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

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Abstract

Recently, we reported that C-reactive protein (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. Seven healthy white 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 crossover design. C-reactive protein 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 that was a direct consequence of increased gluconeogenesis. In conclusion, 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.

1. 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 [1]. 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 [2]. However, because 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 procoagulant and inflammatory pathways [3]. In addition to this, clinical data also suggest that lowering of CRP translates into further cardiovascular benefit [4]. 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

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study whether pathophysiologically relevant concentrations of CRP, as seen in patients with an acute cardiovascular event, exerts direct effects on glucose handling in humans.

2. Materials and methods

2.1. Ethical issues and safety experiments

The study protocol was approved by the institutional review board of the Academic Medical Center in Amsterdam as well as the Central Committee on Research involving Human Subjects in the Netherlands. In view of the fact that the present study was performed before the European Union Clinical Trial Directive came into law (March 1, 2006) in the Netherlands, the study was approved according to the procedures with national Dutch laws. Consequently, the institutional review board of our hospital together with the Central Committee on Research involving Human Subjects of our country functioned as the competent authority that evaluated at that time such clinical studies and carefully reviewed the study protocol that eventually lead to approval. In accordance with European Union Clinical Trial Directive regulations, national Dutch laws also require prehuman toxicology testing. Therefore, we performed these tests in mice and rabbits with CRP concentrations more than 4 times higher than peak concentrations obtained in humans, in which we observed no toxicologic effects [3].

2.2. Purification and safety control of the rhCRP solution

The rhCRP (BiosPacific, Emeryville, CA) was supplied in 20 mmol/L Tris, 140 mmol/L NaCl, 2 mmol/L $CaCl_2$ (pH 7.5), and 0.05% (wt/vol) sodium azide and revealed a single 23-kd band (>99%) after Coomassie Brilliant Blue R-250 staining (1 μg; sodium dodecyl sulfate–polyacrylamide gel). Before purification, the host cell protein concentration was 85 ppm, as determined by a high-sensitive enzyme-linked immunosorbent assay in accordance with manufacturer's instructions (Cygnus Technologies, Southport, NC). Subsequently, the rhCRP was purified using size exclusion chromatography to remove contaminants including endotoxin and sodium azide (Univalid, Leiden, The Netherlands). Purity and stability were 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 less than 1.5 endotoxin units per milliliter as evaluated by limulus assay (turbidimetric kinetic method; ACC, East Falmouth, MA). The rhCRP was stored in a $CaCl_2$-containing buffer (pH 8.5) at 0°C to 4°C, and all experiments were performed within 4 weeks after rhCRP purification.

2.3. Study design

Seven healthy white 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 before 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 g carbohydrates for 3 days before the study.

2.4. Study procedures

Approximately 14 1/2 hours before rhCRP or diluent infusion, participants were instructed to have their last meal. At $t = -14$ 1/2 hours, blood was drawn for the background enrichment of ^2H in body water, followed by ingestion of 1 g/kg body water ^2H_2O (99% pure, Cambridge Isotopes, Cambridge, MA) 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 AM ($t = -2$ 1/2 hours), blood was drawn for assessment of background enrichment of [6,6-$^2H_2$]glucose. Subsequently, a primed (1.6 mg/kg), continuous (1.2 mg/kg per hour) infusion of [6,6-$^2H_2$]glucose (99% enriched, Cambridge Isotope Laboratories) dissolved in sterile isotonic saline was initiated using a calibrated syringe pump (Perfusorâ Secura FT, B. Braun, Melsungen, Germany) through a Millipore filter (size, 0.2 mm; Minisart, Sartorius, Göttingen, Germany). From 10:10 AM ($t = -20$ minutes), 3 blood samples were collected at intervals of 10 minutes for determination of plasma glucose concentration, [6,6-$^2H_2$]glucose enrichment, and ^2H_2O enrichment in body water. Blood samples for the measurement of gluco- and counterregulatory hormones were also collected. At 10:30 AM (0 hours), a bolus of rhCRP (1.25 mg/kg body weight) or CRP-free diluent was administered intravenously. Hereafter, blood samples were collected at $t = 1, 2, 3, 4, 6, 8$, and 9 hours. After the last blood withdrawal at 7:30 PM (9 hours), the study ended.

2.5. Laboratory analysis

Blood samples for measurement of gluconeogenesis were deproteinized by adding an equal amount of 10% perchloric acid. Blood for [6,6-$^2H_2$]glucose enrichment and hormone concentration measurements was collected in heparinized tubes. For the determination of levels of free fatty acids (FFAs), 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-$^2H_2$]glucose, ^2H_2O, and deuterium at the C5 position of glucose were determined as previously described [5]. Briefly, plasma samples for glucose enrichment of [6,6-$^2H_2$]glucose and plasma glucose concentration was measured as the aldonitril pentaacetate 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-$^2H_2$]glucose was determined by dividing

Table 1
Baseline characteristics of the 7 study subjects on both study days

	Control study day	CRP study day
Age (y)	39.3 ± 16.9	–
BMI (kg/m^2)	27.3 ± 5.2	27.4 ± 5.2
Body fat (%)	24.7 ± 9.9	24.8 ± 10.2
Body temperature (°C)	36.9 ± 0.5	36.8 ± 0.5
Heart rate (bpm)	82 ± 11	79 ± 10
Systolic BP (mm Hg)	123 ± 8	124 ± 11
Fasting plasma glucose (mmol/L)	5.3 ± 0.6	5.4 ± 0.4
Fasting plasma insulin (pmol/L)	90 ± 43	88 ± 45
Fasting plasma glucagon (ng/L)	78 ± 28	73 ± 25
Fasting plasma cortisol (nmol/L)	394 ± 83	374 ± 87
C-peptide (pmol/L)	907 ± 180	881 ± 374
FFA (mmol/L)	0.44 ± 0.15	0.45 ± 0.14
Epinephrine (nmol/L)	0.08 ± 0.01	0.19 ± 0.07
Norepinephrine (nmol/L)	1.15 ± 0.57	1.60 ± 1.03
hsCRP (mg/L)	1.9 ± 2.0	1.8 ± 1.9
LDL cholesterol (mmol/L)	3.1 ± 1.1	3.1 ± 1.1
HDL cholesterol (mmol/L)	1.3 ± 0.2	1.3 ± 0.1
Triglycerides (mmol/L)	1.0 ± 0.2	0.9 ± 0.2

Data are expressed as means ± SD. BMI indicates 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.

the peak area of *m*/*z* 189 by the peak area of *m*/*z* 187 and correcting for natural enrichments. Glucose was converted to hexamethylenetetramine [6] to measure deuterium enrichment at the C5 position. Hexamethylenetetramine was injected into a gas chromatograph mass spectrometer. Separation was achieved on an AT-Amine column (Breda, NB, The Netherlands; 30 m × 0.25 mm; d_f, 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).

Plasma insulin concentration was determined with a chemiluminescent immunometric assay (Immulite 2000, Diagnostic Products, Los Angeles, CA). C-peptide was determined by radioimmunoassay (RIA-coat C-peptide, Byk-Sangtec Diagnostica, Dietzenbach, Hesse, Germany). Cortisol was measured by enzyme immunoassay on an Immulite analyzer (DPC, Nutley, NJ) (intra-assay coefficient of variation, 2%-4%). Glucagon was determined by radioimmunoassay (Linco Research, St Louis, MO). Norepinephrine and epinephrine were determined by an in-house high-performance liquid chromatography method. Plasma FFAs were measured by an enzymic method (NEFAC; Wako Chemicals, Richmond, VA).

C-reactive protein was measured by a high-sensitivity immunoturbidimetric assay (Roche Diagnostics, Basel, Switzerland), whereas CRP concentrations in excess of 10 mg/L (after rhCRP infusion) were assayed by immunonephelometry (P800 analyzer, Roche Diagnostics). Circulating cytokine concentrations were assessed with the luminex method (Bioplex Human Cytokines 1 × 96 wells, catalog no. X500000 FFS, Bio-Rad Laboratories, San Diego, CA).

2.6. Calculations

Glucose appearance rate (glucose R_a) was calculated from the dilution of labeled glucose in plasma. The Steele equation for steady-state conditions was used for the measurement of glucose R_a 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 gluconeogenesis was calculated by multiplying the glucose R_a by fractional gluconeogenesis. The fractional gluconeogenesis = 100% × ([^{2}H] enrichment on C5 of glucose)/([^{2}H] enrichment in plasma water). The rationale has been discussed in detail by Landau et al [5]. In brief, glucose

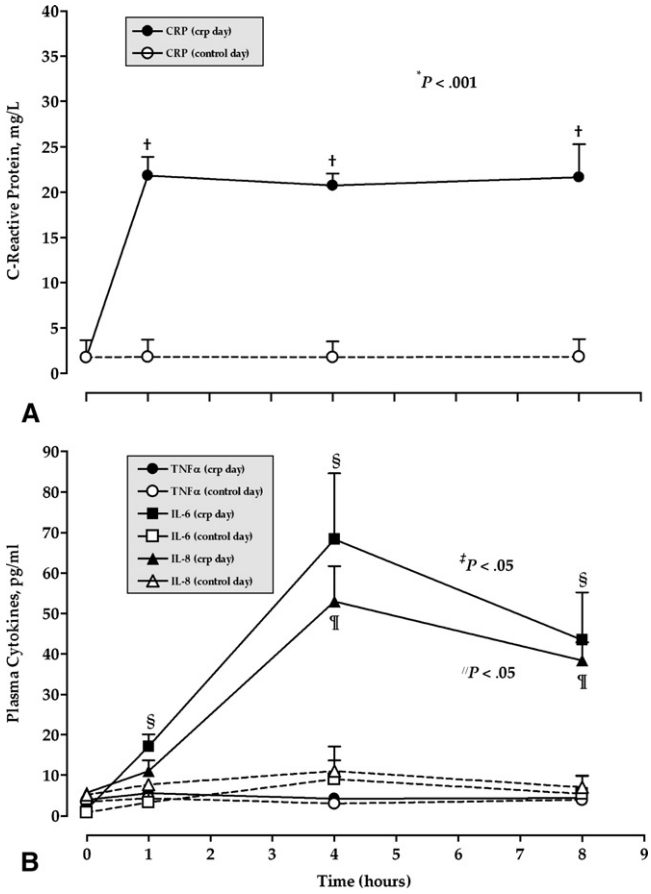

Fig. 1. Effect of CRP infusion on inflammation activation. A, Plasma CRP concentrations. *P < .001 indicates difference between the CRP infusion day (●) and the control day (○) by analysis of variance for repeated measures; †P < .01, difference between time points by Wilcoxon test. B, Plasma cytokine concentrations: TNF-α (nonsignificant difference between interventions and time points on the CRP infusion day [●] and the control day [○]), IL-6 (‡P < .05 indicates difference between the CRP infusion day [■] and the control day [□]; §P < .05, difference between time points), and IL-8 (‖P < .05 indicates difference between the CRP infusion day [▲] and the control day [△]; ¶P < .05, difference between time points). Values are expressed as means ± SD.

produced during plasma ^2H_2O 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 ^2H_2O enrichment. A requirement for the latter method is the complete equilibration of plasma ^2H_2O enrichment with C2 enrichment of glucose.

2.7. Statistical analysis

Descriptive statistics between CRP and diluent infusion were compared by 2-tailed paired *t* tests, or a nonparametric test (Wilcoxon test) was used in case of nonnormal distribution. Statistical analysis of glucose metabolism parameters for individual subjects between CRP and diluent infusion over time was performed using analysis of variance 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, Chicago, IL). Data are expressed as means ± SD.

3. Results

3.1. Clinical characteristics

Baseline characteristics were determined before CRP and diluent infusion (Table 1). The 7 healthy white 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.

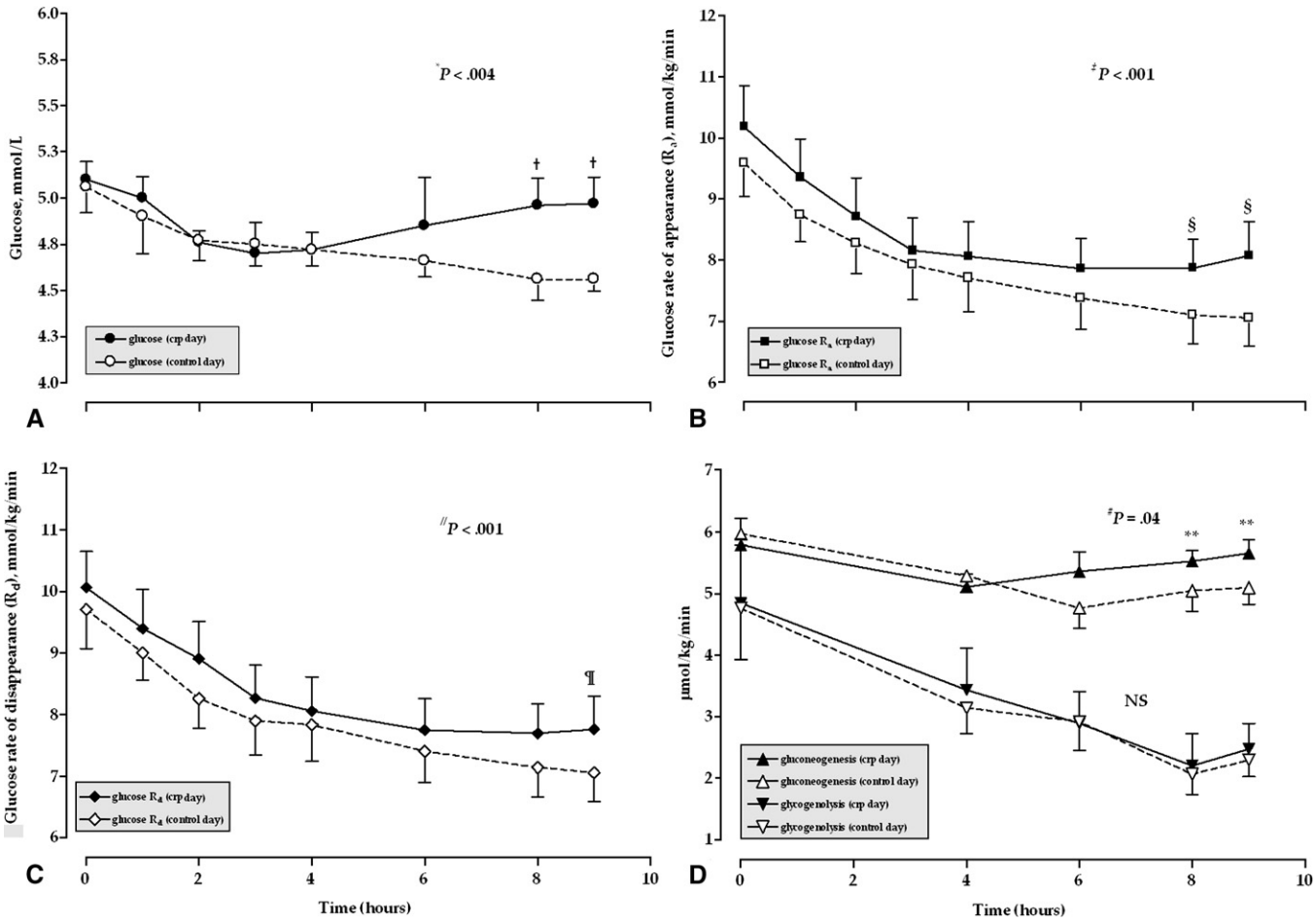

Fig. 2. Effect of CRP infusion on glucose metabolism. A, Plasma glucose concentration. **P* = .004 indicates difference between the CRP infusion day (●) and control day (○) by analysis of variance for repeated measures; †*P* < .05, difference between time points by Wilcoxon test. B, Glucose R_a. ‡*P* < .001 indicates difference between the CRP infusion day (■) and the control day (□); §*P* = .02, difference between time points. C, Glucose R_d. ‖*P* < .001 indicates difference between the CRP infusion day (♦) and the control day (◇); ¶*P* < .05, difference between time points. D, Gluconeogenesis and glycogenolysis. #*P* = .04 indicates difference between the CRP infusion day (▲) and the control day (△); ***P* = .02, difference between time points; nonsignificant (NS), difference between interventions and time points on the CRP infusion day (▼) and on the control day (▽). Values are expressed as means ± SD.

3.2. Plasma CRP and cytokine concentrations

After CRP infusion, plasma concentrations of CRP increased to 23.9 ± 4.2 mg/L at 1 hour (Fig. 1A). Tumor necrosis factor α (TNF-α) concentrations did not change upon CRP infusion (Fig. 1B), whereas a transient rise in interleukin (IL)–6 as well as IL-8 was observed, peaking at 4 hours (Fig. 1B).

3.3. Glucose metabolism

After CRP infusion, plasma glucose concentrations increased with 10% from 8 hours onward compared with diluent infusion (Fig. 2A). Baseline values for glucose R_a were comparable on both study days (Fig. 2B). At the same time, there was a modest increase in glucose R_a of 11% compared with the control experiments, which showed a continuous decline in glucose R_a. Concomitantly, glucose R_d increased slightly by 4% after CRP infusion (Fig. 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 (Fig. 2D). C-reactive protein infusion did not affect glycogenolysis (Fig. 2D).

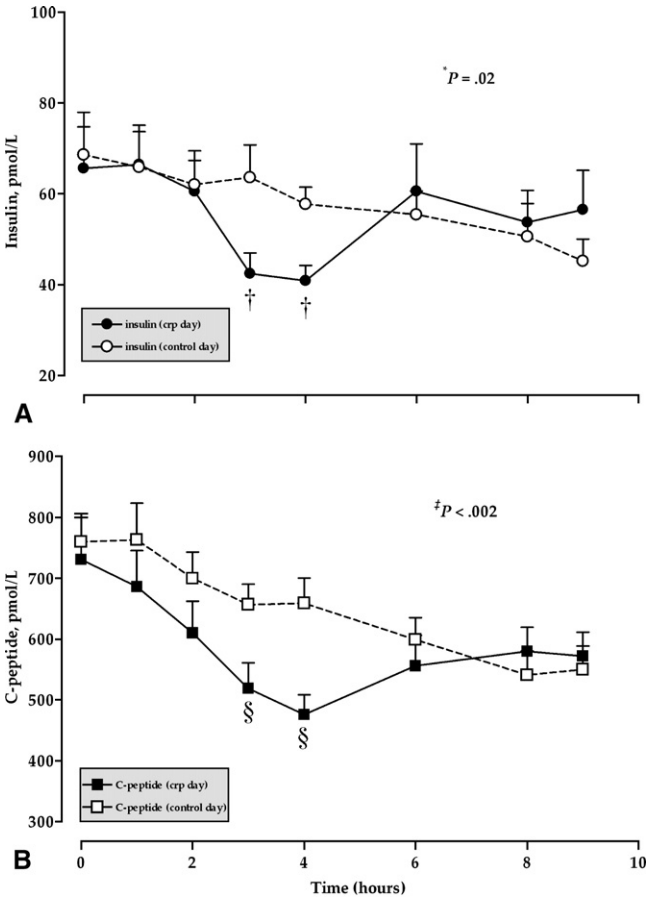

Fig. 3. Effect of CRP infusion on insulin secretion. A, Plasma insulin concentrations. *P = .02 indicates difference between the CRP infusion day (●) and the control day (○) by analysis of variance for repeated measures; †P < .02, difference between time points by Wilcoxon test. B, Plasma C-peptide. ‡P = .002 indicates difference between the CRP infusion day (■) and control the day (□); §P < .05, difference between time points. Values are expressed as means ± SD.

3.4. Gluco- and counterregulatory hormones

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

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

3.5. Other measurements

Baseline FFA and adiponectin concentrations were comparable on both study days and were unaffected upon CRP administration.

4. 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 because of 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 3 to 4 hours after CRP. These findings suggest that CRP may have a direct effect on glucose handling in vivo.

4.1. 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 that in control experiments. However, from 6 hours onward, plasma glucose concentrations rose significantly in CRP-infused subjects, preceded by an 11% increase in glucose R_a. Only a small increase in glucose R_d was observed. The combination of 10% increase in glucose, 11% increase in glucose R_a, 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.

4.2. 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

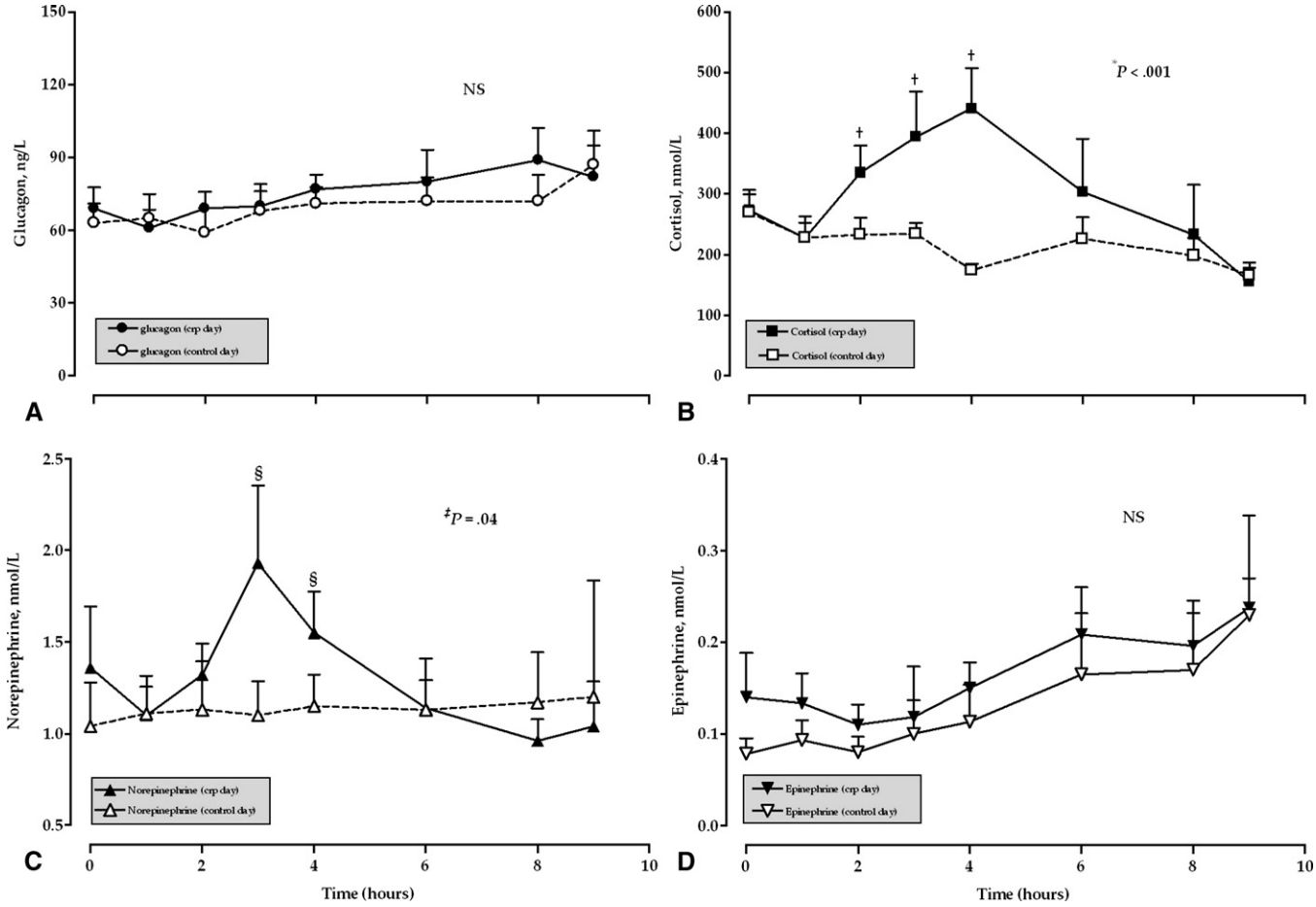

Fig. 4. Effect of CRP infusion on counter-glucoregulatory hormones. A, Plasma glucagon concentrations. NS, difference between interventions and time points on the CRP infusion day (●) and the control day (○). B, Plasma cortisol concentrations. *$P < .001$ indicates difference between the CRP infusion day (■) and the control day (□); †$P < .05$, difference between time points. C, Norepinephrine. ‡$P = .04$ indicates difference between the CRP infusion day (▲) and the control day (△); §$P < .05$, difference between time points. D, Epinephrine. NS, difference between interventions and time points on the CRP infusion day (▼) and the control day (▽). Values are expressed as means ± SD.

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 [7,8]. Although norepinephrine has a relatively short duration of action, some studies have shown that infusion with norepinephrine may immediately increase hepatic glucose production [9,10]. Thus, cortisol together with norepinephrine provides a plausible explanation for the CRP-induced effects on glucose metabolism [11,12]. Most likely, the increases in plasma cortisol and norepinephrine concentrations are secondary to increased IL-6 release peaking at 4 hours [13-15]. Because CRP levels remained elevated throughout the CRP study day, a direct effect of CRP on cortisol and norepinephrine seems unlikely but cannot be ruled out with all certainty. Notably, in contrast to other inflammatory stimuli such as TNF-α and IL-6, CRP infusion did not affect glucagon concentrations [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 [13]. These effects of IL-6 can be observed from concentrations of 400 to 600 pg/mL onward, whereas IL-6 concentrations in the present study did not exceed the 60 to 80 pg/mL range. The latter makes it unlikely that IL-6 had a major contribution to the effect of CRP on glucose handling.

4.3. 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 [17]. Therefore, we used a modified purification procedure [18]. In line with this, 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 TNF-α increase. Second, the trace amounts of endotoxin present in the purified rhCRP solution (1.5 endotoxin units per kilogram) did not induce inflammatory changes in humans, thereby excluding a causal role for endotoxin [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 [20].

4.4. Study limitations

Chronic elevations of CRP in the lower range (eg, 1-5 mg/L) have been associated with metabolic sequelae and adverse cardiovascular outcome [21]. Because repeated administration of rhCRP is not ethical in view of potential sensitization, we performed immediate CRP-infusion experiments only, aiming at pathophysiologic CRP concentrations approximating those found in patients with an acute coronary syndrome (20-25 mg/L) [1]. Therefore, the current effects of CRP on glucose metabolism must be interpreted as pathophysiologic 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.

4.5. 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 bioactivity, particularly in subjects characterized by increased risk for development of insulin resistance or type 2 diabetes mellitus.

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