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C-Reactive Protein Does Not Impair Insulin Suppression of Glucose Release in Primary Hepatocytes

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Abstract

Recent studies have suggested that CRP may interfere with insulin signaling in skeletal muscle and endothelial cells. The aim of this study was to determine whether highly purified CRP increased the rate of glucose appearance in primary hepatocytes in the absence or presence of insulin. Primary rat hepatocytes were provided glucose-free media containing 10 mM lactate, 1mM pyruvate, 0, 1 or 10 nM insulin, and 0 or 10 µg/ml of purified CRP for 6h. Purified CRP did not increase glucose release in the absence of insulin and did not reduce the ability of insulin to suppress glucose release.

Keywords

Liver; insulin resistance; inflammation; obesity; cardiovascular disease

INTRODUCTION

Obesity is associated with the development of insulin resistance, a characteristic feature of metabolic syndrome and type 2 diabetes (1). The cellular and molecular mechanisms linking obesity to insulin resistance remain unclear, in part due to the complexity of the obese state. Obesity is associated with a state of chronic inflammation characterized by the abnormal production of proinflammatory cytokines and acute phase reactants (1,2). Nonalcoholic fatty liver disease (NAFLD) represents a spectrum of disorders ranging from simple steatosis to steatohepatitis, advanced fibrosis and cirrhosis (3). The prevalence of NAFLD in the obese population is very high and NAFLD and the metabolic syndrome share several clinical characteristics, such as obesity, dyslipidemia and insulin resistance (3,4). Several studies have reported significant associations among the acute phase reactant, C-reactive protein (CRP), insulin resistance and obesity in NAFLD (5–7). Although recent studies in L6 myotubes and endothelial cells have demonstrated that CRP can directly impair insulin signaling (8,9), the ability of CRP to directly influence insulin action in hepatocytes has not been examined. Therefore, the purpose of the present study was to determine whether CRP interferes with insulin regulation of glucose metabolism in hepatocytes in the absence of steatosis or the confounding influence of the obese state.

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METHODS

Purification of *Escherichia coli*-derived CRP

Human recombinant *Escherichia coli*-derived CRP (CRP), supplied in 140 mM NaCl, 2 mM CaCl₂, 20 mM Tris, 0.05% (weight/vol) NaN₃, pH 7.5, was purchased from Calbiochem (La Jolla, CA). CRP was dialyzed three times (1 hr per time) against ~600 mL NaCl/CaCl₂/Tris buffer using a Thermo Scientific (Rockford, IL) Slide-A-Lyzer® Dialysis Cassette. Endotoxin was removed using a Thermo Scientific (Rockford, IL) Detoxi-Gel™ Endotoxin removing column to a level that was < 0.1 endotoxin units/ml based on Limulus assay (BioWhittaker, Walkersville, MD). SDS-PAGE of purified, dialyzed CRP revealed a single 23-kDa band on silver staining (Fig. 1A).

Isolation of hepatocytes & primary cell culture

Hepatocytes were isolated by collagenase perfusion (10) from male Wistar Crl(WI)BR rats (~120 g), purchased from Charles River Laboratories (Wilmington, MA). Cells were first incubated in RPMI media (HyClone, Logan, UT) containing 11 mM glucose, 10⁻⁷ M dexamethasone, 10⁻⁷ M insulin and 5% fetal bovine serum on collagen-coated plates for 4 hrs (attachment period). The medium was then changed to one containing RPMI, 8 mM glucose, 10⁻⁷ dexamethasone, and 10⁻⁸ insulin. The following morning cells were rinsed 3 times with PBS and then incubated in RPMI media and 0 or 10 µg/ml of purified CRP for 30 min. The media was then replaced with RPMI containing 10 mM lactate, 1 mM pyruvate, 0, 1, or 10 nM insulin, and 0 or 10 µg/ml of purified CRP for a period of 6 hrs. Cell viability at the beginning of experiments was >95% and did not change significantly over the 6 hr experimental period, based on trypan blue exclusion and media lactate dehydrogenase concentrations. Each independent experiment was performed in triplicate and a total of 7 independent experiments were performed. In some experiments (n=3), the cell permeable inhibitor of c-Jun NH₂ terminal kinase (JNK; 10 µM), SP600125 (Calbiochem, San Diego, CA) was used. To demonstrate that insulin resistance could be induced in primary hepatocytes experiments identical to those described above for CRP were performed but in which palmitate (200 µM) complexed to albumin (125 µM) was used in place of CRP (n=3).

Sample Collection

Media samples were taken at 0, 2, 4 and 6 hrs and stored at -80°C for later analysis of glucose concentrations (Sigma Kit # GAHK-20) and lactate dehydrogenase (Sigma). At 6 hrs, cells were rinsed 3 times with ice-cold PBS, harvested and processed for western blot analysis as described previously (11).

Western blot analysis

Western blot analysis was performed as described in detail previously (12). Membranes were exposed to the following primary antibodies: total and phosphorylated Akt (serine 473), total and phosphorylated (Serine 9 & 21) glycogen synthase kinase-α/β (GSK), total and phosphorylated (Serine 307) insulin receptor substrate-1 (IRS1), total and phosphorylated c-Jun NH₂ terminal kinase (JNK) (Cell Signaling, Danvers, MA). Proteins were detected with horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia Biotech, Piscataway, NJ) and an enhanced chemiluminescence reagent (Santa Cruz Biotechnology, Santa Cruz, CA). Optical density of individual western blots was determined using a UVP Bioimaging system (Upland, CA).

Data Analysis

Glucose appearance over 6 hrs was normalized to total protein content for each experiment and reported as nmol/mg protein/6h. Statistical comparisons were calculated using analysis

of variance and post-hoc comparisons among means using the Scheffe's or Tukey test. Statistical significance was set at $p < 0.05$. All data are reported as the mean $\pm$ SE.

RESULTS

Effects of purified CRP on glucose release in primary hepatocytes

The presence of CRP did not have a significant effect on glucose appearance in the media in the absence or presence of insulin (Fig. 1B).

Effects of purified CRP on insulin signaling in primary hepatocytes

Insulin increased phosphorylation of Akt and GSK; however the presence of CRP did not have a significant effect on the level of phosphorylation of these two proteins (Fig. 2A–B). Activation of the serine/threonine kinase, JNK promotes phosphorylation of IRS1 on serine³⁰⁷ and can impair insulin signaling (13, 14). Therefore, to examine whether CRP was bioactive, we examined phosphorylation of JNK and IRS1. CRP increased the phosphorylation of JNK and IRS1 on serine³⁰⁷ in the absence of insulin, however this was not maintained when insulin was present (Fig. 3A–B). Inclusion of the cell permeable JNK inhibitor, SP600125, blocked the CRP-mediated increase in phosphorylation of IRS1 on serine³⁰⁷ in the absence of insulin (Fig. 3C).

Effects of palmitate on glucose release in primary hepatocytes

Due to the lack of effect of CRP on insulin action in primary hepatocytes we wanted to determine whether a reduction in insulin suppression of glucose release could be induced in our hepatocyte model. Indeed, palmitate increased glucose release in the presence of insulin (Fig. 4).

DISCUSSION

CRP is elevated in individuals with obesity, cardiovascular disease and metabolic syndrome, conditions also characterized by inflammation and insulin resistance (15–18). However, the extent to which CRP is causally linked to the inflammatory process and/or insulin resistance is unclear. Recent studies have demonstrated that purified CRP can reduce insulin signaling in L6 myotubes and endothelial cells (9,19). The present study was undertaken to examine the effects of CRP on glucose release and insulin signaling in primary hepatocytes. In contrast to previous observations, CRP did not significantly influence glucose release in the absence or presence of insulin or insulin-stimulated phosphorylation of Akt or GSK in primary liver cells.

The purity of commercially obtained recombinant CRP derived from *Escherichia coli* is a concern in studies that attempt to examine the ability of CRP to induce inflammation or insulin resistance (20–22). In the present study, the purification protocol resulted in a CRP preparation containing less than 0.1 endotoxin units/ml based on limulus assay. These values are similar to or less than what has been reported in previous investigations (19). In addition, lipopolysaccharide, one of the contaminants present in commercially obtained CRP from *Escherichia coli*, appears to suppress glucose production (23), which was not observed in the present studies. Therefore, the negative results obtained in the present series of studies do not appear to be due to contaminants that remain in the purified CRP preparation.

Activation of the stress kinase, JNK, leads to phosphorylation of IRS1 on serine³⁰⁷ and impaired insulin signaling (13,14). Activation of JNK and increased phosphorylation of IRS1 on serine³⁰⁷ was previously observed in both L6 myotubes and endothelial cells upon exposure to CRP (9,19). In the present study, CRP increased phosphorylation of JNK and

IRS1 on serine³⁰⁷ in the absence of insulin. In addition, inclusion of a cell permeable inhibitor of JNK abolished CRP-mediated phosphorylation of IRS1 on serine³⁰⁷ in the absence of insulin. This result is consistent with previous studies (9,19) and demonstrates that the purified CRP preparation used in the present study was active. Importantly, CRP-mediated phosphorylation of IRS1 on serine³⁰⁷ did not result in increased glucose release by primary hepatocytes in the absence of insulin. In addition, CRP did not influence the ability of insulin to phosphorylate two insulin signaling target proteins, Akt or GSK, or to suppress glucose release. Consistent with these observations, insulin also abolished the CRP-mediated differences in phosphorylation of IRS1 on serine³⁰⁷ that were observed in the absence of insulin. Thus, in primary hepatocytes CRP does not accelerate glucose release in the absence or presence of insulin. Reasons for the discrepancies between the present study and previous studies (9,19) may include the duration of exposure, cell type and/or concentration of CRP employed.

NAFLD and the metabolic syndrome share several clinical characteristics, including obesity, dyslipidemia, inflammation and insulin resistance (24). Several studies have reported significant associations among CRP, insulin resistance and obesity in NAFLD (5–7). Data from the present study would suggest that CRP alone may not be causally linked to insulin resistance in hepatocytes. Thus, CRP may represent a biomarker of inflammation or be linked to extra-hepatic consequences, such as insulin resistance in muscle and/or cardiovascular complications (8,9,25,26). Alternatively, CRP may work in concert with dyslipidemia and cytokines to exacerbate insulin resistance in the liver. Finally, it cannot be ruled out that more prolonged exposure to CRP could provoke insulin resistance in hepatocytes.

In summary, the present study demonstrates that purified CRP does not alter glucose release in the absence of insulin or the ability of insulin to suppress glucose release in primary hepatocytes.

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Figure 1.

(A) Silver stain of purified, dialyzed CRP and (B) glucose appearance in the media normalized to total cellular protein in primary hepatocytes incubated in the absence (Control) or presence of 10 $\mu\text{g/ml}$ of C-reactive protein (CRP) and in the absence (0 Insulin) or presence of 1 or 10 nM insulin for 6 hours. At the start of experiments media was devoid of added glucose but contained 10 mM lactate and 1 mM pyruvate. Final glucose concentrations following the 6 hour incubations ranged from 160–350 μM . Lane 1 of the gel is molecular weight marker and lanes 2 and 3 are duplicate CRP samples. Data in figure 1B is presented as the mean $\pm$ SE for 7 independent experiments. *, significantly ($P < 0.05$) different from respective no insulin condition.

Figure 2.

(A) Ratio of phosphorylated Akt to total Akt, (B) ratio of phosphorylated GSK to total GSK in primary hepatocytes incubated in the absence (Control) or presence of 10 μ g/ml of CRP and in the absence (0 Insulin) or presence of 1 or 10 nM insulin for 6 hours. Data in figures is presented as the mean $\pm$ SE for 4 independent experiments and representative gels are shown. *, significantly ($P < 0.05$) different from respective 0 insulin condition.

Figure 3.

(A) Ratio of phosphorylated JNK to total JNK, (B) ratio of serine³⁰⁷ phosphorylation on IRS1 to total IRS1, (C) ratio of serine³⁰⁷ phosphorylation of IRS1 to total IRS1 in primary hepatocytes incubated in the absence (Control) or presence of 10 μ g/ml of CRP and in the absence (0 Insulin) or presence of 1 or 10 nM insulin for 6 hours. In figure C the JNK inhibitor, SP600125 was also included in the incubation media (CRP + SP600125). Data in figures is presented as the mean $\pm$ SE for 4 independent experiments and representative gels are shown. +, significantly ($P < 0.05$) from all other groups.

Figure 4.

Glucose appearance in the media normalized to total cellular protein in primary hepatocytes incubated in the absence (Control) or presence of albumin-bound palmitate (200 μ M) and in the absence (0 Insulin) or presence of 1 or 10 nM insulin for 6 hours. Data in figures is presented as the mean $\pm$ SE for 3 independent experiments. *, significantly ($P < 0.05$) from control group at similar insulin concentration.