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Effects of red wine polyphenolic compounds on paraoxonase-1 and lectin-like oxidized low-density lipoprotein receptor-1 in hyperhomocysteinemic mice☆

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Abstract

Hyperhomocysteinemia, or abnormally high plasma homocysteine (Hcy) concentration, has often been associated with vascular thrombosis and the development of premature atherosclerosis. Many studies have shown that moderate wine consumption has potential beneficial effects related to the prevention of atherosclerosis, in part attributed to the biological properties of polyphenolic components, mainly flavonoids. The aim of the present study is to determine the effects of a red wine polyphenolic extract (PE) administration on hyperhomocysteinemia due to cystathionine β-synthase (CBS) deficiency and on the associated biochemical markers of hepatic and endothelial dysfunctions in mice. Red wine PE was added for 4 weeks to the drinking water of heterozygous CBS-deficient mice fed a high-methionine diet, a murine model of hyperhomocysteinemia. Red wine PE supplementation at low dose significantly reduced plasma Hcy levels and restored the hepatic and plasma-decreased paraoxonase-1 activity induced by chronic hyperhomocysteinemia. Moreover, aortic expression of proinflammatory cytokines and adhesion molecules and levels of soluble lectin-like oxidized low-density lipoprotein receptor-1 were reduced in hyperhomocysteinemic mice fed the red wine PE supplementation. These findings suggest that red wine PE administration in low quantities has beneficial effects on biochemical markers of endothelial dysfunction due to hyperhomocysteinemia.

Keywords: Polyphenols; Homocysteine; Mice; Aorta; Cystathionine β-synthase; Adhesion molecules

1. Introduction

Homocysteine (Hcy) is a thiol-containing amino acid produced during methionine metabolism. Once Hcy is formed, it may be recycled to methionine after remethylation or also undergo condensation with serine to form cystathionine. Conversion of Hcy to cystathionine is catalyzed by the vitamin B_6-dependent enzyme cystathionine β-synthase (CBS), the first enzyme involved in the transsulfuration pathway [1]. Normal concentrations of total Hcy (tHcy) in plasma range from 5 to 15 μM, and an elevated plasma Hcy level is denoted hyperhomocysteinemia. An inborn error of metabolism, CBS deficiency, results in elevated levels of Hcy in plasma [2].

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Elevated plasma Hcy level is now recognized as an important vascular risk factor for atherosclerosis in the coronary, cerebrovascular and peripheral arterial circulation [3–5], even if the degree of hyperhomocysteinemia is moderate. Experimental evidence suggests that hyperhomocysteinemia leads to endothelial dysfunction [6], which plays a crucial role in the pathogenesis of atherothrombotic vascular disease, both as a target and as a mediator of the disease process. Animal models of moderate and intermediate hyperhomocysteinemia induced by the combination of genetic and dietary approaches, heterozygous CBS-deficient mice [7] fed a methionine-enriched diet, show that a mild increase in Hcy alone is sufficient to induce endothelial dysfunction [8,9].

Moderate wine consumption appears to have potential beneficial effects related to the prevention of atherosclerosis [10]. This is in part attributed to the biological properties of polyphenolic compounds, mainly flavonoids and resveratrol [10,11]. It has been demonstrated that some flavonoids, like quercetin and catechin, increase serum paraoxonase-1 (PON1) activity in mice [12], due to their antioxidant properties. PON1 is synthesized in the liver and secreted into the serum as a high-density lipoprotein (HDL)-associated protein and plays a major role in the protective role of HDL against coronary artery disease [13]. In the vessel lumen, PON1 hydrolyzes oxidized cholesteryl esters and phospholipids in oxidized lipoproteins, thereby inhibiting the lipid peroxidation products from binding the low-density lipoproteins (LDLs) [14,15]. As we have found a reduced activity of PON1 associated with a reduced gene expression in liver of CBS-deficient mice [16,17], we have recently studied the effects of quercetin and catechin on the impaired PON1 gene expression induced by hyperhomocysteinemia *in vivo*. We showed that catechin, but not quercetin, counteracts Hcy-induced impairment of PON1 gene expression and activity in liver of hyperhomocysteinemic mice due to CBS deficiency [18]. Moreover, catechin administration to CBS-deficient mice reduced plasma Hcy level [18]. However, since food products contain different polyphenols, it seems to be relevant to continue our study with a natural source of polyphenols.

The aim of the present study was to analyze the effects of a red wine polyphenolic extract (PE), which contains, notably, catechin [19], on hyperhomocysteinemia and on the associated biochemical markers of hepatic dysfunction and biochemical markers of endothelial dysfunctions in mice. We applied mice model of hyperhomocysteinemia and triggered hyperhomocysteinemia by combination of genetic (heterozygous CBS-deficient mice) and dietary (feeding with high-methionine diet) approaches. Then, we have investigated outcomes of PE administration on Hcy level, on CBS and PON1 activities and on the expression level of markers of endothelial dysfunction, adhesion molecules, proinflammatory cytokines and the lectin-like oxidized LDL (OxLDL) receptor-1 (LOX-1).

2. Methods and materials

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) [7]. *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 [7].

Mice were fed a standard laboratory diet (A03, Safe-UAR, Augy, France) ad libitum. 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, France) in drinking water for 3 months; (c) high-methionine diet with 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}) [19]. 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. 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 1/10 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 and aorta were harvested, snap-frozen and stored at −80°C until use. Plasma tHcy was assayed by using the fluorimetric high-performance liquid chromatography method described by Fortin and Genest [20]. Levels of LOX-1 were determined using an ELISA from R&D Systems Inc. (R&D Systems Europe, Lille, France).

2.3. Determination of CBS activity

CBS activity assay was performed on 400 μg of total proteins obtained from liver samples, determined by Bradford method, as previously described [21]. Proteins were incubated for 1 h at 37°C with 1 mM of propargylglycine (Sigma-Aldrich), 0.2 mM of pyridoxal 5′-phosphate (Sigma-Aldrich), 10 mM of L-serine (Sigma-Aldrich), 10 mM of DL-Hcy (Sigma-Aldrich) and 0.4 mM of *S*-adenosylmethionine (Sigma-Aldrich), using 5,5′-dithiobis-(2-nitrobenzoic acid)-based assay (Sigma-Aldrich).

2.4. Determination of PON1 activity

PON1 activity assay was performed on 200 μg of total proteins obtained from liver samples or 5 μL of plasma. PON1 arylesterase activity toward phenyl acetate was quantified spectrophotometrically using 20 mM of Tris–HCl, pH 8.3, with 1 mM of $CaCl_2$ and 10 mM of phenyl acetate (Sigma-Aldrich). The reaction was performed at room temperature for 1 min by measuring the appearance of phenol at 270 nm with the use of a continuous and automated recording spectrophotometer. All values were corrected for nonenzymatic hydrolysis.

2.5. RNA extraction and determination of mRNA levels

mRNA was prepared from liver or aorta with the Micro-FastTrack mRNA isolation kit (Invitrogen, Cergy-Pontoise). The quantity and purity of the RNA were assessed by measuring absorbance at 260 and 280 nm. Reverse transcription was carried out on 150 ng mRNA as described by the manufacturer (Ambion, UK). 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 FastStart DNA Master SYBR Green I Kit, Roche Diagnostics) containing a final concentration of 3 mM $MgCl_2$ and 0.5 μM of primers in a final volume of 10 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 duplicate. ΔΔCp analysis of the results allows to assess the ratio of the target mRNA versus control mRNA [22].

2.6. Lipid peroxidation

The lipid peroxidation content of liver homogenates and plasma was estimated by assessing the malondialdehyde (MDA) level using a Lipid Hydroperoxide Assay kit (Cayman, USA).

2.7. Data analysis

Statistical analysis was done with one-way ANOVA followed by Student's unpaired *t* test using Statview software. The results are expressed as mean±S.E.M. Data were considered significant when $P<.05$.

3. Results

3.1. Effects of low and high doses of red wine PE administration on plasma tHcy level and hepatic PON1 activity in wild-type mice

In order to determine the proper dose that will show the most beneficial effect in mice, we have applied two different concentrations of PE based on the catechin content. The daily intake was 3 mg of polyphenols for low PE (LPE), which contains 25 μg of catechin, and 12 mg of polyphenols for high PE (HPE), which contains 100 μg of catechin. In order to determine tHcy levels in male and female wild-type [*Cbs* (+/+)] mice fed the diet supplemented with LPE or HPE in the drinking water, we analyzed the serum. Even if the tHcy level in male *Cbs* (+/+) mice was lower than that in female *Cbs* (+/+) mice, we found the same effect with the LPE or HPE supplementation in male and female *Cbs* (+/+) mice. *Cbs* (+/+) mice fed the diet supplemented with LPE showed a nonsignificant decrease of plasma tHcy levels (Fig. 1A), while *Cbs* (+/+) mice fed the diet supplemented with HPE showed a significant increase of plasma tHcy levels (Fig. 1B).

Table 1
Primer sequences for Q-PCR

Gene	Left primer	Right primer
E-selectin	AGCTACCCATGGAACACGAC	CGCAAGTTCTCCAGCTGTT
Fez-1	CCAGCTGCAGGTGTTCAGT	TCGCTGGCCTTAGTGTTCACC
Icam-1	GAGTTTTACCAGCTATTTATTGAGTACCC	CTCTCACAGCATCTGCAGCAG
IL-6	CCACGGCCTTCCCTACTTCA	TGCAAGTGCATCATCGTTGTTC
Lox-1	GACTGGCTCTGGCATAAAGA	CCTTCTTCTGACATATGCTG
Pon1	TCCAGGCTTACTGGGATCGAAA	CCTCGTGGGACTGGTGTTGG
Tnf-α	CGGTGCCTATGTCTCAGCCTCT	CACTCCAGCTGCTCCTCCACTT
Vcam-1	TTAAAGTCTGTGGATGGCTCGTAC	CTTAATTGTCAGCCAACTTCAGTCTT
Sod1	TGGGGACAATACACAAGGCTGT	TTTCCACCTTTGCCCAAGTCA

All primers are listed 5′ to 3′.

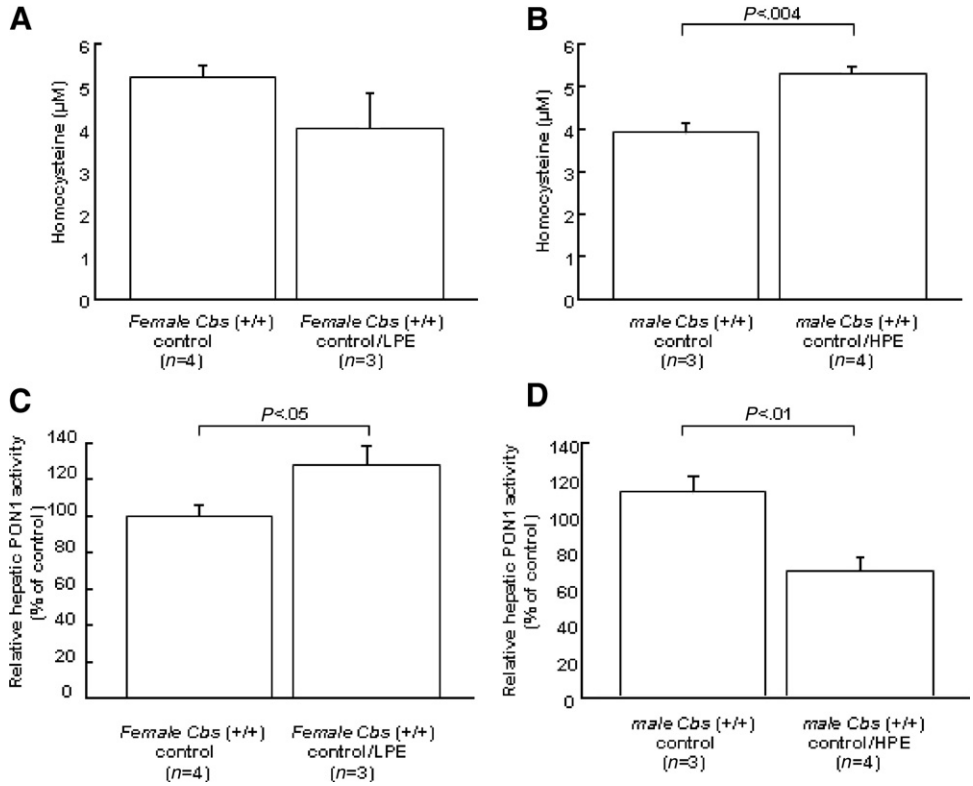

Fig. 1. Plasma tHcy levels (A and B) and hepatic PON1 activity (C and D) in wild-type [*Cbs* (+/+)] mice fed the control diet supplemented with low (LPE) (A and C) or high (HPE) (B and D) red wine PE. The hepatic activity values are mean±S.E.M. of *n* mice normalized to the mean of *Cbs* (+/+) mice fed the control diet. Statistical analysis was done with one-way ANOVA followed by Student's unpaired *t* tests.

As we have previously demonstrated that PON1 activity was negatively correlated with plasma tHcy levels in mice [16,23], we assayed the effect of diet supplemented with LPE and HPE on hepatic PON1 activity in hyperhomocysteinemic mice. *Cbs* (+/+) mice fed the diet supplemented with LPE showed a significant increase of hepatic PON1 activity (Fig. 1C), while *Cbs* (+/+) mice fed the diet with HPE showed a significant decrease of hepatic PON1 activity (Fig. 1D). As we found a deleterious effect of HPE and a beneficial effect of LPE in *Cbs* (+/+) mice, we then used LPE in order to investigate the beneficial effects of PE administration on hyperhomocysteinemia.

3.2. Effects of low red wine PE administration on Hcy metabolism in hyperhomocysteinemic mice

We have found that chronic administration of catechin significantly reduced plasma tHcy level in hyperhomocysteinemic mice by increasing the rate of catabolism of Hcy [18]. Therefore, we also determined if LPE administration has the same effect on hyperhomocysteinemia in mice. As expected, *Cbs* (+/−) mice fed the high-methionine diet (Met) showed a significant decrease of CBS activity (Fig. 2) and a significant increase in plasma tHcy level (Table 2) when compared with *Cbs* (+/−) mice fed the control diet (control), suggesting that the low activity of CBS might be associated, at least in part, with the hyperhomocysteinemia in *Cbs* (+/−) mice fed the high-methionine diet. LPE administration augmented the hepatic activity of CBS (Fig. 2) and

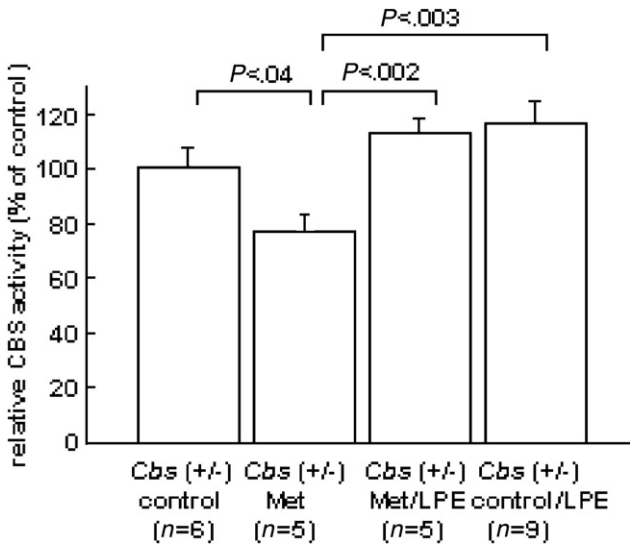

Fig. 2. Comparison of relative hepatic CBS activity obtained from female heterozygous [*Cbs* (+/−)] mice fed the control diet supplemented with (LPE) or without (control) low red wine PE or a high-methionine diet supplemented with (Met/LPE) or without (Met) low red wine PE. The values are mean±S.E.M. of *n* mice normalized to the mean of *Cbs* (+/−) mice fed the control diet. Statistical analysis was done with one-way ANOVA followed by Student's unpaired *t* tests.

Table 2
Plasma tHcy levels in female heterozygous CBS-deficient mice fed the control diet supplemented with (LPE) or without low red wine PE (control) or high-methionine diet supplemented with (Met/LPE) or without low red wine PE (Met)

Diet	tHcy (μM), mean±S.E.M. (range)
Control (*n*=6)	10.1±1.4 (5.6–14.3)
Met (*n*=5)	30±4.3** (18.3–45)
Met/LPE (*n*=4)	19.6±1.8*,† (14.7–23.1)
Control/LPE (*n*=9)	11.3±1.1††,‡ (6.3–15.5)

Statistical analysis was done with one-way ANOVA followed by Student's unpaired *t* tests.
* *P*<.003 (vs. control diet).
** *P*<.001 (vs. control diet).
† *P*<.08 (vs. Met diet).
†† *P*<.0002 (vs. Met diet).
‡ *P*<.002 (vs. Met/LPE diet).

diminished the plasma tHcy level (Table 2) in *Cbs* (+/−) mice fed the high-methionine diet (Met/LPE, Fig. 2), even if the decrease is statistically nonsignificant. However, the plasma tHcy levels remained statistically elevated as compared with *Cbs* (+/−) mice fed the control diet (control, Table 2). We also found that LPE administration alone did not influence CBS activity (Fig. 2) and plasma tHcy level (Table 2) in *Cbs* (+/−) mice fed the control diet (control/LPE). These results show a beneficial effect of LPE supplementation on Hcy metabolism in hyperhomocysteinemic mice.

3.3. Effects of low red wine PE administration on hepatic activity and gene expression of PON1 in hyperhomocysteinemic mice

We showed that catechin supplementation counteracts Hcy-induced impairment of PON1 gene expression and activity in liver of hyperhomocysteinemic mice due to CBS deficiency [18]. Therefore, to investigate whether LPE supplementation can counteract decrease in PON1 activity triggered by hyperhomocysteinemia, we analyzed the hepatic enzyme activity and gene expression of PON1. The mean hepatic activity of PON1 in *Cbs* (+/−) mice fed the high-methionine diet (Met, Fig. 3A) was approximately 40% lower than that in *Cbs* (+/−) mice fed the control diet (control, Fig. 3A). Even if the LPE-supplemented diet (control/LPE, Fig. 3A) did not show statistical difference with the control diet (control, Fig. 3A), PE administration significantly augmented the hepatic activity of PON1 in *Cbs* (+/−) mice fed the high-methionine diet (Met/LPE, Fig. 3A). To determine the effect of diet supplemented with LPE on *Pon1* mRNA expression in liver of hyperhomocysteinemic mice, we isolated mRNA from livers from the four groups of *Cbs* (+/−) mice and we assayed mRNA expression of *Pon1* using Q-PCR (Fig. 3B). The *Sod1* mRNA was used as endogenous control [16,17]. Commensurate with the difference in hepatic PON1 activity, the *Cbs* (+/−) mice fed the high-methionine diet (Met, Fig. 3B) showed a 40% reduction of *Pon1* mRNA level as compared with the group of *Cbs* (+/−) mice fed the control diet (control, Fig. 3B). LPE administration to the high-methionine diet (Met/LPE, Fig. 3B) up-regulated the *Pon1* mRNA expression by 45%, as compared with the methionine-alone group (Met; Fig. 3B). Moreover, even if the difference is not statistically significant, LPE administration alone (LPE, Fig. 3B) up-regulated the *Pon1* mRNA expression as compared with the control group (control; Fig. 3B).

3.4. Effects of low red wine PE administration on hepatic oxidative modifications in hyperhomocysteinemic mice

Previous results showed an enhanced lipid peroxidation due to oxidative stress in liver of CBS-deficient mice [24]. Interaction of free radical species with polyunsaturated fatty acids results in the production of a variety of aldehydes, such as MDA, thus providing a convenient index of lipid peroxidation. As we found a beneficial effect of LPE supplementation on hepatic PON1 activity, an enzyme that

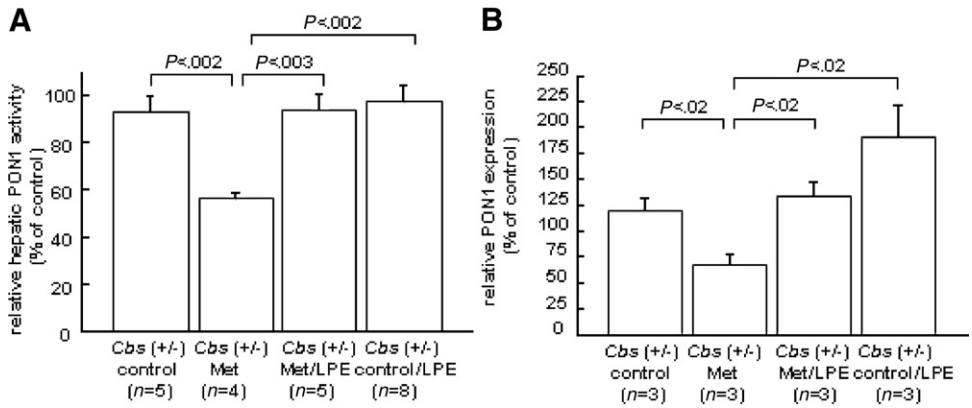

Fig. 3. Comparison of relative hepatic PON1 activity (A) and relative hepatic expression of *Pon1* gene based upon Q-PCR data (B) obtained from female heterozygous [*Cbs* (+/−)] mice fed the control diet supplemented with (LPE) or without (control) low red wine PE or a high-methionine diet supplemented with (Met/LPE) or without (Met) low red wine PE. The values are mean±S.E.M. of *n* mice normalized to the mean of *Cbs* (+/−) mice fed the control diet. Statistical analysis was done with one-way ANOVA followed by Student's unpaired *t* tests.

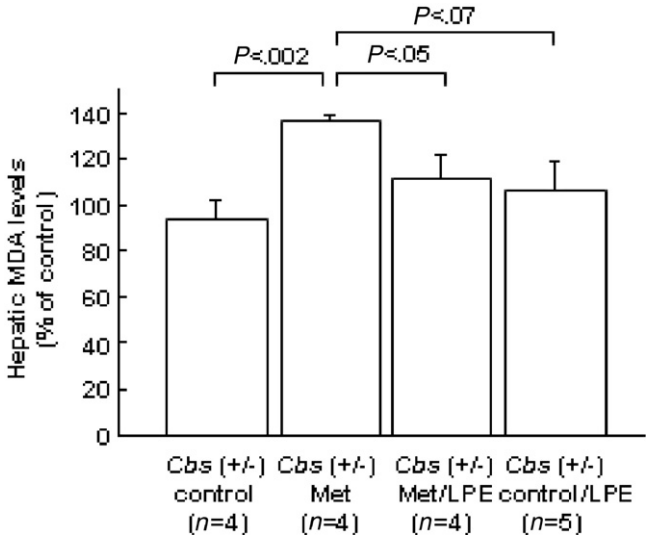

Fig. 4. Comparison of hepatic MDA levels obtained from female heterozygous [*Cbs* (+/−)] mice fed the control diet supplemented with (LPE) or without (control) low red wine PE or a high-methionine diet supplemented with (Met/LPE) or without (Met) low red wine PE. The values are mean±S.E.M. of *n* mice normalized to the mean of *Cbs* (+/−) mice fed the control diet. Statistical analysis was done with one-way ANOVA followed by Student's unpaired *t* tests.

has antioxidant properties, we then analyzed the effect of LPE supplementation on MDA levels in liver of hyperhomocysteinemic mice. As expected, the levels of MDA in liver of *Cbs* (+/−) mice fed the high-methionine diet (Met, Fig. 4) were 45% higher than that in liver of *Cbs* (+/−) mice fed the control diet (control, Fig. 4). Even if LPE

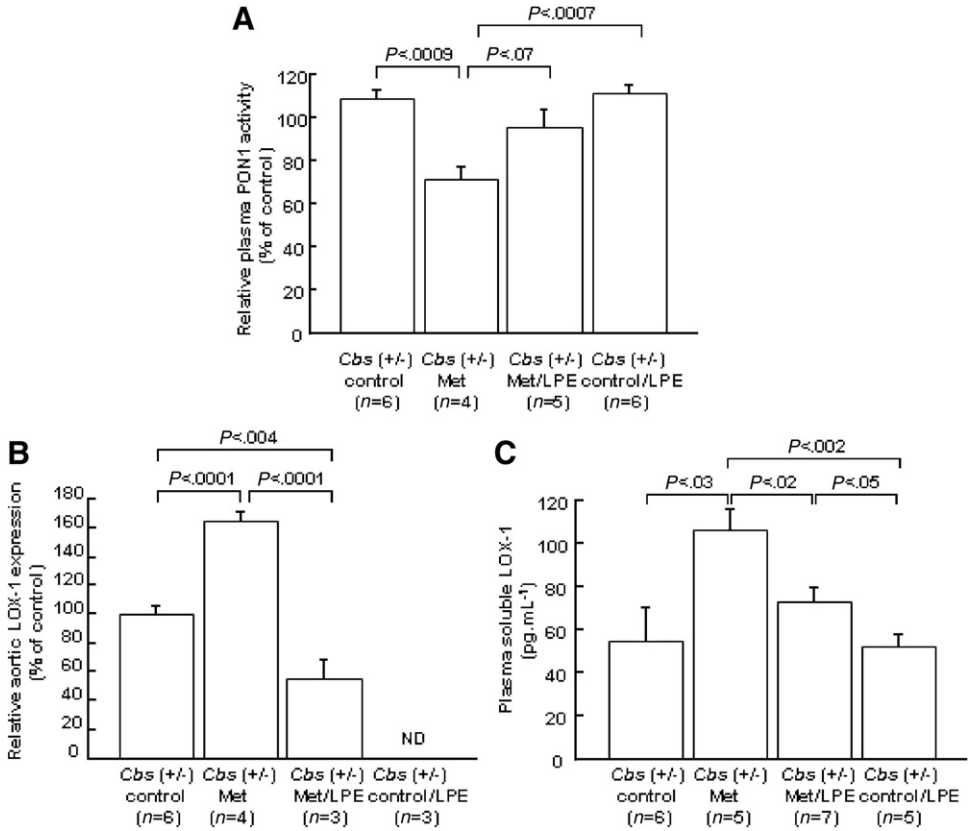

Fig. 5. Comparison of relative plasma PON1 activity (A), aortic expression of *Lox-1* gene based upon Q-PCR data (B) and plasma levels of soluble LOX-1 (C) obtained from female heterozygous [*Cbs* (+/−)] mice fed the control diet supplemented with (LPE) or without (control) low red wine PE or a high-methionine diet supplemented with (Met/LPE) or without (Met) low red wine PE. The values are mean±S.E.M. of *n* mice normalized to the mean of *Cbs* (+/−) mice fed the control diet. Statistical analysis was done with one-way ANOVA followed by Student's unpaired *t* tests. ND, not determined.

administration alone (control/LPE, Fig. 4) did not influence the MDA levels in *Cbs* (+/−) mice fed the control diet (control, Fig. 4), LPE supplementation diminished the hepatic MDA levels by 20% in *Cbs* (+/−) mice fed the high-methionine diet (Met/LPE, Fig. 4).

3.5. Effects of low red wine PE administration on plasma levels of soluble LOX-1 and PON1 activity and on the aortic expression of LOX-1 in hyperhomocysteinemic mice

The decreased levels of the antioxidant enzyme PON1 in the liver of CBS-deficient mice associated with an oxidative stress indicate a possible mechanism of enhanced generation of OxLDLs in hyperhomocysteinemic mice. PON1 not only is synthesized in the liver but is secreted into the serum as well [13]. Therefore, activity of plasma PON1 and aortic LOX-1 expression, a receptor for OxLDLs on endothelial cells [25], were also examined in serum of *Cbs* (+/−) mice fed the high-methionine diet with or without LPE supplementation. The mouse *Sod1* and the *Fez-1* mRNA were used as endogenous controls for aortic mRNA expression. As expected, *Cbs* (+/−) mice fed the high-methionine diet (Met, Fig. 5A) showed a significant decrease of plasma PON1 activity compared to mice fed the control diet (control, Fig. 5A). Commensurate with the decreased plasma PON1 activity, aorta from *Cbs* (+/−) mice fed the high-methionine diet (Met, Fig. 5B) showed a 1.5-fold overexpression of *Lox-l* mRNA as compared to aorta from *Cbs* (+/−) mice fed the control diet (control, Fig. 5B). Even if LPE supplementation alone (control/LPE, Fig. 5A) did not influence the plasma PON1 activity in *Cbs* (+/−) mice fed the control diet (control, Fig. 5A), it greatly diminished the aortic *Lox-l* mRNA expression that it was not quantifiable (Fig. 5B). Moreover, LPE supplementation (Met/LPE) to the high-methionine diet (Met) not only increased the plasma PON1 activity, also not significantly (Fig. 5A), but also decreased *Lox-l* mRNA expression (Fig. 5B). In addition, levels of plasma-soluble LOX-1 in *Cbs* (+/−) mice fed the high-methionine diet (Met) were twofold increased compared to *Cbs* (+/−) mice fed the control diet (control, Fig. 5C). However, even if no difference was found between *Cbs* (+/−) mice fed the LPE supplementation alone (control/LPE, Fig. 5C) and *Cbs* (+/−) mice fed the control diet (control, Fig. 5C), the levels of plasma-soluble LOX-1 were decreased by the LPE supplementation to the high-methionine diet (Met/LPE, Fig. 5C) compared to *Cbs* (+/−) mice fed the high-methionine diet (Met, Fig. 5C).

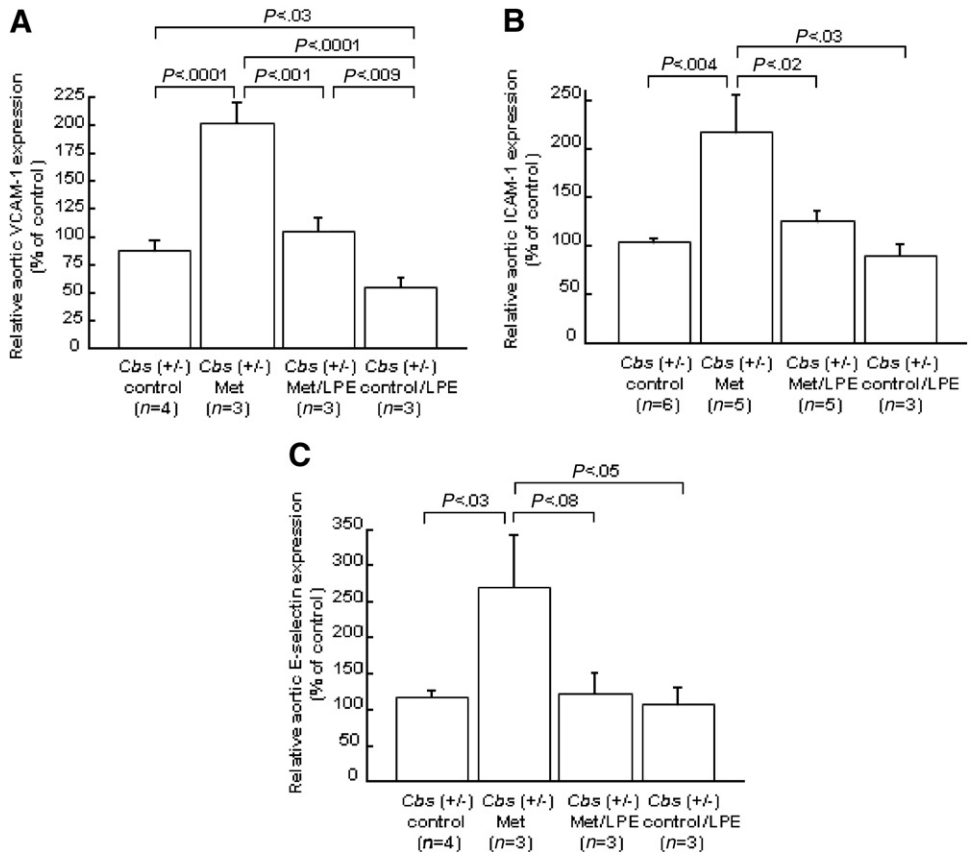

Fig. 6. Comparison of relative aortic expression of *Vcam-1* (A), *Icam-1* (B) and *E-selectin* (C) genes based upon Q-PCR data obtained from female heterozygous [*Cbs* (+/−)] mice fed the control diet supplemented with (LPE) or without (control) low red wine PE or a high-methionine diet supplemented with (Met/LPE) or without (Met) low red wine PE. The values are mean±S.E.M. of *n* mice normalized to the mean of *Cbs* (+/−) mice fed the control diet. Statistical analysis was done with one-way ANOVA followed by Student's unpaired *t* tests.

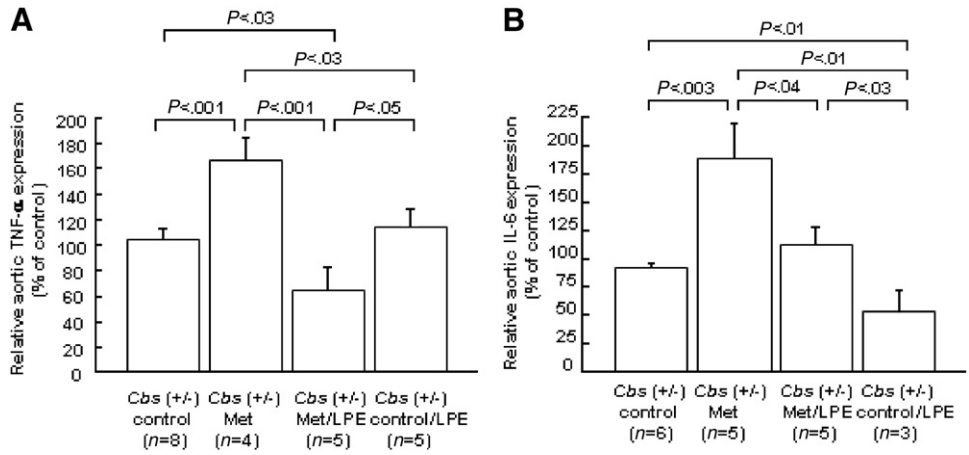

Fig. 7. Comparison of relative aortic expression of *Tnf-α* (A) and *Il-6* (B) genes based upon Q-PCR data obtained from female heterozygous [*Cbs* (+/−)] mice fed the control diet supplemented with (LPE) or without (control) low red wine PE or a high-methionine diet supplemented with (Met/LPE) or without (Met) low red wine PE. The values are mean±S.E.M. of *n* mice normalized to the mean of *Cbs* (+/−) mice fed the control diet. Statistical analysis was done with one-way ANOVA followed by Student's unpaired *t* tests.

3.6. Effects of low red wine PE administration on the aortic expression of biochemical markers of endothelial dysfunction in hyperhomocysteinemic mice

It has been demonstrated that activation of LOX-1 by OxLDLs induces an up-regulation of adhesion molecules like intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) expression [26], which are known to be characteristic of endothelial dysfunction. Therefore, we have investigated the effects of LPE administration on the expression of biochemical markers of endothelial dysfunction in aorta of hyperhomocysteinemic mice. *Cbs* (+/−) mice fed the high-methionine diet (Met, Fig. 6) showed a twofold increase of *Vcam-1* (Fig. 6A), *Icam-1* (Fig. 6B) and *E-selectin* (Fig. 6C) mRNA level as compared with the group of *Cbs* (+/−) mice fed the control diet (control, Fig. 6). LPE administration to the high-methionine diet (Met/LPE, Fig. 6) down-regulated the *Vcam-1* (Fig. 6A), *Icam-1* (Fig. 6B) and *E-selectin* (Fig. 6C) mRNA expression as compared with the methionine-alone group (Met, Fig. 6). However, the difference was not statistically significant for *E-selectin* mRNA expression. Moreover, LPE administration alone (control/LPE, Fig. 6A) down-regulated *Vcam-1* mRNA expression as compared with the control group (control, Fig. 6A).

3.7. Effects of low red wine PE administration on the aortic expression of proinflammatory cytokines in hyperhomocysteinemic mice

Overexpression of adhesion molecules (VCAM-1, ICAM-1, E-selectin) results in enhanced recruitment of inflammatory cells to the endothelium surface, which are known to release proinflammatory cytokines like tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) [27]. We then investigated the effects of LPE administration on the expression of proinflammatory cytokines in aorta of hyperhomocysteinemic mice. The *Cbs* (+/−) mice fed the high-methionine diet (Met, Fig. 7) showed a 1.7-fold increase of *Tnf-α* (Fig. 7A) and *Il-6* (Fig. 7B) mRNA level as compared with the group of *Cbs* (+/−) mice fed the control diet (control, Fig. 7). LPE administration to the high-methionine diet (Met/LPE, Fig. 7) down-regulated the *Tnf-α* (Fig. 7A) and *Il-6* (Fig. 7B) mRNA expression as compared with the methionine-alone group (Met, Fig. 7). LPE administration alone (control/LPE, Fig. 7B) down-regulated the *Il-6* mRNA expression as compared with the control group (control, Fig. 7B).

4. Discussion

Hyperhomocysteinemia is now considered to be an independent risk factor for cardiovascular disease, even if the level is moderate [3]. Heterozygous CBS-deficient mice fed a high-methionine diet showed an increase of plasma tHcy level, from moderate to intermediate, which is sufficient to induce endothelial dysfunction [8,9]. We previously demonstrated that a supplementation of catechin, a polyphenol found in red wine, could reduce the concentration of plasma tHcy via action on hepatic CBS activity [18]. In view of this result, we decided to examine the influence of PE, a natural extract of polyphenols that notably contains catechin, on hyperhomocysteinemia due to CBS deficiency and on biochemical markers of the associated endothelial dysfunction. Homozygous CBS deficiency induces a decrease in glutathione level [28] resulting from the complete blockade of the transsulfuration pathway. Therefore, we used heterozygous Cbs-deficient mice, an intermediate model in which Hcy-independent perturbations are minimal. Moreover, as heterozygous CBS-deficient mice fed a methionine-enriched diet present a plasma thiol compounds profile more closely related to human than that of wild-type mice fed a

methionine-enriched diet [29], we decided to study the effects of PE on Hcy level in this combined genetic and dietary model of hyperhomocysteinemia.

We first determined the dose that shows the most beneficial effect on Hcy level in mice and found a deleterious effect of HPE and a beneficial effect of LPE in wild-type mice. Our results are similar to those obtained with antioxidant vitamin supplementation studies. Although the antioxidant effects of vitamin E have been well documented, high-dose vitamin E supplementation may possess prooxidant effects. This may be in part due to vitamin E displacing other fat-soluble antioxidants at such doses [30]. Moreover, the PE contains not only catechin but also caffeic acid, a common coffee polyphenol [19]. Since high coffee consumption raises plasma tHcy level [31,32], our results emphasize the dose–effect relationship with polyphenolic components on hyperhomocysteinemia.

We then used LPE and found that LPE administration significantly diminished plasma tHcy level that was increased under methionine treatment, as catechin supplementation alone [18]. Moreover, LPE supplementation, like catechin supplementation, can normalize impairment of hepatic CBS activity by the high-methionine diet, which is consistent with plasma tHcy concentration measurement. In order to show if LPE administration can protect liver from Hcy toxicity, PON1 activity and lipid peroxidation were analyzed in liver of mice fed the high-methionine diet supplemented with LPE. We observed a significant reduction in *Pon1* expression and PON1 activity in hyperhomocysteinemic mice liver and an increase of MDA levels, in agreement with previous reports [16,17,24]. LPE administration normalized the hepatic decreased expression and activity of PON1 to control level and attenuated the lipid peroxidation in liver of mice fed the high-methionine diet. Our results are in agreement with the observed *Pon1* expression and PON1 activity under catechin treatment [18]. However, we have not tested the other PE polyphenolic compounds.

We can put forward the hypothesis that enhancing hepatic PON1 activity may be one of the mechanisms by which polyphenols act as antioxidative agents and protect liver against damage under hyperhomocysteinemia. However, the data obtained for the hepatic MDA levels, also found in plasma (data not shown), do not significantly support antioxidant properties of PE, but rather the variation of Hcy levels. In this sense, we did not find any variations of plasma superoxide anion levels (data not shown). LPE administration not only augmented the hepatic activity of PON1 in mice fed the high-methionine diet but also attenuated the decreased plasma PON1 activity. Previous results also reported correction of plasma PON1 activity under red wine extract supplementation in apolipoprotein E-deficient mice [33]. Previous studies on apolipoprotein E-deficient mice revealed that consumption of red wine flavonoids preserves plasma PON1 hydrolytic activity towards lipid peroxides in OxLDLs [12]. Oxidative hypothesis of atherosclerosis assumes that OxLDL generation is necessary for the foam cells and atherosclerotic plaque formation. It is known that to enter the cell barrier, OxLDLs have to attach to a specific receptor, LOX-1. LOX-1 exists in activated vascular endothelial and smooth muscle cells as well as in macrophages, and its expression can be induced by oxLDLs as well as proinflammatory cytokines [34,35]. Thus, LOX-1 can be viewed as a mediator of endothelial dysfunction. We then analyzed whether LPE administration influences expression of *Lox-1* in aorta of hyperhomocysteinemic mice. We found that under high-methionine diet, not only *Lox-1* expression but also plasma levels of soluble LOX-1 were about twofold higher, when compared to mice fed the control diet. Previous results found that hyperhomocysteinemic subjects have enhanced expression of LOX-1 in mononuclear cells [36]. Moreover, it has been demonstrated that Hcy enhanced *Lox-1* mRNA expression in cultured aortic endothelial cells [37]. Here, we found that LPE supplementation decreased not only *Lox-1* mRNA expression in aorta of mice fed the high-methionine diet but also plasma levels of soluble LOX-1, suggesting diminished uptake of OxLDLs by endothelial cells. Other results in apolipoprotein E-deficient mice revealed that administration of red wine reduces the cellular LDL uptake by macrophages [32].

Lox-1 gene expression is notably regulated by cytokines. In resting vascular tissues, LOX-1 mRNA and protein levels are low. However, they increased under proinflammatory disease states [38]. Endothelial dysfunction, oxidative stress and chronic inflammation were proposed as mechanisms by which Hcy promotes atherosclerosis [39]. Then, we have also analyzed the effects of LPE administration on the expression of proinflammatory cytokines and biochemical markers of endothelial dysfunction in aorta of hyperhomocysteinemic mice. As expected, *Vcam-1*, *Icam-1*, *E-selectin*, *Tnf-α*, and *Il-6* mRNA expressions were found to be higher in aorta of mice fed the high-methionine diet when compared to mice fed the control diet, which confirms previous results by immunostaining of aortic sections and ELISA [9,40–42]. Moreover, LPE supplementation to high-methionine diet caused a decrease in these genes' expressions. Previous study also found a beneficial effect of the same PE with a similar dose on induced hypertension and endothelial dysfunction in rats [43].

LPE supplementation led to a diminution of plasma Hcy levels accompanied by a reduction in aortic expression of proinflammatory cytokines, as well as biochemical markers of endothelial dysfunction in mice fed a high-methionine diet. However, as LPE supplementation to the control diet did not influence plasma Hcy level but diminished the aortic expression of *Lox-1*, *Vcam-1* and *Il-6* in *Cbs* (+/−) mice, we can also expect a direct effect of LPE administration on expression of biochemical markers of endothelial dysfunction. In this sense, two recent studies have found a down-regulation of inflammatory markers of atherosclerosis after moderate red wine consumption, without changes in plasma tHcy levels [44,45].

In conclusion, PE administration in low quantities to CBS-deficient mice is able to block the rise of plasma tHcy levels and has beneficial effects on biochemical markers of hepatic and endothelial dysfunction due to hyperhomocysteinemia.

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References

[1] Selhub J. Homocysteine metabolism. Annu Rev Nutr 1999;19:217–46.
[2] Mudd SH, Levy HL, Kraus JP. Disorders of transsulfuration. In: Scriver CR, Beaudet AL, Sly WS, Valle D, Childs B, Kinzler KW, Vogelstein B, editors. The metabolic and molecular bases of inherited disease. New York: McGraw Hill Inc; 2001. p. 2007–56.
[3] Welch GN, Loscalzo J. Homocysteine and atherothrombosis. N Engl J Med 1998;338:1042–50.
[4] Eikelboom JW, Lonn E, Genest Jr J, Hankey G, Yusuf S. Homocyst(e)ine and cardiovascular disease: a critical review of the epidemiologic evidence. Ann Intern Med 1999;131:363–75.
[5] Temple ME, Luzier AB, Kazierad DJ. Homocysteine as a risk factor for atherosclerosis. Ann Pharmacother 2000;34:57–65.
[6] Lentz SR. Mechanisms of thrombosis in hyperhomocysteinemia. Curr Opin Hematol 1998;5:343–9.
[7] Watanabe M, Osada J, Aratani Y, Kluckman K, Reddick R, Malinow MR, et al. Mice deficient in cystathionine β-synthase: animal models for mild and severe homocyst(e)inemia. Proc Natl Acad Sci 1995;92: 1585–9.
[8] Eberhardt RT, Forgione MA, Cap A, Leopold JA, Rudd MA, Trolliet M, et al. Endothelial dysfunction in a murine model of mild hyperhomocyst (e)inemia. J Clin Invest 2000;106:483–91.
[9] Weiss N, Heydrick S, Zhang YY, Bierl C, Cap A, Loscalzo J. Cellular redox state and endothelial dysfunction in mildly hyperhomocysteinemic cystathionine beta-synthase-deficient mice. Arterioscler Thromb Vasc Biol 2002;22:34–41.
[10] Aviram M, Fuhrman B. Wine flavonoids protect against LDL oxidation and atherosclerosis. Ann N Y Acad Sci 2002;957:146–61.
[11] Frankel EN, Kanner J, German JB, Parks E, Kinsella JE. Inhibition of oxidation of human low-density lipoprotein by phenolic substances in red wine. Lancet 1993;341:454–7.
[12] Fuhrman B, Aviram M. Preservation of paraoxonase activity by wine flavonoids: possible role in protection of LDL from lipid peroxidation. Ann N Y Acad Sci 2002;957:321–4.
[13] Durrington PN, Mackness B, Mackness MI. Paraoxonase and atherosclerosis. Arterioscler Thromb Vasc Biol 2001;21:473–80.
[14] Watson AD, Berliner JA, Hama SY, La Du BN, Faull KF, Fogelman AM, et al. Protective effect of high density lipoprotein associated paraoxonase. Inhibition of the biological activity of minimally oxidized low density lipoprotein. J Clin Invest 1995;96: 2882–91.
[15] Aviram M, Rosenblat M, Bisgaier CL, Newton RS, Primo-Parmo SL, La Du BN. Paraoxonase inhibits high-density lipoprotein oxidation and preserves its functions. A possible peroxidative role for paraoxonase. J Clin Invest 1998;101:1581–90.
[16] Robert K, Chasse JF, Santiard-Baron D, Vayssettes C, Chabli A, Aupetit J, et al. Altered gene expression in liver from a murine model of hyperhomocysteinemia. J Biol Chem 2003;278:31504–11.
[17] Janel N, Robert K, Chabert C, Ledru A, Gouedard C, Barouki R, et al. Mouse liver paraoxonase-1 gene expression is downregulated in hyperhomocysteinemia. Thromb Haemost 2004;92:221–2.
[18] Hamelet J, Demuth K, Dairou J, Ledru A, Paul JL, Dupret JM, et al. Effects of catechin on homocysteine metabolism in hyperhomocysteinemic mice. Biochem Biophys Res Commun 2007;255:221–7.
[19] Auger C, Caporiccio B, Landrault N, Teissedre PL, Laurent C, Cros G, et al. Red wine phenolic compounds reduce plasma lipids and apolipoprotein B and prevent early aortic atherosclerosis in hypercholesterolemic golden Syrian hamsters (*Mesocricetus auratus*). J Nutr 2002;132:1207–13.
[20] Fortin LJ, Genest J. Measurement of homocyst(e)ine in the prediction of atherosclerosis. Clin Biochem 1995;28:155–62.
[21] Miller JW, Nadeau MR, Smith D, Selhub J. Folate-deficiency-induced homocysteinaemia in rats: disruption of *S*-adenosylmethionine's coordinate regulation of homocysteine metabolism. Biochem J 1994;298: 415–9.
[22] Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001;29:e45.
[23] Hamelet J, Aït-Yahya-Graison E, Matulewicz E, Noll C, Badel-Chagnon A, Camproux AC, et al. Homocysteine threshold value based on cystathionine beta synthase and paraoxonase 1 activities in mice. Eur J Clin Invest 2007;37:933–8.
[24] Robert K, Nehmé J, Bourdon E, Pivert G, Friguet B, Delcayre C, et al. Cystathionine beta synthase deficiency promotes oxidative stress, fibrosis and steatosis in mice liver. Gastroenterology 2005;128: 1405–15.
[25] Sawamura T, Kume N, Aoyama T, Moriwaki H, Hoshikawa H, Aiba Y, et al. An endothelial receptor for oxidized low-density lipoprotein. Nature 1997;386:73–7.
[26] Li D, Chen H, Romeo F, Sawamura T, Saldeen T, Mehta JL. Statins modulate oxidized low-density lipoprotein-mediated adhesion molecule expression in human coronary artery endothelial cells: role of LOX-1. J Pharmacol Exp Ther 2002;302:601–5.
[27] Libby P. Molecular bases of the acute coronary syndromes. Circulation 1995;91:2844–50.
[28] Vitvitsky V, Dayal S, Stabler S, Zhou Y, Wang H, Lentz SR, et al. Perturbations in homocysteine-linked redox homeostasis in a murine model for hyperhomocysteinemia. Am J Physiol Regul Integr Comp Physiol 2004;287:R39–46.
[29] Likogianni V, Janel N, Ledru A, Beaune P, Paul JL, Demuth K. Thiol compounds metabolism in mice, rats and humans: comparative study and potential explanation of rodents protection against vascular diseases. Clin Chim Acta 2006;372:140–6.
[30] Miller ER, Pastor-Barriuso R, Dalal D, Riemersma RA, Appel LJ, Guallar E. Meta-analysis: high-dosage vitamin E supplementation may increase all-cause mortality. Ann Intern Med 2005; 142:37–46.
[31] Nygard O, Refsum H, Ueland PM, Stensvold I, Nordrehaug JE, Kvale G, et al. Coffee consumption and plasma total homocysteine: the Hordaland Homocysteine Study. Am J Clin Nutr 1997;65: 136–43.
[32] Panagiotakos DB, Pitsavos C, Zampelas A, Zeimbekis A, Chrysohoou C, Papademetriou L, et al. The association between coffee consumption and plasma total homocysteine levels: the "ATTICA" study. Heart Vessels 2004;19:280–6.
[33] Hayek T, Fuhrman B, Vaya J, Rosenblat M, Belinky P, Coleman R, et al. Reduced progression of atherosclerosis in apolipoprotein E-deficient mice following consumption of red wine, or its polyphenols quercetin or catechin, is associated with reduced susceptibility of LDL to oxidation and aggregation. Arterioscler Thromb Vasc Biol 1997;17:2744–52.
[34] Kume N, Murase T, Masaki T, Kita T. Inducible expression of lectin-like oxidized low density lipoprotein receptor-1 in vascular endothelial cells. Circ Res 1998;83:322–7.
[35] Li D, Mehta J. Upregulation of endothelial receptor for oxidized LDL (LOX-1) by oxidized LDL and implications in apoptosis of human coronary artery endothelial cell. Arterioscler Thromb Vasc Biol 2000; 20:1116–22.
[36] Holven KB, Scholz H, Halvorsen B, Aukrust P, Ose L, Nenseter MS. Hyperhomocysteinemic subjects have enhanced expression of lectin-

like oxidized LDL receptor-1 in mononuclear cells. J Nutr 2003;133: 3588–91.
[37] Nagase M, Ando K, Nagase T, Kaname S, Sawamura T, Fujita T. Redox sensitive regulation of LOX-1 gene expression in vascular endothelium. Biochem Biophys Res Commun 2001;281: 720–5.
[38] Kataoka H, Kume N, Miyamoto S, Minami M, Moriwaki H, Murase T, et al. Expression of lectinlike oxidized low-density lipoprotein receptor-1 in human atherosclerotic lesions. Circulation 1999;99: 3110–7.
[39] Sharma P, Senthilkumar RD, Brahmachari V, Sundaramoorthy E, Mahajan A, Sharma A, et al. Mining literature for a comprehensive pathway analysis: a case study for retrieval of homocysteine related genes for genetic and epigenetic studies. Lipids Health Dis 2006;5: 1–19.
[40] Wang G, Woo CW, Sung FL, Siow YL, O K. Increased monocyte adhesion to aortic endothelium in rats with hyperhomocysteinemia: role of chemokine and adhesion molecules. Arterioscler Thromb Vasc Biol 2002;22:1777–83.
[41] Li M, Chen J, Li YS, Feng YB, Gu X, Shi CZ. Folic acid reduces adhesion molecules VCAM-1 expression in aortic of rats with hyperhomocysteinemia. Int J Cardiol 2006;106:285–8.
[42] Hofmann MA, Lalla E, Lu Y, Gleason MR, Wolf BM, Tanji N, et al. Hyperhomocysteinemia enhances vascular inflammation and accelerates atherosclerosis in a murine model. J Clin Invest 2001;107: 675–83.
[43] Sarr M, Chataigneau M, Martins S, Schott C, El Bedoui J, Oak MH, et al. Red wine polyphenols prevent angiotensin II-induced hypertension and endothelial dysfunction in rats: role of NADPH oxidase. Cardiovasc Res 2006;71:794–802.
[44] Sacanella E, Vázquez-Agell M, Mena MP, Antúnez E, Fernández-Solá J, Nicolás JM, et al. Down-regulation of adhesion molecules and other inflammatory biomarkers after moderate wine consumption in healthy women: a randomized trial. Am J Clin Nutr 2007;86:1463–9.
[45] Vázquez-Agell M, Sacanella E, Tobias E, Monagas M, Antúnez E, Zamora-Ros R, et al. Inflammatory markers of atherosclerosis are decreased after moderate consumption of cava (sparkling wine) in men with low cardiovascular risk. J Nutr 2007;137:2279–84.