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Journal of Nutritional Biochemistry 22 (2011) 856–864
Journal of Nutritional Biochemistry

Protection and reversal of hepatic fibrosis by red wine polyphenols in hyperhomocysteinemic mice☆

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Received 23 November 2009; received in revised form 17 July 2010; accepted 20 July 2010

Abstract

Hyperhomocysteinemia leads to several clinical manifestations and, particularly, liver disease. Lowering homocysteine through nutrition or other means might offer preventive or therapeutic benefits. Polyphenols are natural compounds known for their antioxidant and healing properties for vessels. In a previous study we have shown a beneficial effect of a red wine polyphenolic extract (PE) administration on plasma homocysteine level in cystathionine beta synthase deficient mice, a murine model of hyperhomocysteinemia. These mice also develop hepatic fibrosis. As increased matrix metalloproteinase (MMP) 2 has been shown to be involved in the development of hepatic fibrosis, we then focused on the effect of PE administration on expression and activity of MMP-2 in liver of hyperhomocysteinemic mice and its impact on hepatic fibrosis development. PE was added for four weeks to the drinking water of heterozygous cystathionine beta synthase-deficient mice fed a high-methionine diet. Effects of PE administration were examined by histological analysis with Sirius red staining, zymography, immunobloting, real-time quantitative reverse transcriptase polymerase chain reaction, peroxynitrite level, catalase activity and nicotinamide adenine dinucleotide phosphate oxidase activity. We show that administration of PE had a beneficial effect (i) on MMP-2 expression via modulation of nitrotyrosine-modified total protein level and (ii) on MMP-2 activity via modulation of its activator/inhibitor balance. We also demonstrated a reversal effect of PE supplementation on hepatic fibrosis development. Our results demonstrate a preventive action of PE administration on biomarkers of hepatic dysfunction due to hyperhomocysteinemia.

Keywords: Homocysteine; Fibrosis; Polyphenols; Catalase; e-NOS; Matrix metalloproteinase-2; Zymography

1. Introduction

Homocysteine is a thiol-containing amino acid produced during methionine metabolism. Once formed, it may be recycled to methionine via the remethylation pathway that involves methionine synthase, a vitamin B_{12}-dependent enzyme that uses 5-methyltetrahydrofolate as the methyl donor. The 5-methyltetrahydrofolate is generated by 5,10-methylene tetrahydrofolate reductase. Another pathway, that occurs in the liver and the kidney, involves the betaine-homocysteine methyltransferase. Otherwise, homocysteine could also undergo condensation with serine to form cystathionine via the vitamin B_6-dependent cystathionine beta synthase (CBS), the first enzyme involved in the transsulfuration pathway [1]. Hyperhomocysteinemia corresponds to an elevation of total plasma homocysteine level and exists under three forms: moderate (15–30 μM), intermediate (30–100 μM) and severe (above 100 μM). Hyperhomocysteinemia is now well recognized as an important vascular risk factor associated with atherosclerosis in the coronary, cerebrovascular and peripheral arterial circulation, even if the degree of hyperhomocysteinemia is moderate [2].

Hyperhomocysteinemic patients develop perivascular hepatic fibrosis [3]. We observed hepatic fibrosis in homozygous CBS-deficient mice [4], a severe murine model of hyperhomocysteinemia [5]. In this model, Ovechkin et al. demonstrated an increase in matrix metalloproteinases (MMPs) expression in aorta [6]. MMPs and particularly MMP-2, have been involved in the development of fibrosis [7] via a MMP-2-induced oxidative stress mechanism [8]. MMPs are a large family of proteolytic zinc-containing enzymes expressed as proenzymes that need to be activated by cleavage. This activation is regulated by mechanisms involving a balance between

☆ Grants and funding sources: This work was supported in part by an EU grant AnEUploïdie and by Viniflhor, Ministère de l'Agriculture, programme Vin et Santé, Pathologie et biologie vasculaires. Christophe Noll and Ludovic Benard are supported by a fellowship from the Ministère de l'Enseignement supérieur et de la Recherche.
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doi:10.1016/j.jnutbio.2010.07.010

tissue inhibitor of metalloproteinases (TIMPs) and membrane-type (MT) MMP. In the case of MMP-2, a gelatinase involved in the development of thrombosis [9], the activation occurs on cell surface by interaction between proMMP-2 and TIMP-2 and the recruitment of the complex by MT1-MMP, leading to the formation of a ternary complex [10].

Polyphenols are organic aromatic compounds found in nuts, fruits and red wine. Red wine polyphenols, catechin and (−)epigallocatechin-3-gallate have been described to suppress MMP-2 expression and activation by inhibiting MT1-MMP activity [11–14]. Red wine polyphenols have been shown for their protective effects on angiotensin II-induced MMP-2 expression in aortic wall [15]. In previous studies, we have shown that catechin or red wine polyphenolic extract (PE) administration to CBS-deficient mice reduced plasma homocysteine level [16,17]. Therefore, the aim of the present study was to determine the effect of red wine polyphenolic extract onto MMP-2 activity and its repercussion onto development of hepatic fibrosis in case of hyperhomocysteinemia. We applied mice model of hyperhomocysteinemia and triggered hyperhomocysteinemia by combination of genetic (heterozygous CBS-deficient mice) and dietary approaches (feeding with high-methionine diet), which allow us to discriminate between the effects of hyperhomocysteinemia and those due to the loss of CBS expression by genetic deletion.

2. Materials and methods

2.1. Mice, genotyping and experimental protocol

Mice were maintained in a controlled environment with unlimited access to food and water on a 12-h light/dark cycle. All procedures were carried out in accordance with internal guidelines of the French Agriculture Ministry for animal handing. Number of mice and suffering were minimized as possible. Mice heterozygous for targeted disruption of the *Cbs* gene [*Cbs* (+/−)] were generously donated by Dr. N. Maeda (Department of Pathology, University of North Carolina, Chapel Hill, NC, USA) [5]. *Cbs* (+/−) mice, on a C57BL/6 background, were obtained by mating male *Cbs* (+/−) mice with female wild-type C57BL/6 [*Cbs* (+/+)] mice. DNA isolated from 4-week-aged mice tail biopsies was subjected to genotyping of the targeted CBS allele using polymerase chain reaction assay [5].

Mice were fed a standard laboratory diet (A03, Safe-UAR, Augy, France) ad libitum. Female three-month-old Cbs (+/−) mice from the same litter were divided into four groups and maintained for 3 months on the following diets before the experiments: (a) control diet (control), consisting of the standard A03 rodent diet; (b) high methionine diet (Met), consisting of a control diet supplemented with 0.5% L-methionine (Sigma-Aldrich, Saint-Quentin Fallavier, France) in drinking water for 3 months; (c) high methionine diet with red wine polyphenolic extract (PE) for the last month; (d) control diet with PE for the last month. The PE dry powder (provided by the Faculté d'oenologie, Talence, France) represents the polyphenolic compounds isolated from red wine and involves 8.6 $mg.g^{-1}$ catechin, 8.7 $mg.g^{-1}$ epicatechin, dimers (B1: 6.9 $mg.g^{-1}$, B2: 8.0 $mg.g^{-1}$, B3: 20.7 $mg.g^{-1}$ and B4: 0.7 $mg.g^{-1}$), anthocyanins (malvidin-3-glucoside: 11.7 $mg.g^{-1}$, peonidin-3-glucoside: 0.66 $mg.g^{-1}$ and cyanidin-3-glucoside: 0.06 $mg.g^{-1}$) and phenolic acids (gallic acid: 5.0 $mg.g^{-1}$, caffeic acid: 2.5 $mg.g^{-1}$ and caftaric acid: 12.5 $mg.g^{-1}$) [18]. For mice fed the standard diet, the daily methionine intake is 21 mg, and for mice fed the high methionine diet, it is 36 mg. Animals were given fresh portion of supplemented diet twice a week in drinking water. Dietary supplementation did not affect the growth or food consumption of the mice during the experimental feeding period.

2.2. Preparation of serum samples, tissue collection and plasma assays

At the time of sacrifice, blood samples were collected into tubes containing a one-tenth volume of 3.8% sodium citrate, placed on ice immediately. Plasma was isolated by centrifugation at 2500×*g* for 15 min at 4°C. Liver was harvested, snap-frozen and stored at −80°C until use. Plasma total homocysteine was assayed by using the fluorimetric high-performance liquid chromatography method as previously described [17].

2.3. RNA extraction and determination of mRNA levels

Total RNA was prepared from liver with the Nucleospin RNA II kit (Macherey-Nagel, Hoerdt). The quantity and purity of the RNA were assessed by measuring absorbance at 260 and 280 nm. Reverse transcription was carried out on 1 μg of total RNA. Total RNA was heated for 5 min at 70°C with random decamer primers (Ambion) and then ice cooled. Reverse transcription was performed in presence of RT buffer, 0.5 mM dNTP, 20U RNasine (Promega), 8 U M-MulV reverse transcriptase (Promega) and incubated at 42°C for 1 h. Reverse transcriptase was heat inactivated at 95°C for 5 min. The mRNA levels of individual mice were assessed by real-time quantitative reverse transcription-polymerase chain reaction (Q-PCR). cDNA (0.4 μl) was diluted with PCR mix (Light Cycler 480 SYBR Green I Master, Roche Diagnostics) containing a final concentration of 3 mM $MgCl_2$ and 0.5 μM of primers in a final volume of 7 μl. The primers were designed by Primer 3 software. The primer pairs were selected to yield a single amplicon based on dissociation curves. The mouse superoxide dismutase 1 (*Sod1*) and the fasciculation and elongation protein zeta 1 (*Fez-1*) mRNA were used as endogenous controls. Primer sequences are given in Table 1. The thermal cycler parameters were as follows: hold for 8 min at 95°C for one cycle followed by amplification of cDNA for 40 cycles with melting for 5 s at 95°C, annealing for 5 s at 65°C and extension for 10 s at 72°C. Each reaction was performed in triplicate. ΔΔCp analysis of the results allows to assess the ratio of the target mRNA versus control mRNA [19].

2.4. Western blotting

Liver protein extracts were prepared by homogenizing 100 mg of liver in 500 μl phosphate-buffered Saline (PBS) with a cocktail of proteases inhibitors (1 mM Pefabloc SC, 5 μg/ml E64 and 2.5 μg/ml leupeptin). Homogenates were centrifuged at 13000×*g* for 15 min at 4°C. Supernatants were then assayed for protein concentrations with the Bio-Rad Protein Assay reagent (Bio-Rad). Protein preparations were subjected to sodium dodecyl sulfate (SDS) electrophoresis on 10% acrylamide gels under reducing conditions and transferred to Hybond-C Extra membrane (GE Healthcare Europe). After transfer, membranes were blocked in 10 % nonfat dry milk in Tris-saline buffer (1.5 mM Tris, 5 mM NaCl, 0.1 % Tween-20) and probed overnight at 4°C with primary antibody. The antibodies were purchased as followed: MMP-2 (Abcam, ab7032), MT1-MMP (Lifespan Biosciences, LS-C9080), TIMP-2 (Abcam, ab1828). Horseradish peroxidase-conjugated secondary antibodies and Western Blotting Luminol Reagent (Santa Cruz Biotechnology) were used to detect specific proteins. β-actin was used as an internal control. Blots were developed with a LAS-3000 imaging system (Fujifilm), and densitometry was performed with UnScan It software (Silk Scientific).

2.5. Nitrotyrosine-modified protein level determination

Ten micrograms of protein under reducing conditions were blotted on Hybond-C Extra membrane (GE Healthcare Europe GmbH) using Bio-Dot SF Microfiltration Apparatus (Bio-Rad). Membranes were blocked in 10% nonfat dry milk in Tris-saline buffer (1.5 mM Tris, 5 mM NaCl, 0.1% Tween-20) and probed overnight at 4°C with anti-3-nitrotyrosine antibody (Fisher Scientific, 2a12). Horseradish peroxidase-conjugated secondary antibodies and Western Blotting Luminol Reagent (Santa Cruz Biotechnology) were used to detect nitrotyrosine-modified proteins. Ponceau-S (Sigma) coloration was used as an internal control. Blots were developed with a LAS-3000 imaging system (Fujifilm) and densitometry was performed with UnScan It software (Silk Scientific).

2.6. Gelatin zymography

One hundred micrograms of liver protein extracts were purified by affinity with 100 μl of gelatin-agarose (Sigma) in 1 ml of distilled water. After 1 h of agitation at 4°C, tubes were centrifugated at 10,000×*g* for 1 min at 4°C. Gelatin-agarose beads were resuspended in non-reducing Laemmli and incubated at room temperature for 30 min. After centrifugation at 14,000×*g* for 2 min at 4°C, 10 μl of surpernatant were loaded on 7.5 % SDS-polyacrylamide gel electrophoresis gel. After migration, gels were washed three times in renaturation buffer (50 mM Tris HCl pH 7.5, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 2.5 % Triton X-100) for 40 min to remove SDS followed by 10 min of distilled water 2 times. Then, gels were incubated at 37°C overnight in activity buffer (50 mM Tris Hcl pH 7.5, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 1 % Triton X-100). Gels were stained with amido black (Sigma) for 20 min to visualize proteolytic bands. Densitometry was performed with UnScan It software (Silk Scientific).

2.7. Catalase activity

Catalase activity was measured on liver extract corresponding to 25 μg of total proteins in 1.5 ml of 50 mM Tris buffer pH 7.8, containing 2 mM $MgCl_2$ and 2 mM $CaCl_2$. The maximum catalase activity was determined with a sensitive Clark electrode measuring dioxygen produced by the dismutation of H_2O_2. 2–8 μl H_2O_2 (100 mM) were progressively injected, in order to obtain the maximum production rate of dioxygen.

Table 1
Primer sequences for real-time Q-PCR

Gene	Left primer	Right primer
eNOS	GCTTCAGGAAGTGGAGGCTGAG	GGAACACTGTGATGGCTGAACG
FEZ-1	CCAGCTGCAGGTGTTCAGT	TCGCTGGCCTTAGTGTTCACC
MMP-2	AACGATGGCAAGGTGTGGTG	GCTCCTGGATCCCCTTGATG
SOD1	TGGGGACAATACACAAGGCTGT	TTTCCACCTTTGCCCAAGTCA

All primers are listed 5′ to 3′.

Table 2
Plasma total homocysteine levels in female heterozygous CBS-deficient mice fed the control diet supplemented with PE or without PE (control) or high-methionine diet supplemented with (Met/PE) or PE (Met)

Diet	tHcy (μM), mean±S.E.M. (range)
Control (n=8)	10.88±1.3 (5.8–16.3)
Met (n=4)	39.4±7.5 * (27.1–59)
Met/PE (n=9)	23.3±2.3 **,† (13.8–33.5)
PE (n=7)	12.1±1.5 ††,‡ (6.3–19.6)

Statistical analysis was done with one-way ANOVA followed by Student's unpaired t-tests.
* P<.0004 (versus control diet).
** P<.0003 (versus control diet).
† P<.03 (versus Met diet)
†† P<.001 (versus Met diet)
‡ P<.002 (versus Met/PE diet).

2.8. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity

NADPH oxidase activity was measured by monitoring the oxidation rate of cytochrome c as described by Iwai et al. [20] with 20 μg of total proteins. NADPH oxidase activity was quantified from the absorbance at 540, 550 and 560 nm as previously described [21].

2.9. Determination of fibrosis

Liver was fixed in 4% paraformaldehyde solution in PBS, then embedded in paraffin. Paraffin-embedded sections (10 μm) were stained with the collagen specific Sirius red stain (0.5% in saturated picric acid). The ratio of interstitial collagen surface-to-total vessel surface was determined with IPLab software. Quantification of fibrosis was performed in a masked fashion on at least 20 areas per liver.

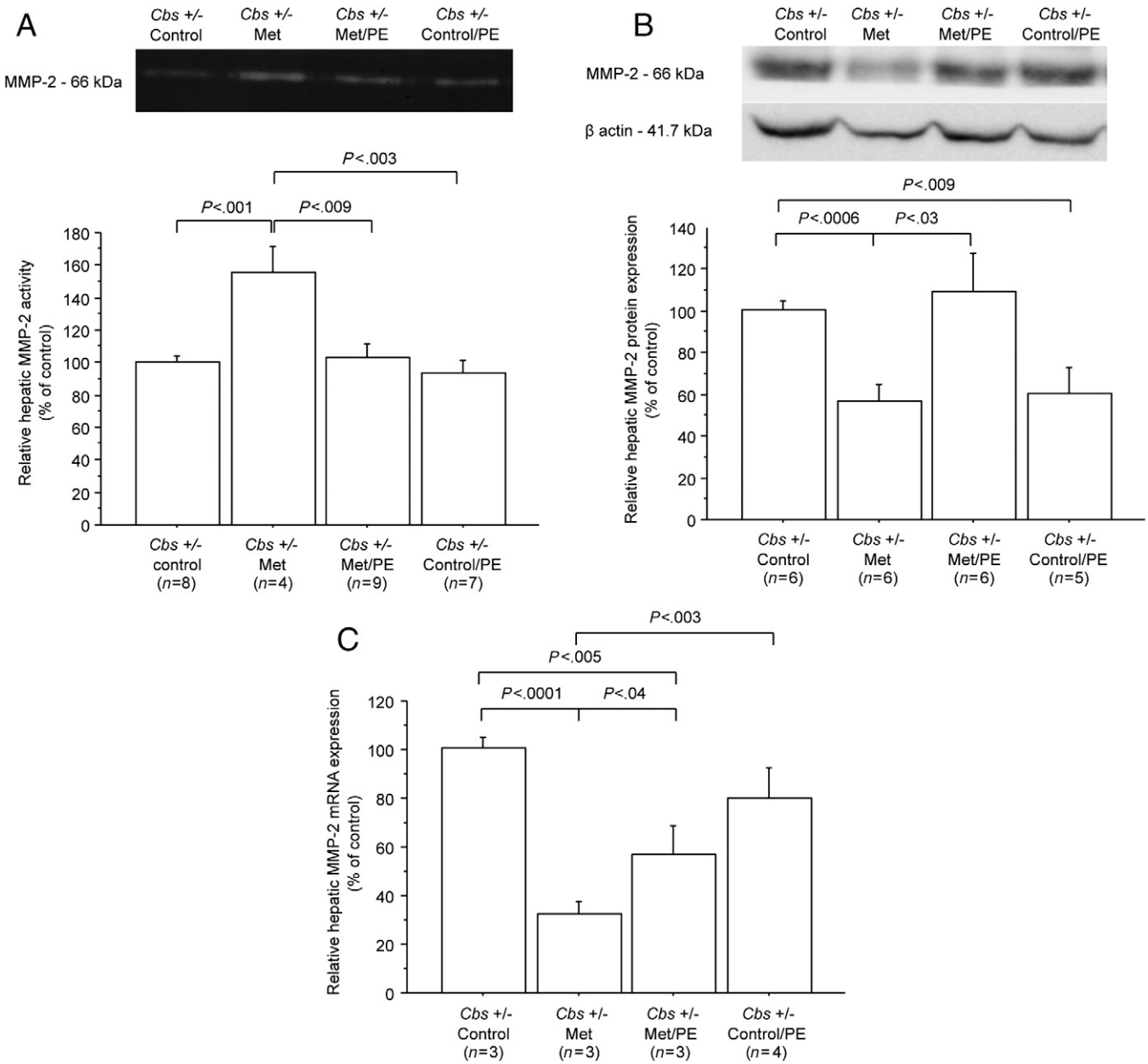

Fig. 1. Effects of PE administration on hepatic MMP-2 activity and expression in hyperhomocysteinemic mice. Comparison of relative hepatic MMP-2 activity (A), hepatic MMP-2 protein expression (B) and hepatic MMP-2 mRNA expression (C) obtained from heterozygous *Cbs* deficient (*Cbs* +/−) mice fed a control diet supplemented with (Control/PE) or without (Control) red wine polyphenol extract or a high-methionine diet supplemented with (Met/PE) or without (Met) red wine polyphenol extract. The values are mean±S.E.M. of n (number of mice) mice normalized to the mean of *Cbs* +/− mice fed a control diet. Statistical analysis was done with one-way ANOVA followed by Student's unpaired t tests.

2.10. Data analysis

Statistical analysis was done with one-way analysis of variance (ANOVA) followed by Student's unpaired *t* test using Statview software. The results are expressed as mean ±S.E.M. Correlation between homocysteine level and hepatic MMP-2 activity was determined by using Bravais-Pearson correlation test as data were normally distributed according to Shapiro-Wilk test. Data were analyzed using R software (http://www.R-project.org) and considered significant at $P<.05$.

3. Results

3.1. Effects of PE administration on hepatic MMP-2 activity and expression in hyperhomocysteinemic mice

As expected, *Cbs* +/− mice fed the high-methionine diet (Met) showed a significant increase in plasma total homocysteine level (Table 2) when compared with *Cbs* +/− mice fed the control diet (control). We also found that, as previously described [17], PE administration diminished the plasma total homocysteine level (Table 2) in *Cbs* +/− mice on the high-methionine diet.

In order to determine the impact of PE supplementation on MMP-2, we analyzed MMP-2 activity by gelatin zymography and its mRNA and protein expression. We found an increase of MMP-2 activity in liver of *Cbs* +/− mice fed a methionine-enriched diet in comparison to *Cbs* +/− mice fed a control diet (Fig. 1A, $P<.001$). PE administration decreased MMP-2 activity in *Cbs* +/− mice fed a methionine-enriched diet (Fig. 1A, $P<.009$) but had no effect on *Cbs* +/− mice fed a control diet (Fig. 1A). mRNA expression was determined by Q-PCR. We found a decrease of MMP-2 expression in liver of *Cbs* +/− mice fed a methionine-enriched diet in comparison to *Cbs* +/− fed a control diet (Fig. 1C, $P<.0001$). PE administration increased hepatic MMP-2 expression in *Cbs* +/− mice fed a methionine-enriched diet (Fig. 1C, $P<.04$) but has no effect on MMP-2 expression in *Cbs* +/− mice fed a control diet. These effects were also found at the protein level. We found a decrease of MMP-2 protein level in liver of *Cbs* +/− mice fed a methionine-enriched diet in comparison to *Cbs* +/− fed a control diet (Fig. 1B, $P<.0006$). The administration of PE increased MMP-2 protein level in *Cbs* +/− mice fed a methionine-enriched diet (Fig. 1B, $P<.03$) but decreased MMP-2 protein level in *Cbs* +/− mice fed a control diet (Fig. 1B, $P<.009$).

3.2. Correlation between hepatic MMP-2 activity and plasma homocysteine level

With regard to the changes of hepatic MMP-2 activity and plasma homocysteine level, we observed differences between types of hyperhomocysteinemia. Below 15 μM, there was no correlation between these two parameters (Fig. 2A, $r=-0.36$, $P<.2$). Between 15 and 30 μM, i.e., in moderate hyperhomocysteinemia, we found a correlation between the small increase in plasma homocysteine level and the strong increase in hepatic MMP-2 activity (Fig. 2B, $r=0.69$, $P<.04$). Above 30 μM, i.e., in intermediate hyperhomocysteinemia, a correlation was found again between the moderate increase in plasma homocysteine level and the moderate increase in hepatic MMP-2 activity (Fig. 2C, $r=0.92$, $P<.03$).

3.3. Effects of PE administration on MMP-2 metabolism via a modulation of TIMP-2/MT1-MMP balance

To explain the results of PE administration on MMP-2 activity in hyperhomocysteinemic mice, we analyzed the expression of protein involved in its activation pathway. We found an increase of MT1-MMP protein level in liver of *Cbs* +/− mice fed a methionine-enriched diet in comparison to *Cbs* +/− mice fed a control diet (Fig. 3A, $P<.003$) with a decrease of TIMP-2 protein level (Fig. 3B, $P<.02$) resulting in an increase of 150% of the MT1-MMP/TIMP-2 ratio (Fig. 3C, $P<.002$). PE administration led to a decrease of MT1-MMP protein level in *Cbs* +/− mice fed a methionine-enriched diet (Fig. 3A, $P<.02$) with an increase of TIMP-2 protein level (Fig. 3B, $P<.003$) resulting in a decrease of the MT1-MMP/TIMP-2 ratio (Fig. 3C, $P<.003$). In *Cbs* +/− mice fed a control diet supplemented with PE, no effect on MT1-MMP protein and on TIMP-2 protein level was found, leading to no variation of MT1-MMP/TIMP-2 ratio. The modulation of TIMP-2 protein level by methionine-enriched diet and PE administration is also found at mRNA level. In *Cbs* +/− mice fed a high methionine-enriched diet, we found a decrease of TIMP-2 mRNA expression in comparison to *Cbs* +/− mice fed a control diet (84.2±3.8% vs. 100.2±3.8%, $P<.04$). PE administration increased TIMP-2 mRNA expression in *Cbs* +/− mice fed a high methionine-enriched diet (126.9±10.0 % vs. 84.2±3.8 %, $P<.03$) but had no

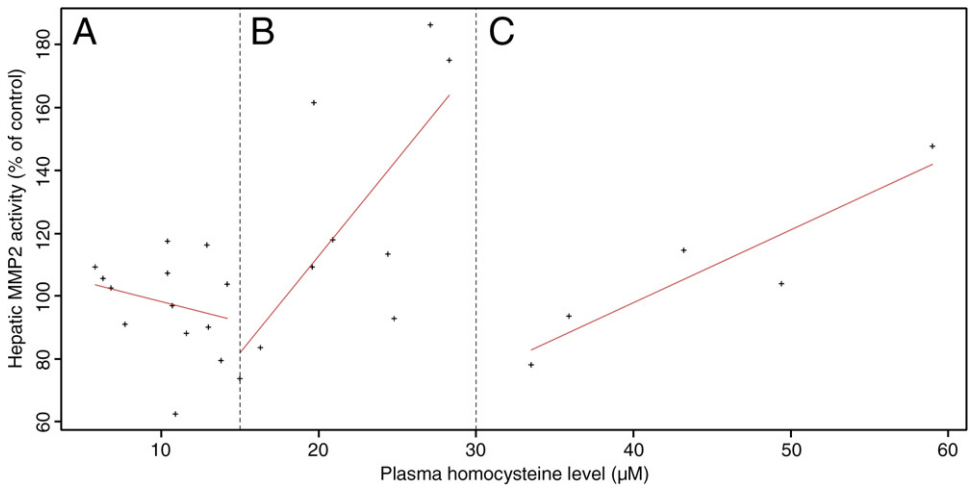

Fig. 2. Correlation between hepatic MMP-2 activity and plasma homocysteine level. Plasma homocysteine level is correlated to hepatic MMP-2 activity in function of the type of hyperhomocysteinemia. The results are presented as followed: normal condition (A), moderate hyperhomocysteinemia (B) and mild hyperhomocysteinemia (C). In normal condition, plasma Hcy level is not correlated to hepatic MMP-2 activity with a cor=−0.36 and $P<.2$, but in moderate and mild hyperhomocysteinemia, plasma homocysteine level and hepatic MMP-2 activity are positively correlated with a cor=0.69 and cor=0.92 respectively and $P<.04$ and $P<.03$, respectively.

effect on *Cbs* +/− mice fed a control diet (100.0±3.7 % vs. 100.2±3.8 %, *P*<.98). However, we found no variation for MT1-MMP mRNA expression (data not shown).

3.4. Effects of PE administration on hepatic peroxynitrite level in hyperhomocysteinemic mice

Nitrotyrosine levels have been shown to modulate MMP-2 mRNA expression in human aortic smooth muscle cell [22]. Nitrotyrosines are generally produced by reaction of peroxynitrite ($ONOO^-$) with tyrosine residues. We analyzed the level of nitrotyrosine-modified protein as a biomarker of peroxynitrite level. We found a reduced level in liver of *Cbs* +/− mice fed a methionine-enriched diet in comparison to *Cbs* +/− mice fed a control diet (Fig. 4, *P*<.0002). PE administration significantly increased nitrotyrosine-modified protein level in *Cbs* +/− mice fed a methionine-enriched diet (Fig. 4, *P*<.04) but had no effect on *Cbs* +/− mice fed a control diet (Fig. 4).

3.5. Effects of PE administration on hepatic biomarkers of oxidative stress in hyperhomocysteinemic mice

Peroxynitrite is the product of a reaction between nitric oxide (NO) and the superoxide anion (O_2^-), or between nitrite (NO_2^-, a derivative of NO) and hydrogen peroxide (H_2O_2). In order to determine the mechanism of peroxynitrite modulation, we analyzed several biomarkers of production or catabolism of these species. As

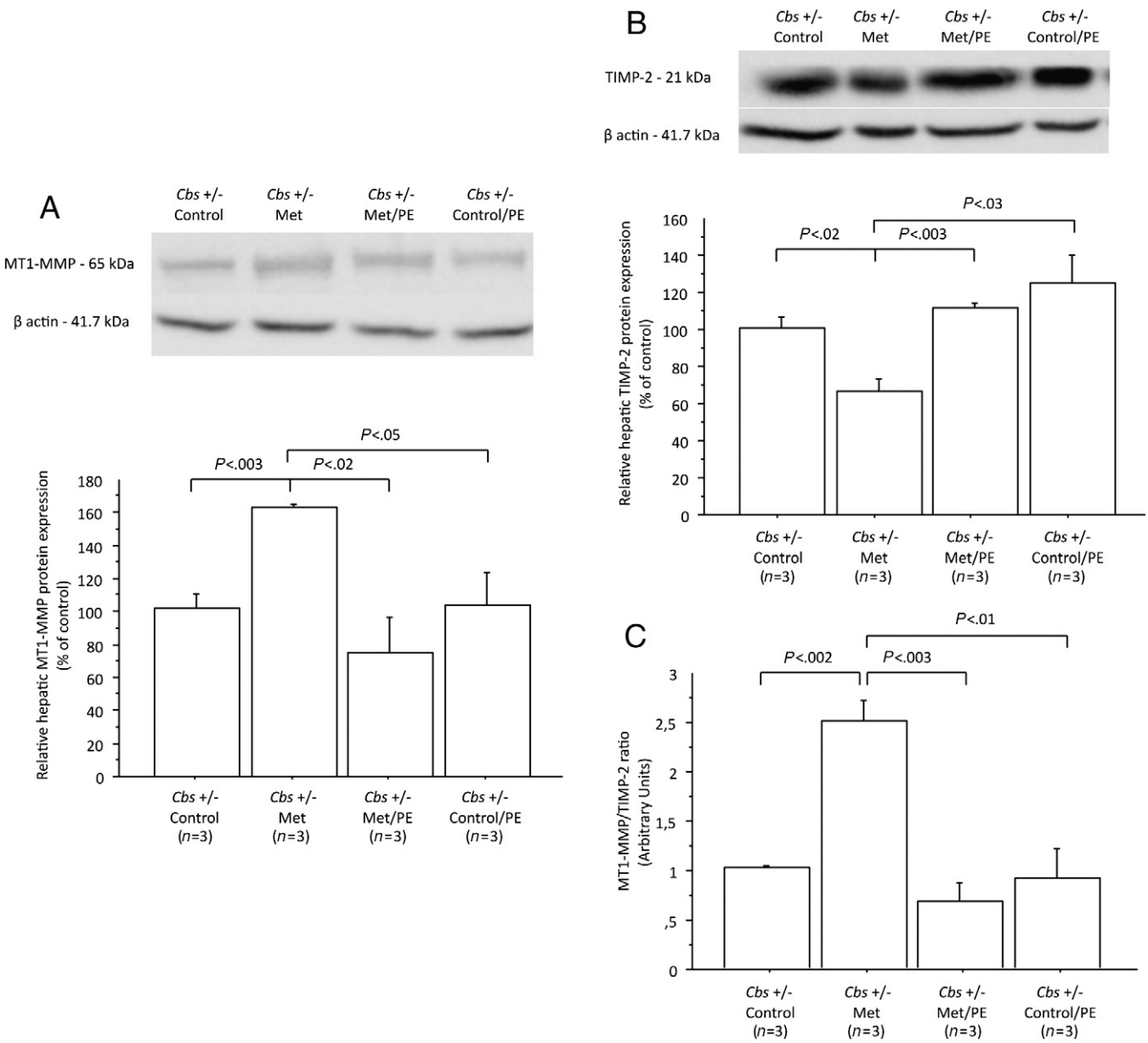

Fig. 3. Effects of PE administration on MMP-2 metabolism via a modulation of TIMP-2/MT1-MMP balance. Comparison of relative hepatic MT1-MMP protein expression (A), hepatic TIMP-2 protein expression (B) and hepatic MT1-MMP/TIMP-2 ratio (C) obtained from heterozygous *Cbs* deficient (*Cbs* +/−) mice fed a control diet supplemented with (control/PE) or without (control) red wine polyphenol extract or a high-methionine diet supplemented with (Met/PE) or without (Met) red wine polyphenol extract. The values are mean±S.E.M. of *n* (number of mice) mice normalized to the mean of *Cbs* +/− mice fed a control diet. Statistical analysis was done with one-way ANOVA followed by Student's unpaired *t* tests.

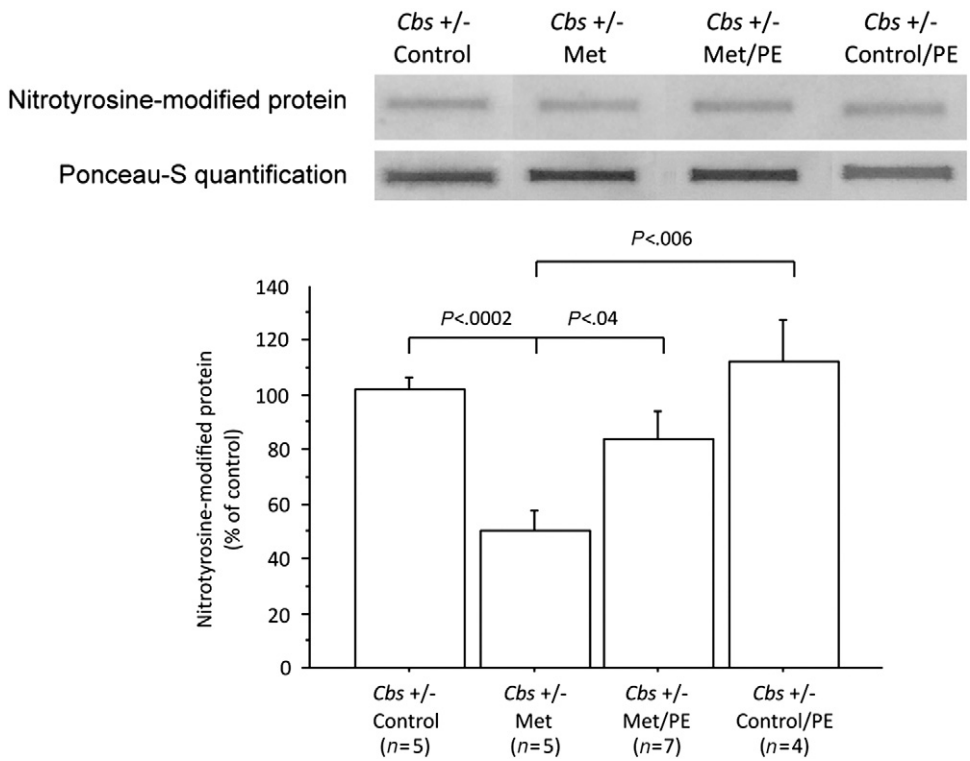

Fig. 4. Effects of PE administration on hepatic peroxynitrite level in hyperhomocysteinemic mice. Comparison of relative nitrotyrosine-modified protein level obtained from heterozygous *Cbs* deficient (*Cbs* +/−) mice fed a control diet supplemented with (control/PE) or without (control) red wine polyphenol extract or a high-methionine diet supplemented with (Met/PE) or without (Met) red wine polyphenol extract. The values are mean±S.E.M. of *n* (number of mice) mice normalized to the mean of *Cbs* +/− mice fed a control diet. Statistical analysis was done with one-way ANOVA followed by Student's unpaired *t* tests.

catalase is the major enzyme involved in H_2O_2 detoxification, we analyzed its hepatic activity. Hepatic catalase activity was approximately 20 % lower in *Cbs* +/− mice fed a methionine-enriched diet than in *Cbs* +/− mice fed a control diet (Fig. 5A, $P<.006$). PE administration significantly restored hepatic catalase activity in *Cbs* +/− mice fed a methionine-enriched diet (Fig. 5A, $P<.03$) but had no effect on *Cbs* +/− mice fed a control diet (Fig. 5A). Moreover, this modulation did not occur at the mRNA level (data not shown). As NADPH oxidase is the major enzyme of O_2^- production, we analyzed its hepatic activity. We found no effect of methionine-enriched diet and PE administration on NADPH oxidase activity (Fig. 5B). We analyzed endothelial NO synthase (eNOS) gene expression. We observed a decrease of eNOS gene expression in *Cbs* +/− mice fed a methionine-enriched diet in comparison to *Cbs* +/− mice fed a control diet (Fig. 5C, $P<.0009$). PE administration increased eNOS mRNA expression in *Cbs* +/− mice fed a methionine-enriched diet (Fig. 5C, $P<.002$). However, PE administration decreased eNOS mRNA expression in *Cbs* +/− mice fed a control diet (Fig. 5C, $P<.0003$).

3.6. Effect of PE administration on hepatic fibrosis in hyperhomocysteinemic mice

The effect of PE administration on hepatic fibrosis was analyzed on Sirius red stained paraffin-embedded liver sections. We found a strong increase in collagen deposit around vessels in liver of *Cbs* +/− mice fed a high methionine-enriched diet (Fig. 6B) in comparison to *Cbs* +/− mice fed a control diet (Fig. 6A and E, $P<.007$). PE supplementation reversed the development of hepatic fibrosis in *Cbs* +/− mice fed a high methionine-enriched diet (Fig. 6C, E, $P<.006$) and diminished also collagen deposit in *Cbs* +/− mice fed a control diet even if this decrease was not significant (Fig. 6D and E, $P<.18$).

4. Discussion

Hyperhomocysteinemia leads to several clinical manifestations and particularly liver disease. Kinsell et al. found that methionine metabolism deficiency has been associated with hepatic fibrosis development [23]. Later, homocysteine has been shown to be the key mediator of methionine metabolism deficiency in the development of hepatic fibrosis [24]. Homozygous CBS-deficient mice, a mouse model of severe hyperhomocysteinemia, present some liver injuries such as inflammation and fibrosis [4]. Here, we also found a strong increase of hepatic fibrosis in heterozygous *Cbs*-deficient mice fed a high methionine-enriched diet, which showed an increase of plasma total homocysteine level from moderate to intermediate. Moreover, the comparison of thiol compounds between humans and mice showed that heterozygous *Cbs* deficient mice fed the methionine-enriched diet present a plasma thiol compounds profile more closely related to human one than that of wild type mice fed a methionine-enriched diet [25]. Hyperhomocysteinemia-induced hepatic fibrosis is mediated by the enhanced activity of MMP-2, which has also been found activated in aorta of hyperhomocysteinemic mice [6] and in cultured human vascular smooth cells [26]. However, the increased activity of MMP-2 was not due to its increased expression in liver of hyperhomocysteinemic mice. MMPs are a large family of zinc-containing calcium-dependent endopeptidase involved in several pathologies such as cancer, atherosclerosis or fibrosis [27]. They degrade extracellular matrix proteins and collagen fibers leading to cell invasion. They are secreted as proenzymes that need to be activated by cleavage. MMP-2, a gelatinase involved in thrombosis development [9] is activated by MT1-MMP at the cell surface and inhibited by TIMP-2. Here we found not only an increased expression of MT1-MMP, but also a decreased expression of TIMP-2 in liver of hyperhomocysteinemic mice, which led to an increased MT1-MMP/

TIMP-2 ratio. This increased ratio could explain the increased MMP-2 activity despite the decreased mRNA and protein level.

We report that the correlation between hepatic MMP-2 activity and plasma homocysteine level depends on the severity of hyperhomocysteinemia. In normal conditions, there is no correlation between these two parameters, but in moderate and mild hyperhomocysteinemia, there were different correlations between plasma homocysteine level and hepatic MMP-2 activity. The change of correlation at the threshold value of 30 μM, between moderate and mild hyperhomocysteinemia, may be explained by the direct effect of homocysteine onto MMP-2 protein as explained by Bescond et al [28]. They proposed a model, in vitro, where homocysteine could directly interact and made a disulfur bond with free cysteine at the surface level of the protein leading to a non-cleaved activated pro-MMP-2 form. But with an increase of homocysteine level, the generation of another disulfur bound leads to a non-cleaved inactivated pro-MMP-2 forms. We hypothesized that there is a competition between homocysteine level and MT1-MMP/TIMP-2 balance to activate MMP-2.

In aortic smooth muscle cell culture, nitrotyrosine has been found to modulate MMP-2 mRNA level [22]. Nitrotyrosine is generally obtained from the reaction between peroxynitrite ($ONOO^-$) and tyrosine residue. Peroxynitrite is the product of reaction between superoxide anion (O_2^-) or hydrogen peroxide (H_2O_2) with NO. We found a nitrotyrosine-modified protein profile similar to MMP-2 mRNA profile leading us to consider that in liver of hyperhomocysteinemic mice, MMP-2 expression is controlled by nitrotyrosine level. To explain this decrease in hyperhomocysteinemic mice, we analyzed several biomarkers of oxidative stress that promote peroxynitrite generation. Catalase activity, which is the major enzyme involved in H_2O_2 detoxification, has been shown to be up-regulated in liver of *Cbs* +/− mice in comparison to *Cbs* +/+ mice in order to detoxify liver from O_2^- generated by increased NADPH oxidase activity [29]. Catalase activity and eNOS expression have been found to be diminished in liver of hyperhomocysteinemic mice, and we also found an increase of H_2O_2 in liver of hyperhomocysteinemic mice (unpublished data). A small increase of H_2O_2 has been shown to induce the interaction of SH3 domains of Arg and c-abl, two non-receptor tyrosine kinases, with the proline-rich (PFNP) motif at the surface of catalase leading to its phosphorylation and activation in vitro [30]. However, these authors showed in the same study that a stronger increase of H_2O_2 level leads to a dissociation of Arg and c-abl from catalase resulting to a decreased enzymatic activity. Although we found an increase of H_2O_2 and no variation of hepatic NADPH oxidase activity in liver of hyperhomocysteinemic mice, a decrease of eNOS mRNA expression was also noted leading to consider a decrease in NO metabolites and thus its availability. Reduced eNOS mRNA level has been associated with an increase of tumor necrosis factor α (TNF-α) level in lung tissues [31]. We have shown an increase of TNF-α mRNA level in liver [4] and aorta [17] of hyperhomocysteinemic mice. Furthermore, homocysteine has been shown to reduce NO availability in bovine aortic endothelial cells [32] even if in this study they found no variation of eNOS protein expression and activity. Taken together, these results emphasized the diminution of peroxynitrite formation in liver of hyperhomocysteinemic mice.

Beyond the antioxidant properties of polyphenols, it has been described a beneficial effect of green tea catechins on rat hepatic stellate cells via an inhibition of collagen production and MMP-1 collagenase activity [33] or MMP-2 activation [14]. In a previous study, we have shown a beneficial effect of PE, which contains catechins, onto homocysteine metabolism via a modulation of CBS

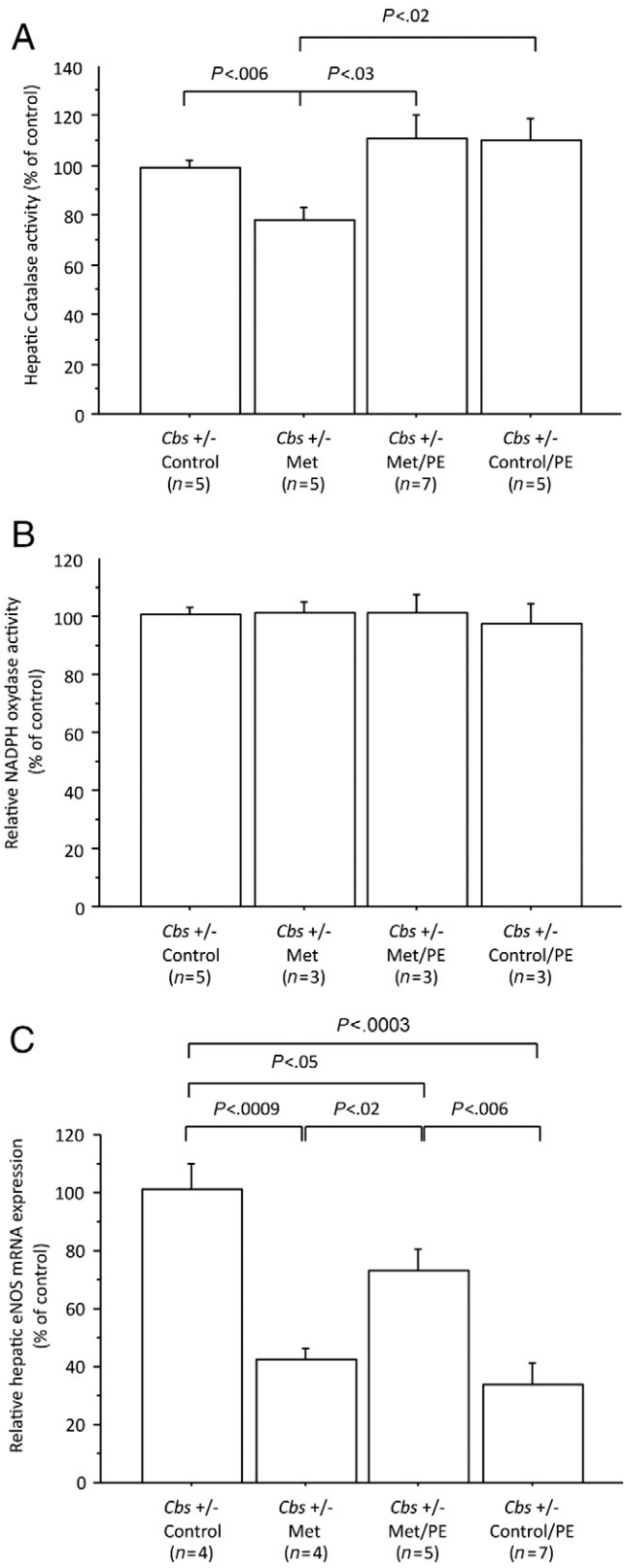

Fig. 5. Effects of PE administration on hepatic biomarkers of oxidative stress in hyperhomocysteinemic mice. Comparison of relative hepatic Catalase activity (A), relative hepatic NADPH oxidase activity (B) and relative hepatic eNOS mRNA expression (C) obtained from heterozygous *Cbs* deficient (*Cbs* +/−) mice fed a control diet supplemented with (control/PE) or without (control) red wine polyphenol extract or a high-methionine diet supplemented with (Met/PE) or without (Met) red wine polyphenol extract. The values are mean±S.E.M. of *n* (number of mice) mice normalized to the mean of *Cbs* +/− mice fed a control diet. Statistical analysis was done with one-way ANOVA followed by Student's unpaired *t* tests.

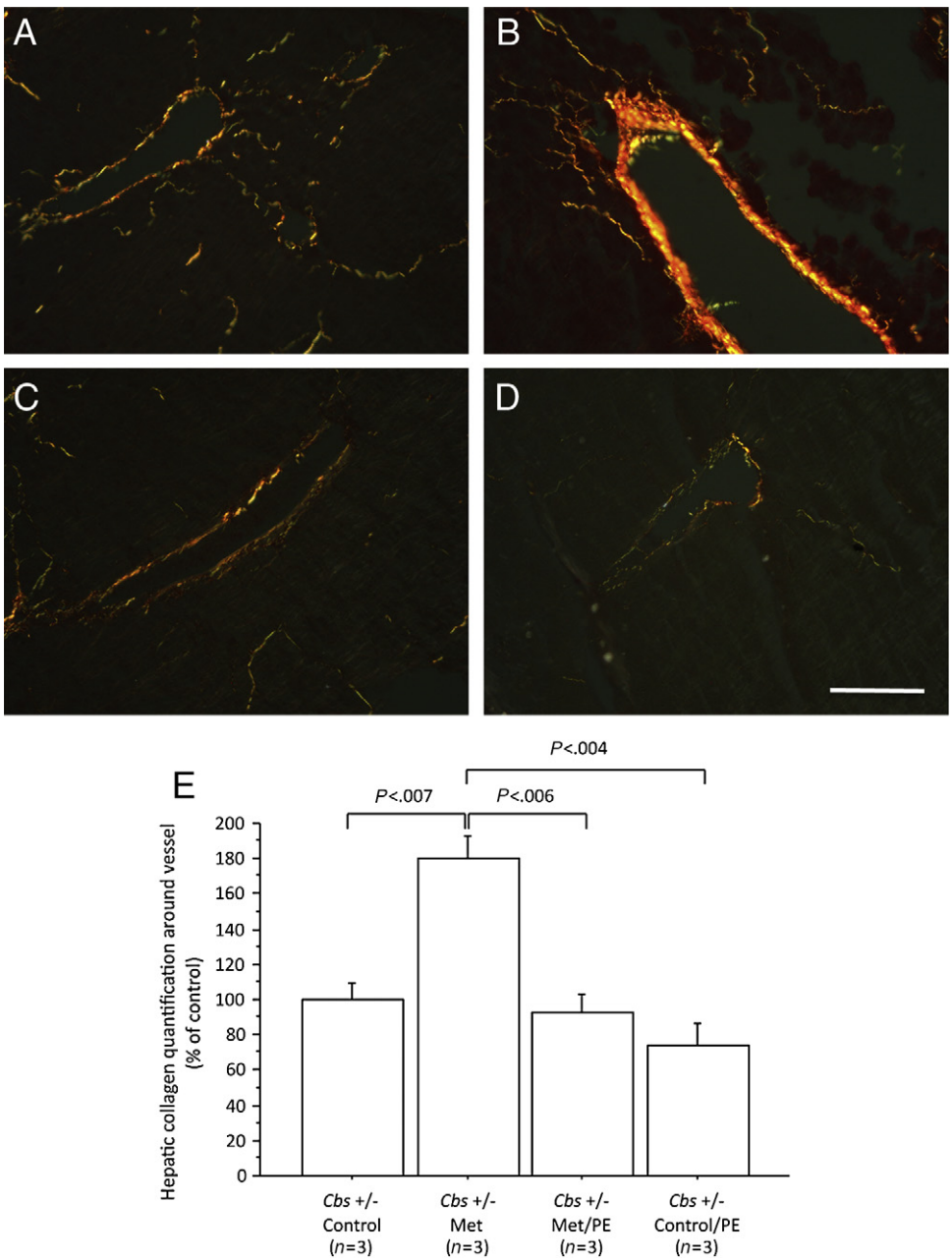

Fig. 6. Effect of PE administration on hepatic fibrosis in hyperhomocysteinemic mice. Paraffin-embedded section of liver from heterozygous *Cbs* deficient (*Cbs* +/−) mice fed a control diet supplemented with (D, control/PE) or without (A, control) red wine polyphenol extract or a high-methionine diet supplemented with (C, Met/PE) or without (B, Met) red wine polyphenol extract were stained for collagen determination by Red Sirius. Scale bar=100 μm. Fibrosis quantification (E) was done on polarized light using iPLab software. The values are mean±S.E.M. of *n* (number of mice) mice normalized to the mean of *Cbs* +/− mice fed a control diet. Statistical analysis was done with one-way ANOVA followed by Student's unpaired *t* tests.

activity in liver of hyperhomocysteinemic mice [17]. A study using the same PE has demonstrated a modulation of MMP-2 activation via direct inhibition of MT1-MMP in vitro [11]. However, none is known about the effect of a polyphenolic supplementation onto MT1-MMP in vivo neither onto TIMP-2 in vitro and in vivo. Here we show, for the first time, a beneficial effect of PE onto MMP-2 activity in vivo via a modulation of MT1-MMP/TIMP-2 balance. We show that administration of PE reduces the MMP-2 activation in liver of hyperhomocysteinemic mice, and counteracts the decrease of mRNA and protein levels. However, there is a discrepancy between mice fed the control diet supplemented with or without PE in MMP-2 protein expression. Nevertheless, the MMP-2 activity is the same in both groups.

The decrease of hepatic MMP-2 activity is followed by a reversal of hepatic fibrosis development in hyperhomocysteinemic mice. The increase of hepatic MMP-2 mRNA and protein level follow the increase of peroxynitrite level. Indeed, we found an increase of hepatic catalase activity and an increase of hepatic eNOS mRNA expression which could lead to an increase of NO availability. However, there is a discrepancy between mice fed the control diet supplemented with or without PE in eNOS mRNA expression. Nevertheless, the decreased mRNA expression of eNOS in mice fed the control diet supplemented with PE has no effect on peroxynitrite level. Polyphenols have been found to modulate expression and activity of catalase and eNOS in several tissues. Resveratrol, a red wine

polyphenol, increases catalase activity in guinea pig cardiac tissue [34] or rat liver [35] while red wine polyphenols extract increases phosphorylation of eNOS in porcine coronary arteries [36] and in aorta and carotid artery of Zucker Fatty rat, a model of obesity, with an increase of NO bioavailability [37].

Taken together, our results show that the development of hepatic fibrosis is prevented by red wine PE supplementation via a modulation of MMP-2 activity in hyperhomocysteinemic mice. They also emphasize the beneficial effect of red wine PE supplementation in case of hyperhomocysteinemia.

Acknowledgment

We thank Dr. N. Maeda (Department of Pathology, University of North Carolina, Chapel Hill, NC) for providing heterozygous *Cbs* mice. We thank C. Imbert, F. Lequellec, and A. Djemat for technical assistance. We acknowledge the technical plateform "Séparation, caractérisation et quantification de biomolécules" (Unité de Biologie Fonctionnelle et Adaptative, Université Paris Diderot-Paris 7, CNRS EAC 4413) for provision of Q-PCR facilities.

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