Interleukin-6 Induces Cellular Insulin Resistance in Hepatocytes

Joseph J. Senn,[1] Peter J. Klover,[2] Irena A. Nowak,[2] and Robert A. Mooney[3]

Interleukin (IL)-6 is one of several proinflammatory cytokines that have been associated with insulin resistance and type 2 diabetes. A two- to threefold elevation of circulating IL-6 has been observed in these conditions. Nonetheless, little evidence supports a direct role for IL-6 in mediating insulin resistance. Here, we present data that IL-6 can inhibit insulin receptor (IR) signal transduction and insulin action in both primary mouse hepatocytes and the human hepatocarcinoma cell line, HepG2. This inhibition depends on duration of IL-6 exposure, with a maximum effect at 1–1.5 h of pretreatment with IL-6 in both HepG2 cells and primary hepatocytes. The IL-6 effect is characterized by a decreased tyrosine phosphorylation of IR substrate (IRS)-1 and decreased association of the p85 subunit of phosphatidylinositol 3-kinase with IRS-1 in response to physiologic insulin levels. In addition, insulin-dependent activation of Akt, important in mediating insulin's downstream metabolic actions, is markedly inhibited by IL-6 treatment. Finally, a 1.5-h preincubation of primary hepatocytes with IL-6 inhibits insulin-induced glycogen synthesis by 75%. These data suggest that IL-6 plays a direct role in insulin resistance at the cellular level in both primary hepatocytes and HepG2 cell lines and may contribute to insulin resistance and type 2 diabetes. ***Diabetes*** **51:3391–3399, 2002**

Type 2 diabetes and the insulin resistance syndrome have recently been hypothesized to represent an acute phase response (1,2). This is in part due to the strong correlation between elevations in the local and circulating proinflammatory cytokines (tumor necrosis factor [TNF]-α, interleukin [IL]-1, interferon-γ, and IL-6) and insulin resistance (1,3–7). It is well known that these cytokines are elevated in cases of cancer, cachexia, and severe infection, and insulin resistance frequently accompanies these clinical conditions (8). Importantly, the proinflammatory cytokines have also been shown to be elevated in type 2 diabetes. Of these proinflammatory cytokines, IL-6 has been shown to have the strongest correlation with insulin resistance and type 2 diabetes (5,9,10). Plasma levels of IL-6 are two- to threefold higher in patients with obesity and type 2 diabetes than in lean control subjects (5). This elevation is highly related to increased blood glucose, decreased glucose tolerance, and decreased insulin sensitivity (5,9).

Genetic studies in individuals with insulin resistance have found a high degree of correlation between insulin sensitivity and a particular allele of the IL-6 gene that is associated with higher expression of IL-6 (11). Individuals with this G/G allele of the IL-6 gene have a polymorphism that leads to increased IL-6, increased circulating insulin, and higher blood glucose than those individuals who have the C/C allele without the polymorphism (11).

Several interesting observations provide further evidence that IL-6 plays an important role in insulin resistance. The predominant site of IL-6 production has, until recently, been thought to be macrophages and peripheral mononuclear cells. However, recent evidence suggests that both the adipose and muscle tissue are important sites of IL-6 production. Adipose tissue has been shown to produce 10–35% of IL-6 in a resting individual, and this production increases with increased adiposity (12). This suggests that adipose tissue is a source of the increased circulating IL-6 observed in obesity. Exercise also increases circulating IL-6, occuring through increased production by the muscle (13,14). The liver may be a critical target of adipose- and muscle-derived IL-6. IL-6 has been shown to increase blood glucose through elevated hepatic glucose output (15,16). Increased IL-6 levels have been linked to inhibition of hepatic glycogen synthase, activation of glycogen phosphorylase and lipolysis, and increased triglyceride production (16,17). As a result of these observations, it has been hypothesized that IL-6 plays a role as a glucoregulatory hormone (18).

While IL-6 appears to play an important role in mediating insulin resistance in the liver, no mechanism has been proposed to explain such a role. The present study examines the effect of IL-6 on insulin signal transduction in liver cells and demonstrates that IL-6 can cause cellular insulin resistance in both the HepG2 cell line and primary hepatocytes. IL-6 is shown to exert an inhibitory effect on both early insulin receptor (IR) signal transduction and downstream insulin action, specifically glycogen synthesis. These data are the first complete characterization of IL-6–induced insulin resistance at the cellular level.

From the [1]Graduate Program in Pharmacology and Physiology, University of Rochester School of Medicine and Dentistry, Rochester, New York; the [2]Graduate Program in Biochemistry, University of Rochester School of Medicine and Dentistry, Rochester, New York; and the [3]Department of Pathology and Laboratory Medicine, University of Rochester School of Medicine and Dentistry, Rochester, New York.

Address correspondence and reprint requests to Robert A. Mooney, Department of Pathology and Laboratory Medicine, University of Rochester School of Medicine and Dentistry, 601 Elmwood Ave., Rochester, NY 14642. E-mail: robert_mooney@urmc.rochester.edu.

Received for publication 28 March 2002 and accepted in revised form 9 September 2002.

DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; IL, interleukin; IκB, NF-κB inhibitor; IR, insulin receptor; IRS, IR substrate; NF, nuclear factor; PI, phosphatidlyinositol; SOCS, suppressors of cytokine signaling; TNF, tumor necrosis factor.

RESEARCH DESIGN AND METHODS

Reagents and materials. Porcine insulin and recombinant human IL-6 were purchased from Sigma (St. Louis, MO) and R&D Systems (Minneapolis, MN), respectively. Cell culture reagents such as Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Life Technologies (Burlington, ON, Canada). Anti–IR antibody (α-IR-1) was a gift from Dr. S. Jacobs (Wellcome Research Laboratories, Research Triangle Park, NC). The phospho-Akt (Ser-473) antibody was purchased from Cell Signaling Technology (Beverly, MA). Antiphosphotyrosine antibody (4G10), polyclonal rabbit anti-p85, and anti–IR substrate (IRS)-1 were from Upstate Biotechnology (Lake Placid, NY). [C^{14}]glucose was purchased from NEN (Boston, MA). Male C57/BL6 mice were from Charles River Laboratories (Wilmington, MA).
Cell culture. HepG2 human hepatocarcinoma cells were obtained from American Type Tissue Culture Collection (Rockville, MD). Cells were maintained at 37°C in a humidified 5% CO_2 environment in DMEM/high glucose supplemented with 10% FBS.
IL-6 treatment of HepG2 cells. HepG2 cells were grown to confluence in 10-cm dishes before overnight treatment in serum-free DMEM/high glucose containing 1% BSA. Cells were then treated with recombinant human IL-6 at 20 ng/ml for 0–8 h. At selected time points, cells were stimulated for 3 min with insulin at the indicated concentrations. After two washes with cold PBS, cells were lysed in lysis buffer A containing 50 mmol/l Tris, pH 7.4, 140 mmol/l NaCl, 1% Triton X-100, 50 mmol/l NaF, 10 mmol/l tetrasodium pyrophosphate, 25 mmol/l benzamidine, protease inhibitor mixture (Calbiochem), 2.5 μmol/l pervanadate, 2 mmol/l phenylmethylsulfonyl fluoride, and 10% glycerol. Lysates were passed 10 times through an 18-gauge needle, centrifuged at 10,000*g* for 10 min, and then adjusted to equal amounts of protein as determined by the Bradford method (19). IRs were immunoprecipitated using an anti–human IR antibody (α-IR-1) bound to protein G-Sepharose. Immune complexes were washed four times with wash buffer (1% Triton X-100, 100 mmol/l Tris-HCl, pH 7.4, and 150 mmol/l NaCl) and separated by SDS-PAGE on 5–20% gradient gels (20). In experiments in which IRS-1 was immunoprecipitated, the same protocol was followed, except protein A Sepharose was used. After separation by SDS-PAGE, the samples were subjected to Western blot analysis using appropriate antibodies at 4°C overnight. To determine the amount of phosphorylated Akt, lysates from HepG2 cells, treated as previously described, were separated by SDS-PAGE and subjected to Western blot analysis using anti–phospho Akt antibody. Blots were visualized by enhanced chemiluminescence (Amersham Pharmacia). Quantitation was performed using a ChemiImager (Alpha Innotech, San Leandro, CA).
Mouse hepatocyte isolation. Mouse hepatocytes were prepared for primary culture by nonrecirculating collagenase perfusion, as adapted from Klaunig et al. (21). In brief, the liver was initially perfused with Ca^{2+}-free Hank's buffer and then dissociated with collagenase type IV (1 mg/ml; Sigma) in Hank's buffer plus 5 mmol/l $CaCl_2$. Isolated hepatocytes were resuspended in Hank's buffer containing 1.2 mmol/l $CaCl_2$ and 0.6 mmol/l $MgSO_4$. Cells were then filtered through 90-micron nylon mesh, counted, and tested for viability using trypan blue exclusion.
Analysis of glycogen synthesis. Primary mouse hepatocytes were seeded onto collagen-coated 6-well tissue culture plates (Corning, Corning, NY) at 0.5×10^6 cells/well in serum-free Waymouth's media supplemented with 100 units/ml penicillin/streptomycin and 10 nmol/l dexamethasone. A total of three wells were seeded for each condition. The cells were incubated overnight at 37°C in a humidified atmosphere containing 5% CO_2. After 12 h, cells were pretreated for 90 min with IL-6 (20 ng/ml) in serum-free low-glucose DMEM. Cells were then treated with 1.5 μCi [^{14}C]D-glucose (300 mCi/mmol/l) and a final concentration of either 0, 1, or 100 nmol/l insulin. After 3 h, the reaction was terminated by washing the cells three times with ice-cold PBS. Cells were solubilized with 0.3 ml 10N KOH. Cold glycogen carrier (4 mg) was added to the lysates, and samples were boiled for 30 min. Glycogen was precipitated with two volumes ethanol overnight on ice. Precipitated glycogen was centrifuged at 10,000*g* for 10 min. Pellets were washed once with 66% ethanol, resuspended in 0.5 ml water, and counted by scintillation counting. All procedures involving laboratory animals were approved by the University of Rochester Committee on Animal Resources.
Statistics. Data were analyzed using a Student's *t* test (Stataquest) to compare results between time points. $P < 0.05$ was considered statistically significant.

RESULTS

Time-dependent effects of IL-6 on IR signal transduction in HepG2 cells. To examine the effect of IL-6 on IR signal transduction, HepG2 cells were serum starved overnight and then exposed to IL-6 (20 ng/ml) for 0–8 h. This concentration of IL-6 has been shown by Paysant et.al. (22) to produce a near-maximum effect (fibrinogen production) in HepG2 cells. At the indicated time points after initiation of IL-6 exposure, the HepG2 cells were subjected to a 3-min treatment with 0.5 or 100 nmol/l insulin. The effect of IL-6 alone was also assessed. Immunoprecipitates of the IR (Fig. 1) were subjected to Western blot analysis using an antiphosphotyrosine antibody to determine IR autophosphorylation. As expected, insulin increased autophosphorylation of the IR at both the physiologic and supraphysiologic concentrations, but IL-6 had no effect on IR autophosphorylation in the absence or presence of insulin at the time points examined. Similarly, IL-6 had no effect on tyrosine phosphorylation of IRS-1 in the absence of insulin or in response to 100 nmol/l insulin (Fig. 2). However, IL-6 did inhibit tyrosine phosphorylation of IRS-1, which was initiated by the physiologic insulin concentration (0.5 nmol/l). Tyrosine phosphorylation of IRS-1 decreased to 43 ± 10% of control levels at 1 h after IL-6 treatment. A partial recovery was observed at 2 h, followed by a return to suppression of tyrosine phosphorylation (55 ± 20% of control levels) at 4 h. Such a transient loss of the IL-6 response in HepG2 cells has been reported by Wang et.al. (23) when examining c-*jun* protein expression in response to IL-6 at 10 ng/ml.

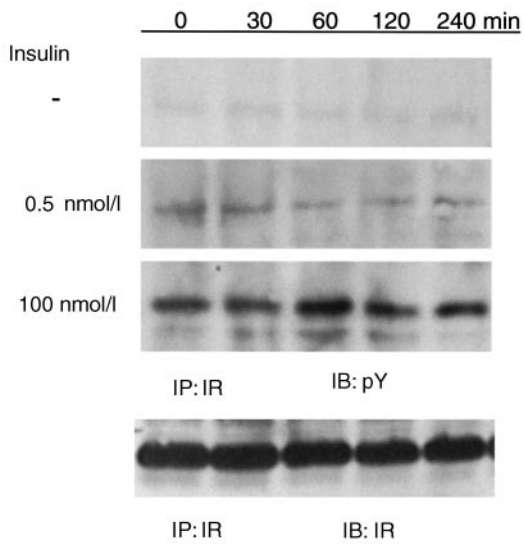

FIG. 1. Time-dependent effects of IL-6 on tyrosine phosphorylation of the IR. Confluent HepG2 cells were serum starved overnight before treatment with 20 ng/ml IL-6 for 0–4 h. At the indicated times, cells were treated with insulin (0.5 and 100 nmol/l) or without insulin for 3 min, harvested, and lysates-protein normalized. IR was immunoprecipitated, separated by SDS-PAGE, and subjected to Western blot analysis with an antiphosphotyrosine antibody (4G10). A comparable IR immunoprecipitate from 0.5 nmol/l insulin-treated cells was probed for IR mass. Results were visualized by enhanced chemiluminescence (Amersham Pharmacia). Data are representative of three independent experiments.

Association of the p85 subunit of phosphatidylinositol (PI)-3 kinase with IRS-1 is a rapid and essential response to insulin-dependent tyrosine phosphorylation of IRS-1. Both 0.5 and 100 nmol/l insulin increased this association, as detected by Western blot analysis of IRS-1 immunoprecipitates (Fig. 3). Once again, IL-6 suppressed this insulin-dependent effect only in cells exposed to the physiological insulin concentration. A small suppression was seen at 120 min with 100 nmol/l insulin but was not statistically significant. As with IRS-1 phosphorylation, the inhibition

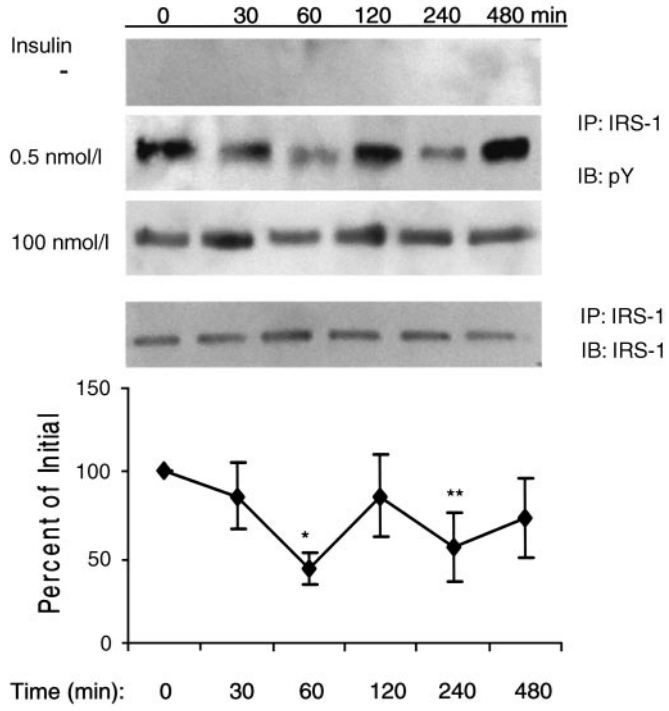

FIG. 2. Time-dependent effects of IL-6 on tyrosine phosphorylation of IRS-1. Confluent HepG2 cells were treated as in Fig. 1. IRS-1 was immunoprecipitated, separated by SDS-PAGE, and subjected to Western blot analysis with an antiphosphotyrosine antibody (Upstate Biological). A comparable IRS-1 immunoprecipitate from 0.5 nmol/l insulin-treated cells was probed for IRS-1 mass. Results were visualized by enhanced chemiluminescence (Amersham Pharmacia). Representative blots of three independent experiments are shown. The 0.5 nmol/l blot required longer exposure times to obtain comparable signal intensity to the 100 nmol/l insulin blot. The data for 0.5 nmol/l insulin from the three experiments were quantitated by densitometry and expressed as percent of initial ± SD (no IL-6, time zero). *$P < 0.01$ and **$P < 0.05$ as compared with control (no IL-6, time zero).

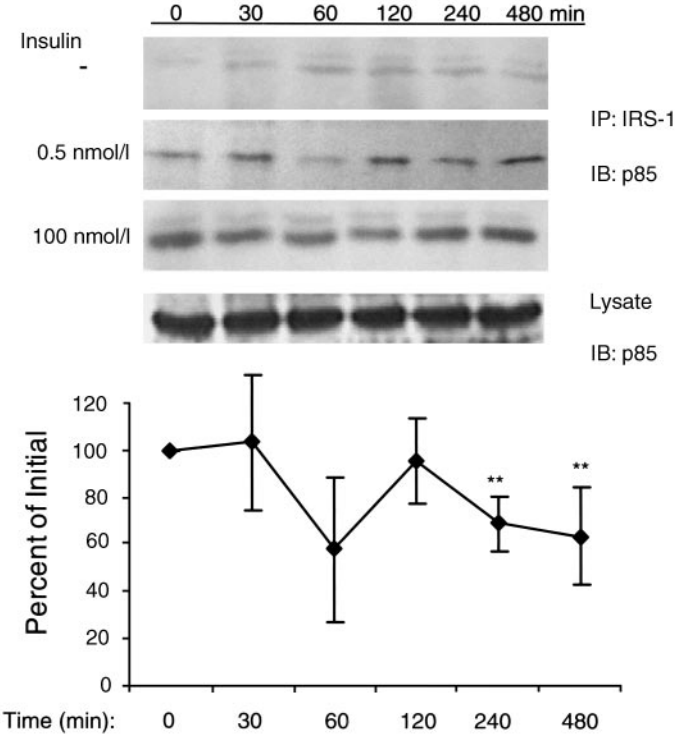

FIG. 3. Time-dependent effects of IL-6 on association of the p85 subunit of PI 3-kinase with IRS-1. Confluent HepG2 cells were treated as in Fig. 1. IRS-1 was immunoprecipitated, separated by SDS-PAGE, and subjected to Western blot analysis with an anti-p85 antibody (Upstate Biological). A comparable lysate from 0.5 nmol/l insulin-treated cells was probed for p85 mass. Results were visualized by enhanced chemiluminescence (Amersham Pharmacia). Representative blots of three independent experiments are shown. The data for 0.5 nmol/l insulin from the three experiments were quantitated by densitometry and expressed as percent of initial ± SD (no IL-6, time zero). **$P < 0.05$ as compared with control (no IL-6, time zero).

of p85 association was maximum at 60 min, with a decrease to 58 ± 31% of controls. After a loss of the IL-6–dependent inhibition of IRS-1/p85 association at 2 h, association again was inhibited at 4 and 8 h. IL-6 alone was also observed to increase association of the p85 subunit with IRS-1, although the magnitude of the effect was substantially less than that of the physiologic insulin concentration.

Finally, lysates from insulin- and IL-6–treated cells were assayed for the presence of activated Akt using Western blot analysis (Fig. 4). Insulin at both 0.5 and 100 nmol/l activated Akt, as assessed by detection of Akt phosphorylation at ser473. In HepG2 cells exposed to 0.5 nmol/l insulin, IL-6 again produced an inhibition, with a statistically significant suppression of Akt activation to 52 ± 12% of controls at 1 h and comparable inhibition at 4 and 8 h. In agreement with data in Figs. 2 and 3, a transient diminution of this inhibition was observed at the 2-h time point. In contrast to the other parameters of IR signaling that were unaffected by IL-6 when activated by 100 nmol/l insulin, activation of Akt in response to 100 nmol/l insulin was suppressed by IL-6. This inhibition was more gradual, beginning at 60 min but progressing through 8 h. Finally, IL-6 alone had no effect on the activation state of Akt, despite its modest increase in IRS-1/p85 association. In some experiments, basal Akt activity was detectable (Fig. 8), but this was not affected by IL-6.

Effect of IL-6 on dose-dependent insulin signaling. The above data suggested that IL-6–dependent inhibition of IR signaling was dependent on the insulin concentration. To more thoroughly characterize the effect of IL-6 on dose-dependent insulin signaling, HepG2 cells were pretreated with IL-6 (20 ng/ml) and then stimulated with various insulin concentrations. A 90-min time point was chosen as a compromise between the time to maximum IL-6–dependent inhibition of Akt with physiologic insulin (60 min) and the apparently longer time course with supraphysiologic concentrations (≥2 h). IL-6 had no effect on dose-dependent IR autophosphorylation under these conditions (data not shown), confirming data in Fig. 1. However, tyrosine phosphorylation of IRS-1 exhibited decreased sensitivity to insulin as a result of IL-6 exposure—it was undetectable until 10 nmol/l insulin (Fig. 5). This is somewhat different from the results in Fig. 2, which show that IL-6 did not totally suppress IRS-1 phosphorylation induced by 0.5 nmol/l insulin. These results suggest that the true nadir in IRS-1 phosphorylation is at 90 min (Fig. 5), not 60 min (Fig. 2). Control cells exhibited tyrosine phosphorylation of IRS-1 in response to 0.3 nmol/l insulin and threefold maximum phosphorylation at 10 nmol/l. Although basal phosphorylation of IRS-1 was sometimes detected, it did not appear to be altered by the presence of IL-6.

No effect of IL-6 on tyrosine phosphorylation of the IR or

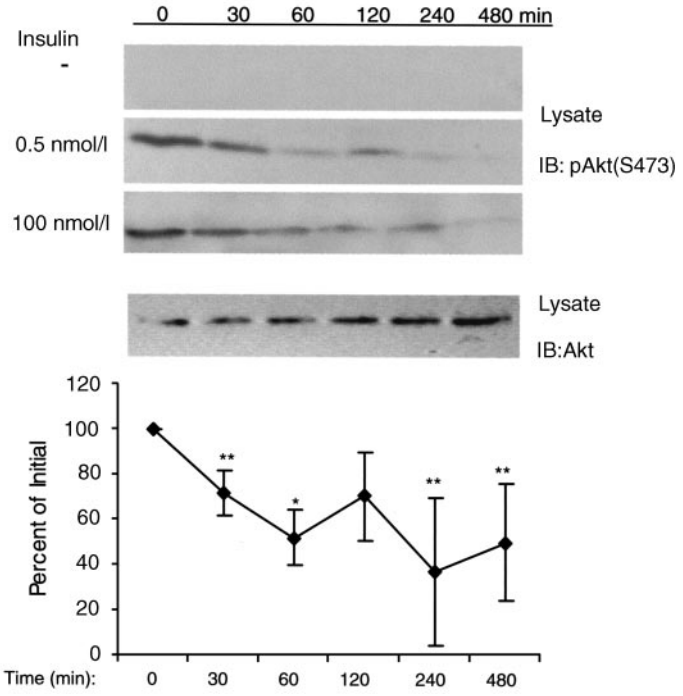

FIG. 4. Time-dependent effects of IL-6 on insulin-dependent activation of Akt. Confluent HepG2 cells were treated as in Fig. 1. Cell lysates were separated by SDS-PAGE and subjected to Western blot analysis with an anti-phosphoAkt (ser473) antibody (Cell Signaling Technology) or an anti-Akt antibody (Cell Signaling Technology). The latter was used to probe lysates from 0.5 nmol/l insulin-treated cells to determine Akt mass. Results were visualized by enhanced chemiluminescence (Amersham Pharmacia). Representative blots of three independent experiments are shown. The data for 0.5 nmol/l insulin from the three experiments were quantitated by densitometry and expressed as percent of initial ± SD (no IL-6, time zero). **$P < 0.05$ as compared with control (no IL-6, time zero).

IRS-1 in the absence of insulin was detected. However, IL-6 did affect basal p85 association with IRS-1 (Fig. 6). This was also observed in Fig. 3. The data in Fig. 6 show that cells exposed to IL-6 without insulin exhibit a 1.7-fold increase in IRS-1/p85 complexes when compared with controls. The association of p85 with IRS-1 also increased in response to insulin in both IL-6–treated and control cells. The amount of IRS-1/p85 complexes was comparable until 100 nmol/l insulin. At this concentration, control cells showed an additional response to insulin, whereas IL-6–treated cells did not. When IRS-1/p85 complex formation in IL-6–treated and control cells was normalized to the individual insulin-free basal levels (Fig. 6 inset), insulin-dependent p85 association was clearly inhibited by at least 50% at all concentrations. The apparent increased sensitivity to insulin of p85/IRS-1 association versus IRS-1 tyrosine phosphorylation in the presence of IL-6 (Fig. 6 versus Fig. 5) is most likely due to the more sensitive Western blot method for detection of p85 at these low insulin concentrations.

Finally, insulin increased phosphorylation of Akt at 0.3 nmol/l in untreated cells and reached a maximum of sixfold at 10 nmol/l. Cells pretreated with IL-6 exhibited a complete loss of Akt activation in response to all insulin concentrations (Fig. 7). These results again suggest that a maximum effect of IL-6 is attained at 90 min. This is the optimum time point for all insulin concentrations under the conditions used in this study. As with IRS-1 tyrosine phosphorylation, basal activation of Akt was sometimes observed but not affected by IL-6 treatment. These data suggest that IL-6 induces a decrease in both the sensitivity and maximum effect of insulin on p85 association with IRS-1 and activation of Akt in HepG2 cells.

IL-6 inhibits insulin signaling in primary hepatocytes. While HepG2 cells are a useful model to investigate aspects of hepatic insulin action, primary hepatocytes remain a more physiologic model. The objective in the following experiments was to confirm that IL-6 inhibits IR signal transduction as well as inhibits glycogen synthesis, a metabolic end point of insulin action. Primary hepatocytes were isolated from mice and plated on collagen-coated dishes overnight in serum-free media. Paysant et al. (22) have shown that IL-6 at 20 ng/ml elicits a near-maximum response in HepG2 cells. To examine IL-6 sensitivity in primary hepatocytes, the concentration-dependent effect of IL-6 on STAT-3 tyrosine phosphorylation was examined. As shown in Fig. 8*A*, STAT-3 phosphorylation increased in response to 20 ng/ml IL-6 and showed further response at 60 ng/ml. These data indicated that the sensitivity to IL-6 was similar in HepG2 cells and primary hepatocytes. Therefore, 20 ng/ml IL-6 was used in the following experiments with primary hepatocytes to examine the effect of IL-6 on IR signaling.

Akt activation in response to a series of insulin concentrations was markedly inhibited by IL-6 (Fig. 8*B*). This inhibition was not as profound as the inhibition observed in HepG2 cells, but clearly demonstrated that IL-6 can inhibit IR signaling in primary hepatocytes. As shown in Fig. 8*C*, IL-6 inhibited activation of glycogen synthesis in response to physiologic insulin concentrations. In control cells, 1 nmol/l insulin increased glycogen synthesis by ~50%. IL-6 inhibited this increase in glycogen synthesis by nearly 75%. The level of glycogen synthesis at 1 nmol/l insulin with an IL-6 treatment was no greater than IL-6 alone. A supraphysiological insulin concentration (100 nmol/l) overcame the IL-6–dependent inhibition of glycogen synthesis. The discordance between the ability of IL-6 to inhibit Akt activation in response to 100 nmol/l insulin, and the inability of IL-6 to inhibit glycogen synthesis at this insulin concentration has several possible explanations. First, the level of Akt activity that is reached with 100 nmol/l insulin, even in the presence of IL-6, may be sufficient to activate glycogen synthesis. Second, the glycogen synthesis assay requires exposure to IL-6 for 90 min followed by insulin for 3 h. Thus, the acute activation state of Akt after 3 min of insulin treatment may not reflect time-averaged glycogen synthesis over 3 h. Third, supraphysiologic insulin concentrations may be activating glycogen synthase through an IL-6–insensitive pathway such as the insulin-responsive serine/threonine phosphatase PP1. Finally, phosphoAkt (Ser473) may not reflect the potential of insulin to activate glycogen synthesis. Nonetheless, these data with primary hepatocytes support our results in HepG2 cells and provide evidence that IL-6 alters a major metabolic end point of insulin action in the liver.

DISCUSSION

Proinflammatory cytokines are thought to contribute to insulin resistance in both humans and animals (1–6).

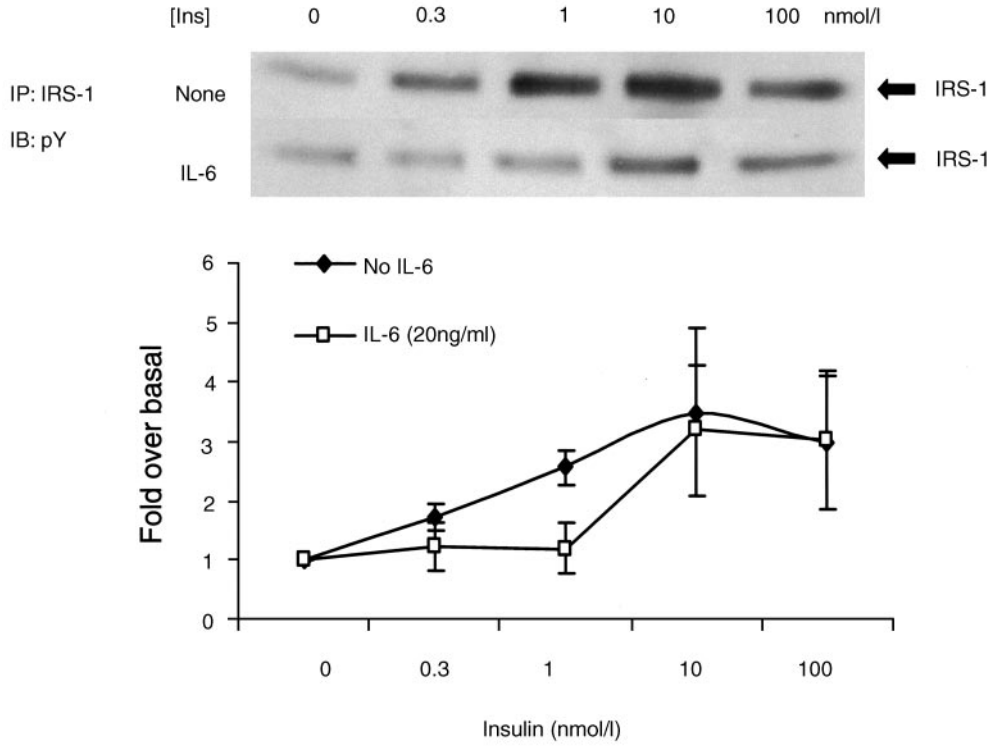

FIG. 5. Effect of IL-6 on sensitivity of HepG2 cells to insulin-induced IRS-1 tyrosine phosphorylation. Confluent HepG2 cells were serum starved overnight before pretreatment for 90 min with or without IL-6 (20 ng/ml) and subsequent treatment with insulin at the indicated concentrations for 3 min. IRS-1 was immunoprecipitated from lysates, separated by SDS-PAGE, and subjected to Western blot analysis with antiphosphotyrosine antibody. Results of four independent experiments were quantitated by densitometry and expressed as the fold increase over IL-6–free basal ± SD. A representative Western blot is shown.

However, the molecular mechanism of cytokine-induced insulin resistance is not well characterized. The present study clearly demonstrates that IL-6 affects both the proximal and distal events in hepatic IR signal transduction. IL-6–induced inhibition was not an immediate event, but was observed after an exposure of 1–2 h, perhaps suggesting that protein synthesis is required. Work is ongoing to address the mechanism of IL-6–mediated inhibition. Insulin signal transduction was not inhibited at the level of IR autophosphorylation. However, insulin-dependent tyrosine phosphorylation of IRS-1, association of the p85 subunit of PI 3-kinase with IRS-1, and activation of Akt were all comparably inhibited, both temporally and in magnitude, by IL-6 exposure in HepG2 cells at physiological concentrations of insulin. This suggests a site of action of IL-6 at the level of IR/IRS-1 interaction. IL-6 was also capable of causing inhibition of insulin action in primary hepatocytes. Pretreatment of primary hepatocytes with IL-6 markedly inhibited both insulin-induced Akt activation and glycogen synthesis. This suggests that hepato-

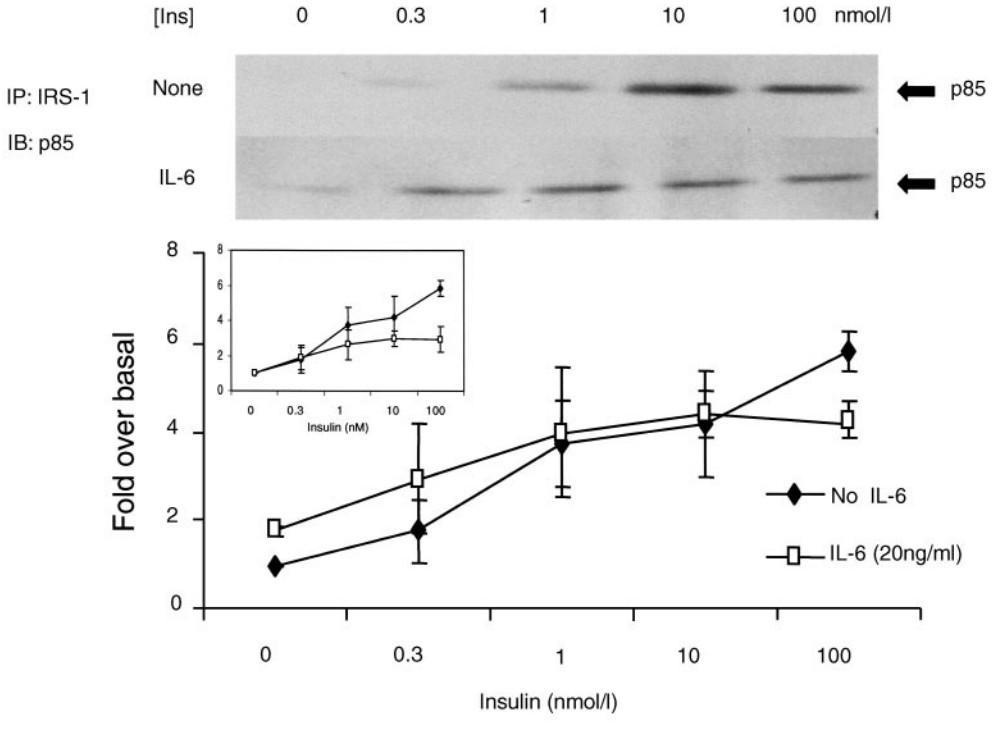

FIG. 6. IL-6 inhibits insulin-induced formation of p85/IRS-1 complexes. HepG2 cells were treated as in Fig. 5. Western blots of IRS-1 were probed with anti-p85 antibody. A representative blot of four independent experiments is shown. Results of the four experiments were quantitated by densitometry and expressed as fold increase over IL-6–free basal ± SD. Inset: data were expressed as fold increase of control and IL-6–treated cells relative to their respective insulin-free basal levels.

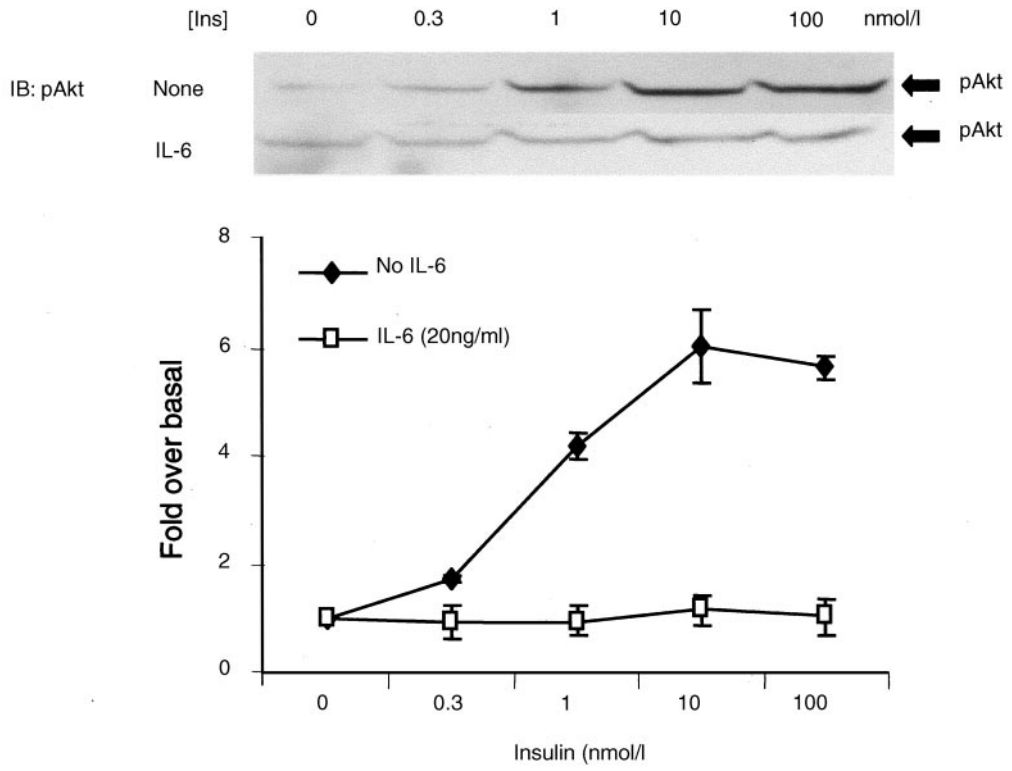

FIG. 7. Effect of IL-6 on insulin-dependent Akt activation. Confluent HepG2 cells were serum starved overnight before pretreatment for 90 min with or without IL-6 (20 ng/ml) and subsequent treatment with insulin at the indicated concentrations for 3 min. Lysates were separated by SDS-PAGE and subjected to Western blot analysis with anti–pSer473-Akt antibody. A representative blot of four independent experiments is shown. Results of the four experiments were quantitated by densitometry and expressed as fold increase ± SD of control and IL-6–treated cells relative to their respective insulin-free basal levels.

cytes, in general, are physiologic targets for the inhibitory effect of IL-6 on insulin signaling.

While the effect of IL-6 on all the measured parameters of IR signaling was closely correlated in the presence of physiologic insulin concentrations, a lack of correlation was found between insulin-dependent p85/IRS-1 complex formation and Akt activation at supraphysiologic concentrations of insulin. The only aspect of IR signaling that was inhibited by IL-6 under these conditions was activation of Akt. Additionally, in HepG2 cells, IL-6 caused an association of the p85 regulatory subunit with IRS-1 in the absence of insulin, but this association was not sufficient to cause activation of Akt. A review of recent literature provides several examples of discordance between IRS-1/p85 association and Akt activation (24–27). Takano et al. (24) observed that growth hormone inhibited insulin-induced Akt activation without affecting insulin-dependent p85 association with IRS-1. Their data suggest that altered localization of p85/IRS-1 complexes was associated with an inability to activate Akt. Perrault et al. (27) observed that cytokine-induced iNOS expression decreased insulin sensitivity in insulin-sensitive tissues. In adipocytes with increased iNOS expression, insulin induced p85 association with IRS-1 and activitation of the p110 catalytic subunit without activating Akt (27). Therefore, even when PI 3-kinase is associated with IRS-1, it does not always activate downstream signaling components. IRS-1 has multiple phosphotyrosine binding sites where p85 can associate. It is possible that all p85/IRS-1 complexes are not equal in their ability to activate Akt. It is also possible that the observed dissociation between the presence of IRS-1/p85 complexes and activation of Akt reflects IL-6–dependent changes at the level of PDK1 or -2 activity, which is proposed to link PI 3-kinase to Akt activation. Thus, our results in Figs. 3 and 6 agree with several other reports that p85/IRS-1 association is not sufficient for Akt activation. In the case of IL-6, a second explanation is possible. While one site of inhibition by IL-6 may be at the level of IR/IRS-1 interaction, there may be a second site of action of IL-6 that inhibits Akt activation distal to IRS-1/PI 3-kinase assocation. It may be this second site of IL-6–dependent Akt inhibition that cannot be overcome by acute exposure to supraphysiological insulin concentrations (Figs. 4 and 7).

Each of the proinflammatory cytokines, TNF-α, IL-6, and IL-1, has recently been implicated in the development of insulin resistance and, possibly, type 2 diabetes (1–6). Many studies have investigated the role of TNF-α–induced insulin resistance in cell lines, animal models, and humans. Hotamisligil et al. (28) provided evidence that serine/threonine phosphorylation of IRS-1 is increased by TNF-α. They also provide evidence that serine/threonine-phosphorylated IRS-1 is an inhibitor of IR signaling (28). Multiple studies using muscle and adipose models support this hypothesis (7,29–35). The validity of this hypothesis in the liver is less certain. In both cell line and animal studies, the effect of TNF-α on hepatic IR signal transduction is frequently small or absent (7,29,36,37). While TNF-α may well have an effect on the liver, an alternate mechanism may be required.

Circulating IL-6 has been shown to be elevated two- to threefold in insulin-resistant states (5,10). The degree of correlation between these levels and severity of insulin resistance is actually higher than that of TNF-α (5). Interestingly, recent evidence suggests that some of the observed effects of TNF-α may be mediated by its ability to induce IL-6 and IL-6 receptor expression in tissues such as the liver and muscle (38,39). Our current report together with a growing body of literature argue that IL-6 plays a role in regulating insulin sensitivity. A recent study with

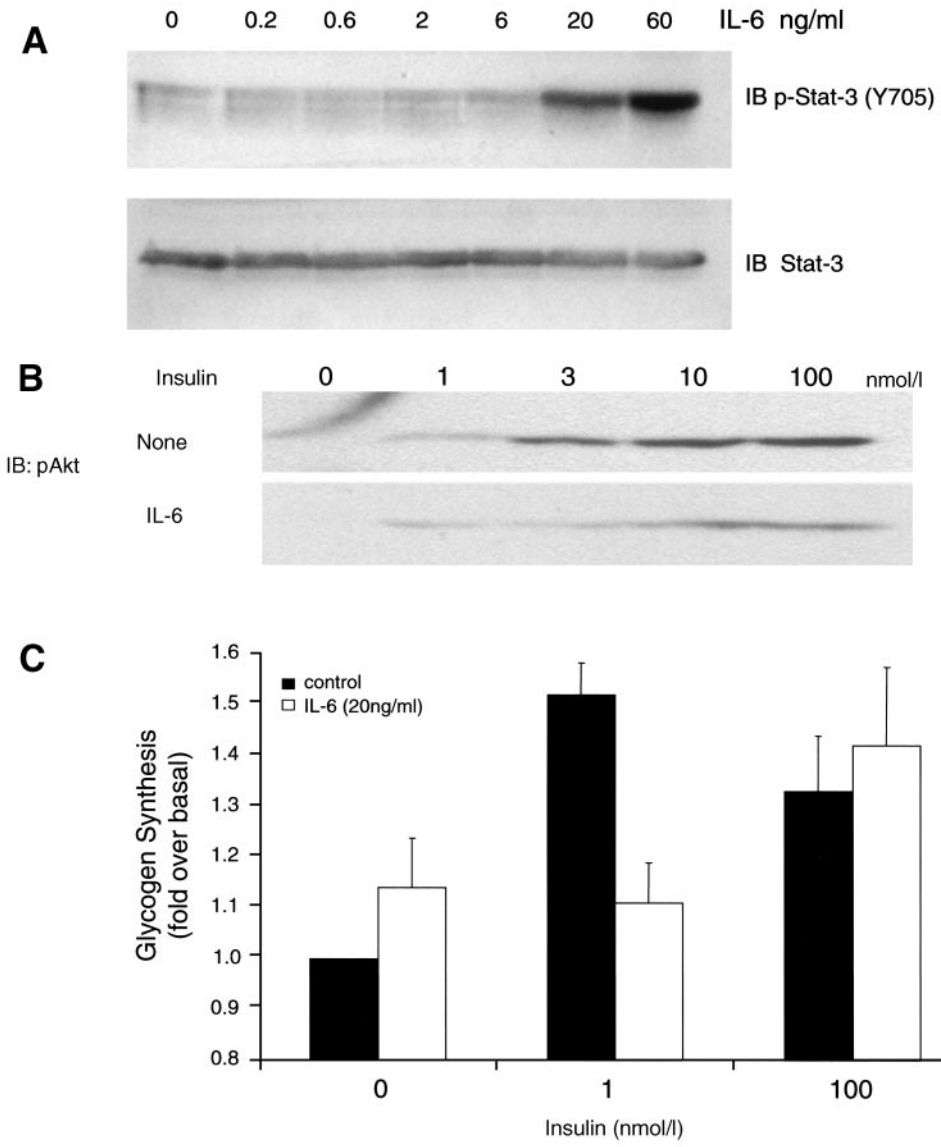

FIG. 8. Effect of IL-6 on STAT-3 activation, insulin-dependent Akt activation, and glycogen synthesis in primary hepatocytes. Primary mouse hepatocytes were incubated overnight in serum-free media. *A*: Cells were treated with or without IL-6 at the indicated concentrations for 10 min. Cells were harvested, and Western blots of lysates were probed with either an anti–phosphoSTAT-3 (pY705) antibody (Cell Signaling Technology) or an anti–STAT-3 antibody (Cell Signaling Technology). *B*: After a 90-min pretreatment with or without IL-6 (20 ng/ml), cells were treated with insulin for 3 min. Cells were harvested, and Western blots of lysates were probed with the anti–pSer473-Akt antibody. These results are representative of three experiments. *C*: After a 90-min pretreatment with or without IL-6 (20 ng/ml) in low glucose media, cells were treated with [^{14}C]D-glucose and insulin at the indicated concentrations. After 3 h, the cells were harvested and glycogen isolated as described in RESEARCH DESIGN AND METHODS. Results represent the means ± SE of four experiments, each performed in triplicate.

IL-6 knockout mice, however, demonstrates the potential complexities of IL-6 responses in vivo. Wallenius et al. (40) observed that IL-6$^{-/-}$ mice developed maturity-onset obesity and decreased glucose tolerance compared with lean wild-type controls. Intracerebroventicular but not intraperitoneal IL-6 replacement improved energy expenditure and obesity in these mice. The authors conclude that IL-6 in this model is acting centrally to influence obesity. How does the decreased glucose tolerance in obese IL-6–deficient mice not contradict our hypothesis that IL-6 plays a role in obesity-mediated insulin resistance? It is highly likely that increased adiposity in this IL-6–deficient mouse model releases several factors that contribute to insulin resistance (TNF-α, free fatty acids, etc). Only IL-6 was absent from the array of adipose tissue–derived factors. Thus, the insulin resistance in these animals, compared with lean controls, was not surprising. A comparison of glucose homeostasis and hepatic insulin sensitivity in high-fat diet–induced obese IL-6$^{-/-}$ mice versus comparably obese controls would have directly addressed the effect of obesity-derived IL-6 on insulin sensitivity in insulin target tissues. This study does demonstrate, however, that IL-6 has complex systemic effects. By using HepG2 cells and primary hepatocytes, the experiments described in the current study isolated the effects on IL-6 on hepatic insulin sensitivity. While this study demonstrates that IL-6 inhibits IR signaling, the molecular mechanism of IL-6–dependent inhibition remains undefined.

IL-6 is known to induce a family of negative regulators of cytokine signal transduction, the suppressors of cytokine signaling (SOCS) (41). Our laboratory and others have shown that SOCS-1, -3, and -6 can directly interact with the IR or IRS-1 and inhibit insulin signal transduction when overexpressed in hepatocytes and other cell lines (42–45). Preliminary investigations in our laboratory have indicated that IL-6–dependent induction of SOCS-3 mRNA and protein in the HepG2 cells corresponds with the time course reported here for IL-6–induced insulin resistance (J.J.S., R.A.M., unpublished observations). Our working hypothesis is that IL-6–dependent insulin resistance is mediated, at least in part, through induction of SOCS protein expression. This model not only can provide a

potential mechanism to explain IL-6–dependent inhibition of the IR, but also the transient decrease in IL-6–dependent inhibition of IR signaling at the 2-h time point. IL-6 induces expression of SOCS proteins (specifically SOCS-3 in HepG2 cells), which increase in concentration at ~1 h. We believe that SOCS proteins are direct mediators of IL-6–dependent inhibition of IR (42). The rise in SOCS concentration correlates with maximum IR inhibition by IL-6 (1 h). SOCS proteins, as their name implies, also suppress IL-6 receptor signaling by antagonizing Janus kinase activation by the receptor. This negative-feedback loop is well characterized (46). Thus, as SOCS proteins suppress IR and IL-6 receptors, they suppress induction of additional SOCS protein. SOCS expression decreases after 1 h and, consequently, we would argue that IL-6–dependent suppression of IR is decreased and IR signaling rebounds at 2 h. We have observed that SOCS3 mRNA peaks at 1 h (approximately eightfold), decreases toward basal levels at 2 h (less than twofold elevated), and increases again to two- to threefold by 4 h, consistent with a return of IL-6–dependent inhibition of the IR signaling. SOCS3 protein levels similarly peak at 1 h. Although not proven, we believe that IL-6–dependent SOCS expression provides a molecular explanation for IL-6–dependent inhibition of IR signaling and the temporary diminution of the IL-6 effect at 2 h.

Shoelson and coworkers (47,48) have provided evidence that salicylates can alleviate obesity-dependent and TNF-α–induced insulin resistance. Based on evidence that salicylates inhibit NF-κB inhibitor (IκB) kinase, a serine/threonine kinase in the nuclear factor (NF)-κB regulatory pathway, these investigators examined obesity-dependent insulin resistance in IκB knockout mice (48). They observed significantly decreased insulin resistance in the knockout mice model and proposed that IκB kinase is essential in mediating the serine/threonine phosphorylation of IRS-1 induced by TNF-α. While these studies are an important advance, an alternate interpretation of the results is possible. The TNF-α receptor activates IκB kinase, which releases the NF-κB transcription factor from inhibition. TNF-α–dependent induction of IL-6 is mediated through the NF-κB pathway. An interesting possibility is that the ability of salicylates to alleviate insulin resistance is, at least in part, mediated through its inhibition of TNF-α–dependent IL-6 induction. The inhibition of this induction may in turn lower expression of the SOCS proteins.

The data presented in this study indicate that chronic exposure to IL-6 inhibits IR signal transduction in both the HepG2 hepatocarcinoma cell line and primary mouse hepatocytes. The IL-6 effect is characterized by an inhibition of physiologic insulin-dependent IRS-1 tyrosine phosphorylation and association with the p85 subunit of PI 3-kinase, as well as a near complete inhibition of insulin-induced Akt activation in the HepG2 cell line. In the primary hepatocyte, IL-6 also inhibits Akt activation in response to insulin and inhibits a key physiological response to insulin, glycogen synthesis. While these data suggest that one site of action of IL-6 is at the level of IR/IRS-1 interaction, the precise mechanism for IL-6–dependent insulin resistance in hepatocytes has not been defined.

ACKNOWLEDGMENTS

This work was supported by U.S. Public Health Service Grant R01-38138 (to R.A.M.).

REFERENCES

1. Pickup JC, Mattock MB, Chusney GD, Burt D: NIDDM as a disease of the innate immune system: association of acute-phase reactants and interleukin-6 with metabolic syndrome X. *Diabetologia* 40:1286–1292, 1997
2. Fernandez-Real JM, Ricart W: Insulin resistance and inflammation in an evolutionary perspective: the contribution of cytokine genotype/phenotype to thriftiness. *Diabetologia* 42:1367–1374, 1999
3. Ventre J, Doebber T, Wu M, MacNaul K, Stevens K, Pasparakis M, Kollias G, Moller DE: Targeted disruption of the tumor necrosis factor-α gene: metabolic consequences in obese and nonobese mice. *Diabetes* 46:1526–1531, 1997
4. Shiba T, Higashi N, Nishimura Y: Hyperglycaemia due to insulin resistance caused by interferon-gamma. *Diabet Med* 15:435–436, 1998
5. Kern PA, Ranganathan S, Li C, Wood L, Ranganathan G: Adipose tissue tumor necrosis factor and interleukin-6 expression in human obesity and insulin resistance. *Am J Physiol Endocrinol Metab* 280:E745–E751, 2001
6. Yamakawa T, Tanaka S, Yamakawa Y, Kiuchi Y, Isoda F, Kawamoto S, Okuda K, Sekihara H: Augmented production of tumor necrosis factor-alpha in obese mice. *Clin Immunol Immunopathol* 75:51–56, 1995
7. Uysal KT, Wiesbrock SM, Marino MW, Hotamisligil GS: Protection from obesity-induced insulin resistance in mice lacking TNF-alpha function. *Nature* 389:610–614, 1997
8. Nelson KA, Walsh D, Sheehan FA: The cancer anorexia-cachexia syndrome. *J Clin Oncol* 12:213–225, 1994
9. Pickup JC, Chusney GD, Thomas SM, Burt D: Plasma interleukin-6, tumour necrosis factor alpha and blood cytokine production in type 2 diabetes. *Life Sci* 67:291–300, 2000
10. Pradhan AD, Manson JE, Rifai N, Burin JE, Ridker PM: C-Reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus**.** *JAMA* 286:327–334, 2001
11. Fernandez-Real JM, Broch M, Vendrell J, Gutierrez C, Casamitjana R, Pugeat M, Richart C, Ricart W: Interleukin-6 gene polymorphism and insulin sensitivity. *Diabetes* 49:517–520, 2000
12. Mohamed-Ali V, Goodrick S, Rawesh A, Katz DR, Miles JM, Yudkin JS, Klein S, Coppack SW: Subcutaneous adipose tissue releases interleukin-6, but not tumor necrosis factor-alpha, in vivo. *J Clin Endocrinol Metabol* 82:4196–4200, 1997
13. Del Aguila LF, Krishnan RK, Ulbrecht JS, Farrell PA, Correll PH, Lang CH, Zierath JR, Kirwan JP: Muscle damage impairs insulin stimulation of IRS-1, PI 3-kinase, and Akt-kinase in human skeletal muscle. *Am J Physiol Endocrinol Metab* 279:E206–E212, 2000
14. Pedersen BK, Steensberg A, Schjerling P: Muscle-derived interleukin-6: possible biological effects. *J Physiol* 536:2:329–337, 2001
15. Sundgren-Andersson AK, Ostlund P, Bartfai T: IL-6 is essential in TNF-alpha-induced fever. *Am J Physiol* 275:R2028–R2034, 1998
16. Tsigos C, Papanicolaou DA, Kyrou I, Defensor R, Mitsiadia CS, Chrousos GP: Dose-dependent effects of recombinant human interleukin-6 on glucose regulation. *J Clin Endocrinol Metab* 82:4167–4170, 1997
17. Kanemaki T, Kitade H, Kaibori M, Sakitani K, Hiramatsu Y, Kamiyama Y, Ito S, Okumura T: Interleukin 1β and interleukin 6, but not tumor necrosis factorα, inhibit insulin-stimulated glycogen synthesis in rat hepatocytes. *Hepatology* 27:1296–1303, 1998
18. Stouthard JM, Oude Elferink RP, Sauerwein HP: Interleukin-6 enhances glucose transport in 3T3–L1 adipocytes. *Biochem Biophys Res Commun* 220:241–245, 1996
19. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254, 1976
20. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685, 1970
21. Klaunig JE, Goldblatt PJ, Hinton DE, Lipsky MM, Chacko J, Trump BF: Mouse liver cell culture. I. Hepatocyte isolation. *In Vitro* 17:913–925, 1981
22. Paysant J, Blanque R, Vasse M, Soria C, Soria J, Gardner CR: Factors influencing the effect of the soluble IL-6 receptor on IL-6 responses in HepG2 hepatocytes. *Cytokine* 12:774–779, 1999
23. Wang S, Evers BM: Cytokine-mediated differential induction of hepatic activator protein-1 genes. *Surgery* 123:191–198, 1998
24. Takano A, Haruta T, I: wata M, Usui I, Uno T, Kawahara J, Ueno E, Sasaoka T. Kobayashi, M: Growth hormone induces cellular insulin resistance by uncoupling phosphatidylinositol 3-kinase and its downstream signals in 3T3–L1 adipocytes. *Diabetes* 50:1891–1900, 2001

25. Balbis A, Baquiran G, Bergeron JJ, Posner BI: Compartmentalization and insulin-induced translocations of insulin receptor substrates, phosphatidylinositol 3-kinase, and protein kinase B in rat liver. *Endocrinology* 141:4041–4049, 2000
26. Tremblay F, Marette A: Amino acid and insulin signaling via the mTOR/p70 S6 kinase pathway. *J Biol Chem* 276:38052–38060, 2001
27. Perreault M, Marette A: Targeted disruption of inducible nitric oxide synthase protects against obesity-linked insulin resistance in muscle. *Nat Med* 7:1138–1143, 2001
28. Hotamisligil GS, Peraldi P, Budavari A, Ellis R, White MF, Spiegelman BM: IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNFα and obesity-induced insulin resistance. *Science* 271:665–668, 1996
29. Hotamisligil GS, Budavari A, Murray D, Spiegelman BM: Reduced tyrosine kinase activity of the insulin receptor in obesity-diabetes central role of tumor necrosis factor-α. *J Clin Invest* 94:1543–1549, 1994
30. Feinstein R, Kanety H, Papa MZ, Lunenfeld B, Karasik A: Tumor necrosis factor-alpha suppresses insulin-induced tyrosine phosphorylation of insulin receptor and its substrates. *J Biol Chem* 268:26055–26058, 1993
31. Gokhan S, Hotamisligil D, Murray DL, Choy LN, Spiegelman BM: Tumor necrosis factor α inhibits signaling from the insulin receptor. *Proc Natl Acad Sci U S A* 91:4854–4858, 1994
32. Del Aguila LF, Claffey KP, Kirwan JP: TNF-α impairs insulin signaling and insulin stimulation of glucose uptake in C2C12 muscle cells. *Endocrinol Metab* 39:E849–E855, 1999
33. Aguirre V, Werner ED, Giraud J, Lee YH, Shoelson SE, White MF: Phosphorylation of SER307 in IRS-1 blocks interactions with the insulin receptor and inhibits insulin action. *J Biol Chem* 277:1531–1537, 2002
34. Rui L, Aguirre V, Kim JK, Shulman GI, Lee A, Corbould A, Dunaif A, White MF: Insulin/IGF-1 and TNF-alpha stimulate phosphorylation of IRS-1 at inhibitory Ser307 via distinct pathways. *J Clin Invest* 107:181–189, 2001
35. Tanti JF, Gremeaux T, van Obberghen E, Le Marchand-Brustel Y: Serine/threonine phosphorylation of insulin receptor substrate 1 modulates insulin receptor signaling. *J Biol Chem* 269:6051–6057, 1994
36. Cheung AT, Ree D, Kolls JK, Fuselier J, Coy DH, Bryer-Ash M: An in vivo model for elucidation of the mechanism of tumor necrosis factor-α (TNF-α)-induced insulin resistance: evidence for differential regulation of insulin signaling by TNF-α. *Endocrinology* 139:4928–4935, 1998
37. Cheung AT, Wang J, Ree D, Kolls JK, Bryer-Ash M: Tumor necrosis factor-α induces hepatic insulin resistance in obese Zucker (*fa/fa*) rats via interaction of leukocyte antigen-related tyrosine phosphatase with focal adhesion kinase. *Diabetes* 49:810–819, 2000
38. Yamada Y, Webber EM, Kirillova I, Peschon JJ, Fausto N: Analysis of liver regeneration in mice lacking type 1 or type 2 tumor necrosis factor receptor: requirement for type 1 but not type 2 receptor. *Hepatology* 28:959–970, 1998
39. Campbell JS, Prichard L, Schaper F, Schmitz J, Stephenson-Famy A, Rosenfeld ME, Argast GM, Heinrich PC, Fausto N: Expression of suppressors of cytokine signaling during liver regeneration. *J Clin Invest* 107: 1285–1292, 2001
40. Wallenius V, Wallenius K, Ahren B, Rudling M, Carlsten H, Dickson S, Ohlsson C, Jansson J-O: Interleukin-6 deficient mice develp mature-onset obesity. *Nature Med* 8:75–79, 2002
41. Starr R, Willison TA, Viney EM, Murray LJL, Rayner JR, Jenkins BJ, Gonda TJ, Alexander WS, Metcalf D, Nicola NA, Hilton DJ: A family of cytokine-inducible inhibitors of signalling. *Nature* 387:917–921, 1997
42. Mooney RA, Senn J, Cameron S, Inamdar N, Boivin LM, Shang Y, Furlanetto RW: Suppressors of cytokine signaling-1 and -6 associate with and inhibit the insulin receptor. *J Biol Chem* 276:25889–25893, 2001
43. Emanuelli B, Peraldi P, Filloux C, Sawka-Verhelle D, Hilton D, Van Obberghen E: SOCS-3 is an insulin-induced negative regulator of insulin signaling. *J Biol Chem* 275:15985–15991, 2000
44. Kawazoe Y, Naka T, Fujimoto M, Kohzaki H, Morita Y, Narazaki M, Okumura K, Saitoh H, Nakagawa R, Uchiyama Y, Akira S, Kishimoto T: Signal transducer and activator of transcription (STAT)-induced STAT inhibitor 1 (SSI-1)/suppressor of cytokine signaling 1 (SOCS1) inhibits insulin signal transduction pathway through modulating insulin receptor substrate (IRS-1) phosphorylation. *J Exp Med* 193:263–269, 2001
45. Emanuelli B, Peraldi P, Filloux C, Chavey C, Freidinger K, Hilton DJ, Hotamisligil GS, Van Obberghen E: SOCS-3 inhibits insulin signaling and is upregulated in response to TNF-α in the adipose tissue of obese mice. *J Biol Chem* 276:47944–47949, 2001
46. Krebs D, Hilton D: SOCS: physiological suppressors of cytokine signaling. *J Cell Sci* 113:2813–2819, 2000
47. Kim JK, Kim YJ, Fillmore JJ, Chen Y, Moore I, Lee J, Yuan M, Li ZW, Karin M, Perret P, Shoelson SE, Shulman GI: Prevention of fat-induced insulin resistance by salicylate. *J Clin Invest* 108:437–446, 2001
48. Yuan M, Konstantopoulos N, Lee J, Hansen L, Li ZW, Karin M, Shoelson SE: Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of Ikkβ. *Science* 293:1673–1677, 2001