Free Radical Research, February 2010; 44(2): 181–190

informa
healthcare

Cyp4a14 overexpression induced by hyperoxia in female CBA mice as a possible contributor of increased resistance to oxidative stress

SANDRA SOBOČANEC[1], TIHOMIR BALOG[1], ANA ŠARIĆ[1], VIŠNJA ŠVERKO[1], NEVEN ŽARKOVIĆ[1], ANA ČIPAK GAŠPAROVIĆ[1], KAMELIJA ŽARKOVIĆ[2], GEORG WAEG[3], ŽELJKA MAČAK-ŠAFRANKO[1], BORKA KUŠIĆ[1] & TANJA MAROTTI[1]

[1]*Division of Molecular Medicine, Ruder Bošković Institute, Bijenička 54, 10000 Zagreb, Croatia,* [2]*School of Medicine, University of Zagreb, Department of Neuropathology Clinical Medical Center, Zagreb, Croatia, and* [3]*Karl Franz University, Graz, Austria*

(Received date: 26 May 2009; in revised form date: 9 September 2009)

Abstract

The beneficial effects of hyperoxia have been noted in treatment of several diseases and pathological states. However, the excessive production of ROS under hyperoxic conditions can directly damage cellular macromolecules if the imbalance in antioxidant status exists. Cytochrome P450 (Cyp) 4a14 has an important role in the metabolism of lipids and as a source of ROS in oxidative stress. This study investigated the oxidant/antioxidant status as a response to hyperoxia treatment in liver of young CBA/Hr mice of both sexes and whether the observed response is mediated by Cyp4a14 via PPAR isoforms in a sex-dependent manner. The overexpression of Cyp4a14, lack of both LPO and of 4-hydroxynonenal(HNE)-protein adducts revealed by immunohistochemical analysis in hyperoxia-treated females indicates their greater resistance to hyperoxia compared to males, which is parallelled to changes in PPARβ/δ and PPARγ expression. These results suggest the presence of sex-dependent changes in all investigated parameters, which points out sex-related susceptibility towards oxidative stress and hyperoxia treatment of various pathological conditions and diseases.

Keywords: *Cyp4a14, mice, hyperoxia, lipid peroxidation, antioxidant enzymes, HNE, PPAR*

Abbreviations: *LPO, lipid peroxidation; PPAR, peroxisome proliferator-activated receptor; MDA, malondialdehyde; HNE, 4-hydroxy-2-nonenal; ROS, reactive oxygen species; Cyp, Cytochrome P450.*

Introduction

The beneficial effects of hyperoxia have been noted in treatment of several diseases, including acute carbon monoxide poisoning [1] and decompression sickness [2]. On the other hand, supplemental oxygen therapy may contribute to the development of neonatal diseases such as bronchopulmonary dysplasia (BPD), which is a major cause of mortality in premature infants [3]. Exposure to hyperoxia causes inflammatory response, which aggravates oxygen toxicity [4]. The excessive production of reactive oxygen species (ROS) under hyperoxic conditions can directly damage cellular macromolecules, resulting in cell damage and death [5]. The hyperoxia-induced increase in lipid peroxidation and its end products MDA and HNE is well known in numerous studies [6–8]. Both MDA and HNE are therefore considered as biomarkers of lipid peroxidation [9]. Moreover, hyperoxia induced increase of ROS and RNS, which are considered as a side-effect of breathing hyperbaric oxygen [10]. Thus, hyperoxia is considered to be the effective method for physiological increase of ROS/RNS production in biological systems. Both malondyaldehyde and four-hydroxy-2-nonenal (HNE) are

Correspondence: Sandra Sobočanec, Division of Molecular Medicine, Ruđer Bošković Institute, Bijenička 54, 10000 Zagreb, Croatia. Tel: +385-1-4561172. Fax: +385-1-4561-010. Email: ssoboc@irb.hr

ISSN 1071-5762 print/ISSN 1029-2470 online
DOI: 10.3109/10715760903390820

among the end products of lipid peroxidation (LPO) and are found to be involved in various pathological and physiological processes. Four-hydroxy-2-nonenal (HNE) is also considered as a 'second toxic messenger of free radicals' [11] as well as the indicator of oxygen free-radical mediated membrane injury. It has been proposed that once the oxidant/antioxidant imbalance is established following hyperoxic conditions, the effective protection results on the ability of the tissue to increase its antioxidant enzyme activity levels [12,13]. It is known that young animals are tolerant to hyperoxic exposure, while the adult animals are regularly susceptible to the conditions of hyperoxia; the reason for the loss of protective biochemical response to hyperoxia that comes with ageing is still unknown [14]. In our previous study we demonstrated the presence of a different correlation pattern of antioxidant enzyme activity in young and old mice and sex-related correlation in young mice [15].

Based on these observations, we wanted to compare this status with fatty acid metabolism and systems related to lipid peroxidation (LPO), such as Cyp4a14 expression, which is found to be differentially regulated in males and females [16]. Potential involvement of cytochrome P450 system as a source of ROS generation has also been demonstrated, but with conflicting results [17,18]. Cyp enzymes are heme-containing mono-oxygenases involved in the metabolism of endogenic substrates such as steroids, prostaglandins, bile acids, fatty acids and foreign xenobiotic compounds including most of the therapeutic drugs and environmental pollutants [19,20]. Recent studies have revealed direct effect of oxidative stress on the induction [21,22] and the repression of the Cyp genes [23]. Cyp4a14 has been found to be involved in hepatic fatty acid disposal and therefore is important in regulation of LPO and oxidative stress [24]. The association between oxidative stress and Cyp4a14 expression via peroxisome proliferator-activated receptor (PPAR) isoforms, which play key roles in lipid and glucose metabolism, has been observed in several studies [25,26]. PPARα regulates expression of various genes involved in lipid oxidation, mainly in liver. Also, PPARα regulates genes involved in maintenance of lipid homeostasis. PPARβ/δ is implicated in membrane lipid synthesis and other basic cellular functions [27,28], as well as protection of the cell from oxidant damage by the inhibition of ROS production and lipid intermediates or by increasing degradation of these molecules [29]. PPARγ regulates processes of differentiation, inhibits tumour cell growth and regulates tissue inflammation processes [30]. These parameters are part of a broader cellular response that has been associated with ageing and longevity [31]. In this study we demonstrate the presence of sex-dependent changes in all investigated parameters, which points out sex-related susceptibility towards oxidative stress and hyperoxia treatment.

Materials and methods

Animals and experimental design

For the purpose of the study, we used male and female CBA/Hr mice aged 1 month from the breeding colony of the Ruđer Bošković Institute (Zagreb, Croatia). The animals were maintained under the following laboratory conditions: three to a cage; light on from 06:00 to 18:00; 22 ± 2°C room temperature; access to food pellets and tap water *ad libitum* and divided into two groups of nine animals each. The first group of animals was the control group. The other group was subjected to normobaric oxygen (100% O_2 for 18 h) in a hyperbaric chamber. Normobaric oxygen conditions were carried out by flushing the chamber (Đuro Đaković, Slavonski Brod, Croatia) by pure oxygen (25 L/min for 10 min) to replace room air. Each group consisted of nine mice divided into three pools of three animals per each pool. A total of 36 animals was used for the experiment. Animals were euthanized by inhalation of ether (Kemika, Zagreb, Croatia), following cervical dislocation. The experiments were performed in accordance with the current laws of the Republic of Croatia and with the guidelines of the European Community Council Directive of 24 November 1986 (86/609/EEC).

Microsomal preparation

Microsomal fractions were prepared as described previously [32]. Briefly, the liver was homogenized with TRIS-HCl (10 mM) and sucrose (0.25 M, pH = 7.4, 10% w/v) using an ice-jacketed Potter-Elvehjem homogenizer (1300 × g). During the homogenization procedure the homogenizer was immersed into ice slurry. Whole liver homogenates were fractionated in a Beckman coulter refrigerated ultracentrifuge. All operations were conducted at 4°C. Subcellular fractionation was achieved by differential centrifugation. Samples were centrifuged at 1000 × g for 10 min. The supernatant was fractionated at 8000 × g for 20 minutes and then at 15 000 × g for 15 minutes. The resulting supernatant was centrifuged at 105 000 × g for 1 h and the microsomal pellet resuspended in 0.1 M potassium phosphate buffer (pH = 7.4), quickly frozen in liquid nitrogen and stored at −80°C.

Lipid peroxidation assay

The LPO assay was carried out on liver microsomes from control and mice treated with 100% oxygen using a LPO assay kit (Bioxytech® LPO-586™, OXIS International, Inc. Foster City, CA) according to the protocol. Polyunsaturated fatty acid peroxides generate malondialdehyde (MDA) and 4-hydroxyalkenals (HNE) upon decomposition. Measurement of MDA and HNE has been used as an indicator of lipid peroxidation [11].

Although the MDA is only one of several increased biomarkers of lipid peroxidation [33] it has high sensitivity for detecting low-level polyunsaturated acid (PUFA) oxidation [34]. The LPO-586™ method is designed to assay either MDA alone or MDA in combination with 4-hydroxyalkenals. In this study the MDA alone was measured.

Catalase and glutathione peroxidase activities

Catalase (EC 1.11.1.6.) activity was determined according to Aebi [35] by measuring changes in absorbance in the reaction mixture using the final concentrations of 10 mM H_2O_2 and 50 mM phosphate buffer (pH = 7.0) at 240 nm during the time interval of 30 s after addition of the sample. One unit of CAT was defined as the amount of enzyme which liberates half the peroxide oxygen from H_2O_2 solution in 30 s at 25°C. The activity was expressed as U/mg protein.

Glutathione peroxidase (EC 1.11.1.6.) activity was measured by Gpx assay kit (Ransel, Randox, San Diego, CA) based on the method of Paglia and Valentine [36]. Gpx catalyses the oxidation of glutathione at a concentration of 5 mmol by cumene hydroperoxide. The absorbance was monitored for 3 min at 340 nm. The Gpx unit was defined as the enzyme activity necessary to convert 1 mmol of NADPH to NADP in 1 min. Gpx activity was expressed as U/mg protein.

RNA isolation and quantitative real-time PCR analysis

Total RNA was extracted from a pool of three individual mouse livers in each group using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Total RNA was reverse transcribed using the Superscript first-strand synthesis system for RT-PCR kit (Invitrogen) according to the manufacturer's instructions. For the first strand cDNA synthesis, the reverse transcription reaction was performed on 1 μg of total RNA prepared from each pool in a final volume of 20 μl using SuperScript™ II Rnase H^- Reverse Transcriptase (Invitrogen, Carlsbad, CA). Real-time PCR analysis was carried out on an ABI 7300 sequence detection system (Applied Biosystems, Foster City, CA) to quantify relative P450 mRNA expression of catalase, glutathione peroxidase, Cyp4a14, PPARα, PPARβ/δ and PPARγ in liver of mice. Primer length and Assay ID used for the analysis are shown in Table I. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control to normalize the total amount of cDNA in each sample. Reactions were carried out in a total volume of 20 μl using TaqMan® Gene Expression Master Mix reagent (Applied Biosystems, Foster City, CA) and 5 μl of cDNA for template with forward and reverse primers. All reactions were carried out in triplicate. Relative gene expression of each gene was calculated by using the Relative Expression Software Tool (REST©) which uses the Pair Wise Fixed Reallocation Randomization Test© to calculate result significance [37].

Table I. Assay ID used for the real time PCR analysis.

Gene	Assay ID	Product size (bp)
CYP4A14	Mm00484132_m1	71
CAT	Mm00437992_m1	64
GPX-1	Mm00656767_g1	134
PPARα	Mm00627559_m1	86
PPARβ/δ	Mm01305434_m1	72
PPARγ	Mm00440940_m1	63
GAPDH	Mm99999915_m1	107

Protein measurement

Protein concentration in all samples was determined with a standardized method by Lowry et al. [38], using bovine serum albumin (Sigma, St. Louis, MO) as a standard.

SDS-PAGE and Western blotting

Total cellular proteins (75–125 μg per lane) were separated using denaturing polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli [39]. Ten per cent polyacrylamide gels were mounted in the Mini-PROTEAN 3 Electrophoresis Module AsSEbly (BIO-RAD, Hercules, CA) and proteins were then transferred to Immun-Blott® PVDF membrane (BIO-RAD) and blocked overnight at 4°C in 5% non-fat dry milk in 50 mM phosphate buffer (pH = 7.8 and 0.1% Tween 20). The membranes were washed three times with PBS and incubated for 3 h at room temperature with specific primary antibody for CYP4A14 diluted 1:500 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), for CAT diluted 1:2000 (ab16731, Abcam, Cambridge, UK), GPX diluted 1:2000 (ab16798, Abcam) or ERK-2 (C-14, Santa Cruz Biotechnology, Inc.). After being washed, the membranes were incubated with anti-goat horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Inc.) diluted 1:5000 for 1 h at room temperature or anti-rabbit horseradish peroxidase-conjugated secondary antibody (Amersham, Buckinghamshire, UK) diluted 1:5000 for 1 h at room temperature. Blots were washed three times and bands were visualized using BM Chemiluminescence blotting substrate (POD) (Roche Applied Science, Mannheim, Germany), exposed to film and digitized. For CYP4A14 we revealed specific bands of expected size (56 kDa), for CAT at 60 kDa, for GPX at 22 kDa and for ERK-2 the band of 42 kDa. Expression of the mouse ERK-2 protein was examined as an

internal control in both sexes. The blots were repeated at least three times and one representative bolt is presented in the figures.

Immunohistochemical analyses

Immunohistochemistry for the HNE-modified proteins was carried out on formalin-fixed paraffin embedded samples of mice livers obtained from three animals pre respective group. A genuine antibody specific for the HNE-histidine epitope in HNE-protein (peptide) conjugates that gives only 5% cross-reactivity with HNE-lysine and 4% with HNE-cysteine [40] was obtained from culture medium of the clone 'HNE 1g4', produced by a fusion of Sp2-Ag8 myeloma cells with B-cells of a BALB-c mouse immunized with HNE-modified keyhole limpet haemocyanine.

Immunohistochemistry was done in a three step procedure as described before [41,42] using a multi link LSAB kit (DAKO, Denmark) where the first step was incubation with anti-HNE monoclonal antibodies (dilution 1:10) during 12 h in a refrigerator. The second step was incubation with biotinylated secondary rabbit anti-mouse immunoglobulins (AB2) during 30 min. The third step was incubation with streptavidin peroxidase during 30 min. Finally, the reaction was visualized by a DAB (3,3-diaminobenzidine tetrahydrochloride in organic solvent, Sigma) giving brown colour after 10 min, using haematoxylin (Kemika, Croatia) contrast staining (blue). Negative control was done without application of HNE-histidine specific monoclonal antibodies.

All immunohistochemical analyses were done by a pathologist experienced in the HNE-immunohistochemistry without prior knowledge of the study group design.

Statistical analysis

Data were analysed using the statistical package SPSS for Windows (v.16.0.) and reported as mean ± SEM from three pools of three mice per each sex and each treatment. Statistical analysis of the data was done according to the Student's *t*-test for independent samples; $p < 0.05$ was considered significant.

Results

Effect of hyperoxia on MDA level in liver microsomes of male and female CBA mice

Hyperoxia induced a statistically significant increase of malondialdehyde (MDA) concentration ($p = 0.004$) in liver microsomes of male mice in comparison with the control group. However, in females MDA level did not accumulate under hyperoxia and was even slightly below the control level. The difference in MDA level between control groups of both sexes was not significant (Figure 1).

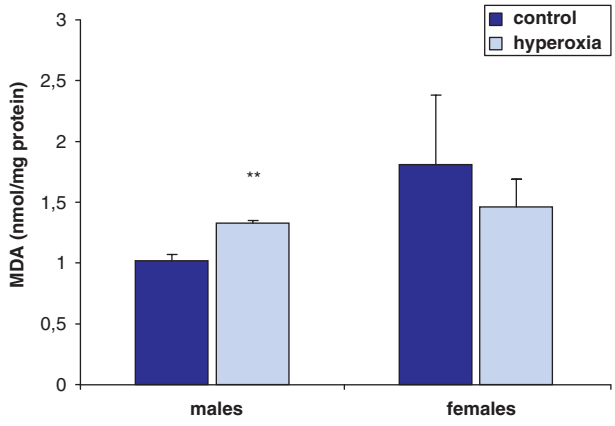

Figure 1. Effect of hyperoxia on MDA concentration in liver of male and female CBA mice. Data are mean ± SEM from three pools of three mice per each sex and each treatment. $**p = 0.004$ in male liver, control compared to hyperoxia.

Effect of hyperoxia on CAT and Gpx activity in liver of male and female CBA mice

CAT activity showed marked increase in hyperoxia-treated male liver ($p = 0.011$), while in females hyperoxia had no effect on CAT activity (Figure 2A). GPX activity had a tendency to increase in hyperoxia-treated animals of both sexes; however, it has reached significance only in females ($p = 0.038$), when compared to the control group (Figure 2B).

Effect of hyperoxia on CAT and Gpx mRNA and protein level in liver of male and female CBA mice

Real-time PCR analysis showed that hyperoxia had no effect on either CAT or Gpx mRNA level in the liver of both sexes (Figure 3). However, we observed marked differences between males and females in CAT protein level: control females had overall higher CAT protein level than control males ($p = 0.001$), even with the significant decrease observed in hyperoxia treated females compared to control ($p = 0.0056$). In males CAT protein level was significantly increased upon hyperoxia treatment ($p = 0.0018$). There was no change in GPX protein level in either of the sexes upon treatment (Figures 4A and B).

Effect of hyperoxia on Cyp4a14 gene and protein expression in liver

Real-time PCR analysis showed that hyperoxia induced sex-dependent changes in the gene expression of Cyp4a14, with the increase in females more than 16-fold (16.7, $p = 0.0325$) and decrease in males to 5-fold when compared to control (0.199; $p = 0.033$) (Figure 5). Protein content of Cyp4a14 followed the pattern of gene expression in both sexes

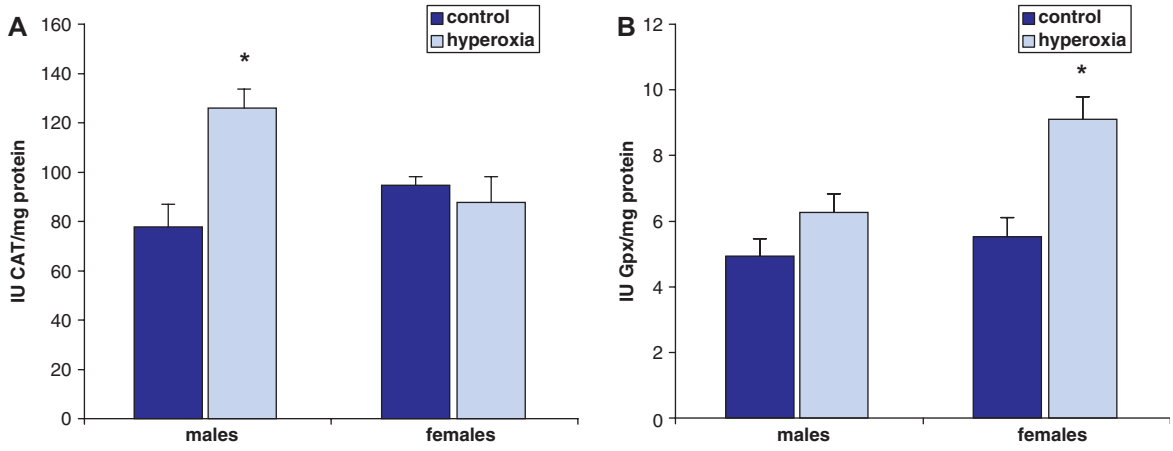

Figure 2. Effect of hyperoxia on hepatic CAT (A) and Gpx (B) activity in male and female CBA mice. Data are mean ± SEM from three pools of three mice per each sex and each treatment. For CAT in males $*p = 0.011$ compared to control; for Gpx in females $*p = 0.038$ compared to control.

with doubled decrease in males ($p = 0.001$) and significant increase in females ($p = 0.002$) in hyperoxia treated animals (Figure 6).

Effect of hyperoxia on PPARα, PPARβ/δ and PPARγ gene expression in liver

Hyperoxia induced diverse changes in the gene expression of all three PPAR isoforms in the liver of CBA mice, as revealed by real-time PCR analysis. We found down-regulation of PPARβ/δ in both sexes, with a significant decrease of almost 3-fold in females ($F = 0.345$; $p = 0.001$); PPARγ also exhibited a strong decrease of 2.5-fold in males, but without significance due to great individual variations, while in females there was no significant change in the expression upon hyperoxia, although the fold-change was increased about 1.5 when compared to control animals, due to great individual variations. Also, there was no statistically significant changes between hyperoxia-treated males and females ($F = 2.423$; $p = 0.113$). PPARα isoform remained unchanged in both sexes when compared to control animals (Figure 7).

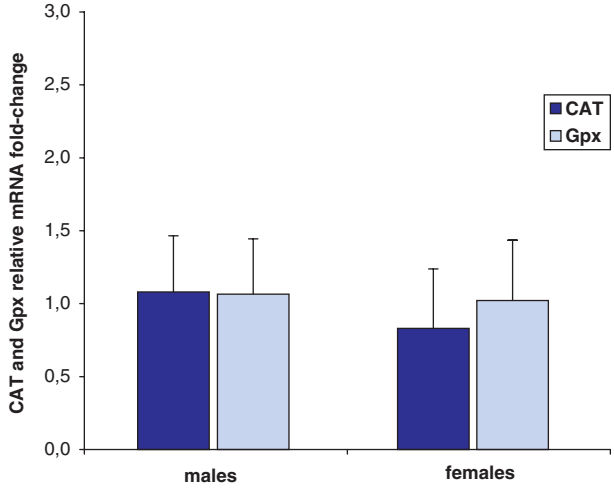

Figure 3. Real-time PCR analysis of CAT and Gpx gene expression in liver of male and female CBA mice exposed to hyperoxia. Data are mean ± SEM from three pools of three mice per each sex and each treatment. The relative fold-change compared to control (defined as 1) was calculated using the $2^{-\Delta\Delta CT}$ method as described in Material and methods.

Effect of hyperoxia on 4-hydroxynonenal(HNE)-protein adducts in liver

Immunohistochemical findings of 4-hydroxynonenal (HNE)-protein adducts in the livers of control mice and of the experimental group exposed to hyperoxia are presented in Figure 8. In control male mice occasionally HNE-protein adducts were noticed in the endothelium, mostly of the central vein, and in perivascular hepatocytes. In control female mice, HNE-protein adducts were even less present than in males and were less pronounced. Hyperoxia did not cause any particular changes in female mice, while in males the presence of HNE-protein adducts was mostly noticed within the blood vessels, as indicated in Figure 8.

Discussion

In this study, we investigated the involvement of Cyp4a14 and PPAR isoforms with respect to different oxidant/antioxidant status followed by hyperoxia treatment in liver of 1 month old CBA mice of both sexes. Although we found sex-dependent differences in MDA level, the differences in HNE-protein adducts in livers exposed to hyperoxia were observed only to a minor extent and were considered unsignificant. Therefore,

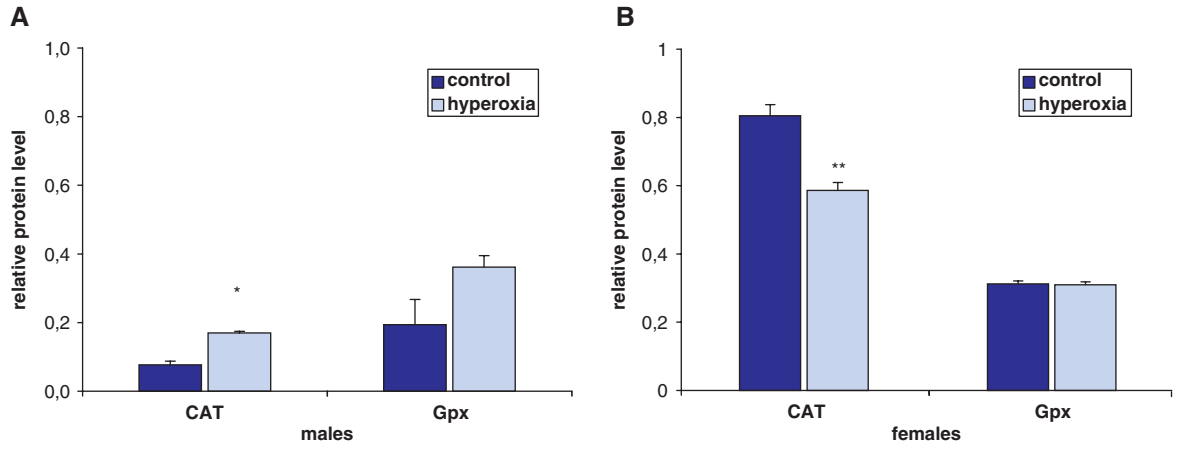

Figure 4. Effect of hyperoxia on hepatic CAT and Gpx (A, B) protein level in male and female CBA mice revealed by western blots analysis. Data are mean ± SEM from three pools of three mice per each sex and each treatment. For CAT in males $*p = 0.018$ compared to control; for CAT in females $**p = 0.0056$ compared to control.

we may conclude from these experiments that we found no pathological changes in the liver of mice upon hyperoxia treatment revealed by immunohistochemical examination. However, the sex-related difference was found at the physiological level by observing the significant differences in the MDA level and CAT activity, which are usually increased as a consequence of oxidative stress [43,44]. Males had higher MDA content followed by a marked increase in CAT activity and protein level, while in females there was no change neither in MDA nor CAT level upon hyperoxia treatment. The observed increase in CAT level implies the presence of oxidative stress in males because CAT plays the important role in organism's coping with the increased generation of ROS [45] as opposed to Gpx, which has a major antioxidant role in physiological conditions [46]. The observed discrepancy between the RNA, protein and activity of CAT and Gpx in our study could be the result of post-transcriptional processes of mRNA by which target gene expression could be modulated [47,48]. However, this phenomenon cannot be explained solely by the alteration in the individual antioxidant enzyme status. Rather, their cooperation and mutual relationship in response to hyperoxia as well as the contribution of other factors should be taken into account. MDA and HNE, as the end products of LPO, are both considered as biomarkers of lipid peroxidation and oxidative stress. Since MDA is found to be elevated in hyperoxia conditions [8,49], we determined the levels of MDA as the end product of lipid peroxidation

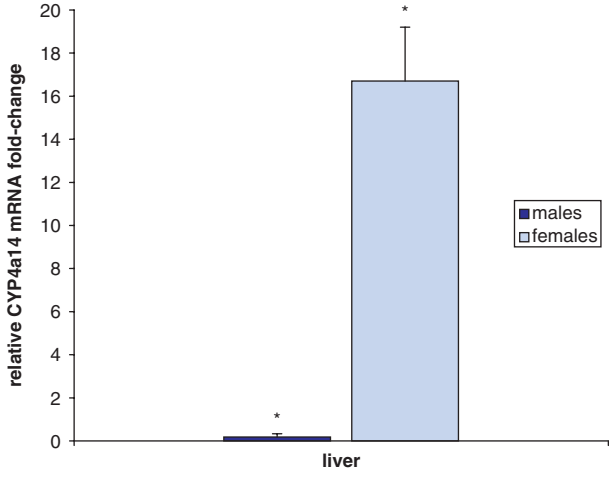

Figure 5. Effect of hyperoxia on CYP4a14 gene expression in liver of male and female CBA mice. Data are mean ± SEM from three pools of three mice per each sex and each treatment. The relative fold-change compared to control (defined as 1) was calculated using the $2^{-\Delta\Delta CT}$ method as described in Materials and methods. For males, $*p = 0.0325$; for females $*p = 0.033$ compared to control.

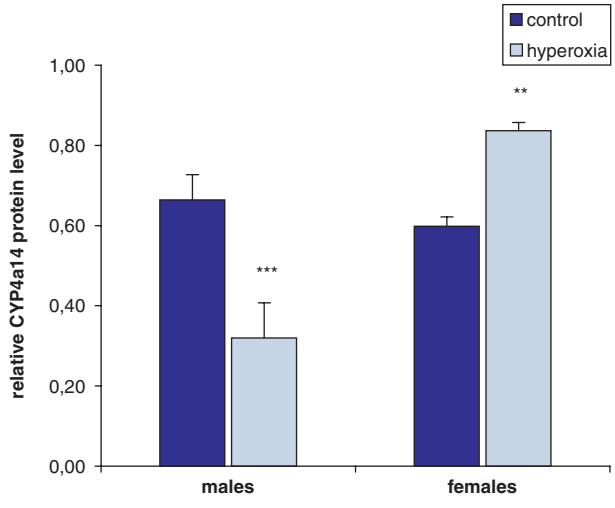

Figure 6. Effect of hyperoxia on CYP4a14 protein level in male and female CBA mice revealed by western blot analysis. Data are mean ± SEM from three pools of three mice per each sex and each treatment. For males $***p = 0.001$; for females $**p = 0.002$ compared to control. Expression of the mouse ERK-2 protein was examined as an internal control.

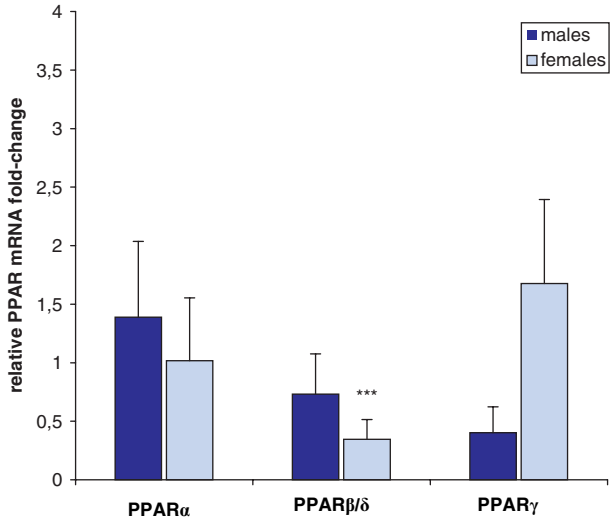

Figure 7. Effect of hyperoxia on the expression of PPARα, PPARβ/δ and PPARγ gene expression in male and female CBA mice. Data are mean ± SEM from three pools of three mice per each sex and each treatment. The relative fold-change compared to control (defined as 1) was calculated using the $2^{-\Delta\Delta CT}$ method as described in Materials and methods. For PPARβ/δ in females $***p = 0.001$ compared to control.

and biomarker of oxidative stress since MDA has high sensitivity for detecting low-level polyunsaturated acid (PUFA) oxidation. HNE is found to be involved in various pathological and physiological processes and is also considered as the indicator of oxygen free-radical mediated membrane injury. The concept of HNE as a toxic messenger was changed to the concept of HNE as a bioactive marker of LPO and oxidative stress following development of new methods [50]. Hyperoxia did not have very prominent effects on the presence of the HNE-protein adducts in the liver. Most likely, that is due to the intense liver metabolism which prevents development of HNE in amounts exceeding its enzymatic (by aldehyde and alcohol dehydrogenases) or non-enzymatic (by glutathione) removal. Namely, we evaluated the possible presence of HNE-protein adducts in various normal tissues of both humans and animal origin, among which normal liver tissue appears to be almost free from the HNE-protein adducts [51]. The possibility that liver eliminates HNE-protein adducts rapidly could also explain why in male mice hyperoxia resulted in development of pronounced HNE-immunopositivity within the blood vessels, but not in the hepatocytes. Therefore, the possible role of the blood in removal of the cytotoxic products of lipid peroxidation from the affected tissues, in particular the liver, should be further studied. This could help better understanding of the beneficial effects of hyperoxia, which are yet not understood as in the case of rats exposed to the focal brain ischemia treated afterwards by hyperbaric

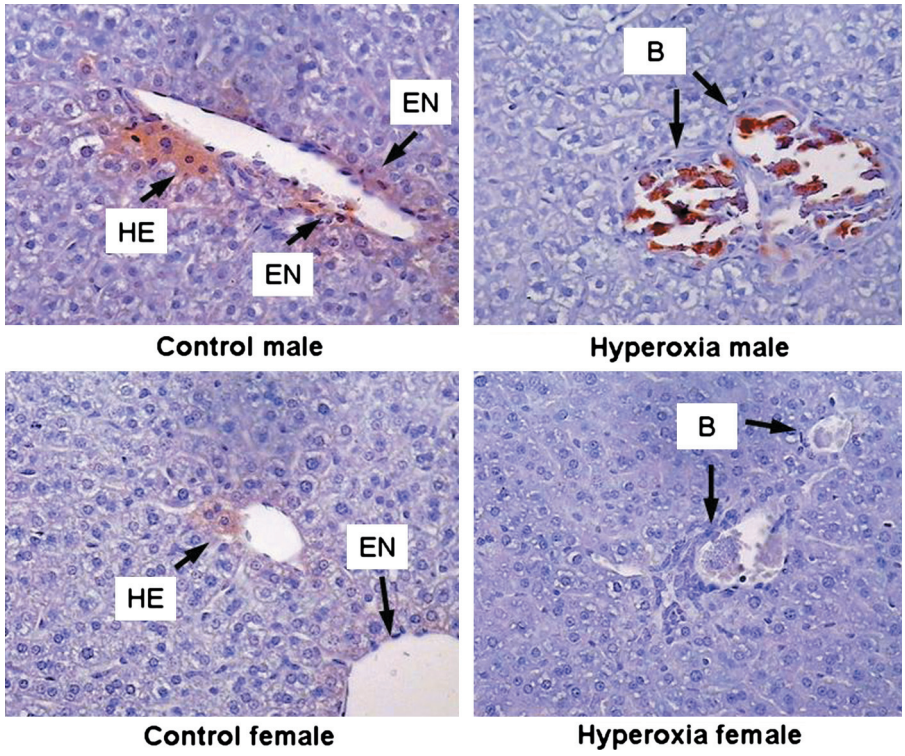

Figure 8. Immunohistochemical findings of 4-hydroxynonenal(HNE)-protein adducts in the liver. In control male mice occasionally HNE-protein adducts were noticed in the endothelium, mostly of the central vein, and in perivascular hepatocytes. In control female mice, HNE-protein adducts were even less present than in males and were less pronounced. Hyperoxia did not cause any particular changes in female mice, while in males the presence of HNE-protein adducts was mostly noticed within the blood vessels, as indicated on the figure (PAP-DAB staining giving yellow to brown immunopositivity with the blue contrast staining, magnification 400×). HE, hepatocytes; EN, endothelial cells; B, blood vessel content.

oxygenation [52]. Interestingly, in these animals also HNE-protein adducts were only present moderately and were mostly associated with the blood vessels specimens, being influenced only to a minor extent by hyperoxia, in spite of the beneficial effects of hyperoxia for the brain functional recovery. Hence, further studies on the possible effects of both hypoxia and hyperoxia on the metabolism of the HNE-protein adducts and biological effects of the aldehyde are needed to help us understand oxidative homeostasis and the role of HNE-protein adducts in it.

The expression of Cyp4a14 exhibited sex-dependent changes upon hyperoxia treatment, with significant suppression of both gene (20% of control) and protein level (15% of control) in males. However, in females hyperoxia had the opposite effect, where it strongly increased mRNA level ~ 9-fold, which was followed by the increase in protein level of 44%. This finding emphasizes the sex-dependent pattern of Cyp4a14 expression in regulation of oxidant status upon hyperoxic exposure: males exhibited the reduction of Cyp4a14 on both RNA and protein level which resulted in increased LPO and susceptibility towards the oxidative stress, while unchanged LPO in females could be a consequence of strong upregulation of Cyp4a14, proposed to be involved in hepatic fatty acid disposal, which depletes the liver of substrate for LPO [24]. The observed sex-related differences in Cyp4a14 expression are the consequence of a sexually dimorphic pituitary growth hormone secretory pattern which regulates many sex-dependent liver genes and several Cyp genes as well [16,53]. Also, Cyp4a14 proteins are important intermediates in an adaptive response to the imbalance of hepatic lipid metabolism [54]. The upregulated expression of Cyp4a14 was established in the liver of young mutant long-lived Snell mice and was maintained in the middle-aged and aged mutants as well, which suggests that the increased activity of Cyp4a14 among other genes constitutes favourable physiological status for increased longevity [55]. These and other data provide evidence for a positive relationship in animals with higher Cyp4a14 content and their greater ability to cope with oxidative stress, which could also be linked with the greater resistance of female mice to oxidative stress observed in our study. Cyp4a14 metabolizes fatty acid derivatives and is modulated via PPAR. PPARα isotype is abundant in liver and activated by fasting and by chronic high-fat dieting [56]. PPARα also controls oxidative stress and inflammation in the cardiovascular system [57]. Real-time PCR analysis in our study showed neither induction nor suppression of PPARα in both sexes. However, PPARβ/δ was found to be down-regulated, with marked decrease in females. Since this isoform may protect the cell from oxidant damage either by inhibition of ROS production or inhibition of lipid intermediates [29], its down-regulation might be the possible cause of increased LPO in males. However, this explanation does not fit for females, where the decrease in the expression of PPARβ/δ is not followed by increased LPO. There is a possible link between reduction of PPARγ expression and increased LPO in males, since it is known that alterations in oxidant metabolism resulting in oxidative stress down-regulate PPARγ expression and are inversely correlated with LPO [58]. In accordance with Li et al. [59], we found sex-related differences in PPAR activation. However, it seems that Cyp4a14 up-regulation demonstrated in this study is not mediated by the activation of PPARα, but rather by sex-related down-regulation of PPARβ and PPARγ expression, but further experiments have to be done in order to confirm this hypothesis. Nevertheless, this could indicate the possible role of these two isoforms on the sex-dependent expression of Cyp4a14 and therefore different susceptibility towards oxidative stress in male and female young mice.

Our study points out the sex-dependent susceptibility towards oxidative stress, which could have further impact on designing various strategies in supplemental oxygen therapy treatment in males and/or females. However, additional studies are required to clarify the possible relationships between PPAR isoforms, Cyp4a14 and antioxidant enzyme system with respect to sex-related responses to oxidative stress. These findings may play an important role in understanding the question of the mechanisms of these physiological functions in relation to hyperoxia treatment of various pathological conditions and diseases.

Acknowledgements

We thank Iva Pešun-Međimorec for her excellent technical assistance. The research is funded by Croatian Ministry of Science, Education and Sports, Grant No. 0982464-1647 within collaborations of the COST B35 Action.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

[1] Weaver LK, Hopkins RO, Chan KJ, Churchill S, Elliott CG, Clemmer TP, Orme JF Jr, Thomas FO, Morris AH. Hyperbaric oxygen for acute carbon monoxide poisoning. N Engl J Med 2002;347:1057–1067.

[2] Coles C, Williams M, Burnet N. Hyperbaric oxygen therapy. Combination with radiotherapy in cancer is of proved benefit but rarely used. BMJ 1999;318:1076–1077; author reply 1077–1078.

[3] Northway WH Jr, Moss RB, Carlisle KB, Parker BR, Popp RL, Pitlick PT, Eichler I, Lamm RL, Brown BW Jr. Late

pulmonary sequelae of bronchopulmonary dysplasia. N Engl J Med 1990;323:1793–1799.

[4] Zaher TE, Miller EJ, Morrow DM, Javdan M, Mantell LL. Hyperoxia-induced signal transduction pathways in pulmonary epithelial cells. Free Radic Biol Med 2007;42:897–908.

[5] Li Y, Zhang W, Mantell LL, Kazzaz JA, Fein AM, Horowitz S. Nuclear factor-kappaB is activated by hyperoxia but does not protect from cell death. J Biol Chem 1997;272: 20646–20649.

[6] Kwak DJ, Kwak SD, Gauda EB. The effect of hyperoxia on reactive oxygen species (ROS) in rat petrosal ganglion neurons during development using organotypic slices. Pediatr Res 2006;60:371–376.

[7] Mori H, Oikawa M, Tamagami T, Kumaki H, Nakaune R, Amano J, Akinaga Y, Fukui K, Abe K, Urano S. Oxidized proteins in astrocytes generated in a hyperbaric atmosphere induce neuronal apoptosis. J Alzheimers Dis 2007;11: 165–174.

[8] D'Agostino DP, Olson JE, Dean JB. Acute hyperoxia increases lipid peroxidation and induces plasma membrane blebbing in human U87 glioblastoma cells. Neuroscience 2009;159: 1011–1022.

[9] Loiseaux-Meunier MN, Bedu M, Gentou C, Pepin D, Coudert J, Caillaud D. Oxygen toxicity: simultaneous measure of pentane and malondialdehyde in humans exposed to hyperoxia. Biomed Pharmacother 2001;55:163–169.

[10] Dean JB, Mulkey DK, Henderson RA 3rd, Potter SJ, Putnam RW. Hyperoxia, reactive oxygen species, and hyperventilation: oxygen sensitivity of brain stem neurons. J Appl Physiol 2004;96:784–791.

[11] Esterbauer H, Schaur RJ, Zollner H. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Radic Biol Med 1991;11:81–128.

[12] Barazzone C, Horowitz S, Donati YR, Rodriguez I, Piguet PF. Oxygen toxicity in mouse lung: pathways to cell death. Am J Respir Cell Mol Biol 1998;19:573–581.

[13] Freeman BA, Mason RJ, Williams MC, Crapo JD. Antioxidant enzyme activity in alveolar type II cells after exposure of rats to hyperoxia. Exp Lung Res 1986;10:203–222.

[14] Frank L. Developmental aspects of experimental pulmonary oxygen toxicity. Free Radic Biol Med 1991;11:463–494.

[15] Sobocanec S, Balog T, Kusic B, Sverko V, Saric A, Marotti T. Differential response to lipid peroxidation in male and female mice with age: correlation of antioxidant enzymes matters. Biogerontology 2008;9:335–343.

[16] Waxman DJ, O'Connor C. Growth hormone regulation of sex-dependent liver gene expression. Mol Endocrinol 2006;20: 2613–2629.

[17] Bhakta KY, Jiang W, Couroucli XI, Fazili IS, Muthiah K, Moorthy B. Regulation of cytochrome P4501A1 expression by hyperoxia in human lung cell lines: implications for hyperoxic lung injury. Toxicol Appl Pharmacol 2008;233: 169–178.

[18] Hazinski TA, Noisin E, Hamon I, DeMatteo A. Sheep lung cytochrome P4501A1 (CYP1A1): cDNA cloning and transcriptional regulation by oxygen tension. J Clin Invest 1995;96: 2083–2089.

[19] van der Weide J, Steijns LS. Cytochrome P450 enzyme system: genetic polymorphisms and impact on clinical pharmacology. Ann Clin Biochem 1999;36:722–729.

[20] Omura T. Mitochondrial P450s. Chem Biol Interact 2006; 163:86–93.

[21] Konstandi M, Johnson E, Lang MA, Camus-Radon AM, Marselos M. Stress modulates the enzymatic inducibility by benzo[alpha]pyrene in the rat liver. Pharmacol Res 2000;42: 205–211.

[22] Barouki R, Morel Y. Repression of cytochrome P450 1A1 gene expression by oxidative stress: mechanisms and biological implications. Biochem Pharmacol 2001;61:511–516.

[23] Morel Y, Barouki R. Repression of gene expression by oxidative stress. Biochem J 1999;342:481–496.

[24] Ip E, Farrell GC, Robertson G, Hall P, Kirsch R, Leclercq I. Central role of PPARalpha-dependent hepatic lipid turnover in dietary steatohepatitis in mice. Hepatology 2003;38: 123–132.

[25] Maher JM, Aleksunes LM, Dieter MZ, Tanaka Y, Peters JM, Manautou JE, Klaassen CD. Nrf2- and PPAR alpha-mediated regulation of hepatic Mrp transporters after exposure to perfluorooctanoic acid and perfluorodecanoic acid. Toxicol Sci 2008;106:319–328.

[26] Leclercq IA, Farrell GC, Field J, Bell DR, Gonzalez FJ, Robertson GR. CYP2E1 and CYP4A as microsomal catalysts of lipid peroxides in murine nonalcoholic steatohepatitis. J Clin Invest 2000;105:1067–1075.

[27] Braissant O, Wahli W. Differential expression of peroxisome proliferator-activated receptor-alpha, -beta, and -gamma during rat embryonic development. Endocrinology 1998;139: 2748–2754.

[28] Escher P, Wahli W. Peroxisome proliferator-activated receptors: insight into multiple cellular functions. Mutat Res 2000;448: 121–138.

[29] Coleman JD, Prabhu KS, Thompson JT, Reddy PS, Peters JM, Peterson BR, Reddy CC, Vanden Heuvel JP. The oxidative stress mediator 4-hydroxynonenal is an intracellular agonist of the nuclear receptor peroxisome proliferator-activated receptor-beta/delta (PPARbeta/delta). Free Radic Biol Med 2007;42:1155–1164.

[30] Duez H, Fruchart JC, Staels B. PPARS in inflammation, atherosclerosis and thrombosis. J Cardiovasc Risk 2001;8: 187–194.

[31] Corton JC, Apte U, Anderson SP, Limaye P, Yoon L, Latendresse J, Dunn C, Everitt JI, Voss KA, Swanson C, Kimbrough C, Wong JS, Gill SS, Chandraratna RA, Kwak MK, Kensler TW, Stulnig TM, Steffensen KR, Gustafsson JA, Mehendale HM. Mimetics of caloric restriction include agonists of lipid-activated nuclear receptors. J Biol Chem 2004;279:46204–46212.

[32] Shertzer HG, Clay CD, Genter MB, Schneider SN, Nebert DW, Dalton TP. Cyp1a2 protects against reactive oxygen production in mouse liver microsomes. Free Radic Biol Med 2004;36:605–617.

[33] Lefevre G, Beljean-Leymarie M, Beyerle F, Bonnefont-Rousselot D, Cristol JP, Therond P, Torreilles J. [Evaluation of lipid peroxidation by measuring thiobarbituric acid reactive substances]. Ann Biol Clin (Paris) 1998;56:305–319.

[34] Liu J, Yeo HC, Doniger SJ, Ames BN. Assay of aldehydes from lipid peroxidation: gas chromatography-mass spectrometry compared to thiobarbituric acid. Anal Biochem 1997;245:161–166.

[35] Aebi H. Catalase *in vitro*. Methods Enzymol 1984;105:121–126.

[36] Paglia DE, Valentine WN. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J Lab Clin Med 1967;70:158–169.

[37] Pfaffl MW, Horgan GW, Dempfle L. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 2002;30:e36.

[38] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265–275.

[39] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227: 680–685.

[40] Waeg G, Dimsity G, Esterbauer H. Monoclonal antibodies for detection of 4-hydroxynonenal modified proteins. Free Radic Res 1996;25:149–159.

[41] Borovic S, Rabuzin F, Waeg G, Zarkovic N. Enzyme-linked immunosorbent assay for 4-hydroxynonenal-histidine conjugates. Free Radic Res 2006;40:809–820.

[42] Zarkovic K, Zarkovic N, Schlag G, Redl H, Waeg G. Histological aspects of sepsis-induced brain changes in a baboon model. In: Schlag G, Redl H, Traber DL, editors. Shock, sepsis and organ failure, 5th Wiggers Bernard Conference. Heidelberg: Springer-Verlag; 1997. p. 146–160.

[43] Coto-Montes A, Boga JA, Tomas-Zapico C, Rodriguez-Colunga MJ, Martinez-Fraga J, Tolivia-Cadrecha D, Menendez G, Hardeland R, Tolivia D. Physiological oxidative stress model: Syrian hamster harderian gland-sex differences in antioxidant enzymes. Free Radic Biol Med 2001;30: 785–792.

[44] Mates JM, Perez-Gomez C, Nunez de Castro I. Antioxidant enzymes and human diseases. Clin Biochem 1999;32: 595–603.

[45] Hunt CR, Sim JE, Sullivan SJ, Featherstone T, Golden W, Von Kapp-Herr C, Hock RA, Gomez RA, Parsian AJ, Spitz DR. Genomic instability and catalase gene amplification induced by chronic exposure to oxidative stress. Cancer Res 1998;58: 3986–3992.

[46] Brigelius-Flohe R. Tissue-specific functions of individual glutathione peroxidases. Free Radic Biol Med 1999;27: 951–965.

[47] Wilson DO, Johnson P. Exercise modulates antioxidant enzyme gene expression in rat myocardium and liver. J Appl Physiol 2000;88:1791–1796.

[48] Miao L, St Clair DK. Regulation of superoxide dismutase genes: implications in disease. Free Radic Biol Med 2009;47: 344–356.

[49] Sifringer M, Brait D, Weichelt U, Zimmerman G, Endesfelder S, Brehmer F, Haefen CV, Friedman A, Soreq H, Bendix I, Gerstner B, Felderhoff-Mueser U. Erythropoietin attenuates hyperoxia-induced oxidative stress in the developing rat brain. Brain Behav Immun 2009; [Epub ahead of print].

[50] Zarkovic N. 4-hydroxynonenal as a bioactive marker of pathophysiological processes. Mol Aspects Med 2003;24: 281–291.

[51] Zarkovic N, Cipak Gasparovic A, Cindric M, Waeg G, Borovic Sunjic S, Mrakovcic L, Jaganjac M, Kolenc D, Andrisic L, Gveric Ahmetasevic S, Katusic A, Cherkas A, Juric Shekar G, Wildburger R, Zarkovic K. The 4-hydroxynonenal-protein adducts as biomarkers of oxidative stress, lipid peroxidation and oxidative homeostasis. In: Proceedings of the European Society for Free Radical Research Congress. Caporossi D, Pigozzi F, Sabatini S, (editors). Rome. Bologna: Medimont; 2009. p. 37–43.

[52] Schabitz WR, Schade H, Heiland S, Kollmar R, Bardutzky J, Henninger N, Muller H, Carl U, Toyokuni S, Sommer C, Schwab S. Neuroprotection by hyperbaric oxygenation after experimental focal cerebral ischemia monitored by MRI. Stroke 2004;35:1175–1179.

[53] Clodfelter KH, Holloway MG, Hodor P, Park SH, Ray WJ, Waxman DJ. Sex-dependent liver gene expression is extensive and largely dependent upon signal transducer and activator of transcription 5b (STAT5b): STAT5b-dependent activation of male genes and repression of female genes revealed by microarray analysis. Mol Endocrinol 2006;20:1333–1351.

[54] Leone TC, Weinheimer CJ, Kelly DP. A critical role for the peroxisome proliferator-activated receptor alpha (PPARalpha) in the cellular fasting response: the PPARalpha-null mouse as a model of fatty acid oxidation disorders. Proc Natl Acad Sci USA 1999;96:7473–7478.

[55] Boylston WH, DeFord JH, Papaconstantinou J. Identification of longevity-associated genes in long-lived Snell and Ames dwarf mice. Age 2006;28:125–144.

[56] Patsouris D, Reddy JK, Muller M, Kersten S. Peroxisome proliferator-activated receptor alpha mediates the effects of high-fat diet on hepatic gene expression. Endocrinology 2006;147:1508–1516.

[57] Chen R, Liang F, Moriya J, Yamakawa J, Takahashi T, Shen L, Kanda T. Peroxisome proliferator-activated receptors (PPARs) and their agonists for hypertension and heart failure: are the reagents beneficial or harmful? Int J Cardiol 2008;130: 131–139.

[58] Macias-Gonzalez M, Cardona F, Queipo-Ortuno M, Bernal R, Martin M, Tinahones FJ. PPARgamma mRNA expression is reduced in peripheral blood mononuclear cells after fat overload in patients with metabolic syndrome. J Nutr 2008;138: 903–907.

[59] Li AC, Brown KK, Silvestre MJ, Willson TM, Palinski W, Glass CK. Peroxisome proliferator-activated receptor gamma ligands inhibit development of atherosclerosis in LDL receptor-deficient mice. J Clin Invest 2000;106:523–531.

This paper was first published online on Early Online on 11 November 2009.