ELSEVIER

Pharmacological Research 51 (2005) 523–532

Pharmacological research

www.elsevier.com/locate/yphrs

Differential effect of antioxidant treatment on plasma and tissue paraoxonase activity in hyperleptinemic rats

Jerzy Beltowski *, Anna Jamroz-Wiśniewska, Ewelina Borkowska, Grażyna Wójcicka

Department of Pathophysiology, Medical University, ul. Jaczewskiego 8, 20-090 Lublin, Poland

Accepted 31 January 2005

Abstract

Recent studies suggest that adipose tissue hormone, leptin, is involved in atherogenesis, especially in obese subjects. Previously, we have demonstrated that experimentally induced hyperleptinemia decreases plasma paraoxonase 1 (PON1) activity. The aim of this study was to investigate whether treatment with synthetic antioxidant, Tempol, modulates the effect of leptin on plasma and tissue PON1 in the rat. Leptin was administered at a dose of 0.25 mg kg^{-1} s.c. twice daily for 7 days and Tempol was added to the drinking water at a concentration of 2 mM. Leptin reduced plasma PON1 activity toward paraoxon, phenyl acetate and γ-decanolactone to 71.1, 72.3 and 57.1% of control, respectively. In addition, leptin decreased PON1 activity toward paraoxon in aorta, renal cortex and medulla to 78.6, 49.2 and 48.0% of control, respectively, but had no effect on PON1 in heart, lung and liver. PON1 activity toward phenyl acetate was lower following leptin treatment only in aorta. Leptin increased plasma concentration and urinary excretion of isoprostanes as well as malonyldialdehyde + 4-hydroxyalkenals level in aorta, renal cortex and renal medulla. Coadministration of Tempol prevented leptin-induced oxidative stress and normalized PON1 activity in aorta and kidney. However, Tempol had no effect on plasma PON1 in leptin-treated rats. These data indicate that hyperleptinemia decreases tissue PON1 activity through oxidative stress-dependent mechanism. In contrast, leptin-induced downregulation of plasma PON1 is not mediated by oxidative stress.
© 2005 Elsevier Ltd. All rights reserved.

Keywords: Leptin; Paraoxonase; Antioxidants; Obesity; Atherosclerosis

1. Introduction

Leptin is a recently described peptide hormone secreted by white adipose tissue, which acts on hypothalamic centres and regulates food intake and energy expenditure. Leptin receptors are also expressed in many tissues outside the brain including cardiovascular system. Plasma leptin concentration is proportional to the amount of adipose tissue and is markedly increased in obese subjects as well as in animals with dietary-induced obesity. Recent studies suggest that hyperleptinemia may contribute to the pathogenesis of obesity-associated pathologies including arterial hypertension and atherosclerosis [1,2]. Leptin exerts many potentially atherogenic effects such as stimulation of inflammatory cells [3], migration and proliferation of vascular smooth muscle cells [4], increase in platelet activity [5,6] and stimulation of arterial thrombosis [7]. Leptin increases production of proatherosclerotic endothelin-1 [8], induces vascular calcification [9] and regulates lipid metabolism in macrophages and foam cells [10,11]. Neointima formation after endovascular injury is markedly reduced in leptin deficient ob/ob mice and leptin-resistant db/db mice in comparison to wild-type littermates [12,13]. Leptin and its receptor are expressed in mice and human atherosclerotic lesions [14,15]. Finally, some clinical studies indicate that high plasma leptin correlates with reduced arterial distensibility, elevated markers of endothelial damage and increased carotid artery intima-media thickness [16–18], as well as is an independent predictor of acute cardiovascular events [19,20], hemorrhagic stroke [21] and restenosis after coronary angioplasty [22].

Oxidative stress, i.e. imbalance between the amount of reactive oxygen species (ROS) and antioxidant defence

* Corresponding author. Tel.: +48 81 7425837; fax: +48 81 7425828.
E-mail address: jerzybel@hotmail.com (J. Beltowski).

1043-6618/$ – see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phrs.2005.01.007

mechanisms, plays an important role in atherogenesis [23]. Several in vitro studies have demonstrated that leptin stimulates ROS production by inflammatory cells [11], endothelial cells [24,25] and other cell types [26,27]. In contrast, little is known about the effect of leptin on antioxidant mechanisms. Paraoxonase 1 (PON1, EC 3.1.1.2) is synthesized in the liver and circulates attached to high-density lipoproteins (HDL). PON1 protects plasma lipoproteins from oxidative modification. The enzyme inhibits atherogenesis by hydrolyzing bioactive lipid hydroperoxides (esterase activity), reducing peroxides to the respective hydroxides (peroxidase activity) and hydrolyzing homocysteine thiolactone, which prevents protein homocysteinylation (lactonase activity) [28]. Knockout of PON1 gene increases, whereas transgenic PON1 overexpression decreases the size of atherosclerotic lesions in mice [29,30]. In humans, low PON1 activity is an independent predictor of acute coronary events [31].

Recently, we have demonstrated that experimental hyperleptinemia induced in lean rats by administration of exogenous leptin induces systemic oxidative stress and decreases plasma PON1 activity [32]. However, the causal relationship between these two effects remains unclear. ROS and lipid peroxidation products decrease PON1 production in the liver [33] and inactivate HDL-bound enzyme [34,35]. On the other hand, reduced PON1 activity could cause increase in plasma lipid peroxidation products. Therefore, in the present study, we investigated whether treatment with exogenous antioxidant attenuates the effect of leptin on plasma PON1. In addition, because PON1 is contained not only in plasma but also in tissues where it may confer local antioxidant protection [36,37], we also examined the effect of leptin on its activity in the liver and selected extrahepatic tissues.

2. Materials and methods

2.1. Animals and experimental protocol

The study was performed in 48 adult male Wistar rats weighing 168 ± 5 g (mean ± S.E.M.) before the experiment. The study protocol was approved by the Bioethical Committee of the Lublin Medical University. The animals were kept at a temperature of 20 ± 2 °C with a 12-h light:12-h dark cycle (lights on at 7:00 a.m.). After 2 weeks of acclimation, they were randomized into six groups ($n = 8$ each): (1) control group fed standard rat chow ad libitum and receiving tap water to drink, (2) leptin-treated group, which received leptin injections (0.25 mg kg^{-1} twice daily s.c. for 7 days) and tap water to drink, (3) pair-fed group, in which the amount of food served was adjusted to food intake in the leptin group, (4) ad libitum-fed group receiving Tempol, (5) leptin-treated group receiving Tempol and (6) pair-fed group receiving Tempol. Animals not treated with leptin (groups 1, 3, 4 and 6) received s.c. injections of 0.25 mg kg^{-1} BSA every 12 h. Tempol (4-hydroxy-2,2,6,6-tetramethyl piperidinoxyl) is a synthetic membrane-permeable antioxidant and superoxide dismutase mimetic which catalytically scavenges superoxide anion radical (O_2^{-}). Tempol was added to the drinking water at a concentration of 2 mM, which corresponded to a dose of ≈250 μmol kg^{-1} day^{-1}. Before starting their respective treatments, 24-h urine collection was made in all animals kept in individual metabolic cages. The second urine collection was performed during the last 24 h of treatment. Urine was collected into sterile containers with 10 μl of 2 mM EDTA and 10 μl of 0.01% butylated hydroxytoluene (BHT) to prevent formation of isoprostanes in vitro.

The animals were anaesthetized with pentobarbital (50 mg kg^{-1}) 6 h after the last injection. Blood was withdrawn from the abdominal aorta into heparinized tubes (for PON1 assay) and into EDTA-containing tubes (for PAF-AH, plasma leptin, lipid profile and isoprostanes). Then, kidneys, liver, heart, thoracic and abdominal aorta and lungs were excised and frozen in liquid nitrogen. Plasma, urine and tissue samples were stored at −80 °C until analysis. For the assay of isoprostanes, plasma and urine samples were stored in the presence of 0.05% BHT.

2.2. PON1 activity in plasma

Plasma PON1 activity was assayed using three synthetic substrates: paraoxon (diethyl-*p*-nitrophenyl phosphate), phenyl acetate and γ-decanolactone. PON1 activity toward paraoxon was determined by measuring the initial rate of substrate hydrolysis to *p*-nitrophenol, which absorbance was monitored at 412 nm in the assay mixture (800 μl) containing 2.0 mM paraoxon, 2.0 mM $CaCl_2$ and 20 μl of plasma in 100 mM Tris–HCl buffer (pH 8.0). The blank sample containing incubation mixture without plasma was run simultaneously to correct for spontaneous substrate breakdown. The enzyme activity was calculated from E_{412} of *p*-nitrophenol (18,290 M^{-1} cm^{-1}) and was expressed in U ml^{-1}; 1 U of enzyme hydrolyses 1 nmol of paraoxon per min [38].

Enzyme activity toward phenyl acetate (arylesterase activity) was determined by measuring the initial rate of substrate hydrolysis in the assay mixture (3 ml) containing 2 mM substrate, 2 mM $CaCl_2$ and 10 μl of plasma in 100 mM Tris–HCl (pH 8.0). The absorbance was monitored for 3 min at 270 nm and the activity was calculated from $E_{270} = 1310$ M^{-1} cm^{-1}. The results are expressed in U ml^{-1}; 1 U of arylesterase hydrolyses 1 μmol of phenyl acetate per min [38].

PON1 activity toward γ-decanolactone was measured in the 2 mM HEPES buffer (pH 8.0) containing 1 mM $CaCl_2$, 0.004% phenol red, 0.005% BSA and 1 mM substrate. The increase in absorbance was monitored at 422 nm after addition of sample and the rate of hydrolysis was calculated from the calibration curve obtained using known amounts of HCl. The activity was expressed in μmol of acid produced by 1 ml

of serum during 1 min [39]. All PON1 measurements were made at 25 °C.

2.3. PON1 activity in tissues

Tissue samples were thawed and the kidney was separated into cortex and medulla. Tissue slices were homogenized in 10 volumes of 50 mM Tris–HCl (pH 8.0) containing 2 mM $CaCl_2$ and the homogenates were centrifuged at 10,000 × *g* for 15 min. Then, supernatants were incubated for 15 min in the presence of 10 μM paraoxon to inhibit B-type esterases, which could interfere with PON1 assay [40]. PON1 activity toward phenyl acetate in tissue supernatants was assayed by the same method as in plasma. The amount of supernatant used for the assay was chosen to fit within the linear range of the relationships between sample volume and the measured activity (liver: 10 μl, renal cortex and medulla: 30 μl, heart, lung and aorta: 50 μl). PON1 activity toward paraoxon was measured in a microplate reader (EL × 800, Bio-Tek Instruments, USA). Ten microliters of homogenate was mixed with 300 μl of the assay buffer containing 2 mM substrate and the increase in absorbance at 405 nm was monitored for 5 min in 1-min intervals (liver) or for 30 min in 5-min intervals (other tissues). The buffer used to measure tissue PON1 activity toward both substrates contained 2 mM of phenylmethyl sulfonyl fluoride (PMSF) to inhibit serine esterases. Enzyme activity in tissues was expressed in U per mg protein. Protein concentration was assayed by the method of Lowry et al. [41].

2.4. Platelet activating factor acetylhydrolase (PAF-AH) activity

Plasma platelet activating factor acetylhydrolase (PAF-AH), a member of phospholipase A_2 family, is produced by macrophages and is contained in LDL in humans and HDL in rodents. Like PON1, PAF-AH hydrolyzes oxidized phospholipids and may be involved in atheroprotection. Plasma PAF-AH activity was measured by two methods. In one of them [42], PAF-AH hydrolyses 1-myristoyl-2-(4-nitrophenylsuccinyl)phosphatidylcholine producing 4-nitrophenyl succinate. This compound degrades spontaneously in aqueous solutions and liberates 4-nitrophenol, whose formation is monitored spectrophotometrically at 405 nm. The activity is expressed in U per liter, 1 U of PAF-AH hydrolyses 1 μmol of substrate during 1 min. This assay was performed using commercially available kit (Azwell Inc., Osaka, Japan). In the second method (PAF-AH assay kit, Cayman Chemical, Ann Arbor, MI, USA), 2-thio-PAF is used as a synthetic substrate. After hydrolysis of the acetyl thioester bond at the sn-2 position by PAF-AH, free thiols are detected using 5,5-dithiobis(2-nitrobenzoic acid) (DTNB; Ellman's reagent). Enzyme activity is calculated from the rate of increase in absorbance at 405 nm using DTNB extinction coefficient (10.0 mM^{-1} cm^{-1}), and is expressed in U per liter; 1 U hydrolyses 1 μmol of 2-thio-PAF per min.

2.5. Indices of oxidative stress

The level of oxidative stress was assessed by measuring plasma concentration and urinary excretion of F_2-isoprostanes—the products of nonenzymatic peroxidation of arachidonic acid by reactive oxygen species. Isoprostanes were assayed by enzyme immunoassay (EIA) using Cayman Chemical 8-isoprostane EIA kit. Plasma or urine samples were mixed with ethanol and centrifuged to remove particulate matter. Ethanol was evaporated from the supernatant under a stream of nitrogen. Then, supernatant was acidified with acetate buffer to pH 4.0 and isoprostanes were extracted using C-18 SPE cartridges (Waters Corporation, Milford, MA). Cartridges were activated by rinsing with 5 ml methanol and 5 ml H_2O and then the sample was passed through the cartridge. The cartridge was rinsed with 5 ml of water, dried and rinsed with 5 ml of HPLC grade hexane. 8-Isoprostanes were eluted with 5 ml ethyl acetate containing 1% methanol. The solvent was evaporated to dryness; the sample was dissolved in 450 μl of EIA buffer and used for the analysis. All samples were assayed in duplicate; before purification one set of samples was spiked with 8-isoprostane standard contained in the kit to correct for individual recovery. The limit of the sensitivity of the assay was 5 pg ml^{-1} and the intraassay coefficient of variation was 8%.

In addition, the level of malonyldialdehyde + 4-hydroxyalkenals (MDA + 4-HNE) was measured in liver, heart, lung, renal cortex, renal medulla and aorta using Bioxytech LPO-586 assay kit (Oxis International, Portland, OR, USA). After thawing, tissues were homogenized in 20 mM Tris buffer (pH 7.4) containing 5 mM BHT and 20 mM EDTA to prevent lipid peroxidation in vitro. The homogenate was centrifuged at 10,000 × *g* for 10 min and MDA + 4-HNE level was measured in the supernatant. The results are expressed in pmol per mg protein.

2.6. Other assays

Plasma leptin was assayed using Leptin Enzyme Immunoassay Kit (Cayman Chemical). Plasma triglycerides, total cholesterol and HDL-cholesterol were measured using routine enzymatic methods (Thermo Electron Co., Noble Park, Victoria, Australia).

2.7. Reagents

Recombinant human leptin was purchased from R&D Systems (Abingdon, Oxon, UK). As recommended by the manufacturer, the vial containing 5 mg was dissolved in 2.5 ml of 15 mM HCl and then 1.5 ml of 7.5 mM NaOH was added to bring pH to 5.2. This solution was diluted with the 15 mM HCl/7.5 mM NaOH mixture (5:3, v/v), frozen, stored at −40 °C and thawed immediately before use. BSA was dissolved in this HCl/NaOH mixture and administered as a vehicle into animals not treated with leptin. Other reagents were obtained from Sigma–Aldrich (St. Louis, MO, USA).

2.8. Statistics

Data are reported as mean ± S.E.M. from eight animals in each group. Baseline and post-treatment urinary excretion of isoprostanes in a given group was compared by repeated-measures ANOVA. Comparisons between different groups were performed by ANOVA followed by Student–Newman–Keuls post hoc test. A $P < 0.05$ was considered significant.

3. Results

3.1. Characteristics of experimental groups

There were no significant differences in food intake, water intake and body weight between groups before treatment. Administration of vehicle solution into control animals had no effect on these variables. Food intake was significantly lower in leptin-treated animals only on sixth and seventh day of the experiment; however, final body weight was similar in all groups. Tempol had no effect on food intake in ad libitum-fed, leptin-treated and pair-fed animals (Table 1). Plasma leptin concentration in leptin-treated rats increased to 318.2% of control, whereas in pair-fed group plasma leptin decreased to 48.5% of control. Plasma leptin concentration in ad libitum-fed, leptin-treated and pair-fed animals receiving Tempol did not differ from the respective groups receiving tap water without this antioxidant.

3.2. Plasma lipid profile

Total plasma cholesterol tended to be lower in leptin-treated and in pair-fed animals than in ad libitum-fed rats, both in those which received tap water and in those which received Tempol. In addition, total plasma cholesterol tended to be lower in groups receiving Tempol than in the respective groups, which received tap water. However, neither of these differences reached the level of significance. In animals, which were not treated with Tempol, leptin decreased plasma HDL-cholesterol to 81.3% of control. In pair-fed group, HDL-cholesterol tended to be lower than in control group, but this effect was not significant. However, due to numerically lower HDL-cholesterol in pair-fed than in control group, the difference between leptin-treated and pair-fed group was not significant. Among Tempol-treated rats, leptin had no effect on HDL-cholesterol. Plasma HDL-cholesterol in ad libitum-fed animals receiving Tempol was 79.9% of control ($P < 0.01$). In contrast, Tempol had no effect on HDL-cholesterol in either leptin-treated or pair-fed rats. Plasma triglycerides did not differ between groups (Table 1).

3.3. Plasma PON1 activity

Leptin decreased plasma PON1 activity toward paraoxon, phenyl acetate and γ-decanolactone to 71.1, 72.3 and 57.1% of control, respectively, and to 74.1, 76.7 and 66.7% of activity toward these substrates observed in pair-fed group (Fig. 1). Tempol administration did not change PON1 activity in ad libitum-fed or pair-fed animals. Moreover, Tempol did not attenuate the inhibitory effect of leptin on plasma PON1. PON1 activity toward paraoxon, phenyl acetate and γ-decanolactone in leptin-treated animals receiving Tempol was 83.1, 80.4 and 48.7% of activity in ad libitum-fed animals receiving Tempol, respectively, and 83.3, 74.7 and 53.9% of the respective activities observed in pair-fed group receiving Tempol (Fig. 1).

3.4. PON1 activity in tissues

PON1 activity in the liver and heart was similar in all experimental groups. In the renal cortex of leptin-treated animals, PON1 activity toward paraoxon was 49.2% of control ($P < 0.01$) and 44.2% ($P < 0.001$) of activity in pair-fed group (Fig. 2). Administration of Tempol had no effect on renal cortical PON1 activity in ad libitum-fed and pair-fed animals, but normalized its activity in leptin-treated rats. Leptin decreased PON1 activity toward paraoxon also in the renal medulla to 48.0% of control ($P < 0.001$) and to 46.5% ($P < 0.001$) of activity in pair-fed group. Co-treatment with Tempol prevented leptin-induced decrease in paraoxon-hydrolyzing activity in renal cortical homogenates (Fig. 2). PON1 activity toward phenyl acetate tended to be lower in the renal cortex of leptin-treated than of control rats; however, the difference was not

Table 1
The effect of leptin on food intake, body weight, plasma leptin and lipid profile

	Control	Leptin-treated	Pair-fed	ad libitum + Tempol	Leptin + Tempol	Pair-fed + Tempol
Food intake ($g\,day^{-1}$)[a]	21 ± 1	15 ± 1*	15 ± 2*	20 ± 1	14 ± 2*	14 ± 2**
Body weight (g)[b]	209 ± 2	207 ± 6	208 ± 6	207 ± 7	216 ± 8	218 ± 6
Plasma leptin ($ng\,ml^{-1}$)	3.69 ± 0.15	11.74 ± 0.76***	1.79 ± 0.13*	3.53 ± 0.13*	11.56 ± 0.86***	1.49 ± 0.16*
Total cholesterol (mM)	2.58 ± 0.33	2.04 ± 0.21	2.16 ± 0.30	2.04 ± 0.27	1.64 ± 0.19	1.77 ± 0.17
HDL-cholesterol (mM)	1.39 ± 0.05	1.13 ± 0.04**	1.23 ± 0.04	1.11 ± 0.08**	0.95 ± 0.04**	1.07 ± 0.08**
Triglycerides (mM)	0.76 ± 0.09	0.73 ± 0.07	0.75 ± 0.07	0.70 ± 0.19	0.84 ± 0.2	0.77 ± 0.06

[a] During 7th day of treatment.
[b] After 7 days of treatment.
* $P < 0.05$, compared to control group.
** $P < 0.01$, compared to control group.
*** $P < 0.001$, compared to control group.

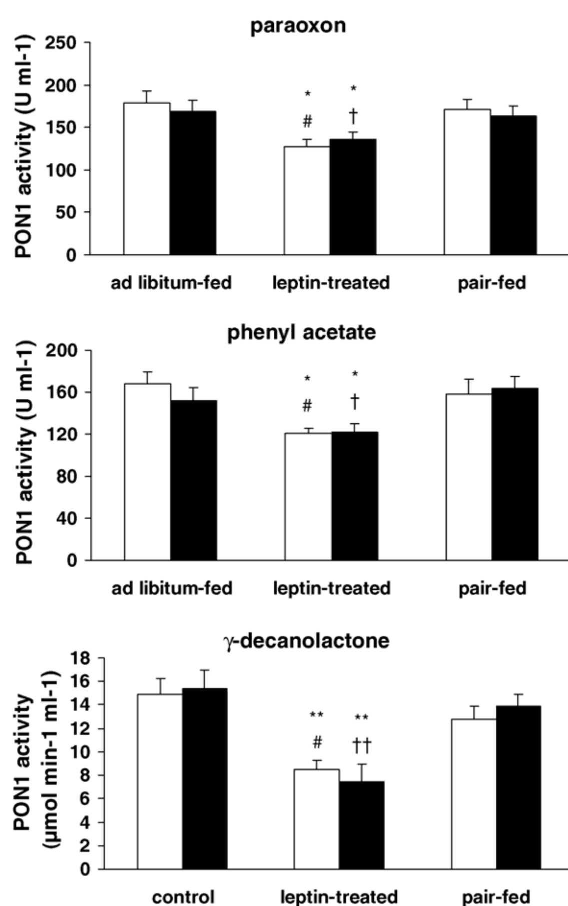

Fig. 1. Plasma paraoxonase (PON1) activity toward different substrates in ad libitum-fed, leptin-treated and pair-fed rats receiving tap water (open bars) or 2 mM Tempol (closed bars) to drink. Leptin was administered s.c. at a dose of 0.25 mg kg^{-1} twice daily for 7 days. PON1 was assayed in heparinized plasma. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control group, $^{\#}P < 0.05$ vs. pair-fed group, $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ vs. pair-fed group receiving Tempol.

significant. There were no significant differences in phenyl acetate-hydrolyzing activity in the renal medulla between experimental groups. Aortic PON1 activity toward paraoxon and phenyl acetate decreased in leptin-treated animals to 78.6 and 71.1% of control, respectively, and to 77.0 ($P < 0.05$) and 77.6% ($P < 0.05$) of activities toward these substrates in pair-fed group. This effect was abolished by Tempol (Fig. 2). In lung homogenates, PON1 activity toward both substrates was similar in all groups.

3.5. Plasma PAF-AH

Consistently with our previous study [32], plasma PAF-AH activity toward 1-myristoyl-2-(4-nitrophenylsuccinyl)phosphatidylcholine was not different between groups (control: 368 ± 35 U l^{-1}, leptin-treated group: 348 ± 11 IU l^{-1}, pair-fed group: 347 ± 12 IU l^{-1}, ad libitum-fed group receiving Tempol: 464 ± 18 IU l^{-1}, leptin-treated group receiving Tempol: 338 ± 39 IU l^{-1}, pair-fed group receiving Tempol: 428 ± 21 IU l^{-1}). Moreover, PAF-AH activity toward 2-thio-platelet activating factor was similar in all groups (control: 56.5 ± 5.9 U l^{-1}, leptin-treated group: 47.6 ± 4.5 U l^{-1}, pair-fed group: 50.3 ± 5.2 U l^{-1}, ad libitum-fed group receiving Tempol: 51.7 ± 3.1 U l^{-1}, leptin-treated group receiving Tempol: 43.5 ± 6.5 U l^{-1}, pair-fed group receiving Tempol: 46.3 ± 4.1 U l^{-1}).

3.6. Lipid peroxidation products

Urinary excretion of isoprostanes was similar in all groups before treatment and did not change in the control group following vehicle administration (not shown). Plasma concentration and urinary excretion of 8-ispoprostanes increased following leptin treatment to 163.4 and 180.9% of control, respectively (Fig. 3). Plasma and urinary isoprostanes in pair-fed group did not differ from control. Tempol had no effect on plasma and urinary 8-isoprostanes in either ad libitum-fed or in pair-fed group. However, Tempol prevented increase in plasma concentration and urinary excretion of 8-isoprostanes induced by leptin treatment.

Oxidative stress in tissues was assessed by measuring MDA + 4-HNE concentration. The level of these lipid peroxidation products in liver and heart did not differ between groups. Leptin increased MDA + 4-HNE concentration in the renal cortex and medulla to 139.6 and 146.9% of control, respectively. Renal MDA + 4-HNE in pair-fed group did not differ from control. Tempol had no effect on MDA + 4-HNE either in ad libitum-fed or in pair-fed groups; however, it prevented increase in MDA + 4-HNE induced by leptin. Similarly, leptin treatment increased MDA + 4-HNE in aortic homogenates to 139.5% of control and this effect was prevented by coadministration of Tempol (Fig. 4).

4. Discussion

The results of this study confirm our previous observation [32] that experimentally induced hyperleptinemia decreases plasma PON1 activity. In addition, leptin reduced enzyme activity in the kidney and aorta but had no effect on PON1 in the liver, heart and lung. Treatment with Tempol ameliorated leptin-induced oxidative stress and normalized PON1 activity in tissues but not in plasma.

Although leptin increases ROS generation in isolated or cultured cells [24–27], little is known about its effect on oxidative stress in vivo. Herein, we demonstrate that leptin induces systemic oxidative stress as evidenced by increase in plasma and urinary isoprostanes. In addition, MDA + 4-HNE level increased in the kidney and aorta following leptin treatment. Recent study has demonstrated that leptin administered at a dose of 0.23 mg kg^{-1} every alternate day for 2 weeks had no effect on lipid peroxidation products and antioxidant enzymes in mice liver and kidney [43]. Nevertheless, leptin augmented oxidative stress induced by ethanol, which suggests that leptin per se had some subthreshold prooxidant activity. It is unclear at present

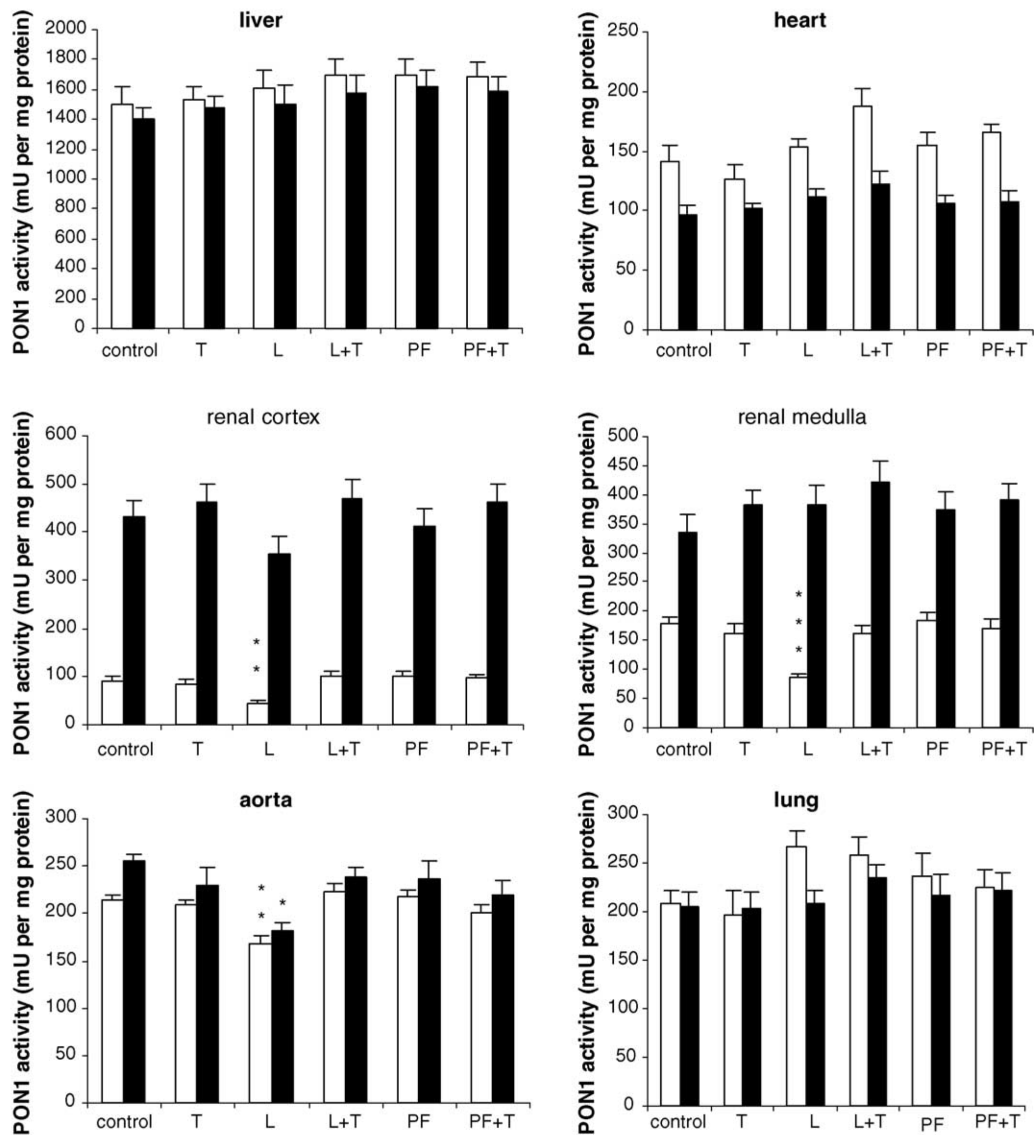

Fig. 2. Tissue PON1 activity toward paraoxon (open bars) and phenyl acetate (closed bars). T, ad libitum-fed group receiving Tempol; L, leptin-treated group; L + T, leptin-treated group receiving Tempol; PF, pair-fed group; PF + T, pair-fed group receiving Tempol. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. control group.

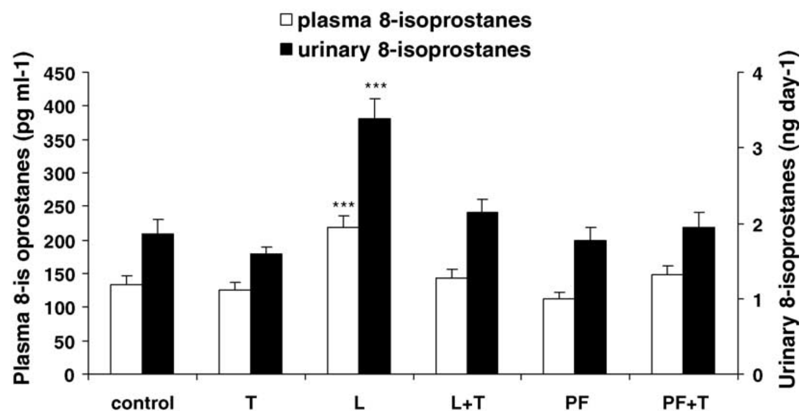

Fig. 3. Plasma concentration (left scale) and urinary excretion (right scale) of 8-isoprostanes in experimental groups. T, ad libitum-fed group receiving Tempol; L, leptin-treated group; L + T, leptin-treated group receiving Tempol; PF, pair-fed group; PF + T, pair-fed group receiving Tempol. $^{***}P < 0.001$ vs. control group.

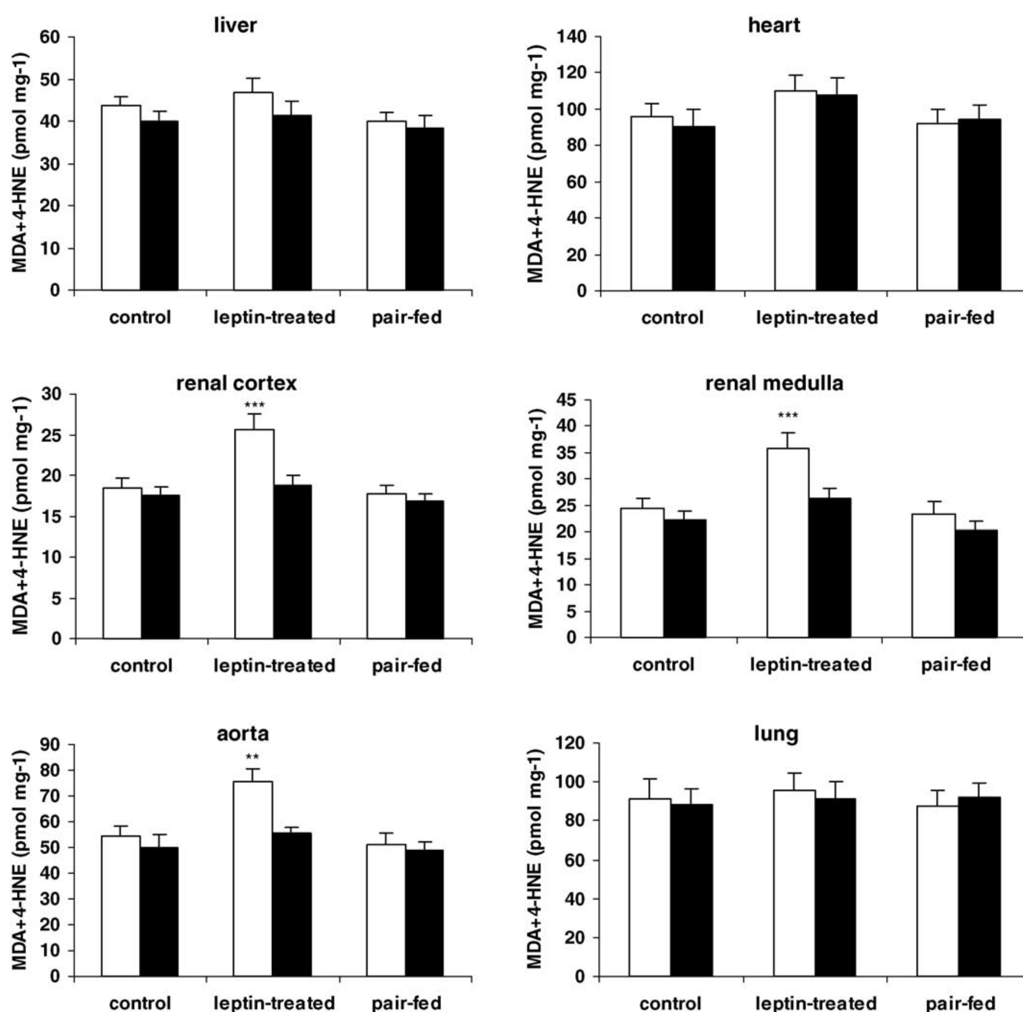

Fig. 4. The effect of leptin on lipid peroxidation products: malonyldialdehyde and 4-hydroxyalkenals (MDA + 4-HNE) in tissues of control (ad libitum-fed), leptin-treated and pair-fed rats not treated with Tempol (open bars) or receiving this antioxidant (closed bars). $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control group.

why leptin-induced oxidative stress only in kidney and aorta in the present study. Leptin receptors are expressed in the vascular wall and leptin stimulates ROS production in cultured endothelial cells [24,25]. In the kidney, leptin stimulates the expression of prooxidant cytokine, transforming growth factor-β (TGF-β) and its receptor in glomerular endothelial and mesangial cells [44–46]. Chronic leptin infusion-induced glomerulosclerosis resembles nephropathy often observed in obesity [45]. Thus, oxidative stress may be involved in the pathogenesis of renal lesions associated with hyperleptinemia and obesity. It remains to be established whether more severe and/or longer lasting hyperleptinemia could stimulate oxidative stress also in other tissues.

It should be noted that leptin decreased PON1 activity only in tissues in which it stimulated oxidative stress, i.e. in aorta and kidney. Tempol ameliorated leptin-induced oxidative stress and normalized PON1 activity in these organs. These data suggest that leptin-induced downregulation of tissue PON1 is accounted for by enzyme inactivation by ROS and/or lipid peroxidation products. Interestingly, in the kidney leptin decreased PON1 activity only toward paraoxon but not toward phenyl acetate. It should be noted that the ratio between phenyl acetate- and paraoxon-hydrolyzing activities was close to 1 in plasma and other tissues but was much higher in the kidney (≈2:1 in the renal medulla and ≈4:1 in the renal cortex). This may indicate that in the kidney phenyl acetate hydrolysis is catalyzed also by other enzyme(s) in addition to PON1 [47]. In particular, PON3 is a PON1-related enzyme, which hydrolyzes phenyl acetate but not paraoxon [48]. Whereas plasma PON3 activity is about two orders of magnitude lower than PON1, PON3 is expressed in substantial amounts in the kidney [49]. PON3 activity is inhibited by ROS and lipid peroxidation products, but in contrast to PON1 its gene expression is not sensitive to oxidative stress [50,51]. One may speculate that in the kidney phenyl acetate-hydrolyzing activity is partially accounted for by PON3, which is less sensitive to oxidative stress than PON1. Alternatively, paraoxon-hydrolyzing activity of renal PON1 might be more sensitive to leptin-induced oxidative stress than its activity toward phenyl acetate.

The effect of antioxidants on plasma PON1 is controversial. In vitro studies suggest that antioxidants protect isolated PON1 from free radical-induced inactivation [34]. Jarvik et al. [52] have reported positive correlation between dietary intake of Vitamins C and E and plasma PON1 activity. However, other studies demonstrate no changes [53,54] or even suggest negative effect [55] of dietary antioxidants on plasma PON1. In the present study, Tempol had no effect on plasma PON1 in either control or leptin-treated rats, although it ameliorated oxidative stress in hyperleptinemic animals. These data suggest that in contrast to aorta and kidney, decrease in plasma PON1 activity in leptin-treated rats is not accounted for by oxidative stress. Previously, we have suggested several possible mechanisms through which leptin could downregulate plasma PON1, such as altered composition of HDL particles, stimulation of inflammatory cytokines and/or acute phase proteins and activation of peroxisome proliferator activated receptor-α [see [32] and Refs. therein]. It seems that at least one more possibility should be considered, i.e. that the effect of leptin is mediated by ghrelin. Ghrelin is a gastric peptide, which induces hunger and stimulates somatotropin secretion from anterior pituitary. Recently, it has been demonstrated that ghrelin specifically binds to PON1-containing HDL subfraction [56]. In addition, antighrelin antibodies inhibit PON1 activity toward paraoxon and phenyl acetate, suggesting that ghrelin may directly stimulate PON1. Plasma ghrelin concentration increases during caloric restriction and Barazzoni et al. [57] have reported that this effect is prevented by leptin treatment. Thus, it may be speculated that caloric restriction per se decreases plasma PON1 activity, but in pair-fed animals this effect is counteracted by hyperghrelinemia. In contrast, leptin treatment could unmask PON1 inhibition by preventing increase in plasma ghrelin induced by caloric restriction. Further studies are needed to verify this hypothesis.

In contrast to our previous study [32], leptin decreased HDL-cholesterol in the present experiments. The reason of this discrepancy is unclear. In this study, we used smaller rats (mean body weight 168 g versus 237 g in the previous study). Increasing age and adiposity is associated with reduced peripheral activities of leptin, including its effects on lipid metabolism [58]. Thus, lower body weight could enhance leptin's impact on HDL in our study. Other experimental studies have demonstrated stimulatory effect of leptin on HDL catabolism and reduced plasma HDL following leptin treatment [59,60]. Reducing effect of leptin on HDL-cholesterol could result, at least in part, from caloric restriction since pair-feeding also tended to decrease HDL-cholesterol. However, decrease in plasma HDL could not solely explain reduced plasma PON1 activity in leptin-treated rats, because Tempol also decreased HDL-cholesterol but had no effect on PON1. In addition, leptin decreased plasma PON1 activity also in animals receiving Tempol, in which it had no effect on HDL-cholesterol.

In conclusion, we have demonstrated that 7-day leptin administration markedly decreases PON1 activity not only in plasma but also in aorta, renal cortex and renal medulla. Antioxidant treatment with Tempol prevents leptin-induced oxidative stress and normalizes PON1 activity in tissues but not in plasma. These data indicate that hyperleptinemia downregulates plasma and tissue PON1 through divergent mechanisms. PON1 deficiency and oxidative stress may contribute to accelerated atherogenesis as well as to renal complications in hyperleptinemic obese individuals.

Acknowledgement

This study was supported by Grant PW 444 from Medical University in Lublin.

References

[1] Ren J. Leptin and hyperleptinemia—from friend to foe for cardiovascular function. J Endocrinol 2004;181:1–10.
[2] Rahmouni K, Haynes WG. Leptin and the cardiovascular system. Recent Prog Horm Res 2004;59:225–44.
[3] Loffreda S, Yang SQ, Lin HZ, Karp CL, Brengman ML, Wang DJ, et al. Leptin regulates proinflammatory immune responses. FASEB J 1998;12:57–65.
[4] Oda A, Taniguchi T, Yokoyama M. Leptin stimulates rat aortic smooth muscle cell proliferation and migration. Kobe J Med Sci 2001;47:141–50.
[5] Nakata M, Yada T, Soejima N, Maruyama I. Leptin promotes aggregation of human platelets via the long form of its receptor. Diabetes 1999;48:426–9.
[6] Konstantinides S, Schafer K, Koschnick S, Loskutoff DJ. Leptin-dependent platelet aggregation and arterial thrombosis suggests a mechanism for atherothrombotic disease in obesity. J Clin Invest 2001;108:1533–40.
[7] Bodary PF, Westrick RJ, Wickenheiser KJ, Shen Y, Eitzman DT. Effect of leptin on arterial thrombosis following vascular injury in mice. JAMA 2002;287:1706–9.
[8] Quehenberger P, Exner M, Sunder-Plassmann R, Ruzicka K, Bieglmayer C, Endler G, et al. Leptin induces endothelin-1 in endothelial cells in vitro. Circ Res 2002;90:711–8.
[9] Parhami F, Tintut Y, Ballard A, Fogelman AM, Demer LL. Leptin enhances the calcification of vascular cells: artery wall as a target of leptin. Circ Res 2001;88:954–60.
[10] O'Rourke L, Yeaman SJ, Shepherd PR. Insulin and leptin acutely regulate cholesterol ester metabolism in macrophages by novel signaling pathways. Diabetes 2001;50:955–61.
[11] Maingrette F, Renier G. Leptin increases lipoprotein lipase secretion by macrophages: involvement of oxidative stress and protein kinase C. Diabetes 2003;52:2121–8.
[12] Stephenson K, Tunstead J, Tsai A, Gordon R, Henderson S, Dansky HM. Neointimal formation after endovascular arterial injury is markedly attenuated in db/db mice. Arterioscler Thromb Vasc Biol 2003;23:2027–33.
[13] Schafer K, Halle M, Goeschen C, Dellas C, Pynn M, Loskutoff DJ, et al. Leptin promotes vascular remodeling and neointimal growth in mice. Arterioscler Thromb Vasc Biol 2004;24:112–7.
[14] Kang SM, Kwon HM, Hong BK, Kim D, Kim IJ, Choi EY, et al. Expression of leptin receptor (Ob-R) in human atherosclerotic lesions: potential role in intimal neovascularization. Yonsei Med J 2000;41:68–75.
[15] Chaldakov GN, Fiore M, Stankulov IS, Hristova M, Antonelli A, Manni L, et al. NGF, BDNF, leptin, and mast cells in human coronary atherosclerosis and metabolic syndrome. Arch Physiol Biochem 2001;109:357–60.

[16] Singhal A, Farooqi IS, Cole TJ, O'Rahilly S, Fewtrell M, Kattenhorn M, et al. Influence of leptin on arterial distensibility: a novel link between obesity and cardiovascular disease? Circulation 2002;106:1919–24.
[17] Porreca E, Di Febbo C, Fusco L, Moretta V, Di Nisio M, Cuccurullo F. Soluble thrombomodulin and vascular adhesion molecule-1 are associated to leptin plasma levels in obese women. Atherosclerosis 2004;172:175–80.
[18] Ciccone M, Vettor R, Pannacciulli N, Minenna A, Bellacicco M, Rizzon P, et al. Plasma leptin is independently associated with the intima-media thickness of the common carotid artery. Int J Obes Relat Metab Disord 2001;25:805–10.
[19] Soderberg S, Ahren B, Jansson JH, Johnson O, Hallmans G, Asplund K, et al. Leptin is associated with increased risk of myocardial infarction. J Intern Med 1999;246:409–18.
[20] Wallace AM, McMahon AD, Packard CJ, Kelly A, Shepherd J, Gaw A, et al. Plasma leptin and the risk of cardiovascular disease in the west of Scotland coronary prevention study (WOSCOPS). Circulation 2001;104:3052–6.
[21] Soderberg S, Ahren B, Stegmayr B, Johnson O, Wiklund PG, Weinehall L, et al. Leptin is a risk marker for first-ever hemorrhagic stroke in a population-based cohort. Stroke 1999;30:328–37.
[22] Piatti P, Di Mario C, Monti LD, Fragasso G, Sgura F, Caumo A, et al. Association of insulin resistance, hyperleptinemia, and impaired nitric oxide release with in-stent restenosis in patients undergoing coronary stenting. Circulation 2003;108:2074–81.
[23] Harrison D, Griendling KK, Landmesser U, Hornig B, Drexler H. Role of oxidative stress in atherosclerosis. Am J Cardiol 2003;91:7A–11A.
[24] Bouloumie A, Marumo T, Lafontan M, Busse R. Leptin induces oxidative stress in human endothelial cells. FASEB J 1999;13:1231–8.
[25] Yamagishi SI, Edelstein D, Du XL, Kaneda Y, Guzman M, Brownlee M. Leptin induces mitochondrial superoxide production and monocyte chemoattractant protein-1 expression in aortic endothelial cells by increasing fatty acid oxidation via protein kinase A. J Biol Chem 2001;276:25096–100.
[26] Savini I, Catani MV, Rossi A, Duranti G, Ranalli M, Melino G, et al. Vitamin C recycling is enhanced in the adaptive response to leptin-induced oxidative stress in keratinocytes. J Invest Dermatol 2003;121:786–93.
[27] Cao Q, Mak KM, Ren C, Lieber CS. Leptin stimulates tissue inhibitor of metalloproteinase-1 in human hepatic stellate cells: respective roles of the JAK/STAT and JAK-mediated H_2O_2-dependant MAPK pathways. J Biol Chem 2004;279:4292–304.
[28] Mackness MI, Mackness B, Durrington PN. Paraoxonase and coronary heart disease. Atheroscler Suppl 2002;3:49–55.
[29] Shih DM, Xia YR, Wang XP, Miller E, Castellani LW, Subbanagounder G, et al. Combined serum paraoxonase knockout/apolipoprotein E knockout mice exhibit increased lipoprotein oxidation and atherosclerosis. J Biol Chem 2000;275:17527–35.
[30] Tward A, Xia YR, Wang XP, Shi YS, Park C, Castellani LW, et al. Decreased atherosclerotic lesion formation in human serum paraoxonase transgenic mice. Circulation 2002;106:484–90.
[31] Mackness B, Durrington P, McElduff P, Yarnell J, Azam N, Watt M, et al. Low paraoxonase activity predicts coronary events in the caerphilly prospective study. Circulation 2003;107:2775–9.
[32] Beltowski J, Wojcicka G, Jamroz A. Leptin decreases plasma paraoxonase 1 (PON1) activity and induces oxidative stress: the possible novel mechanism for proatherogenic effect of chronic hyperleptinemia. Atherosclerosis 2003;170:21–9.
[33] Van Lenten BJ, Wagner AC, Navab M, Fogelman AM. Oxidized phospholipids induce changes in hepatic paraoxonase and ApoJ but not monocyte chemoattractant protein-1 via interleukin-6. J Biol Chem 2004;276:1923–9.
[34] Aviram M, Rosenblat M, Billecke S, Erogul J, Sorenson R, Bisgaier CL, et al. Human serum paraoxonase (PON 1) is inactivated by oxidized low density lipoprotein and preserved by antioxidants. Free Radic Biol Med 1999;26:892–904.
[35] Nguyen SD, Kim JR, Ree KM, Sok JT, Sok DE. Copper ions and hypochlorite are mainly responsible for oxidative inactivation of paraoxon-hydrolyzing activity in human high density lipoprotein. Toxicol Lett 2004;147:201–8.
[36] Rodrigo L, Hernandez AF, Lopez-Caballero JJ, Gil F, Pla A. Immunohistochemical evidence for the expression and induction of paraoxonase in rat liver, kidney, lung and brain tissue. Implications for its physiological role. Chem Biol Interact 2001;137:123–37.
[37] Ferre N, Camps J, Cabre M, Paul A, Joven J. Hepatic paraoxonase activity alterations and free radical production in rats with experimental cirrhosis. Metabolism 2001;50:997–1000.
[38] Ayub A, Mackness MI, Arrol S, Mackness B, Patel J, Durrington PM. Serum paraoxonase after myocardial infarction. Arterioscler Thromb Vasc Biol 1999;19:330–5.
[39] Billecke S, Draganov D, Counsell R, Stetson P, Watson C, Hsu C, et al. Human serum paraoxonase (PON1) isozymes Q and R hydrolyze lactones and cyclic carbonate esters. Drug Metab Dispos 2000;28:1335–42.
[40] Pond AL, Chambers HW, Coyne CP, et al. Purification of two rat hepatic proteins with A-esterase activity toward chlorpyrifos-oxon and paraoxon. J Pharmacol Exp Ther 1998;286:1404–11.
[41] Lowry OH, Rosenbrough NI, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem 1951;193:265–75.
[42] Kosaka T, Yamaguchi M, Soda Y, Kishimoto T, Tago A, Toyosato M, et al. Spectrophotometric assay for serum platelet-activating factor acetylhydrolase activity. Clin Chim Acta 2000;296:151–61.
[43] Balasubramaniyan V, Kalaivani Sailaja J, Nalini N. Role of leptin on alcohol-induced oxidative stress in Swiss mice. Pharmacol Res 2003;47:211–6.
[44] Wolf G, Hamann A, Han DC, Helmchen U, Thaiss F, Ziyadeh FN, et al. Leptin stimulates proliferation and TGF-β expression in renal glomerular endothelial cells: potential role in glomerulosclerosis. Kidney Int 1999;56:860–72.
[45] Han DC, Isono M, Chen S, Casaretto A, Hong SW, Wolf G, et al. Leptin stimulates type I collagen production in db/db mesangial cells: glucose uptake and TGF-β type II receptor expression. Kidney Int 2001;59:1315–23.
[46] Jiang Z, Seo JY, Ha H, Lee EA, Kim YS, Han DC, et al. Reactive oxygen species mediate TGF-β1-induced plasminogen activator inhibitor-1 upregulation in mesangial cells. Biochem Biophys Res Commun 2003;309:961–6.
[47] Rodrigo L, Gil F, Hernandez AF, Pla A. Identification of two rat liver proteins with paraoxonase activity: biochemical evidence for the identity of paraoxonase and arylesterase. Chem Biol Interact 1999;119–120:263–75.
[48] Rodrigo L, Gil F, Hernandez AF, Lopez O, Pla A. Identification of paraoxonase 3 in rat liver microsomes: purification and biochemical properties. Biochem J 2003;376:261–8.
[49] Draganov DI, Stetson PL, Watson CE, Billecke SS, La Du BN. Rabbit serum paraoxonase 3 (PON3) is a high density lipoprotein-associated lactonase and protects low density lipoprotein against oxidation. J Biol Chem 2000;275:33435–42.
[50] Reddy ST, Wadleigh DJ, Grijalva V, Ng C, Hama S, Gangopadhyay A, et al. Human paraoxonase-3 is an HDL-associated enzyme with biological activity similar to paraoxonase-1 protein but is not regulated by oxidized lipids. Arterioscler Thromb Vasc Biol 2001;21:542–7.
[51] Rosenblat M, Draganov D, Watson CE, Bisgaier CL, La Du BN, Aviram M. Mouse macrophage paraoxonase 2 activity is increased whereas cellular paraoxonase 3 activity is decreased under oxidative stress. Arterioscler Thromb Vasc Biol 2003;23:468–74.
[52] Jarvik GP, Tsai NT, McKinstry LA, Wani R, Brophy VH, Richter RJ, et al. Vitamin C and E intake is associated with increased paraoxonase activity. Arterioscler Thromb Vasc Biol 2002;22:1329–33.

[53] Arrol S, Mackness MI, Durrington PN. Vitamin E supplementation increases the resistance of both LDL and HDL to oxidation and increases cholesteryl ester transfer activity. Atherosclerosis 2000;150:129–34.

[54] Sarandol E, Serdar Z, Dirican M, Safak O. Effects of red wine consumption on serum paraoxonase/arylesterase activities and on lipoprotein oxidizability in healthy-men. J Nutr Biochem 2003;14:507–12.

[55] Kleemola P, Freese R, Jauhiainen M, Pahlman R, Alfthan G, Mutanen M. Dietary determinants of serum paraoxonase activity in healthy humans. Atherosclerosis 2002;160:425–32.

[56] Beaumont NJ, Skinner VO, Tan TM, Ramesh BS, Byrne DJ, MacColl GS, et al. Ghrelin can bind to a species of high density lipoprotein associated with paraoxonase. J Biol Chem 2003;278:8877–80.

[57] Barazzoni R, Zanetti M, Stebel M, Biolo G, Cattin L, Guarnieri G. Hyperleptinemia prevents increased plasma ghrelin concentration during short-term moderate caloric restriction in rats. Gastroenterology 2003;124:1188–92.

[58] Steinberg GR, Dyck DJ. Development of leptin resistance in rat soleus muscle in response to high-fat diets. Am J Physiol Endocrinol Metab 2000;279:E1374–82.

[59] Silver DL, Jiang XC, Tall AR. Increased high density lipoprotein (HDL), defective hepatic catabolism of ApoA-I and ApoA-II, and decreased ApoA-I mRNA in ob/ob mice. Possible role of leptin in stimulation of HDL turnover. J Biol Chem 1999;274:4140–6.

[60] Matsuoka N, Ogawa Y, Masuzaki H, Ebihara K, Aizawa-Abe M, Satoh N, et al. Decreased triglyceride-rich lipoproteins in transgenic skinny mice overexpressing leptin. Am J Physiol Endocrinol Metab 2001;280:E334–9.