin-like activity and Hsp90 overexpression was able to maintain the level of proteasome activity in harsh oxidizing conditions, suggesting a proteasome protective effect [23, 24]. Therefore, these observations, in agreement with previous findings [8, 13, 64], provide ample evidence that oxidative conditions impair proteasome activity.

So, the data indicate that while mildly oxidized, actin in association with Hsp90 is conveyed to the 20S proteasome for degradation. However, under too high or prolonged oxidative conditions, the proteasome function becomes impaired, Hsp90 is cleaved and actin crosslink establishment is promoted. These events converge into a time-related, reduced availability of functional Hsp90 and enhanced conditions for actin insoluble cytoplasmic aggregates deposition. As the proteasome degrades oxidized but not aggregated proteins [57], another process ought to be activated to preserve cell viability.

Intracellular aggregate deposits were long ago suggested to be a stage antedating their degradation at the lysosome compartment [69] and the

concept gained strength along time. In fact, following the proteasome functional decrement, such degradation by autophagocytosis has been shown in primary cells [70] and cell lines ([55], [71], [72]) including, now, a T cell line.

Autophagy is a fundamental cell process that is triggered to remove intracellular abnormal molecules or damaged organelles. Also named macroautophagy [73], it refers to membrane encircling (phagophore) of intracellular material in an autophagosome and its submission to lysosome digestion. Autophagosome formation has been described as following successive stages [74] [38]and to foster its course, an increasing number of proteins has been uncovered. Some are activated at the initiation stage and others are more relevant at a nucleation or expansion stage. There, it is especially important the cytosolic soluble mammalian LC3/LC3-I protein (Atg8 in yeast) that reacts with a phosphoglyceride, as the phosphatidylethanolamine, is converted into the LC3-II compound and incorporated in the membrane of the autophagosome. The continued conversion allows LC3-II to bind more membrane and promote its growth to surround the target cellular material to be destroyed; LC3 members are also thought to contribute to the final closure of the phagophore membrane [74].

The current investigation shows that by 9h of stress, LC3II/LC3I ratio is mounting and is remarkable at 24h, when the proteasome function is substantially decreased, the aggregates are established and cells are in a proliferation arrest state [25]. Interestingly, along this time, the LC-II/I ratio had a transient increase in the soluble fraction, in agreement with previous observations [54], before it increased in the insoluble fraction and reached high levels at 24hours. At this time, either the phagophore LC3 marker or the lysosome LAMP-1 marker co-localized with the aggregates, indicating that the cell had attained late phagocytic stages.

It is noteworthy that the degradative process shift from proteasome to autophagocytosis, here reported for Jurkat cell line, had been noticed in cultured oligodendrocytes [75] and other lines [76]; so, the data support the view that autophagocytosis activation is a compensatory process to proteasome functional decrement[76]. Interesting too is the reverse effect reported in colon cancer cells, where inhibition or silencing of a variety of autophagocytosis genes resulted in enhanced proteasome activity[77], thus

enriching the crosstalk ability of these degradation systems.

The proliferative arrest and reduced functional capacity of the proteasome observed in this cell Jurkat cell line has a parallel with a previous report with primary T cells [26, 27], whose changes were interpreted as consequent to immune cells ageing. In fact, there is substantial evidence in favor of an age related and cell senescence related decrement in proteasome activity [78] As the silencing of important genes involved in autophagocytosis results in enhanced cell vulnerability to the oxidative insult [57, 79] and in fact the phagocytic process ameliorates cell resistance to apoptosis and other stresses [80, 81], we are convinced (shown in Fig.7) that early Hsp90 chaperoning ability loss upon cleavage and proteasome reduced functioning are the primer events that lead to protein aggregates formation, which in turn results, in the activation of autophagocytosis as protective mechanism of a larger process for cell survival.

LEGENDS TO FIGURES

Figure 1. In oxidative stressful conditions, Hsp90c accumulates in actin containing protein aggregates.

In a previous report [25] it was shown that submitting Jurkat cells to 100 μM hydrogen peroxide for 24h results in the formation of insoluble actin aggregates. The conditions were repeated and compared to 0h exposure controls.

(A) At 24h of stress, fluorescence detection of Hsp90 protein (green labeling) and cell aggregate (red labeling) revealed a remarkable co-localization, this was not seen at 0h.

(B) Hsp90 accumulates in actin aggregates as evidenced by confocal microscopy detection of Hsp90 (green) and actin (red). While Hsp90 exhibits a fairly homogeneous, cell-wide distribution, actin is mostly localized to the periphery, near the cell membrane (left panels). Upon 24h of hydrogen peroxide use, Hsp90 and actin co-localize and frequently exhibit aggregates (arrows at right panels).

Figure 2. Oxidative stress promoted Hsp90 chaperone cleavage into Hsp90c is dependent on iron and oxidant intensity.

(A) Jurkat cells were exposed to 100 μM hydrogen peroxide for 24h and compared to controls (0h exposure). Immunoblots were prepared with antibodies against full length 84 kDa Hsp90 (top image), and their respective

densitomety was quantified (bottom chart). Upon exposure, Hsp90 level decreased ~50% and a new 73 KDa band appeared, consequent to Hsp90 N-terminal cleavage, identified as Hsp90c. Quantification of Hsp90 and Hsp90c is the result of three independent experiments (Mean ± standard deviation, SD).

(B) Extracts of Jurkat cells submitted to 100 μM hydrogen peroxide for 24h were immunobloted employing Hsp90 full length and Hsp90 N-terminal specific antibodies. Detection was made with secondary antibodies bearing different fluorochromes. Full length antibody evidenced two bands, as in (A), whereas the N-terminal antibody, demonstrated one single 84 kDa band, indicating that Hsp90c band resulted from N-terminal Hsp90 cleavage. Overlay signal co-localizes only in the 84 kDa band, thus showing that the 73 KDa protein lacks the N-terminal fraction. Membrane fluorescence was detected with Li-Cor Odyssey.

(C) Iron is required for Hsp90 cleavage. Cells depleted in iron upon DFO use do not exhibit Hsp90c even when exposed to hydrogen peroxide for 24h (right lane). In contrast, exposed cells without iron depletion, showed the expected double band pattern (left lane).

(D) Hsp90 cleavage in a cell free system. Hsp90 was subjected to hydrogen peroxide at two different concentrations (0.1 and 0.2 mM) in the presence of ADP and $FeCl_3$. Oxidant concentration dependent cleavage is evidenced again at this simple system and confirms that Hsp90c results from N-terminal Hsp90 cleavage.

Figure 3. Hsp90c, accumulates as insoluble aggregates

(A) Hsp90c is present in aggregates. Upon Jurkat cells exposured to 100 μM hydrogen peroxide in a medium without or with the aggregate dissolving agent DTT, western blots were probed with the full length anti Hsp90 antibody. In conditions without DTT, a band is retained at the stacking gel and the 73KDa protein is faint or missing (left lane); when DTT is added, aggregates dissolve and migrate into the resolving gel (right lane), where they exhibit the 84 KDa and the 73 KDa bands, indicating that the Hsp90c previously retained in aggregates migrated to the stacking gel. Blot is representative of 3 different experiments.

(B) Hsp90c is localized to the insoluble fraction. Jurkat cells were exposed to 100 μM hydrogen peroxide for 24h and compared to controls (0h exposure).

Total protein extracts samples from time-point 0h and 24h of oxidative challenge were separated as soluble (SF) and as insoluble (IF) fractions. Hsp90c shows a >3-fold content increase in the insoluble fraction upon oxidative stress (lower chart) supporting the view that it binds to protein aggregates. Also noteworthy, is the increased actin level in the IF after 24h of hydrogen peroxide. Blot is representative of 3 different experiments.

Figure 4. Oxidative stress related Hsp90c appearance is time-dependent and localizes in the cytoplasmic insoluble fraction.

Upon cell exposure to 100 µM hydrogen peroxide, samples were collected at 3h, 6h, and 9h and compared to controls (0h).

(A) Extracts from controls and treated cells were prepared for separation of soluble and insoluble fractions. Oxidative conditions promote Hsp90 cleavage, already evident at 3h. Hsp90c form remains in the cytoplasmic soluble fraction until 6h, when it shifts towards insolubility, which is notorious at 9h post stress. Ponceau S staining (PS, bottom image) and western blots with antibodies against full length Hsp90 (middle image) and actin (top image) are depicted in this representative image.

(B) Quantification of Hsp90c at the soluble fraction confirms that it is already enhanced at 3h.

(C) Quantification of relative % of actin (right graph) and Hsp90c (left graph) in soluble (SF) and insoluble (IF) fractions. Actin level increases in the insoluble fraction, after time point 6h, when Hsp90c had already accumulated considerably and when aggregate formation is likely to begin. It thus indicates that Hsp90 cleavage and accumulation at the IF precedes actin accumulation. Graphic bars result from three independent experiments (Mean ± SD are shown).

(D) Hsp90 enhances proteasome activity *in vitro*. In a cell free system, proteasome activity was measured after adding to the medium actin, oxidized actin, oxidized actin plus Hsp90 and Hsp90 only. Activity was normalized to 100% in the condition of actin only and compared. Oxidized actin promotes proteasome activity 2 fold (200%), further enhanced to 3 fold when oxidized actin is incubated along with Hsp90.

Figure 5. Oxidative stress promoted protein aggregates lead to autophagosome formation.

Jurkat cells were exposed to 100 μM hydrogen peroxide for up to 24h; samples were collected at 3h, 6h, 9h and 24h and compared to controls (0h).

(A) Cell samples were processed for soluble (SF) and insoluble (IF) cytoplasmic fractions; immunoblots (upper figure) were prepared against LC3 (LC3-I) protein that converts into its lipidated form (LC3-II) when autophagy is up-regulated. Data show that LC3-I conversion into LC3-II is time related, as evidenced by comparing 0h (basal) through 24h (stress time), and also shows that LC3-II shifts from the SF to the IF, suggesting its binding to the phagophore. The perception from the visual inspection of the blot is confirmed upon densitometric assessment of LC3-II/LC3-I ratios in SF and IF (charts on the right).

(B) Immunocytochemical study of Jurkat cells at 0h (upper images) and upon 24h (bottom images) exposure to hydrogen peroxide. **(B1)** Cells were labeled for LC3 (left images) and aggregate marker (middle images) and co-localization was verified (right). At 0h, LC3 protein has an uniform distribution inside the cell and aggregate labeling is residual whereas at 24h (bottom images) LC3 protein concentrates in dense areas that co-localize with the aggregate marker. **(B2)** The lysosome marker LAMP-1 (left images) and the aggregate marker (middle) co-localize (right image) at 24h upon hydrogen peroxide treatment. The data indicate that aggregate formation results in autolysosome build-up, as evidenced by promotion of LC3 protein conversion into LC3-II insoluble form and its co-localization with the lysosomal LAMP-1. This is strong evidence in favor of autophagosome-lysosome axis activation after protein aggregates formation.

Figure 6. Desferoxamine (DFO) prevents Hsp90 cleavage and autophagy up-regulation.

Hsp90 cleavage prevention was achieved by adding DFO 500μM to the media 18h before inducing oxidative stress with 100 μM hydrogen peroxide.

(A) DFO prevented Hsp90 cleavage (upper blot) and strongly inhibited the conversion of LC3-I to LC3-II (bottom blot); Ponceau S (PS) image is at the right. The data show that iron is necessary for Hsp90 cleavage, which appears to be part of a process that results in protein aggregate accumulation, proteasome inhibition and promotes autophagy up-regulation.

(B) Depleting iron with DFO before oxidative stress, prevents the increase in carbonylated proteins. This can be

followed after 3h, 9h, 24h of oxidative stress with or without DFO.

(C) Jurkat cell controls (left image) or exposed to 100 μM hydrogen peroxide for 24h, without (center image) or with (right image) previous addition of DFO. Upon oxidative challenge, aggregates accumulate (red labeling, center image), which is prevented by previous DFO treatment.

Figure 7. Proposed events for protein aggregates, proteasome inhibiton and autophagy upregulation. When Hsp90 is fully functional (full length protein) it will assist and enhance oxidized proteins degradation (oxidized actin included) by the 20S proteasome avoiding or simply delaying their accumulation as protein aggregates. However, if Hsp90 gets cleaved during oxidative stress it will become less functional. Therefore, it will bind oxidized proteins, but because it lacks the N-terminal it cannot assist correctly oxidized proteins turnover. They start to accumulate along with cleaved Hsp90 originating protein aggregates, which later on, will bind to proteasome surface motifs, inhibiting its enzymatic activity. In an attempt to solve or compensate proteasome activity decrease, cells upregulate autophagy shown by the formation of the phagophore that will engulf protein aggregates. They are forward to lysosomes to clear the toxic cargo.

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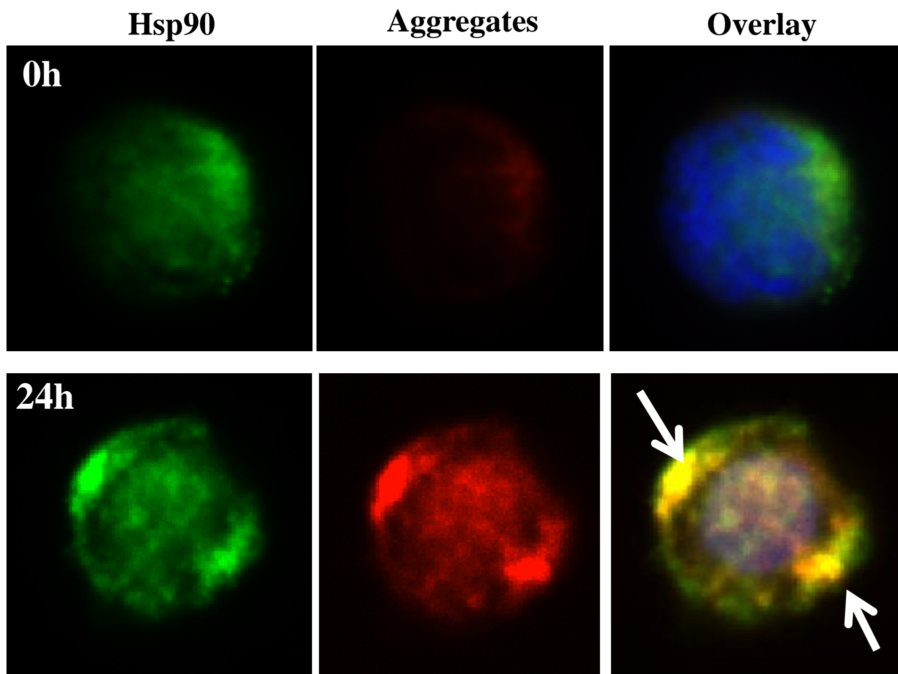
IN OXIDATIVE STRESSFUL CONDITIONS, HSP90 ACCUMULATES IN ACTIN CONTAINING PROTEIN AGGREGATES

A. HSP90 ACCUMULATES IN AGGREGATES AT 24H OF STRESS



B. HSP90 ASSOCIATES TO ACTIN AGGREGATES

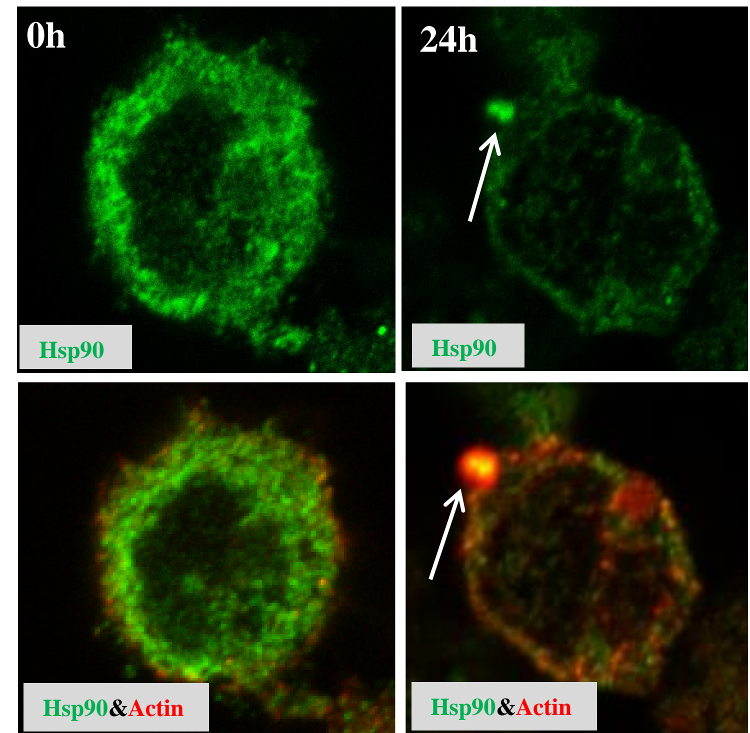
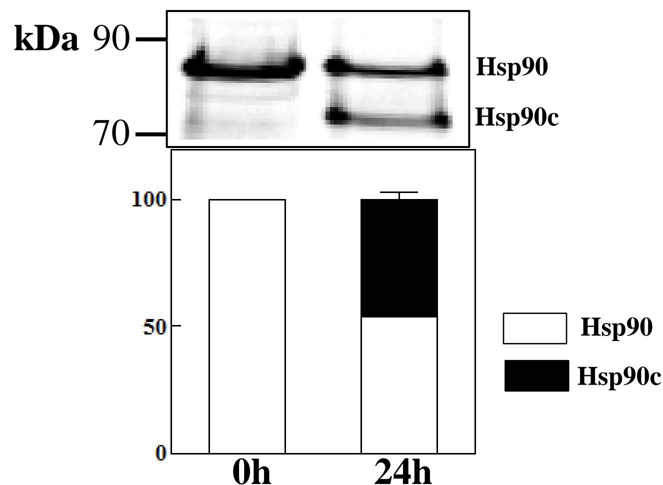


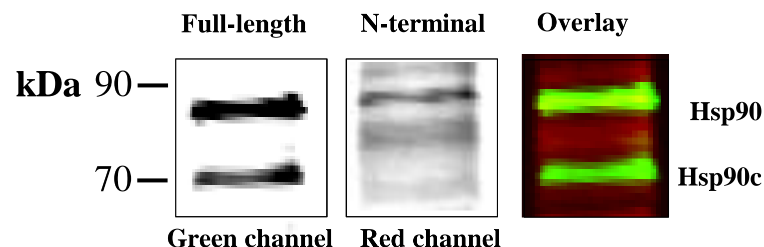
Figure 1. Castro, Reeg, Botelho, Almeida, Grune

HSP90 IS CLEAVED UPON OXIDATIVE STRESS

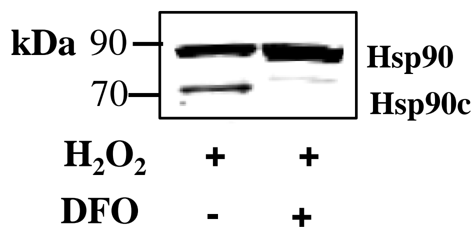
A. OXIDATIVE STRESS CLEAVES HSP90 IN JURKAT CELLS



B. OXIDATIVE STRESS CLEAVAGE IS N-TERMINAL SPECIFIC

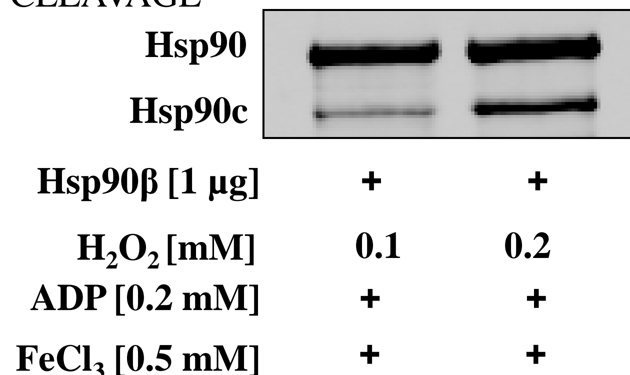


C. HSP90 CLEAVAGE IS IRON DEPENDENT



H_2O_2	+	+
DFO	-	+

D. CELL FREE HSP90 N-TERMINAL CLEAVAGE

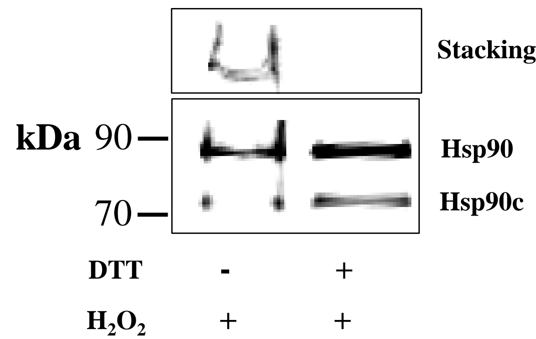


Hsp90β [1 μg]	+	+
H_2O_2 [mM]	0.1	0.2
ADP [0.2 mM]	+	+
$FeCl_3$ [0.5 mM]	+	+

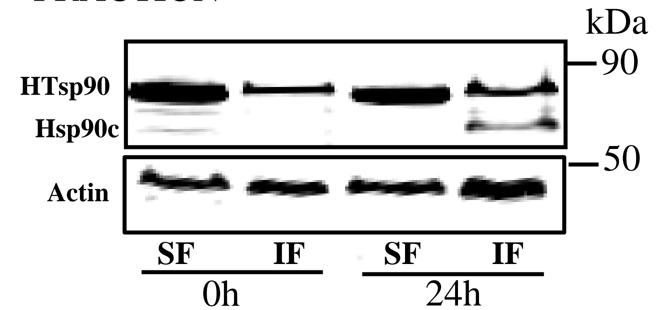
Figure 2. Castro, Reeg, Botelho, Almeida, Grune

CLEAVED HSP90C ACCUMULATES AS INSOLUBLE PROTEIN AGGREGATES

A. HSP90C IS MOSTLY IN PROTEIN AGGREGATES



B. HSP90C FRAGMENT PREDOMINATES IN THE INSOLUBLE FRACTION



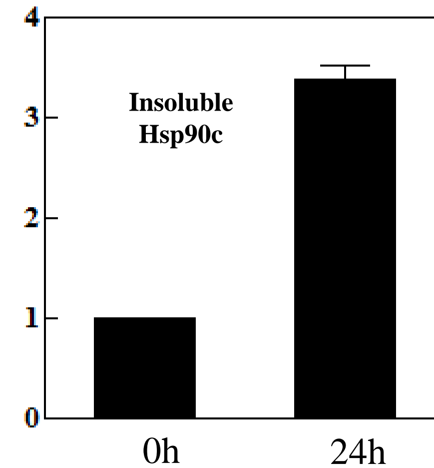
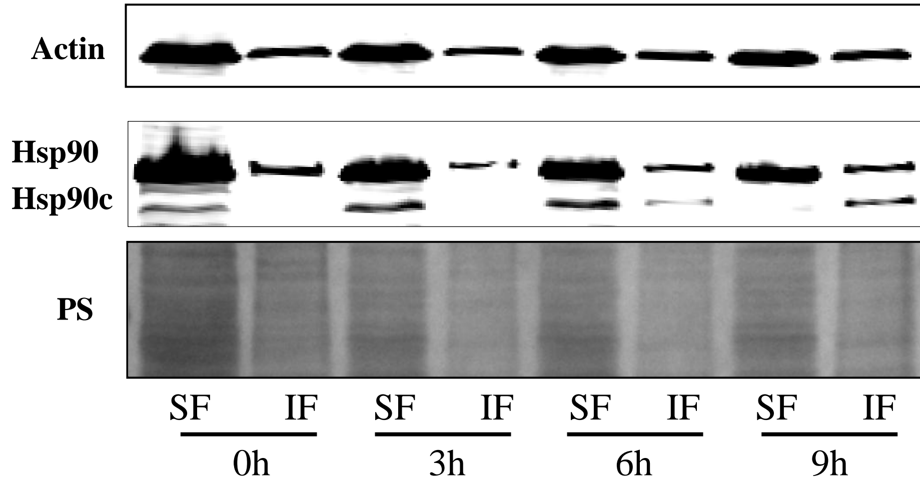


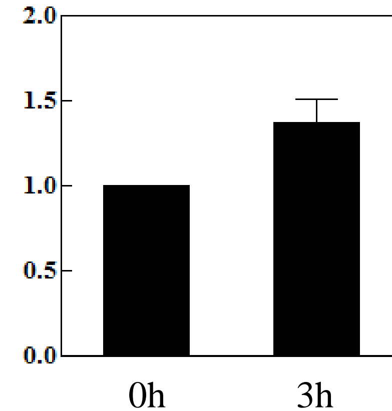
Figure 3. Castro, Reeg, Botelho, Almeida, Grune

HSP90 CLEAVAGE IS TIME-DEPENDENT AND PRECEDES INSOLUBLE ACTIN ACCUMULATION

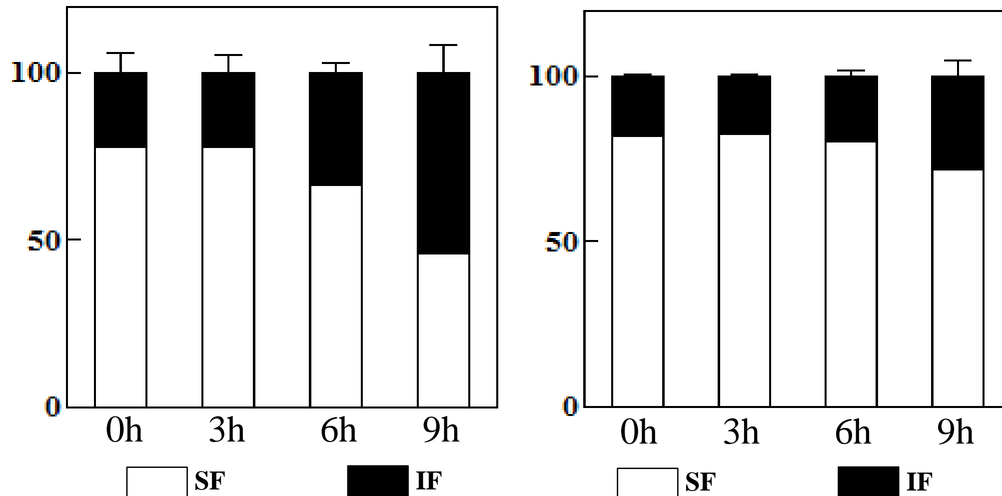
A. ACTIN AND HSP90C ENHANCED INSOLUBILITY ALONG THE TIME



B. HSP90C AT THE SF IS ENHANCED SINCE EARLY TIME



C. % HSP90C (LEFT) AND % ACTIN (RIGHT) AT SF AND IF ALONG TIME



D. DEGRADATION OF OX. ACTIN IS ENHANCED BY HSP90 *IN VITRO*

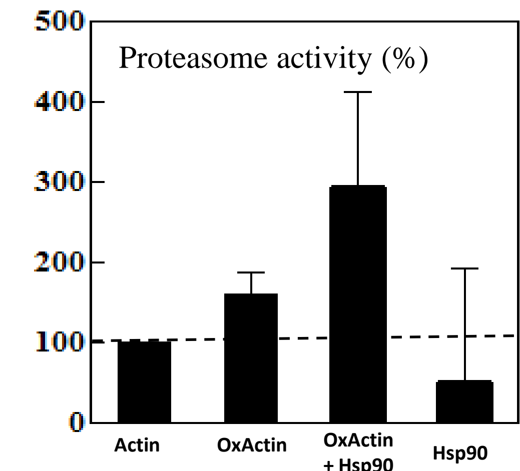


Figure 4. Castro, Reeg, Botelho, Almeida, Grune

PROTEIN AGGREGATES LEAD TO AUTOPHAGOSOME FORMATION

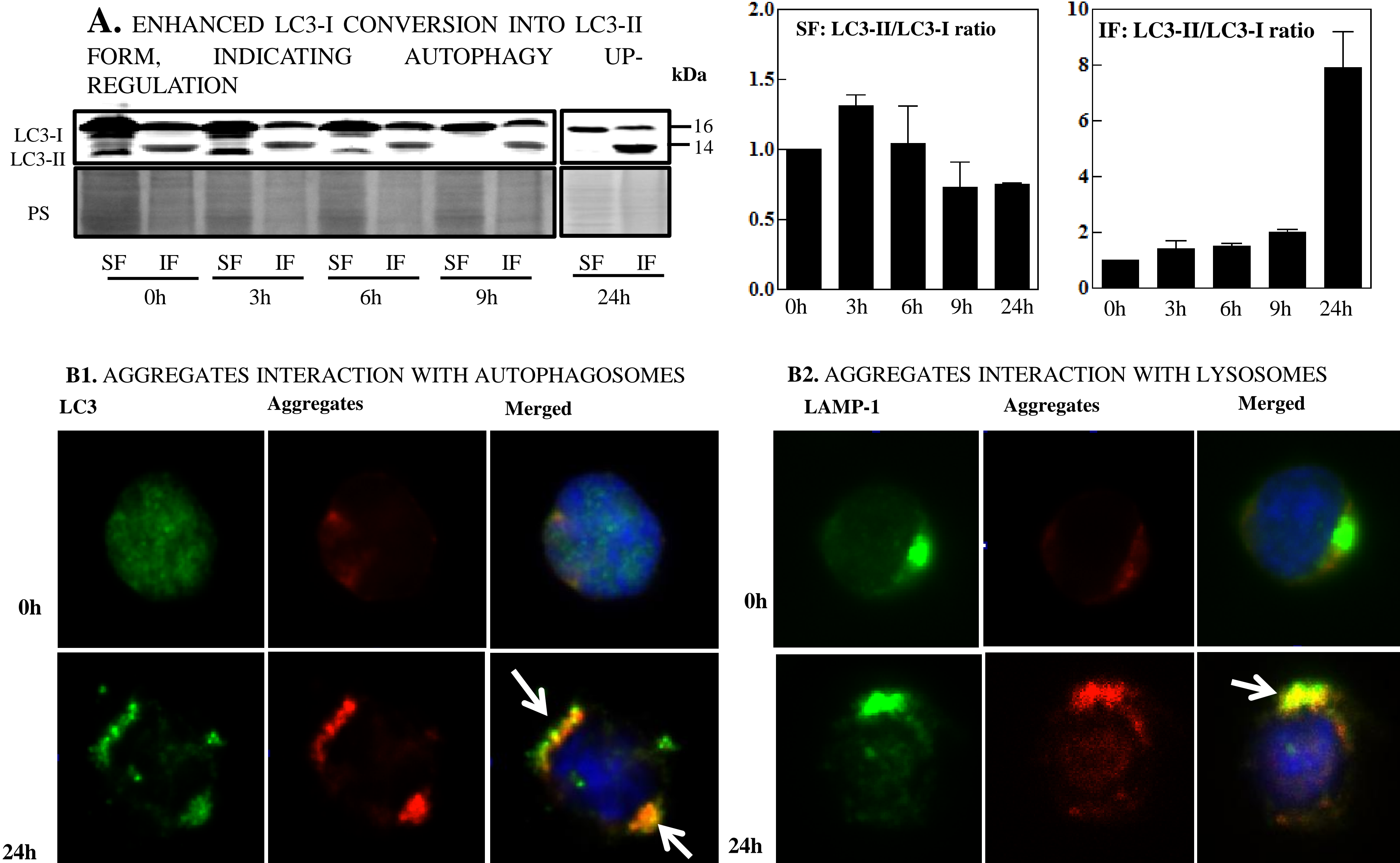
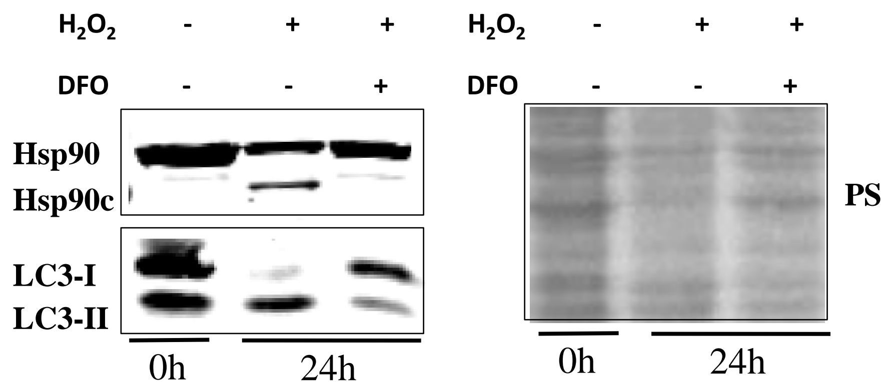


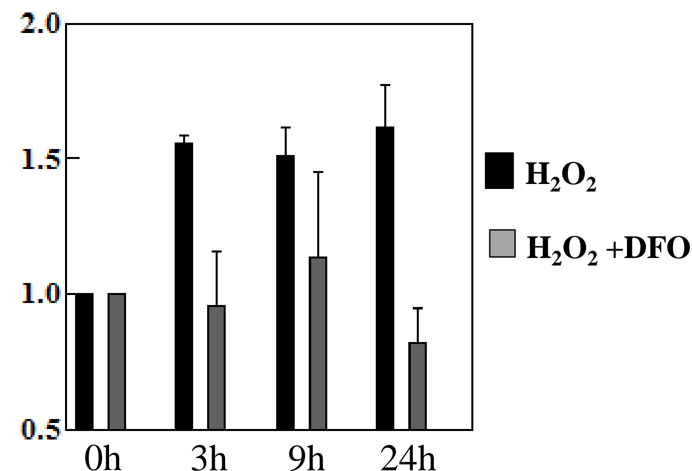
Figure 5. Castro, Reeg, Botelho, Almeida, Grune

HSP90 CLEAVAGE PREVENTION BLUNTS AGGREGATE FORMATION AND AUTOPHAGY UP-REGULATION

A. HSP90 CLEAVAGE PREVENTION BLUNTS AUTOPHAGY UP-REGULATION



B. OXIDIZED PROTEIN FORMATION AND ACCUMULATION IS IRON DEPENDENT. CARBONYLATION NORMALIZED IN ARBITRARY UNITS (AUS)



C. HSP90 CLEAVAGE BLOCK CORRELATES WITH NO PROTEIN AGGREGATES

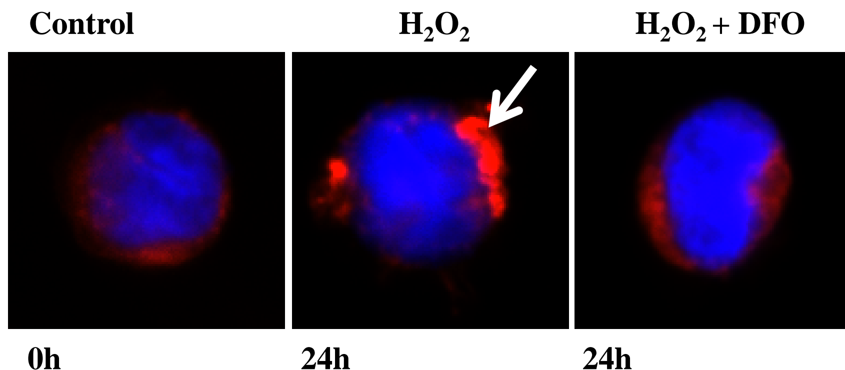


Figure 6. Castro, Reeg, Botelho, Almeida, Grune

HSP90 CLEAVAGE LEADS TO OXIDIZED PROTEINS ACCUMULATION, PROTEASOME INHIBITION AND AUTOPHAGY UPREGULATION

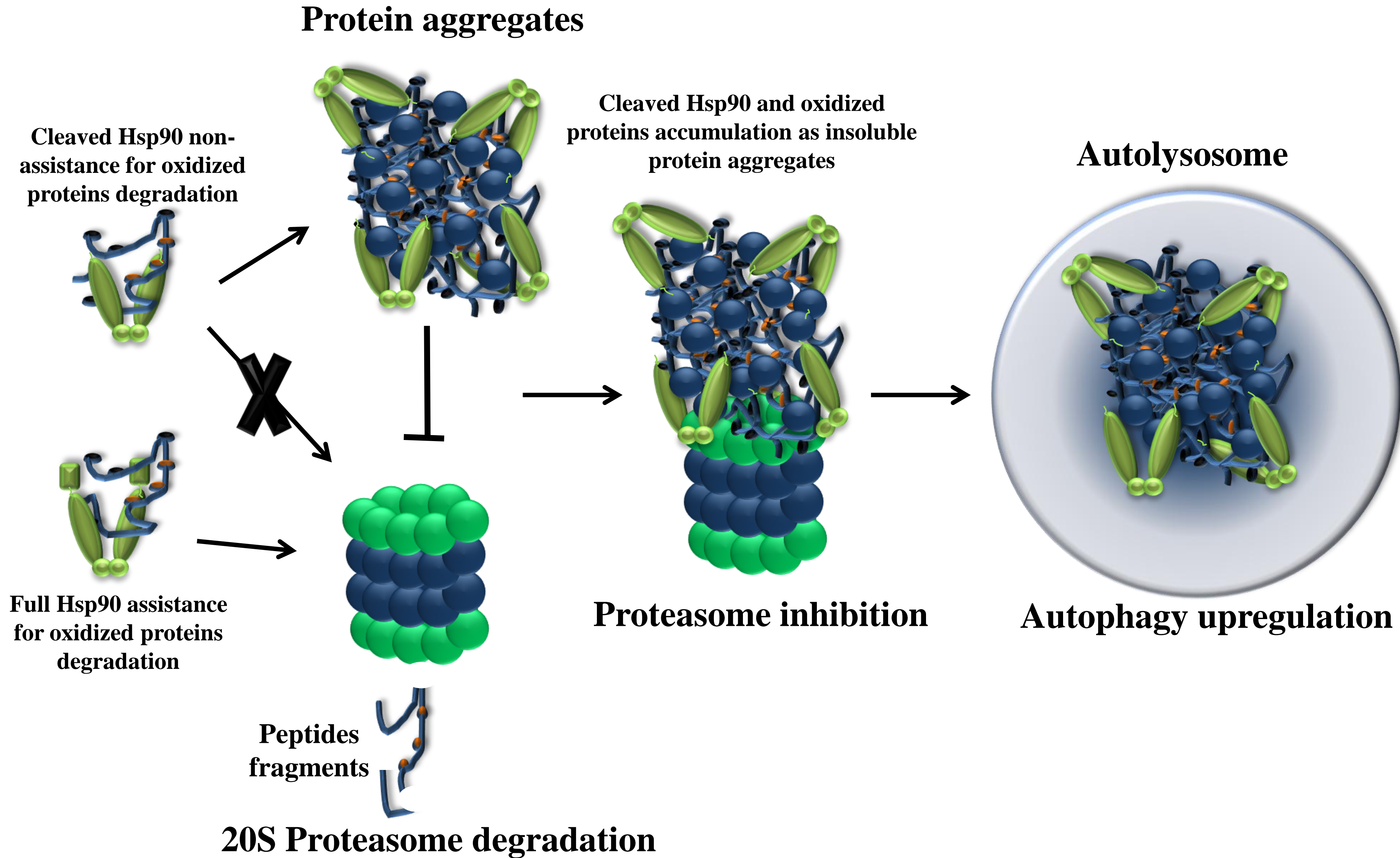


Figure 7. Castro, Reeg, Botelho, Almeida, Grune

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DISCUSSION

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DISCUSSION

Oxidative stress occurs when there is a disruption in the balance between oxidants and anti-oxidants, in the favor of the former [1]; its consequences have fulfilled much of the life sciences scientific agenda in the last decades. Why did such theme become so interesting to science? The main reason is the implication of oxidative stress in ageing progression and disease [2]. It affects biomolecules such as DNA, sugars, lipids and, most importantly, proteins; indeed when oxidative stress impinges on them, it impairs their functions and causes cellular disorders, which can range from reversible to irreversible. A frequent outcome of irreversible oxidation is protein carbonylation, a non-enzymatic event [2-4], that is able to accumulate and, therefore, be harmful.

1.1 Oxidized actin aggregates formation and proteasome impairment

Protein carbonylation increase is a recurrent phenomenon in human ageing or disease. Aggregates, following protein oxidation, have been deeply correlated with ageing across different models such as bacteria [5, 6], yeast [7], *C. elegans* [8] and even mammalian cells [9]. Their formation, previously described in *Introduction Section 2*, may be thus elicited by oxidative stressful conditions as those employed in the current investigation.
To prevent aggregates formation and assure proteostasis, cells degrade carbonylated proteins employing the proteasome, considered the most efficient mechanism for their turnover [10, 11]. Therefore, if the proteasome is impaired or carbonylated proteins formation rate overwhelms the proteasome capacity for degradation, they can aggregate and accumulate [12]. This is an important issue because aggregates are harmful due to their ability to interfere with cellular metabolism, cause changes in gene expression, additional proteasome inhibition and lead to cell senescence or cell death [13-15].
I found that upon Jurkat cells hydrogen peroxide challenging, there is formation of oxidized actin aggregates in a time and dose dependent pattern. In fact, while absent at three hours upon oxidative stress, they accumulate at 24h, a time when proliferation arrest and proteasome inhibition were evidenced too [12]. Such changes may result from a direct effect of aggregates, noticed when I incubated cells with *in vitro* made oxidized actin aggregates without added hydrogen peroxide.
Thus, if not degraded efficiently by the proteasome, oxidized proteins accumulate as insoluble high molecular weight protein aggregates that interfere with important cellular functions [13-15]. Interestingly, the accumulation of abnormal molecules, proteasome

reduced functioning and proliferation arrest are major findings in human senescent skin fibroblasts [16, 17]. The similar changes evidenced in Jurkat cells thus favor the view that they are also occurring in *in vivo*, aged T lymphocytes. In fact, senescent cells increase in organisms over time [18], and immune cells appear to accompany so. In humans, such changes include reduction of naïve T-cell production, either $CD4^+$ and $CD8^+$, and clonal expansion of specific CD8 T-lymphocyte subsets as the $CD8^+$ $CD28^-$ [19]. Such $CD8^+$ cells are particularly prone to oxidation [20] and when cultured, they acquire a senescent like phenotype including growth arrest [21].

Adding to these changes, I found that proteasome function was impaired, which is another feature of aged and senescent T cells i*n vivo* and was proposed as contributor to immunosenescence mechanisms [22, 23]. Similarly, carbonylated proteins accumulation and less rounds of proliferation were found in lymphocytes of *ad libitum* fed aged mice when compared to animals submitted to calorie restriction, a condition known to extend lifespan [24].

There is thus a considerable amount of data that relate cell senescence to oxidized protein accumulation, proteasome functional decrement and proliferative impairment. I noticed parallel findings in the oxidatively stressed model of Jurkat cells, therefore supporting the view that this model mimicks important effects of ageing on cells.

1.2 Hsp90 cleavage leads to insoluble actin accumulation

Although protein aggregates accumulation is a frequent finding upon oxidative stress, and the proteasome impairment is a contributor, the mechanism why cells fail to degrade them is still debatable.

To avoid proteostasis disruption, cells have molecular chaperones that promote oxidized proteins destruction. An example of a key player is the highly conserved and abundant molecular chaperone Hsp90 [25]. It is composed of three distinct domains, the N-terminal, a middle domain and C-terminal. The N-terminal is responsible for ATP hydrolysis, required for substrate remodeling, and the middle domain has a role in substrate binding, whereas the C-terminal is necessary for Hsp90 dimerization and co-chaperones binding [25].

Hsp90 has a high affinity to its clientele proteins but the nature of interaction can be diverse because it binds to unfolded, native proteins, as well as damaged proteins, and requires additional factors, as co-chaperones [25].

Hsp90 is itself sensitive to oxidative stress and is cleaved by several oxidants at a highly conserved N-terminal amino acid motif [26] which originates a N-terminal deprived 73kDa fragment (Hsp90c unable to bind ADP or ATP). This was observed in intact cells, cell lysates, and when purified recombinant proteins were employed, indicating an unlikely protease involvement [26]. This point was verified in the current investigation with Jurkat cells extracts and cell free experiments, but it was also shown that Hsp90 cleavage is dependent on iron [26] (Fig. 5) because when DFO, iron is chelated and the cleavage is prevented.

The accumulation of oxidized proteins can be attributed not only to their hydrophobic residues binding, but also to lesser 20S proteasome degradation events, shown to be enhanced when Hsp90 is present [27]. In fact, in my *in vitro* studies with the proteasome, I found that oxidized actin degradation rate was two-fold higher when compared to non-oxidized actin; however, when purified Hsp90 was added to the experiment, the rate was even higher, providing evidence in favor of Hsp90 role in assisting and enhancing oxidized proteins turnover. But such role likely extends to the proteasome itself. Interestingly, when the stress was imposed in Hsp90 overexpressing cells or onto purified 20S proteasome or cellular extracts artificially contaminated with Hsp90, the proteasome activity was found conserved, suggesting that Hsp90 is a proteasome protector [28-30]. Because the data do not favor the cleavage as amenable to intracellular regulation, it is thus a major challenge to cells. Indeed, Hsp90 cleavage appears to be determinant for decreased proteasome protection and for reduced or abnormal transportation of oxidized proteins to be degraded, conditions that make of protein aggregates an inevitable consequence.

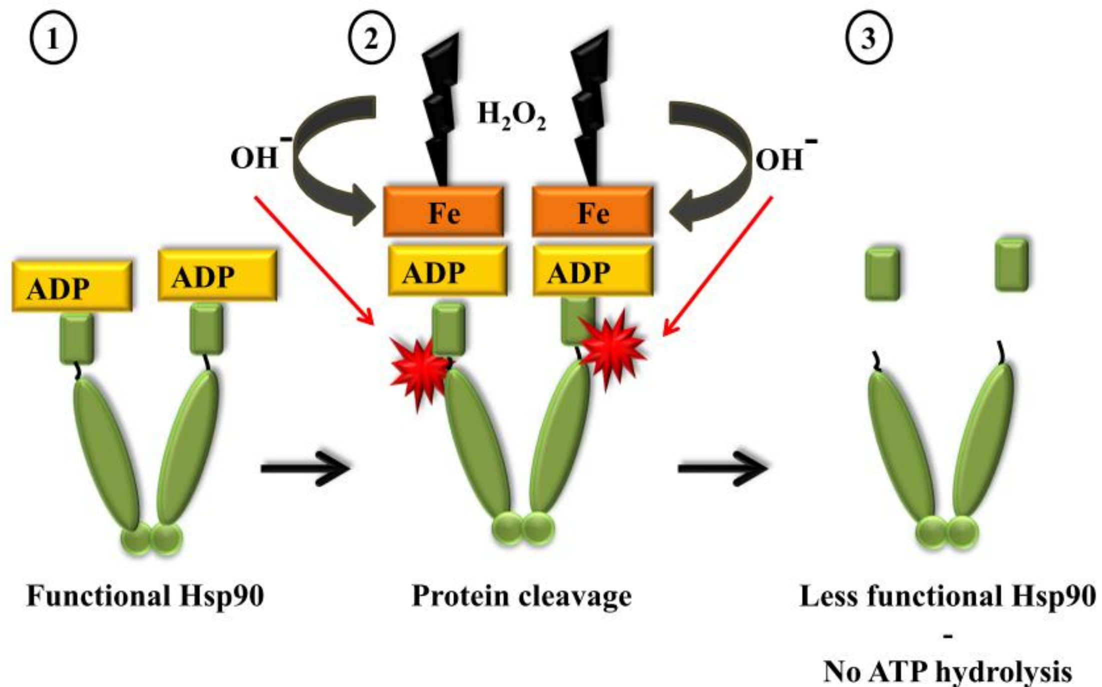

Figure 5. Hsp90 cleavage leads to a less functional protein. Hsp90 N-terminal binds ADP which converts into ATP when the chaperone activity is required (**1**). ADP has affinity for iron that, under oxidative stressful conditions acts as a co-factor in the H_2O_2 conversion into OH^- (Fenton reaction); the reactivity of OH^- impinges on some amino acids in the N-terminal generation a 73kDa protein fragment (**2**). As it lacks ATP binding domain, it is no longer fully functional (**3**). If iron is depleted from cells using iron chelators such as DFO, the cleavage is prevented.

1.3 Proteasome inhibition and protein aggregates lead to autophagy upregulation

The two major proteolytic degradation systems are the proteasome and the lysosome, which has a major role in the autophagy mechanism (described in the Introduction subchapter "*Crosstalk between proteasome and lysosomal autophagy*", and in [31]). In addition, there is evidence that autophagosome formation becomes upregulated when the proteasome is inhibited, although the opposite may not always be true.

Fortun *et al.* demonstrated that after proteasome inhibition, Peripheral Myelin Protein 22 (PMP22) aggregates form and recruit autophagosomes for lysosome mediated autophagy clearance [32]. Moreover, similarly to my own observations, Lazaro-Diegues *et al* have shown that jasplakinolide induced F-actin aggregates inhibited the chymotrypsin-like activity of the proteasome and were able to trigger the lysosomal autophagic system [33]. Adding to this, 17-AAG, a proteasome inhibitor, induced

cytoplasmic α-synuclein aggregates, that were cleared by the induction of autophagy [34]. These studies account in support of intertwined turnover mechanisms.

Autophagy is a fundamental cell process, triggered to remove intracellular abnormal molecules or damaged organelles [35]. Autophagosome formation has been described as following successive stages [31, 36] and to foster its course, an increasing number of proteins has been uncovered. Some are activated at the initiation stage whereas others are more relevant at a nucleation or expansion stage. At that point, it is especially important the cytosolic soluble mammalian LC3/LC3-I protein (Atg8 in yeast) that reacts with a phosphoglyceride, as the phosphatidylethanolamine, is converted into the LC3-II compound and is incorporated in the membrane of the autophagosome. The continued conversion allows LC3-II to bind more membrane and promote its growth to surround the cellular material targeted to be destroyed; LC3 members are also thought to contribute to the final closure of the phagophore membrane [31].

My investigation showed that by 9h of stress, LC3II/LC3I ratio is mounting to become remarkable at 24h, when the proteasome function is substantially decreased, the aggregates are established and cells are in a proliferation arrest state [12]. Interestingly, the LC-II/I ratio had an early transient increase in the soluble fraction, in agreement with previous observations [37], before it increased in the insoluble fraction. At this later time, either the phagophore LC3 marker or the late phagocytic stage lysosome LAMP-1 marker co-localized with the aggregates. Similarly, to our cells, in cultured oligodendrocytes with functionally impaired proteasome, it was reported that the autophagic marker LC3-II was upregulated and was recruited to the growing protein aggregates [38]. In addition, in $CD8^+$ replicative senescent lymphocytes accumulating lipofuscin [39] and in other aged cells with reduced proteasome activity [35, 40, 41] autophagy was found activated.

All these findings, in different experimental conditions, favor the view that when the proteasome becomes impaired, there is compensatory autophagy activation. Why should this occur is currently unknown but it may be related to intracellular sensors of proteasome impairment or protein aggregate accumulation that are activated in order to avoid further damage.

Autophagy requires a considerable amount of proteins whose study has shown to be far more important than previously thought. For example, Atg5 protein deficient cells, obtained by silencing or knocking out technical approaches, exhibit remarkable autophagy impairment and lipofuscin accumulation. This non-degradable material

present in the cytosol leads to increased ROS production and cell viability loss [42]. In contrast, autophagy enhancement was found to rescue Hela cells undergoing apoptosis [43]. Therefore, macroautophagy is not only a protective mechanism to prevent protein aggregates toxic effects [42] but also appears to be a fundamental survival process that is activated under extreme conditions [43].

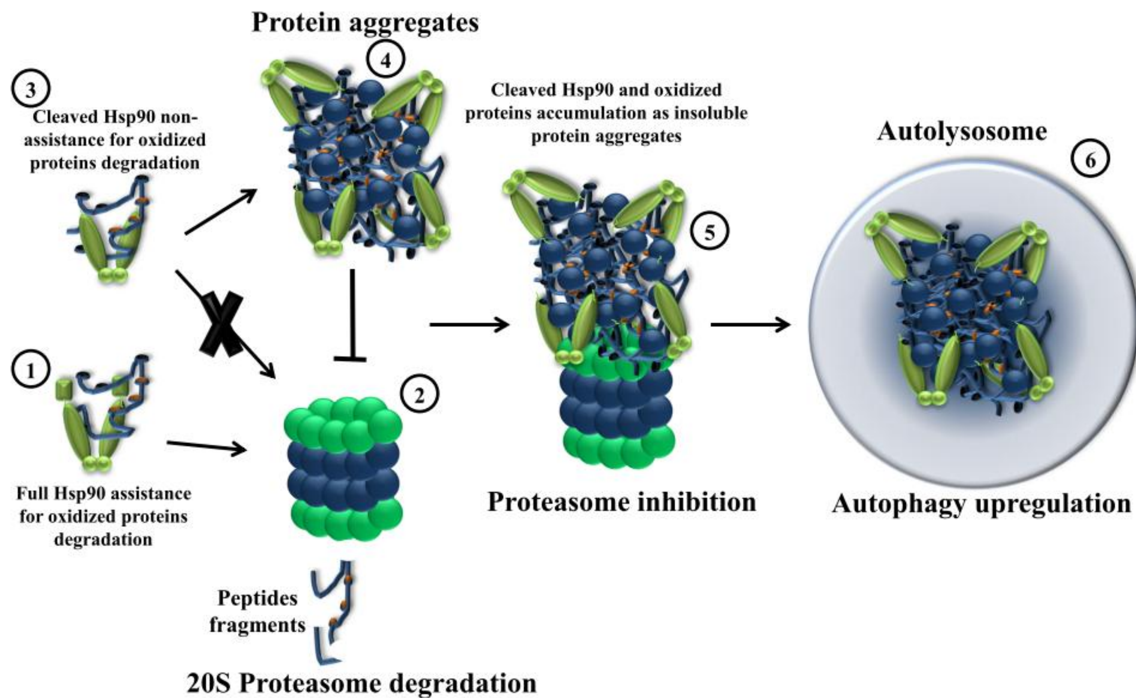

Figure 6. Oxidative promoted Hsp90 cleavage correlates with protein aggregates, proteasome inhibiton and autophagy upregulation. Supported by data our hypothesis points to a chain of events, starting with Hsp90 cleavage. In fact, functional Hsp90 **(1)** assists oxidized proteins (including actin) degradation **(2)** thus by the 20S proteasome delaying or avoiding their accumulation as protein aggregates. However, if Hsp90 gets N-terminally cleaved during oxidative stress **(3),** it loses functional capacities, although it binds to oxidized proteins **(4).** Hsp90c bound oxidized proteins accumulate which originates protein aggregates that later bind proteasome surface motifs and inhibit its enzymatic activity **(5**). To compensate the activity decrease, cells upregulate autophagy and form the phagosome that will engulf aggregates and fuses with lysosomes **(6**).

In short, for the first time, I report that the oxidation sensitive actin forms aggregates following Hsp90 cleavage and proteasome inhibition. These events lead to proliferation arrest and autophagy up-regulation as depicted in Fig.6. Although, here described in a lymphocyte cell line model, I am convinced that they are extensive to other cells and therefore relevant for the study of cellular senescence.

MAIN CONCLUSIONS

I. Oxidative stressful conditions result in Jurkat cell decreased functionality as evidenced by impairment of proteostasis and cell proliferation without viability loss

II. Time and intensity dependent oxidative stressful conditions lead to proteasome reduced activity, enhanced actin carbonylation and susceptibility to aggregate formation

III. Aggregate formation and accumulation are the likely result of actin (and other oxidized proteins) chaperone inability to convey oxidized actin for destruction at the 20S proteasome, consequent to oxidative stress dependent cleavage into the non-functional Hsp90c form

IV. Proteasome dependent proteostasis impairment antedates autophagy increase as a likely compensatory mechanism.

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