Journal of Endocrinological Investigation

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

--Manuscript Draft--

Manuscript Number:	JENI-D-14-00227R2
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	Journal of Endocrinological Investigation Editorial Office att. Dr. LUIGI BARTALENA, Editor-in-chief Varese Italy July 7th, 2014 Dear Editors, We hereby submit the revised manuscript entitled „The effect of 17β-estradiol on the expression of dipeptidyl peptidase III and heme oxygenase 1 in liver of CBA/H mice" by Željka Mačak Šafranko, Sandra Sobočanec, Ana Šarić, Nina Jajčanin Jozić, Željka Krsnik, Gorana Aralica, Tihomir Balog and Marija Abramić. We kindly ask you to consider this revised manuscript for publication in Journal of Endocrinological Investigation. Sincerely, Sandra Sobočanec, PhD, Corresponding Author
Response to Reviewers:	ANSWERS TO REVIEWERS' COMMENTS REVIEWER #1: We have corrected all the grammar and syntax errors throughout the Manuscript, according to the Reviewer's suggestions. Since there were many of the typographic errors and places where we had to omit/change/erase some words, we did not find relevant to specifically mark them, as they were only single letters or parts of the letters. This is the reason why Manuscript is without any underlined word/part of the sentence. We hope that the Reviewer will find this appropriate for the revised version of the Manuscript. But if the Reviewer wants us to mark all typo changes in the Manuscript, please let me know. Some of the "major" changes are also corrected, such as conclusion section of the abstract, and modification of the sentences regarding immunohistochemistry, according to the reviewer's suggestion. Also, the fold-change instead of f.c. is now on y axis of all the graphs. Please let me know if the reviewer is not able to see the corrected axis. Also, all the abbreviations are now next to each experimental group. Thank you very much in advance, Dr. Sandra Sobocanec
Suggested Reviewers:	Vito Turk, Professor Jožef Štefan Institute Vito.Turk@ijs.si Professor Vito Turk is the expert in this field of research.
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The effect of 17β-estradiol on the expression of dipeptidyl peptidase III and heme oxygenase 1 in liver of CBA/H mice

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Running title: E_2 upregulates DPP III protein in mice liver

Abstract

Background 17β-estradiol (E_2) 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 signaling 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_2 on the expression of DPP III and heme oxygenase 1 (HO-1) in liver of adult CBA/H mice of both sexes.

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

Results Ovariectomy diminished expression of DPP III and HO-1 proteins. E_2 administration abolished this effect, and even increased these proteins above the control. A significant enhancement in DPP III protein was found in E_2-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 localised in the pericentral areas of hepatic lobules (Kupffer cells and hepatocytes), while DPP III showed a uniform distribution within hepatic tissue.

Conclusions We demonstrate for the first time that E_2 influences the protein level of DPP III *in vivo*, and confirm earlier finding on HO-1 gene upregulation by17β-estradiol. These results additionally confer new insights into complexity of protective action of E_2.

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-β estradiol (E_2) 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_2 is a powerful endogenous antioxidant that is able to reduce lipid peroxidation in liver and blood [4]. Its deficiency leads to increased vascular reactive oxygen species (ROS) production resulting in endothelial dysfunction, while E_2 replacement therapy can prevent these pathological changes [5]. It was shown that at physiological concentrations E_2 does not act as an chemical antioxidant *per se*, due to its phenolic structure, but through mechanisms that involve oestrogen receptors and tyrosine kinase signalling through MAP kinase and NFκB [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 heme 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 hydrolyzes 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±2^{o}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/xilazyne anesthesia. 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 5 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 μg, 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 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 [38], to quantify relative mRNA expression of DPP III and HO-1. Using the $2^{-\Delta\Delta Ct}$ method, data are presented as the fold-change in DPP III and HO-1 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.

Table 1 here

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 (1300×g). After sonification (3 x 30sec), whole liver homogenates were centrifuged at 16 000g for 20 min in refrigerated centrifuge. Supernatant was collected and total cellular proteins (100 μg 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 (50mM TRIS, 150mM NaCl, pH=7.4) overnight, incubated with primary polyclonal rabbit antibody against DPP III (antiserum diluted 1:200 and incubated 3 hours at room temperature) or with primary polyclonal rabbit anti-mouse HO-1 antibody (Abcam, Cambridge, UK, diluted 1:200 and incubated 18 hours at +4°C), followed by incubation with donkey anti-rabbit IgG, horseradish

peroxidase-conjugated, secondary antibody (Amersham Biosciences Inc., USA) for 3 hours 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 titer 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, 3-9 months 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 (12µm) and deparaffinised 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_2O_2 in PBS and blocking in 5% normal goat serum to prevent nonspecific background staining, rabbit anti-Heme 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 visualisation of peroxidase activity, 3,3'-diaminobenzidine tetrahydrochloride (DAB) with metal enhancer ($CoCl_2$, 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 Kruskall-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

In order to establish whether sex-related differences in the basal expression of hepatic DPP III exist, we measured DPP 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, 1B).** 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 (**Figure 2A**). However, there was a marked increase in DPP III protein level in cme, compared to cm group ($p=0.001$) (**Figure 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 (**Figure 2C**). However, western blot analysis showed that ovariectomy decreased DPP III protein level ($p=0.048$ for ovariectomized vs. control females), while E_2

administration abolished the effect of ovariectomy and increased DPP III expression even higher than control level (p=0.007 for ovx vs. ovxe) (**Figure 2D).**

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, 3B)**. Similarly to DPP III, we wanted to determine whether HO-1 gene and protein expression were influenced by E_2 in males. No change of expression in HO-1 mRNA and protein level between cm and cme group was found **(Fig. 3C, 3D)**. 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 (**Figure 4A**). Western blot has shown that ovariectomy decreased HO-1 protein level (p=0.042 for ovx vs. cf), while E_2 abolished the effect of ovariectomy and increased HO-1 expression compared with cf (p=0.002 ovx vs. ovxe) (**Figure 4B).**

Immunohistochemical analysis of DPP III and HO-1 localisation 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, while 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, while strong DPP III immunoreactivity was detected, in addition to the cytoplasm, in nuclei and plasma membranes **(Figure 5).**

Discussion

In this study the effect of E_2 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_2, 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 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. By using immunohistochemistry, we identified that HO-1 localises in the pericentral areas of hepatic lobules, praticularly 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 localisation 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 localised in the cytoplasm, plasma membranes and nuclei of cells (http://www.proteinatlas.org/).

Our present data clearly show for the first time that E_2 influences the level of DPP III protein in mice of both sexes. It seems that E_2 affects DPP III at a post-transcriptional level. This is in accordance with up to date knowledge on DPP III gene expression, since no estrogen 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, while 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_2 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_2-treated animals. It is known that E_2 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_2 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 among 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 [36].

Considering that males have lower serum E_2 levels compared to intact females, and our finding that E_2 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_2 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 estrogen-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_2 has well-established cytoprotective effect during oxidative stress and its depletion contributes to the pathogenesis of some age-related diseases [37]. We have previously demonstrated that the resistance to hyperoxia-induced oxidative stress appears to be female-biased [38, 39] and that E_2 deficiency caused an increase in oxidative damage in females exposed to hyperoxia, while E_2 administration abolished this effect. Moreover, E_2 treatment significantly decreased oxidative damage in males [40]. These results strongly suggested a beneficial effect of E_2 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_2 in mice.

Although gender-specific differences in hepatic HO expression and activity have been shown in previous studies [41] here we demonstrate for the first time that E_2 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.

Acknowledgements

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.

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

References

1. Wright AF, Jacobson SG, Cideciyan AV, Roman AJ, Shu X, Vlachantoni D, McInnes RR, Riemersma RA (2004) Lifespan and mitochondrial control of neurodegeneration. Nat Genet 36:1153-1158. 10.1038/ng1448

2. Stirone C, Duckles SP, Krause DN, Procaccio V (2005) Estrogen increases mitochondrial efficiency and reduces oxidative stress in cerebral blood vessels. Mol Pharmacol 68:959-965. 10.1124/mol.105.014662

3. Vina J, Borras C, Gambini J, Sastre J, Pallardo FV (2005) Why females live longer than males? Importance of the upregulation of longevity-associated genes by oestrogenic compounds. FEBS Lett 579:2541-2545. 10.1016/j.febslet.2005.03.090

4. Shimizu I (2003) Impact of oestrogens on the progression of liver disease. Liver Int 23:63-69. 10.1034/j.1600-0676.2003.00811.x

5. Wassmann S, Baumer AT, Strehlow K, van Eickels M, Grohe C, Ahlbory K, Rosen R, Bohm M, Nickenig G (2001) Endothelial dysfunction and oxidative stress during estrogen deficiency in spontaneously hypertensive rats. Circulation 103:435-441. 10.1161/01.CIR.103.3.435

6. Borras C, Gambini J, Gomez-Cabrera MC, Sastre J, Pallardo FV, Mann GE, Vina J (2005) 17beta-oestradiol up-regulates longevity-related, antioxidant enzyme expression via the ERK1 and ERK2[MAPK]/NFkappaB cascade. Aging Cell 4:113-118. 10.1111/j.1474-9726.2005.00151.x

7. Yu J, Zhao Y, Li B, Sun L, Huo H (2012) 17beta-estradiol regulates the expression of antioxidant enzymes in myocardial cells by increasing Nrf2 translocation. J Biochem Mol Toxicol 26:264-269. 10.1002/jbt.21417

8. Ferrandiz ML, Devesa I (2008) Inducers of heme oxygenase-1. Curr Pharm Des 14:473-486. 10.2174/138161208783597399

9. Dore S, Takahashi M, Ferris CD, Zakhary R, Hester LD, Guastella D, Snyder SH (1999) Bilirubin, formed by activation of heme oxygenase-2, protects neurons against oxidative stress injury. Proc Natl Acad Sci U S A 96:2445-2450. 10.1073/pnas.96.5.2445

10. Li N, Venkatesan MI, Miguel A, Kaplan R, Gujuluva C, Alam J, Nel A (2000) Induction of heme oxygenase-1 expression in macrophages by diesel exhaust particle chemicals and quinones via the antioxidant-responsive element. J Immunol 165:3393-3401. 10.4049/jimmunol.165.6.3393

11. Hancock R, Bertrand HC, Tsujita T, Naz S, El-Bakry A, Laoruchupong J, Hayes JD, Wells G (2012) Peptide inhibitors of the Keap1-Nrf2 protein-protein interaction. Free Radic Biol Med 52:444-451. 10.1016/j.freeradbiomed.2011.10.486

12. Hast BE, Goldfarb D, Mulvaney KM, Hast MA, Siesser PF, Yan F, Hayes DN, Major MB (2013) Proteomic analysis of ubiquitin ligase KEAP1 reveals associated proteins that inhibit NRF2 ubiquitination. Cancer Res 73:2199-2210. 10.1158/0008-5472.CAN-12-4400

13. Abramić M, Zubanović M, Vitale L (1988) Dipeptidyl peptidase III from human erythrocytes. Biol Chem Hoppe Seyler 369:29-38.

14. Abramić M, Šimaga S, Osmak M, Čičin-Šain L, Vukelić B, Vlahoviček K, Dolovčak L (2004) Highly reactive cysteine residues are part of the substrate binding site of mammalian dipeptidyl peptidases III. Int J Biochem Cell Biol 36:434-446.

15. Baršun M, Jajčanin N, Vukelić B, Špoljarić J, Abramić M (2007) Human dipeptidyl peptidase III acts as a post-proline-cleaving enzyme on endomorphins. Biol Chem 388:343-348. 10.1515/BC.2007.039

16. Liu Y, Kern JT, Walker JR, Johnson JA, Schultz PG, Luesch H (2007) A genomic screen for activators of the antioxidant response element. Proc Natl Acad Sci U S A 104:5205-5210. 10.1073/pnas.0700898104

17. Zhan H, Yamamoto Y, Shumiya S, Kunimatsu M, Nishi K, Ohkubo I, Kani K (2001) Peptidases play an important role in cataractogenesis: an immunohistochemical study on lenses derived from Shumiya cataract rats. Histochem J 33:511-521. 10.1023/A:1014943522613

18. Šimaga S, Babić D, Osmak M, Šprem M, Abramić M (2003) Tumor cytosol dipeptidyl peptidase III activity is increased with histological aggressiveness of ovarian primary carcinomas. Gynecol Oncol 91:194-200. 10.1016/S0090-8258(03)00462-1

19. He M, Mangiameli DP, Kachala S, Hunter K, Gillespie J, Bian X, Shen HC, Libutti SK (2010) Expression signature developed from a complex series of mouse models accurately predicts human breast cancer survival. Clin Cancer Res 16:249-259. 10.1158/1078-0432.CCR-09-1602

20. Persky AM, Green PS, Stubley L, Howell CO, Zaulyanov L, Brazeau GA, Simpkins JW (2000) Protective effect of estrogens against oxidative damage to heart and skeletal muscle in vivo and in vitro. Proc Soc Exp Biol Med 223:59-66. 10.1046/j.1525-1373.2000.22308.x

21. Einecke G, Fairhead T, Hidalgo LG, Sis B, Turner P, Zhu LF, Bleackley RC, Hadley GA, Famulski KS, Halloran PF (2006) Tubulitis and epithelial cell alterations in mouse kidney transplant rejection are independent of CD103, perforin or granzymes A/B. Am J Transplant 6:2109-2120. 10.1111/j.1600-6143.2006.01483.x

22. Špoljarić J, Tomić A, Vukelić B, Salopek Sondi B, Agić D, Tomić S, Abramić M (2011) Human dipeptidyl peptidase III: The role of Asn406 in ligand binding and hydrolysis. Croatica Chemica Acta 84:259-268. 10.5562/cca1808

23. Haas CA, Deller T, Krsnik Z, Tielsch A, Woods A, Frotscher M (2000) Entorhinal cortex lesion does not alter reelin mRNA expression in the dentate gyrus of young and adult rats. *Neuroscience* 97: 25-31

24. Shin SM, Yang JH, Ki SH (2013) Role of the Nrf2-ARE pathway in liver diseases. Oxid Med Cell Longev. 2013:1-9.

25. Cichoż-Lach H, Michalak A (2014) Oxidative stress as a crucial factor in liver diseases. World J Gastroenterol 20: 8082-8091.

26. Su H, van Dam GM, Buis CI,Visser DS, Hesselink JW, Schuurs TA, Leuvenink HGD, Contag CH, Porte RJ (2006) Spatiotemporal expression of Heme Oxygenase 1 detected by in vivo bioluminiscence after hepatic ischemia in HO-1/Luc mice. Liver Transplanation 12:1634-1639.

27. Levonen AL, Hill BG, Kansanen E, Zhang J, Darley-Usmar VM (2014) Redox regulation of antioxidants, autophagy, and the response to stress: Implications for electrophile therapeutics. Free Rad Biol Med (*in press*)

28. Shukla AA, Jain M, Chauhan SS (2010) Ets-1/Elk-1 is a critical mediator of dipeptidyl-peptidase III transcription in human glioblastoma cells. FEBS J 277:1861-1875. 10.1111/j.1742-4658.2010.07603.x

29. Singh R, Sharma MC, Sarkar C, Singh M, Chauhan SS (2014) Transcription factor C/EBP-beta mediates downregulation of dipeptidyl-peptidase III expression by interleukin-6 in human glioblastoma cells. FEBS J 281:1629-1641. 10.1111/febs.12728

30. Fukasawa K, Fukasawa KM, Kanai M, Fujii S, Hirose J, Harada M (1998) Dipeptidyl peptidase III is a zinc metallo-exopeptidase. Molecular cloning and expression. Biochem J 329 (Pt 2):275-282.

31. Abramić M, Schleuder D, Dolovčak L, Schroder W, Strupat K, Sagi D, Peter-Katalini J, Vitale L (2000) Human and rat dipeptidyl peptidase III: biochemical and mass spectrometric arguments for similarities and differences. Biol Chem 381:1233-1243. 10.1515/BC.2000.151

32. Bezerra GA, Dobrovetsky E, Viertlmayr R, Dong A, Binter A, Abramic M, Macheroux P, Dhe-Paganon S, Gruber K (2012) Entropy-driven binding of opioid peptides induces a large domain motion in human dipeptidyl peptidase III. Proc Natl Acad Sci U S A 109:6525-6530. 10.1073/pnas.1118005109

33. Liang K, Yang L, Yin C, Xiao Z, Zhang J, Liu Y, Huang J (2010) Estrogen stimulates degradation of beta-amyloid peptide by up-regulating neprilysin. J Biol Chem 285:935-942. 10.1074/jbc.M109.051664

34. Ogawa M, Yamaji R, Higashimura Y, Harada N, Ashida H, Nakano Y, Inui H (2011) 17beta-estradiol represses myogenic differentiation by increasing ubiquitin-specific peptidase 19 through estrogen receptor alpha. J Biol Chem 286:41455-41465. 10.1074/jbc.M111.276824

35. Rochefort H, Chalbos D, Cunat S, Lucas A, Platet N, Garcia M (2001) Estrogen regulated proteases and antiproteases in ovarian and breast cancer cells. J Steroid Biochem Mol Biol 76:119-124. 10.1016/S0960-0760(00)00142-4

36. Danielsen JM, Sylvestersen KB, Bekker-Jensen S, Szklarczyk D, Poulsen JW, Horn H, Jensen LJ, Mailand N, Nielsen ML (2011) Mass spectrometric analysis of lysine ubiquitylation reveals promiscuity at site level. Mol Cell Proteomics 10:M110 003590. 10.1074/mcp.M110.003590

37. Manolagas SC (2010) From estrogen-centric to aging and oxidative stress: a revised perspective of the pathogenesis of osteoporosis. Endocr Rev 31:266-300. 10.1210/er.2009-0024

38. Sobočanec S, Balog T, Šarić A, Šverko V, Žarković N, Čipak Gašparović A, Žarković K, Waeg G, Mačak Šafranko Ž, Kušić B, Marotti T (2010) Cyp4a14 overexpression induced by hyperoxia in female CBA mice as a possible contributor of increased resistance to oxidative stress. Free Radic Res 44:181-190. 10.3109/10715760903390820

39. Šarić A, Sobočanec S, Mačak Šafranko Ž, Popović-Hadžija M, Aralica G, Korolija M, Kušić B, Balog T (2014) Female headstart in resistance to hyperoxia-induced oxidative stress in mice. Acta Biochim Pol (*in press*).

40. Šarić A, Sobočanec S, Balog T, Popović Hadžija M, Kušić B, Marotti T. Female headstart in key factors responsible for mitochondrial functioning as a protective mechanism towards hyperoxia oxidative injury. Free Radic Biol Med (2012) 53, Supplement 1:S223.

41. Toth B, Yokoyama Y, Kuebler JF, Schwacha MG, Rue LW 3rd, Bland KI, Chaudry IH (2003) Arch Surg 138(12):1375-1382.

Legends to figures

Figure 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 β-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 by using AmidoBlack **(B).** All data are mean ± SD from 3 individual mice per group.

Figure 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 β-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 by using AmidoBlack. All data are mean ± SD from 3 individual mice per group.

Figure 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_2-treated male (cme) CBA/H mice **(C).** Fold-change in gene expression was calculated with $2^{-\Delta\Delta CT}$ 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_2-treated male (cme) CBA/H mice **(D).** Bands are normalized by using AmidoBlack. All data are mean ± SD from 3 individual mice per group.

Figure 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 β-actin as endogenous control **(A).** Western blot analysis of HO-1 protein expression 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 by using AmidoBlack. All data are mean ± SD from 3 individual mice per group.

Figure 5. Immunohistochemical localisation 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 localised in hepatocytes (H) and uniformly distributed throughout the hepatic tissue.

Figure 1
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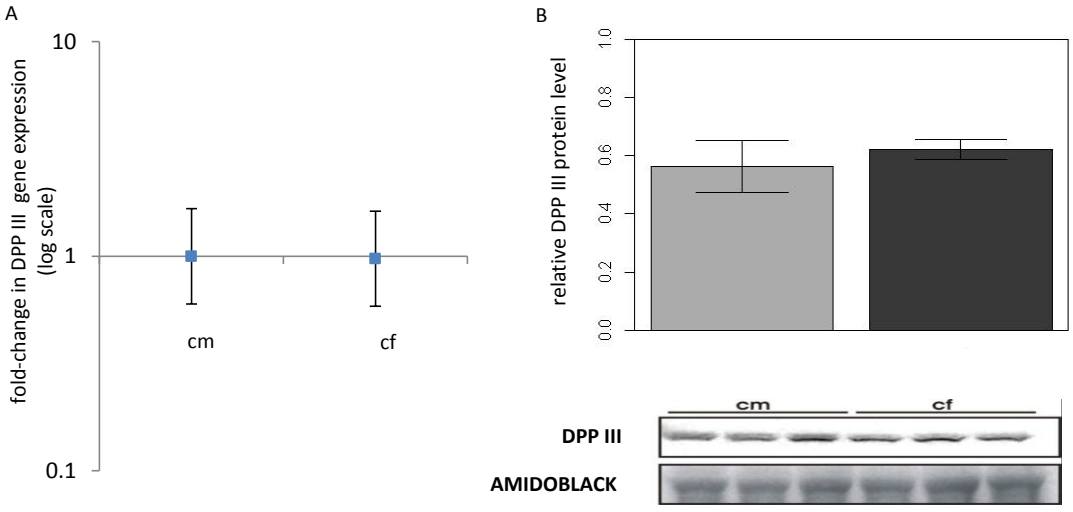

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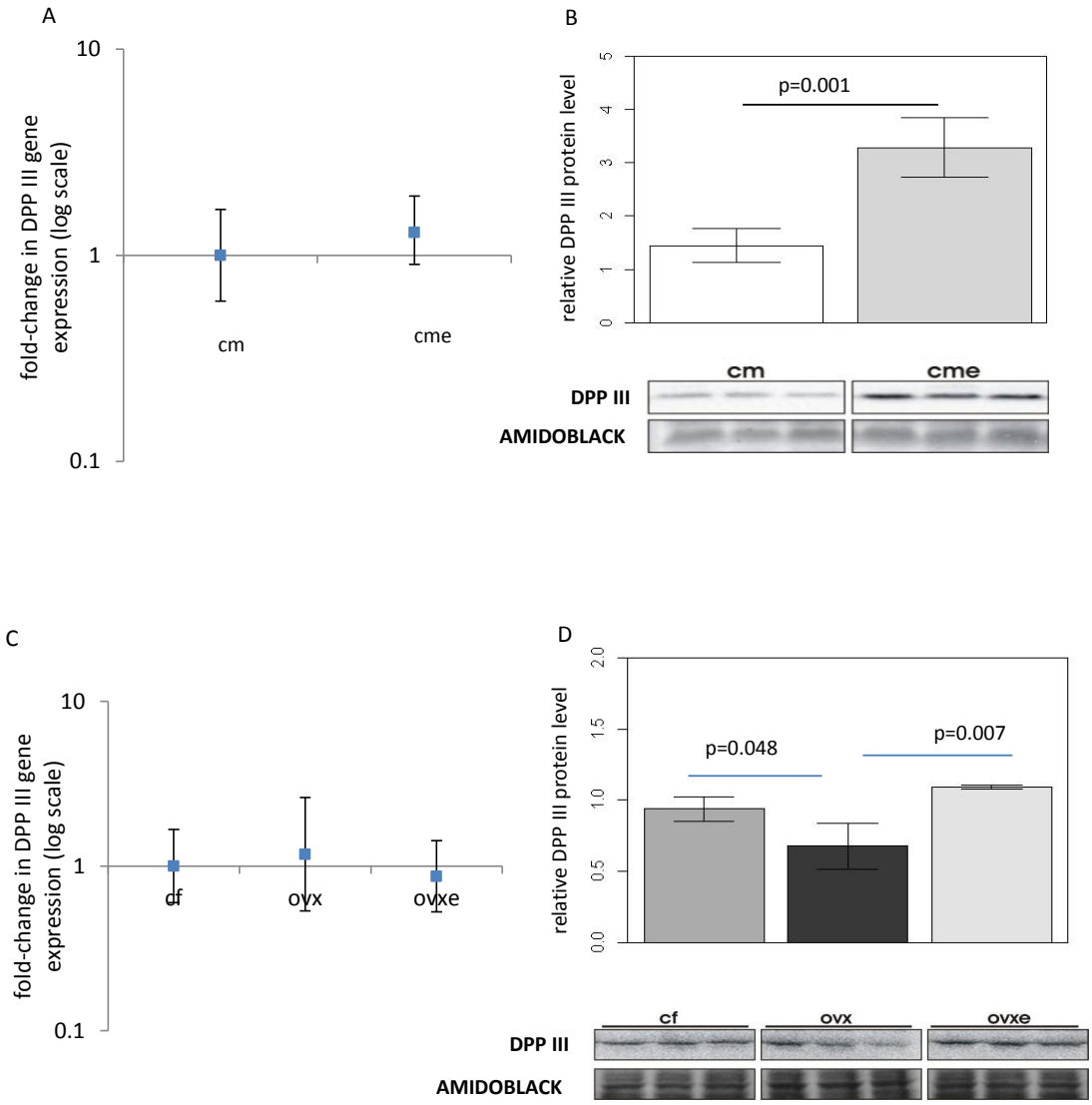

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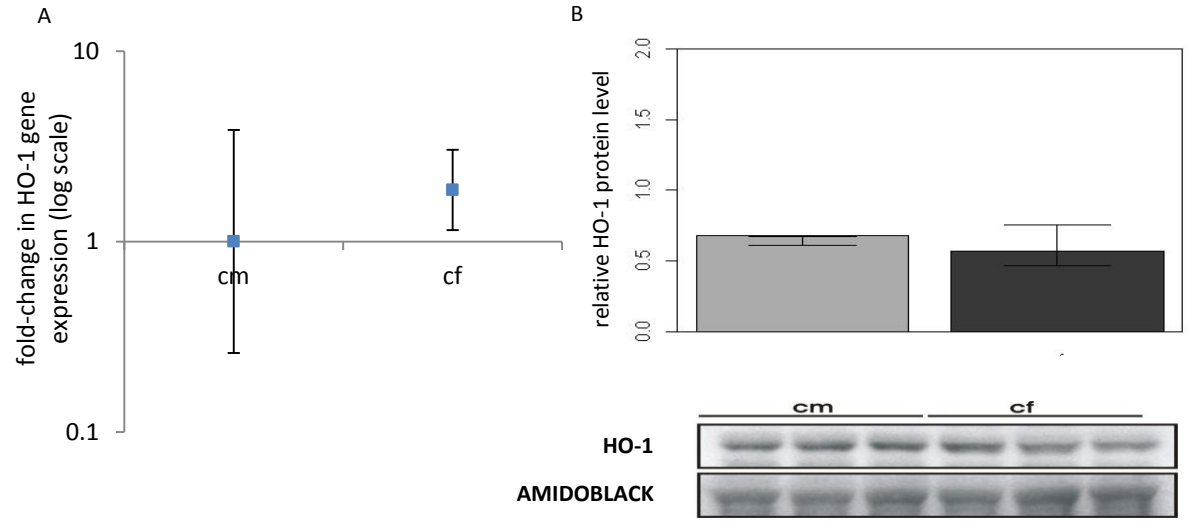

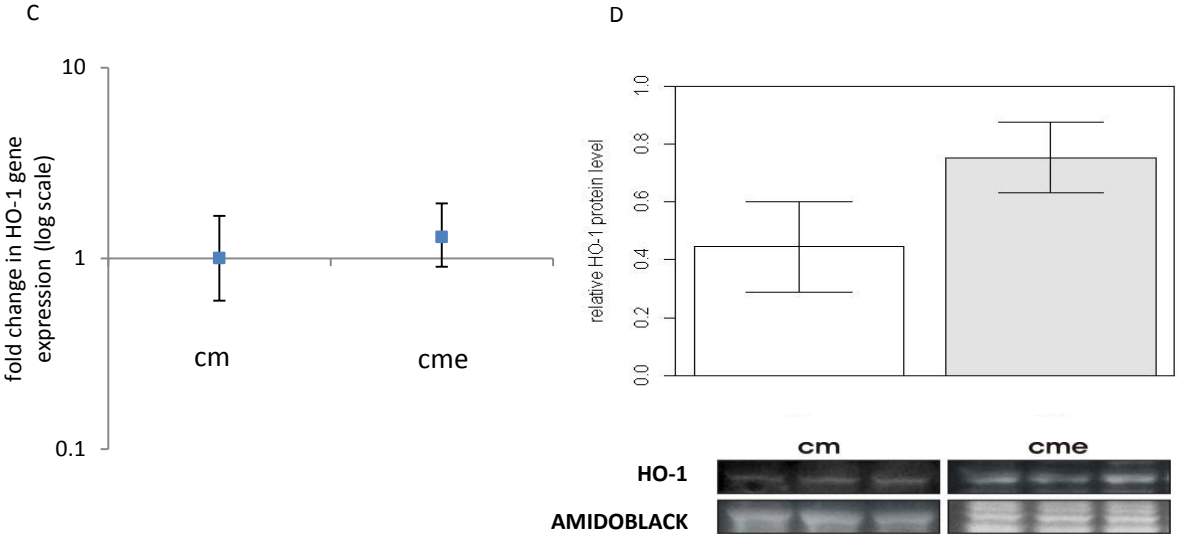

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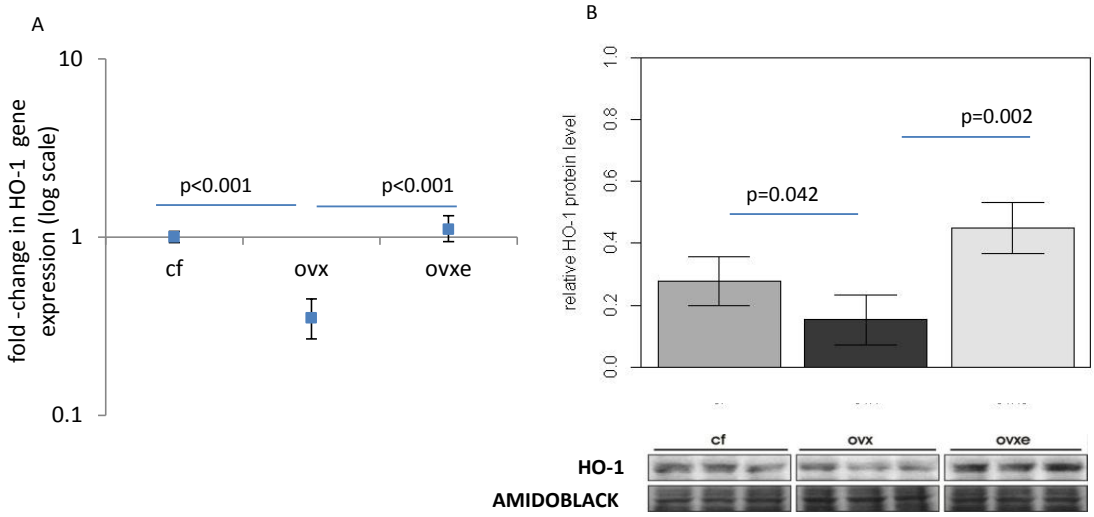

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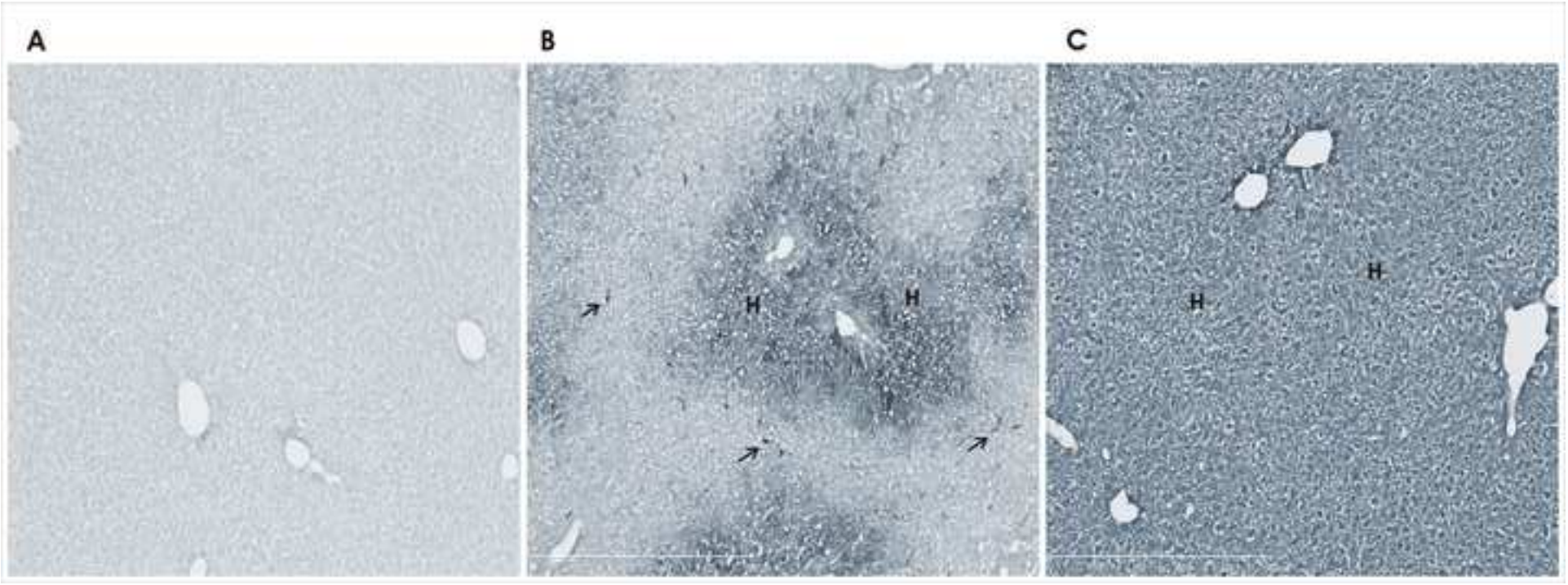

Table 1. Probes used for quantitative real-time PCR analysis.

Gene	Assay ID	Product size (bp)
β-actin	Mm00607939_s1	115
dipeptidyl peptidase III	Mm00505462_m1	89
heme oxygenase 1	Mm00516007_m1	92