Human C-reactive protein exacerbates metabolic disorders in association with adipose tissue remodelling

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Aims
C-reactive protein (CRP) expression is increased with metabolic alterations. We sought to clarify the effect of CRP on the development of obesity-induced metabolic disorders using human CRP-overexpressing transgenic mice (CRPTG).

Methods and results
CRPTG and their non-transgenic littermates (CON) were fed a standard diet (STD) or a high-fat diet (HFD) from 6 weeks of age. Oral glucose tolerance and intraperitoneal insulin tolerance tests 12 weeks after starting the diets showed deterioration of glucose tolerance and insulin sensitivity in HFD/CRPTG compared with HFD/CON. Hepatocellular ballooning, oil droplets, and peri-sinusoidal fibrosis were more prominent in HFD/CRPTG than in HFD/CON. In HFD/CRPTG, hepatic triglyceride content was higher and serum adiponectin levels lower than in HFD/CON. Epididymal adipose tissue mRNA expression of mucin-like, hormone receptor-like 1, monocyte chemotactic protein-1, and tumour necrosis factor-α in HFD/CRPTG was up-regulated compared with that in HFD/CON. Immunohistochemical staining of epididymal adipose tissue showed that the number of Mac-3⁺ macrophages was higher in HFD/CRPTG than in HFD/CON.

Conclusion
Human CRP overexpression facilitated the development of insulin resistance and hepatosteatosis with HFD in association with adiponectin down-regulation and enhancement of macrophage infiltration and expression of pro-inflammatory cytokines in epididymal adipose tissue, suggesting its pathogenic role in the development of obesity-induced metabolic disorders.

Keywords
Biomarker • Cytokine • Inflammation • Insulin resistance • Macrophage • Obesity

1. Introduction
Obesity has become a worldwide pandemic public health problem and is a strong risk factor for metabolic disorders, including insulin resistance, diabetes, dyslipidaemia, and hepatosteatosis. An excess of visceral fat associated with obesity is an important source of molecules inducing metabolic disorders. Inflammatory cytokines produced in visceral fat cause elevation of serum C-reactive protein (CRP), which is reported to be positively correlated with the number of metabolic alterations. Several lines of evidence have suggested a pivotal role of inflammation in the development of atherosclerosis and cardiovascular events. Ridker et al. reported that elevation of CRP is a strong prognostic factor in patients with obesity and metabolic syndrome, suggesting that CRP may reflect systemic inflammation provoked by visceral fat accumulation and may induce cardiovascular complications. However, recent experimental studies have shown that CRP per se might have various direct effects in various pathological conditions by altering the expression of inflammatory cytokines, thrombogenic factors, and matrix metalloproteinase. In addition, Xu et al. reported that CRP induces endothelial insulin resistance and dysfunction by attenuating insulin signalling and endothelial nitric oxide synthase.
CRP exacerbates adipose tissue remodelling

However, the pathological role of human CRP in metabolic disorders in vivo has not been fully elucidated. We hypothesized that human CRP promotes adipose tissue remodelling in the setting of obesity and exaggerates insulin resistance. To test this hypothesis, we generated human CRP-overexpressing transgenic mice and examined the effect of human CRP on obesity-associated insulin resistance and hepatosteatosis, focusing on adipose tissue remodelling.

2. Methods

2.1 Animals

Transgenic mice with human CRP expression (CRPTG, C57BL/6 background) were generated under direction of the CAG promoter to induce ubiquitous transgene expression.6 Male CRPTG at 6 weeks of age and their age-matched, male transgene-negative littermates (CON) were used. Gene presence was confirmed using genomic DNA purified from clipped tail tips (Figure 1A). Protein expression of human CRP in adipocytes, adipose stromal vascular cells in epididymal adipose tissue, and hepatocytes in liver was confirmed by immunohistochemical staining using antibodies against human CRP (Eptomiks, Inc., Burlingame, CA, USA; Figure 1B–E). Mice were housed with free access to food and water and exposed to a 12 h–12 h light–dark cycle. All procedures were performed in accordance with the Keio University animal care guidelines, which conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996).

2.2 Experimental protocol

CRPTG at 6 weeks of age were fed a standard diet (CLEA Japan, Inc., Tokyo, Japan; STD/CRPTG, n = 10) or a high-fat diet (CLEA Japan, Inc.; HFD/CRPTG, n = 20). CON fed a standard diet (STD/CON, n = 10) or a high-fat diet (HFD/CON, n = 20) served as controls. Twelve weeks after starting the diets, mice were killed after non-invasive measurements of blood pressure and heart rate using the tail-cuff method, in a conscious state, as previously described (BP-98A-L; Softron, Tokyo, Japan).8 Liver and epididymal adipose tissue were excised, and each was weighted separately. These tissues were snap frozen in liquid nitrogen, and then preserved at −80°C. For pathological analyses, tissue was fixed with 4% paraformaldehyde and embedded in paraffin. For Oil Red O staining, tissue was embedded in OCT compound.

2.3 Measurements of serum CRP, serum amyloid A, lipids and plasma adiponectin levels

A blood sample was taken from the right ventricle at the time of killing. Serum CRP was measured by latex photometric immunoassay (Nonopia®, CRP; Sekisui Medical Co., Tokyo, Japan). Serum amyloid A level was measured by ELISA (Life Diagnostics, West Chester, PA, USA). Serum total cholesterol and triglycerides were analysed by enzymatic methods using an automatic analyser (JCA-BM8060; JEO Ltd, Tokyo, Japan). Plasma adiponectin level was measured using an ELISA kit (Otsuka Pharmaceutical Co., Tokyo, Japan).

2.4 Metabolic testing

After feeding mice the diets for 12 weeks, an oral glucose tolerance test was performed preceded by 6 h fasting. Blood glucose concentration was measured before and 30, 60, 90, and 120 min after oral glucose administration (1 g/kg), using a cyclic GB sensor (Sanko Junyaku Co., Tokyo, Japan). An intraperitoneal insulin tolerance test was carried out in animals after overnight fasting. After an intraperitoneal bolus injection of recombinant human regular insulin (1 U/kg; Novolin R; Novo Nordisk Inc., Bagsværd, Denmark), blood glucose concentration was measured before and 30, 60, 90, and 120 min after injection.

2.5 Measurement of hepatic triglyceride content

Liver tissue (100 mg) was homogenized for 5 min in 4 mL isopropanol (isopropyl alcohol) with a Polytron disrupter. The homogenate was centrifuged at 2000g for 10 min, and 10 μL of the resulting supernatant was dried with a Speedvac System (Thermo Electron, Holbrook, NY, USA). The dry residue was dissolved in 100 μL isopropanol, and its triglyceride content was measured by an enzymatic method using an automatic analyser (JCA-BM8060; JEO Ltd.).

2.6 Histopathological study of liver and epididymal adipose tissue

Formalin-fixed liver tissue was processed, and 6-μm-thick paraffin sections were stained with haematoxylin and eosin or Masson’s trichrome for histological analysis. Liver tissue embedded in OCT compound was used for Oil Red O staining.

Haematoxylin and eosin staining of epididymal adipose tissue samples was performed to measure mean adipocyte size. Paraffin section-embedded specimens were stained with antibodies against Mac-3 (BD Biosciences, San Jose, CA, USA).

Formalin-fixed liver sections were examined by a pathologist blinded to genotype and kind of diet and scored using the non-alcoholic fatty liver disease (NAFLD) activity score system as described previously.9

2.7 Real-time quantitative RT-PCR

Total RNA was isolated from epididymal adipose tissue by acid–phenol extraction in the presence of chaotropic salts (TRizol; Invitrogen, Carlsbad, CA, USA) and subsequent isopropanol–ethanol precipitation as described previously.10 Real-time quantitative RT-PCR of each sample was carried out with a TaqMan RNA PCR kit and ABI Prism™7500 Fast Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The Taqman assays were performed using an experimental growth factor-like module containing mucin-like, hormone receptor-like 1 (Emr1; Mm00802530_m1), monocyte chemotactic protein-1 (MCP-1; Mm99999056_m1), tumour necrosis factor-α (TNF-α; Mm00443258_m1), CD16 (Mm00556657_m1), and mannose receptor (Mm00485184_m1) from Applied Biosystems. For each sample, the Ct value was divided by that of the housekeeping gene, glycolaldehyde-3-phosphate dehydrogenase (GAPDH), to generate a standardized Ct value. Primer pairs and the probe for GAPDH were as follows: forward primer, AACTCCCTCAAGATTTGCACCA; reverse primer, GTGTCATGAGCCTTCCA; and Taqman probe, CTGACCACCA ACTGCTTAGCCCC.

2.8 Insulin signalling and c-Jun N-terminal kinase (JNK) activity in liver and skeletal muscles

The portal vein of mice starved for 24 h was exposed, and 5 mL/kg of normal saline (0.9% NaCl) with insulin (5 U/kg) was injected. The liver was removed 1 min later, and hindlimb skeletal muscles were removed 3 min later. The dissected specimens were immediately homogenized with a Polytron homogenizer in six volumes of solubilization buffer. Then the extracts were centrifuged at 15 000 g for 30 min at 4°C, and the supernatants were used as samples for immunoprecipitation or immunoblotting studies. Supernatants containing equal amounts of protein (500 μg) were incubated with anti-insulin receptor β subunit (IRβ) antibody (#3025; Cell Signaling Technology, Beverly, MA, USA) and then incubated with 20 μL of protein A–sepharose (GE Healthcare, Piscataway, NJ, USA). The samples were washed and then boiled in sample buffer containing 10 mM dithiothreitol. Total lysates or immunoprecipitated subjects were subjected to western blotting, blotted with anti-IRβ, 4G10 (#05-321; Millipore, Temecula, CA, USA), anti-phosphatidylinositol-3-kinase (PI3K), anti-phospho-PI3K (#4249 and #4228; Cell Signaling}
Figure 1 Human C-reactive protein (CRP)-overexpressing transgenic mice (CRPTG). (A) Gene expression was confirmed by RT-PCR using genomic DNA extracted from clipped tails. Lane 1, positive control; lanes 2–5, negative for CRP transgene in non-transgenic littermates (CON); lanes 6–9, positive. (B–E) Immunohistochemical staining for human CRP. Human CRP was not expressed in epididymal adipose tissue (B) or liver (D) of wild-type mice; however, human CRP was expressed in epididymal adipose tissue (C) and liver (E) of CRPTG. Black arrows indicate adipocytes expressing human CRP. Red arrow indicates adipose stromal vascular cell expressing human CRP. Scale bars indicate 50 μm (B–E).
Technology), anti-Akt and anti-phospho-Akt (#9272 and #9271; Cell Signaling Technology) and anti-phospho-JNK (JNK, #4668; Cell Signaling Technology). GAPDH (sc-25778; Santa Cruz Biotechnology, Santa Cruz, CA, USA) immunoblot was used for protein loading control. The immunoblots were developed by the enhanced chemiluminofluorescence method. The signals were quantified by densitometry (GS-800; Bio-Rad, Hercules, CA, USA).

2.9 Statistical analysis
All continuous data were expressed as mean values ± SEM. The statistical significance of differences between multiple groups was determined using ANOVA and post hoc analysis with Bonferroni test. Statistical significance was defined as a P-value of <0.05. All statistical analyses were performed using SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA).

3. Results

3.1 Body and organ weights and haemodynamic parameters
At 6 weeks of age, there was no significant difference in body weight between CRPTG and CON. At 18 weeks of age, body, liver, and epididymal adipose tissue weights in mice fed a high-fat diet were higher than those of mice fed a standard diet. However, they were comparable between CRPTG and CON. Organ weights, including epididymal adipose tissue and liver, were comparable between HFD/CRPTG and HFD/CON (Table 1).

Haemodynamic parameters, including systolic and diastolic blood pressure and heart rate before and after a high-fat or standard diet, were comparable among the four groups (Table 1).

3.2 Serum CRP, amyloid A, lipid levels, plasma adiponectin level, and CRP expression in epididymal adipose tissue and liver
Serum CRP level was markedly elevated in CRPTG compared with that in CON. Serum CRP level did not differ between STD/CRPTG and HFD/CRPTG. There was no significant difference in fasting glucose or total cholesterol level between CRPTG and CON after a standard diet. Serum amyloid A level was increased in HFD/CON compared with STD/CON. Moreover, serum amyloid A level tended to be higher in HFD/CRPTG compared with HFD/CON.

Fasting triglyceride level was significantly higher in STD/CRPTG than in STD/CON. Fasting glucose and triglyceride levels in HFD/CON were higher than those in STD/CON. These differences were further augmented in CRPTG (Table 1).

Serum adiponectin level was lower in STD/CRPTG than in STD/CON. A high-fat diet in CON decreased serum adiponectin level compared with that in STD/CON. In HFD/CRPTG, serum adiponectin level was further decreased compared with that in HFD/CON (Table 1).

3.3 Glucose and insulin resistance tests
No difference was observed in fasting plasma glucose level between CON and CRPTG after a standard diet or a high-fat diet. In mice with a standard diet, the oral glucose tolerance test showed that plasma glucose levels and the area under the curve (AUC) of plasma glucose change were not significantly different between CRPTG and CON (Figure 2A). However, after a high-fat diet, CRPTG showed a significant increase of plasma glucose level at 90 and 120 min after oral glucose intake. The AUC of plasma glucose change was higher in CRPTG compared with that in CON (Figure 2B). Meanwhile, the intraperitoneal insulin tolerance test showed that plasma glucose levels after oral glucose intake and the AUC of plasma glucose change were comparable between STD/CRPTG and STD/CON (Figure 2C). After a high-fat diet, plasma glucose at 90 and 120 min after insulin injection and the AUC of plasma glucose change in CRPTG were higher than those in CON (Figure 2D).

3.4 Assessment of hepatosteatosis
After a standard diet, liver weight was comparable between CRPTG and CON. After a high-fat diet, liver weight was increased compared with that in mice fed a standard diet, and it tended to be higher in CRPTG than in CON (Table 1).

Table 1  Body and tissue weight, haemodynamic parameters, plasma glucose, and serum lipids

<table>
<thead>
<tr>
<th></th>
<th>STD/CON (n = 8)</th>
<th>STD/CRPTG (n = 8)</th>
<th>HFD/CON (n = 8)</th>
<th>HFD/CRPTG (n = 8)</th>
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<tr>
<td>Body weight at 6 weeks (g)</td>
<td>20.3 ± 1.5</td>
<td>20.6 ± 2.5</td>
<td>20.5 ± 2.9</td>
<td>21.5 ± 2.7</td>
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<tr>
<td>Body weight at 18 weeks (g)</td>
<td>30.5 ± 0.5</td>
<td>31.7 ± 0.6</td>
<td>39.7 ± 2.9*</td>
<td>42.2 ± 3.3*</td>
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<tr>
<td>Liver weight (g)</td>
<td>1.2 ± 0.1</td>
<td>1.3 ± 0.1</td>
<td>2.1 ± 0.4*</td>
<td>2.3 ± 0.3*</td>
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<tr>
<td>Epididymal adipose tissue weight (g)</td>
<td>0.3 ± 0.03</td>
<td>0.3 ± 0.03</td>
<td>1.7 ± 0.2*</td>
<td>1.9 ± 0.2*</td>
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<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>111 ± 3</td>
<td>105 ± 9</td>
<td>111 ± 12</td>
<td>113 ± 16</td>
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<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>78 ± 11</td>
<td>79 ± 4</td>
<td>72 ± 10</td>
<td>71 ± 9</td>
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<tr>
<td>Heart rate (beats/min)</td>
<td>649 ± 24</td>
<td>632 ± 34</td>
<td>667 ± 44</td>
<td>627 ± 30</td>
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<tr>
<td>C-reactive protein (mg/L)</td>
<td>&lt;0.001 ± 0</td>
<td>31.8 ± 3.6*</td>
<td>&lt;0.001 ± 0</td>
<td>32.2 ± 4.1*</td>
</tr>
<tr>
<td>Amyloid A (μg/mL)</td>
<td>5.7 ± 0.5</td>
<td>6.5 ± 0.8</td>
<td>7.0 ± 0.4*</td>
<td>10.0 ± 0.7*</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>131 ± 7</td>
<td>156 ± 10</td>
<td>251 ± 8*</td>
<td>298 ± 9†</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>102 ± 21</td>
<td>94 ± 10</td>
<td>200 ± 18*</td>
<td>217 ± 38*</td>
</tr>
<tr>
<td>Triglyceride (mg/dL)</td>
<td>11 ± 2</td>
<td>27 ± 8*</td>
<td>39 ± 6*</td>
<td>66 ± 12†</td>
</tr>
<tr>
<td>Adiponectin (μg/mL)</td>
<td>37 ± 3</td>
<td>25 ± 1*</td>
<td>18 ± 1*</td>
<td>11 ± 1†</td>
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</table>

Data are expressed as means ± SEM. *P < 0.05 vs. STD/CON; †P < 0.05 vs. HFD/CON.
Haematoxylin and eosin staining showed that hepatocellular ballooning was more commonly observed in the liver of HFD/CON compared with that of STD/CON and STD/CRPTG; however, these findings were more prominent in HFD/CRPTG (Figure 3A–D). Masson’s trichrome staining showed that peri-sinusoidal fibrosis was rare in STD/CON, STD/CRPTG, and HFD/CON, but prominent in HFD/CRPTG (Figure 3E–H). Oil Red O staining showed that oil droplets were increased in HFD/CON compared with those in STD/CON and STD/CRPTG. Oil droplets were more prominent in HFD/CRPTG than in HFD/CON (Figure 3I–L). Hepatic triglyceride content was increased in HFD/CON compared with that in STD/CON. CRPTG had 1.5-fold higher hepatic triglyceride content than that in CON after a high-fat diet (Figure 3M).

Assessment of hepatosteatosis by histological scoring system (NAFLD activity score) revealed that CRP overexpression itself did not cause hepatic injury in mice fed the standard diet. However, in mice fed the high-fat diet, the severity of hepatic injury was pronounced in HFD/CRPTG compared with HFD/CON (Table 2).

### 3.5 Assessment of adipose tissue remodelling

Haematoxylin and eosin staining of epididymal adipose tissue showed that the mean area of adipocytes was enlarged in HFD/CON compared with that in STD/CON and STD/CRPTG. It was comparable between HFD/CON and HFD/CRPTG (Figure 4A–D and I). Immunohistochemical staining for Mac-3 showed that the fraction of Mac-3+
Macrophages (Mac-3+ cells/total cells) was increased in HFD/CON compared with that in STD/CON. It was further increased in HFD/CRPTG compared with that in HFD/CON (Figure 4E–H and J).

Real-time RT-PCR showed that the expression of Emr1, MCP-1, and TNF-α was up-regulated in HFD/CON compared with that in STD/CON and STD/CRPTG. It was further increased in HFD/CRPTG (Figure 5A–C). The mRNA expression of CD165 and mannose receptor was comparable between STD/CON and STD/CRPTG. However, feeding the high-fat diet decreased the expression of both CD165 and mannose receptor. The mRNA expression of CD165 and mannose receptor tended to be lower in HFD/CRPTG than in HFD/CON (see Supplementary material online, Figure S1).

3.6 Insulin signalling and JNK activity in liver and muscles
Phosphorylation of IRβ, PI3K and Akt was attenuated in both liver and skeletal muscles of HFD/CRPTG compared with HFD/CON (see Supplementary material online, Figures S2 and S3); however, expression of phospho-JNK in liver was higher in HFD/CRPTG than in HFD/CON (see Supplementary material online, Figure S4).

**Figure 3** Haematoxylin and eosin, Masson’s trichrome and Oil Red O staining of liver. Haematoxylin and eosin staining showed that hepatocellular ballooning was more commonly observed in liver of non-transgenic littermates (CON) on a high-fat diet (HFD; C) compared with those on a standard diet (STD; A) and human CRP-overexpressing transgenic mice (CRPTG) with STD (B). However, they were more prominent in HFD/CRPTG (D). Masson’s trichrome staining showed that peri-sinusoidal fibrosis was rare in STD/CON (E), STD/CRPTG (F), and HFD/CON (G), but prominent in HFD/CRPTG (H). Oil Red O staining showed that oil droplets were increased in HFD/CON (K) compared with STD/CON (I) and STD/CRPTG (J). Oil droplets were more prominent in HFD/CRPTG (L) than in HFD/CON. Hepatic triglyceride (TG) content was increased in HFD/CON compared with that in STD/CON. The difference was further augmented in HFD/CRPTG (M). Scale bars indicate 25 μm. Data are shown as means ± SEM. *P < 0.05 vs. STD/CON, †P < 0.05 vs. HFD/CON.
4. Discussion

In the present study, we found that human CRP exacerbated insulin resistance and hepatosteatosis in mice fed a high-fat diet, and this was associated with adverse adipose tissue remodelling, including down-regulation of adiponectin, enhanced macrophage infiltration, and expression of pro-inflammatory cytokines. These findings suggest that human CRP may play a pivotal role in the development of obesity-induced metabolic disorders.

CRP is a highly conserved acute-phase protein, secreted predominantly by the liver in response to pro-inflammatory cytokines, such as interleukin (IL)-6 and TNF-α, and activates monocytes.

<table>
<thead>
<tr>
<th>Table 2 Assessment of hepatosteatosis by histological scoring system</th>
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</tr>
<tr>
<td>Steatosis</td>
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<tr>
<td>Lobular inflammation</td>
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<tr>
<td>Hepatocyte ballooning</td>
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<tr>
<td>NAFLD activity score</td>
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Steatosis: 0, <5%; 1, 5–33%; 2, >33–66%; 3, >66%.
Lobular inflammation: 0, no foci; 1, 1–2 foci/×200 field; 2, 2–4 foci/×200 field; 3, >4 foci/×200 field.
Hepatocyte ballooning: 0, none; 1, few balloon cells; 2, many cells/prominent ballooning.
NAFLD (non-alcoholic fatty liver disease) activity score: total of the scores of steatosis, lobular inflammation, and hepatic ballooning.
Data are means ± SEM. *P < 0.05 vs. STD/CON, †P < 0.05 vs. HFD/CON.

Figure 4 Haematoxylin and eosin and immunohistochemical staining of epididymal adipose tissue. Haematoxylin and eosin staining showed that adipocytes were enlarged in non-transgenic littermates (CON) fed a high-fat diet (HFD; C) compared with CON fed a standard diet (STD; A) and human CRP-overexpressing transgenic mice (CRPTG) fed STD (B). They were comparable between HFD/CON and HFD/CRPTG (D). Immunohistochemical staining for Mac-3 showed that Mac-3+ macrophages were increased in HFD/CON (G) compared with those in STD/CON (E) and STD/CRPTG (F). They were more prominent in HFD/CRPTG (H). Mean area size of adipocytes was enlarged in HFD/CON compared with that in STD/CON and STD/CRPTG. It was comparable between HFD/CON and HFD/CRPTG (I). The fractions of Mac-3+ cells (J) were increased in HFD/CON compared with those in STD/CON and further increased in HFD/CRPTG. Scale bars indicate 50 μm. Data are shown as means ± SEM. *P < 0.05 vs. STD/CON, †P < 0.05 vs. HFD/CON.
CRP exacerbates adipose tissue remodelling

Macrophages, neutrophils, and endothelial cells by binding to their IgG Fcγ receptor (FcyR) I and FcyRIIb, which further augment the production of pro-inflammatory cytokines, including IL-6 and TNF-α, via a nuclear factor (NF)-κB-dependent mechanism.12–14 Our previous study using a mouse transverse aortic constriction model revealed that CRP overexpression resulted in a significant increase in the number of infiltrating macrophages, NF-κB-p65-expressing myocardial cells, nuclear NF-κB–DNA-binding activity, and pro-inflammatory cytokine expression in the left ventricle, preceding the development of deteriorated left ventricular remodelling after the transverse aortic constriction operation, whereas in sham-operated mice, in which inflammatory cell infiltration was almost absent, CRP overexpression did not show any adverse effect on cardiac function.15 These findings suggest that the effects of CRP may be mediated through activation of immunocompetent cells infiltrating into the pressure-overloaded myocardium. In the present study, CRP overexpression itself did not induce insulin resistance, hepatosteatosis, and inflammatory response in adipose tissue when a standard diet was fed. Increased serum amyloid A level was observed in CRPTG on a high-fat diet, but not on a standard diet. CRP is reported to increase MCP-1 expression on monocytes16 and promote macrophage proliferation.17 Macrophage infiltration and expression of inflammation-related genes, such as MCP-1 and TNF-α, in the adipose tissue are reported to precede the development of insulin resistance in an animal model of obesity.18,19 Therefore, systemic CRP elevation in obesity might facilitate the development of metabolic disorders through enhanced adipose tissue inflammation.

Obesity induces accumulation of visceral fat and various changes in adipose tissue, so-called ‘adipose tissue remodelling’, including proliferation and hypertrophy of adipocytes, dysregulation of adipokines, and chronic inflammation. Among them, macrophage accumulation in adipose tissue is a hallmark of adipose tissue remodelling and largely contributes to the development of insulin resistance.18,20,21 Suganami et al. reported that macrophages and enlarged adipocytes established a vicious cycle that aggravated inflammatory changes in adipose tissue, through a paracrine loop involving free fatty acids and TNF-α.21 Macrophages infiltrated into epididymal adipose tissue were recruited by MCP-1 in obese adipose tissue. Two previous studies with transgenic mice overexpressing MCP-1 in the adipose tissue and MCP-1-deficient mice showed that MCP-1 plays a pivotal role in the recruitment of macrophages into obese adipose tissue.22,23

The present study showed that mRNA expression of MCP-1 and infiltration of macrophages in adipose tissue were increased in CRPTG compared with CON on a high-fat diet. A recent study revealed that macrophages infiltrated into obese adipose tissue have heterogeneity, including M1 or classically activated (pro-inflammatory) macrophages and M2 or alternatively activated (anti-inflammatory) macrophages, and exhibit the phenotypic change from M2 to M1 polarization in obese adipose tissue, thereby accelerating adipose tissue inflammation.24 In the present study, Emr1, which is a macrophage-specific marker, was up-regulated in HFD/CRPTG compared with HFD/CON. Meanwhile, CD163 and mannose receptor, which are specific markers for M2 macrophages, tended to be down-regulated in HFD/CRPTG compared with HFD/CON, suggesting that human CRP may induce a pro-inflammatory state in adipose tissue by increased infiltration of M1 macrophages.

Adiponectin is an adipocytokine that is protective against cardiovascular events by various mechanisms, including anti-inflammatory and anti-atherosclerotic effects, and is decreased in association with adipocyte enlargement. Meanwhile, the expression of adiponectin in adipocytes was reduced by TNF-α in a dose-dependent manner by suppressing its promoter activity.25 Expression analysis of macrophage and non-macrophage cell populations isolated from adipose tissue demonstrated that adipose tissue macrophages are responsible for almost all the adipose tissue TNF-α expression.18 Furthermore, a previous in vitro study revealed that CRP treatment down-regulates adiponectin mRNA expression in adipocytes.26 These data are consistent with our results showing a decreased plasma adiponectin level in CRPTG compared with CON on the standard diet. However, plasma adiponectin level was further decreased compared with CON on the high-fat diet, in association with increased expression of TNF-α, which is mainly produced by macrophages in adipose tissues.

Several studies reported that human CRP directly suppressed insulin signalling, resulting in deteriorated insulin sensitivity.2,5 We demonstrated that insulin signalling, including insulin receptor phosphorylation, PI3K activation, and Akt activation, in liver and skeletal muscles was diminished in CRPTG compared with CON on the high-fat diet. In addition, JNK activity in liver was higher in HFD/CRPTG than in HFD/CON. JNK is known to play a central role in the development of insulin resistance.27 Human CRP might deteriorate insulin signalling in liver and skeletal muscles, at least in part, by increased JNK activity.

Figure 5 mRNA expression in epididymal adipose tissue. Expression of Emr1 (A), MCP-1 (B), and TNF-α (C) was up-regulated in non-transgenic littermates (CON) fed a high-fat diet (HFD) compared with CON fed a standard diet (STD) and human CRP-overexpressing transgenic mice (CRPTG) fed STD. It was further increased in HFD/CRPTG (A–C). Data are shown as means ± SEM. *P < 0.05 vs. STD/CON, †P < 0.05 vs. HFD/CON.
Several lines of evidence have indicated that insulin resistance is closely associated with the pathogenesis of hepatosteatosis by impairing the ability of insulin to suppress lipolysis and increasing the delivery of free fatty acids to the liver.28,29 Yoneda et al. reported that serum CRP elevation is an independent clinical feature of non-alcoholic steatohepatitis (NASH) and also of the severity of fibrosis in NASH.30 Concomitantly with these data, we found that an increase of hepatic triglyceride content and histological changes of hepatosteatosis, including hepatic cell ballooning, infiltration of inflammatory cells, marked oil droplets, and sinusoidal fibrosis, after a high-fat diet were more prominent in CRPTG compared with CON. Kanda et al. demonstrated that increased MCP-1 mRNA expression in adipose tissue contributed to the development of hepatosteatosis in diabetic db/db mice and in wild-type mice fed a high-fat diet, along with adipose tissue inflammation and insulin resistance.2 Thus, increased CRP may induce the development of hepatosteatosis, as well as adipose tissue remodelling, deteriorating insulin resistance, in obesity, and could be related to the worse clinical outcome of patients with metabolic syndrome.

We recognize that the present study has some study limitations. CRP is not an acute-phase reactant in mice, in which CRP is very scarce.11 Therefore, the physiological role of CRP in mice is unclear. However, human CRP is reported to bind to rodent and human IgG Fc receptors.12 Furthermore, several studies using mice demonstrated that human CRP had a pathogenic role in vascular injury models,31–33 atherosclerosis-prone models,34 and heart failure models.12,13 Hence, our CRPTG may be suitable for investigating the in vivo role of CRP in the development of obesity-associated metabolic disorders. As there is considerable variability among species with respect to the biological and pathological functions of CRP, it would be premature to conclude that CRP is involved in the pathogenesis of obesity-induced metabolic disorders in humans. Moreover, the serum CRP level of the CRPTG was far higher than that seen in patients with metabolic disorders. Thus, it remains unclear whether mild CRP elevation, which is seen in patients with obesity, exerts adverse effects on adipose tissue remodelling and metabolic disorders. However, the present study provides evidence for a possible direct in vivo effect of human CRP on obesity-induced metabolic disorders. Further studies are needed to confirm this deleterious effect of CRP and to clarify the target organ of CRP in these phenomena.

Conventionally, serum CRP elevation has been thought to be a strong predictor of metabolic syndrome and cardiovascular events; however, its causative effect has not been clarified. The present study suggested that human CRP might be an attractive therapeutic target in metabolic syndrome and cardiovascular disease.

In conclusion, human CRP overexpression facilitated the development of insulin resistance and hepatosteatosis on a high-fat diet, in association with adiponectin down-regulation, enhancement of macrophage infiltration, and expression of pro-inflammatory cytokines in epididymal adipose tissue, suggesting its pathogenic role in the development of obesity-induced metabolic disorders.

Conflict of interest: none declared.

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References

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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