Statins induce suppressor of cytokine signaling-3 in macrophages

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Abstract Our previous study has shown that lipophilic 3-hydroxy-3-methyl-glutaryl coenzyme A reductase inhibitors of statins can inhibit interferon-γ-induced inducible nitric oxide synthase gene expression in RAW264.7 macrophages. In this study, we showed that lovastatin and fluvastatin are able to upregulate the mRNA expression of the suppressor of cytokine signaling-3 (SOCS-3) gene. This effect is specific for SOCS-3 and could be blocked by mevalonate, farnesyl pyrophosphate and geranylgeranyl pyrophosphate, while it was not affected by inhibitors of protein kinase C and A, mitogen-activated protein/extracellular signal-regulated kinase kinase, p38 mitogen-activated protein kinase, c-Jun N-terminal kinase, Src, Raf and Rho kinase. SOCS-3 expression results in the inhibition of interferon-γ-, interleukin-6- and macrophage colony-stimulating factor-elicited signal transducer and activator of transcription phosphorylation, suggesting a novel anti-inflammatory mechanism of statins to down-modulate the functions of interferon-γ-activated macrophages.

Key words: Statin; Suppressor of cytokine signaling-3; Signal transducer and activator of transcription; Interferon-γ; Macrophage

1. Introduction

Signal transducer and activator of transcription (STAT) proteins are a family of latent transcription factors that are produced in many cell types and become activated by tyrosine phosphorylation and dimerization in response to a wide variety of extracellular cytokines and growth factors [1,2]. This rapid membrane-to-nucleus signaling system accounts for the fundamental effects of cytokines in proliferation, differentiation, survival, inflammation and immunity. The suppressor of cytokine signaling (SOCS) proteins are a growing family of suppressor of cytokine signaling molecules that are feedback inhibitors of cytokine signaling pathways [3–6]. The SOCS proteins act as negative regulators of the Janus kinase (JAK)-STAT pathway either by binding and inhibiting JAK tyrosine kinases or by inhibiting binding of STAT factors to the cytoplasmic domains of the respective receptors [3,4,7]. Not only do they uncouple the STAT pathway, SOCSs play an important role in terminating the inflammatory response to lipopolysaccharide (LPS) [8–10] and tumor necrosis factor-α [11].

SOCS proteins can be positively induced through cytokine-elicited STAT signaling [12]. Evidence suggests that multiple signal pathways except JAK-STAT are involved in the transcriptional regulation of SOCS. These involve the actions of LPS [13–16], tumor necrosis factor-α [14], interleukin (IL)-1 [17], phorbol 13-myristate 12-acetate, basic fibroblast growth factor [18] and bacterial CpG-DNA [19]. In this context, SOCS-3 expression can be induced by different mechanisms, particularly of extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) [14,18,19].

Statins are inhibitors of 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase and are widely used as lipid-lowering agents [20]. Besides the therapeutic use in hyperlipidemia, the anti-inflammatory and immunomodulatory benefits of statins have been recently reported in many aspects, although its mechanisms are not yet completely defined [21]. Most identified anti-inflammatory benefits of statins rely on the reduction of cellular levels of mevalonate, the direct product of HMG-CoA reductase, and mevalonate-derived isoprenoids (farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), which are involved in post-translational modification of several small G proteins, such as Rho, Rac, Cdc42 and Ras [22,23]. Several studies have shown the abilities of statins to inhibit the immunological functions of interferon-γ (IFN-γ) [21,24,25]. In our previous study, we found that statins are able to inhibit inducible nitric oxide synthase (NOS) gene expression caused by IFN-γ in murine RAW264.7 macrophages. This action of statins is attributed to the rapid interruption of STAT-1 phosphorylation at tyrosine 701 [26]. Since the understanding and evaluation of the pharmacological effects of statins are increasing and accelerating their clinical importance and validity, in this study we further explore the mechanism by which statins uncouple JAK-STAT signaling of cytokines.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, penicillin, and streptomycin were obtained from Gibco BRL (Grand Island, NY, USA). Rabbit polyclonal antibodies against active STAT-

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular signal-regulated kinase; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; IFN-γ, interferon-γ; IL-6, interleukin-6; JAK, Janus kinase; MAPK, mitogen-activated protein kinase; M-CSF, macrophage colony-stimulating factor; MEK, mitogen-activated protein/ERK kinase; NOS, nitric oxide synthase; PKA, protein kinase A; PKC, protein kinase C; RT-PCR, reverse transcription-polymerase chain reaction; SOCS, suppressor of cytokine signaling; STAT, signal transducer and activator of transcription

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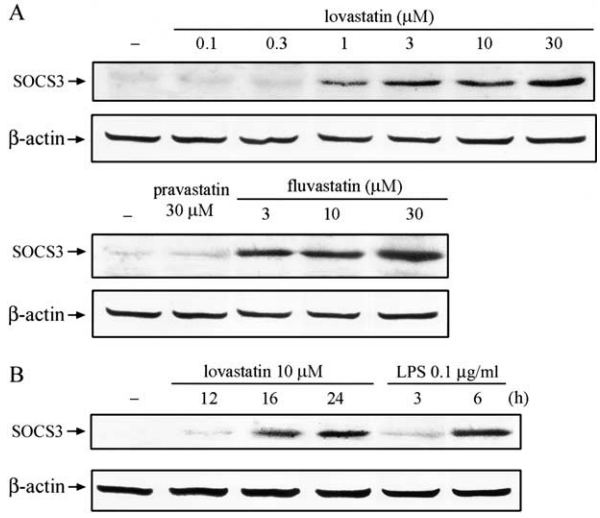

Fig. 1. Lovastatin and fluvastatin induced SOCS-3 expression in RAW264.7 macrophages. RAW264.7 macrophages were incubated with the indicated concentrations of statin for 24 h (A) or for different periods (B). In some experiments LPS (0.1 μg/ml) was added for 3 or 6 h (B). The protein levels of SOCS-3 and β-actin were measured in the cell lysates by Western blot. The results are representative of three different experiments.

1 (Y701), STAT-3 (Y705), and STAT-5 (Y694) were purchased from New England Biolabs (Beverly, MA USA). Rabbit polyclonal antibodies specific for SOCS-1, -2, 3, -5, -6, STAT-1, -3, -5, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). IFN-γ, IL-6, and macrophage colony-stimulating factor (M-CSF) were purchased from R&D (Minneapolis, MN, USA). Horseradish peroxidase-coupled anti-rabbit antibody, and the ECL detection agent were purchased from Amersham Biosciences (Piscataway, NJ, USA). Lovastatin, mevalonate, FPP, GGPP, KT5720 and GW5074 were purchased from Sigma Aldrich (St. Louis, MO, USA). PD98059, PP2, SB203580, Ro318220, GF109203X, herbimycin A, Y27632, and FPT inhibitors (FPTI) were purchased from Calbiochem (San Diego, CA, USA). Fluvastatin and pravastatin were respectively provided by Novartis (Basel, Switzerland) and Sankyo (Tokyo, Japan). All materials for sodium dodecyl sulfate–polyacrylamide gel electrophoresis were obtained from Bio-Rad Laboratories (Hercules, CA, USA).

2.2. Cell culture

Murine RAW264.7 macrophages obtained from the American Type Culture Collection (Manassas, VA, USA) were grown at 37°C in 5% CO_2 using DMEM containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin.

2.3. Immunoblotting analysis

Immunoblotting analysis was performed as in our previous study [26]. Equal protein levels of cell lysates were denatured in sodium dodecyl sulfate (SDS), electrophoresed on SDS/polyacrylamide gel, and transferred to a nitrocellulose membrane. Non-specific binding was blocked with TBST containing 5% non-fat milk. After incubation with the appropriate first antibodies, membranes were washed three times with TBST. The secondary antibody was incubated for 1 h. Following three washes with TBST, the protein bands were detected with the ECL reagent.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

To amplify SOCS-3 mRNA, macrophages were homogenized in RNAzol B reagent, and total RNA was extracted by acid guanidinium thiocyanate–phenol–chloroform extraction. RT was performed using a StrataScript RT-PCR kit following the manufacturer's recommended procedures. RT-generated cDNAs encoding SOCS-3 and β-actin genes were amplified using PCR. The oligonucleotide primers used corresponded to SOCS-3 (5′-GGA CCA GCG CCA CTT CTT CAC-3′ and 5′-TAC TGG TCC AGG AAC TCC CGA-3′) and mouse β-actin (5′-GAC TAC CTC ATG AAG ATC CT-3′ and 5′-CCA CAT CTG CTG GAA GGT GG-3′). PCR was performed as follows: initial denaturing for 2 min at 94°C, 35 cycles of amplification (94°C for 45 s, 58°C for 45 s, and 72°C for 2 min), and 10 min extension at 72°C. PCR products were analyzed on 2% agarose gels. The mRNA of β-actin served as an internal control for sample loading.

3. Results

3.1. Statins selectively induced SOCS-3 gene expression in RAW264.7 macrophages

Immunoblotting analysis with SOCS-3 antibody indicated that after 24 h incubation lovastatin and fluvastatin could increase SOCS-3 protein level (Fig. 1A). The effect of lovastatin displayed a concentration dependence in the range of 0.1–30 μM. Fluvastatin at concentrations in the range of 3–30 μM caused a similar extent of SOCS-3 induction as 10 μM lovastatin. In contrast, pravastatin did not induce such an effect at 30 μM. The stimulatory effect of lovastatin (10 μM) occurred at 12 h incubation and became obvious after 24 h incubation (Fig. 1B). In contrast, LPS (0.1 μg/ml)-induced SOCS-3 expression began at 3 h, and became prominent at 6 h (Fig. 1B). To extend the understanding of the effects on other SOCS members, we also determined SOCS-1, -2, -5, and -6. However, unlike the upregulation of SOCS-3, lovastatin and fluvastatin at 30 μM did not affect these protein levels after 24 h treatment (Fig. 2). Of these SOCSs, only SOCS-1 and -2 can be upregulated by LPS.

To understand whether the effect of statins is dependent on gene transcription, we performed RT-PCR and found that the steady-state level of SOCS-3 mRNA was time-dependently increased by both lovastatin and fluvastatin (Fig. 3).

3.2. SOCS-3 induction by statin is dependent on protein isoprenylation

To explore whether cholesterol deficiency resulting from HMG-CoA reductase inhibition might contribute to SOCS-3 induction, we supplied cells with the cholesterol precursor substrates mevalonate, FPP and GGPP. Results indicated that in the presence of each substrate, lovastatin- and fluvastatin-induced SOCS-3 induction was significantly reduced (Fig. 4A). At the same concentration (10 μM) tested, the inhibition by GGPP was more obvious than FPP. This suggests that a process mediated by protein isoprenylation might balance the gene expression of SOCS-3. Interrupting this balance by statins positively modulates SOCS-3 mRNA level.

Since ERK and p38 MAPK activation have been implicated

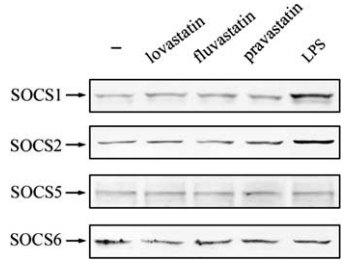

Fig. 2. Lovastatin and fluvastatin did not affect SOCS-1, -2, -5, and -6 expression in RAW264.7 macrophages. RAW264.7 macrophages were incubated with 30 μM statins for 24 h, or 0.1 μg/ml LPS for 6 h. Then the protein levels of SOCSs and β-actin were measured in the cell lysates by Western blot. The results are representative of three different experiments.

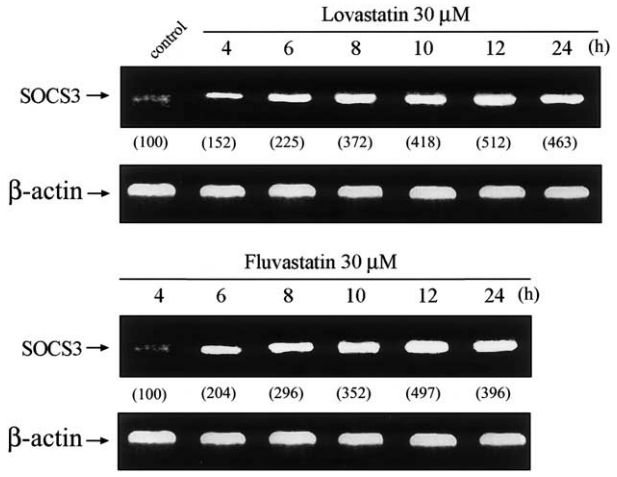

Fig. 3. Statins induced SOCS-3 expression at the transcriptional level. RAW264.7 macrophages were incubated with 30 μM statins for the indicated time periods. Subsequently, total RNA was prepared and subjected to RT-PCR analysis for SOCS-3 mRNA level. β-Actin mRNA level was used as the internal control. The SOCS-3 mRNA level, which was measured by densitometry and normalized to the level of β-actin mRNA, was calculated as a percentage of the control response without statin treatment.

for SOCS gene expression [12,17,18], we examined the possible signaling cascades underlying the action of statins. Results indicated that the upregulation of SOCS-3 by lovastatin and fluvastatin was not affected by inhibitors of protein kinase C (PKC) (Ro318220, GF109203X), protein kinase A (PKA) (KT5720), mitogen-activated protein/ERK kinase (MEK) (PD98059), p38 MAPK (SB203580), JNK (SP600125), Src family members (PP2), tyrosine kinases (herbimycin A), Raf (GW5074), Ras (FPTI), or Rho kinase (Y27632). Neither did the PKA activator dibutyryl cAMP (100 μM) alter the stimulatory effect of statins (Fig. 4B).

3.3. SOCS-3 induction interrupts cytokine-induced STAT phosphorylation

To understand the outcome of SOCS-3 induction on STAT signaling, we examined the signaling events of three cytokines that activate different STATs in macrophages. We treated RAW264.7 macrophages with statin for 24 h, replaced the culture with fresh medium to wash out statin, and immediately stimulated cells with IFN-γ, IL-6 or M-CSF. Results from immunoblotting revealed that in cells expressing large amounts of SOCS-3 following treatment with lovastatin and fluvastatin, the phosphorylated forms of STAT-1, STAT-3, and STAT-5, respectively induced by these cytokines, were markedly reduced. In contrast, pravastatin-treated cells still responded to cytokines as control group (Fig. 5). After 24 h incubation with statins, protein levels of STAT-1, STAT-3 and STAT-5 were not changed. These results indicate that the upregulation of SOCS-3 functions as a negative regulator for cytokine signaling.

4. Discussion

It has been established that transcripts encoding SOCSs are often present in cells at low or undetectable levels, but are upregulated by a broad spectrum of agonists, including cytokines, hormones, and infectious agents in different tissues [5].

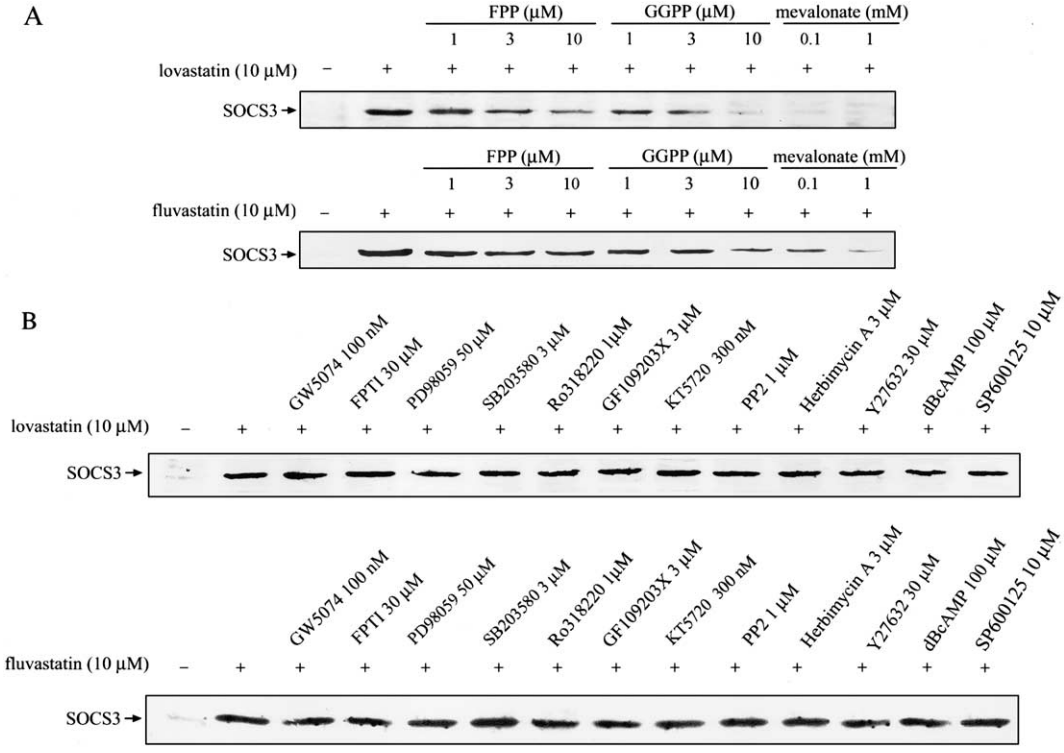

Fig. 4. Mevalonate and its metabolites reversed SOCS-3 induction by statins. RAW264.7 macrophages were pretreated with vehicle, mevalonate (100 μM, 1 mM), FPP (1–10 μM), GGPP (1–10 μM) (A), or indicated kinase inhibitors or dibutyryl cAMP (B) at the concentrations indicated for 30 min. Lovastatin or fluvastatin (each at 10 μM) was then added and incubated for 24 h. Protein levels of SOCS-3 were measured in the cell lysates by Western blot. The results are representative of three separate experiments.

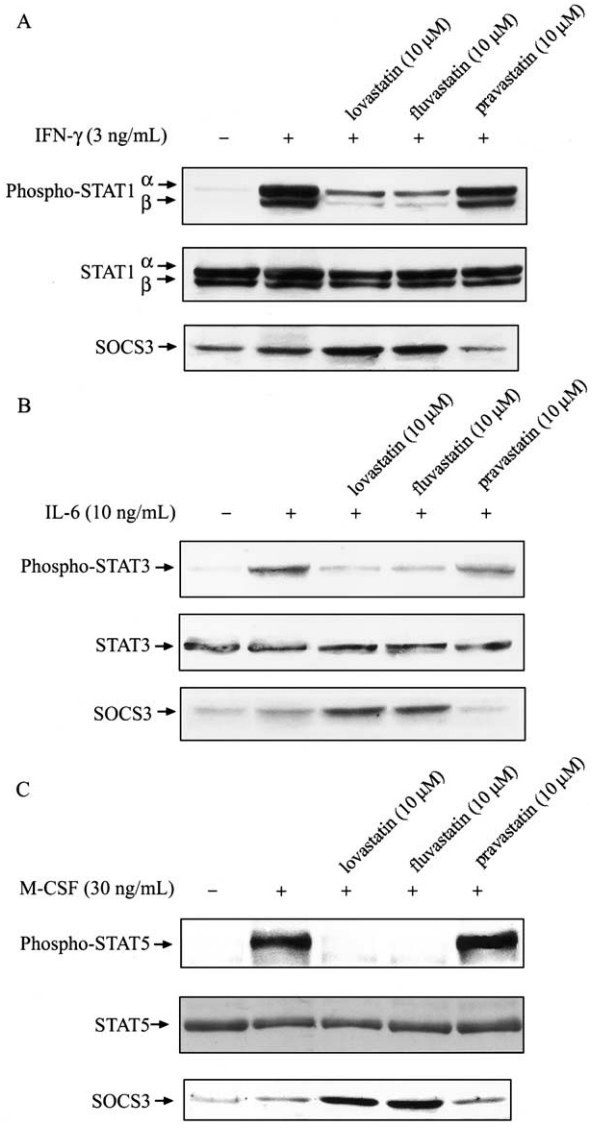

Fig. 5. Statin-induced SOCS-3 induction inhibited the signaling of STATs. Cells were treated with vehicle or statins (each at 10 μM) for 24 h. Culture medium was then washed, and cells were stimulated with IFN-γ (3 ng/ml), M-CSF (30 ng/ml) or IL-6 (10 ng/ml) for 30 min. Protein levels of STAT-1, STAT-3, STAT-5, and SOCS-3 were measured in the cell lysates by Western blot. The results are representative of three separate experiments.

Usually the expression of SOCS family members tends to vary with the cell line studied. The corresponding SOCS proteins thus play a negative feedback role in cytokine signaling pathways and serve as a negative cross-talk between cytokines and other proinflammatory stimuli. The signaling of IFN-γ can be inhibited by both SOCS-1 [3] and SOCS-3 [27], the signaling of LPS and tumor necrosis factor-α can be inhibited by SOCS-1 [9–11], while that of IL-6 can be inhibited by SOCS-3 [13,28,29]. Based on these results, induction of SOCS is a therapeutic strategy for treating inflammatory diseases [30]. In this study, we demonstrated a novel anti-inflammatory mechanism of statins. We observed that among SOCS family members examined in murine RAW264.7 macrophages, lipophilic statins (lovastatin and fluvastatin) could induce only SOCS-3 gene expression, which has been recognized to be the most important for regulative effects in immune cells [31]. The lack of effect of pravastatin might be due to its lower lipid solubility as has been previously shown in many anti-inflammatory aspects [26,32,33].

Previous studies have indicated that the anti-inflammatory effects of statins result from the depletion of mevalonate products [34]. Our present results indicate that the effect of statins on SOCS-3 induction might rely on the increased mRNA level of SOCS-3, and is dependent on the reduction of intracellular levels of mevalonate products. This implies the possible involvement of a protein isoprenylation-dependent event for the regulation of SOCS-3 gene expression. To address the signaling pathway(s) underlying this action of statins, we examined several pharmacological agents. The concentrations of kinase inhibitors tested were sufficient to inhibit specific targets by our previous studies in macrophages [35,36]. Present results excluded the roles of PKC, PKA, Ras/Raf/MEK, p38 MAPK, JNK, Src family members, and Rho kinase. Furthermore, as SOCS can be upregulated by cytokines transducing STAT signaling pathways [12], we examined this possibility in the action of statins. In the RAW264.7 macrophage system, we did not detect any changes of tyrosine-phosphorylated active forms of STAT-1, STAT-3, STAT-5, and STAT-6 by statin treatment alone. Moreover, the non-selective tyrosine kinase inhibitor herbimycin A, which can inhibit JAK activity and thus interrupt STAT signaling, also did not change the statin-induced SOCS-3 regulation. Although a recent report demonstrating SOCS-3 to be an NF-κB-induced gene [37], this possibility underlying the action of statins seems impossible. This is because our previous study proved the inhibitory effect of statins on NF-κB activation in macrophages [26]. To date, the identity of the isoprenylating target and the linkage between the isoprenylation step and SOCS-3 gene expression remain unknown.

In this study we also suggest the signaling role of statin-induced SOCS-3 expression in negative regulation of cytokine action. However, we found that RAW264.7 cells, which were primed with statins for 24 h and then washed with fresh medium, had low responsiveness to IFN-γ, IL-6 and M-CSF, in terms of activation of STAT-1, STAT-3 and STAT-5, respectively. This finding implies the non-specific action of SOCS-3 upregulation to uncouple the signaling pathways of different cytokines. This result also provides an explanation to support previous observations with statins in inhibition of many biological actions of IFN-γ, such as antigen presentation, T cell differentiation to the Th1 phenotype, and NOS induction [21,24,26,38].

Previous studies have reported that macrophages in response to LPS are able to release SOCS-inducing cytokines, such as IFN-α/β and IL-10 [8,16,39]. Moreover, based on reports showing the ability of IL-1 [17], IL-6 [13,40,41], and tumor necrosis factor-α [14,42] in SOCS induction, we wonder whether the action of statins might indirectly result from the release of cytokines, which then function as autocrines or paracrines to modulate SOCS-3 expression. Since these cytokines' production is regulated at the transcriptional level followed by the translation step, we treated cells with the protein synthesis inhibitor cycloheximide, to clear up this possibility. The data showing no changes of statin-induced increase of SOCS-3 mRNA level in the presence of cycloheximide ruled out this possibility (data not shown).

In conclusion, we demonstrated a novel indirect effect of statins to uncouple JAK-STAT signaling of cytokines through

the induction of SOCS-3. These results suggest that the capacity of statins to exert anti-inflammatory action partially results from the induction of SOCS-3, leading to the down-modulation of the effector functions of IFN-γ-activated macrophages.

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