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ARTICLE

C-reactive protein induces phosphorylation of insulin receptor substrate-1 on Ser^{307} and Ser^{612} in L6 myocytes, thereby impairing the insulin signalling pathway that promotes glucose transport

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Abstract
Aims/hypothesis C-reactive protein (CRP) is associated with insulin resistance and predicts development of type 2 diabetes. However, it is unknown whether CRP directly affects insulin signalling action. To this aim, we determined the effects of human recombinant CRP (hrCRP) on insulin signalling involved in glucose transport in L6 myotubes.
Materials and methods L6 myotubes were exposed to endotoxin-free hrCRP and insulin-stimulated activation of signal molecules, glucose uptake and glycogen synthesis were assessed.
Results We found that hrCRP stimulates both c-Jun *N*-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK)1/2 activity. These effects were paralleled by a concomitant increase in IRS-1 phosphorylation at Ser^{307} and Ser^{612}, respectively. The stimulatory effects of hrCRP on IRS-1 phosphorylation at Ser^{307} and Ser^{612} were partially reversed by treatment with specific JNK and ERK1/2 inhibitors, respectively. Exposure of L6 myotubes to hrCRP reduced insulin-stimulated phosphorylation of IRS-1 at Tyr^{632}, a site essential for engaging p85 subunit of phosphatidylinositol-3 kinase (PI-3K), protein kinase B (Akt) activation and glycogen synthase kinase-3 (GSK-3) phosphorylation. These events were accompanied by a decrease in insulin-stimulated glucose transporter (GLUT) 4 translocation to the plasma membrane, glucose uptake and glucose incorporation into glycogen. The inhibitory effects of hrCRP on insulin signalling and insulin-stimulated GLUT4 translocation were reversed by treatment with JNK inhibitor I and the mitogen-activated protein kinase inhibitor, PD98059.
Conclusions/interpretation Our data suggest that hrCRP may cause insulin resistance by increasing IRS-1 phosphorylation at Ser^{307} and Ser^{612} via JNK and ERK1/2, respectively, leading to impaired insulin-stimulated glucose uptake, GLUT4 translocation, and glycogen synthesis mediated by the IRS-1/PI-3K/Akt/GSK-3 pathway.

Keywords CRP · Glucose transport · Insulin resistance · Insulin signalling

Abbreviations

Akt	protein kinase B
CRP	C-reactive protein
ERK	extracellular signal-regulated protein kinase
FcγR	FC γ receptor
GLUT	glucose transporter
GSK-3	glycogen synthase kinase-3
hrCRP	human recombinant C-reactive protein
JNK	c-Jun *N*-terminal kinase
MAPK	mitogen-activated protein kinase
MEK	MAPK kinase
P85	p85 subunit of PI-3K
PI-3K	phosphatidylinositol-3 kinase
SYK	spleen tyrosine kinase

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Introduction

Increasing evidence suggests that chronic, low-grade, inflammatory states may have a pathogenetic role in insulin resistance and type 2 diabetes [1, 2]. Several plasma markers of inflammation have been evaluated as potential tools to predict the risk of type 2 diabetes and glucose disorders and, among these, the most reliable and accessible for clinical use is currently high-sensitive C-reactive protein (CRP) [3, 4]. Cross-sectional studies have shown that high-sensitive CRP is associated with IGT, IFG, type 2 diabetes, insulin resistance, obesity, and other features of the metabolic syndrome [4–7]. Moreover, prospective studies have demonstrated that elevated high-sensitive CRP predicts the subsequent development of type 2 diabetes [3, 4, 8]. One mechanism linking this inflammatory response to the development of insulin resistance involves activation of serine/threonine 'stress kinases,' which may be activated in response to inflammatory cytokines. Once activated, these stress kinases affect insulin signalling cascade by phosphorylating the IRS proteins on serine residues within insulin-sensitive cell types such as hepatocytes, adipocytes and myocytes [9–14]. Increased serine phosphorylation of IRS-1 inhibits its ability to be tyrosine-phosphorylated by the insulin receptor and to bind and activate phosphatidylinositol 3-kinase (PI-3K) [9–11]. Among these stress kinases, it as been shown that c-Jun *N*-terminal kinase (JNK) phosphorylates IRS-1 at Ser^{307}, and extracellular signal-regulated kinase (ERK)1/2 at Ser^{612}, respectively [9–14]. Results from targeted disruption of the genes encoding IRS-1 and IRS-2 in mice have provided compelling results on the pivotal role of these molecules in regulating glucose homeostasis [15–17]. In addition, defects in IRS-1 and IRS-2 expression and function have been reported in insulin-resistant states such as obesity and type 2 diabetes [18–20], and polymorphisms in the genes encoding IRS have been identified, which are associated with insulin resistance [21–23]. Thus, demonstrating novel cross-talk between pro-inflammatory molecules such as CRP and insulin signalling molecules such as IRSs, which are involved in glucose homeostasis, could help elucidate their role in the pathogenesis of insulin resistance and type 2 diabetes and identify potential new therapeutic targets in the treatment of insulin resistance.

In addition to being a sensitive marker of inflammation, recent studies have shown that CRP has direct proinflammatory effects [24]. CRP has been shown in vascular smooth muscle cells to increase inducible nitric oxide production and increase nuclear factor-κb, JNK and mitogen-activated protein kinase (MAPK) activities [25, 26]. Although it has been demonstrated that CRP levels associate with insulin resistance and predict type 2 diabetes, and although many reports on the cellular and biological phenomena induced by CRP in vascular cells have appeared, it is unknown whether CRP directly affects insulin signalling and action in insulin target cells. In this study, we addressed the question of whether CRP affects insulin signalling involved in glucose transport in rat L6 myotubes. In addition, because it has been shown that in vascular smooth muscle cells CRP stimulates both JNK and MAPK, we tested the hypothesis that these kinases mediate the inhibitory effect of CRP on insulin signalling.

Materials and methods

Materials

Rat L6 skeletal muscle cells were from the American Type Culture Collection (Rockville, MD, USA). The MAPK kinase (MEK) inhibitor PD98059, and the cell-permeable JNK inhibitor I were from Calbiochem (La Jolla, CA, USA). All cell culture reagents were from Invitrogen (Carlsbad, CA, USA). 2-Deoxy-D-[^{3}H]glucose and D-[U-^{14}C]glucose were from GE Healthcare Bio-Sciences (Little Chalfont, UK). 2-Deoxy-D-glucose was from Sigma (St. Louis, MO, USA). Antiphosphotyrosine antibody PY20 was from Becton Dickinson (San Diego, CA, USA). Antibodies for the p85 subunit of PI-3K (p85), IRS-1, and IRS-2 were from Upstate Biotechnology (Lake Placid, NY, USA). Antibodies against phospho-ERK1/2 (Thr^{202}/Tyr^{204}), ERK1/2, phospho-JNK (Thr^{183}/Tyr^{185}), JNK, phospho-IRS-1 (Ser^{307}), phospho-IRS-1 (Ser^{612}), phospho-protein kinase B (Akt; Ser^{473}), phospho-Akt (Thr^{308}), Akt, phospho-glycogen synthase kinase-3 (GSK3; $Ser^{21/9}$) and GSK3 were from Cell Signaling Technology (Beverly, MA, USA). Anti-phospho IRS-1 (Tyr^{632}) and anti-glucose transporter (GLUT) 4 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Removal of sodium azide and endotoxin from commercial CRP preparation

Commercial human recombinant CRP (hrCRP) was purchased from Calbiochem (La Jolla, CA, USA). Given the concern surrounding the potential contaminating presence of NaN_3 in commercial CRP preparations, hrCRP was dialysed twice against a buffer containing Tris/NaCl/$CaCl_2$, without NaN_3 at 4°C using a 10-kDa molecular-weight cutoff membrane. Endotoxin was removed from hrCRP using a Detoxigel column (Pierce, Rockford, IL, USA) and quantified to be <0.125 endotoxin units per milliliter by Limulus assay (BioWhittaker, Walkersville, MD, USA). SDS-PAGE of purified dialysed hrCRP revealed a single 23-kDa band on silver staining.

Cell culture

L6 myoblasts were propagated in DMEM containing 25 mmol/l glucose and supplemented with 10% fetal bovine serum and 1% antibiotic–antimycotic mixture in an atmosphere of 5% CO_2 at 37°C. Differentiation was induced by switching confluent cells to fusion medium (DMEM containing 5 mmol/l glucose and 2% horse serum). Experiments were performed with fully differentiated myotubes 12–14 days after confluence, as deduced by a marked increase in expression of myosin heavy chain, a marker of terminally differentiated myotubes (data not shown). However, it is still possible that some myoblasts may have been present at the end of the differentiation process, thus reducing the metabolic effects of insulin.

Measurement of 2-deoxy-D-glucose uptake

After 5 h of serum starvation, myotubes were incubated in the presence or absence of hrCRP (10 mg/l) for 15 min in the presence or absence of PD98059 (50 μmol/l), JNK inhibitor I (20 μmol/l), or a combination of both and subsequently stimulated with insulin (100 nmol/l) for 30 min. Cells were rinsed three times in HEPES-buffered saline solution (140 mmol/l NaCl, 5 mmol/l KCl, 2.5 mmol/l $MgCl_2$, 1 mmol/l $CaCl_2$, 20 mmol/l Hepes, pH 7.4) and were subsequently incubated for 20 min with 11.1 kBq/ml 2-deoxy-D-[3H]glucose (1 mCi/ml, 5–20 Ci/mmol) and 0.1 mmol/l 2-deoxy-D-glucose in the same buffer. After incubation in the uptake buffer, cells were washed three times with ice-cold saline solution, and then lysed by adding 50 mmol/l NaOH. Cell-associated radioactivity was determined by scintillation counting. Cytochalasin B at 10 μmol/l was used to estimate carrier-independent glucose transport. Aliquots of cell lysates were used for protein content determination.

Measurement of glycogen synthesis

Following 5 h of serum starvation, L6 myotubes were incubated in the presence or absence of hrCRP (10 mg/l) for 15 min in the presence or absence of PD98059 (50 μmol/l), JNK inhibitor I (20 μmol/l), or a combination of both and subsequently stimulated with insulin (100 nmol/l) for 2 h in medium containing 18.5 kBq/ml D-[U-14C]glucose (200 μCi/ml, 2–4 mCi/mmol). Cells were washed three times with ice-cold saline solution and lysed in 1 mol/l KOH. Cell lysates were precipitated overnight with ethanol, and precipitated glycogen was then dissolved in water and transferred to scintillation vials for radioactivity measurement. Aliquots of cell lysates were used to determine protein content.

Subcellular fractionation and western blot analysis of GLUT4

Serum-starved L6 myotubes were exposed for 15 min to hrCRP (10 mg/l) in the presence or absence of PD98059 (50 μmol/l), JNK inhibitor I (20 μmol/l), or a combination of both and subsequently stimulated with insulin (100 nmol/l) for 20 min and lysed. Plasma membranes and intracellular membrane from lysed cells were fractionated according to previous reports [27]. Equal amounts of plasma membrane and intracellular membrane proteins were separated on to NuPAGE 4–12% Bis–Tris gels, and proteins were transferred to nitrocellulose membranes, and immunoblotted with GLUT4 antibody.

Immunoprecipitation of IRS-1 and IRS-2 and western blot analyses

Serum-starved L6 myotubes were exposed for 15 min to hrCRP (10 mg/l) in the presence or absence of PD98059 (50 μmol/l), JNK inhibitor I (20 μmol/l), and subsequently stimulated with insulin (100 nmol/l) for 10 min. In all experiments with protein kinase inhibitors, the inhibitors were added to cells 1 h before hrCRP addition. Cells were then lysed for 45 min at 4°C in lysis buffer supplemented with protease and phosphatase inhibitor cocktails. Equal amounts of lysate proteins were immunoprecipitated with anti-IRS-1 or anti-IRS-2 antibodies, and rocked overnight at 4°C. Immune complexes were collected by incubation with protein A Sepharose for 2 h at 4°C and resuspended in Laemmli buffer. Immunoprecipitates were subjected to SDS-PAGE under reducing conditions. Resolved proteins were then transferred to nitrocellulose membranes and immunoblotted with the indicated antibodies. Proteins were detected by enhanced chemiluminescence and band densities quantified by densitometry. To normalise the blots for protein levels, blots were stripped and reprobed with IRS-1 or anti-IRS-2 antibodies after immunoblotting with anti-phospho-specific antibodies.

Analysis of serine phosphorylation of IRS-1 and ERK1/2 and JNK phosphorylation status

Proteins were extracted from L6 serum-starved myotubes incubated in the presence or absence of hrCRP (10 mg/l) for indicated times. Cells were then lysed for 45 min at 4°C in lysis buffer supplemented with protease and phosphatase inhibitor cocktails. Equal amounts of lysate proteins were separated on to NuPAGE 4–12% Bis–Tris gels, and proteins were transferred to nitrocellulose membranes and immunoblotted with the indicated antiphospho-specific antibodies. Proteins were detected by enhanced chemilu-

minescence and band densities quantified by densitometry. To normalise the blots for protein levels, blots were stripped and reprobed with the appropriate primary antibodies after being immunoblotted with antiphospho-specific antibodies.

Analysis of Akt and GSK3 phosphorylation status

Proteins were extracted from L6 serum-starved myotubes incubated in the presence or absence of hrCRP (10 mg/l) for 15 min and subsequently stimulated with insulin (100 nmol/l) for 10 min. Cells were then lysed for 45 min at 4°C in lysis buffer supplemented with protease and phosphatase inhibitor cocktails. Equal amounts of lysate proteins were separated on to NuPAGE 4–12% Bis–Tris gels, and proteins were transferred to nitrocellulose membranes and immunoblotted with the indicated antiphospho-specific antibodies. Proteins were detected by enhanced chemiluminescence and band densities quantified by densitometry. To normalise the blots for protein levels, blots were stripped and reprobed with the appropriate primary antibodies after being immunoblotted with anti-phospho-specific antibodies.

Statistical analysis

Data are mean±SEM. For statistical comparison, data were analysed by ANOVA, followed by Bonferroni's post hoc test. (GraphPad Prism version 4; GraphPad, San Diego, CA, USA). $p<0.05$ was considered statistically significant.

Results

Effect of hrCRP on ERK1/2 and JNK phosphorylation and site-specific serine phosphorylation of IRS-1

It has been shown that both JNK and ERK1/2 are activated by hrCRP [25, 26]. To determine the time-dependence of the effect of hrCRP on L6 myotubes, we examined its effect on phosphorylation of either JNK at Thr^{183}/Tyr^{185} or ERK1/2 at Thr^{202}/Tyr^{204}, since such site-specific phosphorylation is required for activation of JNK and ERK1/2, respectively [28, 29]. Exposure of L6 myotubes to CRP resulted in a time-dependent increase in JNK (p54/p46) and ERK1/2 (p44/p42) phosphorylation, with maximal effect occurring after 15 min of incubation followed by a gradual reduction after 1 h, which was more marked with JNKp46 for reasons as yet unknown (Fig. 1).

It has also been shown that JNK phosphorylates murine IRS-1 at Ser^{307}, and ERK1/2 at Ser^{612}, respectively [9–11]. Since hrCRP has been shown to activate JNK and ERK1/2 [25, 26], we tested whether hrCRP induces serine phosphorylation of IRS-1 in L6 myotubes. Exposure of L6 myotubes to hrCRP resulted in IRS-1 phosphorylation at both Ser^{307} and Ser^{612}. These effects of hrCRP on IRS-1 phosphorylation at Ser^{307} and Ser^{612} were reversed by treatment with JNK inhibitor I and PD98059, a reversible MEK inhibitor, respectively (Fig. 2a,c). JNK inhibitor I did not affect Ser^{612} phosphorylation and PD98059 did not affect Ser^{307} phosphorylation (Fig. 2b,d).

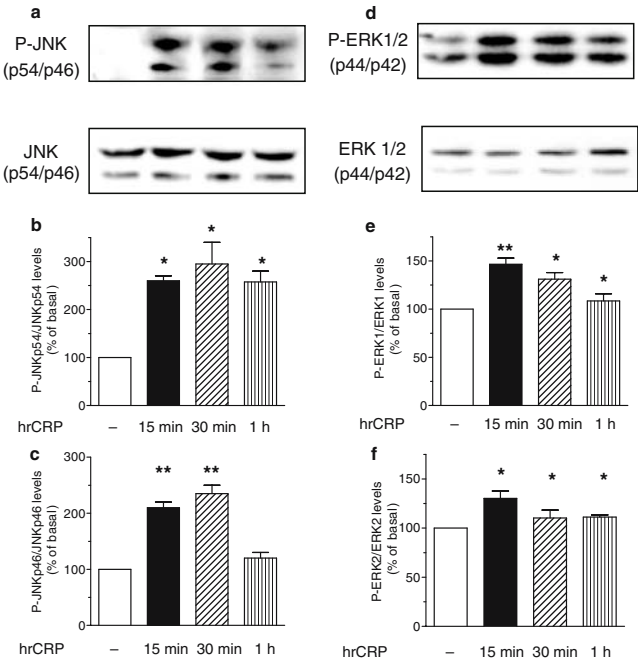

Fig. 1 Effect of human recombinant C-reactive protein (*hrCRP*) on JNK and ERK1/2 phosphorylation. Phosphorylation (*P*) of p54 and p46 isoforms of JNK (**a–c**) and p42 and p44 isoforms of ERK (**d–f**) induced in L6 myotubes by hrCRP (10 mg/l) exposure for indicated times. Representative immunoblots of three experiments are shown. *Bars* represent the means ± SEM for three independent experiments. $*p<0.05$, $**p<0.01$ for differences vs basal

Effect of hrCRP on insulin-stimulated tyrosine phosphorylation of IRS-1 and IRS-2 and their binding to PI-3K

Since hrCRP induces IRS-1 phosphorylation at Ser^{307} and Ser^{612}, we tested whether hrCRP affects insulin-stimulated tyrosine phosphorylation of IRS-1 in L6 myotubes. Exposure of L6 myotubes to hrCRP resulted in 27% inhibition of insulin-stimulated tyrosine phosphorylation of IRS-1 (Fig. 3). Because association of p85 with tyrosine-phosphorylated IRS-1 is pivotal to promotion of downstream signalling, the effect of hrCRP on IRS-1/p85 docking was examined by immunoprecipitation of IRS-1 from cell lysates, followed by immunoblotting with anti-p85 antibody. Insulin stimulated by 2.7-fold the binding of IRS-1 to the p85 subunit (Fig. 3b,d). hrCRP treatment reduced by 30% insulin-stimulated binding of IRS-1 to the p85 subunit.

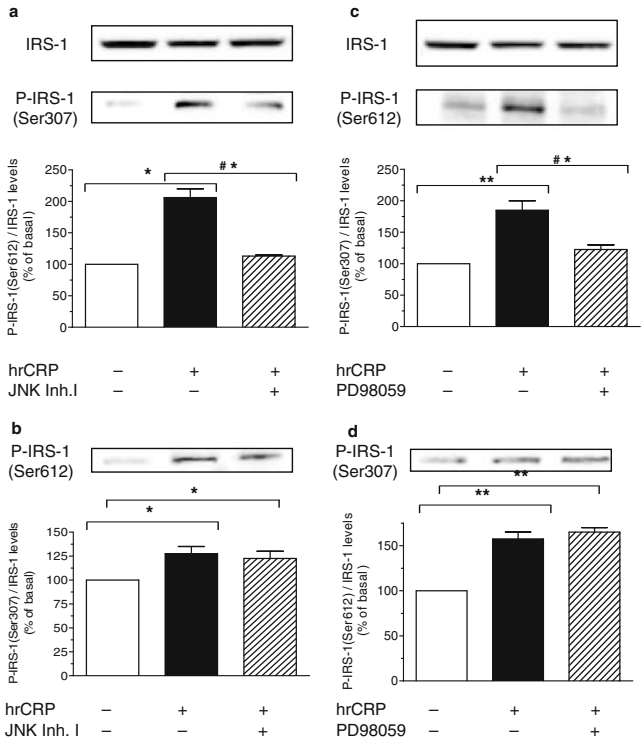

Fig. 2 Phosphorylation (*P*) of IRS-1 at Ser[307] and Ser[612] induced by human recombinant C-reactive protein (*hrCRP*; 10 mg/l, 15 min) in L6 myotubes incubated in the presence or absence of the cell permeable JNK inhibitor I (*JNK Inh.I*) (20 μmol/l) (**a**, **b**) or PD98059 (50 μmol/l) (**c**, **d**). Representative immunoblots of three experiments are shown. *Bars* represent the means ± SEM for three independent experiments. *$p<0.05$; **$p<0.01$ for differences vs basal; #*$p<0.05$ for difference vs hrCRP treatment

Similarly, exposure of L6 myotubes to hrCRP resulted in 40% inhibition of insulin-stimulated tyrosine phosphorylation of IRS-2 and in 23% inhibition of insulin-stimulated binding of IRS-2 to p85 subunit (Fig. 4a,b). The inhibitory effect of hrCRP on tyrosine phosphorylation of IRS-1 and IRS-2 was partially reversed by treatment with either JNK inhibitor I or PD98059. Co-treatment with both inhibitors did not completely reverse the inhibitory effect of hrCRP. Next, we tested the possibility that impaired IRS-1/p85 association induced by hrCRP was related to changes in phosphorylation of the tyrosine residue at position 632 of IRS-1, which plays a major role in engaging p85 [30]. Insulin stimulated by 4.5-fold over basal IRS-1 phosphorylation at Tyr[632] as determined by immunoblotting with phosphor-specific anti-Tyr[632] IRS-1 antibody (Fig. 5). hrCRP treatment reduced by 29% insulin-stimulated phosphorylation of Tyr[632] and this effect was partially reversed by treatment with JNK inhibitor I or PD98059. Co-treatment with both inhibitors did not completely reverse the inhibitory effect of hrCRP.

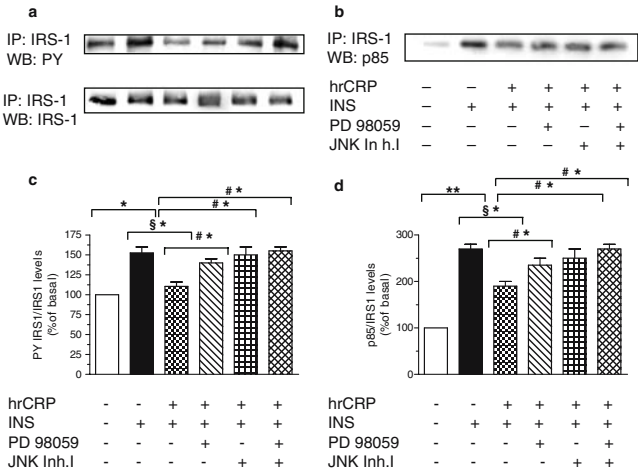

Fig. 3 Effect of human recombinant C-reactive protein (*hrCRP*) on insulin-stimulated tyrosine phosphorylation of IRS-1 and IRS-1/p85 association. IRS-1 phospho-tyrosine levels (**a**, **c**) and IRS-1/p85 association (**b**, **d**) induced by insulin (*INS*) stimulation (100 nmol/l, 10 min) in L6 myotubes incubated in the presence or absence of hrCRP (10 mg/l, 15 min) and pre-treated with or without PD98059 (50 μmol/l) or JNK inhibitor I (*JNK Inh.I*) (20 μmol/l) or a combination of both. Representative immunoblots of four experiments are shown. *Bars* represent the means ± SEM for four independent experiments. *$p<0.05$ and **$p<0.01$ for difference vs basal; §*$p<0.05$ for difference vs insulin stimulation; #*$p<0.05$ for difference vs hrCRP treatment. *WB*, western blot; *IP*, immunoprecipitation; *PY*, phospho-tyrosine

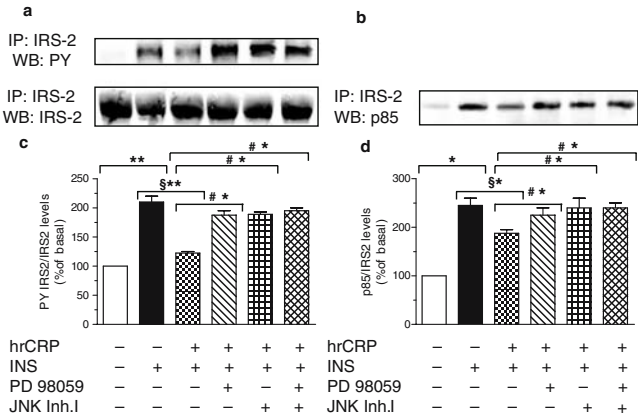

Fig. 4 Effect of human recombinant CRP (*hrCRP*) on insulin-stimulated tyrosine phosphorylation of IRS-2 and IRS-2/p85 association. IRS-2 phospho-tyrosine levels (**a**, **c**) and IRS-2/p85 association (**b**, **d**) induced by insulin (*INS*) stimulation (100 nmol/l, 10 min) in L6 myotubes incubated in the presence or absence of hrCRP (10 mg/l, 15 min) and pre-treated with or without PD98059 (50 μmol/l) or JNK inhibitor I (*JNK Inh.I*) (20 μmol/l) or a combination of both. Representative immunoblots of three experiments are shown. *Bars* represent the means ± SEM for three independent experiments. *$p<0.05$ and **$p<0.01$ for difference vs basal; §*$p<0.05$ and §**$p<0.01$ for difference vs insulin stimulation; #*$p<0.05$ for difference vs hrCRP treatment. *WB*, western blot; *IP*, immunoprecipitation; *PY*, phospho-tyrosine

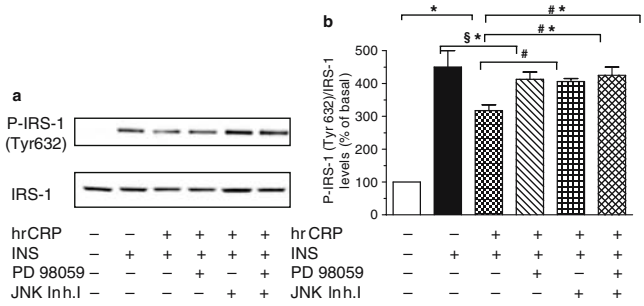

Fig. 5 Effect of human recombinant CRP (*hrCRP*) on insulin-stimulated phosphorylation (*P*) of IRS-1 at Tyr632. **a**, **b** IRS-1 phospho-tyrosine[632] levels induced by insulin (*INS*) stimulation (100 nmol/l, 10 min) in L6 myotubes incubated in the presence or absence of hrCRP (10 mg/l, 15 min) and pre-treated with or without PD98059 (50 μmol/l) or JNK inhibitor I (*JNK Inh.I*) (20 μmol/l) or a combination of both. Representative immunoblots (**a**) of four experiments are shown. *Bars* (**b**) represent the means ± SEM for four independent experiments. $*p<0.05$ for difference vs basal; $\S*p<0.05$ for difference vs insulin stimulation; $\#*p<0.05$ for difference vs hrCRP treatment

Effect of hrCRP on insulin-stimulated activation of Akt and GSK-3

PI-3K-dependent activation of the serine/threonine kinase Akt mediates several metabolic responses induced by insulin including stimulation of glucose transport, and translocation of GLUTs to the cell surface [31, 32]. As shown in Fig. 6, hrCRP treatment reduced insulin-stimulated Akt Ser[473] phosphorylation by 42% and Akt Thr[308] phosphorylation by 38% (Fig. 6a,b). Treatment with JNK inhibitor I or PD98059 partially reversed the inhibitory effects of hrCRP, but co-treatment with the two inhibitors did not completely reverse them. Consistent with these results, insulin-stimulated phosphorylation of GSK-3β, a substrate of Akt implicated in the regulation of glycogen synthesis through a mechanism involving its phosphorylation and inactivation, was decreased by 45% in L6 myotubes exposed to hrCRP as compared with control cells (Fig. 6c). The inhibitory effects of hrCRP were

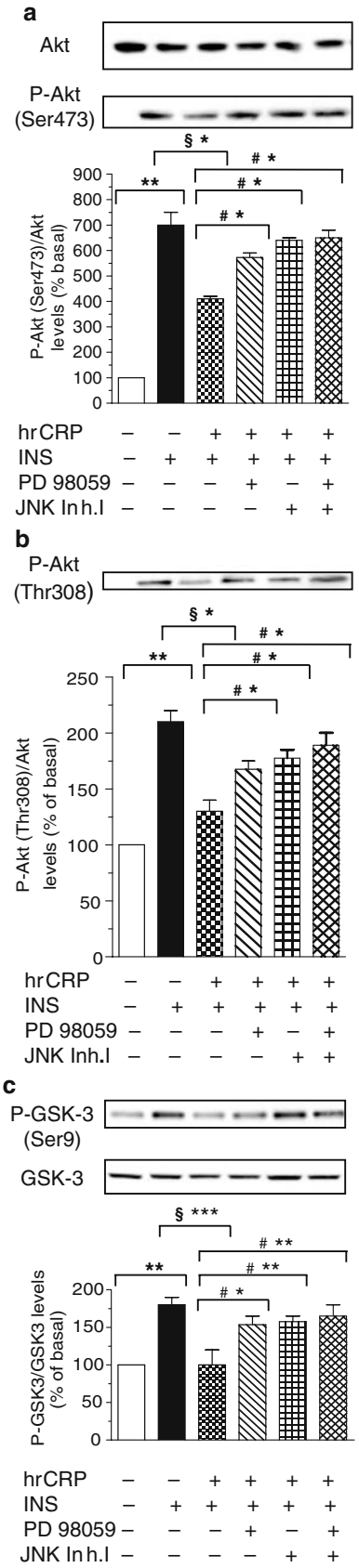

Fig. 6 Effect of human recombinant CRP (*hrCRP*) on insulin-stimulated phosphorylation of Akt and GSK-3. Akt Ser[473] (**a**), Akt (Thr[308]) (**b**) and GSK-3 Ser[9] (**c**) phosphorylation (*P*) induced by insulin (*INS*) stimulation (100 nmol/l, 15 min) in L6 myotubes incubated in the presence or absence of hrCRP (10 mg/l, 15 min) and pre-treated with or without PD98059 (50 μmol/l) or JNK inhibitor I (*JNK Inh.I*) (20 μmol/l) or a combination of both. Representative immunoblots of four experiments are shown. *Bars* represent the means ± SEM for four independent experiments. $**p<0.01$ for difference vs basal; $\S*p<0.05$ and $\S***p<0.001$ for difference vs insulin stimulation; $\#*p<0.05$ and $\#**p<0.01$ for difference vs hrCRP treatment

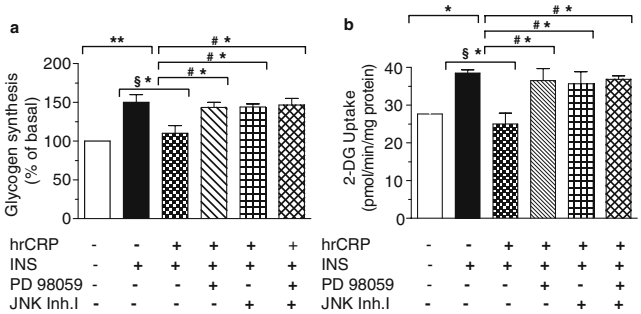

Fig. 7 Effect of human recombinant CRP (*hrCRP*) on insulin-stimulated glycogen synthesis and glucose transport. Glycogen synthesis (**a**) and glucose uptake (**b**) induced by insulin (*INS*) stimulation (100 nmol/l) in L6 myotubes incubated in the presence or absence of hrCRP (10 m g/l, 15 min) as described above. *Bars* represent the means ± SEM for four independent experiments. *$p<0.05$ and **$p<0.01$ for difference vs basal; §*$p<0.05$ for difference vs insulin stimulation; #*$p<0.05$ for difference vs hrCRP treatment. *JNK Inh.I*, JNK inhibitor I

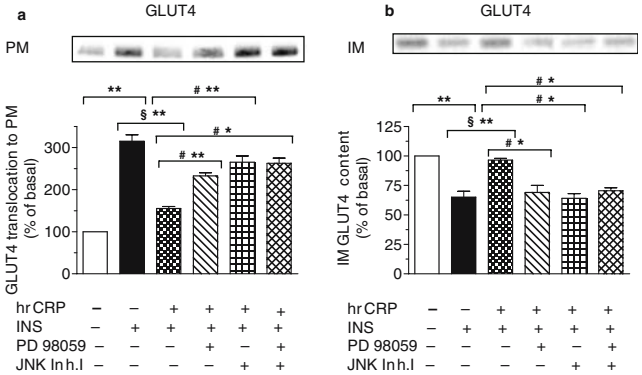

Fig. 8 Effect of human recombinant CRP (*hrCRP*) on insulin-stimulated GLUT4 translocation to plasma membrane. GLUT4 levels in plasma membrane (*PM*) (**a**) and intracellular membrane (*IM*) (**b**) induced by insulin (*INS*) stimulation (100 nmol/l) in L6 myotubes incubated in the presence or absence of hrCRP (10 mg/l, 15 min) as described above. *Bars* represent the means ± SEM for four independent experiments. ** $p<0.01$ for difference vs basal; §** $p<0.01$ for difference vs insulin stimulation; #* $p<0.05$ and #** $p<0.01$ for difference vs hrCRP treatment. *JNK Inh.I*, JNK inhibitor I

partially reversed by treatment with JNK inhibitor I or PD98059, whereas co-treatment with the two inhibitors did not completely reverse those inhibitory effects. Taken together, these results indicate that hrCRP impairs activation of the IRS-1/PI-3K/Akt/GSK-3 signalling pathway in response to insulin. Accordingly, insulin-stimulated glucose incorporation into glycogen was markedly impaired in L6 myotubes exposed to hrCRP (Fig. 7a). Treatment with either JNK inhibitor I or PD98059 partially reversed the inhibitory effects of hrCRP on insulin-stimulated glycogen synthesis. Co-treatment with the two inhibitors did not completely reverse the inhibitory effects of hrCRP.

Effect of hrCRP on insulin-stimulated glucose transport and GLUT4 translocation to plasma membrane

As shown in Fig. 7b, insulin stimulated glucose uptake by 1.4-fold over basal in control L6 myotubes, an extent similar to that observed in previous studies [33, 34]. Exposure of cells to hrCRP resulted in a marked inhibition of insulin-stimulated glucose uptake. Treatment with either JNK inhibitor I or PD98059 partially reversed the inhibitory effects of hrCRP on insulin-stimulated glucose uptake, but co-treatment with the two inhibitors did not completely reverse these inhibitory effects. Insulin-stimulated glucose uptake in myocytes is mediated by translocation of insulin-sensitive GLUT4 from the intracellular compartment to the plasma membrane and its subsequent activation. In control L6 myotubes, the amount of GLUT4 translocated to the plasma membrane upon insulin stimulation was increased by threefold as compared with the myotubes not treated with insulin, with a corresponding reduction in GLUT4 levels in the intracellular membranes (Fig. 8a,b). Exposure of cells to hrCRP resulted in a 50% reduction in GLUT4 translocation in response to insulin with a corresponding increase in GLUT4 levels in the intracellular membranes. The inhibitory effects of hrCRP were partially reversed by treatment with the cell permeable JNK inhibitor I and PD98059, whereas co-treatment with the two inhibitors did not completely reverse the inhibitory effects of hrCRP.

Discussion

Acute phase CRP levels are associated with insulin resistance, glucose disorders, features of the metabolic syndrome and also possibly predict the risk of type 2 diabetes mellitus [3–8]. CRP is a member of the highly conserved pentraxin family and is produced mainly by hepatocytes in response to cytokines such as IL-6, IL-1, and TNF-α. CRP binds to the IgG receptors FC γ receptor (FcγR)I and FcγRII on the surface of cells [35, 36] thus inducing their tyrosine phosphorylation, recruitment of spleen tyrosine kinase, and activation of Rho/Rho-kinase and MAPKs including MEK and ERK1/2, JNK, and p38MAPK [37]. The specific signalling pathway leading to upstream activation of JNK and MAPKs upon binding of CRP to FcγRI and FcγRII receptors is still undefined. Because the molecular mechanism(s) by which CRP affects insulin action in target tissues is still unknown, we investigated whether hrCRP induces alterations in insulin signalling in myocytes, thus contributing to insulin resistance. We provide evidence that exposure of L6 myotubes to hrCRP resulted in inhibition of insulin-stimulated glucose transport and translocation of insulin-sensitive

GLUT4 to the plasma membrane. Of note, the increase in insulin-stimulated glucose transport is lower than the increase in translocation of GLUT4 to the plasma membrane. Possible explanations for this discrepancy include: (1) the contribution of GLUT1 to glucose transport; (2) contamination of plasma membrane preparations with other membranes enriched in GLUT4; or (3) differences in the sensitivity between assays. The impairment of insulin-stimulated glucose transport was associated with altered tyrosine phosphorylation of both IRS-1 and IRS-2 and their binding to p85 subunit of PI-3K, resulting in defective activation of Akt. The impaired insulin-induced activation of the IRS-1/2/PI-3K/Akt pathway in L6 myotubes exposed to hrCRP was not due to alterations in IRS-1, IRS-2, p85 subunit or Akt protein levels, but rather it was associated with reduced phosphorylation of IRS-1 at Tyr632, which plays a major role in the binding of IRS-1 with the SH2 domains of p85 subunit of PI-3K. Accordingly, it is also possible that hrCRP affects phosphorylation of IRS-2 at important functional tyrosine residues, but we were unable to test this possibility due to the lack of available phospho-site-specific anti-IRS-2 antibodies. This issue will require further study in light of the important role of IRS-2 in conveying the insulin signal to glucose transporters and the glycogen synthetic machinery in the L6 myotubes [38].

GSK-3 has been implicated in the regulation of glycogen synthesis in response to insulin through a mechanism involving its phosphorylation and inactivation by Akt [31, 32]. Consistent with this idea, we found that exposure of L6 myotubes to hrCRP caused a significant impairment in insulin-induced phosphorylation and inactivation of GSK-3, which was associated with a decrease in insulin-stimulated glucose incorporation into glycogen. However, we cannot exclude the possibility that the impairment of insulin-stimulated glycogen synthesis provoked by hrCRP is secondary to the impairment of insulin-stimulated glucose transport since both defects were of the same order of magnitude.

Accumulating evidence indicates that serine phosphorylation of IRS-1 can affect its phosphorylation on tyrosine residues, hence impairing downstream events of insulin signalling. Several serine residues of IRS-1 have been reported to be phosphorylated by proinflammatory cytokines through activation of different kinases including JNK, which has been shown to phosphorylate IRS-1 at Ser^{307}, and ERK1/2, which has been shown to phosphorylate IRS-1 at Ser^{612}. Recent reports have pointed out the importance of IRS-1 phosphorylation at Ser^{307} and Ser^{612} in the negative regulation of insulin signalling [9–14]. Ser^{307} is located close to the phosphotyrosine-binding domain of IRS-1 and its phosphorylation has been demonstrated to inhibit insulin-stimulated tyrosine phosphorylation of IRS-1 and subsequent activation of PI-3K [11]. Ser^{612} is located in close vicinity to the Tyr residues involved in the binding of PI-3K to IRS-1, and phosphorylation of Ser^{612} by ERK1/2 was shown to inhibit insulin-induced IRS-1 tyrosine phosphorylation and its subsequent association with PI-3K [9, 10]. JNK1 plays an important role in the modulation of insulin action, and is a major component of the pathogenesis of obesity, fatty liver disease, and type 2 diabetes [13, 14]. JNK activity is increased in obese rodents and human adipose tissue. The cell-permeable JNK-inhibitory peptide used in the present studies was shown to improve glucose tolerance and insulin sensitivity when administered to diabetic mice and mice with diet-induced insulin resistance [39]. Deletion of the gene encoding JNK1 protects mice against insulin resistance and metabolic deterioration induced by high-fat diet, and in obese mice, deletion of this gene results in reversal of the obesity-induced increase in JNK activity and IRS-1 Ser^{307} phosphorylation [13, 14]. Basal ERK1/2 phosphorylation is increased in both primary cultured myocytes and adipocytes isolated from patients with type 2 diabetes [40–43]. Mice lacking ERK1 have decreased adiposity and are protected from obesity and insulin resistance induced by high-fat diet [44]. We found that exposure of L6 myotubes to hrCRP resulted in increased phosphorylation of either JNK or ERK1/2 that was associated with a concomitant increase in IRS-1 phosphorylation at Ser^{307} and Ser^{612}, respectively. We further demonstrated the cause-effect relationship between these two events by using inhibitors of JNK or MEK, which partially reversed the stimulatory effects of hrCRP on Ser^{307} and Ser^{612} IRS-1 phosphorylation, respectively. In addition, we observed that inhibition of either JNK or MEK1 partially reversed the negative effects of hrCRP on insulin-stimulated translocation of GLUT4 to the plasma membrane. It is noteworthy that inhibition of JNK did not affect Ser^{612} phosphorylation and inhibition of ERK1/2 did not affect Ser^{307} phosphorylation induced by hrCRP, thus indicating that the two kinases contribute independently to serine phosphorylation of IRS-1, and, thereby, to cellular insulin resistance. In addition, inhibition of both JNK and MEK1 activity did not completely reverse the negative effect of hrCRP on insulin signalling and glucose metabolism, suggesting incomplete inhibition of JNK and MEK1 by their corresponding inhibitors or, alternatively, that other serine kinases may be involved in impairment of insulin action induced by hrCRP. Among these, mammalian target of rapamycin is a strong candidate whose role needs to be addressed in future studies [45].

Activation of ERKs is important for cell growth, and therefore hrCRP, by stimulating ERK1/2, may induce proliferative effects in L6 myoblasts [28]. Although we did not directly measure cell proliferation in our study, it is

unlikely that exposure of differentiated L6 myocytes (12–14 days after confluence) to CRP for 15 min would induce proliferative effects in L6 myoblasts even in a mixed blast/tube culture.

The cellular effects of hrCRP remain controversial and the issue of whether the observed cellular responses are direct effects of hrCRP itself or caused by contaminants or additives in the hrCRP solution has been a subject of debate. Indeed, it has been reported that most of the responses were induced by the contaminants lipopolysaccharide or sodium azide [45–47]. On the other hand, in vivo infusion of pure CRP, free from lipopolysaccharide and sodium azide, into humans was reported to activate inflammation and coagulation. In the present study, contaminating lipopolysaccharide was almost completely removed using Detoxigel column, by which the contaminating lipopolysaccharide in CRP solution was reduced to <0.125 endotoxin units per milliliter. Sodium azide was removed from the preparation by extensive dialysis. Furthermore, in control experiments in which insulin was diluted in buffer containing the same NaN_3 concentration (0.05%) found in the commercial hrCRP preparation, we found that insulin-stimulated Akt phosphorylation was not affected by the presence of NaN_3 (data not shown). All these results indicate that hrCRP itself has biological activity and can induce insulin resistance in myocytes.

The concentration of hrCRP (10 μg/ml) used in our study is seen in subjects with obesity, type 2 diabetes and cardiovascular disease [3–8], thus suggesting that the present findings may be clinically relevant. Clinical studies have shown that insulin sensitisers such as metformin and thiazolidinediones reduce CRP levels in subjects with insulin resistance, IGT or type 2 diabetes [48–50]. The present novel findings showing that hrCRP inhibits insulin signalling and action in myocyte-specific pathways may help to explain in part the mechanisms by which the agents named above improve insulin sensitivity and prevent the development of type 2 diabetes, which are both incompletely explained by the hypoglycaemic action of metformin and thiazolidinediones.

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