J Physiol Sci (2011) 61:437–442
DOI 10.1007/s12576-011-0165-8

SHORT COMMUNICATION

L-2-oxothiazolidine-4-carboxylate influence on age- and heat exposure-dependent redox changes in rat's blood plasma

Nikola Hadzi-Petrushev · Nikola Jankulovski · Kiril Hristov · Mitko Mladenov

Received: 10 November 2010 / Accepted: 3 July 2011 / Published online: 24 July 2011

Abstract In the present study, we investigated both the age- and heat exposure-related redox changes of blood plasma by analyzing GSH, thiol status and carbonyl groups. Our results clearly indicated that the plasma redox balance shifted toward oxidation during both aging and acute heat exposure. To further confirm this age- and heat exposure-related redox shift, we quantified the changes in thiol content. The total thiol level was found to be significantly decreased in the aged group. A similar pattern can be explained by low levels of serum GSH in old rats compared to young rats. The significance of the present study are the data showing increased oxidative stress in plasma during aging, attributed to a decrease in major antioxidant components in serum. OTC treatment, in relation to C=O regarded as a marker of oxidative damage was probably much more effective in increasing of GSH synthesis than in prevention of protein oxidation.

Keywords Aging · Heat exposure · Blood plasma · Thiol groups · Carbonyl groups · Wistar rats

N. Hadzi-Petrushev · M. Mladenov (✉)
Faculty of Natural Sciences and Mathematics,
Institute of Biology, Ss, Cyril and Methodius University,
P.O. Box 162, Skopje 1000, Macedonia
e-mail: mitkom@pmf.ukim.mk

N. Jankulovski
Medical Faculty, Ss, Cyril and Methodius University,
Skopje 1000, Macedonia

K. Hristov · M. Mladenov
Department of Membrane Ion Channels,
Institute of Biophysics, Bulgarian Academy of Sciences,
1113 Sofia, Bulgaria

Abbreviations

ROS	Reactive oxygen species
GSH	Glutathione
SH	Thiol groups
C=O	Carbonyl groups
OTC	L-2-oxothiazolidine-4-carboxylate

Introduction

Many authors have demonstrated that in rat liver hyperthermia could induce reactive oxygen species (ROS) generation, cellular hypoxia and metabolic stress [1]. In vivo, hyperthermia-generated ROS have been shown to oxidize carboxyl and thiol groups of amino acid residues [2, 3], thus altering protein structure and function [4].

Glutathione is the most widespread cellular thiol and an important intracellular antioxidant. Reduced glutathione (GSH) inhibits free radical-mediated injury by eliminating toxic peroxides and protects protein sulfhydryl groups from oxidation by serving as a biological redox agent [5, 6]. GSH also preserves cellular levels of other antioxidants [7] and participates in the detoxification of xenobiotics that cause cellular injury by generating free radicals [8]. Evidence for GSH deficiency has been found in a variety of diseases, and different stress states including heat exposure and aging [9]. We have previously reported low levels of plasma glutathione in rats undergoing both heat exposure and aging [9]. Although the mechanisms of this depletion in plasma glutathione are unclear, decreased antioxidant capacity may contribute to increased oxidative injury associated with both heat exposure and aging [9, 10].

In light of the prevention of glutathione depletion, direct administration of glutathione is limited, because it must be delivered parenterally to prevent hydrolysis and because it

is inefficiently transported into cells [11]. L-2-oxothiazolidine-4-carboxylic acid (OTC) is a cysteine prodrug that raises cellular glutathione levels by providing a source of cellular cysteine, the rate-limiting substrate for glutathione biosynthesis [12, 13]. OTC is stable in plasma and is readily transported into cells, where it is converted into cysteine by the enzyme 5-oxo-L-prolinase [12]. In vitro, OTC raises cellular glutathione and reduces cellular damage due to free radicals generated by ionizing radiation or other sources [13]. OTC has also been shown to be effective as a nontoxic cysteine delivery system for glutathione synthesis in neonatal pigs, chicks, and rats on a cysteine-free diet [14, 15]. All this evidence indicates that supplementation of blood glutathione levels by cysteine prodrug may be useful in the oxidative injury protection in aged rats affected by heat exposure.

Although many studies have reported findings on tissue redox status during the aging process, blood plasma has not been extensively investigated to date. Also, there are no appropriate data for plasma protein oxidation during acute heat exposure. To the best of our knowledge, no in vivo study linking the rate of plasma carbonyl protein formation, OTC treatment, exposure to heat and aging has as yet been launched. In this paper, we report our findings about the protective effect of L-2-oxothiazolidine-4-carboxylate in vivo in the function of aging and acute heat exposure in rats.

Materials and methods

In vivo treatment and exposure

All experimental procedures were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals approved by the Macedonian Center for Bioethics. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the Institutional Animal Care and Use Committee, April 1997, Oakland University, MI, USA. Anesthetics were applied according to the standards given by the guide of the Oakland University. Male Wistar rats ($n = 80$) were used for all protocols and were maintained on a 12:12 h light:dark cycle and fed standard rat chow and water ad libitum. All animals were divided into eight groups. Four groups consisted of 35-day-old rats. They were further divided depending on treatment with OTC and heat exposure into young saline-treated heat-unexposed (YSHU), young saline-treated heat-exposed (YSHE), young OTC-treated heat-unexposed (YTHU) and young OTC-treated heat-exposed (YTHE) rats. The other four groups included 18-month-old rats and were divided in a similar manner into old saline-treated heat-unexposed (OSHU), old saline-treated heat-exposed (OSHE), old OTC-treated heat-unexposed (OTHU) and old OTC-treated heat-exposed (OTHE) animals [9, 10]. Treated rats were injected intraperitoneally with OTC (6.5 mmol per kg body mass) on the day of sacrifice, 5 h prior to heat exposure. OTC was dissolved in physiological salt solution with pH = 7.0 (adjusted with NaOH). During the treatment period, all animals were housed at 20 ± 2°C.

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 30–40%. During heat exposure, colorectal (co-re) temperature (T_{co}) was read by an electrical thermometer (Ellab TE 3) every 10 min until it reached the temperature of the chamber, then the readings continued to be taken at 2-min intervals. The exposure was terminated when T_{co} reached 42 ± 0.5°C. The animals that did not survive the exposure term of 2 h were excluded from the study.

Blood collection

Because plasma GSH concentration can be altered by release of GSH from lysed erythrocytes or by oxidation of GSH, analysis needed to be rapid and without damage to erythrocytes during their separation from the plasma. Blood was taken from abdominal artery and collected in Vacutaner tubes with ethylenediamine tetra-acetate as an anticoagulant. Blood samples were taken at 11:00 hours on the day of sacrifice and maintained at room temperature until processed. After separation of plasma by centrifugation (15 min, 3,000 rpm), the plasma was frozen at −20°C, and then stored at −70°C until analyses were performed.

Biochemical measurements

Assay for GSH

The plasma GSH content was determined by test reagents manufactured by Sigma: Glutathione Assay Kit, Product Code (CS0260). The plasma samples were first deproteinized with 5% 5-sulfosalicylic acid solution, centrifuged to remove the precipitated proteins, and then assayed for glutathione (as it was described in the Sample Preparation from the kit description). The measurement of GSH uses a kinetic assay in which catalytic amounts (nmoles) of GSH cause a continuous reduction of 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) to 5-thio-2-nitrobenzoic acid (TNB) and the GSSG formed is recycled by glutathione reductase and NADPH. The GSSG present will also react to give a positive value in this reaction. The yellow product, TNB, was measured spectrophotometrically at 412 nm. The results were expressed as mg/dl plasma for GSH.

Assays for SH and COO

The method for determination of SH and C=O groups, as indices of protein oxidation products, were determined by colorimetric assays [16]. Color reactions were read on spectrophotometer *Cintra 6*. The results were expressed as μmol/l plasma for SH, and nmol/ml plasma for C=O groups, respectively.

Statistical analysis

Variables are reported as mean values ± standard error of the mean (SEM). The statistical software package SPSS 16.0 for Windows (SPSS, Chicago, IL, USA) was used for all analyses. Data were analyzed by univariate ANOVA by considering age as a fixed factor and hyperthermic exposure as a random factor. Mean comparisons were analyzed using Student's *t* test of independent samples to compare means between group pairs. Linear multiple regression analysis (backward step) was implemented to assess the determinants of oxidative stress indices in blood plasma. The level of significance was set at $p \leq 0.05$.

Drugs

L-2-oxothiazolidine-4-carboxylate was purchased from Sigma.

Results

Influence of aging, OTC treatment and acute heat exposure on the plasma GSH levels

The OTC treatment caused marginal significant elevation in plasma GSH at young and old heat-unexposed groups ($p = 0.05$ and $p = 0.048$, respectively). Acute heat-exposure caused significant reduction of plasma GSH content in both young and old saline-treated rats ($p < 0.05$). Treatment with OTC did not prevent the reduction of plasma GSH levels caused by acute heat exposure only in old rats ($p < 0.05$), while in young rats comparison between YSHU and YTHE shows nonsignificant changes ($p = 0.092$), (Fig. 1).

Influence of aging, OTC treatment and acute heat exposure on the plasma SH levels

Data presented in Fig. 2 show that aging caused significant decrease of plasma SH levels ($p < 0.001$). OTC treatment had a statistically significant elevating effect on plasma SH concentrations only in young rats ($p < 0.05$). Heat exposure significantly decreased plasma SH content ($p < 0.001$) for both young and old rats. Despite the increasing effect of

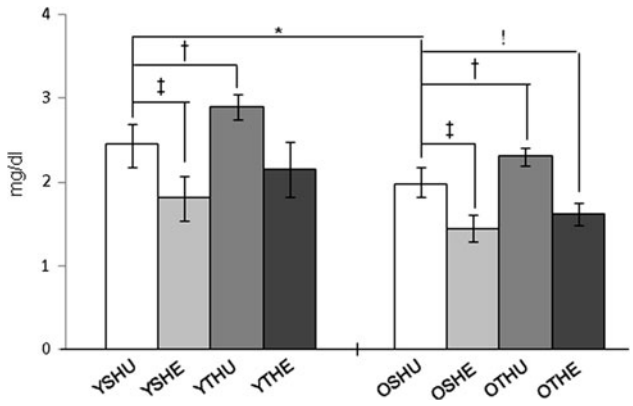

Fig. 1 Plasma total glutathione levels (GSH, mean ± SE). *Effect of aging, †effect of L-2-oxothiazolidine-4-carboxylate treatment, ‡effect of heat exposure, !effect of L-2-oxothiazolidine-4-carboxylate treatment and heat exposure. $^{*,\dagger,\ddagger,!}p < 0.05$

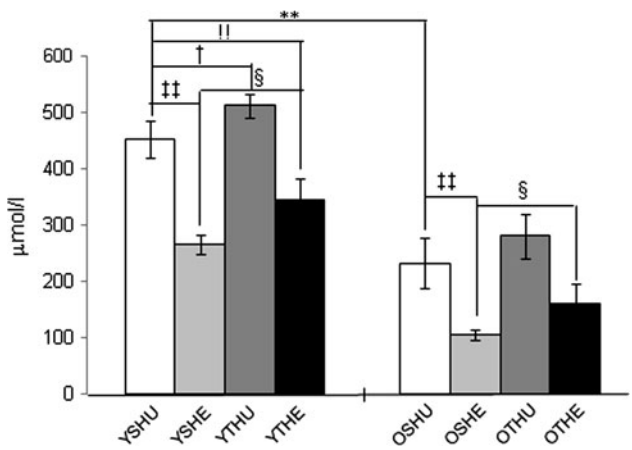

Fig. 2 Plasma sulfhydryl groups concentrations (SH, mean ± SE). *Effect of aging, †effect of L-2-oxothiazolidine-4-carboxylate treatment, ‡effect of heat exposure, §effect of L-2-oxothiazolidine-4-carboxylate treatment in heat-exposed rats, !effect of L-2-oxothiazolidine-4-carboxylate treatment and heat exposure. $^{\dagger,\S}p < 0.05$, $^{**,\ddagger\ddagger,!!}p < 0.001$

OTC treatment on plasma SH, acute heat exposure still overloads homeostatic mechanisms involved in diminishing of its effect. This is evident from significance between OTHE and OTHU ($p < 0.05$ for both young and old animals, respectively). Moreover, it is evident that treatment with OTC did not prevent the reduction of plasma SH levels caused by acute heat exposure only in young rats ($p < 0.001$), while in aged rats, comparison between OSHU and OTHE shows nonsignificant changes ($p = 0.061$).

Influence of aging, OTC treatment and acute heat exposure on the plasma C=O levels

Aging resulted in considerable increase of plasma C=O levels ($p < 0.001$). Also, acute heat exposure led to

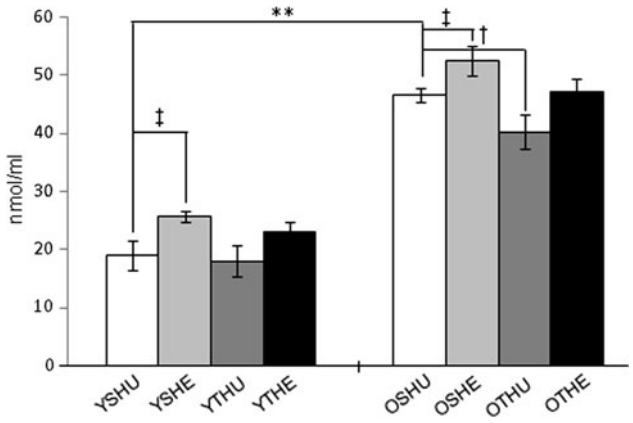

Fig. 3 Plasma carbonyl groups levels (C=O, mean ± SE). *Effect of aging, †effect of L-2-oxothiazolidine-4-carboxylate treatment, ‡effect of heat exposure. †,‡$p < 0.05$, **$p < 0.001$

substantial increase of the mean plasma C=O reactive products in both young ($p = 0.046$) and old ($p < 0.05$) saline-treated rats. However, significant changes in plasma C=O concentration caused by acute heat exposure, diminished after OTC treatment ($p = 0.082$ and $p = 0.071$ for both young and old rats, respectively) (Fig. 3).

Multiple regression analysis between heat unexposed groups

Multiple backward regression analysis with GSH and SH as explanatory variables showed GSH and SH to be the variables determinant of C=O level for young and old heat-unexposed rats (Table 1).

Discussion

In the present study, we investigated the protein oxidation of rat blood plasma during aging and acute heat exposure. Recently, many studies on age-related oxidative stress are documented by evidence of increased induction of protein oxidation during aging [9, 10, 17]. To further support this possibility, we assessed plasma thiol levels, a major contributing factor to the maintenance of the redox state, particularly in plasma, because of the relatively low levels of antioxidant enzymes in plasma [17]. Our findings indicated that lower thiol levels in plasma of elderly animals and heat exposure both coincided with lowered GSH content. Low levels of plasma GSH in aged rats could therefore indicate impaired GSH transport to tissues and directly contribute to oxidative injury associated with poor antioxidant status. From comparison of plasma GSH concentrations in controls and OTC-treated animals, we assume that OTC contributes to the sustained elevation in plasma GSH in both young and old rats. Thus, as a result of involvement in hepatic synthesis and release of GSH [18], it seems that OTC can significantly affect the plasma GSH pool. Moreover, we assume that OTC uptake from hepatocytes during acute heat exposure is increased as a compensatory mechanism of increased GSH efflux. The last assumption is corroborated with the fact that hepatic GSH efflux is stimulated by α_1-adrenergic agonists [19], and probably the extent of GSH efflux may depend from the plasma levels of glucocorticoids [20], the concentration of which significantly increased during acute heat exposure [21]. In this direction, we reasoned that prophylactic OTC treatment could partially be involved in balance of GSH efflux, caused by increased glucocorticoid effect on hepatocytes during acute heat exposure. Also, variation in the GSH-to-GSSG ratio as a result of heat exposure can affect metabolism and transport processes of GSH [22], which might be important in the decreasing of plasma GSH. This system during acute heat exposure could be partially reversed by allowing GSH synthesis to repletion of erythrocyte GSH concentrations [23–25]. In light of this type of control, regulation of GSH synthesis by outer sources like OTC may be especially important. Additional experiments have to be done in future to resolve OTC influence on blood cells in different conditions based on its kinetics of uptake.

It has to be noted that a similar decrease in plasma GSH level during acute heat exposure in both groups of animals indicates that an age-independent mechanisms may exist to protect against a marked increase in thiol-to-disulfide ratio. This is especially evident in OTC-treated groups, in which potential for sustainable GSH levels in blood plasma for a prolonged time is almost equally pronounced. This might be the result of hepatocyte "slow" uptake of OTC-cysteine [18] as a sustainable source for the synthesis of GSH and unavoidable mechanisms activated as a consequence of acute heat stress [26].

On the other hand, it is well known that GSH transport into epithelial cells of lungs, kidneys, and intestine allows GSH to be maintained better than by synthesis alone and improves the function of GSH-dependent detoxication systems. Thus, increasing plasma GSH concentrations as a consequence of the OTC treatment can increase the availability of GSH for transport into these tissues. This provides the basis for the prophylactic use of OTC against a wide variety of changed physiological conditions. Conditions where OTC treatment may be useful include physiological states that adversely affect plasma GSH concentrations, such as hepatic dysfunction [27], or those that affect the epithelial cells which can utilize exogenous GSH for protection [28–31]. However, clinical trials will be needed to determine the effectiveness of therapeutic OTC treatment in humans.

We will not discuss the relationship between OTC treatment and production of SH groups because the

Table 1 Multiple backward step regression analysis of oxidative stress in plasma

Dependent variables	Adjusted r^2	p	Determinant variable (s)[a]	β	p
Oxidative stress indices					
Young rats					
Plasma COO	0.42	<0.0078	GSH	−0.69	0.0005
	0.62	<0.001	SH	−0.87	0.0001
Old rats					
Plasma COO	0.41	<0.0056	GSH	−0.74	0.0005
	0.64	<0.001	SH	−0.90	0.0001

[a] Explanatory variables used in the regression analysis were: plasma levels of GSH (glutathione) and SH (protein carbonyls)

involvement of SH groups of proteins in GSH-typical reactions has been regarded as marginal in quantitative terms [32]. Even if protein thiols are more concentrated than GSH in blood plasma as a consequence of OTC treatment, the fact that their reactivity is usually 2–4 orders of magnitude lower [33] indicates GSH as a main protective player in stress conditions.

Also, using multivariable linear regression analysis, we found that GSH and SH together represent the strongest determinants of protein oxidation in old heat-unexposed rats as measured by plasma C=O levels. Protein carbonyl content was found significantly elevated in the plasma of old rats. The age-related accumulation of oxidatively modified proteins is either due to the excessive oxidation of proteins [17] or to the decreased capacity of cells to clear up oxidatively damaged molecules. Concerning heat exposure, in our study, the importance of OTC treatment in preventing protein oxidation was evident in both groups of young and old heat-exposed rats, in which, upon treatment, the elevating effect of heat stress on C=O levels was diminished.

In conclusion, OTC administration to heat-exposed rats resulted in a significant increase in blood plasma glutathione. The elevation of plasma glutathione is consistent with the mechanism of action of this drug, which is to raise cellular glutathione by increasing the supply of intracellular cysteine. Further investigation will be required to determine whether this increase in plasma glutathione will have benefits related to the protection of other tissues by improving cellular antioxidant status. Acute heat exposure markedly stimulated the process of protein oxidation in both young and aged rats. OTC treatment, in relation to C=O and GSH which are regarded as markers of oxidative damage and protective endogenous antioxidant, respectively, was almost equally effective in prevention of protein oxidation as in increasing GSH synthesis.

Acknowledgments The authors wish to thank Prof. Dr. Hristo Gagov from Department of Human and Animal Physiology, Faculty of Biology, University of Sofia, for assistance with manuscript preparation. Also, the authors wish to thank Oliver Nikolovski and Olga Rusimovich for technical assistance. This work was supported by the Biological Institute in the Faculty of Natural Sciences at the University ''St Cyril and Methodius'' in Skopje.

Conflict of interest None.

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