Effects of Human C-Reactive Protein on Pathogenesis of Features of the Metabolic Syndrome

Michal Pravenec, Takashi Kajiya, Václav Zídek, Vladimír Landa, Petr Mlejnek, Miroslava Šimáková, Jan Šilhavý, Hana Malínská, Olena Oliyarnyk, Ludmila Kazdová, Jianglin Fan, Jiaming Wang, Theodore W. Kurtz

See Editorial Commentary, pp XX–XX

Abstract—Major controversy exists as to whether increased C-reactive protein (CRP) contributes to individual components of the metabolic syndrome or is just a secondary response to inflammatory disease processes. We measured blood pressure and metabolic phenotypes in spontaneously hypertensive rats (SHRs) in which we transgenically expressed human CRP in the liver under control of the apolipoprotein E promoter. In transgenic SHRs, serum levels of human CRP approximated the endogenous levels of CRP normally found in the rat. Systolic and diastolic blood pressures measured by telemetry were 10 to 15 mm Hg greater in transgenic SHRs expressing human CRP than in SHR controls (P<0.01). During oral glucose tolerance testing, transgenic SHRs exhibited hyperinsulinemia compared with controls (insulin area under the curve: 36±7 versus 8±2 nmol/L per 2 hours, respectively; P<0.05). Transgenic SHRs also exhibited increased serum levels of interleukin 6 (36.4±5.2 versus 18±1.7 pg/mL; P<0.005) and increased hepatic and renal thiobarbituric acid reactive substances (1.2±0.9 versus 0.8±0.7 and 1.5±0.1 versus 1.1±0.5 mmol/L per milligram of protein, respectively; P<0.01), suggesting that oxidative stress may be mediating adverse effects of increased human CRP. These findings are consistent with the hypothesis that increased CRP is more than just a marker of inflammation and can directly promote multiple features of the metabolic syndrome. (Hypertension. 2011;57:00-00.) ● Online Data Supplement

Key Words: C-reactive protein ▪ metabolic syndrome ▪ oxidative stress ▪ transgenic spontaneously hypertensive rat

There is mounting interest in the role of inflammation in the pathogenesis of obesity, metabolic disturbances, diabetes mellitus, and cardiovascular disease. Among the various biomarkers of inflammation that are associated with increased risk for these disorders, C-reactive protein (CRP) has attracted the most attention.1,2 However, major controversy exists as to whether increased CRP contributes to disease pathogenesis or is just a secondary response to inflammatory disease processes.3 Although most of the experimental focus has been on the relationship between CRP and risk for coronary heart disease, increased CRP levels have also been reported to correlate with increased risk for diabetes mellitus and multiple features of the metabolic syndrome, including obesity, insulin resistance, dyslipidemia, and increased blood pressure.4,5 However, a huge knowledge gap exists as to whether CRP is just a marker or an active mediator of any of these inflammatory metabolic and cardiovascular disorders.

Although the results of recent “mendelian randomization” studies have failed to support a role for common CRP gene polymorphisms in the pathogenesis of coronary heart disease, they have not addressed whether genetic or nongenetic variation in CRP levels can influence risk for other forms of cardiovascular disease or features of the metabolic syndrome and diabetes mellitus.6,7 Because metabolic and hemodynamic disturbances affect millions of people and significantly increase the risk for a variety of cardiovascular disorders and for diabetes mellitus, there is intense interest in understanding...
the significance of increased CRP in the pathogenesis of features of the metabolic syndrome and related conditions. However, research progress on CRP has been hampered by the lack of effective animal models for studying the role of human CRP in pathogenesis of the metabolic syndrome or its components and for preclinical testing of novel CRP inhibitors. In the current studies, we sought to investigate whether increased levels of human CRP, per se, can promote increases in blood pressure and disturbances in glucose and lipid metabolism characteristic of the metabolic syndrome. To accomplish this goal, we transgenically expressed human CRP in the spontaneously hypertensive rat (SHR), a widely studied animal model of hypertension that is genetically predisposed to the development of multiple features of the metabolic syndrome.

Methods

Animals

We transgenically expressed human CRP in a highly inbred strain of SHRs (SHR/OlaIpcv) that has been brother×sister mated for well over 130 generations. Transgenic SHRs were derived by microinjections of zygotes with a previously described construct containing the cDNA for human CRP under control of the apolipoprotein E promoter8 with the objective of driving expression of the CRP transgene in liver where CRP is normally produced. Because hypertension begins to develop at a relatively young age in SHRs, whereas metabolic disturbances can take longer to become apparent, we performed blood pressure studies in 3-month-old transgenic SHRs (N=9) and age-matched nontransgenic controls (N=8) and metabolic studies in 13-month-old transgenic and control animals (N=8 per group). In all of the experiments, we studied male CRP transgenic SHRs together with male, age-matched, nontransgenic SHR controls. The rats were housed in an air-conditioned animal facility and allowed free access to standard diet and water. All of the experiments were performed in agreement with the Animal Protection Law of the Czech Republic (311/1997) and were approved by the ethics committee of the Institute of Physiology, Academy of Sciences of the Czech Republic.

Expression of the Transgene for Human CRP and the Endogenous Gene for Rat CRP Determined by Real-Time PCR

Total RNA was extracted using TRizol reagent (Invitrogen), and cDNA was prepared and analyzed by real-time PCR testing using QuantiTect SYBR Green reagents (Qiagen, Inc) on an Opticon continuous fluorescence detector (MJ Research). Gene expression levels were normalized relative to the expression of peptidylprolyl isomerase A (Ppia) (cyclophilin) gene, which served as the internal control for the endogenous CRP in the SHR controls. These findings confirm that the transgene is being functionally expressed in the transgenic SHR strain and in transgene expressing tissues. The expression levels are similarly expressed in tissues where the transgene is abundantly expressed in the liver with relatively little or no expression in other tissues, as anticipated given use of the apolipoprotein E promoter. In the SHR controls, the endogenous CRP levels in the transgenic rats were similar to serum levels of human CRP in the offspring. The real-time PCR measures the endogenous CRP gene with the objective of driving expression of the CRP transgene in liver where CRP is normally produced. The level of albumin in urine was analyzed by high-performance liquid chromatography method with UV-visible spectroscopy detection according to Contois et al.9 Urine albumin was adjusted for creatinine concentration (in milligrams per gram of creatinine). Creatinine was measured by Jaffe rate assay (Pliva-Lachema). The activities of antioxidant enzymes and lipoperoxidation products were determined as described previously.10 Detailed descriptions of the methods are available in the online Data Supplement.

Results

Derivation of Transgenic Rats and Expression of Transgenic and Endogenous CRP Proteins

Because the human CRP transgene was directly introduced on the highly inbred genetic background of the SHR, the transgenic SHR and nontransgenic SHR controls are genetically identical except for the presence of the human CRP transgene. The data are expressed as mean±SEM. Individual groups were compared by unpaired Student t test. The 24-hour mean values of systolic blood pressure were analyzed by repeated-measures ANOVA with group effect of strain and repeated measurements in time. Statistical significance was defined as P<0.05.

Urine Collection, Microalbuminuria, cGMP, and Parameters of Oxidative Stress

Rats were placed into metabolic cages for 6 hours to obtain urine samples for analysis of urinary excretion of albumin and cGMP. The level of albumin in urine was analyzed by high-performance liquid chromatography method with UV-visible spectroscopy detection according to Contois et al.9 Urine albumin was adjusted for creatinine concentration (in milligrams per gram of creatinine). Creatinine was measured by Jaffe rate assay (Pliva-Lachema). The activities of antioxidant enzymes and lipoperoxidation products were determined as described previously.10 Detailed descriptions of the methods are available in the online Data Supplement.

Parameters of Glucose and Lipid Metabolism and Blood Pressure Measurements

The methods for oral glucose tolerance testing, assessment of skeletal muscle insulin sensitivity, all of the biochemical measurements in serum and tissue, and telemetric blood pressure measurements are described in the online Data Supplement (please see http://hyper.ahajournals.org).

Transgenic Expression of Human CRP Promotes Multiple Features of the Metabolic Syndrome

Having established functional expression of the human CRP transgene, we next phenotyped the transgenic SHR and
nontransgenic SHR controls for key clinical features of the metabolic syndrome. Figure 2 shows that transgenic expression of human CRP significantly increased blood pressure in the SHR. The daily 24-hour average systolic and diastolic blood pressures measured by radiotelemetry in conscious, unrestrained transgenic SHRs expressing human CRP were significantly greater than in transgene-negative SHR controls (Figure 2). The average mean arterial pressure was significantly greater in the CRP transgenic SHR than in the transgene negative SHR controls (Figure 2). The average mean arterial pressure was significantly greater in the CRP transgenic SHR than in the transgene negative controls, 156±3 mm Hg versus 142±4 mm Hg, respectively (P<0.0001), with blood pressures being elevated in both the daytime and nighttime hours (data not shown). Supplemental dietary sodium chloride induced similar increases in blood pressure in the transgenic SHRs and in the transgene negative controls. In addition, we have found that the levels of the major NO second messenger, cGMP were significantly reduced in urine of the CRP transgenic rats when compared with controls (cGMP:creatinine ratio: 0.177±0.021 versus 0.235±0.015 nmol/mmol, respectively; P<0.05), suggesting that transgenic CRP might be influencing blood pressure regulation through NO-related pathways.

In the transgenic SHRs, fasting glucose levels before oral glucose loading were similar to those in the transgene negative controls (Figure 3A). During the oral glucose tolerance test, there was a tendency for increased serum glucose levels in the transgenic SHRs, but the results did not achieve statistical significance. However, serum insulin levels were significantly increased in the transgenic SHRs compared with controls during the glucose tolerance test (Figure 3B). These findings are consistent with an impairment in insulin action and are further supported by the observation of skeletal muscle insulin resistance in the transgenic SHRs versus transgene negative SHR controls (Figure 3B). These findings are consistent with an impairment in insulin action and are further supported by the observation of skeletal muscle insulin resistance in the transgenic SHRs versus transgene negative SHR controls (Figure 3B). These findings are consistent with an impairment in insulin action and are further supported by the observation of skeletal muscle insulin resistance in the transgenic SHRs versus transgene negative SHR controls (Figure 3B). Transgenic expression of human CRP was associated with increased serum levels of triglycerides and liver triglyceride concentrations, whereas muscle triglyceride concentrations were not significantly different (Table 1). High-density lipoprotein cholesterol levels were similar between the 2 groups (Table 1). The above findings show that transgenic expression of human CRP in the SHR can promote multiple features of the metabolic syndrome, including insulin resistance, hypertriglyceridemia, and increased blood pressure. In addition, we have found that transgenic expression of human CRP
induces decreases in serum adiponectin levels compared with those in nontransgenic SHR controls (Table 1). These findings establish that the SHR strain provides a favorable genetic background for investigating the adverse effects of human CRP on both hemodynamic and biochemical components of the metabolic syndrome.

Figure 3. Oral glucose tolerance test (OGTT). A, Serum glucose levels during the glucose tolerance test. B, Serum insulin levels during the OGTT. *P<0.01 and **P<0.001, respectively.

Table 1. Metabolic and Inflammatory Parameters in Nonfasted Rats

<table>
<thead>
<tr>
<th>Trait</th>
<th>Control SHR</th>
<th>CRP Transgenic SHR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight</td>
<td>412±13</td>
<td>410±4</td>
</tr>
<tr>
<td>Serum adiponectin, ng/mL</td>
<td>4.32±0.59</td>
<td>2.37±0.31*</td>
</tr>
<tr>
<td>Serum triglycerides, mmol/L</td>
<td>0.64±0.03</td>
<td>0.84±0.05*</td>
</tr>
<tr>
<td>Liver triglycerides, μmol/g</td>
<td>4.30±0.57</td>
<td>7.62±0.90*</td>
</tr>
<tr>
<td>Muscle triglycerides, μmol/g</td>
<td>1.00±0.73</td>
<td>1.45±0.38</td>
</tr>
<tr>
<td>Serum NEFA, mmol/L</td>
<td>0.44±0.07</td>
<td>0.36±0.02</td>
</tr>
<tr>
<td>Serum HDL cholesterol, mmol/L</td>
<td>1.35±0.05</td>
<td>1.36±0.06</td>
</tr>
<tr>
<td>Serum interleukin 6, pg/mL</td>
<td>18±1.8</td>
<td>36.4±5.2*</td>
</tr>
<tr>
<td>Serum TNF-α, pg/mL</td>
<td>0.94±0.25</td>
<td>0.82±0.19</td>
</tr>
<tr>
<td>Serum ALT, U/L</td>
<td>104±3</td>
<td>120±5*</td>
</tr>
<tr>
<td>Serum AST, U/L</td>
<td>403±20</td>
<td>376±22</td>
</tr>
<tr>
<td>Serum creatinine, μmol/L</td>
<td>43.6±2.5</td>
<td>45.9±2.2</td>
</tr>
</tbody>
</table>

NEFA indicates nonesterified fatty acids; HDL, high-density lipoprotein; TNF, tumor necrosis factor; ALT, alanine transaminase; AST, aspartate transaminase. *P<0.05.

Transgenic Expression of Human CRP Promotes Inflammation and Oxidative Tissue Damage in the SHR Model

It has been proposed recently that CRP might be influencing risk for hypertension, diabetes mellitus, and cardiovascular disease by increasing oxidative stress in tissues involved in the pathogenesis of these disorders. It has long been suspected that increased oxidative stress in the liver might influence glucose metabolism and risk for diabetes mellitus and that increased oxidative stress in the kidney might play a role in the pathogenesis of hypertension. Therefore, we measured activities of key antioxidant enzymes in the liver and kidney and assessed tissue levels of thiobarbituric acid reactive substances and conjugated dienes as indexes of tissue oxidative damage in transgenic SHRs. Table 2 shows that expression of transgenic CRP was associated with impaired tissue activity of glutathione peroxidase, reduced glutathione levels, and increased hepatic and renal oxidative tissue damage. As shown in Table 1, serum levels of adiponectin were increased in the transgenic CRP SHR consistent with the findings of oxidative liver damage. Expression of transgenic CRP was also associated with increased levels of interleukin 6, suggesting the presence of an inflammatory state.

In addition to finding direct evidence of renal oxidative damage in the transgenic CRP SHR (as reflected by increased thiobarbituric acid reactive substances and conjugated dienes in kidney tissue shown in Table 2), we found marked increases in microalbuminuria in the CRP transgenic rats (200±35 versus 26±5 mg/g of albumin:creatinine ratio, respectively; P<0.001), which was accompanied by histopathologic changes in the kidneys (Figure 5). These observations raise the possibility that increased levels of CRP might be promoting renal damage not only through effects on blood pressure but also through direct actions of CRP on the kidney. However, it is also possible that renal damage in the CRP transgenic rat could have been mediated by increases in blood pressure coupled with disturbances in glucose and lipid.
metabolism rather than by a direct effect of CRP on the kidney.

**Discussion**

Many epidemiological studies have shown associations between increased CRP levels and increased risk for cardiovascular disease, diabetes mellitus, and multiple components of the metabolic syndrome, including obesity, insulin resistance, dyslipidemia, and increased blood pressure. However, epidemiological studies cannot resolve issues of pathogenesis, and considerable controversy exists as to whether increased CRP functions as a disease marker, a disease mediator, or both. To directly investigate the effects of increased levels of human CRP on disease pathogenesis, it is necessary to determine the impact of actively perturbing CRP levels in the whole organism.

Infusions of highly purified CRP can be used to manipulate CRP levels in humans and in animals; however, such studies are typically performed on a relatively short-term basis and are inconvenient for studying the pathogenic effects of chronic exposure to increased levels of CRP. Delivery of human CRP to animals by virally mediated gene transfer can facilitate chronic studies but can be complicated by immune reactions to the administration of heterologous forms of CRP. In contrast, transgenic techniques can be used to manipulate CRP levels over an extended period of time in vivo without many of the limitations associated with CRP injections or delivery of CRP by viral vectors.

Given the potential benefits of transgenic approaches for investigating the biological effects of increased levels of CRP, many investigators have used transgenic mice expressing human CRP to study its role in atherosclerosis and cardiovascular function. However, relatively little work has been done in these models with respect to pathogenesis of features of the metabolic syndrome and risk factors for diabetes mellitus. Because of the fact that CRP is not an acute-phase reactant in mice and is synthesized in very low amounts in mice, some investigators have also raised questions about the utility of mouse models for studying the pathogenic effects of human CRP. Recently, several groups have advocated use of the rat as an alternative model for studying the biological actions of human CRP. Because human CRP can activate rat complement and can be proinflammatory in rats, we investigated the blood pressure and metabolic effects of transgenically expressed human CRP in the SHR model that is genetically predisposed to developing multiple features of the metabolic syndrome.

In the current studies, we have found that, in the SHR model, transgenic expression of human CRP in the liver promotes insulin resistance and hypertriglyceridemia, as well as increased blood pressure. The serum level of human CRP in the transgenic rats was similar to the serum level of

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**Table 2. Parameters of Oxidative Stress**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control SHR</th>
<th>CRP Transgenic SHR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SOD, U/mg of protein</td>
<td>0.363±0.038</td>
<td>0.299±0.014†</td>
</tr>
<tr>
<td>GSH-Px, μM GSH per min per mg of protein</td>
<td>636±18</td>
<td>467±22*</td>
</tr>
<tr>
<td>GR, μM NADPH per min per mg of protein</td>
<td>115±14</td>
<td>142±14</td>
</tr>
<tr>
<td>CAT, μM H2O2 per min per mg of protein</td>
<td>798±36</td>
<td>899±21†</td>
</tr>
<tr>
<td>GSH, μM/mg of protein</td>
<td>22.0±1.6</td>
<td>16.9±1.1‡</td>
</tr>
<tr>
<td>TBARS, nM/mg of protein</td>
<td>0.829±0.045</td>
<td>1.328±0.086*</td>
</tr>
<tr>
<td>CD, nM/mg of protein</td>
<td>19.5±1.1</td>
<td>24.2±2‡</td>
</tr>
<tr>
<td>Renal cortex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SOD, U/mg of protein</td>
<td>0.386±0.019</td>
<td>0.304±0.022†</td>
</tr>
<tr>
<td>GSH-Px, μM GSH per min per mg of protein</td>
<td>512±18</td>
<td>292±19*</td>
</tr>
<tr>
<td>GR, μM NADPH per min per mg of protein</td>
<td>236±23</td>
<td>268±19</td>
</tr>
<tr>
<td>CAT, μM H2O2 per min per mg of protein</td>
<td>671±19</td>
<td>595±25‡</td>
</tr>
<tr>
<td>GSH, μM/mg of protein</td>
<td>12.7±0.6</td>
<td>9.0±0.8*</td>
</tr>
<tr>
<td>TBARS, nM/mg of protein</td>
<td>1.111±0.055</td>
<td>1.456±0.102†</td>
</tr>
<tr>
<td>CD, nM/mg of protein</td>
<td>20.8±1.1</td>
<td>26.8±1.6†</td>
</tr>
</tbody>
</table>

SOD indicates superoxide dismutase; GSH-Px, seleno-dependent glutathione peroxidase; GR, glutathione reductase; CAT, catalase; GSH, glutathione; TBARS, thiobarbituric acid reactive substances; CD, conjugated dienes.

*P<0.001.
†P<0.01.
‡P<0.05.
endogenous rat CRP in the transgene negative controls. Accordingly, in the transgenic SHR, the overall CRP level (the level of rat and human CRP combined) was increased by <2-fold compared with the normal CRP level observed in the SHR controls. This relative increase in total CRP level in the transgenic SHR is similar in magnitude to the relative increase in CRP observed in humans with metabolic syndrome. Thus, in the SHR model, serum levels of human CRP that are close to the endogenous levels of CRP normally found in rats can be associated with effects on the main hemodynamic and biochemical features of the metabolic syndrome. However, it should also be noted that the absolute increase in CRP in our rat model was very large compared with the absolute increase of CRP observed in humans with the metabolic syndrome. Therefore, in the transgenic SHRs, as with any animal model, caution should be taken when extrapolating the results to humans.

Insulin resistance is considered to be a central component of the metabolic syndrome, and in the current studies, we have found that transgenic expression of human CRP can promote disturbances in insulin and glucose metabolism, including hyperinsulinemia and impaired insulin-stimulated glucose incorporation into skeletal muscle glycogen. These findings are consistent with recent in vitro studies in which exposure of L6 myocytes to human CRP was found to decrease both insulin-stimulated glucose uptake and glucose incorporation into glycogen.27 The in vitro studies by D’Alessandris et al27 indicate that human CRP affects insulin-stimulated phosphorylation of insulin receptor substrate 1 and suggest that human CRP causes disturbances in glucose metabolism by impairing insulin signaling pathways that regulate cell glucose transport.

In the current studies, transgenic expression of human CRP was also associated with reduced urinary excretion of cGMP, suggesting that increased levels of CRP may be attenuating NO production. It has been reported that CRP might reduce the activity of endothelial NO synthase by affecting the stability of endothelial NO synthase mRNA either directly or through the action of interleukin-6 or tumor necrosis factor-α.28 In addition, it has been shown that CRP can activate NADPH oxidase and cause increases in reactive oxygen species that may decrease NO production by inducing endothelial NO synthase uncoupling.26,29 Consistent with the current findings, Guan et al29 reported recently that virus-mediated expression of human CRP in Wistar rats also induces increases in blood pressure (measured by direct puncture of carotid artery under anesthesia) and decreases in urinary cGMP. In addition, Vongpatanasin et al31 have reported that transgenic expression of rabbit CRP in mice augments pressor responses to angiotensin II and promotes hypertension by inducing a decline in bioavailable NO and secondary downregulation of angiotensin II type 2 receptors in the vasculature. In contrast, alterations in angiotensin II type 1 receptor activity did not appear to play a role in the effects of CRP on blood pressure.31 Taken together, these observations support the possibility that increases in human CRP might be aggravating hypertension and features of the metabolic syndrome in part by reducing NO bioavailability. In future studies, it will be of interest to confirm whether the effects of human CRP on blood pressure in transgenic SHRs are more likely mediated by downregulation of NO activity and angiotensin II type 2 receptors than by activation of angiotensin II type 1 receptors.

Increased oxidative stress represents a well-known mechanism that may mediate some of the adverse effects of increased CRP levels on the risk for hypertension, diabetes mellitus, and cardiovascular disease.12,13,26 It has long been suspected that increased oxidative stress in liver and skeletal muscle might play an important role in the risk for diabetes mellitus and that increased oxidative stress in the kidney may be an important determinant of hypertension. In the current studies, we found that transgenic expression of human CRP causes increased oxidative tissue damage in both the liver and kidney. These observations suggest that increased CRP levels may be influencing risk for the metabolic syndrome by inducing oxidative damage in a variety of tissues. As shown previously by Hein et al,26 activation of NADPH oxidase is one of the principal mechanisms whereby human CRP stimulates production of reactive oxygen species. The current studies suggest that impaired activity of glutathione peroxidase and reductions in glutathione levels could also be contributing to the effects of human CRP on oxidative stress and tissue damage.

It should be emphasized that the current studies on the metabolic effects of human CRP were performed in the SHR model, which is a strain known to be genetically susceptible to developing multiple features of the metabolic syndrome. It is possible that other hypertensive models or strains might differ from the SHR with respect to their susceptibility to the adverse metabolic effects of human CRP. Having established that human CRP can aggravate features of the metabolic syndrome in the most widely used animal model of essential hypertension, future studies can now be planned to determine whether other strains or models exist that are more or less susceptible than the SHR to the adverse metabolic effects of human CRP. If so, this could open the door to linkage mapping of genetic factors that influence metabolic responses to increased levels of human CRP.

**Perspectives**

The current findings are consistent with the proposal that human CRP is more than just a secondary marker of inflammation and that increased levels of human CRP might directly contribute to the pathogenesis of features of the metabolic syndrome and risk for diabetes mellitus. The humanized CRP transgenic SHR represents a new model for investigating mechanisms whereby increased CRP levels may promote multiple components of the metabolic syndrome and could be further used to search for genetic factors that might influence susceptibility to the adverse metabolic effects of human CRP. This transgenic SHR model should also be of interest for testing the therapeutic effects of novel CRP inhibitors and a variety of other drugs, such as antioxidants, anti-inflammatory agents, that might block or attenuate the adverse effects of human CRP on tissues involved in the pathogenesis of the metabolic syndrome, diabetes mellitus, and related cardiovascular disorders.
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Disclosures
None.

References
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Effects of Human C-Reactive Protein on Pathogenesis of Features of the Metabolic Syndrome

Running title: CRP and the metabolic syndrome

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Supplementary Methods

**Oral glucose tolerance testing**
Oral glucose tolerance tests were performed using a glucose load of 300 mg/100 g body weight after overnight fasting. Blood was drawn from the tail without anesthesia before the glucose load (0 min time point) and at 30, 60, and 120 min thereafter.

**Skeletal muscle insulin sensitivity**
Insulin stimulated nonoxidative glucose metabolism was determined in isolated soleus muscle by measuring the incorporation of \(^{14}\text{C-U}\) glucose into glycogen. The soleus muscles were attached to a stainless steel frame in situ at in vivo length by special clips and separated from other muscles and tendons and immediately incubated for 2 h in Krebs-Ringer bicarbonate buffer, pH 7.4, gaseous phase 95\% O\(_2\) and 5\% CO\(_2\) that contained 5.5 mM unlabeled glucose, 0.5 µCi/ml of \(^{14}\text{C-U}\) glucose, and 3 mg/ml bovine serum albumin (Armour, Fraction V) with or without 250 µunits/ml insulin. After 2 h incubation, glycogen was extracted and glucose incorporation into glycogen determined.

**Biochemical analyses**
Rat serum CRP and human serum CRP were measured using ELISA kits (Alpha Diagnostics International, San Antonio, U.S.A.). Blood glucose levels were measured by the glucose oxidase assay (Pliva-Lachema, Brno, Czech Republic) using tail vein blood drawn into 5\% trichloracetic acid and promptly centrifuged. NEFA levels were determined using an acyl-CoA oxidase-based colorimetric kit (Roche Diagnostics GmbH, Mannheim, Germany). Serum triglyceride concentrations were measured by standard enzymatic methods (Pliva-Lachema, Brno, Czech Republic). Serum insulin concentrations were determined using a rat insulin ELISA kit (Mercodia, Uppsala, Sweden). Serum adiponectin was determined with a rat ELISA kit (B-Bridge International, Inc., Mountain View, U.S.A.). Serum IL6 and TNF\(\alpha\) were measured by rat ELISA kits (BioSource International, Inc., Camarillo, U.S.A.).

**Tissue triglyceride measurements**
For determination of triglycerides in liver and soleus muscle, tissues were powdered under liquid N\(_2\) and extracted for 16 h in chloroform:methanol, after which 2\% KH\(_2\)PO\(_4\) was added and the solution was centrifuged. The organic phase was removed and evaporated under N\(_2\). The resulting pellet was dissolved in isopropyl alcohol, and triglyceride content was determined by enzymatic assay (Pliva-Lachema, Brno, Czech Republic).

**Blood pressure measurement**
Arterial blood pressures were measured continuously by radiotelemetry in paired experiments between conscious, unrestrained male rats. All rats were allowed to recover for at least 7 days after surgical implantation of radiotelemetry transducers before the start of blood pressure recordings. Pulsatile pressures were recorded in 5-second bursts every 10 minutes throughout the day and night, and 24-hour averages for systolic and diastolic arterial blood pressure were calculated for each rat for 1 week periods. After measuring blood pressure for two weeks, all rats were given 1\% NaCl for drinking instead of tap water for an additional week of blood pressure measurements to test for effects of human CRP on blood pressure salt-sensitivity. The results from each rat in the same group were then averaged to obtain the group means.

**Parameters of oxidative stress**
The activity of superoxide dismutase (SOD) was analyzed using the reaction of blocking nitrotetrazolium blue reduction and nitroformazan formation. Catalase (CAT) activity measurement was based on the ability of H₂O₂ to produce with ammonium molybdate a color complex detected spectrophotometrically. The activity of seleno-dependent glutathione peroxidase (GSH-Px) was monitored by oxidation of glutathione by Ellman reagent (0.01M solution of 5,5′-dithiobis-2 nitrobenzoic acid). The level of GSH was determined in the reaction of SH-groups using Ellman reagent. Glutathione reductase (GR) activity was measured by the decrease of absorbance at 340 nm using a millimolar extinction coefficient of 6220 M⁻¹cm⁻¹ for NADPH (using Sigma assay kit). The levels of conjugated dienes (CD) were analyzed by extraction in the media (heptane:isopropanol = 2:1) and measured spectrophotometrically in the heptane layer. The levels of thiobarbituric acid reactive substances (TBARS) were determined by the reaction with thiobarbituric acid.

**Histopathological examination.**
Organs from each of three males of SHR-CRP and SHR strains, four months of age were collected immediately after euthanasia with ether overdose. The tissues were fixed in 10% buffered formalin. Samples were processed by the common paraffin technique and histological slices 5 μm thick were stained with hematoxylin and eosin, with blue trichrome and with PAS reaction as indicated.