

Age and Heat Exposure-Dependent Changes in Antioxidant Enzymes Activities in Rat's Liver and Brain Mitochondria : Role of α -tocopherol

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Short title: Changes in Rat's Mitochondrial Antioxidant Enzymes Activities

Age and Heat Exposure-Dependent Changes in Antioxidant Enzymes Activities in Rat's Liver and Brain Mitochondria : Role of α -tocopherol

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Summary

To investigate the role of mitochondrial antioxidant capacity during increased susceptibility to heat accompanied by the aging, young and aged Wistar rats were exposed on heat for 60 min. After heat exposure, hepatic and brain mitochondria were isolated. Our results revealed changes in antioxidant enzyme activities in liver and brain mitochondria from young and to a greater extent in aged rats. Our measurements of MnSOD, GPx and GR activity indicate greater reactive oxygen species production from the mitochondria of aged heat exposed in comparison to young heat exposed rats. Also in the aged rats, the effect of α -tocopherol treatment in the prevention of oxidative stress occurred as a result of heat exposure, is less pronounced. Taken together, our data suggest that mitochondria in aged rats are more vulnerable and less able to prevent oxidative changes that occur in response to acute heat exposure.

Key words

Antioxidant enzymes · mitochondria · liver · brain · aging · heat exposure

Introduction

According to the “mitochondrial theory of aging”, mitochondria are incorporated as critical components that regulate aging. This theory postulates that electrons from the mitochondrial electron transport chain (ETC) produce reactive oxygen species (ROS), which damage components of the ETC, leading to a cyclic increase in intracellular ROS levels and a decline in mitochondrial function (Wallace 2005). The same author published that such a declined mitochondrial function could be the result of superoxide anions, whose production is induced by different stressors. On the other hand, an ameliorative mechanism responsible for correction of superoxide anions, includes conversion to membrane-permeable hydrogen peroxide (H_2O_2) by the action of mitochondrial superoxide dismutase (SOD), dominantly present in the matrix as a (MnSOD) and in inter-membrane space as a (CuZnSOD) (Ozturk *et al.* 2012, Aydin *et al.* 2012, Morrison *et al.* 2005). Further, H_2O_2 can be reduced to water by glutathione peroxidase (GPx) using electrons from glutathione (GSH) (Jaeschke 1990). The last reaction ends with oxidation of GSH into glutathione disulfide (GSSG), as an index for increased H_2O_2 formation in mitochondria (Jaeschke 1990, Knight *et al.* 2001), although extra-mitochondrial sources of ROS cannot be excluded. In the same study (Knight *et al.* 2001), it was reported that despite GPx, catalase (CAT) could be also involved in neutralization of H_2O_2 produced during different acute stress conditions. Hence, they promote CAT as an important player in the regulation of red-ox balance. Another study (Olafsdottir and Reed 1988) indicates that the activity of glutathione reductase (GR) is especially important in preventing protein oxidation in mitochondria during acute stress. However, our review of recently published research showed that there are no scientific data

supporting the studies about the determination of mitochondrial enzyme red-ox mechanisms induced during acute heat exposure and in function of aging in the same time.

The α -tocopherol as a lipid-soluble antioxidant, effectively prevents lipid peroxidation (Knight *et al.* 2001). Few studies, demonstrated that increased level of α -tocopherol in mitochondria is critical against oxidative stress in the same mitochondria (Farris and Zhang 2003, Farris *et al.* 2001, Zhang *et al.* 2001a). In fact, recent findings indicate that enrichment of mitochondria with protective α -tocopherol, weakens production of ROS and antioxidant enzyme activities, especially in hepatic mitochondria (Chow *et al.* 1999, Zhang *et al.* 2001b). Nevertheless, up to date there are no studies that examine preventive role of α -tocopherol on mitochondrial level, during acute heat exposure associated with different ages.

Considering that generation of ROS typically associated with the application of heat stress is exaggerated with advancing age (Hadzi-Petrushev *et al.* 2011), our approach was to determine preventive role of α -tocopherol on mitochondrial antioxidant enzymes, influenced by acute heat exposure, in rats of different ages. Our experiments were focused on liver and brain, because they are very active metabolically, contain the largest number of mitochondria and show age-related increased ROS production (Yen *et al.* 1994). Therefore, the objective of this investigation was to test hypothesis that α -tocopherol, due to its ROS scavenging activity, would aid the maintenance of antioxidant capacity in mitochondria and prevent some of age and heat stress related changes in antioxidant enzyme activities.

Material and Methods

Animals and experimental design

All experimental procedures were conducted in accordance with the Guiding Principles for Care and Use of Laboratory Animals approved by the Macedonian Center for Bioethics. All protocols were approved by the Animal Ethics Committee of the University “Sts. Cyril and Methodius”, Skopje, R. Macedonia in accordance with the International Guiding Principles for Biomedical Research Involving Animals, as issued by the Council for International Organizations of Medical Sciences. Anesthetics were applied according to standards given by the guide of the EC Directive 86/609/EEC. Animals were anesthetized with an intraperitoneal injection of (100 mgkg⁻¹ b.wt., thiopentane sodium BP; Rhone-Poulenc Rorer Limited, Nenagh, Co Tipperary, Ireland). Male Wistar rats (n = 64) 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 eight groups. Four groups which consisted of 15 days old rats were further divided depending on treatment with α -tocopherol and heat exposure into: young placebo heat-unexposed (n=8; YP), young heat-exposed (n=8; YH), young α -tocopherol treated heat unexposed (n=8; YT) and young α -tocopherol treated heat-exposed (n=8; YTH) rats. The other four groups included 12 months aged rats were divided in a similar manner into: aged placebo heat unexposed (n=8; AP), aged heat-exposed (n=8; AH), aged α -tocopherol treated heat unexposed (n=8; AT) and aged α -tocopherol treated heat-exposed (n=8; ATH) rats.

In vivo treatment and heat exposure

For 21 consecutive days rats in α -tocopherol groups were treated intra-gastrally with vegetable oil containing α -tocopherol (0,32 IU kg⁻¹, Sigma) and rats in placebo groups were

injected intra-gastrally with vegetable oil containing 0 mg α -tocopherol. Previously was shown that used dose of α -tocopherol significantly increase the levels of α -tocopherol in several tissues, including liver and plasma in mice (Berg *et al.* 2004, Godbout *et al.* 2004a, Godbout *et al.* 2004b). During the treatment period all animals were housed at 20 ± 2 °C.

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 (rec) temperatures (T_{rec}) were read by an electric thermometer (Ellab TE 3) every 10 min until it reached the temperature of the chamber, then the readings continued to be taken in 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 the young or aged rats, respectively. The exposure was terminated in terms of 60 minutes (Hadzi-Petrushev *et al.* 2012, Aprile and Austin 1981).

Isolation of mitochondria

Animals were sacrificed by ether narcosis, and the appropriate organs were immediately removed. Liver mitochondria were isolated using the method described by [23]. Tissues were homogenized in ice-cold buffer containing 1 mM Tris-HCl, 0.25 M sucrose and 1 mM EDTA, pH 7.4. The homogenate was first centrifuged at 600g for 10 min at 4 °C and the supernatant fractions were collected and further centrifuged at 8000g for 20 min at 4 °C in order to pellet the mitochondria. After washing with 0.25 M sucrose buffer containing 1 mM EDTA and 1 mM Tris-HCl, pH 7.4, mitochondrial pellets were resuspended in 0.25 M sucrose buffer containing 1 mM Tris-HCl, pH 7.4 and stored at -80 °C until further analyses.

Mitochondria in the brain were isolated using the method described by [24]. Tissues were homogenized in ice-cold buffer containing 0.3 M mannitol and 0.1 mM EDTA, pH 7.4. The homogenates were first centrifuged at 600g for 10 min at 4 °C and the supernatant fractions were collected and further centrifuged at 10000g for 10 min at 4 °C in order to obtain the brain mitochondria. The mitochondrial pellets were washed three times with 0.25 M sucrose buffer containing 0.1 mM EDTA, pH 7.4, resuspended in 0.25 M sucrose buffer, pH 7.4 and stored at -80 °C until further analyses. Mitochondria were disrupted by twice freezing and thawing before analyses.

Assay for MnSOD activity

MnSOD activity was determined according to the method described by [25], based on the ability of MnSOD to inhibit the auto-oxidation of pyrogallol. The reaction mixture consisted of 50 mM Tris-HCl, pH 8.2, 1 mM diethylenetriamine pentaacetic acid, and sample. CuZnSOD activity was inhibited by the addition of 0.1 M NaCN to the assay buffer for 15 min. The reaction was initiated by the addition of pyrogallol (final concentration of 0.2 mM) and the absorbance measured kinetically at 420 nm (25 °C) for 3 min. One unit of activity is defined as the amount of sample needed to inhibit pyrogallol oxidation by 50 %. The final results were expressed as U/mg protein.

Assay for total GPx activity

The activity of GPx was determined according to Lawrence and coworkers [26], with some modifications. The activity was assayed by following the oxidation of NADPH at 340 nm for 3 min (25 °C) in the presence of GR and GSH. The reaction mixtures containing 50 mM potassium phosphate, pH 7.0, 1 mM sodium azide, 2 mM GSH, 0.2 mM NADPH, 1 U/ml GR, 1.5 mM cumene hydroperoxide and samples were incubated at 25 °C for 5 min. The reaction was initiated by the addition of cumene hydroperoxide. The unit was defined as the oxidation of 1 μ mol of NADPH/min. Final results were expressed as U/mg protein.

Assay for GR activity

The rate of oxidation of NADPH by GSSG at 30 °C was used as a standard measure of GR activity [27]. The reaction system contained 1 mM GSSG, 0.1 mM NADPH, 0.5 mM EDTA, 100 mM potassium phosphate buffer, pH 7.5 and a suitable amount of sample. The oxidation of 1 μ mol of NADPH/min was defined as a unit of glutathione reductase activity. The specific activity was expressed as U/mg protein.

The mitochondrial protein content was determined using the method described by [28]. Analyses for MnSOD, GPx, and GR were carried out on the ELISA reader (Bio-Rad), while analyses for protein concentration were carried out on the spectrophotometer *Cintra 6*.

Statistical analysis

Variables are reported as mean values \pm standard deviation (SD). Data were analyzed by 3 way ANOVA. The means were compared using the Tukey test. Multiple linear regression analysis was performed to assess independent predictor of GPx and GR activity. Differences

were considered significant at a level of $p < 0.05$. The statistical software package SPSS 16.0 for Windows (SPSS, Chicago, IL, USA) was used for all analyses.

Results

Time course of rectal temperature dynamics in heat-exposed animals

The time course of rectal temperature dynamic in heat-exposed animals is well documented in our previous study (Mladenov *et al.* 2006). In this study we employed the same conditions with shortened duration of exposure. In fact, we terminated exposure in terms of 60 minutes, during which time the rats achieved a second phase known as temperature plateau. Our expectation was that this duration of exposition could be enough to throw the rats in “alarming” high-temperature state, as a suitable condition for investigation of early **mitochondrial events** of temperature shock. Our results revealed that after 30 min of heat exposure as a transitional point between primary hypertermia and temperature plateau (Mladenov *et al.* 2008), α -tocopherol treatment did not cause significant changes ($p < 0.092$ and 0.104) in rectal temperatures in both young and aged animal groups, when compared to placebo rats. Rectal temperatures 30 min after heat exposure significantly increased in both young and aged animals ($p < 0.05$, respectively) in comparison to their unexposed counterparts (Table 1). The rectal temperatures for both treated and placebo heat unexposed groups, were also measured according to the same protocol applied for heat exposed rats and there were no changes in rectal dynamics during proposed interval of measurement (results are not shown).

Influence of aging, α -tocopherol treatment and acute heat exposure on MnSOD activity

MnSOD activity in the liver and brain mitochondria of aged animals was significantly higher compared to the values obtained from young rats ($p < 0.05$ and 0.001 , respectively). α -tocopherol treatment caused significant decrease of MnSOD activity in brain mitochondria of both, young and aged ($p < 0.05$, respectively), and only in liver mitochondria of the aged rats ($p < 0.05$). The preventive effect of α -tocopherol treatment was also observed for MnSOD activity in liver mitochondria of heat-exposed rats ($p < 0.05$, for both young and aged, respectively). However, the activity of MnSOD in brain mitochondria of heat-exposed α -tocopherol treated rats wasn't changed in comparison to the activity in untreated heat-exposed rats, regardless of age ($p = 0.672$ and $p = 0.534$). On average, the brain mitochondria showed lower MnSOD activity compared to the liver mitochondria (Fig. 1A and 1B).

Influence of aging, α -tocopherol treatment and acute heat exposure on GPx activity

Data presented in Fig. 2A and 2B shows that aging caused significant increase in GPx activity in liver and brain mitochondria ($p < 0.05$ and 0.001 , respectively). In the brain mitochondria from aged rats, were registered significant heat-induced deterioration in the GPx activity ($p < 0.05$). The treatment with α -tocopherol caused significant decreasing of GPx activity in aged liver and brain mitochondria in both ages ($p < 0.05$ respectively). Similarly to relations observed in MnSOD activity, the brain mitochondria showed lower average GPx activity compared to the liver mitochondria.

Influence of aging, α -tocopherol treatment and acute heat exposure on GR activity

Aged rats showed significantly higher GR activity in liver and brain mitochondria compared to their younger counterparts ($p < 0.001$, for both). Acute heat exposure caused a

significant elevation of GR activity in aged brain and liver mitochondria of both aging groups ($p < 0.05$, respectively). The α -tocopherol treatment effectively prevents this change in liver mitochondria at both heat-exposed aging groups, which can be concluded checking up the pairs YH/YTH and AH/ATH Fig. 3A. In brain compartment, the enzyme activity in ATH was significantly lower in comparison with AH, while the means of AT and ATH differ non-significantly ($p = 0.121$) (Fig. 3B). On overall, GR activity in liver mitochondria was higher than GR activity in brain mitochondria (Fig. 3A and 3B).

Multiple regression analysis

In order to define relationship between GPx and GR enzyme activity and MnSOD activity as an index of oxidation that precede consequent activation of the above enzymes in mitochondrial level (Wallace 2005), multiple regression analysis was performed with baseline GPx and GR enzyme activity as dependent variable. These results are detailed in Table 2 and show that mitochondrial MnSOD activity determine changes in GPx activity only at α -tocopherol: AT liver mitochondria (adjusted $r^2 = 0,311$, $p < 0,025$ and $\beta = 0,50$ for MnSOD), YT brain mitochondria (adjusted $r^2 = 0,341$, $p < 0,030$ and $\beta = 0,53$ for MnSOD) and AT brain mitochondria (adjusted $r^2 = 0,371$, $p < 0,028$ and $\beta = 0,56$ for MnSOD).

Discussion

Although many investigators have studied heat and age-related changes in mitochondrial antioxidant defense, results are still controversial (Rikans and Hornbrook 1997). For this reason,

we tried to find what happens with the mitochondrial enzymatic antioxidant line during heat exposure in two different aging settings.

In our study, mitochondrial MnSOD activity didn't change significantly as a consequence of heat exposure. Actually, in our opinion applied protocol of heat stress is not long enough to induce such a huge production of superoxide that will overwhelm mechanisms responsible for its neutralization. On the contrary, Djordjevic with cow. (2010) suggested that oxidation as a consequence of heat exposure can be initiated at this stage, especially if subsequent protective enzyme GPx does not work in sequence with the MnSOD. On the other hand, we observed heat-induced deterioration of GPx activity in mitochondria of aged rats, while GR activity was significantly increased in the aged brain and both liver mitochondria. This relationship between GR and GPx, especially in aged mitochondria, indicates for impaired removal of H₂O₂ by GPx. One possible explanation is the high GPx dependence from glutathione (GSH) for its activity, so decreasing of the hepatic GSH level with aging, shown from (Liu *et al.* 2002), may partly be responsible for the lowered GPx activity. Moreover, the renewable capacity of tissues for GSH could become very weak with the aging, which can be an additional reason for GPx injury during acute heat exposure (Liu *et al.* 2002).

The treatment with α -tocopherol caused significant decrease in both GPx and MnSOD activity in the liver and brain mitochondria of the aged rats. These, α -tocopherol induced changes in the activity of GPx can be explained by changes in the activity of MnSOD only in the liver mitochondria of the aged rats. Such a role of α -tocopherol is supported by data about subcellular distribution of α -tocopherol, indicating that liver's mitochondrial fraction contains higher concentrations of α -tocopherol than brain's (Murphy and Mavis 1981). Concerning the changes in GPx activity, as a consequence of α -tocopherol, our regression model shows that MnSOD can

be taken as a determinant variable of GPx in both young and aged brain mitochondria. These results can be explained by an inverse relationship between the concentration of α -tocopherol and GPx activity in rat brain mitochondria (Murphy and Mavis 1981). Further, we did not observe significant α -tocopherol influence on GR activity in both liver and brain mitochondria from young and in liver mitochondria from aged rats. In this direction, Iqbal and coworkers (Iqbal *et al.* 2002), also have shown that α -tocopherol has no effect on GR activity in lung mitochondria. This insignificant effect was additionally corroborated by our regression model, which don't support association between the changes in MnSOD and GR activity. On the other hand, prevented GR activity in the presence of α -tocopherol was outlined only in the aged heat exposed brain mitochondria. Tretter and Adam-Vizi (2000), suggested that such prevention is a reflection of α -tocopherol inhibited GSH depletion.

In summary, the beneficial effect of the treatment with α -tocopherol was observed mainly in prevention of heat-induced alterations in the enzyme activities within aged groups. We showed that influenced antioxidant enzymes by intensive production of ROS, especially in liver mitochondria of aged rats, cannot be fully ameliorated by α -tocopherol. Taking into account the changes of the studied enzyme activities in the brain mitochondrial level, we assume that the ameliorative effect of α -tocopherol is more potentiated on the GPx activity. Actually the fact that our regression model supported determination characteristic of MnSOD only at treated rats and only in relation to GPx, clearly indicate that the relation between these two enzymes is a reflection of α -tocopherol prevented GSH depletion. On the other hand, particularly lowered GR activity in comparison to the GPx activity in brain's mitochondrial compartment in our study, indicated that the activity of this enzyme is more important in ameliorative red-ox processes in the same mitochondria.

Conflict of interest

No conflicts of interest, financial or otherwise, are declared by the authors.

Acknowledgments

We wish to thank Prof. Anton Kossev from the Institute of Biophysics, Bulgarian Academy of Sciences in Sofia for assistance with manuscript preparation. We acknowledge the financial support from the Ss. Cyril and Methodius University in Skopje.

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Table 1. Rectal temperature changes in heat-exposed animals

Group	T _{rec} (°C) ± SD	<i>P</i>	After 30 min of exposure	<i>P</i>	After 60 min of exposure
YH	36.8±0.41	< 0.05	40.1±0.32	> 0.05	40.6±0.22
YTH	36.6±0.38	< 0.05	40.4±0.28	> 0.05	40.5±0.19
AH	36.7±0.41	< 0.05	39.9±0.34	> 0.05	40.3±0.22
ATH	36.5±0.51	< 0.05	39.6±0.21	> 0.05	40.2±0.13

Animals were exposed at 40 °C. *P* value represents modified least squares difference (Bonferroni) test. Data are presented as mean ± SD. YH - young heat exposed; YTH - young α -tocopherol treated heat-exposed; AH - aged heat-exposed; ATH - aged α -tocopherol treated heat-exposed rats.

Table 2. Multiple regression analysis of GPx activity in relation to SOD as an index of oxidation

Dependent Variable/ Oxidation indices	adjusted r^2	<i>p</i>	Determinant variable*	β
AT (Liver GPx)	0.311	< 0.025	SOD	0.50
YT (Brain GPx)	0.341	< 0.030	SOD	0.53
AT (Brain GPx)	0.371	< 0.028	SOD	0.56

*Explanatory variable used in the regression analysis was: mitochondrial level of SOD (superoxide dismutase). Dependent variable is GPx (glutathione peroxidase). YT - young α -tocopherol treated; AT - aged α -tocopherol treated.

Fig. 1. MnSOD activity in liver (A) and brain (B) mitochondria (MnSOD, mean \pm SE). YP - young placebo heat unexposed; YH - young heat exposed; YT - young α -tocopherol treated heat unexposed; YTH - young α -tocopherol treated heat-exposed; AP - aged heat unexposed; AH - aged heat-exposed; AT - aged α -tocopherol treated and ATH - aged α -tocopherol treated heat-exposed rats. * effect of aging, † effect of α -tocopherol treatment, ‡ effect of α -tocopherol treatment in heat-exposed rats, *†,‡p < 0.05; ** p < 0.001

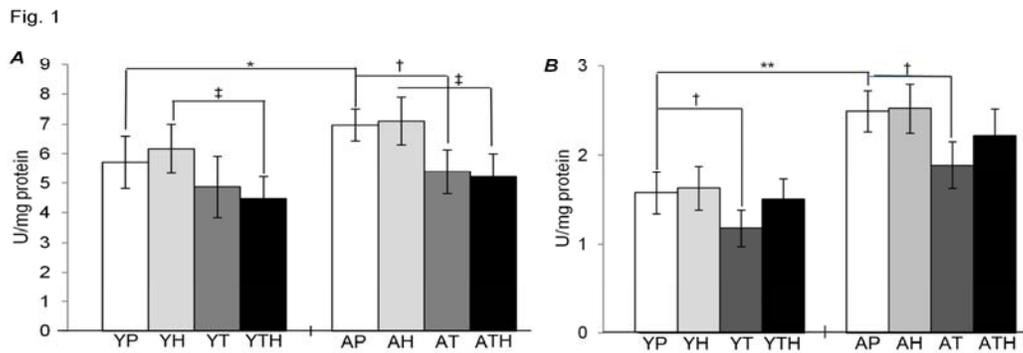


Fig. 2. GPx activity in liver (A) and brain (B) mitochondria (GPx, mean \pm SE). Abbreviations for groups are the same as in Fig. 1, * effect of aging, † effect of α -tocopherol treatment, ‡ effect of heat exposure, *,†,‡ p < 0.05; **, p < 0.001

Fig. 2

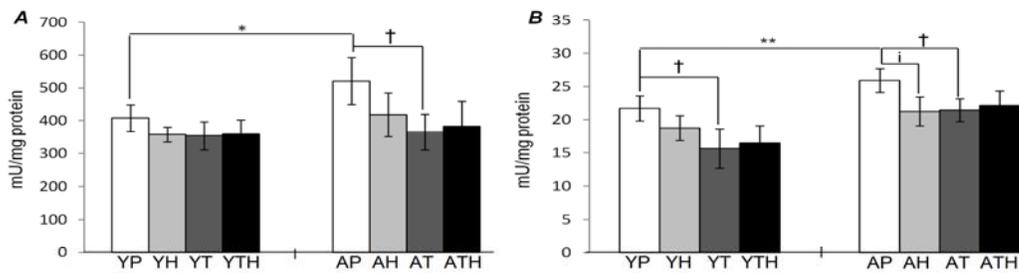


Fig. 3. GR activity in liver (*A*) and brain (*B*) mitochondria (GR, mean \pm SE). Abbreviations for groups are the same as in Fig. 1, *Effect of aging, †effect of α -tocopherol treatment in heat-exposed rats, ‡effect of heat exposure, ⁱ†p < 0.05; ** p < 0.001

