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The effect of 17β-estradiol on sex-dimorphic cytochrome P450 expression patterns induced by hyperoxia in the liver of male CBA/H mice

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Abstract The aim of this study was to determine whether treatment of male CBA/H mice with 17β-estradiol (E_2) had protective effect on survival and hepatic oxidative damage of lipids and proteins against hyperoxia. Furthermore, we wanted to explore the effect of E_2 treatment on the expression of sex-specific cytochrome P450 isoforms, and their possible involvement in E_2-induced resistance to hyperoxia. Lipid peroxidation and protein carbonylation were analysed spectrophotometrically and were used as a measure of lipid and protein oxidative damage. Real-time PCR and western blot analysis were used to measure both gene and protein expression levels of Cyp2E1, Cyp7B1 and Cyp2A4, respectively. We found that treatment of male CBA/H mice with E_2 increased survival upon hyperoxia exposure, and provided protection against hepatic lipid and protein oxidative damage. Hyperoxia had feminizing effect on the expression of sex-specific CYPs, which resembled the lifespan-promoting conditions. E_2 administration had the opposite effect on the expression pattern of these CYPs in hyperoxic versus normoxic conditions. Results of this research proposed possible male strategy in adaptive response to oxidative stress, which may finally result in their longer lifespan.

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Introduction

Exposure to increased concentrations of oxygen (hyperoxia) is routinely used to treat several conditions like hypoxemia, acute respiratory failure and acute carbon monoxide poisoning [1]. However, the prolonged administration of high oxygen concentration results in tissue damage. Reactive oxygen species (ROS) are generated at increased rate in cells and tissues during hyperoxia, causing oxidative damage of biological molecules [2]. Moreover, hyperoxia causes inflammatory response which aggravates oxygen toxicity, resulting in lethality of experimental animals after prolonged exposure [3].

Susceptibility to hyperoxia is found to reflect the longevity potential of the species and hyperoxia-induced gene expression pattern resembles ageing process [4].

Cytochrome P450 enzymes (Cyp) constitute a family of monooxygenases that play important role in the oxidative metabolism and detoxification of pollutants and carcinogens. Also, Cyp enzymes catalyse biosynthesis of endogenous compounds such as fatty acids and steroid hormones [5]. Their regulation is controlled by sex, age, tissue and hormones. Cyp2E1 has important role in the metabolism of ethanol, glycerol and fatty acids and participates in depletion of lipid peroxidation substrates [6] and is implicated hyperoxia susceptibility. Cyp7B1 (oxysterol 7α-hydroxylase) is male-predominant isoform in the liver of adult mice and its sex-related expression is dependent on androgen signalling [7]. Cyp7B1 plays a key role in the metabolism of the cholesterol, oxysterols and bile acids [8]. Cyp2A4 (steroid 15α-hydroxylase) is a

female-predominant isoform responsible for the hydroxylation of testosterone and progesterone, with suppressed expression in male liver [9]. Sex-specific pattern of Cyp7B1 and Cyp2A4 expression in mice is established in puberty by sex-related differences in the secretion of growth hormone (GH) [10]. Female sex hormone 17β-estradiol (E_2) has well-established cytoprotective effect during oxidative stress. Its depletion contributes to pathogenesis of age-related diseases [11]. Also, E_2 is responsible for females' longer lifespan and their overall better protection to oxidative stress [12]. We have shown that female CBA/H mice were more resistant to hyperoxia and had higher survival compared to their male counterparts. The observed differences in survival were rather due to liver oxidative damage found exclusively in males, than to acute lung injury which was not severe enough to induce death [13]. Also, treatment of male mice with E_2 efficiently activated their hepatic antioxidative system in physiological conditions [14]. In this study, we hypothesize that E_2 serves as a protective factor in conditions of acute oxidative stress in the liver of male CBA/H mice. Moreover, we suggest that protective response of E_2 is associated with altered expression of sex-dependent Cyps.

Materials and methods

Animals and experimental design

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). Total of 32 male CBA/H mice aged 4 months from the breeding colony of the Ruđer Bošković Institute (Zagreb, Croatia) were used for all experiments. 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; and access to food pellets and tap water ad libitum.

For the E_2 administration, a pellet containing E_2 (50 µg, Innovative research of America, Sarasota, FL) was placed into the interscapular subcutaneous space releasing a constant dose of 830 ng of E_2 daily for 37 days. Another set of animal was used as an untreated control. After 37 days, E_2 treated and untreated animals were subjected to experimental protocols.

For survival analysis, E_2-treated and E_2-untreated animals ($n = 6$ per group) were placed in oxygen chamber (Đuro Đaković, Slavonski Brod, Croatia) and exposed to oxygen conditions for 48 h, by flushing the chamber with pure oxygen (25 L/min for 10 min) to replace air. Concentration of O_2 in the chamber was determined using O_2 sensor (0–100 % Dräger PacIII, Lübek, Germany)

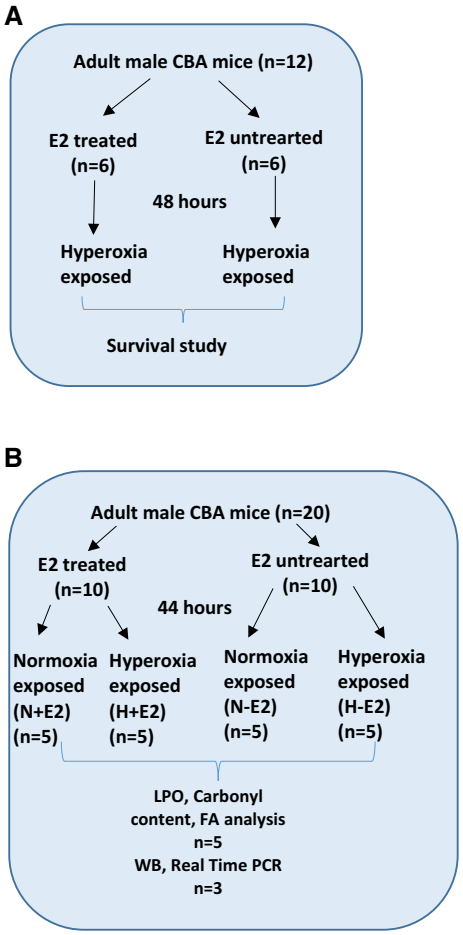

Fig. 1 Scheme of the experimental design, number of animals and experimental groups. **a** Survival study **b** Biochemical analyses

(Fig. 1a). Surviving animals were counted to establish a role of E_2 treatment on survival in hyperoxia conditions and euthanized.

For biochemical analysis, another set of CBA/H mice was used. Animals were divided randomly into E_2-treated and E_2-untreated group. E_2 treatment procedure was made in same fashion as for the survival study. After E_2 treatment, animals were exposed to hyperoxia conditions for 44 h to approach the conditions where we previously noticed significant differences in mortality between male and female mice [13], yet allow survival of all animals in order to perform further analysis. Control animals were placed in hyperoxic chamber exposed to ambient air. After sacrifice, portion of the same liver were used for all analyses as shown in Fig. 1b. Samples were snap frozen and stored on −80 °C until analysis.

Lipid peroxidation (LPO)

Lipid peroxidation was assessed by measurement of malondialdehyde (MDA) reaction with thiobarbituric acid

following the formation of thiobarbituric reactive substances (TBARS), according to Ohkawa et al. [15]. In brief, liver tissue was homogenized (10 % w/v) using an ice-packed Potter–Elvehjem homogenizer (Braun, Biotech. Int., Germany) in RIPA buffer containing protease inhibitors. Homogenates were sonicated for 30 s, and centrifuged on 3000×*g* for 15 min at +4 °C. Supernatants were treated with 10 % trichloroethanoic acid (TCA) (1:2 v/v). After protein precipitation, equal volume of thiobarbituric acid (TBA) was added, and samples were incubated for 60 min at 95 °C. Absorbance of each sample was measured on plate reader at 532 nm. 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.

Protein carbonylation

Protein carbonyls in liver supernatants were determined according to [16]. Samples in PBS with protease inhibitors (Roche Diagnostics, Penzberg, Germany) were supplemented with lipid removal agent (Sigma 13360-U) and incubated at room temperature for 1 h, then centrifuged for 20 min at 13000 rpm. Supernatants were diluted to 10 µg/mL, loaded into Maxisorb wells (Sigma Aldrich, St. Louis, MO, USA) and incubated overnight at 4 °C to allow proteins to adsorb to the surface. Adsorbed proteins were derivatized using 12 µg/mL 2,4-dinitrophenylhydrazine (DNPH). Derivatized dinitrophenol (DNP)-carbonyl was detected by rabbit anti-DNP primary antibody (D9656, Sigma Aldrich, St. Louis, MO, USA) and goat anti-rabbit secondary antibody conjugated to HRP (Jackson ImmunoResearch, West Grove, PA, USA). 1 µg/µL antibody stocks were used at a 1:7000 dilution. Samples were then incubated with enzyme substrate 3,3′,5,5′-tetramethylbenzidine (Sigma Aldrich, St. Louis, MO, USA) until colour developed, and the reaction was stopped using 0.3 M H_2SO_4. Absorbance was measured by a microplate reader at 450 nm.

Fatty acid analysis

For the detection of fatty acids, liver was homogenized in PBS. Total lipids were extracted from homogenates according to [17]. The lipid extract was treated with 0.5 M KOH/MeOH for 20 min at room temperature, and the corresponding fatty acid methyl esters (FAMEs) were formed and analysed by gas chromatography (GC). GC analyses of total fatty acids were performed by Varian 450-GC equipped with a flame ionization detector. A Stabilwax column (crossbond carbowax polyethylene glycol, 60 m × 0.25 mM) was used as a stationary phase at a programmed temperature with helium as the carrier gas. The heating was carried out at a temperature of 150 °C for 1 min followed by an increase of 1 °C/min up to 250 °C. Methyl esters were identified by comparison with the retention times of authentic samples.

RNA isolation and real-time PCR analysis

Total RNA was extracted from individual mouse livers in each group ($n = 3$) using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Reverse transcription and real-time PCR analysis were done as described previously [18], to quantify relative mRNA expression of cyp2E1, cyp7B1 and cyp2A4. Using the $2^{-\Delta\Delta Ct}$ method, data are presented as the fold-change in gene expression normalized to endogenous reference gene (β-actin) and relative to the untreated control. Assays used in this study are listed in Table 1. All reactions were carried out in triplicate.

SDS-PAGE and western blotting

Liver was homogenized with RIPA buffer supplemented with proteinase inhibitors (10 % w/v) using an ice-jacketed Potter–Elvehjem homogenizer (1.300×*g*). After sonification (3 × 30 s), whole liver homogenates were centrifuged at 16.000×*g* for 20 min at +4 °C. Supernatant was collected and total cellular proteins (75 µg per lane) were resolved by denaturing SDS-PAGE, and transferred onto a PVDF membrane (Bio-Rad, Hercules, CA, USA). Membranes were blocked in 5 % nonfat dry milk in TN buffer (50 mM TRIS, 150 mM NaCl, pH = 7.4) overnight at +4 °C. Membranes were incubated with primary polyclonal goat antibody against Cyp7B1 (Santa Cruz Biotechnology Inc, TX, USA) (diluted 1:200 and incubated overnight at +4 °C), followed by incubation with donkey anti-goat IgG, horseradish peroxidase-conjugated secondary antibody (BioRad, Hercules, CA, USA) for 1 h at room temperature. For Cyp2E1 protein detection, membranes were incubated with primary polyclonal rabbit antibody against Cyp2E1 (Abcam, Cambridge, UK), diluted 1:200 and incubated overnight at +4 °C, followed by incubation with donkey anti-rabbit IgG, horseradish peroxidase-conjugated, secondary antibody (Amersham Biosciences Inc., USA) for 1 h at room temperature. For Cyp2A4 protein detection, membranes were incubated with

Table 1 Assays used for real-time PCR analysis

Gene	ID	Product size
Cyp2e1	Mm00491127_m1	83
Cyp7b1	Mm00484157_m1	62
Cyp2a4	Mm00487248_g1	75
Beta-actin	Mm00607939_s1	115

primary polyclonal rabbit antibody against Cyp2A (Santa Cruz Biotechnology Inc., TX, USA) diluted 1:200 and incubated overnight at +4 °C, followed by incubation with donkey anti-rabbit IgG, horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences Inc., USA) for 1 h at room temperature. Equality of loading was confirmed using AmidoBlack (Sigma Aldrich, St. Louis, USA), which was also used for normalization of the bands [19]. The chemiluminescence signals were detected and analysed with the Alliance 4.7 Imaging System (UVITEC, Cambridge, UK). The blots were repeated at least three times and representative blots are presented.

Protein concentration

Protein concentration in all samples was determined using BCA protein assay (Thermo Scientific, Rockford, USA).

Statistical analysis

Statistical analyses of data were performed using R v2.15.3 (CRAN, http://cran.r-project.org) and RStudio for Windows, v 0.97 (http://www.rstudio.com/). All groups were tested for normality of distribution using Shapiro–Wilk test. Since data followed normal distribution, the differences between multiple groups were compared with one-way parametric ANOVA, followed by Tukey's post hoc test for testing differences between multiple groups. For all tests, significance level was set at $p < 0.05$.

Results

The effect of E_2 and hyperoxia on total body mass of male CBA/H mice exposed to normobaric hyperoxia for 44 h

In order to evaluate the efficacy of E_2 implementation, we determined total body masses in all groups of animals on 37th day post-implementation. E_2 induced significant decrease in the average body mass compared to untreated male mice in normoxic conditions ($p < 0.001$, N vs. N+E_2). Hyperoxia-exposed animals had lower body mass compared to their normoxic littermates ($p = 0.020$, N vs. H). Also, E_2 treatment additionally decreased body mass in hyperoxia-treated group, compared to hyperoxia-treated group alone ($p = 0.031$, H vs. H+E_2) (Fig. 2).

The effect of E_2 on survival of male CBA/H mice exposed to normobaric hyperoxia for 44 h

In order to determine whether E_2 has beneficial effect on survival of males subjected to hyperoxia, we have determined the rate of survival of a normobaric hyperoxia treatment for 48 h and the results were evaluated using Pearson χ^2 test. The fraction of male mice treated with E_2 that survived the hyperoxia was higher compared to their corresponding control, but without reaching significance due to small number of samples (χ^2 (1) = 2.4, $p = 0.121$). None of the males untreated with E_2 (H) survived the treatment, while 2 out of 6 males treated with E_2 (H+E_2) survived the treatment.

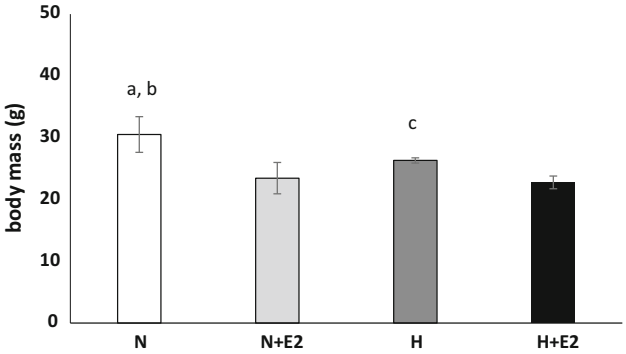

Fig. 2 Effect of hyperoxia and E_2 administration on total body mass of male CBA/H mice on 37th day post-surgery. The results are presented as mean ± SD from 6 animals per group. $^{a}p < 0.001$, N versus N+E_2; $^{b}p = 0.020$, N versus H; $^{c}p = 0.031$, H versus H+E_2

The effect of E_2 on hepatic oxidative stress markers and total fatty acid content of male CBA/H mice exposed to normobaric hyperoxia for 44 h

As a measure of lipid oxidative damage, LPO was evaluated by measuring TBARS level in liver homogenates of 4-month-old male CBA/H mice subjected to hyperoxia. LPO was markedly increased in hyperoxia-exposed males compared to their corresponding normoxic control ($^{a}p = 0.001$, N vs. H). Although E_2 administration decreased LPO in hyperoxia conditions ($^{c}p = 0.011$, H vs. H+E_2), it still remained significantly elevated, when compared to normoxic group alone ($^{b}p = 0.007$, N vs. H+E_2) (Fig. 3a). In order to investigate the effect of E_2 on protein oxidative damage in hyperoxia-treated mice, we determined protein carbonylation. The level of carbonylated proteins markedly increased in hyperoxia-exposed mice, compared to their corresponding normoxic group ($^{a}p = 0.003$, N vs. H). The administration of E_2 in normoxic conditions caused even greater difference between this group and hyperoxia-treated animals ($^{b}p = 0.001$, N+E_2 vs. H). In hyperoxic conditions, E_2 markedly decreased protein oxidative damage, compared to hyperoxia-treated mice alone ($^{c}p = 0.005$, H vs H+E_2) (Fig. 3b). Since we have found that hyperoxia causes substantial increase in LPO damage, we analysed changes in total fatty acid profile in response to hyperoxia and E_2

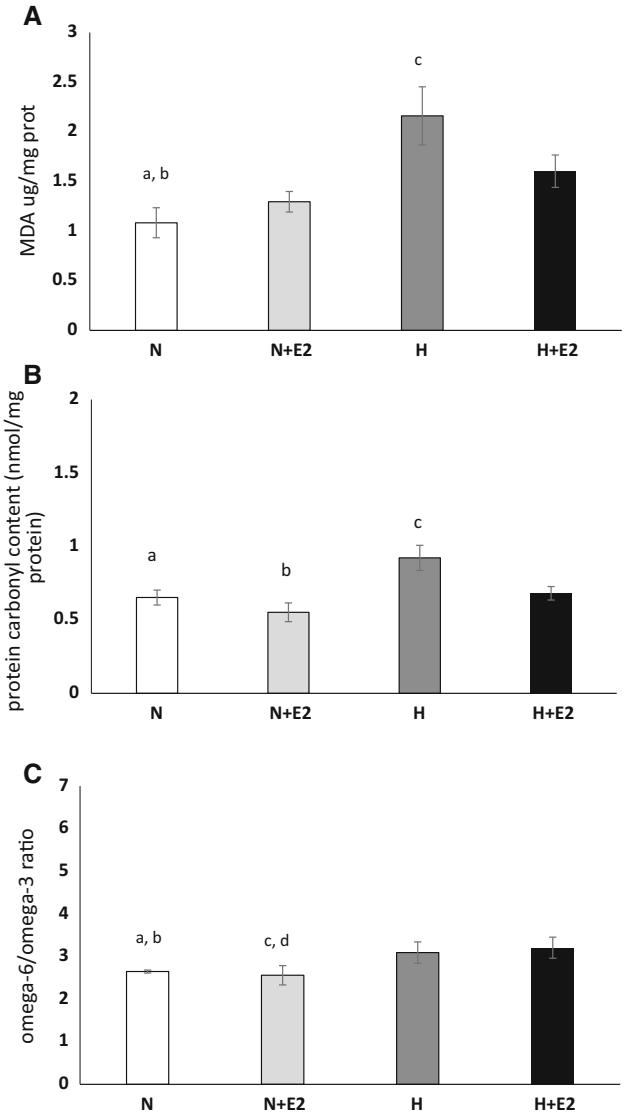

Fig. 3 Effect of E_2 administration on TBARS level in liver supernatants of normoxia- and hyperoxia-exposed male CBA/H mice. Data present mean ± SD from 6 animals per group. N-animals exposed to normoxia untreated with E_2, N+E_2-animals exposed to normoxia treated with E_2, H-hyperoxia-exposed animals untreated with E_2, H+E_2-animals exposed to hyperoxia treated with E_2. $^{a}p = 0.001$, N versus H; $^{b}p = 0.007$, N versus H+E_2; $^{c}p = 0.011$, H versus H+E_2 (**a**). Effect of E_2 administration on protein carbonylation in liver supernatants of normoxia- and hyperoxia-exposed male CBA/H mice. The results are presented as mean ± SD from 6 animals per group. N-animals exposed to normoxia untreated with E_2, N+E_2-animals exposed to normoxia treated with E_2, H-hyperoxia-exposed animals untreated with E_2, H+E_2-animals exposed to hyperoxia treated with E_2. $^{a}p = 0.003$, N versus H; $^{b}p = 0.001$, N+E_2 versus H; $^{c}p = 0.005$, H versus H+E_2 (**b**). Effect of E_2 administration on omega-6/omega-3 fatty acid ratio in liver supernatants of normoxia- and hyperoxia-exposed male CBA/H mice. The results are presented as mean ± SD from 6 animals per group. N-animals exposed to normoxia untreated with E_2, N+E_2-animals exposed to normoxia treated with E_2, H-hyperoxia-exposed animals untreated with E_2, H+E_2-animals exposed to hyperoxia treated with E_2. $^{a}p = 0.040$, N versus H; $^{d}p = 0.008$, N+E_2 versus H+E_2. $^{b}p = 0.026$, N versus H+E_2; $^{c}p = 0.017$, N+E_2 versus H (**c**)

treatment. The percentage of total fatty acids was not significantly changed in any group of mice, compared to normoxic group, mainly due to large intra-variability of the samples (Table 2). However, we found that the omega-6/omega-3 fatty acid ratio, known as a measure of proinflammatory potential, was significantly increased in hyperoxia-exposed group of mice compared to both normoxic ($^{a}p = 0.040$, N vs. H) and E_2-treated normoxic group ($^{c}p = 0.017$, N+E_2 vs. H). In hyperoxic group, E_2 administration failed to decrease omega-6/omega-3 fatty acid ratio back to normoxic group ($^{b}p = 0.026$, N vs. H+E_2; $^{d}p = 0.008$, N+E_2 vs. H+E_2) (Fig. 3c).

The effect of E_2 on Cyp2E1 expression in the liver of male CBA/H mice exposed to normobaric hyperoxia for 44 h

We determined gene expression and protein expression levels of Cyp2E1 to evaluate if beneficial effect of E_2 in hyperoxia conditions has any association with the changes in the expression of this Cyp. We have found marked increase in Cyp2E1 gene expression in E_2-treated normoxic males, compared to their corresponding normoxic group (fold-change 3.09 ± 0.74; $^{a}p = 0.004$, N vs. N+E_2) (Fig. 4a). However, due to large sample variation and small size of the sample, this difference was not followed on protein level, and no change in protein expression level was observed (Fig. 4b).

The effect of E_2 on Cyp7B1 expression in the liver of male CBA/H mice exposed to normobaric hyperoxia for 44 h

We determined gene and protein expression profile of Cyp7B1 upon hyperoxia and E_2 administration using real-time PCR and western blot analysis. Real-time PCR showed significant downregulation of cyp7B1 gene in hyperoxia-treated group, (fold-change −2.26 ± 0.35; $^{a}p = 0.033$, N vs. H) and E_2 administration decreased cyp7B1 mRNA level even more (fold-change −2.49 ± 0.35; $^{b}p = 0.001$, N vs. H+E_2) (Fig. 5a). Protein expression pattern followed mRNA level, with the lowest content in hyperoxia-exposed animals treated with E_2 compared to all other groups ($^{a}p = 0.002$, N vs. H+E_2; $^{b}p = 0.001$, N+E_2 vs. H+E_2; $^{c}p = 0.016$, H vs. H+E_2) (Fig. 5b).

The effect of E_2 on Cyp2A4 expression in the liver of male CBA/H mice exposed to normobaric hyperoxia for 44 h

We determined gene and protein expression profile of Cyp2A4 upon hyperoxia and E_2 administration using real-

Table 2 Total fatty acid content in the liver of male CBA/H mice exposed to normobaric hyperoxia for 44 h

Group	Saturated	Unsaturated	Polyunsaturated
N	37.5 ± 2.3	22.7 ± 6.2	39.8 ± 4.1
N+E_2	37.6 ± 2.2	25.7 ± 5.8	36.6 ± 4.5
H	34.7 ± 2.4	26.8 ± 4.3	38.5 ± 3.5
H+E_2	36.3 ± 3.4	24.7 ± 3.4	38.9 ± 1.6
Group	C-16:0	C-16:1	C-18:0
N	25.1 ± 0.8	2.0 ± 0.7	12.0 ± 2.2
N+E_2	25.4 ± 0.9	2.0 ± 0.6	11.8 ± 2.0
H	23.9 ± 1.1	2.4 ± 0.5	10.3 ± 2.0
H+E_2	24.5 ± 1.1	2.5 ± 0.4	11.6 ± 2.4
Group	C-18:1	C-18:2	C-18:3
N	17.2 ± 4.5	18.5 ± 0.8	0.3 ± 0.1
N+E_2	19.5 ± 4.7	16.7 ± 3	0.3 ± 0.1
H	20.9 ± 3.5	20.6 ± 3.5	0.6 ± 0.3
H+E_2	18.9 ± 2.9	20.1 ± 2.1	0.5 ± 0.2
Group	C-20:4	C-22:5	C-22:6
N	10.2 ± 2.2	0.7 ± 0.0	9.3 ± 1.4
N+E_2	9.5 ± 2.2	0.6 ± 0.1	8.7 ± 1.0
H	8.0 ± 1.9	0.7 ± 0.1	7.6 ± 1.0
H+E_2	9.0 ± 1.7	0.6 ± 0.1	7.8 ± 0.8

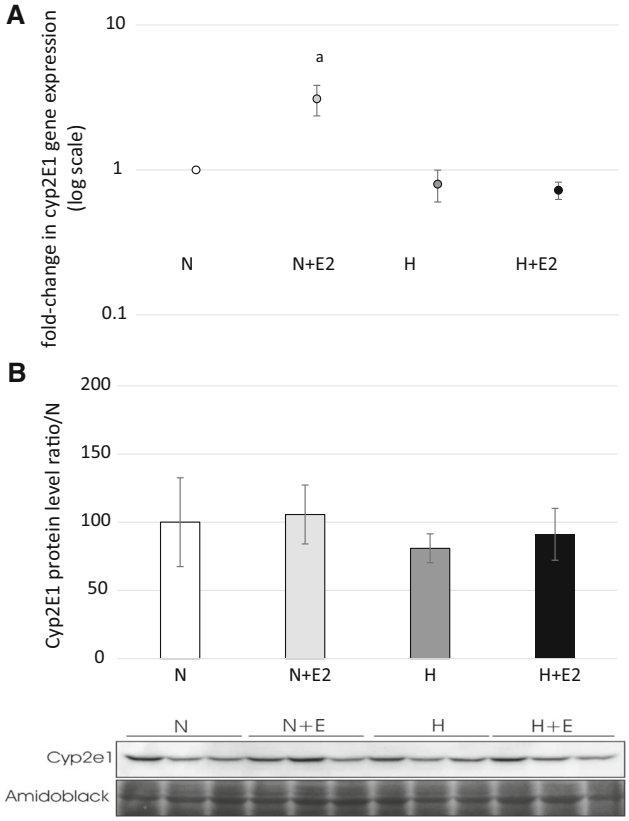

Fig. 4 Effect of E_2 administration on cyp2E1 gene expression in the liver of normoxia- and hyperoxia-exposed male CBA/H mice. The fold-change in gene expression was calculated using the $2^{-\Delta\Delta CT}$ method and β-actin as the endogenous control. The results are presented as fold-change ± SE. $^{a}p = 0.004$, N versus N+E_2 (**a**). Western blot analysis of Cyp2E1 protein level in the liver of normoxia- and hyperoxia-exposed male CBA/H mice. Results are presented as mean ± SD. Amidoblack was used as a loading control. Representative immunoblots are shown (**b**)

time PCR and western blot analysis. Significant increase in cyp2A4 gene expression was found only in hyperoxia-treated group, compared to normoxic group of mice (fold-change 4.27 ± 1.72, $^{a}p = 0.031$, N vs. H), although the tendency towards the increase of cyp2A4 mRNA level was present across all experimental groups (Fig. 6a). Protein level of Cyp2A followed mRNA level in hyperoxia-treated males, but was found to be only marginally increased compared to normoxic group ($^{a}p = 0.050$, N vs. H). However, E_2 administration markedly decreased the level of Cyp2A protein under hyperoxic conditions ($^{d}p = 0.001$, H vs. H+E_2) and was also found significantly lower compared to normoxic group of animals ($^{b}p = 0.003$, N vs. H+E_2; $^{c}p = 0.015$, N+E_2 vs. H+E_2) (Fig. 6b).

Discussion

Hyperoxia presents a useful model for studying ageing, oxidative stress and metabolic disorders. Resistance to hyperoxia is found to be female predominant in adult animals [20]. We found that hyperoxia induced significant sex-related changes in liver oxidative/antioxidative status which were reflected in higher mortality rate of adult male mice [13], and also found that treatment of male mice with E_2 could efficiently boost antioxidative system in the liver [14]. In this study, we have shown that treatment of adult male mice with E_2 increased the survival rate after 48 h of hyperoxia exposure. Moreover, male mice treated with E_2 had lower body mass, compared to their corresponding controls. It is a well known fact that E2 has weight-lowering effects [21]. Namely, weight gain is a common occurrence for women at the time of menopause, characterized by low circulating levels of estrogen. This phenomenon is related with increase in coronary heart disease risk factors observed during this period [22]. This suggests that E_2 may be considered as beneficial since lower body mass accompanies decrease in diabetes and coronary diseases prevalence. Furthermore, we found that E_2 treatment was effective in their protection against oxidative damage of lipids and proteins. Since hyperoxia exposure is associated with changes in lipid metabolism that contributes to oxygen susceptibility and eventually results in liver

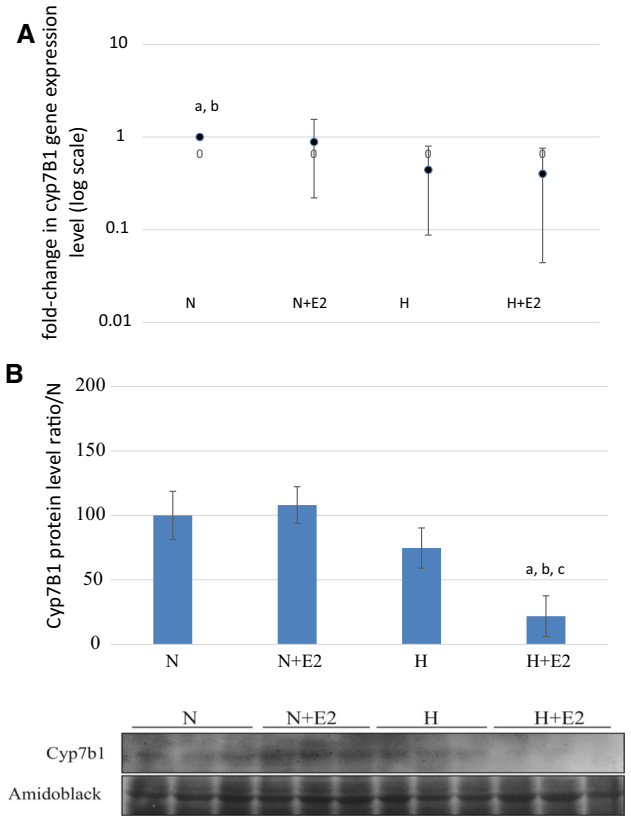

Fig. 5 Effect of E_2 administration on CYP7B1 gene expression in the liver of normoxia- and hyperoxia-exposed male CBA/H mice. The fold-change in gene expression was calculated using the $2^{-\Delta\Delta CT}$ method and β-actin as the endogenous control. The results are presented as fold-change ± SE. $^{a}p = 0.033$, N versus H; $^{b}p = 0.001$, N versus H+E_2 (**a**). Western blot analysis of Cyp7B1 protein level in the liver of normoxia- and hyperoxia-exposed male CBA/H mice. Results are presented as mean ± SD. Amidoblack was used as a loading control. Representative immunoblots are shown. $^{a}p = 0.002$, N versus H+E_2; $^{b}p = 0.001$, N+E_2 versus H+E_2; $^{c}p = 0.016$, H versus H+E_2 (**b**)

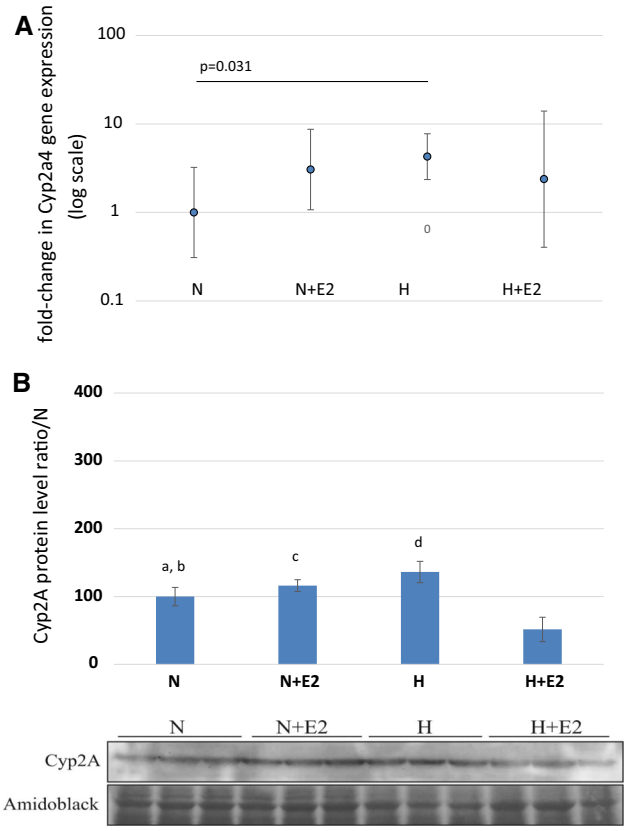

Fig. 6 Effect of E_2 administration on Cyp2A4 gene expression in the liver of normoxia- and hyperoxia-exposed male CBA/H mice. The fold-change in gene expression was calculated using the $2^{-\Delta\Delta CT}$ method and β-actin as the endogenous control. The results are presented as fold-change ± SE. $^{a}p = 0.031$, N versus H (**a**). Western blot analysis of Cyp2A protein level in the liver of normoxia- and hyperoxia-exposed male CBA/H mice. Results are presented as mean ± SD. Amidoblack was used as a loading control. Representative immunoblots are shown. $^{a}p = 0.050$, N versus H; $^{b}p = 0.003$, N versus H+E_2; $^{c}p = 0.015$, N+E_2 versus H+E_2; $^{d}p = 0.001$, H versus H+E_2 (**b**)

pathology, we have investigated the impact of hyperoxia and E_2 treatment on total fatty acid content. Although percentage of total fatty acids remained constant with respect to hyperoxia and/or E_2 administration, we noticed that hyperoxia shifted the ratio of ω-6/ω-3 fatty acids towards proinflammatory profile and E_2 treatment was ineffective in ameliorating this event. This results suggest that hyperoxia may be considered as one of the causing factors for altered lipid metabolism that could finally lead to pathologic conditions and liver disease [23]. Cyp2E1 enzyme plays a major role in fatty acid metabolism. Its activity is usually increased in various pathophysiological states linked with altered lipid metabolism such as diabetes [24] increased caloric intake [25] and in ketosis during excessive fat consumption [26]. Although Cyp2E1 is usually linked with increased ROS production [27], there are several evidences of protective role of Cyp2E1. Furthermore, Cyp2E1 participates in the depletion of lipid peroxidation substrates, and is found to have protective effect on lipid oxidative damage by decreasing amount of lipid peroxidation substrates [28]. We have previously found that the level of hepatic cyp2E1 mRNA expression was higher in hyperoxia-exposed adult female CBA/H mice in comparison to males, and this increase was in association with their lower level of MDA [29]. In our present study, we observed that E_2 treatment of normoxic male mice lead to increase of cyp2E1 mRNA, which was not in correlation with the expression of Cyp2E1 protein. In addition, E_2 had no effect on Cyp2E1 expression level in hyperoxia. The observed discrepancies can be explained with a complex regulation of Cyp2E1 that involves stabilization of mRNA, in addition to RNA expression [30], and protein stabilization with the substrate [31], but further

investigations are needed to explain this mechanism in more detail.

Male-predominant isoform Cyp7B1, in addition to bile acids synthesis, is responsible for the aromatization of sex hormone intermediates, which gives this Cyp isoform an important role in the maintenance of masculine properties [32]. The results of the present study revealed that E_2 administration in normoxic conditions did not result with significant changes in the expression of Cyp7B1. Li-Hawkins et al. [7] have found that E_2 treatment lead to increase of hepatic Cyp7B1 protein in male mice, but these mice received significantly higher concentration of E_2 than concentration used in our study. Hyperoxic exposure induced marked downregulation of Cyp7B1, which represents a male-to-female shift in the expression of this isoform. Even more, in E_2-treated males exposed to hyperoxia, the expression of the Cyp7B1 protein was downregulated to a level of no detection. Similar male-to-female shift in the expression pattern of this gene has been observed in lifespan-promoting conditions, such as in long-lived Ames dwarf mice [33] and in the regime that is known to increase the lifespan in various model organisms [34]. This finding suggests that combination of E_2 treatment and hyperoxia could be used as a beneficial agent in amelioration of age-related diseases and potential elongation of lifespan.

Expression of the female-predominant isoform Cyp2A4 in males was increased after hyperoxia, which also represents the shift towards female phenotype. In normoxic conditions, E_2 administration did not cause significant increase in the expression of Cyp2A, probably due to small sample size, although such trend was noticed. However, in E_2-treated group exposed to hyperoxia, the expression of Cyp2A protein was downregulated in similar manner as observed for Cyp7B1 protein. Other studies also suggested partial feminization of males as one of the protective mechanisms in response to stress in other experimental models [35]. According to some authors [36], shift to feminine gene expression may be responsible for the lifespan extension in conditions of CR. Experiments performed on *C. elegans* confirmed that short-term hyperoxia promoted lifespan [37]. Although mice in our model were subjected to sublethal exposure to hyperoxia, interestingly, CYP expression pattern was similar to that noticed in lifespan-promoting conditions. However, due to excessive exposure to hyperoxia, mice were probably unable to achieve sufficient level of protection. The novelty of this study is the result which suggests that E_2 achieves its protective role by modulating sex-specific Cyp genes towards protective, female-specific pattern of expression against hyperoxia. However, more studies are needed to find possible strategies to achieve efficient level of protection against oxidative stress, in order to retard the ageing process and minimizing deleterious side effects of E_2 administration.

Conclusion

Our study showed that hyperoxia induced hepatic oxidative damage of lipids, proteins and shifted omega-6/omega-3 ratio towards proinflammatory state in male CBA/H mice. E_2 administration protected against hyperoxia by increasing survival and lowering oxidative damage. Hyperoxia induced male-to-female shift in the expression of male-predominant Cyp7B1 and female-predominant Cyp2A4. Combined effect of hyperoxia and E_2 induced additional downregulation in male-predominant Cyp7B1, and unexpectedly, the female-predominant Cyp2A4. Although exact reason for the observed pattern of Cyp2A4 upon combined effect of hyperoxia and E_2 is unknown, the interesting fact is that E_2 has the opposite effect on the expression pattern of these Cyps in hyperoxia, in comparison to physiological conditions. The observed feminization of male-specific Cyps with E_2 administration under the conditions of hyperoxia may be a part of males' attempt in activation of adaptive response to hyperoxia, which may eventually lead to their longer lifespan.

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Compliance with ethical standards

Conflicts of interest No conflicts of interest declared.

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