The Potential Role of SOCS-3 in the Interleukin-1β–Induced Desensitization of Insulin Signaling in Pancreatic β-Cells

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Defects in insulin secretion, resulting from loss of function or destruction of pancreatic β-cells, trigger diabetes. Interleukin (IL)-1β is a proinflammatory cytokine that is involved in type 1 and type 2 diabetes development and impairs β-cell survival and function. Because effective insulin signaling is required for the optimal β-cell function, we assessed the effect of IL-1β on the insulin pathway in a rat pancreatic β-cell line. We show that IL-1β decreases insulin-induced tyrosine phosphorylation of the insulin receptor (IR) and insulin receptor substrate (IRS) proteins as well as phosphatidylinositol 3-kinase (PI3K) activation, and that this action is not due to the IL-1β–dependent nitric oxide (NO) production in RINm5F cells. We next analyzed if suppressor of cytokine signaling (SOCS)-3, which can be induced by multiple cytokines and which we identified as an insulin action inhibitor, was implicated in the IL-1β inhibitory effect on insulin signaling in these cells. We show that IL-1β increases SOCS-3 expression and induces SOCS-3/IR complex formation in RINm5F cells. Moreover, we find that ectopically expressed SOCS-3 associates with the IR and reduces insulin-dependent IR autophosphorylation and IRS/PI3K pathway in a way comparable to IL-1β treatment in RINm5F cells. We propose that IL-1β decreases insulin action in β-cells through the induction of SOCS-3 expression, and that this effect potentially alters insulin-induced β-cell survival. *Diabetes* 53 (Suppl. 3):S97–S103, 2004

Diabetes is the most common metabolic disorder, affecting 150 million people worldwide. It appears upon immune-mediated destruction of the insulin-secreting pancreatic β-cells in type 1 diabetes or upon the inability of these cells to secrete appropriate amounts of insulin to compensate for the hormone resistance in type 2 diabetes. In both cases, a defect in the insulin secretory function of the pancreatic β-cells is the chief cause of the development of the disease. Many efforts have therefore been made in order to clarify the mechanisms responsible for the impairment of β-cell function or the decrease in β-cell mass during disease progression to diabetes.

Proinflammatory cytokines, such as γ-interferon (IFN-γ), interleukin (IL)-1β, or tumor necrosis factor (TNF)-α, are able to induce β-cell death, acting alone or in combination (1). Hence, they have been suggested to play a key role in the pathologic destruction of pancreatic β-cells. It has also been reported that IL-1β impairs the function of these cells by inhibiting glucose-induced insulin secretion (2,3). This cytokine is produced by the activated macrophages during the inflammatory process or by the β-cells themselves in presence of high glucose concentrations (4). The activation of the transcription factor NFκB is a central component of IL-1β action in β-cells. Among its target genes, inducible nitric oxide synthase (iNOS) and Fas have received major attention. Nitric oxide (NO) production induced by IL-1β seems to be an important factor contributing to β-cell dysfunction and leading to cell death (5,6). However, these results are currently debated since β-cells deficient in the iNOS gene remain sensitive to cytokine-induced inhibition of insulin release and cell death (7).

Recently, several reports based on the analysis of knockout animals indicated that insulin and insulin-like growth factor (IGF)-1 play an essential role in the function of the β-cells. The invalidation of their respective receptors selectively in β-cells (βIRKO and βIGF-1RKO mice) results in a deterioration of glucose-induced insulin secretion due to abnormal β-cell physiology (8–10). Deficiency of insulin receptor substrate (IRS) proteins, which are involved in insulin/IGF-1 signaling pathways, also induces defects in β-cell function (11,12). While the importance of IRS proteins in β-cell functioning seems to have reached a consensus, the precise role of insulin itself, especially on β-cell physiology, remains a matter of debate. Indeed, in some studies insulin has been shown to increase insulin gene expression (13–15), while in others the hormone has no effect on isolated islets (16) or leads to decreased insulin gene expression in vivo (17). In addition to their role in the maintenance of β-cell function, insulin and IGF-1 are well-known growth factors for a variety of tissues and cell lines and may thus act on β-cell growth and/or survival. They might thus be involved in the regulation of the mass and the maturation of pancreatic islets.

Considering together the deleterious effect of IL-1β on β-cells and the potential beneficial action of insulin on the

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IL, interleukin; iNOS, inducible nitric oxide synthase; IR, insulin receptor; IRS, insulin receptor substrate; NO, nitric oxide; PI3K, phosphatidylinositol 3-kinase; SOCS, suppressor of cytokine signaling.

same cells, in this study we examined whether IL-1β could interfere with insulin signaling in β-cells. We found that IL-1β treatment decreases insulin-induced insulin receptor (IR) autophosphorylation and that this cytokine has also a strong inhibitory effect on the insulin-dependent IRS/phosphatidylinositol 3-kinase (PI3K) pathway. Our results suggest that the interconnection between IL-1β and insulin pathways in β-cells is not dependent on NO production stimulated by IL-1β, but is mediated by suppressor of cytokine signaling (SOCS)-3. SOCS-3 belongs to the SOCS family of inhibitory proteins, which were originally described as molecules involved in the negative feedback loop of cytokine signaling (18). The SOCS proteins inhibit the Jak-Stat (signal transducer and activator of transcription) pathway by various mechanisms. There are currently eight members of this family (CIS for cytokine-induced SH2-containing protein and SOCS1–7). All of them share an SH2 (src homology 2) domain, which binds to phosphotyrosine residues, and a characteristic SOCS-box, thought to regulate proteosomal degradation of proteins with which the SOCS interact. Finally, SOCS-1 and SOCS-3 possess an extra kinase inhibitory region (KIR) that is able to inhibit Jak tyrosine kinase activity. At least three distinct mechanisms could account for this inhibitory function, depending on biochemical and structural properties of each SOCS family member (19). We hypothesized that the inhibitory action of IL-1β on insulin signaling occurs through the induction of SOCS-3 expression since *1*) IL-1β induces SOCS-3 expression and SOCS-3 interaction with the IR in RINm5F cells occurs with a time course compatible with the inhibitory effect observed on insulin signaling upon IL-1β treatment; and *2*) ectopically expressed SOCS-3 associates with IR and reduces in these cells insulin-induced IRS tyrosine phosphorylation and PI3K activity by decreasing IR autophosphorylation in a way similar to IL-1β.

RESEARCH DESIGN AND METHODS

Cell culture. RINm5F rat insulinoma cells were obtained from ATCC and were grown in RPMI 1640 supplemented with 10% (vol/vol) fetal calf serum. Cells were depleted in RPMI 1640, 1 g/l glucose, and 0.2% bovine serum albumin (wt/vol) before experiments.

Preparation of recombinant adenovirus and infection of cells. The adenovirus vector containing SOCS-3 (SOCS-3-AdV) was constructed as previously described (20). Briefly, mouse SOCS-3 cDNA was subcloned into the shuttle vector pAd Track-CMV. The resultant plasmid was linearized by the restriction endonuclease *Pme*I and cotransformed with the supercoiled adenoviral vector pAd-Easy1 into *Escherichia coli* strain BJ5183. Recombinants were selected by kanamycin resistance and screened by restriction endonuclease digestion. Then, the recombinant adenoviral construct was cleaved with *Pac*I and transfected into the packaging cell-line 293. The adenovirus vector containing the major late promoter with no exogenous gene (GFP-AdV) was used as a control. The adenoviral vectors were propagated in the 293 cell line and stored as described (20).

RINm5F cells were cultured in media containing the adenoviruses (at high multiplicity of infection) for 2 h at 37°C. RPMI 1640 supplemented with 10% (vol/vol) fetal calf serum was added for 24 h; cells were then depleted for 16 h before experiments. The numbers of cells infected was monitored by the observation of GFP fluorescence in cultured cells 24 h after infection and was equivalent in all experiments.

Antibodies, immunoprecipitation, and immunoblotting. Rabbit polyclonal serum against IRS proteins (used for immunoprecipitation) was produced in our laboratory. Monoclonal antibody to phosphotyrosine and polyclonal anti–IRS-1 and anti-$p85^{PI3K}$ antibodies (for Western blot) were purchased from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal anti-SOCS-3 antibodies (used for immunoprecipitation) were prepared by Eurogentec (Belgium). Goat polyclonal anti–SOCS-3 and rabbit polyclonal anti–IRS-2 antibodies (for immunoblotting) were purchased from Santa Cruz (Santa Cruz, CA).

Cells were starved overnight and then stimulated or not with various stimuli as indicated in RESULTS. Cells were washed in phosphate-buffered saline and lysed in ice-cold lysis buffer. Whole-cell lysates were mixed with various antibodies (as described in RESULTS) and protein A or G coupled to agarose beads (Amersham Biosciences, Uppsala, Sweden) for immunoprecipitations. Proteins from whole-cell lysates and immunoprecipitates were resolved by SDS-PAGE, then transferred to Immobilon-P membrane (Millipore, Bedford, MA) and blotted with various antibodies (as specified in RESULTS). Finally, membranes were incubated with horseradish peroxidase–linked anti-rabbit, anti-goat, or anti-mouse antibodies (Santa Cruz Biotechnology), and proteins were revealed by enhanced chemiluminescence (Amersham Biosciences, Uppsala, Sweden) and quantified using Scion Image.

PI3K activity assay. Immunoprecipitates were washed and resuspended in HEPES 20 mmol/l, $MgCl_2$ 5 mmol/l, pH 7.4. They are incubated with 10 μg of phosphoinositides (Sigma-Aldrich, St Louis, MO) and a phosphorylation mix containing [γ-^{32}P]ATP for 20 min. The reaction was stopped by HCl 4N. A mixture of chloroform/methanol (1:1) was added to separate the phosphoinositides from the incubation mix. Phosphoinositides were resolved by thin-layer chromatography and revealed by autoradiography or by a Storm 840 apparatus (Molecular Dynamics; Amersham Biosciences).

Northern blot analysis. Total RNA from cells was isolated with the Trizol reagent (Life Technologies, UK, Inc.) according to the manufacturer's instructions. Ten micrograms of denatured total RNA was separated by electrophoresis on formaldehyde-containing 1.2% (wt/vol) agarose gels and transferred to positively charged nylon membranes (Ambion, Austin, TX). Full-length SOCS-3 cDNA was used as probe. The probe was labeled by random priming with [γ-^{32}P]dCTP using the Rediprime kit (Amersham Biosciences) and purified with the Probequant kit (Amersham Biosciences). A radiolabeled oligonucleotide corresponding to an 18 S RNA specific sequence was used as an internal control for RNA loading and integrity. Hybridizations were performed at 42°C in hybridization buffer (Ambion, Austin, TX). Membranes were washed three times in 0.5× sodium chloride–sodium citrate (SSC), 0.1% (wt/vol) SDS at 42°C and revealed by autoradiography or by a Storm 840 apparatus (Molecular Dynamic; Amersham Biosciences) and quantified using NIH Image.

Real-time quantitative PCR. Total RNA was treated with DNase (Ambion), and 1 μg was reverse-transcribed for 60 min at 42°C using the Reverse Transcription System kit (Promega) in the presence of random primers and oligo$(dT)_{15}$. Quantitative PCR was performed by monitoring in real time the increase in fluorescence of the SYBR green dye on an ABI PRISM 7000 Sequence Detector System (Applied Biosystems, Courtaboeuf, France), according to the manufacturer's instructions. PCR primers for each gene were designed using Primer Express software (Applied Biosystems, Courtaboeuf, France), with a melting temperature at 58–60°C and a resulting product of 100 bp. Each PCR was carried out in triplicate in a 20-μl volume using SYBR Green I Master Mix Plus (Eurogentec, Seraing, Belgium) for 10 min at 95°C for initial denaturing, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min using the ABI Prism 7000 Sequence Detector System (Applied Biosystems). To exclude the contamination of nonspecific PCR products such as primer dimers, melting curve analysis was applied to all final PCR products after the cycling protocols. Values for each gene were normalized to expression levels of HPRT mRNA in rat tissue. Each RT-PCR quantification experiment was performed in triplicate.

Relative quantification of SOCS-3 gene was calculated by using 2-*Ct* formula, as recommended by the manufacturer (Applied Biosystems). Results were expressed relative to the control condition, which was arbitrary assigned a value of 1.

Primers sequences used to quantify SOCS-3 mRNA by real-time RT-PCR were designed by using the Primer Express software from Applied Biosystems. Oligonucleotides used were as follows: rat SOCS-3 sense, 5′-AAAAATCCAGC CCCAATGG-3′, and antisense, 5′-GGCCTGAGGAAGAAGCCTATC-3′; HPRT sense, 5′-AGCCTGGTCATGTTGCCTTT-3′, and antisense, 5′-AAAGAACTTAT AGCCCCCCTTGA-3′.

RESULTS

IL-1β decreases insulin-induced IR autophosphorylation and tyrosine phosphorylation of IRS proteins in RINm5F cells. The first step of insulin signaling is the autophosphorylation of the IRβ subunits on multiple tyrosine residues following insulin binding to the alpha subunits. We thus analyzed the effect of IL-1β on the IR tyrosine phosphorylation in RINm5F cells. We used a

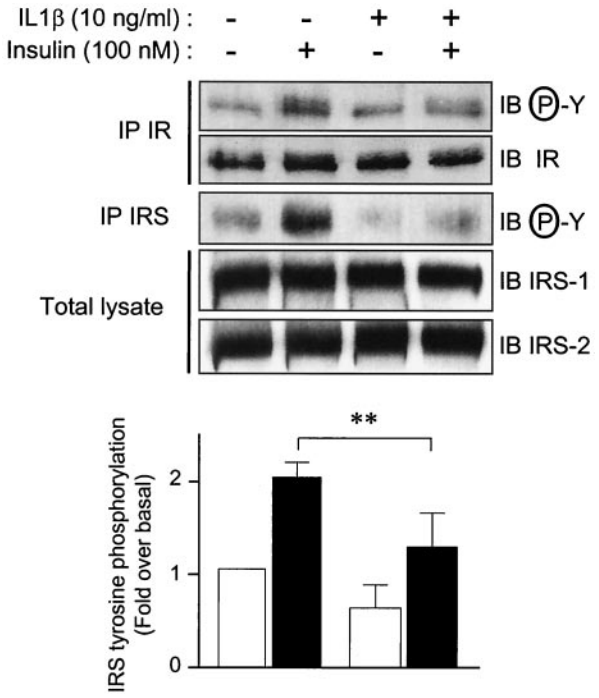

FIG. 1. IL-1β reduces insulin-induced IR autophosphorylation and IRS tyrosine phosphorylation without affecting IR or IRS expression in RINm5F cells. RINm5F cells were starved overnight then pretreated or not with IL-1β (10 ng/ml) for 2 h. Cells were then stimulated or not with insulin (100 nmol/l) for 5 min and lysed. IRβ subunits and IRS proteins were immunoprecipitated with antibodies recognizing IRβ subunits or both IRS-1 and IRS-2, respectively. An antiphosphotyrosine Western blot was then performed to analyze their tyrosine phosphorylation status. The expression levels of IRβ subunits, IRS-1, or IRS-2 were monitored by anti-IRβ, anti–IRS-1, and anti–IRS-2 Western blot of IR immunoprecipitates and total lysate, respectively. Quantification of IRS tyrosine phosphorylation is expressed as a mean ± SD from six independent experiments. **$P < 0.01$.

relatively short time of IL-1β exposure to observe primary events of IL-1β treatment since longer exposure may result in cell death, as previously reported (21). RINm5F cells were pretreated or not with IL-1β and then stimulated or not with insulin. The phosphorylation status of the IRβ subunits was analyzed by Western blot. Interestingly, IL-1β was found to inhibit IR autophosphorylation in response to insulin by ~30% (Fig. 1). Because IL-1β can decrease the IR autophosphorylation, the following experiments examined whether IL-1β was capable of altering insulin signal transduction. The tyrosine phosphorylation of IRS proteins was assessed by immunoprecipitation followed by an antiphosphotyrosine Western blot, as shown in Fig. 1. As expected, tyrosine phosphorylation of the IRS proteins is stimulated by insulin in these cells (Fig. 1). IL-1β pretreatment results in a 40% decrease in insulin-induced tyrosine phosphorylation of IRS proteins. The expression levels of the IRβ subunits and IRS-1 and IRS-2 proteins are not significantly modulated by IL-1β pretreatment, as revealed by anti-IRβ, anti–IRS-1, and anti–IRS-2 Western blot of IR immunoprecipitates and total lysate, respectively (Fig. 1).

These data clearly provide evidence that IL-1β is able to inhibit insulin-dependent tyrosine phosphorylation of IRS proteins by decreasing IR autophosphorylation and without affecting the expression levels of the IRβ subunits and IRS proteins in RINm5F cells.

IL-1β inhibits the insulin-induced association of $p85^{PI3K}$ with IRS proteins and IRS-associated PI3K activity in RINm5F cells. PI3K activation is a crucial component of insulin signaling and is one of the major events following insulin-stimulated IRS tyrosine phosphorylation. It results from the recruitment of the PI3K p85 regulatory subunit to phosphotyrosine residues on IRS proteins that activate the catalytic subunit and bring it close to its substrates. To further document the involvement of IL-1β on the insulin-dependent IRS/PI3K pathway, we examined the ability of IL-1β to modulate the formation of the IRS/$p85^{PI3K}$ complex and PI3K activity stimulated by insulin in RINm5F cells. Figure 2*A* shows that IL-1β exposure reduced by ~50% the IRS/$p85^{PI3K}$ association upon insulin stimulation. Consistent with this, IL-1β reduces by ~40% the IRS-associated PI3K activity in RINm5F cells stimulated by insulin (Fig. 2*B*). These results indicate that IL-1β has a strong inhibitory effect on the insulin-dependent IRS/PI3K pathway in RINm5F cells.

The inhibitory effect of IL-1β on insulin signaling pathway is not dependent on NO production. One of the major events triggered by IL-1β is the production of NO. This is achieved through the induction of iNOS. NO production is a crucial element responsible for the loss of function and death of β-cells induced by IL-1β and has a deleterious effect on insulin action (22,23). To test whether iNOS induction is responsible for the damaging effect of IL-1β on insulin signaling, we used drugs that either mimic (SNAP) or inhibit (NAME) NO production. RINm5F cells were treated or not with IL-1β, in combination or not with NAME or SNAP at the times indicated in Fig. 3. Cells were then stimulated or not with insulin. IRS tyrosine phosphorylation was analyzed by Western blot as shown in Fig. 3. As already observed, IL-1β pretreatment decreases insulin-induced IRS tyrosine phosphorylation. This inhibition is not abolished by the coincubation of RINm5F cells with NAME. NAME pretreatment alone has no effect on insulin-induced IRS tyrosine phosphorylation. Moreover, SNAP pretreatment for 1 or 2 h does not result in the inhibition of insulin-induced IRS tyrosine phosphorylation. These data show that the alteration of insulin signaling by IL-1β is not dependent on NO production. The next step was therefore to determine which factor might be involved in the inhibitory effect of IL-1β on insulin signaling.

IL-1β induces SOCS-3 mRNA expression and the association of SOCS-3 with the IRβ subunits in RINm5F cells. We have previously shown that SOCS-3 is an inhibitor of insulin signaling (24,25). As SOCS-3 expression is induced by a large range of cytokines, we tested the ability of IL-1β to modulate SOCS-3 mRNA expression in RINm5F cells. Total RNA was prepared from RINm5F cells treated with IL-1β for the indicated times and analyzed by Northern blot and RT-PCR. As shown in Fig. 4*A*, the peak of SOCS-3 expression induced by IL-1β is reached within 2 h of treatment in these cells, which corresponds to the duration of IL-1β exposure leading to the occurrence of the inhibitory effect on insulin signaling. By using the more sensitive real-time quantitative PCR assay, we examined the amount of SOCS-3 mRNA in RINm5F cells after 2 h of IL-1β treatment. In these cells, IL-1β induces a 4.3-fold increase in SOCS-3 mRNA expression (Fig. 4*B*). We (24) and Dey et al. (26) also demonstrated by the yeast two-

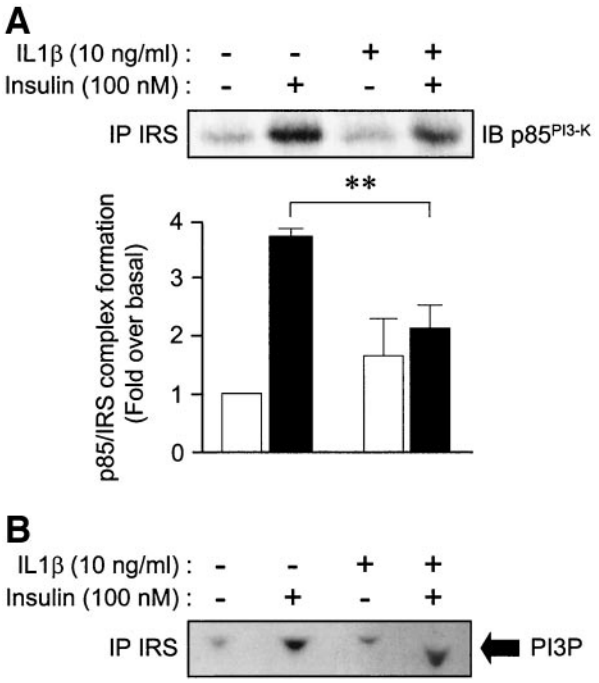

FIG. 2. IL-1β inhibits the insulin-induced IRS/p85^{PI3K} complex formation and IRS-associated PI3K activity in RINm5F cells. RINm5F cells were starved overnight then pretreated or not with IL-1β (10 ng/ml) for 2 h. Cells were then stimulated or not with insulin (100 nmol/l) for 5 min and lysed. IRS proteins were immunoprecipitated and then subjected to Western blot or PI3K activity analysis. *A*: The association of PI3K p85 regulatory subunit with IRS proteins was assessed by an anti-p85^{PI3K} Western blot. Quantification of the presence of PI3K p85 regulatory subunit in IRS immunocomplex is expressed as a mean ± SEM from three independent experiments. **$P < 0.01$ *B*: PI3K assay was performed with IRS immunoprecipitates as described in RESEARCH DESIGN AND METHODS.

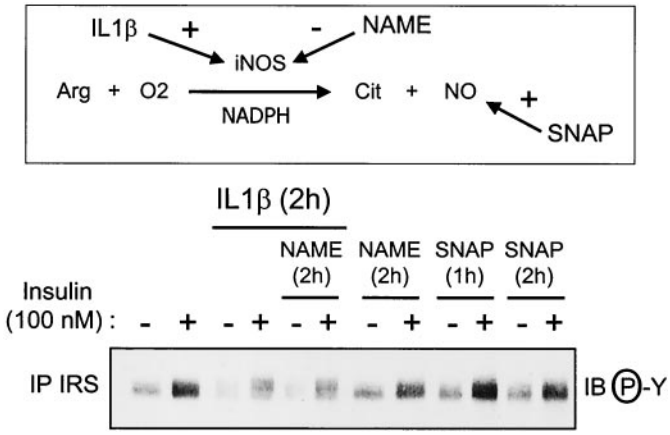

FIG. 3. The inhibitory effect of IL-1β on insulin signaling pathway is not dependent on NO production. RINm5F cells were starved overnight and then submitted to several treatments before insulin stimulation. Cells were pretreated with IL-1β (10 ng/ml) for 2 h in *lanes 3–6*. In *lanes 5 and 6*, IL-1β was coincubated with NAME (2 mmol/l). As a control, cells were incubated with NAME alone for 2 h (*lanes 7 and 8*). Cells were preincubated with SNAP (100 μmol/l) for 1 h (*lanes 9 and 10*) or 2 h (*lanes 11 and 12*). Cells were then stimulated or not with insulin (100 nmol/l) for 5 min and lysed. IRS proteins were immunoprecipitated and tyrosine phosphorylation was assessed by an antiphosphotyrosine Western blot.

hybrid approach and colocalization experiments in cells that SOCS-3 may interact with the IR. Therefore, we investigated whether endogenously expressed SOCS-3 in RINm5F was able to directly interact with the IRβ subunits after IL-1β pretreatment. IR immunoprecipitates were examined by Western blot analysis for the presence of SOCS-3. As indicated in Fig. 4*C*, SOCS-3 can be coimmunoprecipitated with the IRβ subunits after IL-1β pretreatment in the presence or absence of insulin. The increased association of SOCS-3 with IR is independent of IR expression since the expression level of the IRβ subunits was unchanged in the presence or absence of IL-1β, as shown in Fig. 4*C*.

Altogether, these results provide further evidence that the inhibitory effect of IL-1β on insulin signal transduction is associated with a SOCS-3/IR complex formation in RINm5F cells.

SOCS-3 expression decreases both insulin-induced IRS tyrosine phosphorylation and PI3K activity. To further characterize the role played by SOCS-3 in the inhibition of insulin signaling in RINm5F cells, we expressed in these cells exogenous SOCS-3 through adenoviral-mediated gene delivery. Control cells were infected with GFP-AdV. Cells were treated or not with insulin and lysed. Lysates were immunoprecipitated with anti-IRβ, anti-IRS, and anti-SOCS3 antibodies and analyzed by Western blot (Fig. 5*A*). Interestingly, SOCS-3 was found to coimmunoprecipitate with the IRβ subunits independently of insulin stimulation (Fig. 5*A*). Because SOCS-3 can directly interact with the IRβ subunits when ectopically expressed, the next experiments examined whether SOCS-3 was capable of altering insulin signal transduction. We first examined the effect of ectopic expression of SOCS-3 on IR autophosphorylation. As shown in Fig. 5*A*, SOCS-3 inhibits IR tyrosine phosphorylation by ~30% in response to insulin without affecting the total cellular amount of IR in RINm5F cells.

In addition, as compared with noninfected cells, SOCS-3 expression also dramatically decreased insulin-induced IRS tyrosine phosphorylation (up to 45%, Fig. 5*B*). By contrast, control GFP-AdV does not modify IRS tyrosine phosphorylation stimulated by insulin. This SOCS-3–dependent inhibition was seen without detectable changes in IRS-1 or IRS-2 protein levels. As expected, in cells expressing SOCS3, insulin-stimulated association of PI3K p85 regulatory subunit with IRS proteins is decreased accordingly with IRS tyrosine phosphorylation (Fig. 6*A*). Consequently, insulin-stimulated PI3K activity is reduced by ~47% in cells expressing SOCS3 compared with noninfected cells or cells infected by GFP-AdV (Fig. 6*B*).

DISCUSSION

IL-1β is involved in the autoimmune process leading to pancreatic β-cell dysfunction and destruction during type 1 diabetes. This cytokine has also been shown to mediate the effects of glucotoxicity during type 2 diabetes (4). The precise molecular mechanism is still debated, but most of the studies point to a role of enhanced NO production in both loss of function and death of β-cells exposed to this cytokine. Our data presented here show that IL-1β is also able to decrease insulin action in a rat pancreatic β-cell line. This effect is likely to occur before the induction of NO production since it has been revealed within 2 h of treatment. Furthermore, a pharmacological inhibitor and an activator of NO production do not modulate insulin signaling in the absence or presence of IL-1β.

We found that IL-1β is able to repress in a robust fashion

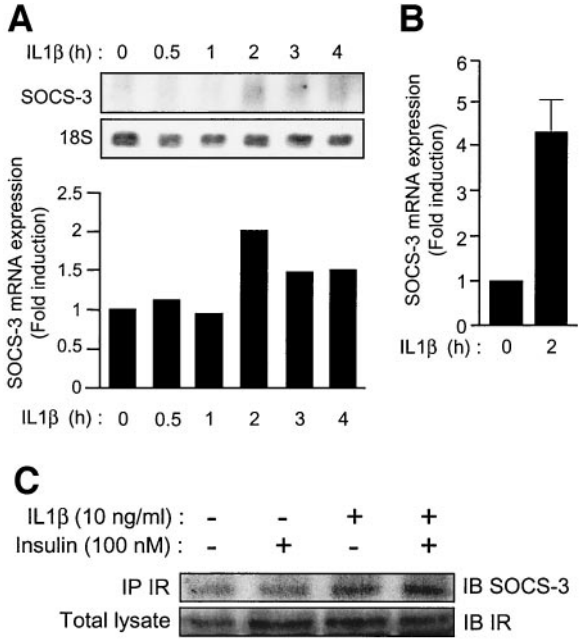

FIG. 4. IL-1β induces SOCS-3 mRNA expression and SOCS-3/IR complex formation in RINm5F cells. RINm5F cells were starved overnight and then stimulated or not with IL-1β (10 ng/ml) for 30 min, 1 h, 2 h, 3 h, or 4 h. *A*: Total RNA was extracted and analyzed by Northern blot using SOCS-3 as a probe. RNA loading and integrity were verified with an 18S ribosomal probe. Quantification of SOCS-3 mRNA (arbitrary units). *B*: After 2 h of treatment, total RNA was extracted and analyzed by real-time quantitative PCR using SOCS-3 primers. mRNA expression was normalized using hypoxanthine guanine phosphoribosyl transferase (HPRT) RNA levels. Results are expressed as a mean ± SEM from three independent experiments. *C*: RINm5F cells were starved overnight then pretreated or not with IL-1β (10 ng/ml) for 2 h. Cells were then stimulated or not with insulin (100 nmol/l) for 5 min and lysed. IRβ subunits were immunoprecipitated with an anti-IRβ antibody. An anti–SOCS-3 Western blot was then performed to analyze the coimmunoprecipitation of SOCS-3 with IRβ subunits. The expression levels of IRβ subunits was monitored by anti-IRβ Western blot of total lysate.

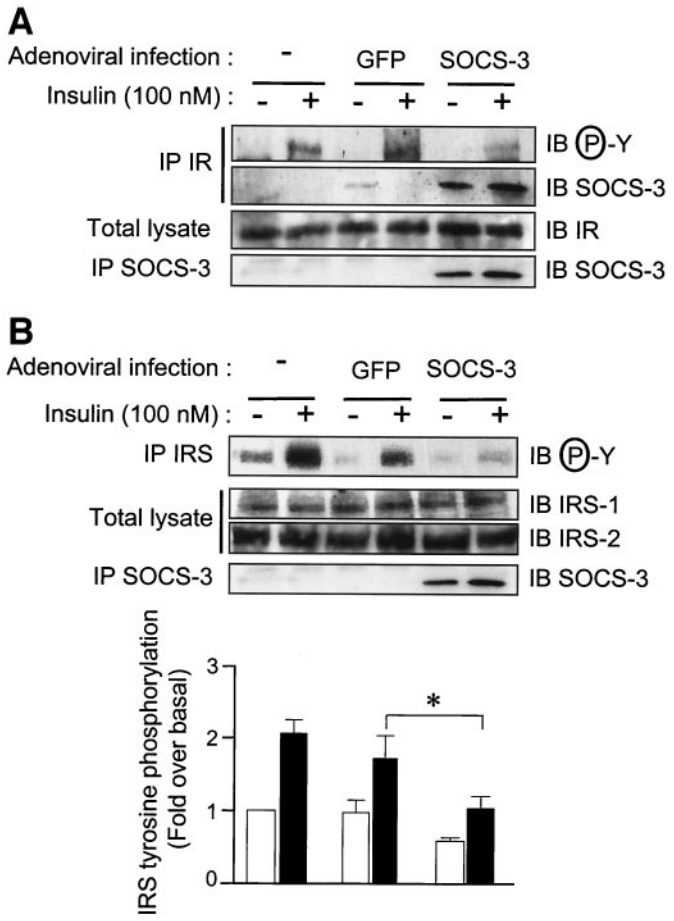

FIG. 5. Ectopically expressed SOCS-3 associates with IRβ subunits and decreases insulin-dependent IR autophosphorylation and IRS tyrosine phosphorylation in RINm5F cells. RINm5F cells were infected or not with comparable amounts of GFP-AdV or SOCS-3-AdV. Forty-eight hours after infection, cells were starved overnight then stimulated or not with insulin (100 nmol/l) for 5 min and lysed. *A*: IRβ subunits were immunoprecipitated with anti-IRβ antibody. Anti–SOCS-3 and antiphosphotyrosine Western blot were then performed. *B*: IRS proteins were immunoprecipitated with an anti-IRS antibody recognizing both IRS-1 and IRS-2. Tyrosine phosphorylation of these proteins was assessed by an antiphosphotyrosine Western blot. Quantification of IRS tyrosine phosphorylation is expressed as a mean ± SEM from five independent experiments. *$P < 0.05$. The expression levels of IRβ subunits, IRS-1, and IRS-2 were monitored by anti-IRβ, anti–IRS-1, and anti–IRS-2 Western blot of total lysate, respectively. Ectopically expressed SOCS-3 was revealed after an immunoprecipitation with a rabbit polyclonal anti–SOCS-3 antibody followed by a Western blot with a goat polyclonal anti–SOCS-3 antibody.

insulin-induced IRS tyrosine phosphorylation and PI3K activity. IRS proteins are essential for insulin action because they act, once phosphorylated on tyrosine residues as docking modules, allowing the recruitment and subsequent activation of signaling molecules, including PI3K. Deficiency of these proteins impairs β-cell function studied in animals and cells. IRS-1–deficient islets and cell lines exhibit insulin secretory defects (12). IRS-2 KO mice develop diabetes induced by defects in both peripheral insulin signaling and pancreatic β-cell function (11). The re-expression of IRS-2 solely in β-cells of these mice is able to revert the diabetic phenotype through the enhancement of β-cell growth, survival, and insulin secretion (27). It is also interesting to note that the most common human polymorphism for IRS-1, a glycine to arginine substitution at codon 972, is associated with a defect in glucose-stimulated insulin secretion. PI3K is a central component of the insulin-signaling pathway. However, its role in β-cells has not been well explored at the level of metabolism and regulation of gene expression involved in this cellular function. The PI3K/Akt pathway is also known to be involved in the regulation of cell survival. This is achieved through the phosphorylation and subsequent activation or inactivation of several cellular components including Bad and transcription factors belonging to the Foxo subfamily (28). In this regard, IRS proteins seem to play distinct roles in β-cells with an involvement of IRS-2 particularly in survival and growth (11,29), while IRS-1 appears to act more selectively in insulin secretion. The incretin hormone GLP1, which promotes islet cells survival, is thought to mediate its effects through the upregulation of IRS-2, which results in increased Akt phosphorylation in response to insulin/IGF-1 (30). The ability of IL-1β to inhibit these early steps of insulin signaling could therefore lead to the impairment of β-cell function or survival stimulated by insulin/IGF-1. In addition to the already well-described negative effects of IL-1β on β-cells, this process may thus further reveal its global damaging action on β-cells. The inhibitory effect of IL-1β on insulin signaling likely involves SOCS-3. We found that IL-1β induces SOCS-3 mRNA and protein expression in these cells, in a time frame compatible with the effect of

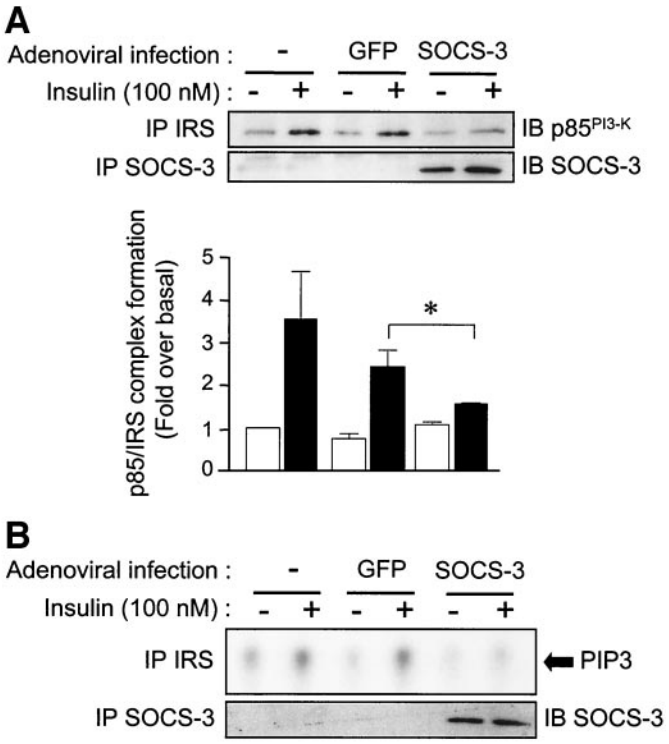

FIG. 6. SOCS-3 expression inhibits the insulin-induced IRS/p85^{PI3K} complex formation and IRS-associated PI3K activity in RINm5F cells. RINm5F cells were infected or not with comparable amounts of GFP-AdV or SOCS-3-AdV. Forty-eight hours after infection, cells were starved overnight then stimulated or not with insulin (100 nmol/l) for 5 min and lysed. *A*: IRS proteins were immunoprecipitated with an antibody recognizing both IRS-1 and IRS-2. The association of PI3K p85 regulatory subunit with IRS proteins was assessed by an anti-p85^{PI3K} Western blot. Quantification of PI3K p85 regulatory subunit amount in IRS immunoprecipitates is expressed as a mean ± SEM from five independent experiments. **$P < 0.01$ *B*: PI3K activity assay has been performed with IRS immunoprecipitates. Ectopically expressed SOCS-3 was revealed as described in Fig. 5.

IL-1β on insulin signaling. Furthermore, an association between the insulin receptor and SOCS-3 can be detected upon IL-1β treatment or ectopic SOCS-3 expression. The mechanism of SOCS-3 expression induced by the cytokine remains unclear. IL-1β is a poor activator of STAT transcription factors, which are usually believed to be the main regulators of SOCS expression, although it has been shown to activate a short STAT3 isoform in β-cells (31). However, recent evidence showed that induction of SOCS expression also occurs in a STAT-independent manner, suggesting the involvement of other pathways. A recent study identified SOCS-3 as an NFκB target gene (32). If this could be verified in pancreatic β-cells, it would constitute a probable link between IL-1β and SOCS-3 expression.

We and others previously identified SOCS-3 as an inhibitor of insulin signaling (24,25,33–35). Our former studies, performed using cotransfection experiments, led us to propose that the mechanism of inhibition of SOCS-3 was due to a competition between SOCS-3 and IR substrates to bind to phosphotyrosine 960 of the IR. Senn et al. (35) also reported that SOCS-3 is able to decrease the IR tyrosine kinase activity in hepatocytes. Our observations on endogenous insulin signaling proteins and endogenous or ectopic expression of SOCS-3 show that SOCS-3 induces a decrease in the IR autophosphorylation in RINm5F cells. However, it is difficult to estimate whether this reduction may be sufficient to account for the reduction in insulin-induced tyrosine phosphorylation of IRS proteins observed upon IL-1β treatment or ectopic SOCS-3 expression. Therefore, the most likely scenario probably includes a combination of these two mechanisms, which together are responsible for the decrease in IRS tyrosine phosphorylation we report here. In contrast to the report of Rui et al. (33), who showed an ubiquitin-dependent degradation of IRS proteins upon SOCS-1 and SOCS-3 expression in hepatocytes, we find that expression of IRS proteins is not altered in a detectable fashion in RINm5F cells treated with IL-1β or overexpressing SOCS-3. Although the involvement of other molecules cannot be fully excluded in this study, it is unlikely that the decrease in IRS tyrosine phosphorylation observed here is linked to an IL-1β–induced IRS serine phosphorylation (a well-established IRS inhibition mechanism [36]). Indeed, as shown in Fig. 1, IL-1β does not induce an IRS molecular mass shift, a hallmark of IRS serine phosphorylation. Furthermore, while the induction of IRS serine phosphorylation occurs rapidly (within a few minutes), we observed that IL-1β treatment has no significant effect before an hour (data not shown).

Taken as a whole, our results highlight, for the first time to the best of our knowledge, an interconnection between IL-1β and insulin signaling pathways involving the protein SOCS-3. Interestingly, recent articles point to the implication of SOCS-3 in β-cell function (37,38). In fact, these studies strongly support a role for SOCS-3 in the regulation of β-cell mass and function, even though they have led to contrasting interpretations. Sustained overexpression of SOCS-3 has been proposed to protect β-cells against cytotoxic effects of IL-1β and IFN-γ (37). By contrast, SOCS-3 is also able to inhibit the ability of growth hormone (GH) to promote β-cell growth and insulin production (38). These apparently contradictory results may be explained by the use of experimental methods resulting in lasting SOCS-3 expression, whereas it is generally transiently expressed. This underlines the urgent need to identify the conditions associated with an upregulation of SOCS-3 expression in β-cells. Analysis of SOCS-3 expression in the β-cells of diabetic rodents as well as in humans would help us to assess the contribution of SOCS-3 in the development of the disease process. Also, the analysis of the phenotype of mice deleted in their SOCS-3 gene, or overexpressing SOCS-3, specifically in pancreatic β-cells, would provide crucial information to apprehend the role of this protein in β-cell function and its involvement to mediate IL-1β action.

While it is well established that type 1 diabetes is due to an uncontrolled proinflammatory process, at the same time evidence is accumulating that suggests a role of inflammation in the development of insulin resistance since increased concentrations of proinflammatory cytokines, including TNF-α and IL-6, are associated with insulin-resistant states. A unifying hypothesis would be that inflammation not only results in insulin resistance at the level of peripheral tissues such as skeletal muscle, adipose tissue, and liver, but also occurs in the pancreas to trigger type 2 diabetes. According to this hypothesis, SOCS-3 might play a central role because it inhibits insulin

signaling and is expressed in response to proinflammatory cytokines.

In conclusion, we found that IL-1β inhibits the insulin signaling pathway in RINm5F cells, through a mechanism involving the newly identified insulin signaling inhibitor SOCS-3. The ability of this proinflammatory cytokine to decrease insulin sensitivity in pancreatic β-cells reveals a potentially new, high-impact means by which it may impair islet functioning in the disease process leading to diabetes.

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