

HEAT-INDUCED OXIDATIVE STRESS AND INFLAMMATION IN RATS IN RELATION TO AGE

UDC:

(Original scientific paper)

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Abstract

Heat stress is implied to be an environmental factor for stimulating reactive oxygen species (ROS) production because of the resemblance in responses observed following heat stress with those occurring in the state of oxidative stress. To investigate the protein oxidation, lipid peroxidation and inflammation related to aging and heat stress we determined the levels of sulfhydryl (SH) groups, carbonyl (CO) groups, malondialdehyde (MDA) and tumor-necrosis factor-alpha (TNF- α) in the plasma of rats at two different ages (14 days and 18 months). The results showed that heat stress increased the plasma levels of CO, MDA and TNF- α and lowered the levels of SH. The concentration of TNF- α and MDA remained unchanged in relation to aging. Moreover the process of aging was characterized by increased protein oxidation, according to the observed decrease in the level of sulfhydryl groups and the increase in carbonyl groups plasma concentration. Our study showed that acute heat stress leads to increased lipid peroxidation, protein oxidation and inflammation in rats. Additionally, it was evident that young rats possess greater ability to cope with heat-induced oxidative stress and inflammation compared to the ability of aged animals, in which a pre-existing state of increased protein oxidation probably augments the cellular damage related to the heat exposure.

Keywords: Heat stress, age, lipid peroxidation, protein oxidation, inflammation, Wistar rats.

Introduction

Reactive oxygen species (ROS) are continuously produced *in vivo* and have many biological functions as well as the potential to induce serious damage to cells. During normal cell metabolism, ROS are produced for specific functions as signaling molecules. One of the primary intracellular ROS production site is the mitochondria (Beal, 2005). This organelle generates ATP through series of processes of oxidative phosphorylation that include reduction of the molecular oxygen to water, with the addition of four electrons. Unfortunately, during this process, some incomplete reductions can occur (using only one or two electrons), leading to the production of superoxide anion and hydrogen peroxide. Other examples of intracellular sources of ROS are the reactions which include the peroxisomal oxidase (Schrader & Fahimi, 2004) and NAD(P)H oxidase (Li et al., 2001). Superoxide anion, hydrogen peroxide and nitric oxide are the three types of ROS essential for normal physiological function, but are also responsible for acceleration of the aging process and play a role in the degenerative processes. Oxidative damage attributed to the ROS, plays a major role in the functional and physiological decline characteristic of aging (Harman, 1972). On the other hand, aging occurs due to loss of the ability to manage stress on a molecular level (Blake et al., 1990). Some of the mechanisms in the physiological changes of aging include: modification of the transcription regulation of the stress-induces genes (Papaconstantinou, 1994), faulty production of the inflammatory cytokines (Ershler, 1993) and insufficient synthesis of protector proteins (e.g. heat-shock proteins) (Kregel & Moseley, 1996). Oxidative stress occurs as a consequence of an imbalance between ROS production and inactivation. Major cellular antioxidant enzymes include: superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and glutathione peroxidase (GPx), as well as the nonenzymatic antioxidant tripeptide glutathione (GSH).

Elevated levels of ROS can lead to radical-mediated chain reactions that unselectively damage proteins (Stadtman & Levine, 2000), lipids (Rubo et al., 1994), carbohydrates (Kaur & Halliwell, 1994) and nucleic acids (Richter et al., 1988; LeDoux et al., 1999). Structurally polyunsaturated fatty acids are very susceptible to damage from ROS due to their multiple double bonds. This process is known as lipid peroxidation. Once initiated, this self-propagating process continues until the terminal products are produced or is interrupted by antioxidants. Radical-mediated peroxidation of the membrane lipids can lead to serious damages and is associated with changes in the biophysical features of the membrane and inactivation of the membrane bound receptors or enzymes, which can lead to inability to perform normal physiological functions. The process of peroxidation of the cell membrane leads to accumulation of the terminal products of the process, such as malondialdehyde (MDA) and F₂ isoprostanes. These highly reactive products of lipid peroxidation have the ability to covalently bond and modify crucial biomolecules, a process that leads to amplification of the cellular damage (Gutteridge & Halliwell, 1990).

Oxidation of the proteins is defined as covalent modification of the molecule induced directly by ROS or indirectly through secondary byproducts of the oxidative stress. There are different types of oxidative modifications of the proteins, including oxidation of the sulfhydryl groups, reduction of disulfides, reaction with aldehydes, protein-protein cross bonding and fragmentation of peptides (Stadtman & Oliver, 2005; Starke-Reed & Oliver, 1989).

Another hallmark of oxidative stress is its relation to the production of cytokines. Tumor necrosis factor alpha (TNF- α) is a multifunctional cytokine involved in many different signaling pathways, such as inflammation. It can show opposing biological effects suggesting complex regulatory mechanisms. TNF- α release is mainly triggered by viral infections, endotoxins, lipopolysaccharides, bacterial components, tissue injury, DNA damage, by interleukin-1 or TNF- α itself. It is produced mainly by activated macrophages, but also by natural killer cells, neutrophils, mast cells, eosinophils and neurons (Old, 1985). The primary role of TNF- α is in the regulation of immune cells, but it is also an endogenous pyrogen that induces fever, induces apoptotic cell death, inhibits tumor genesis and viral replication and responds to infections via interleukin-1 and interleukin-6 producing cells.

The body core temperature could also be increased by exercise (Wenger, 1995). Performing excessive exercise typically increases the instance of muscle injury and development of soreness. Accompanying the muscle damage is a local inflammatory response that includes infiltration of leukocytes into the damaged tissue (Fielding et al., 1993; Tidball et al., 1993). Excess ROS production by accelerated mitochondrial respiration is held responsible for cellular damage upon hyperthermic exposure and heat stress (Abele et al., 1998, 2002). Heat stress is implied to be an environmental factor responsible for stimulating ROS production because of the resemblance in responses observed following heat stress compared with those occurring following exposure to oxidative stress (Slimen et al., 2014). Some findings suggest that the elevated body temperature can induce the metabolic changes that are involved in the induction of oxidative stress (Sawka & Wenger, 1988).

Considering the relations between oxidative stress and inflammation the aim of this study was to follow oxidative stress through protein oxidation, lipid peroxidation and inflammatory response during heat exposure and to determine how age affects these mechanisms. The measurements of carbonyl and sulfhydryl groups were used to assess ROS-mediated protein oxidation, MDA was used as a marker for lipid peroxidation and TNF- α as a marker for inflammation.

Material and methods

Ethics statement

All protocols were approved by the Animal Ethics Committee of the Faculty of Natural Sciences, Skopje, R. Macedonia, in accordance with the International Guiding Principles for Biomedical Research Involving Animals, as issued by the Council for the International Organizations of Medical Sciences. Anesthetic was applied according to the standards given by the guide of the EC Directive 86/609/EEC.

Animals and experimental design

Male Wistar rats (n=32) were used for all protocols and were maintained on a 12:12 light:dark cycle and fed with rat chow containing 0 mg α -tocopherol and water ad libitum. All animals were divided into four groups. Two groups consisted of 15 days

old rats. The other two groups included 18 months old rats. The two groups of young rats were: YHU-young heat unexposed and YHE-young heat exposed. The aged rats were grouped as AHU- aged heat unexposed and AHE-aged heat exposed. The heat exposed rats were housed individually in a special heated

chamber, maintaining a constant temperature of 40 ± 0.5 °C and relative air humidity of 40–50%. During heat exposure, rectal temperatures (T_{rec}) were measured by an electrical thermometer (Ellab TE 3) every 10 min, until they reached the temperature of the chamber, then the readings continued to be taken at 2 min intervals. The temperatures were recorded at various distances from the anal sphincter in dependence from age. Thermistor probes were inserted 25 or 45 mm (Lomax, 1966), into the rectum of young or middle-aged rats, respectively. The exposure was terminated in term of 60 min (Hadzi-Petrushev et al., 2011). Immediately after the heating procedure animals were anesthetized with intraperitoneal injection of (50 mg kg^{-1} b wt.), thiopental sodium BP; Rhone-Poulenc Rorer Limited, Nenagh, Co. Tipperary, Ireland). Shortly after, the rats were decapitated under anesthesia and the abdomen was immediately incised.

Blood collection

Blood was taken from the abdominal aorta and collected in Vacutainer tubes with ethylene-di-amine tetra-acetate as anticoagulant. The blood samples were centrifuged (15 min, 3000 rpm), the plasma was frozen at -20 °C, and then stored at -80 °C until analyses were performed.

Biochemical analysis

TNF- α assay

The plasma levels of TNF- α were determined using commercially available enzyme-linked immunosorbent assay kits (TNF- α Platinum ELISA, Bender Med Systems, Vienna, Austria). The results are expressed as $pg\ mL^{-1}$. All assays were performed as outlined in the protocols enclosed in each kit. Intraassay and inter-assay coefficients of variation were $\leq 10\%$.

Assays for SH and CO groups

The method for determination of SH and CO groups, as indices of protein oxidation products, were determined by colorimetric assays. Color reactions were read on spectrophotometer Cintra 6. The results were expressed as $lmol/l$ plasma for SH, and $nmol/ml$ plasma for CO groups, respectively.

MDA determination

For determination of the concentration of MDA in blood plasma, the method modified by Yagi (1998) was used. To the sample of plasma were added solutions of 50% TCA and 1.3% TBA (dissolved in 0.3% NaOH). After the period of incubation for 20 minutes in water bath at temperature of 90-95°C, the samples were cooled down and were centrifuged and the absorption of the supernatant was measured at 535nm. As a standard was used 1,1,3,3-tetraethoxypropane.

Statistical analysis

Results are presented as means \pm standard error (SE). Data was analysed using One-way analysis of variance (ANOVA). The post hoc test of Bonferroni was performed in selected instances to evaluate further differences between group pairs. All analyses were performed with GraphPad Prism 4.0 (San Diego, CA, USA). $p < 0.05$ was considered statistically significant.

Results

Influence of aging and acute heat exposure on the levels of carbonyl groups (CO)

The levels of carbonyl groups in plasma increased in function of aging. Samples from young heat exposed compared to samples from aged heat exposed were significantly different ($p < 0.05$). The heat exposure did not evoke changes in the concentration of carbonyl groups in the plasma of both young and aged rats.

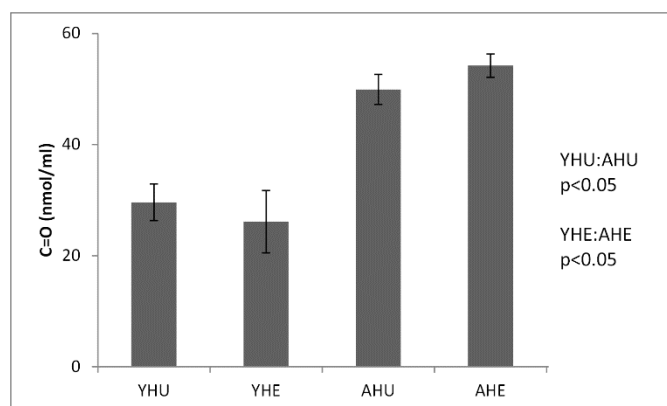


Fig. 1. Levels of carbonyl groups in samples from rat's plasma in function of age and heat exposure in YHU - Young heat unexposed, YHE - Young heat exposed, AHU - Aged heat unexposed, and AHE - Aged heat exposed.

Influence of aging and acute heat exposure on the levels of sulfhydryl groups (SH)

The levels of sulfhydryl groups in the rat plasma samples significantly decreased in function of aging ($p < 0.05$). Heat exposure also decreased the levels of SH in samples taken from young rats ($p < 0.01$). Aged heat unexposed rats compared to aged heat exposed animals had a significant depletion of SH levels ($p < 0.05$). Additionally, aged heat exposed rats had a depletion of plasma SH levels compared to the samples taken from young heat exposed rats ($p < 0.01$).

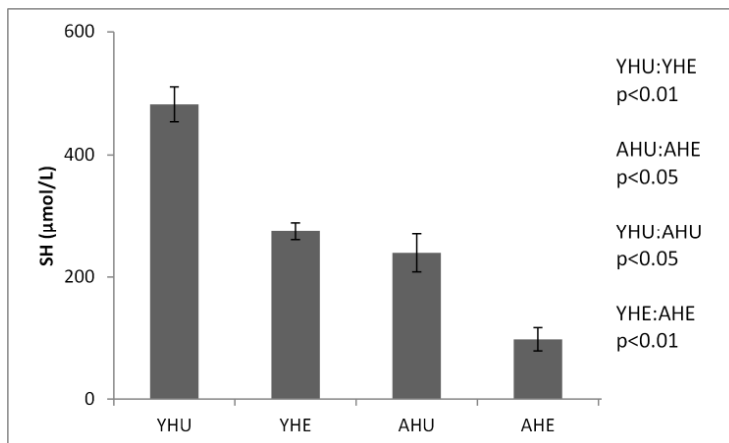


Fig 2. Levels of sulfhydryl groups (SH) in samples from rat's plasma in function of age and heat exposure in YHU - Young heat unexposed, YHE - Young heat exposed, AHU - Aged heat unexposed, and AHE - Aged heat exposed.

Influence of aging and acute heat exposure on the levels of MDA

Levels of MDA remained unchanged in function of aging. Acute heat exposure in young rats significantly increased the plasma concentration of MDA ($p < 0.05$). Samples taken from aged heat exposed rats had significantly greater concentrations of MDA compared to samples taken from aged heat unexposed rats ($p < 0.05$).

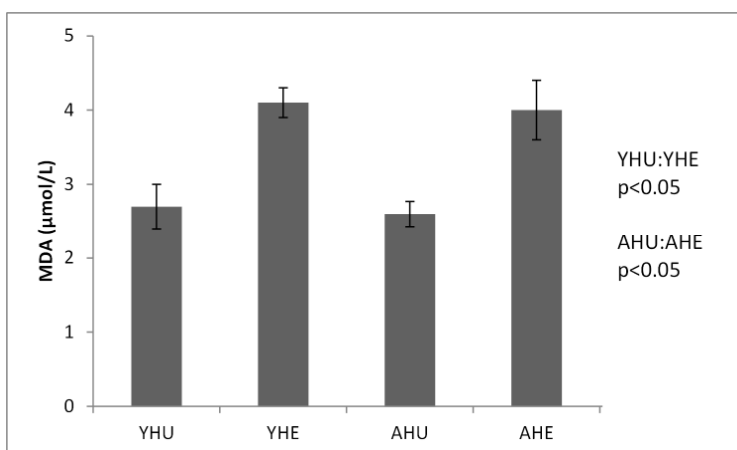


Fig 3. Levels of MDA in samples from rat's plasma in function of age and heat exposure in YHU - Young heat unexposed, YHE - Young heat exposed, AHU - Aged heat unexposed, and AHE - Aged heat exposed.

Influence of aging and acute heat exposure on the levels of TNF-α

The aging process had no significant impact on TNF- α levels. Young heat exposed rats had significantly higher TNF- α levels compared to young heat unexposed rats ($p < 0.05$). Also, the heat exposure caused a significant increase in TNF- α levels in the plasma of aged animals.

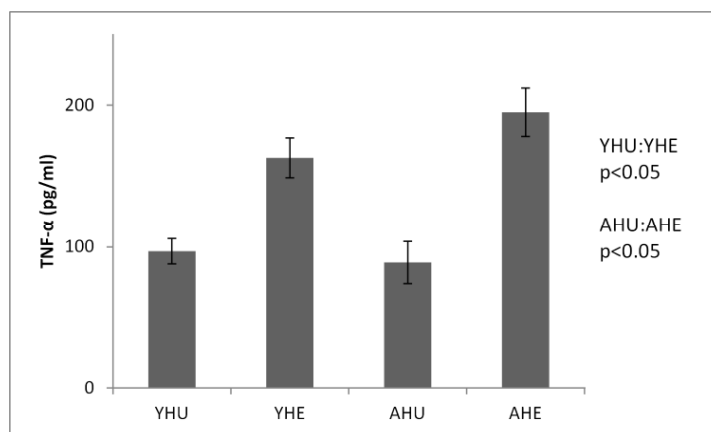


Fig 4. Levels of TNF- α in samples from rat's plasma in function of age and heat exposure in YHU - Young heat unexposed, YHE - Young heat exposed, AHU - Aged heat unexposed, and AHE - Aged heat exposed.

Discussion

The aim of this study was to investigate the effects of aging and acute heat exposure on protein oxidation, lipid peroxidation and inflammation in rats. It is known that acute heat exposure leads to changes in the cardiovascular, endocrine and nervous system (Hutter et al., 1996) and whole body exposition to heat induces reduction of the blood flow in the gastrointestinal tract (Kubes, 1993). This highly specific circulatory adaptation allows large quantities of blood to move towards peripheral circulation, so that the temperature would lower (Rowell et al., 1971), but at the same time this adaptation leads to hypoxia (Hall et al., 1994), production of ROS (Hall et al., 2000) and depletion of ATP. According to the free radical theory of aging, Harman (1956; 1972) contributed the aging due to continuous free radical damage over time. Precisely, it states, that reactive oxygen species that are produced in the mitochondria cause damage to biological macromolecules including lipids, proteins and mitochondrial DNA. This damage causes further mutations which lead to increased production of ROS and enhance the accumulation of free radicals in the cells due to mitochondrial dysfunction (Van Remmen & Richardson, 2001). Also the products of lipid peroxidation contribute to the degenerative changes associated with the process of aging (Kohen & Nyska 2002; Szwedra et al., 2003), initiating chain reactions of the free radicals and inducing oxidative damage to the proteins and DNA (Nakamura & Omaye, 2004; Stocker & Keane, 2004; Vassalle et al., 2004).

The process of aging and acute heat exposure and their effect on protein oxidation was measured through the changes in the levels of carbonyl (C=O) and sulfhydryl (SH) groups in the plasma of the rats. Some amino acids such as lysine, proline, and tyrosine go through changes that lead to formation of carbonyl (CO) groups that can be aldehydes or ketones according to their side chains (Amici et al., 1989). Presence of carbonyl groups in aged animals is a marker for ROS-mediated protein oxidation. This type of oxidation is associated with the process of aging, oxidative stress and a number of pathological states (Levine et al., 1994). The oxidation products of the proteins can covalently bind to other proteins (Hazen et al., 1997). Binding to glutathione can occur in states of oxidative stress, which leads to less effective antioxidant activity (Cabiscol & Levine, 1996). Keeping in mind all the different and unique biological functions of the proteins, their oxidative modification could have vast functional consequences. Oxidative modification of the enzymes leads to inhibition of their activity (Stadtman, 1990). Large number of studies have shown that enzymes go through oxidative modification during the process of aging (Oliver et al., 1987). In this group of enzymes are included glutamine synthetase (Carney et al., 1991), mitochondrial aconitase (Yan et al., 1997), adenine nucleotide translocase (Yan & Sohal, 1998) and carbonic anhydrase (Cabiscol & Levine, 1995). The first three enzymes showed increased CO group presence, while the last enzyme showed affinity to covalent binding to glutathione (Cabiscol & Levine, 1996). Interestingly, in the same study the heat exposure did not evoke changes in the levels of CO groups measured in the rat's plasma. The elevation of CO groups in plasma is the result of normal physiological changes that occurred during the process of aging. In our study the levels of sulfhydryl groups in plasma decreased in function of aging and acute heat exposure. More specifically, during the normal physiological process of aging that is accompanied with

loss of function and modification of biomolecules and during acute heat stress, SH levels in the rat's plasma are depleted. The exact mechanisms of this occurrence need to be further investigated.

Using MDA as an accurate marker for lipid peroxidation, we have confirmed that acute heat exposure induces significant increase in the concentration of MDA in both young and aged animals. These results are similar to the findings of Mladenov et al. (2006), where they confirmed that acute heat exposure induces lipid peroxidation in the liver. Increased levels of MDA correlate with loss of the antioxidant capacity suggesting that the reduced antioxidative activity is the reason for oxidative stress (Rizvi & Maurya, 2007). Accumulation of the oxidized lipids creates suitable conditions for further changes in the cells, according to the fact that oxidized lipids regulate genes that are connected to the process of inflammation, lipid metabolism and cell proliferation (Bochkov et al., 2010).

Our investigation of the inflammation marker THF- α led us to the finding that acute heat exposure induces higher concentration of TNF- α in the plasma. Brenner et al., (1995), also postulated that some physical stressors can lead to higher levels of circulating TNF- α , but they were unable to identify the involving mechanisms. High levels of TNF- α in the blood plasma is correlated with increased levels of IL-6 and C-reactive protein, and together induce a combined activation of the inflammatory cascade (Bruunsgaard et al., 1999). Considering the relation between heat stress and oxidative stress, we are on opinion that the heat-induced increase in TNF- α levels observed in our study could be contributed to the cross-talk that arises between pro-inflammatory cytokines and oxidative stress through activation of mitogen-activated protein kinases (MAPK) and nuclear factor kappaB (NF-kappaB). Pro-inflammatory cytokines, particularly TNF- α , and oxidative stress promote each other often generating a vicious circle (Pereda et al. 2006).

In conclusion, acute heat stress led to increased lipid peroxidation, protein oxidation and inflammation in rats. The process of aging did not change significantly the levels of the markers of lipid peroxidation and inflammation in the plasma, but there was age-related increase in the concentration of carbonyl groups, in parallel to a significant decline in the concentration of sulfhydryl groups. This pre-existing, age-related state of more pronounced protein oxidation is most likely responsible for the observed lower tolerance of aged rats to heat stress compared to the tolerance of young animals. However, the exact mechanisms of these changes needs to be further investigated.

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