Dual Function of Interleukin-1β for the Regulation of Interleukin-6-induced Suppressor of Cytokine Signaling 3 Expression*

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Interleukin-6 (IL-6) exerts pro- as well as anti-inflammatory activities in response to infection, injury, or other stimuli that affect the homeostasis of the organism. IL-6-induced expression of acute-phase protein genes in the liver is tightly regulated through both IL-6-induced feedback inhibitors and the activity of pro-inflammatory cytokines such as tumor necrosis factor α and interleukin-1β. In previous studies mechanisms for how IL-1β counteracts IL-6-dependent acute-phase protein gene induction have been proposed. Herein we analyzed IL-1β-mediated regulation of IL-6-induced expression of the feedback inhibitor SOCS3. In hepatocytes IL-1β alone does not induce SOCS3 expression, but it counteracts SOCS3-promoter activation in long term studies. Surprisingly, short term stimulation revealed IL-1β to be a potent enhancer of SOCS3 expression in concert with IL-6. This activity of IL-1β does not depend on IL-1β-dependent STAT1-serine phosphorylation but on NF-κB-dependent gene induction. Such a regulatory network allows IL-1β to counteract IL-6-dependent expression of acute-phase protein genes without inhibiting IL-6-induced SOCS3 expression and provides a reasonable mechanism for the IL-1β-dependent inhibition of acute-phase gene induction, because reduced SOCS3 expression would lead to enhanced IL-6 activity.

The acute-phase reaction in response to inflammation, infection, or injury starts with the release of pro-inflammatory cytokines such as interleukin (IL)1-β or tumor necrosis factor (TNF)-α. In the later phase, IL-6, the major mediator of acute-phase protein induction in the liver, is expressed and secreted into the circulation. IL-6 exerts pro- as well as anti-inflammatory activities. IL-1β has a co-regulatory function on IL-6-induced gene expression: Several IL-6-induced acute-phase proteins such as serum amyloid A, C-reactive protein, and α1-acid glycoprotein are positively regulated by IL-1β. On the other hand, fibrinogen, α1-antichymotrypsin, α2-macroglobulin, and thiostatin expression are suppressed by IL-1β (for review see Refs. 1 and 2).

IL-6 signaling occurs via JAK/STAT and MAPK cascades (for review see Refs. 3 and 4) and can be negatively regulated by SH2-containing protein-tyrosine phosphatase, by the protein inhibitor of activated STAT3 (PIAS3), or by the suppressors of cytokine signaling SOCS1 and SOCS3 (5). SOCS3 is supposed to act as a negative regulator of JAK/STAT-dependent IL-6-signaling by binding to tyrosine 759 of gp130, resulting in reduced activation/phosphorylation of JAKs, gp130, and STATs (6, 7). Inhibition of signaling by SOCS3 was found to be independent of SH2-containing protein-tyrosine phosphatase, although both proteins bind to the same site within the cytoplasmic part of the signal transducer gp130 (8). Besides being induced by IL-6, SOCS3 expression is also mediated by pro-inflammatory mediators such as lipopolysaccharide, TNFa, and CpG-DNA (9–11). Recent findings demonstrate that in SOCS3-deficient macrophages IL-6 induces an anti-inflammatory response. Thus, the rather restricted view of SOCS3 as an inhibitor of IL-6-induced JAK/STAT signaling should be extended to the role as a modulator of biological functions of IL-6 (12–14).

Additional mechanisms for inhibition of IL-6 signaling by pro-inflammatory cytokines seem to exist, because IL-1β, TNF-α, and lipopolysaccharide inhibit IL-6-dependent STAT activation in macrophages through pathways that are not completely understood (9, 15, 16). Interestingly, both SOCS3-dependent and SOCS3-independent inhibitory mechanisms of pro-inflammatory stimuli require the p38 MAPK (9, 15, 17, 18). The activation of the MKK6/p38 pathway is also involved in IL-6-dependent SOCS3 induction (19).

Recently, it has been shown in hepatocytes that inhibition of IL-6-induced expression of α2-macroglobulin and fibrinogen by IL-1β involves the activation of NF-κB and competition of NF-κB and STAT3 for overlapping binding sites (20–22). It has been suggested that other IL-6-induced proteins such as the α1-antichymotrypsin and SOCS3 may be regulated similarly (22).

With respect to the importance of SOCS3 for the regulation of IL-6 signaling (12–14), we studied the concerted regulation of SOCS3 expression by IL-6 and IL-1β in more detail. Here we show that IL-1β does not induce SOCS3 in hepatocytes. However, a time-dependent dual regulatory function of IL-1β on
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IL-6-induced SOCS3 expression was found: we discovered an enhanced SOCS3 expression by IL-1β immediately after stimulation. In contrast, SOCS3-promoter activation was suppressed by IL-1β at later time points. Both functions of IL-1β were dependent on the activation of NF-κB. Interestingly, in the absence of NF-κB activation early SOCS3 expression even decreased in response to IL-1β. This inhibitory function seems to be compensated by the strong NF-κB-mediated positive regulatory activity in the initial phase of IL-1β/IL-6 co-stimulation and IL-1β-dependent stabilization of SOCS3 mRNA.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes were purchased from Roche Molecular Biochemicals (Mannheim, Germany); Taq polymerase was from Hybaid (Heidelberg, Germany); oligonucleotides were obtained from MWG-Biotech (Ebersberg, Germany); Dulbecco’s modified Eagle’s medium (DMEM) and DMEM/nutrient mix F-12 were from Invitrogen; fetal calf serum was from Seromed (Berlin, Germany); recombinant human IL-6 was prepared as described (23); recombinant human IL-1β was from Roche Applied Science. Recombinant erythropoetin (Epo) was a generous gift of Drs. B. Hilger and K. H. Sellinger (Roche Applied Science—Hamburg, Germany). Antibodies for immunoprecipitation of SOCS3 were kindly provided by Drs. B. Hilger and K. H. Sellinger (Roche Applied Science). The following double-stranded oligonucleotide has been inserted into the BglII/HindIII opened pSuper: fos sequence described by Leung (26), 0.8 μg of vector pCAG5-FLAG-EpoR/gp130 plasmid, and 5 μg/ml Polybrene (Sigma) was added to the filtrate. Thereafter, medium of HepG2 cells plated in 6-well plates was replaced by δNXX cell supernatant containing the IeBo(SA)-CDNA containing retrovirus (giving HepG2-IeBo(SA)) or control virus (giving HepG2-mock). Culture plates were centrifuged at 1000 × g for 3 h. Medium was then replaced, HepG2 cells were cultured with fresh medium, and all cells were positive for GFP.

Retroviral infection of HepG2 cells was performed with supernatants from δNXX producer cells essentially as described for other cells (28). Briefly, supernatants were filtered through a 0.45-μm filter, and 5 μg/ml Polybrene (Sigma) was added to the filtrate. Thereafter, medium of HepG2 cells plated in 6-well plates was replaced by δNXX cell supernatant containing the IeBo(SA)-CDNA containing retrovirus (giving HepG2-IeBo(SA)) or control virus (giving HepG2-mock). Culture plates were centrifuged at 1000 × g for 3 h. Medium was then replaced, HepG2 cells were cultured with fresh medium, and all cells were positive for GFP.

Nuclear Extract Preparation and Electrophoretic Mobility Shift Assays—Preparation of nuclear extracts and EMSAs were performed as described previously (29). The protein concentration of the nuclear extracts was determined with a Bio-Rad reagent. 8 μg of protein was incubated with a surplus of double-stranded 5′-labeled eB site (5′-AGAATCTCTTGAATTCTTGGTCCCAGACTGGGTCGGG-3′) for binding of 45°F-B. The protein-DNA complexes were separated on a 4.5% polyacrylamide gel containing 7.5% glycerol in 0.25-fold TBE (20 mM Tris base, 20 mM boric acid, 0.5 mM EDTA, pH 8.0) at 20 V/cm for 4 h. Gels were fixed in 10% methanol, 10% acetic acid, and 80% water for 1 h, dried, and autoradiographed.

Immunoprecipitation and Immunoblot Analyses—For immunoprecipitation, 10 μg of protein from 10-cm plate were subjected to the selection marker Zeocin (Invitrogen). For immunoprecipitation and Western blot analyses of SOCS3 siRNA-treated cells, HepG2 cells were cultured on 10-cm dishes and transfected with 1 μg of a biotinylated SOCS3-specific primary antibodies and HRP-coupled secondary antibodies (DAKO). SOCS3 detection was performed by biotinylated SOCS3-specific primary antibodies and HRP-coupled streptavidin (1:5000) (Pierce). The membranes were developed with an enhanced chemiluminescence kit from Amersham Biosciences. For immunoprecipitation and Western blot analyses of SOCS3 siRNA-treated cells, HepG2 cells were cultured on 10-cm dishes and transfected with 1 μg of a biotinylated SOCS3-specific primary antibodies and HRP-coupled secondary antibodies (DAKO). SOCS3 detection was performed by biotinylated SOCS3-specific primary antibodies and HRP-coupled streptavidin (1:5000) (Pierce). The membranes were developed with an enhanced chemiluminescence kit from Amersham Biosciences. For immunoprecipitation and Western blot analyses of SOCS3 siRNA-treated cells, HepG2 cells were cultured on 10-cm dishes and transfected with 1 μg of a biotinylated SOCS3-specific primary antibodies and HRP-coupled secondary antibodies (DAKO). SOCS3 detection was performed by biotinylated SOCS3-specific primary antibodies and HRP-coupled streptavidin (1:5000) (Pierce). The membranes were developed with an enhanced chemiluminescence kit from Amersham Biosciences.
Nonidet P-40; and 10% glycerol) supplemented with protease inhibitors for 5 min. Nuclei were centrifuged at 3000 rpm and re-suspended in L2 buffer (50 mM Tris/HCl, pH 8.0; 1% sodium dodecyl sulfate; and 5 mM EDTA). Chromatin was sheared by sonication (4/1100316 s at about 50% output, Branson Sonifier). Debris was eliminated by centrifugation, and the supernatant was diluted (1:10) in dilution buffer (50 mM Tris/HCl, pH 8.0; 0.5% Nonidet P-40; 0.2 M NaCl; and 5 mM EDTA). Immunoprecipitation was performed with specific antibodies overnight. 20/1100210 f Protein A-Sepharose (Amersham Biosciences) was added at 4 °C for 45 min. Immune complexes were washed three times for 5 min with high salt buffer (20 mM Tris/HCl, pH 8.0; 0.1% SDS; 1% Nonidet P-40; 2 mM EDTA; and 0.5 M NaCl). Complexes were washed three times in 1× TE buffer (10 mM Tris/HCl, pH 8.0, 1 mM EDTA) and finally extracted in 1× TE buffer containing 2% SDS. Cross-linking of the protein with DNA was reverted by heating at 65 °C overnight. Supernatants were then incubated with proteinase K (100 µg at 50 °C for 2 h). DNA was extracted with phenol-chloroform, precipitated in ethanol, and resolved in 50 µl of TE buffer. 2.5 µl of DNA was used for PCR, with a specific primer for the human IκBα or SOCS3 promoter. The following promoter-specific primers were used: 5′-GACGACCCCAATTCAATCG-3′ and 5′-TCAGGCTCGGGGAATTTCC-3′ for IκBα promoter and 5′-GCTCAGCCTTTCCTGTC-3′ and 5′-GGAAGGCAGCAGCAGC-3′ for the −131 to +32 region of the human SOCS3 promoter and 5′-CAGGGTTGCAAAGAAC-3′ and 5′-ACCTGGAGAGCCTC-3′ for the −722 to −420 region of the human SOCS3 promoter (numbering relative to the proposed transcription starting site).

The PCR reaction was performed for 35 cycles in a total volume of 25 µl (1.25 unit of Taq DNA polymerase; 100 ng of each primer; 200 µM dNTP; 2.5 µl of 10× Taq buffer). PCR conditions were as follows: the first denaturation was for 94 °C for 180 s then 35 cycles consisting of denaturation (94 °C for 45 s), annealing (60 °C for 60 s), and elongation (72 °C for 60 s); the final elongation was carried out at 72 °C for 10 min. DNA fragments were separated by electrophoresis in a 2% agarose gel. DNA was visualized with ethidium bromide.

RESULTS

IL-1β Counteracts IL-6-dependent SOCS3-promoter Activation—Our own initial experiments suggested that SOCS3 may be negatively regulated by IL-1β (22). To analyze the collabo-
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rative regulation of SOCS3 by IL-6 and IL-1β, we performed promoter/reporter gene assays with an SOCS3-luciferase reporter construct in human HepG2 hepatoma cells. Fig. 1A (left part) shows that the SOCS3 promoter was responsive to stimulation with IL-6 (second bar), whereas the promoter was not inducible by IL-1β (third bar). Instead, IL-1β counteracted IL-6-dependent promoter activation, because co-incubation with IL-1β significantly reduced IL-6-induced reporter activity (compare bars 2 and 4).

Because NF-κB is a major mediator of IL-1β signaling, we asked whether IL-1β may act through NF-κB. Therefore, we tested whether expression of a non-degradable IκBα(S/A) inhibitor could overcome the effect of IL-1β. As shown in the right part of Fig. 1A, stable expression of IκBα(S/A) strongly affected the inhibitory action of IL-1β on SOCS3-promoter-driven reporter expression.

Expression of IκB was monitored by Western blotting. Fig. 1B shows that degradation of endogenous IκBα occurred 2 min after IL-1β treatment of HepG2-mock cells. IL-1β-induced IκBα re-expression was detectable after 20 min (Fig. 1B, left panel). In contrast, expression of the endogenous IκBα as well as of the exogenous IκBα(S/A) mutant was not affected by IL-1β treatment in HepG2-IκBα(S/A) cells (right panel). IL-1β-induced NF-κB DNA-binding activity was monitored by EMSA with a κB-site-containing DNA-probe (Fig. 1C). IL-1β-induced NF-κB binding to the κB site in HepG2-mock cells was detectable already after 1 min and further increased during the following 40 min (left panel). In contrast, no NF-κB binding in response to IL-1β was detectable in HepG2-IκBα(S/A) cells (right panel) demonstrating an efficient blockade of NF-κB activation in these cells. These data suggest that IL-1β acts negatively on IL-6-mediated SOCS3 promoter activation through an NF-κB-dependent pathway.

The Inhibitory Activity of IL-1β on SOCS3-promoter Activation Is Time-dependent—SOCS3 is an immediate early IL-6 response gene. Therefore, we decided to analyze the kinetics of IL-1β-induced inhibition of the SOCS3-reporter in the initial phase of expression. SOCS3-promoter-driven reporter gene expression was assessed 50, 80, 180, and 240 min after stimulation of HepG2 cells with IL-6. Reporter gene activity increased within the first 3 h but did not rise further up to 4 h of IL-6 stimulation. To our surprise, gene induction was most efficiently inhibited by IL-1β in cells stimulated for more than 3 h with IL-6 (Fig. 2, panels 3 and 4), whereas cells stimulated for 80 min were less sensitive to IL-1β (Fig. 2, panel 2). A reproducible slight increase of reporter gene induction was observed in cells stimulated with IL-6 for only 50 min (Fig. 2, panel 1).

IL-1β Mediates Enhanced SOCS3 Expression in Response to IL-6—To confirm the significance of the IL-1β-dependent increase of SOCS3-promoter activation observed in Fig. 2, we extended the duration of IL-1β pre-stimulation up to 40 min prior to addition of IL-6 (Fig. 3A). Stimulation of HepG2 cells with IL-6 for 50 min led to a 2-fold induction of reporter gene expression (left panel). As described in Fig. 2 a small but significant (p = 0.007) increase of reporter gene expression was detectable when IL-1β treatment started 10 min prior to IL-6 stimulation (bars 2 and 4). This increase was more obvious when IL-1β was given 20, 30, or 40 min before addition of IL-6 (bars 6, 8, and 10). These data suggest that IL-1β acts negatively on SOCS3 expression only in response to long term IL-6 stimulation, whereas a positive regulatory function of IL-1β is obvious early after stimulation.

To inspect a putative positive effect of IL-1β on early IL-6-induced SOCS3 induction, we analyzed expression of IL-6-induced endogenous SOCS3 in response to IL-1β. Intentionally, in the following experiments only the initial phase of SOCS3 induction has been analyzed to exclude putative secondary effects mediated by SOCS3 itself.

We investigated whether the supporting activity of IL-1β on IL-6-induced SOCS3 expression also affects SOCS3 mRNA levels in the initial phase of IL-6 stimulation. SOCS3 mRNA was isolated from HepG2 cells stimulated for up to 70 min with IL-6 in the presence or absence of IL-1β and analyzed by Northern
Enhanced SOCS3 expression by IL-1β. A, SOCS3 reporter gene induction was analyzed as in Fig. 2, but pre-stimulation with IL-1β was extended to up to 40 min prior to stimulation with IL-6 for 50 min. B–D, induction of IL-6-dependent expression of endogenous SOCS3 mRNA (B) and SOCS3 protein (C and D) was analyzed in the presence or absence of IL-1β in HepG2 cells. To exclude putative secondary effects mediated by SOCS3 itself, only the initial phase of SOCS3 induction was considered in these experiments. B, kinetics of SOCS3 mRNA expression. HepG2 cells were stimulated with 100 units/ml IL-6 for 15–70 min (left part). For co-stimulation, IL-1β (100 units/ml) was given 10 min prior to IL-6 (right part). Total mRNA was isolated and analyzed by Northern blotting with a specific probe for SOCS3 (upper panel). For loading controls, the membrane was re-probed with a GAPDH-specific probe (lower panel). C, kinetics of SOCS3 protein induction. Cells were treated with 100 units/ml IL-6 or IL-1β for the times indicated. For IL-1β/IL-6 co-stimulation IL-1β was given 10 min prior to IL-6. Cellular extracts were prepared and lysates were incubated with antibodies against SOCS3 (IP: SOCS3). Protein/antibody complexes were separated by SDS-PAGE and analyzed by immunoblotting (IB: SOCS3). D, dose-dependent increase in IL-6-induced SOCS3 expression. HepG2 cells were stimulated with IL-6 (100 units/ml) for 60 min. IL-1β (0, 100, 400, or 800 units/ml) was given 10 min prior to IL-6. SOCS3 protein was measured as described above.

NF-κB Is Crucial for the Enhancing Effect of IL-1β on IL-6-dependent Expression of Endogenous SOCS3—To clarify whether NF-κB is crucial for IL-1β-dependent enhancement of IL-6-induced SOCS3 expression, we monitored the amount of endogenous SOCS3 protein in HepG2 cells stably expressing the non-degradable IκBα(S/A) mutant, which represents an inhibitor of NF-κB activation (Fig. 4A). Consistent with the data in Fig. 3, IL-1β enhanced IL-6-induced SOCS3 expression in HepG2 cells. Stimulation with IL-1β alone did not lead to SOCS3 expression. Surprisingly, in cells expressing IκBα(S/A), IL-1β was not simply ineffective to enhance IL-6-dependent SOCS3 expression but, rather, reduced SOCS3 protein amounts (compare lanes 6 and 8). This finding is not due to an effect of the retroviral gene transfer, because cells infected with control virus behave similar to wild-type cells (lower panel of Fig. 4A).

These observations were also confirmed for SOCS3 mRNA expression (Fig. 4B). IL-6 alone induced SOCS3 mRNA expression in cells expressing the IκBα(S/A) inhibitor 30 min post stimulation (lane 5). After 60 min of IL-6 stimulation SOCS3 mRNA levels further increased (lane 6). In line with SOCS3 protein shown in Fig. 4A, IL-1β reduced SOCS3 mRNA in cells expressing the IκBα(S/A) inhibitor. These data indicate that SOCS3 expression is positively regulated by IL-1β through an NF-κB-dependent mechanism; however, in the absence of NF-κB activation, IL-1β rather represses SOCS3 expression indicating a dual regulatory role of IL-1β on IL-6-induced SOCS3 expression.
**IL-1β-induced Serine Phosphorylation of STAT1 Does Not Depend on NF-xB Activation and Thus Is Not Crucial for the Enhancing Effect of IL-1β on IL-6-induced SOCS3 Expression**—Recently, Stark and colleagues (30) described an IL-1β-induced STAT1 serine phosphorylation that in turn leads to enhanced transcriptional activity of STAT1. We asked whether IL-1β exerts enhanced SOCS3 expression through STAT1-serine phosphorylation. Fig. 5A shows that IL-1β is a strong mediator of STAT1-serine phosphorylation in HepG2 cells (left panel, lane 3), whereas IL-6 mediates STAT1-tyrosine phosphorylation but was only a comparable weak inductor of STAT1-serine phosphorylation (right panel, lane 2). Being aware of the fact that NF-xB activation is crucial for the enhancing effect of IL-1β on SOCS3 expression, we analyzed STAT1-serine phosphorylation in HepG2 cells expressing the non-degradable IxBo(S/A) inhibitor (Fig. 5B). The right part of the figure shows that IL-1β-dependent serine 727 phosphorylation of STAT1 did not depend on NF-xB activation. These data suggest that STAT1-serine phosphorylation is not responsible for enhanced SOCS3 expression after treatment with IL-1β, because the IL-1β-dependent increase in SOCS3 expression, but not the IL-1β-induced STAT1-serine phosphorylation, requires NF-xB activation.

**NF-xB Activated by IL-1β Does Not Act through Binding to SOCS3 Promoter**—Because NF-xB plays a crucial role for IL-1β-dependent enhancement of IL-6-induced SOCS3 expression, it is reasonable to speculate that NF-xB may act directly on the SOCS3 promoter. Two regions within the SOCS3 promoter were found by the MatInspector (Genomatix Software, München, Germany) to harbor putative xB sites. To monitor IL-1β-dependent NF-xB binding to these SOCS3 promoter regions we performed chromatin immunoprecipitation assays with p65-specific antibodies (Fig. 6A). As a positive control, binding of NF-xB to the IxBo promoter was determined (upper panel, left four bands). The applicability of the selected PCR primers was confirmed by the input control (four right bands in both panels). Unspecific binding of promoter fragments was controlled by a ChIP assay without antibodies (w/o antibodies). Fig. 6A shows that no NF-xB binding to the SOCS3 promoter was detectable in response to IL-1β, whereas NF-xB binding was confirmed for the IxBo-promoter. These results suggest that NF-xB does not act directly through binding to the SOCS3 promoter but more likely indirectly by the induction of secondary effector proteins.

**The Enhancing Effect of IL-1β on IL-6-dependent SOCS3 Expression Requires Protein Synthesis**—To further verify the idea that IL-1β does not act directly on the SOCS3 promoter but rather through the induction of downstream effector proteins, we checked whether the enhancing activity of IL-1β on IL-6-dependent SOCS3 expression requires newly synthesized proteins. We analyzed the effect of cycloheximide (CHX) on IL-6-induced SOCS3 mRNA expression in presence of IL-1β. Fig. 6B shows that CHX treatment enhanced IL-6-induced SOCS3 expression probably due to the blockade of SOCS3 feedback inhibition (compare lanes 3 and 7). Furthermore, co-stimulation with IL-1β enhanced IL-6-dependent SOCS3 expression in Me₃SO-treated cells (compare lanes 3 and 4) but not in CHX-treated cells (compare lanes 7 and 8). CHX treatment rather reduced SOCS3 mRNA levels in response to IL-1β, similar as shown in response to the block of NF-xB activation in Fig. 4. To exclude any effect of CHX treatment on IL-1β-induced NF-xB activation, we monitored NF-xB DNA-binding activity in the presence and absence of CHX in EMSA. As shown in Fig. 6C CHX treatment did not affect NF-xB activation. These data support the idea that IL-1β acts through NF-xB to induce the synthesis of one or more regulatory proteins that finally act positively on the SOCS3 expression.

**IL-1β Does Not Affect SOCS3 Protein Degradation but Stabilizes SOCS3 mRNA**—Instead of increasing IL-6-dependent induction of SOCS3, IL-1β could also affect the stability of SOCS3 protein or mRNA to increase SOCS3 levels in the cell. To test whether IL-1β stabilizes SOCS3 protein we compared the time-dependent loss of SOCS3 in the presence or absence of...
IL-1β (Fig. 7A). SOCS3 protein expression in HepG2 cells was induced by stimulation with IL-6 for 40 min. Afterward, IL-6 was removed and cycloheximide was added to the medium to avoid ongoing protein synthesis. The loss of SOCS3 protein within the following 90 min was compared in the absence (left part) or presence (right part) of IL-1β. No obvious effect of IL-1β treatment on the degradation of SOCS3 was observed, suggesting that IL-1β does not act by stabilizing SOCS3 protein.

Another possibility would be that IL-1β stabilizes SOCS3 mRNA. Therefore, we performed analogous experiments as described for Fig. 7A to analyze the decrease of SOCS3 mRNA in the absence and presence of IL-1β (Fig. 7B). SOCS3 mRNA was induced by stimulation of HepG2 cells with IL-6 for 30 min. Ongoing mRNA synthesis was blocked by eliminating IL-6 and adding actinomycin D into the medium. The blockage of SOCS3 transcription in the presence of actinomycin D is shown in the right part of Fig. 7B. The decrease of SOCS3 mRNA within the following 90 min was monitored in the absence (left part) or presence (right part) of IL-1β by Northern blotting (upper panel). Whereas SOCS3 mRNA is rapidly degraded in the absence of IL-1β, SOCS3 mRNA was stabilized in cells treated with IL-1β. These results suggest that IL-1β is able to increase SOCS3 expression by stabilizing its mRNA.

SOCS3 Expression Contributes to the Inhibitory Activity of IL-1β on gp130-mediated Signaling—The role of SOCS3 expression in the context of cross-talk between IL-1β and IL-6 signaling was studied by using SOCS3 siRNA. We transiently transfected HepG2 cells with vectors for SOCS3 siRNA. To be able to specifically stimulate the population of transfected cells, we co-transfected expression vectors for chimeric receptors composed of the extracellular part of the EpoR and the transmembrane and cytoplasmic part of gp130. These well established receptors allowed us to analyze gp130-dependent signaling in response to Epo (26, 31). In Fig. 8A we first confirmed the efficiency of the SOCS3 siRNA construct generated. Stimulation of the transfected HepG2 cells with Epo led to SOCS3 expression (lane 2). In contrast, cells additionally expressing SOCS3 siRNA showed drastically reduced SOCS3 levels in response to Epo, indicating that the SOCS3 siRNA is functional. We then studied the effect of IL-1β on Epo-induced reporter expression in the absence or presence of SOCS3 siRNA (Fig. 8B). Epo stimulation led to a significant induction of the STAT3-driven reporter construct (second bar), whereas the expression was inhibited to 50% the presence of IL-1β (compare second and fourth bars). These results are quite similar to those already presented in Fig. 1A. Cells additionally expressing SOCS3 siRNA showed a drastically increased reporter expression due to the lack of SOCS3-mediated feedback inhibition (compare bars 2 and 6). Interestingly, IL-1β was hardly potent to inhibit expression of the reporter gene in these cells (compare bars 6 and 8) suggesting SOCS3 plays an important role in the IL-1β-dependent inhibition of IL-6-induced gene expression.
**Fig. 6.** **NF-κB** activated by IL-1β does not act directly through binding to the SOCS3 promoter but rather indirectly through the induction of gene expression. A, HepG2 cells were stimulated as described in the legend to Fig. 6. Chromatin immunoprecipitation was performed with antibodies specific for the p65 subunit of NF-κB. Binding of NF-κB to the SOCS3 promoter was tested by PCR with two different pairs of SOCS3 promoter-specific primers. PCR conditions for these primers were tested for amplification of SOCS3 promoter fragments out of the input chromatin without immunoprecipitation. Efficiency of immunoprecipitation of NF-κB-DNA complexes was monitored with PCR primers specific for the IκB promoter. B, after pretreatment with Me2SO (DMSO) or cycloheximide (CHX, 25 μM) for 20 min, HepG2 cells were stimulated with IL-6 (100 units/ml), IL-1β (100 units/ml), or both for an additional 60 min. For IL-1β/IL-6 co-stimulation, IL-1β was given 10 min prior to IL-6. Total mRNA was isolated and analyzed by Northern blotting with a specific probe for SOCS3 (upper panel). For loading controls, the membrane was re-stained with a GAPDH-specific probe (lower panel). C, HepG2 cells were stimulated with 100 units/ml IL-1β in the presence or absence of cycloheximide. Nuclear extracts were prepared and tested for NF-κB DNA binding to the κB-site by EMSA.
DISCUSSION

IL-6 is the major mediator of acute-phase protein gene induction in the liver in response to inflammation, injury, and other stimuli (32, 33). The inflammatory cascade underlying the acute-phase response is largely controlled by the action of different mediators (34, 35). Monocytes and macrophages control hepatic IL-6 signaling through the release of pro-inflammatory cytokines such as IL-1β and TNF-α. Several mechanisms for IL-1β and TNF-α action on IL-6 signaling have been proposed, and additional complexity stems from cell-specific differences. In macrophages, TNF-α has been suggested to mediate repression of IL-6 signaling through the induction of SOCS3 protein expression (9). Additionally, de novo protein synthesis-independent mechanisms for inhibition of IL-6 signal transduction in macrophages have been suggested (15, 16).

In contrast to macrophages, SOCS3 expression in hepatocytes is not induced by IL-1β. Nevertheless, IL-1β affects IL-6-induced acute-phase gene expression. Several groups have proposed a crucial role of NF-κB for IL-1β-dependent repression of IL-6-induced gene induction in hepatocytes and suggest a competitive binding of NF-κB and STAT3 for overlapping binding sites within the respective promoters (20–22). Furthermore, IL-1β counteracts IL-6-dependent STAT3 activation through the activation of p38 MAPK (15). The induction of the SOCS3 feedback inhibitor by IL-6 is also affected by p38 (19).

In the study presented here, we found that IL-1β increases IL-6-induced SOCS3 expression. Blocking SOCS3 expression impairs the inhibitory action of IL-1β on gp130-dependent gene expression (Fig. 5), indicating an important role of SOCS3 for IL-1β-mediated repression of IL-6-induced gene expression.

SOCS3 plays a key role in the regulatory network of pro- and anti-inflammatory cytokines (12–14). On one hand SOCS3 is induced by IL-6, similar to type 2 acute-phase proteins. On the other hand SOCS3 suppresses IL-6 signaling and may thus be a mediator of IL-1β-dependent repression of acute-phase protein gene induction in the liver. Therefore, it was important to analyze the concerted regulation of SOCS3 expression by IL-1β and IL-6 in detail.

IL-1β was found to inhibit IL-6-induced SOCS3 promoter activation by activating NF-κB in human hepatoma cells (Fig. 1). A similar inhibition of IL-6-mediated expression of acute-phase protein genes upon IL-1β stimulation has been reported (20–22, 34). Surprisingly, IL-1β did not exert its inhibitory function on the SOCS3 promoter immediately after stimulation. Instead, IL-1β showed an NF-κB-dependent co-stimulatory activity (Figs. 2 and 3A). Importantly, this IL-1β/IL-6-synergism could also be confirmed for endogenous SOCS3 expression in a time- and dose-dependent manner for IL-1β (Fig. 3). It is intriguing to speculate that the ratio of stimulation and suppression of SOCS3 gene induction may explain the differential regulation of type 1 and type 2 acute-phase protein genes by IL-1β.

Knowing that IL-1β alone does not induce SOCS3 gene expression in hepatocytes, the key question of how IL-1β signal-
Enhanced SOCS3 expression could be a result of enhanced transcriptional activity of STAT1 and/or STAT3. Serine phosphorylation of both transcription factors has been shown to affect transcriptional activity (36, 37). Recently, Stark and collaborators described STAT1 serine phosphorylation in response to IL-1β (30). In respect to this study and the knowledge that NF-κB activation is crucial for the enhancing effect of IL-1β on IL-6-induced SOCS3 expression, we asked whether IL-1β-induced serine phosphorylation of STAT1 is also dependent on NF-κB expression (Fig. 5). We confirmed the results of Nguyen et al. (30) and additionally excluded a requirement of NF-κB for STAT1 serine phosphorylation, suggesting that enhanced SOCS3 expression is not a result of STAT1 serine phosphorylation.

The necessity of NF-κB for the enhancing effect of IL-1β on SOCS3 expression and the computer-aided identification of two putative NF-κB binding regions within the SOCS3 promoter led us to look for NF-κB binding in this promoter area in vivo (Fig. 6A). Although we found IL-1β-dependent NF-κB binding to the IκBα promoter, we did not detect any binding of NF-κB to the SOCS3 promoter. These results argue against a direct function of NF-κB on the SOCS3 promoter but rather for the induction of one or more unknown IL-1β-induced factors that finally, in concert with IL-6, support SOCS3 expression. Indeed, we could show that de novo protein synthesis is required for IL-1β to exert its enhancing effect on SOCS3 gene induction (Fig. 6B). Inhibition of protein biosynthesis counteracted the enhancing activity of IL-1β on SOCS3 expression, similar as observed in response to impaired NF-κB activation (Fig. 4).

Lipopolysaccharide and CpG activate TLR4 and TLR9, re-
IL-1β plays a positive regulatory role on IL-6-driven SOCS3 expression, but enhanced immediate early SOCS3 expression in response to IL-1β/NF-κB results in enhanced feedback inhibition of IL-6 signaling and in turn, later, in reduced SOCS3 expression. What is the clue of such a complex regulatory network? IL-1β counteracts IL-6-driven acute-phase protein gene expression by inhibiting STAT3 activation or by competition of NF-κB and STAT3 for overlapping binding sites in promoters of IL-6-inducible genes. Consequently, the induction of the SOCS3 feedback inhibitor would also be repressed by IL-1β. The lack of feedback inhibition by SOCS3 would finally result in enhanced IL-6 signaling and thus reverse the initial pro-inflammatory function of IL-1β. To overcome this problem enhanced SOCS3 expression in response to IL-1β, especially when STAT3 activation is inhibited by IL-1β, is reasonable. Our study provides evidence that enhanced SOCS3 expression contributes to the inhibitory effect of IL-1β on IL-6 signaling.

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