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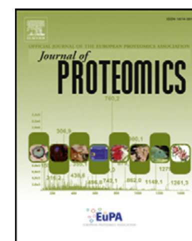

The role of heat stress on the age related protein carbonylation☆

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ABSTRACT

Since the proteins are involved in many physiological processes in the organisms, modifications of proteins have important outcomes. Protein modifications are classified in several ways and oxidative stress related ones take a wide place. Aging is characterized by the accumulation of oxidized proteins and decreased degradation of these proteins. On the other hand protein turnover is an important regulatory mechanism for the control of protein homeostasis. Heat shock proteins are a highly conserved family of proteins in the various cells and organisms whose expressions are highly inducible during stress conditions. These proteins participate in protein assembly, trafficking, degradation and therefore play important role in protein turnover. Although the entire functions of each heat shock protein are still not completely investigated, these proteins have been implicated in the processes of protection and repair of stress-induced protein damage. This study has focused on the heat stress related carbonylated proteins, as a marker of oxidative protein modification, in young and senescent fibroblasts. The results are discussed with reference to potential involvement of induced heat shock proteins.
This article is part of a Special Issue entitled: Protein Modifications.

Biological significance
Age-related protein modifications, especially protein carbonylation take a wide place in the literature. In this direction, to highlight the role of heat shock proteins in the oxidative modifications may bring a new aspect to the literature. On the other hand, identified carbonylated proteins in this study confirm the importance of folding process in the mitochondria which will be further analyzed in detail.
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Abbreviations: ATP, Adenosine triphosphate; BAG-1, Bcl2-associated athanogene 1 protein; BiP, The immunoglobulin heavy chain binding protein; DNPH, 2,4-Dinitrophenylhydrazine; GR, Glutathione reductase; Grp, Glucose-related protein; GS, Glutathione synthetase; HCl, Hydrogen chloride; Hip, Heat shock congate 70 interacting protein; HNE, 4-Hydroxynonenal; Hop, HSP70/HSP90 organizing protein; Hsc, Heat shock congate; NADPH, Nicotinamide adenine dinucleotide phosphate; NP40, Nonyl phenoxypolyethoxylethanol; PD, Population Doubling; PDI, Protein disulfide isomerases; Rpn, Regulatory Particle Non ATPase; Rpt, Regulatory particle tripleA-ATPase; SUMO-2, Small ubiquitin-related modifier 2; TRAP, Tumor necrosis factor receptor-associated protein; UBR4, E3 ubiquitin-protein ligase; Upb, Deubiquitinating enzyme; USP17, Ubiquitin carboxyl-terminal hydrolase 17-like protein

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1. Introduction

Aging is a complicated process characterized by the changes in cellular functions and decline in the physiological conditions. There are hundreds of theories to explain the mechanisms of aging including the mainly accepted free radical theory of aging firstly published by Harman [1]. This theory suggests a leading role of the oxidative modifications of biomolecules during aging. Besides the oxidative modification of other molecules, protein oxidation takes a wide place in this oxidative process. The main problem in the protein oxidation during aging, is the accumulation of damaged proteins because of the reduced efficiency of degradation mechanisms such as the proteasomal system and also the decrease in the efficiency of antioxidant defenses [2–9]. Proteasomal degradation in the cell is regulated by different mechanisms and recently heat shock proteins are believed to be among these regulators [10].

Heat-shock proteins (HSPs) belong to a highly conserved protein family whose expressions are inducible by stress conditions. HSPs account for 1–2% of total protein in unstressed cells and their expression is upregulated to 4–6% during exposure to stress conditions such as heat shock, excessive reactive oxygen species (ROS), drugs or inflammation [11]. Although the entire functions of each HSPs are still not fully clarified, most of the HSPs act as molecular chaperones and perform essential functions such as the proper folding of proteins and repair stress-induced protein damage [12].

The HSP family is encoded by the genes including HSP10, HSP27, HSP40, HSP60, HSP70, HSP90, and HSP110 as molecular chaperones [13,14]. HSP10 and HSP60 function as chaperonins that provide generally the correct folding of mitochondrial proteins, thus preventing aggregation [14]. Molecular chaperones HSP70 and HSP90 bind to unfolded polypeptide sequences in the cytoplasm and functions to the folding of proteins [15]. The chaperone HSP27 from small HSP family also mediates client holding and protein folding in an ATP independent manner. Coordinated activity of each of chaperones implied is important for efficient folding of the proteome [15,16].

In the aging process, damaged proteins undergo aggregation which results in aggregate accumulation and molecular chaperones are essential since protein aggregation is prevented by HSPs [17]. *Escherichia coli* studies show that aggregation during heat shock is prevented by the *E. coli* HSP70 homolog DnaK [15]. On the other hand, many key regulatory proteins including kinases and transcription factors require constant chaperoning by HSP90-containing complexes to have stable conformations for their activity [18] and decline in this protein quality control during aging leads to loss of function.

It was confirmed that all organisms respond to a mild heat shock by the HSPs [13] and it has been proposed that constitutive synthesis of heat shock protein 70 (HSP 70) can be used as a marker for thermal stress [19]. Heat shock protein related protection against stress has been found to be decreased during aging in rat tissues, human skin, and cultured human cell lines including T lymphocytes [20–28].

In the present study, we investigated human skin fibroblasts in an early stage of proliferation (young cells) and in a late stage (senescent cells). The activation of heat shock proteins (HSP40, HSP60, HSP70, HSP90) and protein carbonylation as an indicator of protein oxidation has been tested following heat stress (without and with recovery for 3 h). The results confirmed the previous results for the increase of protein carbonyls in senescence process. When the carbonylated proteins were identified, several interesting proteins were observed related to chaperone proteins and antioxidant proteins related to the repair or removal of oxidized proteins (Table 1).

2. Materials and methods

Cell culture reagents; DMEM, Penicillin–streptomycin solution, FBS, trypsin and PBS were purchased from Hyclone. SYPRO Ruby stain, Bradford protein assay reagent, and nitrocellulose membrane were from Bio-Rad. All HSP antibodies were obtained from cell signaling, anti-DNP (Dinitrophenylhydrazine) was from Sigma and the secondary antibodies were from Bio-Rad. All other chemicals were obtained from Sigma.

2.1. *Cell culture*

Human foreskin fibroblasts were cultured in DMEM supplemented penicillin 100 U/ml, streptomycin 100 μg/ml and 10% FBS in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. Cells initially were seeded at a density of 0.2×10^6 cells/ml and then grown for one week for young cells and three weeks for senescent cells to a density of 3×10^6 cells/ml. Population doubling values of young cells were 25 and of senescent cells were 58. Cell viability was determined using MTT to the corresponding blue formazan by viable cells.

2.2. *Heat treatment of human dermal fibroblast cells*

Cells were treated with mild hyperthermia at 42 °C for 1 h in culture medium and used immediately or following culturing at 37 °C for 3 h for analysis. Cells were washed twice in 1 ml PBS and lysed with buffer containing 10 mM Tris HCl (pH 7.5), 0.9% NP-40, 0.1% SDS, 1 mM Pefabloc, and homogenized by passing through a 25-gage needle ten times. Insoluble material was removed by centrifugation at 14,000 rpm for 20 min at 4 °C. Protein concentration was determined using the Bradford method.

2.3. *Immunoblotting for the determination of heat shock protein expressions*

Following the heat treatments and lysis of fibroblast cells as explained above, 30 μg of total protein in reducing Laemmli-buffer (0.25 M Tris pH 6.8, 8% SDS, 40% glycerol, 0.03% bromophenol blue) was denatured at 95 °C for 5 min and applied to SDS-PAGE of 12% (w/v) acrylamide, followed by electrophoresis and blotted onto nitrocellulose membrane according to standard procedures. Immunodetections were performed with the following antibodies: rabbit or mouse polyclonal anti-HSP90 (C45G5), anti-HSP60 (D307), anti-HSP70 (D69), anti-HSP40, and anti-β-actin (cell signaling) at 1:1000 dilution. After exposure to peroxidase-coupled secondary antibodies (Bio-rad) at 1:5000 dilution, membranes were

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