Increased oxygen concentration (hyperoxia) induces oxidative damage of tissues and organs. Oxygen toxicity in hyperoxia is controlled by factors such as sex, age, tissue, strain and hormones. In most species females show lower incidence of some age-related pathologies linked with oxidative stress, which has been attributed to a beneficial effect of ovarian hormones. In this study we found that hyperoxia induced hepatic oxidative damage exclusively in male CBA/H mice, followed by their decreased survival. Histopathological examination revealed that the observed differences in survival were not the consequence of acute lung injury induced by hyperoxia. Next, we observed that an increased Sirt1 protein level in hyperoxia-exposed female CBA/H mice correlated with their lower PPAR-γ and higher eNOS and Sod2 protein levels. In males, higher PPAR-γ and lower Sod2 protein levels were associated with unchanged Sirt1 expression. Although these results are of a correlative nature only, they clearly show that females show better survival, increased resistance to hyperoxia and have generally more efficient defense systems, which suggests that their headstart in resistance to hyperoxia could be a consequence of the beneficial effect of ovarian hormones.

Key words: hyperoxia, sex-related, mice, ROS, Sirt1, PPAR-γ, eNOS, Sod2

Received: 22 May, 2014; revised: 01 August, 2014; accepted: 29 September, 2014; available on-line: 29 October, 2014

INTRODUCTION

Exposure to increased oxygen concentrations (hyperoxia) has been used for treatment of various pathological conditions (Tibbles & Edelsberg, 1996). The increased oxygen concentration has numerous side effects on the organism and induces oxidative damage of tissues and organs. In addition, hyperoxia causes pathological changes in the liver, associated with an increased level of blood triglycerides (Alhazza & Haffor, 2005). Resistance to hyperoxia reflects the longevity potential, as hyperoxia reduces lifespan, induces same level of oxidative damage and similar gene expression patterns as aging (Landis et al., 2004). For this reason, we employed hyperoxia as a model of acute oxidative stress load that induces pathological changes in metabolism. Oxygen toxicity in hyperoxia conditions is controlled by factors such as sex, age, development, tissue, strain and hormones (Choudhary et al., 2003). In most mammals, including humans, life expectancy is female-biased. Also, females show lower incidence of some age-related pathologies linked with oxidative stress and this sex-difference disappears after the menopause, which has led to the conclusion that this protection is attributed to sex hormones (Vina et al., 2005). Moreover, females were found to be more protected than males in a rat model of ischemia and reperfusion (Lagrangha et al., 2010). Sirtuin (Sirt1) is a conserved NAD-dependent histone deacetylase that has been linked to longevity, apoptosis, inflammation and energy homeostasis (Yamamoto et al., 2007). One of the key functions of Sirt1 is associated with resistance to stress related to ageing and as such Sirt1 appears to be important in suppression of the common diseases of aging (Cheng et al., 2003). Specifically, the overproduction of glucose, chronic hyperglycemia, and increased ROS production in the liver of Sirt1 null mice leads to oxidative stress-mediated impairment in mTORC2/AKT signaling and consequent insulin resistance (Wang et al., 2011). On the other hand, mice with whole-body overexpression of Sirt1 are leaner, more metabolically active, and glucose tolerant (Bordone et al., 2007) and are protected against diseases of aging such as metabolic syndrome and cancer. PPAR-γ and its target genes are involved in lipid and glucose metabolism and insulin resistance (Rangwala & Lazar, 2004). In the liver, PPAR-γ is expressed at a low basal level, but is up-regulated in murine models of obesity and type two diabetes (Boelsterli & Bedoucha, 2002). Sirt1 has been found to repress PPAR-γ activity thus promoting fat mobilization (Guarente, 2013). Sirt1-mediated upregulation of the major mitochondrial antioxidant enzyme superoxide dismutase 2 (Sod2) reduces mitochondrial ROS production, thus diminishing the effects of oxidative stress (Tanno et al., 2010). Results considering hyperoxia and endothelial nitric oxide synthase (eNOS) are controversial. Elevated levels of peroxyamine have been found in hyperoxia, associated with increased eNOS expression and nitrosative stress (Kondrikov et al., 2010). On the other hand, eNOS has a protective role in conditions of oxidative stress and its activation delays endothelial cellular senescence (Hayashi et al., 2006). eNOS has been implicated in a regulation of Sirt1 expression (Nisoli et al., 2005). The role of eNOS in female cardio-protection...
Experimental procedures. The experiments were performed in accordance with the current laws of the Republic of Croatia and with the guidelines of the European Community Directive of November 24, 1986 (86/609/EEC). Male and female CBA/H mice aged 4 months from the breeding colony of the Rudjer Bošković Institute (Zagreb, Croatia) were used for all experiments. Hyperoxic oxygen conditions were carried out by flushing the chamber (Duro Daković, Slavonski Brod, Croatia) with pure oxygen (25 L/min for 10 minutes) to replace air. 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. For survival analysis mice were placed in a hyperoxic chamber for 48 h and allowed to breathe 95% O₂. Normoxic O₂ conditions serving as a control were obtained by keeping mice in the same chamber, but under ambient air. Surviving animals were counted to establish sex-related mortality and euthanized. For biochemical and histopathological analyses, another set of CBA/H male and female mice was divided randomly into four groups of six animals each, i.e., males control (c-m); males hyperoxia (h_m); females control (c-f) and females hyperoxia (h_f). Mice were placed in a hyperoxic chamber for 46 hours to approach the conditions where we previously noticed significant differences in mortality between sexes, yet allow survival of all animals in order to perform further analysis.

Oxidative damage parameters. Lipid peroxidation was assessed by measurement of malondialdehyde (MDA) reaction with thiobarbituric acid following the formation of thiobarbituric reactive substances (TBARS) at 532 nm, according to Ohkawa and coworkers (1979). The results were expressed as nmol TBARS/mg of protein in liver supernatant according to a standard curve which was prepared with serial dilutions of 1,1,3,3-tetramethoxypropane standard. Protein carbonyls in liver tissue were determined using spectrophotometric assay according to (Reznick & Packer, 1994) with slight modifications (Fagan et al., 1999). In brief, liver tissue was homogenized using an ice-packed Potter-Elvehjem homogenizer (Braun, Biotech. Int., Germany) in PBS buffer (pH 7.4) containing 0.1% digitonin, protease inhibitors and 1 mM EDTA. Purified proteins were treated with HCl-acetone to remove interfering chromophores, and treated with 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2.5 M HCl. Following DNPH derivatization, samples were extensively washed with 30%, trichloroacetic acid (TCA), 10% TCA and three washes with ethanol/ethyl acetate. The resulting precipitate was solubilised in 400 μl of 6 M guanidine hydrochloride at 37°C for 30 min. The absorbance of each sample was measured at 375 nm and read against the sample treated with HCl alone, which served as background, and carbonyl content was calculated using the absorption coefficient of 22,000 M⁻¹cm⁻¹. Comet assay was carried out under alkaline conditions, as described by (Singh et al., 1988; Tice et al., 2000). One-hundred randomly selected cells per sample were analyzed by an automatic digital analysis system Comet assay IV, at 250× magnification (Perspective Instruments Ltd., Suffolk, Halstead, UK). To quantify DNA damage, the tail moment (tail length × tail % DNA/100) was evaluated. One-hundred comets were measured on 3 slides per each group. Since the data of the comet assay measurements are usually more or less dispersed, the statistical analysis and graphical presentation of the data were performed using the adequate statistical method that takes into account the non-Gaussian distribution of the data.

RNA isolation and quantitative real-time PCR analysis. Total RNA was extracted from individual mouse livers using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer’s instructions. RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer’s instructions. For first-strand cDNA synthesis, the reverse transcription reaction was performed on 1 μg of total RNA in a final volume of 20 μl. Real-time PCR analysis was carried out on an ABI 7300 sequence detection system using the comparative CT (ΔΔCT) method described in Taqman® Gene Expression Assays Protocol http://docs.appliedbiosystems.com (Applied Biosystems) to quantify relative levels of Sirt1, PPAR-γ, eNOS and Sod2 mRNAs. Primer length and Assay ID used for the analysis are shown in Table 1. Beta actin mRNA was used as a reference. Reactions were carried out in a total volume of 20 μl using TaqMan® Gene Expression Master Mix reagent (Applied Biosystems) and 5 μl of cDNA as template, and forward and reverse primers. All reactions were carried out in triplicate. Relative level of expression of each gene was calculated using the 2^(-ΔΔCT) method with the level in control samples set as one. Fold change was calculated using mean Ct values for each triplicate.

SDS/PAGE and Western blotting. Liver was homogenized in RIPA buffer supplemented with protease inhibitors (10%, w/v) using an ice-jacketed Potter-Elvehjem homogenizer (1300 rpm). After soni-
fication (3 x 30s), the homogenate was centrifuged at 16,000 x g for 20 min in a refrigerated centrifuge. Supernatant was collected and total cellular proteins (100 μg per lane) were resolved by denaturing sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS/PAGE) and transferred onto a PVDF membrane (Bio-Rad, Hercules, CA). Membranes were blocked in 5% nonfat dry milk in 50 mM phosphate buffer (pH 7.8) with 0.1% Tween-20 and incubated with primary antibodies against Sirt1 (diluted 1:500, Santa Cruz Biotechnology, Inc.), Sod2 (diluted 1:2000, Abcam, Cambridge, UK), PPAR-γ (diluted 1:200, Santa Cruz Biotechnology, Inc.) or eNOS (diluted 1:500, Abcam, Cambridge, UK). Anti -Erk-2 (diluted 1:1000, H-102, Santa Cruz Biotechnology, Inc.) was used for normalization. After 1 hour of incubation with horseradish peroxidase-conjugated secondary antibody (1:5000, Amersham, Buckinghamshire, UK) the blots were visualized using Western Lightning-ECL system (Perkin-Elmer, Inc.). The chemiluminescence signals were detected with an Alliance 4.7 Imaging System (UVITEC, Cambridge, UK). Densitometric analysis was done using Image J software. The blots were repeated at least three times and representative blots are presented in the figures.

**Histopathological analysis.** Lungs of animals from all experimental groups were analysed histologically to determine the presence of hyperoxia-induced acute lung injury (ALI). Slides were prepared from formalin-fixed paraffin-embedded lungs stained with haematoxylin and eosin. Due to the patchy nature of ALI, at least 5 random high-power fields (400× total magnification) were independently scored in a blinded fashion for each microscopic slide. The injury scoring of ALI was performed according to (Matute-Bello et al., 2011). All immunohistochemical analyses were done by a pathologist oblivious of the study group design.

**Protein concentration.** Protein concentration in all samples was determined using BCA protein assay.

**Statistical analysis.** Statistical analyses of data were performed using R v2.15.3 (CRAN, http://cran.r-project.org) and RStudio for Windows, v0.97 (http://www.rstudio.com/). Before all analyses samples were tested for normality of distribution using Shapiro-Wilk test. If data followed non-Gaussian distribution, nonparametric analyses were performed: Kruskal-Wallis non-parametric ANOVA, followed by Wilcoxon signed-rank test for testing differences between two related groups. Statistical analysis of comet test results was performed on logarithmically transformed data. In case of normal distributions, parametric tests were performed: one-way ANOVA, followed by Tukey’s post-hoc tests for multiple comparisons of the samples. To determine differences in survival rate, Pearson Chi-Square test was performed. For all tests significance level was set at p<0.05. For determination of sample size in this experiment, the “resource equation” method of determining sample size was used (Festing, 1996) and revealed that six animals per group is sufficient for this experimental design.

**RESULTS**

**Survival of CBA mice exposed to normobaric hyperoxia for 48 hours**

The rate of survival of a normobaric hyperoxia treatment for 48 hours was determined for female and male mice and the results were evaluated using Pearson Chi-Square test. The fraction of female mice that survived the hyperoxia was significantly higher compared with males ($\chi^2 (1) = 7.025, p = 0.008$). Namely, 19 out of 20 females (95%), and 12 out of 20 males survived the treatment (60%).

**Lipid peroxidation and carbonyl content**

Lipid peroxidation (LPO) was evaluated by measuring the level of TBARS in liver homogenates. The LPO was increased significantly only in males ($p<0.001$). In females the increase was non-significant (Fig. 1). As shown in Fig. 2, carbonyl level was significantly higher only in male mice subjected to hyperoxia ($p<0.01$); in females the protein carbonyl content was virtually unaffected.

**DNA damage analysis (comet assay)**

The effect of hyperoxia on the level of DNA damage was assessed by single-cell gel electrophoresis (comet assay). Since the large amount of data of the comet assay are usually more or less dispersed, the scattered pattern of data is typical, with relatively high number of outliers in most of the groups examined. Quantification of the DNA damage expressed as tail moment is presented...
in Fig. 3. There was no significant change in the DNA damage across all experimental groups ($p=0.556$).

**Sirt1, Ppar-$\gamma$, eNOS and Sod2 mRNA levels**

Real time PCR analysis showed a small but significant difference in Sirt1 mRNA level in control females compared to males (1.26-fold higher; $p=0.041$). Ppar-$\gamma$ was downregulated in hyperoxia-exposed males by more than 5-fold (fold-change $-5.018$; $p=0.022$) while Sod2 was significantly downregulated only in hyperoxia-exposed females (fold-change $-1.379$; $p=0.016$). eNOS was significantly upregulated in hyperoxia-exposed groups of both sexes, and difference between sexes was insignificant (Fig. 4).

**Sirt1, Ppar-$\gamma$, eNOS and Sod2 protein expression**

Western blot analysis showed that Sirt1 protein was significantly upregulated ($p=0.039$) in liver of female mice upon hyperoxia treatment, while its level remained unchanged in male mice (Fig. 5A). On the other hand, PPAR-$\gamma$ was significantly upregulated ($p=0.002$) upon hyperoxia in males, and decreased in females ($p=0.014$) (Fig. 5B). The expression of Sod2 was suppressed in males ($p=0.032$) and upregulated in females ($p=0.006$) after hyperoxia (Fig. 5C). eNOS protein level was significantly upregulated in hyperoxia-exposed females ($p=0.038$), while in males the increase in eNOS protein did not reach significance, due to a large variation among individual mice (Fig. 5D).

**Histological evidence of acute lung injury**

Lungs of animals from all experimental groups were analysed histologically to determine whether hyperoxia induced ALI which could have contributed to greater mortality rate in males. In hyperoxia-exposed males, there was a significant accumulation of neutrophils in alveolar ($p=0.011$) and interstitial spaces ($p<0.001$) along with other hallmarks of ALI, such as depositions of hyaline membranes ($p=0.016$) composed of proteinaceous debris ($p<0.001$) as evidence that serum proteins have entered the airspaces when compared to control males (Fig. 6A, 6B). In contrary, hyperoxia-exposed females showed no significant changes in any of the ALI parameters, although there were small amounts of proteina-
Females’ resistance to hyperoxia

ceous debris, occasional interseptal neutrophils and congested blood vessels (Fig. 6C, 6D). Despite the fact that more pronounced changes of the ALI parameters were observed in males, these results suggest that the greater mortality rate upon hyperoxia exposure in males is not due to lung damage, because the severity of the pathological changes induced by hyperoxia was not sufficient to induce serious lung damage and consequent death.

DISCUSSION

The purpose of this study was to determine if the resistance to hyperoxia is sex-related, taking into account that sexual dimorphism in age-related pathologies is linked with oxidative stress (Mikkola et al., 2013). Hyperoxia, a model of acute oxidative stress load, was chosen because it has been shown that excessive production of ROS under hyperoxic conditions has a potential to directly damage cellular macromolecules, although beneficial effects of hyperoxia have also been noted in the treatment of several diseases (Weaver et al., 2002). Even though ROS are normally produced in cells, their excess can overwhelm the antioxidant defenses, inducing tissue injury (Muriel, 2009). Our previous investigations indicated that female mice are better protected from oxidative stress than male mice (Sobočanec et al., 2010). Female CBA/H mice live significantly longer than their male counterparts (Storer, 1966). In this study we have found better female survival in comparison to males (95% vs. 63%) after exposure to hyperoxia for 48 hours. To determine whether the greater male mortality rate was due to ALI as a consequence of breathing pure oxygen, we per-
formed histopathological lung examination in all groups. Although more pronounced changes in ALI parameters were observed in males which is in agreement with (Linggappan et al., 2013), we concluded that the greater mortality rate found in males was not due to lung damage because the degree of the pathological changes induced by hyperoxia was not sufficient to induce serious lung damage and consequent death. For biochemical and histopathological analyses, the animals were subjected to hyperoxia for 46 h only. The time of exposure was chosen based on our preliminary results so as to avoid the hyperoxia-induced mortality while preserving the hyperoxia-induced oxidative damage (see Experimental Procedures). Hyperoxia induced sex-related changes in oxidative stress markers, such as lipid peroxidation and protein carbonylation, with males having higher levels of both LPO and carbonyl levels compared to females. DNA damage was not markedly affected by hyperoxia in either sex. Next, we observed an increased Sirt1 protein level in hyperoxia-exposed females and lower PPAR-γ, and higher eNOS and Sod2 protein levels. On the other hand, in males unchanged Sirt1 was accompanied by higher PPAR-γ, and lower Sod2 and eNOS protein levels. The differences found in the level of protective antioxidant enzymes in the liver could be responsible for the sex-related mortality rate, but this is only speculation since we did not perform a histopathological analysis of the livers from this experimental setup. Sirt1 is being increasingly recognized as a regulator of diverse cellular processes, including stress resistance, senescence, aging and inflammatory response to oxidative stress (Yao et al., 2012). Sirt1 prolongs the lifespan in simple organisms from yeast to flies. Upregulation of Sirt1 can influence redox functions of cells either directly or indirectly, by activation of various transcription factors, such as PGC-1α and FOXO3, which may lead to increased resistance to oxidative stress (Tanno et al., 2010), while Sirt1 deficiency causes increased ROS production (Wang et al., 2011). Sirt1 has been found to repress PPAR-γ activity thus promoting fat mobilization (Picard et al., 2004), and attenuate cellular oxidative stress toxicity through induction of Sod2 expression (Tanno et al., 2010). Upregulated Sirt1 along with decreased PPAR-γ triggers reduction of fat storage, a primary way by which calorie restriction extends lifespan in mammals (Picard et al., 2004), and these data are in accordance with our results since female mice better cope with oxidative stress since a role of Sirt1 in cell-dependency upon estrogen has been shown (Moore & Faller, 2013). PPAR-γ plays an important role in adipogenesis, and as such has been implicated in the pathology of numerous diseases including diabetes, atherosclerosis and cancer (Mansure et al., 2009). Enhanced levels of PPAR-γ have been found in the liver of obese patients (Pettinelli & Videla, 2011), and in animals fed high-fat diet (Chen et al., 2013). The elevated level of PPAR-γ along with increased oxidative damage in males found in this study are in agreement with other reports (Almeida et al., 2009) and suggest that PPAR-γ may contribute to their increased susceptibility. Sod2, the major mitochondrial antioxidant enzyme, is responsible for the reduction of ROS generated during oxidative phosphorylation. Downregulated Sod2 increases the level of ROS and oxidative stress (Tanno et al., 2010), which may account for the increase of hepatic oxidative damage markers and subsequent increase in LPO and carbonyl levels found in males. In contrast, females responded to hyperoxia with a significantly increased Sod2 level which was correlated with the absence of oxidative damage. Data from our laboratory showed sex-related differences in CBA/H mice hepatic oxidant and antioxidant status, with males having constantly 50% more catalase (CAT) and 85% more glutathione peroxidase (Gpx) activity than females. Those data correlated with increased incidence of hepatic tumors found exclusively in old males (Sverko et al., 2004). While males displayed higher CAT and Gpx activities with aging, females showed far less dramatic changes in antioxidant enzyme activities, which may imply that they had less need for detoxication of ROS. Collectively, those data along with our present study suggest that a possible cause of the males’ higher mortality rate could lie in their less effective antioxidant enzyme system unable to cope with the ROS generation during prolonged hyperoxia.

The females’ resistance to hyperoxia could be therefore attributed to estrogen action, since estrogen increases the expression of Sod2, thus leading to a lower efflux of ROS from mitochondria (Vina et al., 2005). Moreover, an increased eNOS protein level was found in hyperoxia-exposed females. Females are protected from cardiovascular injury via an eNOS-mediated mechanism (Cross, 2000). eNOS expression is found to be estrogen-regulated (Kleiner et al., 1998). The upregulation of both eNOS mRNA and protein levels in females upon hyperoxia suggests that the greater resistance of females to hyperoxia could be, at least in part, a consequence of their higher eNOS expression induced by estrogen. It has been reported that oxidative damage rather than defense or repair of such damage controls the aging rate (Sanz et al., 2006), and for this reason we suggest that the higher oxidative damage markers in males could be indicative of their greater susceptibility to oxidative stress which in turn could lead to their decreased survival rate. Moreover, Sirt1, eNOS, PPAR-γ and Sod2 are affected in a sex-dependent manner and as such could contribute to the sex-specific response to hyperoxia. Although these results are of a correlative nature, they clearly show that females exhibit a higher survival rate, increased resistance to hyperoxia and overall better defense systems, which suggests that the female headstart in the resistance to hyperoxia could be a consequence of the beneficial effects of ovarian hormones.

Acknowledgements

We thank Iva Pešun-Medimorec for her excellent technical assistance. The research is funded by the Croatian Ministry of Science, Education and Sports, Grants No. 098-0982464-1647 and 098-0982464-2460.

REFERENCES


Sirt2 homolog (SIRT1)-deficient mice. Proc Natl Acad Sci USA 100: 10794–10799.


