Modulation of paraoxonase 1 and protein N-homocysteinylation by leptin and the synthetic liver X receptor agonist T0901317 in the rat

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Abstract

The adipose tissue hormone leptin and homocysteine (Hcy)-thiolactone are linked to the pathogenesis of atherosclerosis through their interactions with the anti-atherogenic enzyme paraoxonase 1 that has the ability to hydrolyze Hcy-thiolactone and minimizes protein N-homocysteinylation. Here we examined the relationships between hyperleptinemia, Hcy-thiolactonase, and protein N-homocysteinylation in rats. Hyperleptinemia was induced in adult rats by administration of leptin for 7 days (0.25 mg/kg twice daily s.c.). We found that serum Hcy-thiolactonase was lower in hyperleptinemic than in control animals (41.0%, P<0.001). Leptin administration increased the level of N-linked Hcy in plasma proteins (92.9%, P<0.01), but had no effect on plasma total Hcy. These effects were not reproduced by pair-feeding. We also found that the synthetic liver X receptor (LXR) agonist, T0901317 (1 mg/kg per day) normalized Hcy-thiolactonase and protein N-homocysteinylation levels in leptin-treated rats. However, leptin-induced increase in plasma isoprostane levels (a marker of oxidative stress) was not normalized by T0901317. The NADPH oxidase inhibitor apocynin prevented leptin-induced increase in isoprostane levels but did not normalize Hcy-thiolactonase and protein N-homocysteinylation levels. These results suggest that the decreased capacity to metabolize Hcy-thiolactone and concomitant increase in protein N-homocysteinylation contribute to pro-atherogenic effect of chronic hyperleptinemia, independently of oxidative stress. LXR agonists normalize Hcy-thiolactonase levels and decrease protein N-homocysteinylation, especially under conditions associated with excess leptin such as metabolic syndrome.


Introduction

Leptin is a 16 kDa protein secreted by adipose tissue, which acts on hypothalamic centers to suppress food intake and increase energy expenditure (Farooqi & O’Rahilly 2009). Mutations of leptin- or leptin receptor-encoding genes result in severe obesity in rodents and are rare causes of obesity in humans. However, plasma leptin concentration is markedly increased in most overweight/obese humans as well as in animals with obesity induced by high-calorie diet, reflecting greater amount of adipose tissue and (acquired) resistance to anorectic effect of this hormone (Considine et al. 1996).

Obesity and the accompanying metabolic syndrome are the major risk factors of atherosclerosis but the underlying mechanisms are incompletely elucidated. Recent studies have suggested that chronic hyperleptinemia contributes to various complications of the metabolic syndrome including atherosclerosis (Koh et al. 2008). Leptin has many potentially pro-atherogenic effects. It stimulates oxidative stress (Bouloumie et al. 1999), induces endothelial dysfunction (Knudson et al. 2008, Korda et al. 2008), platelet aggregation (Dellas et al. 2008), inflammatory reaction (Singh et al. 2007), and vascular smooth muscle cell hypertrophy/proliferation (Zeidan et al. 2005). Depletion of leptin or its receptor reduces atherosclerosis in animal models such as apolipoprotein E (Chiba et al. 2008) or low-density lipoprotein (LDL) receptor knockout mice (Taleb et al. 2007), whereas administration of leptin in supraphysiological doses accelerates atherosclerosis (Bodary et al. 2005). In addition, high leptin level is an independent predictor of acute cardiovascular events (Sattar et al. 2009, Sodeberg et al. 2009) and ischemic stroke (Sodeberg et al. 2003), the most common complications of atherosclerosis.

Previous studies suggest a link between leptin and an anti-atherogenic enzyme paraoxonase 1 (PON1). We have demonstrated that chronic hyperleptinemia is induced in normal healthy rats by 7-day administration of exogenous leptin decreases PON1 levels in plasma and tissues (Bełtowski et al. 2003, 2005). Subsequently, an independent inverse
correlation between serum leptin and PON1 was reported in some (Ferretti et al. 2005), although not in all (Bajnok et al. 2007) clinical studies. PON1 is a Ca$^{2+}$-dependent esterase synthesized in the liver and carried on high-density lipoproteins (HDL) in the circulation. PON1 inhibits atherogenesis due to its antioxidant and anti-inflammatory properties as well as by beneficially affecting cholesterol balance in macrophages/foam cells (Shih et al. 1998, Tward et al. 2002, Mackness et al. 2006). Low PON1 activity is an independent predictor of acute coronary events (Mackness et al. 2003). In addition to other mechanisms, PON1 inhibits atherogenesis due to its ability to hydrolyze homocysteine (Hcy)-thiolactone and thus minimizes protein damage by N-homocysteinylination (Jakubowski 2000). Hcy-thiolactonase activity of PON1 predicts cardiovascular disease (Domagala et al. 2006). Thus, negative effect on PON1 with subsequent accumulation of Hcy-thiolactone might contribute to proatherogenic effect of leptin.

In our previous study (Beltowski et al. 2003), PON1 activity was measured toward its synthetic substrates, paraoxon, and phenyl acetate. Thus, it is unclear if leptin affects PON1 activity toward one of its physiological substrates, Hcy-thiolactone. In the present study, we examined how leptin administration affects N-Hcy-protein levels and the ability to hydrolyze Hcy-thiolactone in the rat. In addition, we investigated how the liver X receptor (LXR) agonist T0901317, known to inhibit atherogenesis (Terasaka et al. 2003), modulates PON1 levels in the rat model of hyperleptinemia.

Materials and Methods

Animals and experimental protocol

Male Wistar rats weighing 243±7 g were used in the study. The animals were kept at a temperature of 20±2 °C, on a 12 h light:12 h darkness (lights on at 0700 h), and had free access to food and tap water before the experiment. The study protocol was reviewed and approved by the Bioethical Committee of the Medical University in Lublin.

After 2 weeks of acclimation, the animals were randomly divided into the following groups: 1) control, fed standard rat chow ad libitum; 2) leptin-treated group, receiving exogenous leptin (0-25 mg/kg twice daily s.c.) for 7 days; 3) pair-fed group, in which food intake was restricted to match food intake in leptin-treated group; 4) leptin-treated group receiving T0901317; 5) group receiving T0901317 alone; 6) leptin-treated group receiving NADPH oxidase inhibitor, apocynin; and 7) group receiving apocynin alone. The two latter groups were included to test whether potential protective effect of T0901317 results from amelioration of oxidative stress; since leptin stimulates reactive oxygen species (ROS) formation by NADPH oxidase and apocynin prevents this effect (Beltowski et al. 2005).

Leptin was injected into the flank region in 0.5 ml vehicle twice daily between 0700 and 0800 h and between 1900 and 2000 h. Rats not receiving leptin were injected with equal volume of saline. T0901317 was administered orally at a dose of 1 mg/kg per day; the compound was mixed with powdered chow suspended in tap water. The amount of chow served as a powder (about 25% of daily food consumption) was adjusted daily to maintain constant T0901317 intake. This dose of LXR agonist was chosen to avoid the induction of hypertriglycerideremia, liver steatosis, and elevation of liver enzymes observed at higher doses of T0901317 (10–100 mg/kg). In preliminary experiments, we established that T0901317 administered at 1 mg/kg per day had no effect on plasma lipid profile and liver triglyceride concentration. Apocynin was administered in the drinking water at 2 mM; at this dose apocynin effectively prevents leptin-induced oxidative stress (Beltowski et al. 2005).

About 6 h after the last leptin injection or vehicle injection, animals were anesthetized with pentobarbital (50 mg/kg i.p.), and blood was withdrawn from the abdominal aorta into lithium heparin-containing tubes (for PON1) and EDTA-containing tubes (for lipid profile, isoprostanes, aminotransferases, and leptin level). Blood was centrifuged (3000 g for 10 min at 4 °C) and plasma was stored at −80 °C until analysis.

**PON1 activity toward synthetic substrates**

PON1 activity toward paraoxon was determined by measuring the initial rate of p-nitrophenol formation, monitored at 412 nm in the assay mixture (800 μl) containing 2:0 mM paraoxon, 2:0 mM CaCl$_2$, and 20 μl plasma in 100 mM Tris–HCl (pH 8-0). The blank sample containing incubation mixture without plasma was run simultaneously to correct for spontaneous substrate hydrolysis. The enzyme activity was calculated from $E_{412}$ of p-nitrophenol (18 290/M per cm) and was expressed in U/ml; 1 U enzyme hydrolyses 1 nmol paraoxon per min (Ayub et al. 1999).

PON1 activity toward phenyl acetate (arylesterase activity) was determined by measuring the initial rate of phenol formation, monitored at 270 nm, in the assay mixture (3 ml) containing 2 mM phenyl acetate, 2 mM CaCl$_2$, and 10 μl plasma in 100 mM Tris–HCl (pH 8-0). Increase in absorbance at 270 nm was monitored for 3 min and the activity was calculated from $E_{270}$= 1310/M per cm. The results are expressed in U/ml, 1 U arylesterase hydrolyses 1 μmol phenyl acetate per min (Ayub et al. 1999).

**Hcy-thiolactone hydrolase assay**

Hcy-thiolactonase activity of PON1 was assayed by a modified pH-metric method, which measures the amount of H$^+$ ions liberated during hydrolysis of lactone to free acid (Billecke et al. 2000). Although alkaline pH is optimal for PON1 activity, Hcy-thiolactone is unstable in alkaline environment and therefore Hcy-thiolactone hydrolase assay
was carried out at pH 7.0. Heparinized plasma (20 μl) was added to 800 μl of the reaction mixture containing 5 mM HEPES (pH 7.0), 5 mM 1-L-hcy-thiolactone, 0.004% (106 μM) phenol red, 0.005% BSA, and 1.0 mM CaCl₂, and the increase in absorbance at 422 nm was monitored for 4 min. Blank sample (without plasma) was subtracted to correct for spontaneous substrate hydrolysis. Hcy-thiolactonase/PON1 activity was calculated from the standard HCl titration curve and was expressed in nmol H⁺ liberated/1 ml plasma per min. The relationship between the amount of plasma used for the assay and the measured activity was linear within the range of at least 10–50 μl. The detection limit calculated as 0 ± 2 s.d. derived from four blank samples was 58.1 nmol/ml per min. The intra- and inter-assay coefficient of variation (CV) values were 3.5 and 7.4% respectively. The method was further validated by the following observations: 1) no activity was measured in the absence of Ca²⁺ in the buffer or when 2 mM EDTA was added; 2) about sevenfold higher activity was observed in the rabbit and no measurable activity was detected in the chick serum, which is consistent with the absence of Hcy-thiolactonase/PON1 activity in birds and very high Hcy-thiolactonase/PON1 activity in the rabbit (Jakubowski 2000, Jakubowski et al. 2001, James 2006).

**PON1 activity in the liver**

Liver samples were homogenized in ten volumes of 50 mM Tris–HCl (pH 8.0) containing 2 mM CaCl₂, and the homogenate was centrifuged at 10 000 g for 15 min. PON1 activity toward paraoxon was measured as described previously (Belowski et al. 2005). Hcy-thiolactonase is hydrolyzed in tissues by both PON1 and a cysteine protease, bleomycin hydrolase (Jakubowski 2008). To measure PON1-dependent Hcy-thiolactonase hydrolytic activity, the difference between activities measured in CaCl₂-containing buffer and in the buffer containing no Ca²⁺ but 2 mM EDTA was calculated.

**Plasma Hcy assay**

Plasma total Hcy was assayed by enzyme immunoassay using commercially available kit (Axis Shield Diagnostics Ltd, Dundee, UK). Since most of the plasma Hcy occurs in the form of disulfides, the samples are first reduced with dithiothreitol (DTT) to a free thiol form. Hcy is then converted to S-adenosylhomocysteine (SAH) by adding the excess of adenosine and SAH hydrolase. The sample was pipetted into the microwell plates precoated with SAH, and mouse anti-SAH antibody was then added. SAH contained in the sample competes with SAH bound to the wells for these antibodies. During the incubation, anti-SAH antibody binds to SAH attached to the wells; the amount of antibodies bound is thus inversely proportional to the amount of SAH in the sample. After removal of unbound anti-SAH antibody and washing, secondary rabbit anti-mouse antibody labeled with HRP was added together with peroxidase substrate, N-methyl-2-pyrrolidone. After 10-min incubation at 25°C, the reaction was stopped by adding 100 μl of 0.8 M sulfuric acid to each well and the absorbance was measured at 450 nm. Hcy concentration in the sample was calculated from the calibration curve prepared with 2–50 μM of SAH standard. The detection limit of the assay is 0.5 μM, whereas the intra- and inter-assay CV values were 6 and 9% respectively.

**N-Hcy-protein assay**

Hcy bound via an isopeptide bond to ε-NH₂ groups of lysine residues in plasma protein (N-Hcy-protein) is not released by DTT in the procedure used for the measurement of ‘total’ Hcy (Jakubowski et al. 2008). To assay levels of protein-bound Hcy, 100 μl plasma was treated with 10 μl of 10 mM DTT for 5 min to release Hcy bound to plasma proteins by disulfide bonds. Then, 100 μl ethanol was added and the sample was centrifuged at 10 000 g for 10 min. Protein precipitate was washed three times with 1 ml saline and recentrifuged. After the final wash, 1 ml of 6 M HCl was added to the precipitate, and the sample was incubated in sealed tubes at 110°C for 5 h. Protein hydrolysate was then filtered by centrifugation through 10 kDa cut-off filters (Ultrafree 0.5, Millipore, Bedford, MA, USA) and evaporated under nitrogen. Subsequently, the sample was dissolved in 100 μl of 100 mM NaOH to convert Hcy-thiolactone to Hcy, which was then assayed by the method described above.

**Plasma isoprostanes**

Plasma 8-isoprostanes, the products of ROS-mediated peroxidation of arachidonic acid, were measured as a marker of oxidative stress using 8-Isoprostane EIA kit (Cayman Chemical, Ann Arbor, MI, USA). Plasma samples were mixed with ethanol and centrifuged to remove particulate matter. Ethanol was evaporated from the supernatant by a stream of nitrogen. Then, the supernatant was acidified with acetate buffer to pH 4.0 and isoprostanes were extracted using C-18 SPE cartridges (Waters Corporation, Milford, MA, USA). Cartridges were activated by rinsing with 5 ml methanol and 5 ml H₂O, and then the sample was passed through the cartridge. The cartridge was rinsed with 5 ml water, dried, and then rinsed with 5 ml 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 EIA buffer contained in the kit and used for the analysis. All the samples were assayed in duplicate; before purification one set of samples was spiked with 8-isoprostane standard to correct for individual recovery. The recovery averaged 76%. The limit of detection of the assay was 5 pg/ml, the intra- and inter-assay CV values were 6 and 9% respectively.
Other assays

Plasma leptin was assayed using Leptin Enzyme Immunoassay Kit (Cayman Chemical). Plasma triglycerides, total cholesterol, alanine aminotransferase and aspartate aminotransferase were measured by routine laboratory methods. HDL cholesterol was assayed after precipitation of other lipoproteins with the solution containing phosphotungstic acid (6.1 mM) and MgCl₂ (20 mM).

Reagents

Recombinant rat leptin was purchased from R&D Systems (Abingdon, Oxon, UK). T09013 was obtained from Cayman Chemical. Other reagents were from Sigma–Aldrich.

Statistical analysis

Data are reported as mean ± S.E.M. from eight animals in each group. Results were analyzed statistically by one-way ANOVA and Tukey post-hoc test. *P<0.05 was considered significant.

Results

Characteristics of experimental groups

Leptin administration resulted in 15–20% reduction of food intake. However, it had no effect on final body weight of the animals (Table 1). Plasma leptin concentration was 3.8-fold higher in leptin-treated rats than in control animals, whereas pair-feeding lowered leptin level to about half of the control. Neither T09013 nor apocynin had any effect on food intake, body weight, and plasma leptin level in either leptin-treated rats (Table 1) or in animals not receiving this hormone (not shown). Lipid profile and aminotransferases did not differ between the experimental groups.

Hyperleptinemia lowers plasma PON1 levels

PON1 levels, measured by enzymatic assays with paraoxon, phenyl acetate, and Hcy-thiolactone, were lower in leptin-treated animals than in control animals by 27.4, 29.0, and 41.0% respectively (Fig. 1). Pair-feeding had no effect on PON1 levels.

To test whether leptin had any acute effect on PON1 levels, leptin was injected at a single dose (0.25 mg/kg) and blood was withdrawn after 1, 2, 3 or 6 h. Although plasma leptin concentration was markedly increased (1 h: 124 ± 13 ng/ml; 2 h: 78 ± 6.5 ng/ml; 3 h: 44 ± 3.9 ng/ml; 6 h: 15.7 ± 2.6 ng/ml), PON1 levels measured by enzymatic assays with Hcy-thiolactone did not change (1 h: 214 ± 17 nmol/min per ml; 2 h: 228 ± 16 nmol/min per ml; 3 h: 204 ± 19 nmol/min per ml; 6 h: 225 ± 23 nmol/min per ml). Similarly, PON1 levels measured by enzymatic assays with paraoxon or phenyl acetate were not altered after acute leptin administration (not shown). These observations exclude a direct effect of leptin on PON1 activity and indicate that enzyme deficiency is induced only by long-lasting hyperleptinemia.

Table 1

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Leptin-treated</th>
<th>Pair-fed</th>
<th>Leptin-treated + T0901317</th>
<th>Leptin-treated + apocynin</th>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td>248 ± 6</td>
<td>241 ± 7</td>
<td>240 ± 8</td>
<td>259 ± 7</td>
<td>252 ± 9</td>
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<td>Plasma leptin (ng/ml)</td>
<td>3.75 ± 0.39</td>
<td>14.42 ± 1.07†</td>
<td>2.01 ± 0.27*</td>
<td>14.07 ± 1.11†</td>
<td>14.76 ± 1.03†</td>
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<td>Triglycerides (mmol/l)</td>
<td>0.67 ± 0.05</td>
<td>0.55 ± 0.05</td>
<td>0.62 ± 0.06</td>
<td>0.57 ± 0.06</td>
<td>0.52 ± 0.05</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>1.64 ± 0.11</td>
<td>1.60 ± 0.14</td>
<td>1.52 ± 0.112</td>
<td>1.86 ± 0.17</td>
<td>1.63 ± 0.13</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/l)</td>
<td>1.27 ± 0.08</td>
<td>1.18 ± 0.08</td>
<td>1.22 ± 0.10</td>
<td>1.39 ± 0.13</td>
<td>1.16 ± 0.10</td>
</tr>
<tr>
<td>ALT (U/ml)</td>
<td>24.3 ± 1.6</td>
<td>25.2 ± 1.4</td>
<td>22.1 ± 1.8</td>
<td>27.9 ± 1.9</td>
<td>21.3 ± 1.5</td>
</tr>
<tr>
<td>AST (U/ml)</td>
<td>23.7 ± 1.8</td>
<td>24.1 ± 1.6</td>
<td>25.2 ± 1.5</td>
<td>26.7 ± 2.0</td>
<td>22.7 ± 1.8</td>
</tr>
</tbody>
</table>

ALT, alanine aminotransferase; AST, aspartate aminotransferase. *P<0.05, †P<0.001 versus control group.

Figure 1

Effect of leptin (0.25 mg/kg twice daily s.c. for 7 days) administered alone or together with synthetic LXR agonist, T0901317 (1 mg/kg per day p.o.) or NADPH oxidase inhibitor, apocynin (2 mM in the drinking water) on plasma PON1 levels assayed by enzymatic activity measurements with the artificial substrates paraoxon and phenyl acetate (A) and the natural substrate Hcy-thiolactone (B). **P<0.01, ***P<0.001 versus control group.
Hyperleptinemia has no effect on liver PON1

Because plasma HDL-bound PON1 is synthesized in the liver, we also measured enzyme activity in liver homogenates. PON1 activity toward paraoxon did not differ between groups (control: 5.82 ± 0.47 U/mg protein; leptin-treated: 5.26 ± 0.51 U/mg protein; pair-fed: 5.43 ± 0.42 U/mg protein; leptin + T0901317: 5.31 ± 0.37 U/mg protein; leptin + apocynin: 5.71 ± 0.56 U/mg protein; T0901317 alone: 5.23 ± 0.41 U/mg protein; apocynin alone: 5.27 ± 0.42 U/mg protein). Similarly, PON1 activity toward Hcy-thiolactone was comparable in all groups (control: 3.91 ± 0.42 nmol/min per mg protein; leptin-treated: 3.72 ± 0.42 nmol/min per mg protein; pair-fed: 3.51 ± 0.39 nmol/min per mg protein; leptin + T0901317: 3.96 ± 0.44 nmol/min per mg protein; leptin + apocynin: 3.77 ± 0.46 nmol/min per mg protein; T0901317 alone: 4.31 ± 0.57 nmol/min per mg protein; apocynin alone: 4.07 ± 0.49 nmol/min per mg protein).

Hyperleptinemia elevates plasma N-Hcy-protein

Owing to its Hcy-thiolactone-hydrolyzing activity, PON1 has the ability to protect against the accumulation of N-Hcy-protein in vitro (Jakubowski et al. 2000, 2001). To examine whether PON1 protects against the accumulation of N-Hcy-protein in vivo, N-Hcy-protein level was assayed in hyperleptinemic and control rats. We found that the level of N-Hcy-protein was 92.9% higher in leptin-treated animals than in controls (Fig. 2). Pair-feeding had no effect on N-Hcy-protein level. Plasma total Hcy did not differ between groups.

A single leptin injection had no effect on either plasma N-Hcy-protein (1 h: 2.01 ± 0.24 μM; 2 h: 1.87 ± 0.21 μM; 3 h: 2.14 ± 0.21 μM; 6 h: 1.99 ± 0.20 μM) or total Hcy (1 h: 8.21 ± 0.74 μM; 2 h: 7.82 ± 0.84 μM; 3 h: 8.14 ± 0.79 μM; 6 h: 8.51 ± 0.98 μM).

T0901317, but not apocynin, normalizes PON1 and N-Hcy-protein levels

LXR agonists, including T0901317, inhibit the progression of atherosclerosis in animal models (Terasaka et al. 2003), possibly by their positive effects on HDL and PON1 levels (Gouéda et al. 2003). Thus, we set out to test how T0901317 affects PON1 and N-Hcy-protein levels in hyperleptinemic rats. We found that co-administration of T0901317 restored the PON1 (Fig. 1) and N-Hcy-protein levels (Fig. 2) in leptin-treated animals to values observed in the control group.

To determine whether oxidative stress mediates the effects of hyperleptinemia on PON1 and N-Hcy-protein levels, the NADPH oxidase inhibitor apocynin was co-administered into the hyperleptinemic rats. We found that apocynin did not affect PON1 (Fig. 1) and N-Hcy-protein levels (Fig. 2) in the hyperleptinemic rats. The levels of PON1 and N-Hcy-protein in animals receiving T0901317 or apocynin, but not treated with leptin, did not differ from the control (not shown).

Apocynin, but not T0901317, prevents oxidative stress in hyperleptinemia

Leptin stimulates ROS formation by NADPH oxidase abundantly expressed in the cardiovascular system (Korda et al. 2008), and LXR agonists have been shown to ameliorate oxidative stress in some studies (Chang et al. 2007). In addition, PON1 may be inactivated by oxidative stress (Nguyen et al. 2009). To investigate if the normalizing effect of T0901317 on PON1 activity levels results from amelioration of leptin-induced oxidative stress, we measured plasma concentration of isoprostanes in each experimental group. Plasma isoprostane levels were higher in leptin-treated group than in control group, but were unchanged in the pair-fed group (Fig. 3). The NADPH oxidase inhibitor apocynin, but not the LXR agonist T0901317, normalized isoprostane levels in leptin-treated animals (Fig. 3). Neither apocynin nor T0901317 had any effect on plasma isoprostanes in rats not receiving leptin. Leptin administered as a single injection had no effect on plasma isoprostanes (1 h: 137 ± 11 pg/ml; 2 h: 147 ± 13 pg/ml; 3 h: 129 ± 12 pg/ml; 6 h: 136 ± 13 pg/ml). Taken together, these results suggest that oxidative stress does not mediate effects of hyperleptinemia on PON1 and N-Hcy-protein levels in the rat.

Figure 2 Effect of leptin administered alone or together with T0901317 or apocynin on plasma N-Hcy-protein (black bars) and total Hcy (white bars). **P<0.01 versus control group.
Discussion

The principal findings of this study are: 1) experimental hyperleptinemia induced in rats decreases the animals’ ability to metabolize Hcy-thiolactone; 2) hyperleptinemia significantly increases plasma N-Hcy-protein levels but has no effect on plasma total Hcy; 3) effects of leptin on PON1 and N-Hcy-protein levels are not attributable to caloric restriction because they are not reproduced by pair-feeding; 4) the synthetic LXR agonist T0901317 normalizes protein N-homocysteinyltaion by preventing leptin-induced decrease in Hcy-thiolactonase activity of PON1; 5) the NADPH oxidase inhibitor apocynin prevents oxidative stress but does not normalize PON1 levels in leptin-treated rats.

Hcy-thiolactone is synthesized when Hcy is mistakenly selected in place of methionine by methionyl-tRNA synthetase. In contrast to methionine, Hcy is not incorporated into proteins but is converted to highly reactive cyclic thioester, Hcy-thiolactone. Although this mechanism protects from erroneous incorporation of Hcy into proteins, Hcy-thiolactone can post-translationally react with amino groups of protein lysine residues and alter physicochemical properties of proteins and their biological activities (Jakubowski 2008). Protein N-homocystinyltaion has been implicated in atherosclerosis and other complications of hyperhomocysteinemia. For example, N-Hcy-LDL triggers oxidative stress, decreases NO production by endothelial cells, and induces endothelial cell apoptosis (Ferretti et al. 2004, 2006, Vignini et al. 2004). N-homocysteinyltaion of fibrinogen renders it less susceptible to fibrinolysis (Saules et al. 2006). N-Hcy-proteins induce an auto-immune response (Jakubowski 2005) and accumulate in atherosclerotic lesions (Perla-Kajan et al. 2008). The levels of free Hcy-thiolactone and N-Hcy-proteins are increased in hyperhomocysteinemia secondary to genetic or nutritional deficiencies (Jakubowski et al. 2008, 2009) or renal insufficiency (Perna et al. 2006), as well as in patients with ischemic heart disease (Yang et al. 2006). The level of Hcy-thiolactone and N-Hcy-protein is also affected by PON1, which hydrolyzes thiolactone to free Hcy. In the present study, we found that hyperleptinemia lowers the ability of rats to hydrolyze Hcy-thiolactone, which results in significant increase in N-Hcy-protein levels in these animals. These results demonstrate that N-Hcy-protein levels can be increased not only by hyperhomocysteinemic conditions but also by PON1 deficiency. In addition, it has been demonstrated in the recent study (Riederer et al. 2009) that adipose tissue produces Hcy and releases it to the bloodstream. Thus, obesity associated with ‘endogenous’ hyperhomocysteinemia may result in the augmentation of protein homocysteinyltaion due to both excess of Hcy and impaired PON1-dependent metabolism of Hcy-thiolactone.

LXRs are transcription factors, which regulate the expression of target genes. LXRs are activated by oxygenated cholesterol derivatives, e.g. 24(S),25-epoxy-, 24(S)-hydroxy-, 25-hydroxy-, and 27-hydroxycholesterol. LXRs are ‘sterol sensors’, which protect against cholesterol overload by inhibiting intestinal cholesterol absorption, stimulating cholesterol efflux from plasma membrane to HDL, its transport to the liver and biliary excretion. In addition, LXRs have anti-inflammatory activity (Baranowski 2008, Fiévet & Staels 2009). Owing to their effects on cholesterol balance and inflammatory reaction, LXRs are implicated in atherosclerosis and other complications of hyperhomocysteinemia. For example, 22(R)-hydroxycholesterol, increases PON1 expression in cultured hepatocytes (Gouëdard et al. 2003). In the present study, we found that T0901317, administered at a low dose which has no effect on plasma lipid profile, normalizes PON1 activity and decreases N-Hcy-protein levels in leptin-treated rats. Our results suggest that the effects on PON1 and protein N-homocysteinyltaion might contribute to anti-atherogenic properties of LXRs. The mechanism by which T0901317 normalizes PON1 activity is not clear. The observation that the LXR agonist did not affect PON1 levels in animals not treated with leptin suggests that hyperleptinemia decreases PON1 levels by impairing LXR signaling. For example, leptin might induce deficiency of LXR-activating oxysterols. Leptin regulates macrophage cholesterol synthesis in a concentration-dependent manner (Balogh et al. 2007), so it might deplete especially 24(S),25-epoxycholesterol, the product of the ‘shunt pathway’ of the mevalonate cascade, the level of which is particularly vulnerable to inhibition of cholesterol synthesis (Brown 2009). Second, several oxysterols are formed in non-enzymatic reactions in ROS-dependent manner and some of them are potent LXR antagonists (Janowski et al. 1999). Leptin induces oxidative stress and may increase formation of these oxysterols, which then could compete with endogenous LXR antagonists, although the observation that the antioxidant apocynin normalized isoprostanes but not PON1 levels argues against this possibility. Third, the composition of HDL is essential for the uptake of PON1 from hepatocytes, its stability, and activation (Khersonsky & Tawfik 2005, James 2006). Although T0901317 did not change HDL cholesterol, we cannot exclude that as it affected other components of these lipoproteins such as phospholipids. Fourth, LXR activity may be impaired by its phosphorylation by various serine–threonine protein kinases such as protein kinase C (Delvecchio & Capone 2008), which is stimulated by leptin (Fruhbeck 2006). It should be noted that the LXR agonist T0901317 activates also the farnesoid X receptor (Houck et al. 2004). However, it is unlikely that the effect of T0901317 was mediated by farnesoid X receptor since the latter decreases PON1 levels (Shih et al. 2006).
In conclusion, our study identifies a novel pro-atherogenic effect of chronic hyperleptinemia in the rat; increased N-homocysteinylilation of plasma proteins caused by impairment of the animal’s ability to hydrolyze Hcy-thiolactone by PON1. We also found that LXR agonists decrease protein N-homocysteinylilation levels by improving PON1 status, which could be relevant in treatment of conditions associated with hyperleptinemia such as obesity, metabolic syndrome or type 2 diabetes.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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