SOCS1 Inhibits Tumor Necrosis Factor-induced Activation of ASK1-JNK Inflammatory Signaling by Mediating ASK1 Degradation*

Received for publication, November 16, 2005, and in revised form, December 20, 2006 Published, JBC Papers in Press, January 5, 2006, DOI 10.1074/jbc.M512338200

Yun He†§1, Wei Zhang†§1, Rong Zhang†§1, Haifeng Zhang†, and Wang Min‡2

From the †Interdepartmental Program in Vascular Biology and Transplantation, Boyer Center for Molecular Medicine, Department of Pathology, Yale University School of Medicine, New Haven, Connecticut 06510 and the ‡School of Public Health, Sun Yat-Sen University, Guangzhou S1008, Republic of China

We have previously shown that ASK1 undergoes ubiquitination and degradation in resting endothelial cells (EC) and that proinflammatory cytokine tumor necrosis factor (TNF) induces deubiquitination and stabilization, leading to ASK1 activation. However, the mechanism for the regulation of ASK1 stability is not known. In the present study, we have shown that SOCS1, a member of suppressor of cytokine signaling, induces ASK1 degradation. SOCS1 was constitutively expressed in EC and formed a labile complex with ASK1 that can be stabilized by proteasomal inhibitors. The phosphotyrosine-binding SH2 domain of SOCS1 was critical for its association with ASK1. Thus a SOCS1 mutant defective in phosphotyrosine binding failed to bind to and induce ASK1 degradation. Phosphotyrosine of ASK1 was induced in response to growth factors, and TNF induced dephosphorylation and dissociation of ASK1 from SOCS1. ASK1 with a mutation at Tyr-718 diminished the binding to SOCS1, suggesting that the phosphotyrosine-718 of ASK1 is critical for SOCS1 binding. Moreover, ASK1 expression and activity were up-regulated in SOCS1-deficient mice and derived EC, resulting in enhanced TNF-induced activation of JNK, expression of proinflammatory molecules, and apoptotic responses. We concluded that SOCS1 functions as a negative regulator in TNF-induced inflammation in EC, in part, by inducing ASK1 degradation.

The vascular cell that primarily limits the inflammatory and atherosclerotic process is the vascular endothelial cell (EC). Inflammatory cytokines such as tumor necrosis factor-α (TNF) induce EC dysfunction by disturbing normal homeostasis, relaxation, and survival by triggering signal transduction and gene transcription (1). In addition to the NF-κB pathway, the stress-activated MAP kinases c-Jun N-terminal kinase (JNK) and p38 MAPKs have been shown to be critical for TNF-induced gene expression of proinflammatory molecules such as E-selectin and VCAM-1 (2). Apoptosis signal-regulating kinase 1 (ASK1), a member of the mitogen-activated protein kinase kinase (MAP3K) family, is an upstream activator of JNK/p38 MAPK cascades (3). Studies from our laboratory and others have demonstrated that ASK1 functions as an effector in TNF-induced inflammation in EC (4–6). Thus ASK1 can be activated by almost all inflammatory stimuli such as TNF, interleukin-1, and reactive oxygen species. In contrast, anti-inflammatory factors such as antioxidants and shear stress inhibit ASK1 activity (4, 6).

ASK1 is a 170-kDa protein that functionally is composed of an inhibitory N-terminal domain, an internal kinase domain, and a C-terminal regulatory domain. The C-terminal domain of ASK1 binds to the TRAF domain of TRAF2 and TRAF6 (7). We have recently shown that the association with TRAF2 followed by recruitment of AIP1 (ASK1-interacting protein-1), a Ras-GAP (GTPase-activating protein) family protein, is required for ASK1 activation by TNF (8). On the other hand, several cellular inhibitors including thioredoxin (Trx), glutaredoxin, and 14-3-3 bind to and inhibit ASK1 activity in resting cells (8–12). Specifically, redox sensors Trx and glutaredoxin in reduced forms bind to ASK1 and block cytokine/stress-induced ASK1 activation (4, 5, 9). 14-3-3, a phosphoserine-binding molecule, binds to ASK1 specifically via Ser-967 of ASK1 and inhibits ASK1-induced apoptosis (4, 12, 13). The mechanism by which ASK1 activity is regulated in EC is not fully understood. We have previously shown that TNF activates ASK1, in part, by dissociating preexisting complexes of ASK1 with 14-3-3 and Trx. In contrast, atheroprotective laminar flow inhibits TNF-induced ASK1 and JNK activation by preventing the release of ASK1 from 14-3-3 and Trx (4). Furthermore, we have shown that ASK1 is a labile protein and undergoes ubiquitination/degradation in resting EC. Although cellular inhibitors such as Trx promote ASK1 ubiquitination/degradation, proinflammatory cytokines such as TNF induce deubiquitination and stabilization of ASK1 (5). Thus regulation of ASK1 stability is a critical step in ASK1 activation. However, the mechanism for ASK1 degradation is not understood.

SOCS1, a member of the SOCS family of proteins, was first identified as an inhibitor of cytokine signaling. The role of SOCS1 in T cell function has been extensively studied (14, 15). SOCS1 deletion causes perinatal lethality with death by 2–3 weeks due to uncontrolled inflammation. Introducing an interferon-γ (IFN-γ) deficiency or introducing neutralizing antibody to IFN-γ eliminates lethality, suggesting that lymphocyte-produced IFN-γ is critical to SOCS1-associated perinatal lethality (15, 16). Thus SOCS1 functions as a feedback regulator in IFN-γ signaling. Mechanistic studies suggest that SOCS1 via its N-terminal domain binds to and inhibits the kinase activities of all members of JAK kinase family (JAK1–3 and Tyk2), kinases critical for signaling in many cytokines in immune cells (17). Thus SOCS1 is generally consid-
SOCS1 Induces ASK1 Degradation

SOCS1 was identified as an anti-inflammatory molecule by suppressing cytokine production from T cells, macrophages, and antigen presentation from dendritic cells (17). Eight SOCS family members (CIS, SOCS1–7) have been identified and are defined by a characteristic structure composed of a highly variable N-terminal region, a central SH2 domain, and a highly conserved 40–50-amino acid motif (called SOCS box) at the C terminus. SOCS1 also functions as an inhibitor in other cytokine signaling by various mechanisms. For example, SOCS1 attenuates insulin/IGF-1 signaling by binding to the insulin/IGF-1 receptors to inhibit the receptor kinase activity and by targeting insulin/IGF-1 receptor substrate-1 (IRS-1) for proteasome degradation (18, 19). Besides IFN-γ, other inflammatory cytokines such as TNF and interleukin-6 also induce SOCS1 in fat and muscle tissues and inhibit insulin/IGF-1 signaling (18, 19). Thus SOCS1 has been implicated in inflammation-induced insulin resistance. A general model has been proposed for SOCS protein-targeted protein degradation; SOCS box contains a conserved elongin BC-binding motif (BC box) and mediates interaction with elongin BC complex. In turn, the elongin complex associates with the putative ubiquitin ligase cullin-2. Signaling proteins (e.g. JAKs) associated with the N-terminal or SH2 domains of SOCS proteins could be ubiquitinated by cullin-2 and are targeted for degradation by the proteasome (20, 21). Recent data suggest that SOCS1 may also function as an inhibitor in TNF signaling. Thus SOCS1 knock-out (KO) mice or cells derived from the mice are hypersensitive to TNF (22). However, the mechanism by which SOCS1 suppresses TNF signaling is not known.

In the present study, we have shown that SOCS1 via its SH2 domain binds to the phosphotyrosine residues on ASK1 to induce ASK1 degradation in an elongin complex-dependent manner. TNF induced dephosphorylation of ASK1 and dissociation of ASK1 from SOCS1, resulting in ASK1-JNK activation. Moreover, SOCS1-KO mouse tissues and derived EC showed increased ASK1 expression and enhanced TNF-induced ASK1-JNK activation, gene expression of proinflammatory molecules, as well as apoptotic responses. We concluded that SOCS1 functions as a negative regulator in TNF-induced inflammatory signaling in EC.

EXPERIMENTAL PROCEDURES

Plasmid Construction—Expression plasmids for human SOCS1, -2, -3, -6, and CIS expression plasmids were provided by Dr. Robert Mooney (University of Rochester, NY) (19); expression plasmids for elongin B and C were provided by Dr. Paul Rothman (Columbia University, NY) (23). ASK1 constructs were described previously (4, 5). The mutant SOCS1, SOCS3, and ASK1 were constructed by site-directed mutagenesis using QuickChange™ site-directed mutagenesis kit (Stratagene) according to the protocol of the manufacturer.

Cells and Cytokines—Bovine aortic endothelial cells (BAEC) were purchased from Clonetics (San Diego, CA). Human umbilical vein EC (HUVEC) were from Boyer Center for Molecular Medicine Cell Culture Core, Yale University. Human recombinant TNF was purchased from R&D Systems (Minneapolis, MN) and used at 10 ng/ml, and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum.

Cell Transfection—Transfection of BAEC was performed by Lipofectamine 2000 according to the manufacturer’s protocol (Invitrogen). Cells were cultured at 90% confluence in 6-well plates and were transfected with total of 4 μg of plasmid constructs as indicated. Cells were harvested at 36–48 h after transfection, and cell lysates were used for protein assays.

Isolation of SOCS1-deficient Mouse Lung EC (MLEC)—SOCS1<sup>−/−</sup> and SOCS3<sup>−/−</sup> mice were from Dr. James Ihle (St. Jude Children’s Research Hospital, Memphis, TN) (15, 24). MLEC isolation was performed as we described (25) followed by immunoselection and immortalization modified from the protocol described by Lim et al. (26). For immunoselection, 10 μl of beads (per T-75 of mouse lung cells) were washed three times with 1 ml of buffer A (phosphate-buffered saline + 2% fetal bovine serum) and resuspended in 100 μl of buffer A. 10 μl (10 μg) of anti-mouse ICAM-2 or 10 μl (10 μg) of PECAM-1 were added and rocked at 4 °C for 2 h. Beads were washed three times and resuspended in 160 μl of buffer B. Confluent mouse lung cells cultured in a T-75 flask were placed at 4 °C for 5 min and incubated with the beads at 4 °C for 1 h. Cells were then washed with warm phosphate-buffered saline and treated with 3 ml of warm Trypsin/EDTA. When cells were detached, 7 ml of growth media were added. An empty 15-ml tube in the magnetic field was placed on the holder, and the cell suspension (∼10 ml) was added slowly by placing the pipette on the wall of the tube so that the cells pass through the magnetic field. Cells were incubated for 5 min, and the media were carefully aspirated. The 15-ml tube was removed from the magnetic holder, and the beads/cells were resuspended in 10 ml of media. The selected cells were plated on 0.2% gelatin-coated flasks and cultured for 3–7 days. When the cells were confluent, another round of immunoselection was repeated.

Antibody Array Screening—The antibody array membranes were provided by Dr. Y. Eugene Chin (Brown University School of Medicine, Providence, RI) (27). 100 polyclonal or monoclonal antibodies, including those against SOCS family proteins, were immobilized on polyvinylidene difluoride membranes (5 by 5 cm) at predetermined positions. The antibody array membranes were then incubated with 5% milk at room temperature for 2 h followed by incubation with cell lysates from BAEC in the presence or absence of HA-tagged ASK1. After incubation for 2 h, the membranes were washed three times with phosphate-buffered saline with 0.1% Tween 20 and blotted with horseradish peroxidase-conjugated anti-HA antibody (Roche Diagnostics) for 2 h followed by three washes and enhanced chemiluminescence (ECL) detection.

Immunoprecipitation and Immunoblotting—EC (HUVEC, BAEC, or MLEC) after various treatments were washed twice with cold phosphate-buffered saline and lysed in 1.5 ml of cold lysis buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% Triton X-100, 0.75% Brij 96, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM sodium pyrophosphatase, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2 mM phenylmethylsulfonyl fluoride, 1 mM EDTA) for 20 min on ice. Protein concentrations were determined with a Bio-Rad kit. For immunoprecipitation to analyze protein interaction in vivo, 400 μg of cell lystate supernatant were preclarified by incubating with 5 μg of normal rabbit serum plus protein A/G-agarose beads on rotator at 4 °C overnight. The lysates were then incubated with 5 μg of the first protein-specific antisera (e.g. anti-SOCS1 from Medical and Biological Laboratory) for 2 h with 50 μl of protein A/G-agarose beads. The immune complexes were collected after each immunoprecipitation by centrifugation at 14,000 × g for 10 min followed by four washes with lysis buffer. The immune complexes were subjected to SDS-PAGE followed by immunoblot (Immobilon P, Millipore, Milford, MA) with the second protein (e.g. ASK1)—specific antibody (H300, Santa Cruz Biotechnology). The chemiluminescence was detected using an ECL kit according to the instructions of the manufacturer (Amersham Biosciences). For detection of FLAG-tagged proteins (e.g. SOCS proteins), anti-FLAG M2 antibody (Sigma) was used for immunoblot. For detection of HA-tagged proteins (ASK1 and elongin B/C), anti-HA antibody (Roche Diagnostics) was used for immunoblot.

ASK1 and JNK Kinase Assays—ASK1 and JNK assays were performed as described previously (4, 5) using GST-MKK4 and GST-c-Jun(1–80) fusion protein as a substrate, respectively. Briefly, a total of 400 μg of cell
lysates was immunoprecipitated with 5 μg of antibody against ASK1 or JNK1 (Santa Cruz Biotechnology). The immunoprecipitates were mixed with 10 μg of GST-MKK4 or GST-c-Jun-(1–80) suspended in the kinase buffer (20 mM Hepes, pH 7.6, 20 mM MgCl₂, 25 mM β-glycerophosphate, 100 μM sodium orthovanadate, 2 mM dithiothreitol, 20 μM ATP) containing 1 μl (10 μCi) of [γ-³²P]ATP. The kinase assay was performed at 25 °C for 30 min. The reaction was terminated by the addition of Laemmli sample buffer, and the products were resolved by SDS-PAGE (12%) followed by protein transferring to a membrane (Immobilon P). The phosphorylated GST-MKK4 or GST-c-Jun-(1–80) was visualized by autoradiography. The membrane was further used for Western blot with anti-ASK1 or anti-JNK1.

**GST-SOCS1 Pull-down Assay**—GST fusion protein preparation and GST pull-down assay were performed as described previously (4, 5). Briefly, GST-SOCS1 fusion proteins expressed in Escherichia coli XL-1 blue were affinity-purified on glutathione-Sepharose beads (Amersham Biosciences). 400 μg of cell lysates expressing HA-tagged ASK1 were incubated overnight at 4 °C with 10 μg of GST-SOCS1 bound to glutathione-Sepharose in the lysis buffer. The beads were washed four times with the lysis buffer before the addition of boiling Laemmli sample buffer. Bound ASK1 proteins were resolved on SDS-PAGE and detected with the lysis buffer before the addition of 10 μg of antibody against ASK1 or JNK1. This was visualized by autoradiography. The membrane was further used for Western blot with anti-ASK1 or anti-JNK1.

**Quantitation of Apoptotic Cell**—Cell killing assays were performed as described previously with a modification (5, 8, 10, 13). The propidium iodide (PI) exclusion method for loss of integrity of cell membranes was used to assess viability. In brief, cells were suspended in phosphate-buffered saline containing 25 μg/ml PI for 5 min at 37 °C and then subjected to analytic cell flow cytometry on a FACSort (BD Biosciences) immediately after labeling. A light scatter gate was set up to eliminate cell debris from the analysis. The PI fluorescence signal was recorded on the FL3 channel and analyzed by using CellQuest software. Phosphatidylserine translocation, which precedes loss of PI exclusion in apoptotic cell death, was assessed by annexin V-fluorescein isothiocyanate translocation, which precedes loss of PI exclusion in apoptotic cell debris from the analysis. The PI fluorescence signal was recorded on a FACSort (BD Biosciences) immediately after labeling. A light scatter gate was set up to eliminate cell debris from the analysis. The PI fluorescence signal was recorded on the FL3 channel and analyzed by using CellQuest software. Phosphatidylserine translocation, which precedes loss of PI exclusion in apoptotic cell death, was assessed by annexin V-fluorescein isothiocyanate staining kit (Roche Diagnostics) following the manufacturer’s protocol. For nuclear morphology, cells were stained with 4′,6-diamidino-2-phenylindole, and apoptotic cell (nuclei condensation) were visualized under UV microscope.

**RNA Isolation and Quantitative Real-time RT-PCR**—Total RNA was isolated from EC with a Qiagen RNeasy mini kit (Qiagen Inc., Valencia, CA) as recommended by the supplier. Total RNA was quantitated by OD at 260 using a Du-64 spectrophotometer (Beckman Instruments). Using an equal amount of total RNA (200 ng) from EC, stimulated under control or stimulated conditions, cDNA was synthesized from mRNA by TaqMan reverse transcription with MultiScribe reverse transcriptase (Applied Biosystems, Foster, CT) according to the manufacturer’s instructions. The final cDNA product was used for subsequent cDNA amplification by polymerase chain reaction. cDNA was amplified and quantitated by using SYBR Green PCR reagents from Applied Biosystems according to the manufacturer’s instructions. Briefly, the cDNA for the specific genes (E-selectin, VCAM-1, SOCS1) and 18 S rRNA were amplified by AmpliTaq Gold DNA polymerase using specific primers, which were synthesized by Yale Howard Hughes Medical Institute/Keck oligonucleotide synthetic facility (Yale University School of Medicine, New Haven, CT). The cDNA for 18 S rRNA was amplified by using a specific forward primer (5′-TTC CGA TAA CGA ACG AGA CTCT-3′) and a specific reverse primer (5′-TGG CTG AAG GCC ACT TGT C-3′). The following specific forward and reverse primers were used to amplify the gene of interest: SOCS1, 5′-TCC GTT CGC ACG CCG ATT AC-3′ and 5′-TCA ATG CTG GAA GGG GAA GG-3′; E-selectin, 5′-CAT CCA ACG AAC CAA AGC TCG-3′ and 5′-GCC ACT TGC AGG TGT AACTATT-3′; VCAM-1, 5′-AGT TGG GGA TAC GGT GTT TCT TCT-3′ and 5′-CCC CTC ATT CCT TAC CAC CC-3′. The PCR reaction mixture (final volume 25 μl) contained 5 μl of cDNA, 1 μl of 10 μM forward primer, 1 μl of 10 μM reverse primer, 2.5 μl of PCR 10× SYBR Green PCR buffer, 3 μl of 25 mM MgCl₂, 2 μl of dNTP mix (2.5 mM dATP, 2.5 mM dCTP, 2.5 mM dGTP, and 5 mM dUTP), 0.25 μl of AmpliTaq DNA polymerase (1 unit/μl uracil-N-glycosylase), 0.125 μl of AmpliTaq Gold DNA polymerase (5 units/μl of AmpliTaq Gold DNA polymerase), and 10.125 μl of H₂O. The PCR reaction was performed in triplicate (3 wells of C96-well plate). The reaction was amplified with iCycler iQ multi-color real time PCR detector (Bio-Rad) for 37 cycles with melting at 94 °C for 30 s, an annealing at 58 °C for 30 s, and extension at 72 °C for 1 min in iCycler iQ PCR 96-well plates (Bio-Rad). The relative quantification values for the interest gene expression were calculated from the accurate CT, which is the PCR cycle at which an increase in reporter fluorescence from SYBR Green dye can be first detected obtained above a baseline signal. CT values for 18 S rRNA cDNA were subtracted from CT values for the interest gene cDNA for each well to calculate −CT. The triplicate −CT values for each sample were averaged. To calculate the fold induction of the interest gene mRNA in cells treated with cytokines over control cells, the averaged −CT values calculated for control cells were subtracted from −CT values calculated for cytokine-treated cells to calculate −CT. Then, the fold induction for each well was calculated by using the 2−(−CT) formula. The fold induction value for triplicate wells was averaged, and data are presented as the mean ± S.E. of triplicate wells.

**RESULTS**

**SOCS1 Binds to ASK1 and Induces ASK1 Degradation**—To understand the mechanism of ASK1 degradation, we performed antibody arrays using HA-ASK1 as bait (see "Experimental Procedures"). Results showed that ASK1 interacted with several members of the SOCS protein family that have been implicated in protein degradation. This result prompted us to reason that SOCS family protein may target ASK1 to degradation machinery. We first confirmed the interaction of ASK1 with SOCS proteins by co-immunoprecipitation assays. ASK1 was co-transfected with FLAG-tagged SOCS1, -2, -3, -6, or CIS into bovine EC (BAEC). Expression of SOCS and ASK1 was determined by Western blot with anti-FLAG and anti-ASK1 antibody, respectively. SOCS proteins showed similar levels in expression (Fig. 1a). Interestingly, ASK1 expression was dramatically different in the presence of different SOCS proteins. Co-expression of SOCS1 and SOCS3 significantly decreased ASK1 expression (Fig. 1b). Association of ASK1 with SOCS was determined by immunoprecipitation with anti-FLAG antibody followed by Western blot with anti-ASK1 antibody. SOCS1 and SOCS3 strongly bind to ASK1 (Fig. 1c). These data suggest that SOCS1 and SOCS3 might bind to ASK1 and induce ASK1 degradation. We focused on SOCS1 for further studies to determine the role of SOCS1 in ASK1 degradation.

**The SH2 Domain of SOCS1 Is Critical for the Association with ASK1**—To define the critical domain of SOCS1 for ASK1 binding and degradation, we generated SOCS1 truncated mutants in GST- or FLAG-tagged constructs (5). SOCS1-DC contains a deletion of the C-terminal SOCS box, and SOCS1-DN contains a deletion of the N-terminal domain (Fig. 2a). Preliminary data indicated that both SOCS1-DC and SOCS1-DN bind to ASK1 in a GST pull-down assay (not shown), suggesting that the SH2 domain might be critical for ASK1 interaction. We then mutated the Arg-105 (to Lys) within the SH2 domain of SOCS1, which has been previously shown to be crit-
SOCS1 Induces ASK1 Degradation

**FIGURE 1.** SOCS family proteins bind to ASK1 and induce ASK1 degradation. FLAG-tagged SOCS proteins (SOCS1, 2, 3, 6 and CIS) were transfected into BAEC in the presence of ASK1. Expression of SOCS and ASK1 was determined by Western blot with anti-FLAG (a) and anti-ASK1 antibody (b), respectively. Association of ASK1 with SOCS1 was determined by immunoprecipitation with anti-FLAG antibody followed by Western blot with anti-ASK1 antibody. Normal mouse serum (NS) was used as a control (c). SOCS proteins in the immunoprecipitates were determined by Western blot with peroxidase-conjugated anti-FLAG antibody (FLAG-POD). IB, immunoblot. VC, vector control.

**FIGURE 2.** The SH2 domain of SOCS1 is critical for ASK1 binding and degradation. a, schematic diagram of SOCS1 domains and expression constructs. SOCS1-RK contains a mutation at Arg-105 (to Lys) within the SH2 domain. SOCS1-DC has a deletion of the C-terminal SOCS box, and SOCS1-DN contains a deletion of the N-terminal (N) domain. Red, amino acids. b, the SH2 domain of SOCS1 is critical for ASK1 binding in vitro. Cell lysates expressing ASK1 were used in a GST pull-down assay using GST-SOCS1-WT, SOCS1-RK, or SOCS1-DC as bait. Bound ASK1 was determined by Western blot with anti-ASK1 antibody. GST and GST-SOCS1 proteins were determined by Western blot with anti-GST antibody. IB, immunoblot. c, the SH2 domain of SOCS1 is critical for ASK1 binding and degradation in EC. ASK1 was co-transfected with FLAG-SOCS1 constructs (WT, RK, and DC) in EC. Expression of ASK1 and SOCS1 was determined by Western blot with anti-ASK1 antibody, GST and GST-SOCS1 proteins were determined by Western blot with anti-GST antibody. IB, immunoblot. d, elongin B/C binds to SOCS1-WT but not SOCS1-DC. BAEC were co-transfected with HA-elongin B, HA-elongin C, and FLAG-SOCS1 (WT or DC). Expression of elongin B/C and SOCS1 was determined by Western blot with anti-HA antibody. IB, immunoblot. VC, vector control.

**FIGURE 3.** The phosphorylation of ASK1 at tyrosine-718 is critical for the association with SOCS1. a, GST pull-down assay. BAEC were transfected with ASK1 mutants as indicated. Expression of ASK1 in the input was determined by Western blot with anti-ASK1. Association of ASK1 with SOCS1 was determined by a GST-SOCS1 pull-down assay, and bound ASK1 was determined by Western blot with anti-ASK1 antibody. GST-SOCS1 in the pull down was determined by Western blot with anti-GST. IB, immunoblot. b, immunoprecipitation assay. BAEC were co-transfected with ASK1 mutant and SOCS1 (FLAG). Expression of ASK1 in the input was determined by Western blot with anti-ASK1. Association of ASK1 with SOCS1 was determined by immunoprecipitation with anti-FLAG antibody followed by Western blot with anti-ASK1 antibody. SOCS1 protein in the immunoprecipitates was determined by Western blot with anti-FLAG-POD (peroxidase-conjugated anti-FLAG antibody).

The Phosphorylation of ASK1 at Tyrosine-718 Is Critical for the Association with SOCS1—To map the critical phosphotyrosine residue(s) on ASK1 critical for SOCS1 binding, we mutated the tyrosine residues that are potentially phosphorylated (Tyr-321, Tyr-435, Tyr-625, Tyr-718, Tyr-983) to phenylalanine (YF mutants). BAEC were transfected with ASK1-WT or ASK1-YF in the presence of SOCS1, association of ASK1 with SOCS1 was determined by both co-immunoprecipitation (Fig. 3a) and GST-SOCS1 pull-down assays (Fig. 3b). A mutation of ASK1 at Tyr-718 (ASK1-Y718F) diminished the binding to SOCS1 in both assays. In contrast, mutations at other sites on ASK1 retained the ability in SOCS1 binding (Fig. 3, a and b). These data suggest that the Tyr-718 residues on ASK1 are potentially phosphorylated and are critical for the binding to SOCS1.

ER blot with anti-ASK1. Results showed that ASK1 bound to SOCS1-WT and SOCS1-DC, but not SOCS1-RK, suggesting that the SH2 domain in SOCS1 is critical for ASK1 binding (Fig. 2b).

Association of ASK1 and SOCS1 was further determined by co-immunoprecipitation assay. FLAG-tagged SOCS1 was co-transfected with ASK1, and association of ASK1 with SOCS1 was determined by immunoprecipitation with anti-FLAG antibody (for SOCS1) followed by Western blot with anti-ASK1 antibody. Consistent with the observation in Fig. 2b, SOCS1-WT and SOCS1-DC, but not SOCS1-RK, associated with ASK1. Unlike SOCS1-WT, SOCS1-DC failed to induce ASK1 degradation, although it had an ability in binding to ASK1 (Fig. 2c). SOCS box contains a conserved elongin B/C-binding motif (BC box) and mediates interaction with elongin B/C complex. In turn, the elongin B/C complex associates with the putative ubiquitin ligase cullin-2, leading to protein degradation. To confirm that SOCS1-DC is defective in binding to elongin B/C, FLAG-SOCS1, HA-tagged elongin B, and elongin C were co-transfected in EC, and association of SOCS1 with elongin B/C was determined by immunoprecipitation assay with anti-FLAG antibody followed by Western blot with anti-HA antibody. As expected, elongin B/C associated with SOCS1-WT but not with SOCS1-DC (Fig. 2d). Taken together, these data suggest that both SH2 domain (for ASK1 binding) and SOCS box (for elongin B/C binding) in SOCS1 are required for SOCS1-mediated ASK1 degradation.
Regulation of Endogenous ASK1–SOCS1 Complex in EC by TNF—To determine the endogenous ASK1–SOCS1 complex and its regulation in EC, human EC (HUVEC) were cultured in the absence or presence of a proteasomal inhibitor MG132 (20 μM) for 8 h followed by treatment with TNF (10 ng/ml). Protein expression of ASK1 was determined by Western blot with anti-ASK1 antibody. Results showed that ASK1 accumulated by MG132 (Fig. 4a), suggesting that ASK1 in EC is a labile protein. ASK1–SOCS1 complex was determined by immunoprecipitation with anti-SOCS1 antibody followed by Western blot with anti-ASK1 antibody. ASK1–SOCS1 complex was significantly stabilized by MG132 (Fig. 4b, lane 3). However, TNF induced dissociation of ASK1 from SOCS1 (Fig. 4b, lane 4). Similar results were obtained in a reciprocal immunoprecipitation assay (i.e. cell lysates were immunoprecipitated with anti-ASK1 followed by Western blot with anti-SOCS1) (Fig. 4c). Interestingly, TNF-induced dissociation of ASK1 from SOCS1 resulted in stabilization of ASK1 (Fig. 4, a and c, compare −TNF versus + TNF), consistent with a role of SOCS1 in ASK1 degradation.

To determine the mechanism by which TNF induces disruption of ASK1–SOCS1 complex in EC, we reasoned that TNF induces dephosphorylation of ASK1 at tyrosine residues. To test this hypothesis, we first determined the phosphotyrosine of ASK1 in EC. HUVEC were cultured in the absence of growth factors (ECGS) followed by treatment with vascular endothelial growth factor or IGF-1 (50 ng/ml) for 15 min, and the phosphotyrosine of ASK1 was determined by immunoprecipitation by anti-ASK1 followed by Western blot with anti-phosphotyrosine antibody. Vascular endothelial growth factor and IGF-1 strongly induced phosphorylation of ASK1 (Fig. 4d). To determine the effect of TNF on ASK1 tyrosine phosphorylation, HUVEC were cultured in the presence of ECGS followed by treatment with TNF (10 ng/ml) for 15 min. Tyrosine phosphorylation in ASK1 was determined. Results showed that TNF significantly reduced the extent of tyrosine phosphorylation in ASK1 concomitant with increased ASK1 stabilization (Fig. 4e). Taken together, these data suggest that TNF induces dephosphorylation of ASK1, resulting in disruption of ASK1–SOCS1 complex leading to ASK1 stabilization and activation.

ASK1 Expression and Activity Are Elevated in SOCS1−deficient Mice and EC—SOCS1-KO mice usually die at 2–3 weeks after birth due to uncontrolled inflammation and show hypersensitive to TNF (15, 16). We reasoned that hypersensitiveness to TNF is, at least in part, due to increased ASK1 expression and activity. To test this hypothesis, we compared the expression and activity of ASK1 in lung tissues from SOCS1-KO and normal C57BL/6 (WT) mice. The absence of SOCS1 protein in lung tissues from SOCS1-KO mice was confirmed by Western blot with anti-SOCS1 antibody (Fig. 5a). Endogenous ASK1 expression and activity were determined by Western blot with anti-ASK1 and by an in vitro kinase assay using GST-MKK4 as a substrate, respectively. Results showed that ASK1 expression was up-regulated by 2-fold concomitant with a 4-fold increase in ASK1 activity (Fig. 5a). To determine the effect of SOCS-deficiency on ASK1 activity in EC, we isolated MLEC from SOCS1-KO mice and, the absence of SOCS1 expression in SOCS1-KO cells was confirmed by RT-PCR for mRNA (Fig. 5b) and Western blot with anti-SOCS1 antibody for protein (Fig. 5c). Endogenous ASK1 protein level was determined by Western blot with anti-ASK1 antibody. Results showed that the level of ASK1 in SOCS1-KO MLEC was significantly elevated when compared with that in MLEC from normal C57BL/6 mice (WT) (Fig. 5c). In contrast, TRAF2, an upstream activator of ASK1 implicated in TNF signaling and regulated by proteasomal degradation via a distinct (cellular inhibitor of apoptosis (cIAP)-dependent) pathway (30), was not altered in SOCS1-KO cells (Fig. 5c). These data suggest a specificity of SOCS1 for ASK1. TNF-induced ASK1 and JNK activation was also examined in normal and SOCS1-KO MLEC. Cells were treated with TNF (10 ng/ml) for various time points (0, 5, 10, 15, 30 min), and activation of ASK1 and its downstream JNK was determined by an in vitro kinase assay using GST-MKK4 and GST-c-Jun as a substrate, respectively. Consistent with the observation in the tissue, ASK1 expression was up-regulated concomitant with an increase in JNK activation (Fig. 5d).
FIGURE 5. ASK1 expression and ASK1-JNK signaling are elevated in SOCS1-deficient mouse tissue and derived EC. a, ASK1 expression and activity were elevated in SOCS1-KO mouse lung tissue. 50 μg of lung lysates from SOCS1-KO and WT mice were applied to Western blot with anti-SOCS1 and ASK1 antibodies. β-tubulin was used for normalization. ASK1 activity in the lysates was determined by an in vitro kinase assay using GST-MKK4 as a substrate. b, immunoblot. c, SOCS1 expression in MLEC derived from normal and SOCS1-KO mice. Total RNA from normal and SOCS1-KO MLEC were isolated, and expression of SOCS1 was determined by RT-PCR using a pair of SOCS1-specific primers (conserved between human and mouse, see “Experimental Procedures”). A reaction without template was used as a negative control, and a reaction with the human SOCS1 cDNA as a template was used as a positive control. Mr, molecular weight. d, expression of ASK1, JNK, and IκBα was determined by Western blot with anti-ASK1, anti-JNK, and anti-IκBα antibody, respectively.

Critical Roles of SOCS1 in TNF-induced Inflammatory Responses in EC—ASK1-JNK signaling has been shown to be critical for TNF-induced inflammatory responses (5, 8, 10, 13). To determine the role of SOCS1 in TNF-induced inflammatory responses, we determined TNF-induced gene expression of proinflammatory molecules in normal and SOCS1-KO MLEC. MLEC were either untreated or treated with TNF (10 ng/ml) for 4 h. Gene expression of proinflammatory molecules, E-selectin and VCAM-1, the expression of which is known to be mediated by TNF-induced JNK-dependent c-Jun/ATF2 activation, was determined by a real-time RT-PCR. Results show that expression of E-selectin and VCAM-1 was significantly enhanced in SOCS1-KO cells (Fig. 6a). We further determined the protein expression of E-selectin. MLEC were untreated or treated with TNF (10 ng/ml) for 4 h when E-selectin surface expression peaks (31), and endogenous E-selectin surface expression on EC was determined by fluorescence-activated cell sorter analysis with anti-mouse E-selectin. Consistent with previous observations in HUVEC and BAEC (8), TNF (+cycloheximide) strongly induced MLEC apoptosis (~11%) (Fig. 6c). Similar to TNF-induced ASK1-JNK signaling, TNF-induced apoptosis was also significantly enhanced in SOCS1-KO MLEC (~25%) (Fig. 6c). Similar data were obtained by PI exclusion and annexin V-fluorescein isothiocyanate staining assay (data not shown). Taken together, these data confirm that SOCS1 functions as an endogenous inhibitor in TNF-induced inflammatory and apoptotic responses in EC.

DISCUSSION

In this study, we have shown that SOCS1 forms a complex with ASK1 to regulate ASK1 stability and activity in primary EC. SOCS1 via its SH2 domain associated with the phosphotyrosine-718 on ASK1 to induce ASK1 degradation. ASK1 was phosphorylated at tyrosine residues in resting EC, and proinflammatory stimuli such as TNF-induced dephosphorylation and dissociation of ASK1 from SOCS1, leading to stabilization and activation of ASK1. Moreover, TNF-induced ASK1-JNK activity, and the associated inflammatory and apoptotic responses were significantly augmented in SOCS1-KO EC. Our study demonstrates that SOCS1 functions as a negative regulator in TNF-induced inflammatory and apoptotic responses, in part, by inducing ASK1 degradation (Fig. 7).

SOCS1 has been implicated as a suppressor in TNF signaling from previous studies in different cell types. Chong et al. (33) showed that SOCS1 deficiency in islet beta cells augmented TNF (+interferon-γ)-induced gene expression and cytotoxicity. TNF-induced activation of the p38 MAP kinase and p38-dependent inducible nitric oxide synthase expression appear to mediate the TNF effect. However, the mechanism
for the augmented p38 MAPK activation by TNF is not known. We have shown that SOCS1-KO EC have enhanced ASK1 activity, an upstream activator of p38 MAPK signaling. Furthermore, we have shown that SOCS1 directly binds to ASK1 and induces ASK1 degradation. It needs to be further determined whether SOCS1 also inhibits the kinase activity of ASK1. Nevertheless, our study provides the underlying mechanism by which SOCS1-KO mice display hypersensitivity to TNF (22).

Dr. Kishimoto's group (34) reported that SOCS1 blocked TNF-induced apoptosis in murine embryonic fibroblast, and inhibition of TNF-induced JAK kinases by SOCS1 appeared to be critical in this cell type. In contrast to the results from beta cells (33) and EC (our data), TNF-induced p38 MAPK activation is blunted in SOCS1-KO murine embryonic fibroblast (22). It is likely that SOCS1 suppresses TNF-induced apoptosis through multiple signaling pathways in a cell type-dependent manner.

Our data provided strong evidence for a vital role of SOCS1 in the regulation of ASK1 stability. In resting EC, SOCS1 and ASK1 formed a labile complex that can be stabilized by proteasomal inhibitors. Overexpression of SOCS1 in EC strongly induced ASK1 degradation. However, the SOCS1 mutants defective in the binding to ASK1 (SOCS1-R105K) or to elongin B/C (SOC1-DC) failed to do so, suggesting that SOCS1 targets ASK1 to an elongin-dependent degradation machinery. Furthermore, the expression and activity of ASK1 are significantly elevated in SOCS1-deficient EC. More importantly, proinflammatory cytokines such as TNF induced dephosphorylation of ASK1 and dissociation of ASK1 from SOCS1, leading to activation of ASK1-JNK-dependent inflammatory responses.

Our data strongly supported the finding that SOCS1 is an endogenous inhibitor of ASK1.

It has been shown that tyrosine phosphorylations of Jak2 and IRS1/2 are critical for their degradation by SOCS1. Jak2 and IRS1/2 are not phosphorylated in resting state. However, cytokines induce phosphorylation of these signaling molecules, leading to enhanced associations with SOCS1, providing a negative feedback mechanism for the regulation of Jak2 and IRS1/2 (18, 19, 29, 35). A single phosphotyrosine (p-Tyr-1007) on JAK2 is involved in the interaction with SOCS1 (29). Similarly, a single phosphotyrosine on ASK1 (p-Tyr-718) appears to be critical for SOCS1 binding. Phosphotyrosine of ASK1 is readily detected in resting EC, indicating that ASK1 is basally phosphorylated in response to growth factors in the culture media. Moreover, TNF induces dephosphorylation of ASK1 at tyrosine residues, leading to dissociation of ASK1 from SOCS1. The kinase(s) and phosphatase(s) responsible for ASK1 phosphorylation/dephosphorylation at Tyr-718 are not known. It has been recently shown that IGF-1 receptor can directly bind to and phosphorylate ASK1, leading to inhibition of ASK1 activity (36). However, the phosphotyrosine site(s) on ASK1 induced by IGF-1R has not been identified, and the role of IGF-1R in regulating ASK1-SOCS1 complex and ASK1 stability needs to be determined.
SOCS1 Induces ASK1 Degradation

Defining the kinases and tyrosine phosphatases regulating ASK1-SOCS1 interaction will provide important information regarding ASK1 regulation in patho/physiological settings.

We have previously reported that overexpression of the cytosolic form of thioredoxin (Trx1) induced ASK1 ubiquitination/degradation (5). Our recent data suggest that Trx1 associates with ASK1-SOCS1 complex through ASK1. This was demonstrated in a GST-Trx1 pull-down assay in which Trx1 pull down SOCS1 in the presence of ASK1-WT but not of ASK1-C250S (a mutant defective in Trx1 binding). Furthermore, knockdown of Trx1 by RNA interference stabilized ASK1 protein in EC. Based on these data, it is conceivable that Trx1 associates with ASK1-SOCS1 interaction will provide important information regarding ASK1

REFERENCES

1. Madge, L. A., and Pober, J. S. (2001) Exp. Mol. Pathol. 70, 317–325
2. Davis, R. J. (2000) Cell 103, 239–252
3. Matsuzawa, A., Nishitoh, H., Tobiume, K., Takeda, K., and Ichijo, H. (2002) Antioxid. Redox. Signal 4, 415–425
4. Liu, Y., Yin, G., Surapisitchat, J., Berk, B. C., and Min, W. (2001) J. Clin. Investig. 107, 917–923
5. Liu, Y., and Min, W. (2002) Circ. Res. 90, 1299–1266
6. Yamazaki, H., Pan, S., Lee, R. T., and Berk, B. C. (2003) J. Clin. Investig. 115, 733–738
7. Nishitoh, H., Saitoh, M., Mochida, Y., Takeda, K., Nakano, H., Rothe, M., Miyazono, K., and Ichijo, H. (1998) Mol. Cell 2, 389–395
8. Zhang, R., Al-Lamki, R., Bai, L., Streb, J. W., Miano, J. M., Bradley, J., and Min, W. (2004) Circ. Res. 94, 1483–1491
9. Saitoh, M., Nishitoh, H., Fujii, M., Takeda, K., Tobiume, K., Sawada, Y., Kawabata, M., Miyazono, K., and Ichijo, H. (1998) EMBO J. 17, 2596–2606
10. Zhang, R., He, X., Liu, W., Lu, M., Hsieh, J. T., and Min, W. (2003) J. Clin. Investig. 111, 1933–1943
11. Song, J. J., Rhee, J. G., Suntharalingam, M., Walsh, S. A., Spitz, D. R., and Lee, Y. J. (2002) J. Biol. Chem. 277, 46566–46575
12. Zhang, L., Chen, J., and Fu, H. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 8511–8515
13. Zhang, R., Luo, D., Miao, R., Bai, L., Ge, Q., Sesia, W. C., and Min, W. (2005) Oncogene 24, 3954–3963
14. Yasukawa, H., Misawa, H., Sakamoto, H., Masuhara, M., Sasaki, A., Wakioki, T., Ohtsuka, S., Imazumi, T., Matsuda, T., Ilhe, J. N., and Yoshimura, A. (1999) EMBO J. 18, 1309–1320
15. Marine, J. C., Topham, D. J., McKay, C., Wang, D., Parganas, E., Stravopodis, D., Yoshimura, A., and Ilhe, J. N. (1999) Cell 98, 609–616
16. Alexander, W. S., Starr, R., Fenner, J. E., Scott, C. L., Handman, E., Spigg, N. S., Corbin, J. E., Cornish, A. L., Darwiche, R., Owczarek, C. M., Kay, T. W., Nicola, N. A., Hertzog, P. J., Metcalf, D., and Hilton, D. J. (1999) Cell 99, 597–608
17. Alexander, W. S., and Hilton, D. J. (2004) Annu. Rev. Immunol. 22, 503–529
18. Rui, L., Yuan, M., Frantz, D., Shoelster, S., and White, M. F. (2002) J. Biol. Chem. 12, 12
19. Mooney, R. A., Senn, J., Cameron, S., Inamdar, N., Boivin, L. M., Shang, Y., and Furlanetto, R. W. (2001) J. Biol. Chem. 276, 25889–25893
20. Ilgumunaran, S., and Rottapel, R. (2003) Immunol. Rev. 192, 196–211
21. Krebs, D. L., and Hilton, D. J. (2001) Stem Cells (Durham) 19, 578–387
22. Morita, Y., Naka, T., Kawazoe, Y., Fujimoto, M., Narazaki, M., Nakagawa, R., Fukuyama, H., Nagata, S., and Kishimoto, T. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 5405–5410
23. Chen, X. P., Losman, J. A., Coyne, E., Ferrand, M., Voung, B. Q., Nakajima, H., Cappe, D., Cohlan, V. L., and Rothman, P. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 2175–2180
24. Marine, J. C., McKay, C., Wang, D., Topham, D. J., Parganas, E., Nakajima, H., Pendeville, H., Yasukawa, H., Sasaki, A., Yoshimura, A., and Ilhe, J. N. (1999) Cell 98, 617–627
25. Pan, S., An, P., Zhang, R., He, X., Yin, G., and Min, W. (2002) Mol. Cell. Biol. 22, 7512–7523
26. Lim, Y. C., Garcia-Cardena, G., Alport, J. R., Zervoglos, M., Connolly, A. J., Gimbrone, M. A., Jr., and Luscinskas, F. W. (2003) Am. J. Pathol. 162, 1591–1601
27. Wang, T., Wu, T. R., Cai, S., Welte, T., and Chin, Y. E. (2000) Mol. Cell. Biol. 20, 4505–4512
28. De Sepulveda, P., Okkenhaug, K., Rose, J. L., Hawley, R. G., Dubreuil, P., and Rottapel, R. (1999) EMBO J. 18, 904–915
29. Ungureanu, D., Saharin, P., Junttila, I., Hilton, D. J., and Silvennoinen, O. (2002) Mol. Cell. Biol. 22, 3316–3326
30. Li, X., Yang, Y., and Ashwell, J. D. (2002) Nature 416, 345–347
31. Karmann, K., Min, W., Fanslow, W. C., and Pober, J. S. (1996) J. Exp. Med. 184, 173–182
32. Min, W., and Pober, J. S. (1997) J. Immunol. 159, 3508–3518
33. Chong, M. M., Thomas, H. E., and Kay, T. W. (2002) J. Biol. Chem. 277, 27945–27952
34. Kimura, A., Naka, T., Nagata, S., Kishimoto, T., and Ichijo, H. (2004) Int. Immunol. 16, 991–999
35. Emanuelelli, B., Peraldi, P., Filloux, C., Chavey, C., Freidinger, K., Hilton, D. J., Piotr, Misiligi, G. S., and Van Obberghen, E. (2001) J. Biol. Chem. 276, 47944–47949
36. Galvan, V., Logvinova, A., Sperandio, S., Cichijo, H., and Bredesen, D. E. (2003) J. Biol. Chem. 278, 13325–13332