Inflammation and its echo in atherosclerosis
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Chapter 4

A single bolus infusion of C-reactive protein increases gluconeogenesis and plasma glucose concentration in humans

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Abstract

Objective: Recently we reported that CRP elicits inflammatory and procoagulant responses in humans. In addition, CRP has been associated with the development of type 2 diabetes mellitus. To further explore interactions between CRP and glucose handling, we evaluated the effects of CRP infusion on glucose metabolism in humans.

Methods: Seven healthy Caucasian male volunteers (age 39.3 ± 16.9 years) received a single bolus infusion of 1.25 mg/kg purified recombinant human (rh)CRP or CRP-free diluent in a cross-over design.

Results: CRP infusion induced an inflammatory response which was followed by increased plasma concentrations of norepinephrine (3 hours) and cortisol (4 hours). Concomitantly, plasma concentrations of insulin and C-peptide decreased transiently. These metabolic changes increased plasma glucose concentrations from 8 hours after CRP infusion, which was preceded by an increased rate of glucose appearance which was a direct consequence of increased gluconeogenesis.

Conclusions: CRP infusion induces an inflammatory response followed by increased norepinephrine and cortisol levels which results in increased gluconeogenesis. This finding implies that elevated levels of CRP in humans may in fact contribute to altered glucose metabolism and thereby may contribute to the induction of type 2 diabetes mellitus.
Effect of CRP on glucose metabolism in humans

Introduction

Type 2 diabetes mellitus is a major risk factor for atherosclerotic disease. Even, nondiabetic subjects with an acute coronary syndrome exhibit a high prevalence of disturbed glucose tolerance. Both conditions, chronic cardiovascular disease as well as acute coronary syndromes are characterized by increased C-reactive protein (CRP) levels. In fact, CRP has recently emerged as a strong and independent predictor for cardiovascular risk as well as for the development of type 2 diabetes mellitus. However, since these are all observational studies they do not prove a causal relation between CRP and the development of type 2 diabetes mellitus. Interestingly, evidence from experimental studies has accumulated placing CRP within the atherosclerotic plaque, whereas other studies demonstrated that CRP elicits a wide array of atherothrombotic effects. All these findings were verified in human subjects, and infusion of highly purified recombinant human (rh)CRP has pronounced effects on pro-coagulant and inflammatory pathways. In addition to this, clinical data also suggest that lowering of CRP translates into further cardiovascular benefit. Taken together, these data gave birth to the controversial notion that CRP may actually be a mediator in cardiovascular disease, rather than merely a marker of cardiovascular risk. Given the strong base of epidemiological and experimental evidence linking CRP as causal agent of atherosclerosis as well as the development of type 2 diabetes mellitus, combined with the fact that nondiabetic subjects with an acute coronary syndrome and concomitant elevated CRP levels are associated with a high prevalence of glucose intolerance, we explored in a proof-of-principle study whether pathophysiologically relevant concentrations of CRP, as seen in patients with an acute cardiovascular event, exerts direct effects on glucose handling in humans.
Materials and methods

Ethical Issues and Safety Experiments

The study protocol was approved by the Institutional Review Board (IRB) at the Academic Medical Center in Amsterdam as well as the Central Committee on Research involving Human Subjects (CCMO) in the Netherlands. In view of the fact that the present study was performed before the European Union (EU) Clinical Trial Directive (CTD) came into law (1st March 2006) in the Netherlands the study was approved according to the procedures with national Dutch laws. Consequently, the IRB of our hospital together with the CCMO of our country functioned as the competent authority that evaluated at that time such clinical studies and carefully reviewed the study protocol which eventually lead to approval. In accordance with EU CTD regulations, national Dutch laws also require pre-human toxicology testing. Therefore, we performed these tests in mice and rabbits with CRP concentrations more than four times higher than peak concentrations obtained in humans, in which we observed no toxicological effects.3

Purification and safety control of the rhCRP solution

The rhCRP (BiosPacific, Emeryville, CA, USA) was supplied in 20 mM Tris, 140 mM NaCl, 2 mM CaCl₂, pH 7.5 and 0.05% (wt/vol) sodium azide and revealed a single 23 kDa band (>99%) after CBBR-staining (1 μg; SDS-polyacrylamide gel). Before purification, the host cell protein concentration was 85 p.p.m., as determined by a high-sensitive ELISA in accordance with manufacturers’ instructions (Cygnus Technologies Inc., Southport, NC, USA). Subsequently, the rhCRP was purified using size exclusion chromatography to remove contaminants including endotoxin and sodium azide (Univalid Inc., Leiden, The Netherlands). Purity and stability was evaluated using sequential high-performance liquid chromatography and Time-of-Flight mass spectrometry, showing no other protein fractions besides the CRP-pentamer. The final concentration of endotoxin was below 1.5 endotoxin units (EU)/mL as evaluated by Limulus assay (Turbidimetric kinetic method; ACC Inc., East Falmouth, MA, USA).
The rhCRP was stored in a CaCl₂ containing buffer (pH 8.5) at 0–4°C degrees and all experiments were performed within 4 weeks after rhCRP-purification.

**Study Design**

Seven healthy Caucasian male volunteers (age 39.3 ± 16.9 years) were enrolled after written informed consent was obtained. Subjects did not have diabetes mellitus, hypertension, congestive heart failure or febrile illness and did not use any medication. Subjects abstained from alcohol and caffeine-containing beverages for at least 24 hours prior to the study. Subjects were randomly assigned to receive a single bolus of 1.25 mg/kg rhCRP or CRP-free diluent in a crossover design with a period of 4 weeks between both study visits. All subjects followed a diet with at least 250 grams carbohydrates for 3 days prior to the study.

**Study Procedures**

Approximately 14½ hours before rhCRP or diluent infusion, participants were instructed to have their last meal. At t = -14½ hours blood was drawn for the background enrichment of ³H in body water, followed by ingestion of 1 g/kg body water ²H₂O (99% pure, Cambridge Isotopes, Cambridge, MA, USA) at intervals of 30 minutes until a total dose of 5 g/kg body water was reached. The total body water content in males was estimated to be 60% of body weight. The next morning a catheter was inserted into an antecubital vein of each arm. At 8.00 a.m. (t = -2½ hours), blood was drawn for assessment of background enrichment of [6,6-²H₂]glucose. Subsequently, a primed (1.6 mg/kg), continuous (1.2 mg/kg/hr) infusion of [6,6-²H₂]glucose (99% enriched, Cambridge Isotope Laboratories, MA, USA) dissolved in sterile isotonic saline, was initiated using a calibrated syringe pump (Perfusor® Secura FT, B. Braun, Melsungen, Germany) through a Millipore filter (size 0.2 mm; Minisart, Sartorius, Göttingen, Germany). From 10.10 a.m. (t = -20 minutes) three blood samples were collected at intervals of 10 minutes for determination of plasma glucose concentration, [6,6-²H₂]glucose enrichment and ²H₂O enrichment in body water. Blood samples for the measurement of gluco-and counter regulatory
hormones were also collected. At 10.30 a.m. (0 hours), a bolus of rhCRP (1.25 mg/kg body weight) or CRP-free diluent was administered intravenously. Hereafter blood samples were collected on t = 1, 2, 3, 4, 6, 8 and 9 hours. After the last blood withdrawal at 19.30 pm (9 hours) the study ended.

**Laboratory Analysis**

Blood samples for measurement of gluconeogenesis were deproteinized by adding an equal amount of 10% perchloric acid. Blood for [6,6-²H₂]glucose enrichment and hormone concentration measurements was collected in heparinized tubes. For determining levels of free fatty acids (FFA) plasma was collected in K-EDTA tubes. Samples were kept on ice, centrifuged, snap frozen and stored at -20 °C.

Enrichments of plasma [6,6-²H₂]glucose, ²H₂O and deuterium at the C5 position of glucose were determined as previously described. Briefly, plasma samples for glucose enrichment of [6,6-²H₂]glucose and plasma glucose concentration were measured as the aldonitril penta-acetate derivative of glucose in deproteinized plasma using xylose as an internal standard. Glucose was monitored at m/z 187 and 189. The enrichment of [6,6-²H₂]glucose was determined by dividing the peak area of m/z 189 by the peak area of m/z 187 and correcting for natural enrichments. To measure deuterium enrichment at the C5 position, glucose was converted to hexamethylenetetramine (HMT). HMT was injected into a gas chromatograph mass spectrometer. Separation was achieved on an AT-Amine column (30 m x 0.25 mm, d, 0.25 μm). The deuterium enrichment in the plasma water was measured after conversion of water and carbide to acetylene. All isotopic enrichments were measured on a gas chromatograph mass spectrometer (model 6890 gas chromatograph coupled to a model 5973 mass selective detector, equipped with an electron impact ionization mode, Hewlett-Packard, Palo Alto, CA, USA).

Plasma insulin concentration was determined with a chemiluminescent immunometric assay (Immulite 2000, Diagnostic Products Corporation, Los Angeles, CA, USA). C-peptide was determined by RIA (RIA-coat C-peptide, Byk-Sangtec Diagnostica). Cortisol was measured by enzyme-immunoassay on an Immulite analyzer (DPC),
intra-assay CV 2–4%. Glucagon was determined by RIA (Linco Research, Mo, USA). Norepinephrine and epinephrine were determined by an in-house HPLC method. Plasma FFA were measured by an enzymic method (NEFAC; Wako Chemicals, VA, USA). CRP was measured by a high-sensitivity immunoturbidimetric assay (Roche Diagnostics Corporation, Basel, Switzerland), while CRP concentrations in excess of 10 mg/L (after rhCRP- infusion) were assayed by immuno-nephelometry (P800 analyzer, Roche Diagnostic Corporation). Circulating cytokine concentrations were assessed with the luminex method (Bioplex Human Cytokines 1x96 wells, catalog number X500000 FFS, Bio-Rad Laboratories Inc, CA, USA).

**Calculations**

Glucose appearance rate (glucose Ra) was calculated from the dilution of labeled glucose in plasma. The Steele equation for steady-state conditions was used for the measurement of glucose Ra before rhCRP or diluent infusion and non-steady-state calculations were used after rhCRP or diluent infusion. The fraction of total extracellular glucose pool (pV) was assumed to be 40 mL/kg. The rate of GNG was calculated by multiplying the glucose Ra by fractional gluconeogenesis (GNG). The fractional GNG=100x([2H] enrichment on C5 of glucose)/([2H] enrichment in plasma water). The rationale has been discussed in detail by Landau. In brief, glucose produced during plasma 2H₂O enrichment by gluconeogenesis will be labeled with deuterium at the C5 position. Glucose molecules produced by gluconeogenesis and glycogenolysis will be labeled with deuterium at the C2 position. The ratio of C5 and C2 enrichment of glucose constitutes fractional gluconeogenesis. Alternatively, fractional gluconeogenesis can be calculated by the ratio of C5 enrichment of glucose and plasma 2H₂O enrichment. A requirement for the latter method is the complete equilibration of plasma 2H₂O enrichment with C2 enrichment of glucose.
Statistical Analysis

Descriptive statistics between CRP and diluent infusion were compared by means of 2-tailed paired $t$ tests or a non-parametric test (Wilcoxon test) was used in case of non normal distribution. Statistical analysis of glucose metabolism parameters for individual subjects between CRP and diluent infusion over time was performed using analysis of variance (ANOVA) for repeated measures. If such analysis revealed significant differences, a Wilcoxon test was used to locate the specific difference. All statistics were performed with SPSS software (SPSS for Windows 11.5.1, SPSS Inc., Chicago, IL, USA). Data are expressed as means ± SD.

Results

Clinical characteristics

Baseline characteristics were determined prior to CRP and diluent infusion (table 1). The seven healthy Caucasian male volunteers did not experience symptoms or side-effects during the study. Furthermore, body temperature, blood pressure and heart rate remained stable upon CRP infusion.

Plasma CRP and cytokine concentrations

After CRP infusion plasma concentrations of CRP increased to 23.9 ± 4.2 mg/L at 1 hour (figure 1A). TNFα concentrations did not change upon CRP infusion (figure 1B), whereas a transient rise in IL-6 as well as IL-8 was observed, peaking at 4 hours (figure 1B).
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Table 1. Baseline Characteristics of the Seven Study Subjects on Both Study Days

<table>
<thead>
<tr>
<th></th>
<th>Control study day</th>
<th>CRP study day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>39.3 ± 16.9</td>
<td>-</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>27.3 ± 5.2</td>
<td>27.4 ± 5.2</td>
</tr>
<tr>
<td>Body fat, %</td>
<td>24.7 ± 9.9</td>
<td>24.8 ± 10.2</td>
</tr>
<tr>
<td>Body temperature, ºC</td>
<td>36.9 ± 0.5</td>
<td>36.8 ± 0.5</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>82 ± 11</td>
<td>79 ± 10</td>
</tr>
<tr>
<td>Systolic BP, mm Hg</td>
<td>123 ± 8</td>
<td>124 ± 11</td>
</tr>
<tr>
<td>Fasting plasma glucose, mmol/L</td>
<td>5.3 ± 0.6</td>
<td>5.4 ± 0.4</td>
</tr>
<tr>
<td>Fasting plasma insulin, pmol/L</td>
<td>90 ± 43</td>
<td>88 ± 45</td>
</tr>
<tr>
<td>Fasting plasma glucagon, ng/L</td>
<td>78 ± 28</td>
<td>73 ± 25</td>
</tr>
<tr>
<td>Fasting plasma cortisol, nmol/L</td>
<td>394 ± 83</td>
<td>374 ± 87</td>
</tr>
<tr>
<td>C-peptide, pmol/L</td>
<td>907 ± 180</td>
<td>881 ± 374</td>
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<tr>
<td>FFA, mmol/L</td>
<td>0.44 ± 0.15</td>
<td>0.45 ± 0.14</td>
</tr>
<tr>
<td>Epinephrine, nmol/L</td>
<td>0.08 ± 0.01</td>
<td>0.19 ± 0.07</td>
</tr>
<tr>
<td>Norepinephrine, nmol/L</td>
<td>1.15 ± 0.57</td>
<td>1.60 ± 1.03</td>
</tr>
<tr>
<td>hsCRP, mg/L</td>
<td>1.9 ± 2.0</td>
<td>1.8 ± 1.9</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>3.1 ± 1.1</td>
<td>3.1 ± 1.1</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.3 ± 0.2</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.0 ± 0.2</td>
<td>0.9 ± 0.2</td>
</tr>
</tbody>
</table>

Data are expressed as means±SD. BMI, body mass index; BP, blood pressure; bpm, beats per minute; FFA, free fatty acids; HDL, high-density lipoprotein; hsCRP, high sensitive C-reactive protein; LDL, low-density lipoprotein.

Glucose metabolism

After CRP infusion, plasma glucose concentrations increased with 10% from 8 hours onwards compared with diluent infusion (figure 2A). Baseline values for glucose $R_1$ were comparable on both study days (figure 2B). At the same time there was a modest increase in glucose $R_1$ of 11% compared to the control experiments, which showed a continuous decline in glucose $R_1$. Concomitantly, glucose $R_1$ increased slightly by 4% after CRP infusion (figure 2C). These changes were preceded by increased gluconeogenesis from 6 hours after CRP infusion resulting in a 10% increase when compared with the control experiments (figure 2D). CRP infusion did not affect glycogenolysis (figure 2D).
Figure 1. Effect of CRP infusion on inflammation activation.

(A) plasma CRP concentrations ($P < .001$ indicates difference between the CRP infusion day (●) and control day (○) by ANOVA for repeated measures, † indicates difference between time points by Wilcoxon test, $P < .01$, (B) plasma cytokines concentrations: TNFα, non significant (NS) difference between interventions and time points on the CRP infusion day (●) and control day (○), IL-6 ($P < .05$ indicates difference between the CRP infusion day (●) and control day (○), § indicates difference between time points, $P < .05$), IL-8 ($P < .05$ indicates difference between the CRP infusion day (▲) and control day (Δ), ¶ indicates difference between time points, $P < .05$). Values are means±SD.
Figure 2. Effect of CRP infusion on glucose metabolism

(A) plasma glucose concentration (*P = .004 indicates difference between the CRP infusion day (●) and control day (○) by ANOVA for repeated measures, † indicates difference between time points by Wilcoxon test, P < .05), (B) glucose Rₜ (‡P < .001 indicates difference between the CRP infusion day (■) and control day (†), § indicates difference between time points, P = .02), (C) glucose Rₚ (‖P < .001 indicates difference between the CRP infusion day (□) and control day (○), ¶ indicates difference between time points, P < .05) and (D) gluconeogenesis (*P = .04 indicates difference between the CRP infusion day (▲) and control day (Δ), ** indicates difference between time points, P = .02) and glycogenolysis, NS difference between interventions and time points on the CRP infusion day (▼) and on the control day (∇). Values are mean±SD.
Glucose- and counter regulatory hormones

Three hours after CRP infusion, there was a transient decline in plasma insulin concentrations which subsequently returned back to normal (figure 3A). In line, C-peptide concentrations closely followed insulin kinetics (figure 3B).

Glucagon concentrations were unaffected by CRP (Figure 4A). In contrast, plasma cortisol concentrations rose significantly peaking at 4 hours (figure 4B). Concomitantly, norepinephrine concentrations also peaked at 3 hours after CRP infusion (figure 4C). However, epinephrine concentrations were unaffected by CRP (figure 4D).

Other measurements

Baseline FFA- and adiponectin concentrations were comparable on both study days and were unaffected upon CRP administration.
Figure 3. Effect of CRP infusion on insulin secretion

(A) plasma insulin concentrations (*$P = .02$ indicates difference between the CRP infusion day (●) and control day (○) by ANOVA for repeated measures, † indicates difference between time points by Wilcoxon test, $P < .02$), (B) plasma C-peptide (‡$P = .002$ indicates difference between the CRP infusion day (■) and control day (□), § indicates difference between time points, $P < .05$). Values are means±SD.
Figure 4. Effect of CRP infusion on counter glucoregulatory hormones

(A) plasma glucagon concentrations, NS difference between interventions and time points on the CRP infusion day (●) and control day (○), (B) plasma cortisol concentrations (*P < .001 indicates difference between the CRP infusion day (■) and control day (□), † indicates difference between time points, P < .05), (C) norepinephrine (‡P = .04 indicates difference between the CRP infusion day (▲) and control day (Δ), § indicates difference between time points, P < .05) and (D) epinephrine, NS difference between interventions and time points on the CRP infusion day (▼) and control day (∇). Values are means±SD.
Discussion

In the present study we show that a single bolus infusion of CRP affects glucose metabolism *in vivo* as illustrated by increased glucose production due to increased gluconeogenesis as well as an increase in plasma glucose concentration. Preceding these metabolic changes, CRP elicited an inflammatory response as well as an increase in counter-glucoregulatory hormones with a transient decline in insulin three to four hours after CRP. These findings suggest that CRP may have a direct effect on glucose handling *in vivo*.

Glucose metabolism

The changes in glucose metabolism during the control experiments were reflected by an initial decline in plasma glucose concentration and glucose $R_a$. After CRP infusion, the decrease in plasma glucose concentration during the first hours was similar to control experiments. However, from 6 hours onwards plasma glucose concentrations rose significantly in CRP-infused subjects, preceded by an 11% increase in glucose $R_c$. Only a small increase in glucose $R_d$ was observed. The combination of 10% increase in glucose, 11% increase in glucose $R_c$ and a 10% increase in gluconeogenesis suggests that increased glucose production was the predominant factor responsible for the rise in plasma glucose levels after CRP infusion.

Glucose production

Several factors may have contributed to the observed increase in glucose production. First, CRP infusion induced a transient decrease in insulin and C-peptide concentrations, known to be associated with increased hepatic glucose production. Second, CRP infusion significantly increased plasma cortisol and norepinephrine concentrations. Particularly cortisol and to a lesser extent norepinephrine is known to induce increased glucose production. Although norepinephrine has a relatively short duration of action, some studies have shown that infusion with norepinephrine may acutely increase hepatic glucose production. Thus, cortisol together with
norepinephrine provide a plausible explanation for the CRP-induced effects on glucose metabolism.\textsuperscript{11,12} Most likely, the increases in plasma cortisol and norepinephrine concentrations are secondary to increased IL-6 release peaking at 4 hours.\textsuperscript{13-15} Since CRP levels remained elevated through the CRP study day, a direct effect of CRP on cortisol and norepinephrine seems unlikely but cannot be excluded with all certainty. Notably, in contrast to other inflammatory stimuli such as TNFα and IL-6, CRP infusion did not affect glucagon concentrations.\textsuperscript{14,16} Finally, CRP infusion induced a modest but significant cytokine response. Especially, IL-6 has been shown to result in decreased insulin- and C-peptide.\textsuperscript{13} These effects of IL-6 can be observed from concentrations of 400-600 pg/mL onwards, whereas IL-6 concentrations in the present study did not exceed the 60-80 pg/mL range. The latter makes it unlikely that IL-6 had a major contribution to the effect of CRP on glucose handling.

**Purity of the rhCRP solution**

Recently, the purity of commercially available rhCRP has been criticized in view of potential contamination of rhCRP with endotoxin and sodium azide.\textsuperscript{17} Therefore, we used a modified purification procedure.\textsuperscript{18} In line, several findings argue against a role of contaminants in the present study. First, we show a clear disparity in cytokine profiles between that mediated by CRP and endotoxin, lacking TNFα increase. Second, the trace amounts of endotoxin present in the purified rhCRP solution (1.5 EU/kg) did not induce inflammatory changes in humans, thereby excluding a causal role for endotoxin.\textsuperscript{19} Third and foremost, the metabolic changes in the present study are slow, which is in contrast to the acute metabolic changes upon infusion of endotoxin.\textsuperscript{20}

**Study Limitations**

Chronic elevations of CRP in the lower range (e.g. 1 to 5 mg/L) have been associated with metabolic sequelae and adverse cardiovascular outcome.\textsuperscript{21} Since repeated administration of rhCRP is not ethical in view of potential sensitization, we performed acute CRP-infusion experiments only, aiming at pathophysiological CRP concentrations approximating those found in patients with an acute coronary syndrome.
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(20-25 mg/L). Therefore the current effects of CRP on glucose metabolism must be interpreted as pathophysiological effects. These current methodological impediments need to be addressed in future studies, including dose-response experiments as well as evaluation of the impact of CRP infusion in high risk groups, such as severe metabolic syndrome.

Clinical implications
Whereas several observational studies have reported a relation between CRP and insulin resistance, the present study provides the first in vivo evidence that CRP interferes with glucose metabolism in man. Although the association between modestly elevated CRP concentrations and glucose metabolism does not necessarily reflect the effects observed upon single infusion with higher CRP concentrations, our findings lend further support to develop strategies aimed at lowering CRP concentrations and/or CRP bio-activity, particularly in subjects characterized by increased risk for development of insulin resistance or type 2 diabetes mellitus.

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References