

# The effect of 17 $\beta$ -estradiol on the expression of dipeptidyl peptidase III and heme oxygenase 1 in liver of CBA/H mice

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Received: 7 July 2014 / Accepted: 19 November 2014 / Published online: 29 November 2014  
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## Abstract

**Background** 17 $\beta$ -estradiol (E<sub>2</sub>) has well-established cardioprotective, antioxidant and neuroprotective role, and exerts a vast range of biological effects in both sexes. Dipeptidyl peptidase III (DPP III) is protease involved as activator in Keap1–Nrf2 signalling pathway, which is important in cellular defense to oxidative and electrophilic stress. It is generally accepted that oxidative stress is crucial in promoting liver diseases.

**Objective** To examine the effect of E<sub>2</sub> on the expression of DPP III and haeme oxygenase 1 (HO-1) in liver of adult CBA/H mice of both sexes.

**Methods** Gene and protein expressions of studied enzymes were determined by quantitative real-time PCR and Western blot analysis. Immunohistochemistry was performed to analyse the localization of both proteins in different liver cell types.

**Results** Ovariectomy diminished expression of DPP III and HO-1 proteins. E<sub>2</sub> administration abolished this effect, and even increased these proteins above the control. A

significant enhancement in DPP III protein was found in E<sub>2</sub>-treated males, as well. A decrease in the expression of HO-1, but not of the DPP III gene, was detected in the liver of ovariectomized females. HO-1 protein was found localized in the pericentral areas of hepatic lobules (Kupffer cells and hepatocytes), whilst DPP III showed a uniform distribution within hepatic tissue.

**Conclusions** We demonstrate for the first time that E<sub>2</sub> influences the protein level of DPP III in vivo, and confirm earlier finding on HO-1 gene upregulation by 17 $\beta$ -estradiol. These results additionally confer new insights into complexity of protective action of E<sub>2</sub>.

**Keywords** Antioxidant enzyme · Gene and protein expression · Ovariectomy · Sex-related · Steroid hormone · Zinc peptidase · Immunohistochemistry

## Introduction

The level of oxidative damage in an organism is tightly related to development of several age-related diseases, such as cancer, neurodegenerative diseases and diabetes [1]. The resistance to oxidative damage appears to be sex related, with females being more resistant to oxidative damage [2]. Females show lower incidence of some age-related pathologies linked with oxidative stress and this sex difference disappears after menopause, which led to conclusion that this protection is attributed to sex hormones. 17- $\beta$  estradiol (E<sub>2</sub>) plays a key role in development and maintenance of normal sexual and reproductive function, and exerts a vast range of biological effects in the cardiovascular, immune and central nervous systems in both females and males [3]. E<sub>2</sub> is a powerful endogenous antioxidant that is able to reduce lipid peroxidation in liver and blood [4]. Its deficiency leads to

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increased vascular reactive oxygen species (ROS) production resulting in endothelial dysfunction, whilst  $E_2$  replacement therapy can prevent these pathological changes [5]. It was shown that at physiological concentrations  $E_2$  does not act as a chemical antioxidant per se, due to its phenolic structure, but through mechanisms that involve oestrogen receptors and tyrosine kinase signalling through MAP kinase and NF $\kappa$ B [6]. Consequently, several antioxidant enzymes, including manganese superoxide dismutase (MnSOD) and HO-1, were shown to be up-regulated in MCF-7 cells [6]. Increased expression of HO-1 and several other antioxidant enzymes induced in myocardial cells by  $E_2$  treatment was explained by increased nuclear translocation of transcription factor Nrf2 [7]. HO is the rate-limiting enzyme for haeme degradation in mammals. It is a stress-response enzyme which is highly induced by variety of agents causing oxidative stress, hypoxia, hyperoxia, proinflammatory cytokines [8], and as such regarded as sensitive and reliable indicator of cellular oxidative stress. In comparison to the constitutively expressed HO-2, it is presumed that inducible isoform HO-1 makes greater contribution to the maintenance of oxidant/antioxidant homeostasis during changes in cellular environments. In response to oxidative stress, HO-1 induction provides cell protection by promoting the catabolism of pro-oxidant metalloporphyrins to bile pigments (biliverdin and bilirubin) which are considered to have free radical scavenging properties [9]. As such, HO-1 acts as a potent antioxidant. Transcriptional control of the expression of HO-1 is mediated through the antioxidant response element (ARE), a transcriptional regulatory element located in the upstream regulatory region of many phase II enzymes that play important protective role against oxidative toxicity [10]. ARE is activated via translocation of Nrf2 transcription factor into nucleus upon oxidative stress and other stimuli. Keap1 is a substrate adaptor protein for the Cul3–Rbx1 E3 ubiquitin ligase complex that represses Nrf2 by targeting it for proteasomal degradation [11]. Most recently, it was demonstrated that cytosolic protease dipeptidyl peptidase III (DPP III) binds Keap1 to displace Nrf2, thus inhibiting Nrf2 ubiquitination and driving Nrf2-dependent transcription [12]. DPP III is a monozinc exopeptidase that hydrolyses dipeptides from the N-terminal of its substrates consisting of three or more amino acids [13]. In mammalian tissues, it is broadly distributed and thought to contribute in the final steps of normal intracellular protein catabolism [14]. There are strong indications of its role in the endogenous pain-modulation system [15] as well as in the endogenous defense against oxidative stress [16]. Pathophysiological roles of human DPP III are indicated in cataractogenesis [17] and malignant growth [18]. Recent studies showed that DPP III is a member of a six-gene signature that accurately predicts human breast cancer patient survival [19]. All these findings characterize human DPP III as a valuable drug target.

Although the effect of  $E_2$  on HO-1 expression and its actions as an antioxidant have been well documented [6, 7], currently there are no data about the *in vivo* influence of  $E_2$  on sex-related HO-1 expression. Moreover, it is not known whether  $E_2$  influences DPP III. The liver was chosen, because of its high susceptibility to oxidative stress due to high metabolic activity. In addition, Keap1–Nrf2 pathway is indicated to counteract liver diseases. Therefore, the aim of this study was to examine the effect of  $E_2$  on the expression of DPP III, the protease involved in Nrf2–Keap1 signaling pathway—the main pathway responsible for cell defense against oxidative stress [11], and to associate it with the expression of known antioxidant enzyme HO-1 in the liver of adult CBA/H mice of both sexes.

## 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 European Community Council Directive of November 24, 1986 (86/609/EEC). Male and female CBA/H mice aged 4 months from 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;  $22 \pm 2$  °C room temperature; access to food pellets, and tap water *ad libitum*. The experimental groups were as follows: male control (cm), male control treated with  $E_2$  (cme), female sham (cf), ovariectomized (ovx), ovariectomized and treated with  $E_2$  (ovxe) mice. The number of animals was three per each experimental group.

### Ovariectomy procedure and $E_2$ administration

Ovariectomy and sham surgery were performed under ketamine/xilazyme anaesthesia. Since low levels of  $E_2$  are normally detected in ovariectomized females due to other endogenous  $E_2$  sources [20], plasma  $E_2$  levels were not used as indicator of efficiency of ovariectomy. Instead, the success of ovariectomy was checked by analysing vaginal smear during five consecutive days after the surgery (data not shown). In our preliminary experiments, we established that anestrus phase seen on vaginal smear is reflected by uterus atrophy as evaluated post-mortem. Body weight gain was also used as a marker of successful ovariectomy, since ovariectomy-induced body weight gain is established phenomena. For  $E_2$  administration, a pellet containing  $E_2$  (50  $\mu$ g, Innovative Research of America, Sarasota, FL) was placed into the interscapular subcutaneous space releasing a constant dose of 830 ng daily. The procedure was the

**Table 1** Probes used for quantitative real-time PCR analysis

Gene	Assay ID	Product size (bp)
$\beta$ -actin	Mm00607939_s1	115
Dipeptidyl peptidase III	Mm00505462_m1	89
Haeme oxygenase 1	Mm00516007_m1	92

same for both females and males. After 37 days, animals were subjected to experimental protocols.

#### RNA isolation and quantitative real-time PCR analysis

Total RNA was extracted from individual mouse livers in each group 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 [37], to quantify relative mRNA expression of DPP III and HO-1. Using the  $2^{-\Delta\Delta C_t}$  method, data are presented as the fold-change in DPP III and HO-1 gene expression normalized to endogenous reference gene ( $\beta$ -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 in refrigerated centrifuge. Supernatant was collected and total cellular proteins (100  $\mu$ g per lane) were resolved by denaturing SDS-PAGE, transferred onto a PVDF membrane (Bio-Rad, Hercules, CA). Membranes were blocked in 5 % nonfat dry milk in TN buffer (50 mM TRIS, 150 mM NaCl, pH = 7.4) overnight, incubated with primary polyclonal rabbit antibody against DPP III (antiserum diluted 1:200 and incubated 3 h at room temperature) or with primary polyclonal rabbit anti-mouse HO-1 antibody (Abcam, Cambridge, UK, diluted 1:200 and incubated 18 h at +4 °C), followed by incubation with donkey anti-rabbit IgG, horseradish peroxidase-conjugated, secondary antibody (Amersham Biosciences Inc., USA) for 3 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 [21]. 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. A high-titre anti-DPP III polyclonal antibody was custom prepared by Abcore, USA, using purified recombinant human DPP III as antigen [22], injected into New Zealand white rabbits, 3- to 9-month

old. Rabbits were bled from the auricular artery, blood was allowed to coagulate, clot was removed and serum was collected, clarified by centrifugation and stored at –20 °C.

#### Immunohistochemistry

After perfusion, tissue was fixed by immersion in 4 % paraformaldehyde in 0.1 M PBS (pH 7.4), then tissue blocks were embedded in paraffin, sectioned (12  $\mu$ m) and deparaffinized through a graded series of xylol and alcohols. Sections were processed with immunohistochemistry as previously described [23], with minor modifications. In brief, after incubation in 0.3 % H<sub>2</sub>O<sub>2</sub> in PBS and blocking in 5 % normal goat serum to prevent nonspecific background staining, rabbit anti-Haeme Oxygenase 1 (HO-1) monoclonal antibody (Abcam, UK) or anti-DPP III polyclonal antibody (custom made by Abcore, USA) was diluted 1:200 and incubated overnight at 4 °C. Biotinylated anti-rabbit antibody from Vectastain ABC kit (Vector Laboratories Inc) was used according to the manufacturer's protocol. For visualization of peroxidase activity, 3,3'-diaminobenzidine tetrahydrochloride (DAB) with metal enhancer (CoCl<sub>2</sub>, Sigma-Aldrich) was used to produce intense gray staining. Sections were rinsed, air dried, dehydrated and coverslipped with Histamount (National Diagnostic). Negative controls were included in all immunohistochemical experiments by replacing the primary antibody with blocking solution, or 5 % normal goat serum. No immunolabeling was detected in control sections. Tissue sections were photographed by slide scanner NanoZoomer 2.0 (Hamamatsu) and image assembled in CorelDRAW(R)—Version 11.0.

#### Statistical analysis

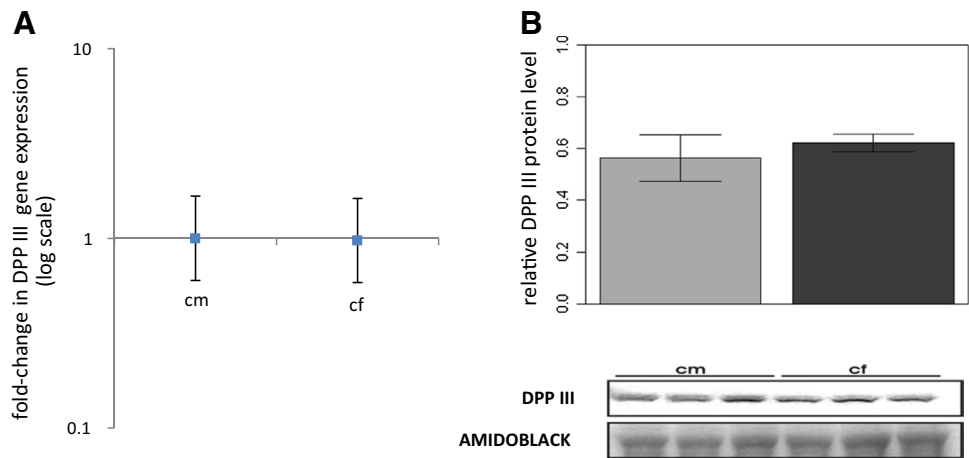
Statistical analyses of data were performed using R v2.15.3 (CRAN, <http://cran.r-project.org>) and RStudio for Windows, v 0.97 (<http://www.rstudio.com/>). For the analysis of real-time PCR data, all groups were tested for normality of distribution using Shapiro–Wilk test. The differences between multiple groups were compared with Kruskal–Wallis non-parametric ANOVA, followed by Wilcoxon signed-rank test for testing differences between two related groups. For all tests significance level was set at  $p < 0.05$ .

## Results

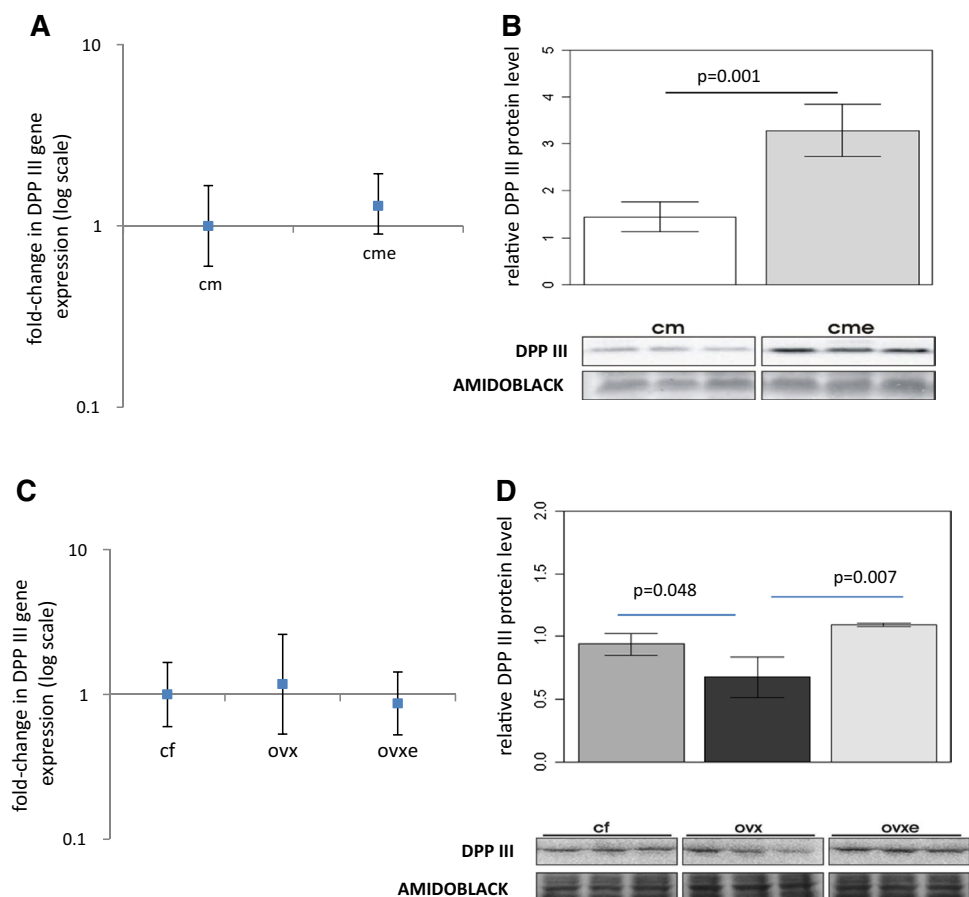
#### DPP III gene and protein expression in the liver of CBA/H mice

To establish whether sex-related differences in the basal expression of hepatic DPP III exist, we measured DPP

**Fig. 1** DPP III expression in liver of control CBA/H mice. Real-time PCR analysis of DPP III gene expression in liver of control male (cm) and female (cf) CBA/H mice. Fold-change in gene expression was calculated with  $2^{-\Delta\Delta CT}$  method, using  $\beta$ -actin as endogenous control (a). Western Blot analysis of DPP III protein expression in liver of control male and female CBA/H mice. Bands are normalized using AmidoBlack (b). All data are mean  $\pm$  SD from three individual mice per group



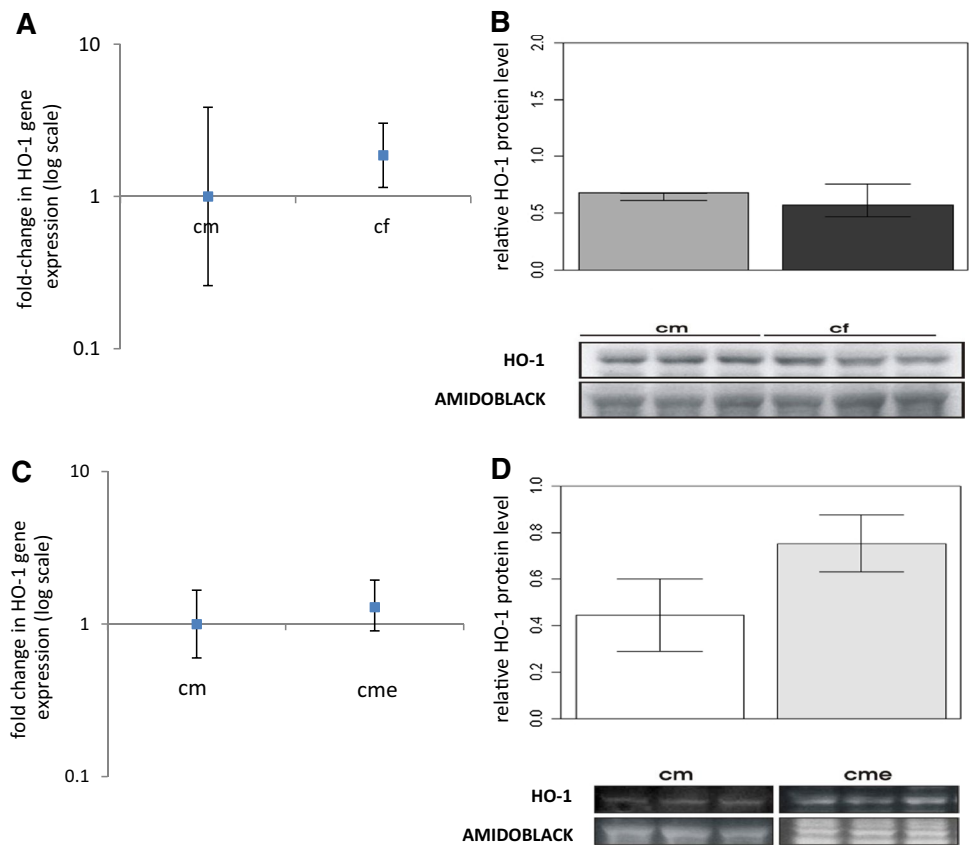
**Fig. 2** Effect of  $E_2$  on DPP III expression in liver of CBA/H mice. Real-time PCR analysis of DPP III gene expression in liver of control (cm) and  $E_2$ -treated male (cme) CBA/H mice (a), and sham (cf), ovariectomized (ovx) and ovariectomized females treated with  $E_2$  (ovxe) (c). Fold-change in gene expression was calculated with  $2^{-\Delta\Delta CT}$  method, using  $\beta$ -actin as endogenous control. Western Blot analysis of DPP III protein level in liver of control and  $E_2$ -treated male CBA/H mice (b:  $p = 0.001$  control vs.  $E_2$ -treated group) and sham, ovariectomized, and ovariectomized females treated with  $E_2$  (d:  $p = 0.048$  control vs. ovariectomized;  $p = 0.007$  ovariectomized vs. ovariectomized treated with  $E_2$ ). Bands are normalized using AmidoBlack. All data are mean  $\pm$  SD from three individual mice per group



III gene and protein expression in intact male and female mice. Both gene and protein expression levels of DPP III were found to be unchanged between sexes, as revealed by real-time PCR and Western blot analysis (Fig. 1a, b). Next, we wanted to determine whether DPP III gene and protein expression were influenced by  $E_2$  in males, who basically have low levels of this hormone. Real-time PCR analysis showed no significant effect of  $E_2$  on DPP III gene

expression in cme, compared to cm group (Fig. 2a). However, there was a marked increase in DPP III protein level in cme, compared to cm group ( $p = 0.001$ ) (Fig. 2b). We then investigated the level of DPP III gene expression in the liver of cf, ovx and ovxe animal group. The transcript level remained unchanged across all experimental groups (Fig. 2c). However, Western Blot analysis showed that ovariectomy decreased DPP III protein level ( $p = 0.048$  for

**Fig. 3** HO-1 expression in liver of CBA/H mice. Real-time PCR analysis of HO-1 gene expression in liver of control male (cm) and female (cf) CBA/H mice (a), and in control (cm) and E<sub>2</sub>-treated male (cme) CBA/H mice (c). Fold-change in gene expression was calculated with 2<sup>-ΔΔCT</sup> method, using β-actin as endogenous control. Western Blot analysis of HO-1 protein expression in liver of control male (cm) and female (cf) CBA/H mice (b), and control male (cm) and E<sub>2</sub>-treated male (cme) CBA/H mice (d). Bands are normalized using AmidoBlack. All data are mean ± SD from three individual mice per group



ovariectomized vs. control females), whilst E<sub>2</sub> administration abolished the effect of ovariectomy and increased DPP III expression even higher than control level ( $p = 0.007$  for ovx vs. ovxe) (Fig. 2b).

### HO-1 gene and protein expression in the liver of CBA/H mice

The expression level of protective enzyme HO-1 in intact mice of both sexes was determined, since there are no such data for CBA/H strain of mice. There was no change in the hepatic expression of HO-1 between control animals of both sexes, as revealed by real-time PCR and Western Blot analysis (Fig. 3a, b). Similarly to DPP III, we wanted to determine whether HO-1 gene and protein expression were influenced by E<sub>2</sub> in males. No change of expression in HO-1 mRNA and protein level between cm and cme group was found (Fig. 3c, d). However, real-time PCR data analysis showed significant decrease in HO-1 gene expression fold-change ( $fc = -2.85$ ,  $p < 0.001$ ) in liver of ovx group compared to both cf and ovxe group (Fig. 4a). Western Blot has shown that ovariectomy decreased HO-1 protein level ( $p = 0.042$  for ovx vs. cf), whilst E<sub>2</sub> abolished the effect of ovariectomy and increased HO-1 expression compared with cf ( $p = 0.002$  ovx vs. ovxe) (Fig. 4b).

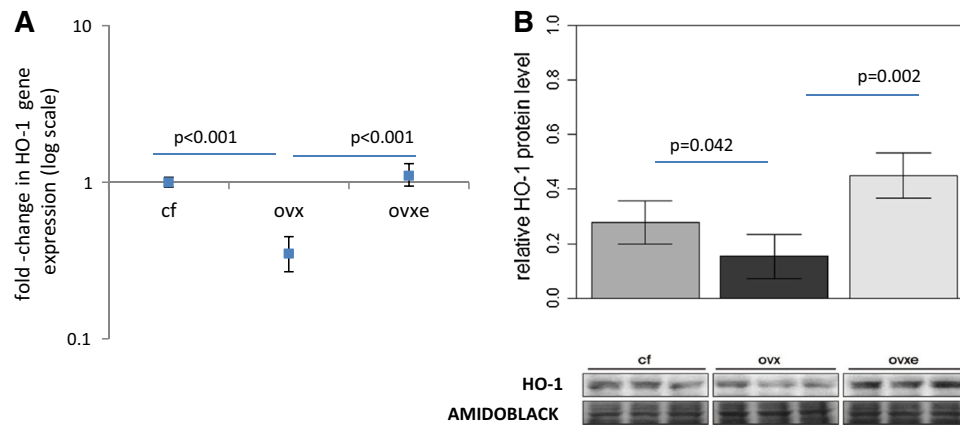
### Immunohistochemical analysis of DPP III and HO-1 localization in mouse liver

Immunohistochemical analysis revealed that in the liver the expression of HO-1 was more pronounced in pericentral areas of hepatic lobules, and particularly in Kupffer cells, whilst DPP III protein was found to be expressed in hepatocytes and uniformly distributed throughout the hepatic tissue. Considering intracellular distribution, HO-1 was found more prominent in the cytoplasm and membranes of organelles, whilst strong DPP III immunoreactivity was detected, in addition to the cytoplasm, in nuclei and plasma membranes (Fig. 5).

### Discussion

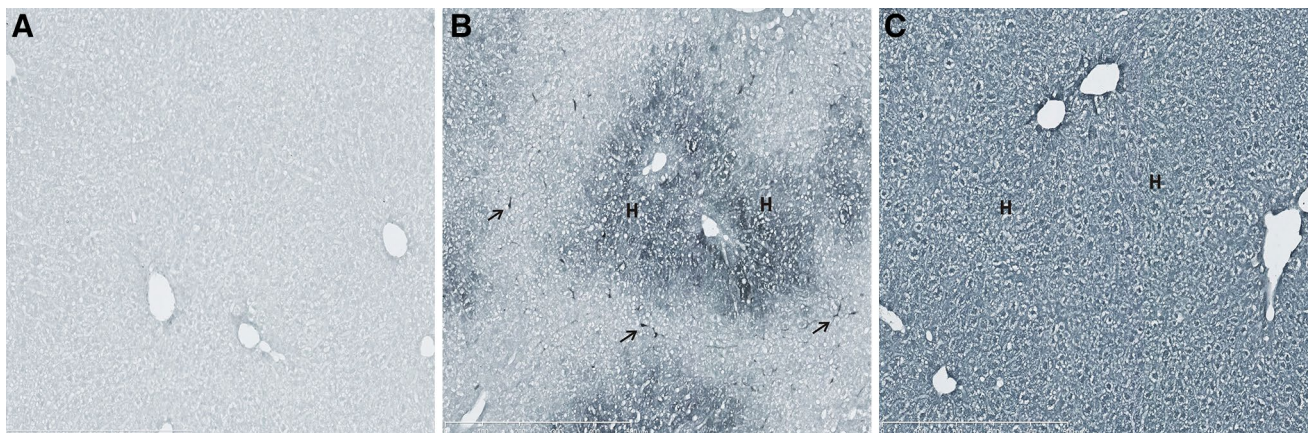
In this study the effect of E<sub>2</sub> on DPP III and HO-1 gene and protein expression level in the liver of 4 month-old male and female CBA/H mice was investigated. The liver is particularly susceptible to oxidative stress, and E<sub>2</sub>, DPP III and HO-1 contribute to cellular antioxidant defense. Reactive oxygen species (ROS) are produced in the liver as byproducts of normal metabolism and detoxification [24]. Hepatic proteins, lipids and DNA are primarily affected by excessive and sustained ROS, which results in structural





**Fig. 4** Effect of  $E_2$  on HO-1 expression in liver of CBA/H mice. Real-time PCR analysis of HO-1 gene expression in liver of sham (cf), ovariectomized (ovx) and ovariectomized treated with  $E_2$  (ovxe);  $p < 0.001$  cf vs. ovx and ovx vs. ovxe. Fold-change in gene expression was calculated with  $2^{-\Delta\Delta CT}$  method, using  $\beta$ -actin as endogenous control (**a**). Western Blot analysis of HO-1 protein expres-

sion in liver of sham (cf), ovariectomized (ovx) and ovariectomized females treated with  $E_2$  (ovxe);  $p = 0.042$  control vs. ovariectomized;  $p = 0.002$  ovariectomized vs. ovariectomized treated with  $E_2$  (**b**). Bands are normalized using AmidoBlack. All data are mean  $\pm$  SD from three individual mice per group



**Fig. 5** Immunohistochemical localization of HO-1 and DPP III protein in mouse liver. **a** Negative control (liver tissue not incubated with primary antibody). **b** HO-1 immunoreactivity is more pronounced in

pericentral areas, inside Kupffer cells (arrows), and in hepatocytes (H). **c** DPP III immunoreactivity is localized in hepatocytes (H) and uniformly distributed throughout the hepatic tissue

and functional abnormalities. The pathogenesis of the oxidative damage involves each hepatic cell type (i.e., hepatocytes and stellate cells, endothelial and Kupffer cells) [25]. It is generally accepted that oxidative stress plays a crucial role in promoting the progression of chronic liver diseases and hepatocellular carcinoma. The DPP III is involved as activator in Keap1–Nrf2–ARE signaling pathway. The protective roles of Nrf2 activation in the pathogenesis of liver diseases have been intensively investigated. In the animal model, Keap1–Nrf2–ARE pathway has been demonstrated to counteract non-alcoholic and alcoholic liver diseases, fibrosis and cancer, and to support liver regeneration [27]. Several Keap1–Nrf2-activating drugs and molecules derived from plants (e.g. sulforaphane) have entered

clinical trials for several disease processes, in which oxidative stress and inflammation play a crucial role [28].

Our present research has shown that ovariectomy markedly lowered the level of both DPP III and HO-1 proteins in liver tissue, and that  $E_2$  administration abolished this effect and increased DPP III and HO-1 protein expression in both sexes. Using immunohistochemistry, we identified that HO-1 localizes in the pericentral areas of hepatic lobules, particularly in Kupffer cells, which is in accordance to previous studies [26]. In our knowledge, the present study is the first to have analysed the immunohistochemical localization of DPP III in the mouse liver. In contrast to HO-1 protein, we have found a uniform expression of DPP III within hepatic tissue, with a more intense signal in

nuclei and membranes of hepatocytes. Interestingly, human DPP III protein is localized in the cytoplasm, plasma membranes and nuclei of cells (<http://www.proteinatlas.org/>).

Our present data clearly show for the first time that E<sub>2</sub> influences the level of DPP III protein in mice of both sexes. It seems that E<sub>2</sub> affects DPP III at a post-transcriptional level. This is in accordance with up to date knowledge on DPP III gene expression, since no oestrogen response element was recognized in its gene promoter, and transcription factors Ets-1/Elk-1 and C/EBP-β have been experimentally confirmed as relevant for human DPP III gene regulation [29, 30]. Human and mouse DPP III share 93 % of amino acid sequence identity. Mouse DPP III (protein) has not been characterized yet biochemically, whilst human and rat orthologs have been the subject of intensive investigation [13, 14, 29–31]. The increased level of DPP III protein observed in this study as a result of E<sub>2</sub> action in vivo could be related to an imbalance between protein synthesis and degradation, i.e. it could be due to a decreased proteolytic degradation in E<sub>2</sub>-treated animals. It is known that E<sub>2</sub> regulates several mammalian peptidases, such as neprilysin, cathepsin D and ubiquitin-specific peptidase 19 (USP 19) [32, 33]. Ogawa and colleagues have shown that E<sub>2</sub> increases the expression of USP 19 in mouse myoblasts and satellite cells, both in vitro and in vivo [34]. As USP 19 does not associate with proteasome, it may release ubiquitin from specific proteins and may have regulatory function, since removal of ubiquitin chain from specific substrate proteins inhibits their degradation by proteasome. At this point, it is not known whether the DPP III is amongst substrates of USP 19 or some other deubiquitinating enzyme. However, several ubiquitination sites can be predicted in DPP III molecule, and experimental evidence on human DPP III ubiquitination has been reported [35].

Considering that males have lower serum E<sub>2</sub> levels compared to intact females, and our finding that E<sub>2</sub> administration increases the DPP III protein content, the observed lack of difference in protein DPP III expression in intact male and female mice is intriguing. This indicates that in vivo the regulation of DPP III protein is rather complex and that the existence of other factors (which are able to up-regulate DPP III) can be postulated. We can speculate that in male mice, androgens could up-regulate DPP III protein. However, up to date there are no data about the effect of testosterone or any other androgenic hormone on the regulation of DPP III.

Our results on the increased expression of HO-1 gene are in agreement with findings of Yu et al. [7] about E<sub>2</sub> effects of transcription factor Nrf2 on myocardial cells. These authors have shown a concomitant upregulation of the transcription factor Nrf2 in nuclear extracts. Moreover, since HO-1 is one of the phase II enzymes with ARE in its promoter, and DPP III was shown to compete with Nrf2 for

binding to the same site on cytosolic repressor Keap1, our finding of an oestrogen-induced increase in HO-1 mRNA and protein content is consistent with the notion that enhanced DPP III protein releases Nrf2 from its repressor and thereby augments the transcription of responsive genes, such as HO-1. E<sub>2</sub> has well-established cytoprotective effect during oxidative stress and its depletion contributes to the pathogenesis of some age-related diseases [36]. We have previously demonstrated that the resistance to hyperoxia-induced oxidative stress appears to be female biased [37, 38] and that E<sub>2</sub> deficiency caused an increase in oxidative damage in females exposed to hyperoxia, whilst E<sub>2</sub> administration abolished this effect. Moreover, E<sub>2</sub> treatment significantly decreased oxidative damage in males [39]. These results strongly suggested a beneficial effect of E<sub>2</sub> in resistance to oxidative stress. Data from our present study clearly show that protein levels of two enzymes involved in endogenous response to oxidative stress, DPP III and HO-1, are increased upon administration of E<sub>2</sub> in mice.

Although gender-specific differences in hepatic HO expression and activity have been shown in previous studies [40] here we demonstrate for the first time that E<sub>2</sub> influences the protein level of DPP III in vivo. These results additionally confer new insights into the complexity of the protective action of this hormone, and in its relationship to Keap1–Nrf2–ARE pathway, which contains molecular targets potentially able to prevent and treat liver diseases.

**Acknowledgments** The authors would like to thank Iva Pešun Međimorec for her excellent technical contribution in performing surgical procedures of ovariectomy and Ana Jagust for her excellent technical assistance in performing immunohistochemical analyses. This research is funded by Croatian Ministry of Science, Education and Sports, Grant No. 098-0982464-1647, Grant No. 098-1191344-2938 and IBRO 2013 RHP.

**Conflict of interest** The authors declare no conflict of interest or any financial interest.

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