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Involvement of the JAK-STAT Pathway and SOCS3 in the Regulation of Adiponectin-Generated Reactive Oxygen Species in Murine Macrophage RAW 264 Cells

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ABSTRACT

Adiponectin is a protein hormone produced by differentiating adipocytes and has been proposed to have anti-diabetic and immunosuppressive properties. We previously reported that the globular form of adiponectin (gAd) induces the generation of reactive oxygen species (ROS) and nitric oxide (NO), followed by caspase-dependent apoptotic cell death in RAW 264 cells. Here, we demonstrate that gAd-induced ROS generation and apoptosis were diminished by suppressor of cytokine signaling 3 (SOCS3). The phosphorylation level of signal transducer and activator of transcription (STAT) 3 detected by Western blotting was highest at 20 min in gAd-treated RAW 264 cells. This phosphorylation was inhibited by AG490, a specific inhibitor of janus-activator kinase (JAK). The gAd-induced ROS and NO were reduced by administration of AG490 and Jak-2-specific siRNA in RAW 264 cells. The gAd stimulation transiently induced SOCS3 mRNA expression and protein production. We examined SOCS3-overexpressing RAW 264 cells to investigate the role of the JAK-STAT pathway in gAd-induced ROS and NO generation. SOCS3 overexpression significantly reduced both ROS and NO generation. Additionally, gAd-induced caspase activation and apoptotic cell death were reduced in SOCS3 transfectants compared with vector control transfectants. These results suggest that the JAK-STAT pathway, which can be suppressed by SOCS3 expression, is involved in gAd-induced ROS and NO generation followed by apoptotic cell death. J. Cell. Biochem. 111: 597–606, 2010.

KEY WORDS: ADIPONECTIN; REACTIVE OXYGEN SPECIES; NITRIC OXIDE; SUPPRESSOR OF CYTOKINE SIGNALING 3; MACROPHAGE

Adiponectin, a 30-kDa protein secreted from adipocytes [Scherer et al., 1995], is closely related with insulin resistance in the regulation of glucose and lipid homeostasis and has potent anti-inflammatory activity [Berg et al., 2002; Ouchi and Walsh, 2007; Huang et al., 2008]. Adiponectin dampens the early phases of the macrophage inflammatory response by inhibiting the growth of myelomonocytic progenitor cells and decreasing the ability of mature macrophages to respond to activation [Yokota et al., 2000; Wulster-Radcliffe et al., 2004]. Adiponectin suppresses lipopolysaccharide (LPS)-stimulated NF-κB and ERK1/2 activation [Yamaguchi et al., 2005; Park et al., 2008; Kamio et al., 2009; Zacharioudaki et al., 2009]. Circulating adiponectin is present as several different isoforms, including low- (trimer), middle- (hexamers), and high-molecular-weight (18 mers) forms [Pajvani et al., 2003], and each adiponectin isoform participates in distinct biological functions. A recent report demonstrated that the high-molecular-weight adiponectin isoform has a greater insulin-sensitizing effect than the other isoforms [Wang et al., 2008]. The globular form of adiponectin (gAd), which is generated via proteolytic cleavage by leukocyte elastase secreted from activated monocytes and/or neutrophils, is much more biologically active than full-length adiponectin [Waki et al., 2005].

Cytokine-mediated signal transduction is naturally transient and closely regulated to maintain systemic and/or local immune homeostasis [Dalpke et al., 2008]. Hematopoietic cytokines act on membrane-bound receptors lacking kinase activity. However, the interleukin (IL)-6 receptor gp130 can be associated with janus-activated kinases (JAKs) [Heim, 1999]. JAKs induce tyrosine phosphorylation within receptor chains, which generate docking sites for signal transducer and activator of transcription (STAT) factors. Cytokine stimulation induces a conformational change in the homo- or hetero-dimers of STATs [Zhong et al., 2005]. In turn, STATs bind to phosphorylated cytokine receptor chains via Src homology 2 domains. After tyrosine phosphorylation by JAKs,

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STAT dimers dissociate from the receptor and translocate to the nucleus, where they act as transcription factors by binding to specific chromosomal DNA sequences. The JAK/STAT signal transduction pathway leads to a very rapid induction of gene activity.

Suppressor of cytokine signaling (SOCS) proteins regulate the intensity and duration of cytokine/growth factor signals and integrate multiple extracellular signals that may converge on target cells. The eight-member SOCS protein family attenuates cytokine signals through interactions with cytokine/growth factor receptors and signaling proteins, leading to proteosomal degradation of the receptor complex [Naka et al., 1998; Yasukawa et al., 1999; Sasaki et al., 2000]. SOCS proteins are rapidly induced in many cell types in response to cytokines, including interferon-γ, IL-1β, and IL-6, or growth factors, including ciliary neurotrophic factor, leukemia inhibitory factor, fibroblast growth factor, and insulin, and the effects of SOCS are transient due to their short half-life [Egwuagu et al., 2002; Yu et al., 2004; Samardzija et al., 2006]. However, constitutive SOCS expression occurs in some tissues owing to unabated stimulation by chronic inflammation or cellular stress; this leads to the silencing of critical cellular pathways and predisposition to organ-specific disease development.

We previously reported that gAd induces reactive oxygen species (ROS) and nitrite oxide (NO) generation followed by apoptosis in RAW 264 cell [Akifusa et al., 2008, 2009]. In the present study, our results indicate that gAd induces ROS and NO generation via the JAK-STAT signal transducing pathway. Additionally, SOCS3 overexpression partially reduced gAd-induced intracellular and mitochondrial ROS production, the amount of NO released, and the apoptotic population in RAW 264 cells.

METHODS

MATERIALS

Propidium iodide (PI), MitoSOX™, and YO-PRO®-1 were purchased from Molecular Probes (Invitrogen, Carlsbad, CA); 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was obtained from Sigma (St. Louis, MO); and 2-cyano-3-(3,4-dihydroxyphenyl)-*N*-(benzyl)-2-propenamide (AG490) were purchased from Calbiochem (Darmstadt, Germany). All cell culture reagents were obtained from Sigma.

CELL CULTURE

The murine macrophage-like cell line RAW 264 (RCB0535; RIKEN Cell Bank, Ibaraki, Japan) was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (Thermo Trace; Thermo Scientific, Rockford, IL), 2 mM L-glutamine, and 50 μg/ml gentamycin, at 37°C in an atmosphere of 5% CO_2. The SOCS3 transfectant and vector control (Neo) were kindly provided by Prof. A. Dalpke (University of Heidelberg, Germany). The RAW264.7 transfectants were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 μg/ml gentamycin, and 450 μg/ml G418 at 37°C in an atmosphere of 5% CO_2 [Dalpke et al., 2001].

PURIFICATION OF RECOMBINANT PROTEIN

A glutathione *S*-transferase (GST) fusion vector, pGEX-6P-1 (GE Amersham Biosciences, Uppsala, Sweden), containing the mouse adiponectin (gAd) globular domain was provided by Dr. I. Shimomura (Osaka University, Japan). Recombinant gAd was prepared as described previously [Akifusa et al., 2008]. Briefly, GST-gAd protein was produced in *Escherichia coli* BL21 and purified with glutathione-Sepharose 4B (GE Amersham Biosciences). GST was cleaved from the GST-gACRP30 protein by PreScission protease (GE Amersham Biosciences), and the purified recombinant gAd was applied to an Affi-Prep polymyxin column (Bio-Rad Laboratories, Hercules, CA) to remove endotoxin contaminants. The concentration of endotoxin in the obtained recombinant gAd was 7.05 pg/μg protein as determined with limulus amebocyte lysate assay (Wako, Tokyo, Japan), having no effect on secretion of IL-6, ROS generation, or apoptosis in RAW 264 cells.

ANALYSIS OF APOPTOSIS

During apoptosis, the cytoplasmic membrane is permeable to the green fluorescent dye YO-PRO, but is not permeable to the red fluorescent dye PI. Thus, the combination of YO-PRO and PI provides a sensitive indicator of apoptosis. Briefly, gAd-treated cells were harvested after incubation and washed in cold phosphate-buffered saline (PBS). The cell density was adjusted to 1×10^6 cells/ml in PBS, and YO-PRO and PI (100 nM each), obtained from a Vybrant apoptosis assay kit (Invitrogen), were added. After incubation for 20 min on ice, the samples were analyzed by flow cytometry, with excitation at 488 nm to visualize the YO-PRO green fluorescence (530/30 bandpass filter) and PI red fluorescence (610/20 bandpass filter). The data obtained from 10,000 cells were analyzed with Expo32 software (Beckman Coulter, Fullerton, CA). Living cells exhibited a low level of green fluorescence, apoptotic cells exhibited incrementally higher levels of green fluorescence, and dead cells revealed both red and green fluorescence.

DNA nick-end labeling of RAW 264 cells was carried out by using an in situ cell death detection kit (Roche Diagnostics GmbH, Mannheim Germany). The labeling target of the in situ cell detection kit is the multitude of new 3′-OH DNA ends generated by DNA fragmentation. Briefly, the cells, fixed with 4% paraformaldehyde in PBS, were blocked with 3% hydrogen peroxide in methanol, and then permialized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice. Apoptotic cells were labeled with terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) reaction mixture as described in the manufacturer's instructions and analyzed by flow cytometry.

CASPASE ASSAY

Caspase activity in gAd-treated cells was measured using colorimetric activity assay kits for caspases-3 and -9 (Chemicon, Rosemont, IL) according to the manufacturer's instructions. These assays are based on spectrophotometric detection of the chromophore *p*-nitroaniline (*p*NA) after cleavage from the labeled substrates DEVD-*p*NA and LEHD-*p*NA for casepases-3 and -9, respectively. Briefly, 1×10^6 cells were harvested and resuspended in 200 μl of chilled cell lysis buffer. The cells were incubated on ice

for 10 min and then centrifuged at 15,000*g* for 5 min. Supernatants were incubated with assay buffer and the respective caspase *p*NA-labeled substrate for 2 h at 37°C, and the release of *p*NA was measured by absorbance at 405 nm using a SpectraMax 340PC^{384} spectrometer (Molecular Devices).

ANALYSIS OF CELLULAR REDOX STATUS

To detect intracellular and mitochondrial ROS formation, measurements of H_2DCFDA and MitoSOX fluorescence were performed by flow cytometry. Cells were incubated for the indicated times at 37°C and 5% CO_2; then PBS containing 5 μM H_2DCFDA and 5 μM MitoSOX was added, and the cells were incubated for another 30 min. The labeled cells were washed twice with PBS and then suspended in PBS for analysis by flow cytometry. The fluorescence intensity of H_2DCFDA was measured using an excitation wavelength of 488 nm and emission wavelength of 580 nm, and MitoSOX was measured using excitation at 510 nm and emission at 580 nm. The resultant histograms were analyzed with the Expo32 program (Beckman Coulter) after subtraction of background fluorescence. Other method for evaluation of O_2^- generation in gAd treated cells was performed with chemiluminescence (CL) using the Diogenes™ Cellular Luminescence Enhancement System for Superoxide Detection reagent (National Diagnostics, Atlanta, GA). The CL was measured over a period of 30 min in a TR717TM Micropated Luminometer (Applied Biosystems, Bedford, MA). The gAd triggered CL was expressed as relative light units (rLU; mean ± SEM of three independent experiments).

ANALYSIS OF NITRITE STATUS

NO release in cultured macrophages was measured by a microplate assay method, as described previously [Park et al., 1996]. To measure nitrite (NO_2^-), 100 μl of macrophage culture supernatant were collected, mixed with an equal volume of Griess reagent (1% sulfanilamide/0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride/2.5% H_3PO_4), and incubated for 10 min at room temperature. The nitrite concentration was determined by measuring the absorbance at 540 nm using a SpectraMax 340PC384 spectrometer (Molecular Devices). For external calibration, $NaNO_2$ was used.

Total NOS activity in cell lysates was measured using NADPH cycling by glucose 6-phosphate dehydrogenase after optimization of the spectrophotometric assay, as described by Ghigo et al. [2006].

REAL-TIME RT-PCR

Total RNA from treated cells was reverse-transcribed to cDNA using Ready-To-Go You-Prime First-Strand Beads (GE Healthcare, Piscataway, NJ). The cDNA was used for TaqMan real-time quantitative PCR in a StepOne™ real-time PCR system (Applied Biosystems, Foster City, CA) following the manufacturer's instructions. PCR conditions were 95°C for 20 s followed by 40 cycles of 95°C for 1 s and 60°C for 20 s. Primer and fluorescent probe sets for SOCS3 (Mm01249143_g1) were purchased from Applied Biosystems. The β-actin primers (Mm00607939_s1) were used as controls in all samples. The critical threshold cycle (C_t) value, reflecting the cycle number at which the DNA amplification was first detected, was determined for each reaction. Relative transcript levels were calculated as $E = 2^{-\Delta C_t}$, where E is the gene expression value, and ΔC_t is the difference in crossing points between β-actin and the test genes.

WESTERN BLOTTING

Cell extracts were resolved by SDS–PAGE before being transferred to PVDF membranes (Bio-Rad Laboratories). After blocking, the membranes were incubated with primary and secondary antibodies then washed thoroughly and examined using ECL Plus (GE Healthcare). The band densities were scanned and quantified by

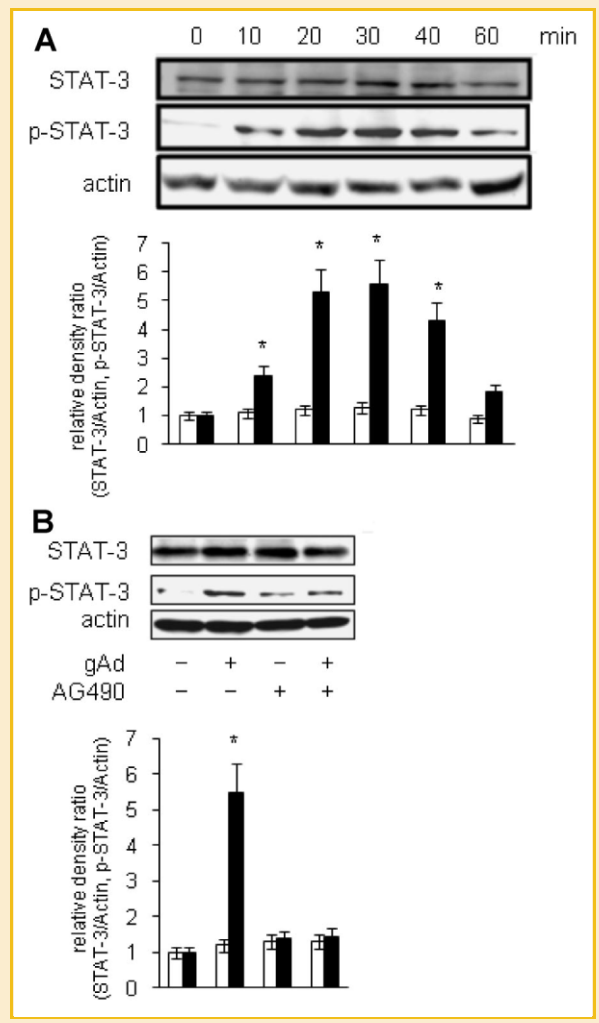

Fig. 1. Effect of gAd stimulation on STAT-3 phosphorylation in RAW 264 cells. A: Effect of gAd treatment on the phosphorylated STAT-3 (p-STAT-3) protein. Cells were exposed to gAd for the lengths of time indicated (0–60 min). Protein levels of STAT-3, p-STAT-3, and actin were analyzed by Western blotting. Quantified data of protein levels indicates in lower panel. B: Effect of AG490 on the gAd-induced phosphorylation of STAT-3. Cells were administrated by 10 μM AG490 for 1 h, and additional 20 min incubation with or without 10 μg/ml gAd were performed. Quantified data of protein levels indicates in lower panel. The relative density ratio at 0 min (A) or in a non-stimulated sample (B) was set at a value of 1.0. The values represent the mean ± standard deviation (SD) of three independent experiments (*$P < 0.05$). White bars indicate STAT-3/actin ratio, and black bars indicate p-STAT-3/actin ratio.

LAS-1000 plus (Fuji Film, Tokyo, Japan). Anti-STAT-3, phospho-STAT-3 (Tyr 705) (Applied Biological Materials, Richmond, ON, Canada), SOCS3-specific antibody (Abcam, Cambridge, MA), and anti-actin antibody (MP Biomedicals, Solon, OH) were used for the primary antibodies. HRP-linked anti-rabbit IgG antibodies (Cell Signaling Technology, Danvers, MA) and HRP-linked anti-mouse IgG antibodies (Zymed Laboratories, Carlsbad, CA) were used as secondary antibodies.

siRNA TRANSFECTION

Small-interfering RNA (siRNA) against Jak-2 and control non-specific siRNA were purchased from Invitrogen. RAW264 cells were transfected with each siRNA 10 pmol) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

STATISTICAL ANALYSIS

Statistical analysis was performed using Student's *t*-test, and $P < 0.05$ was considered significant.

RESULTS

ADIPONECTIN INDUCES STAT3 PHOSPHORYLATION

To investigate whether gAd activates the JAK-STAT pathway, we first conducted a Western blot analysis to detect STAT3 phosphorylation in gAd-treated RAW 264 cells. The time course revealed that STAT3 phosphorylation peaked at 20 min, decreased rapidly, and became undetectable after 50–60 min (Fig. 1A). The gAd-induced STAT3 phosphorylation was significantly reduced by pretreatment

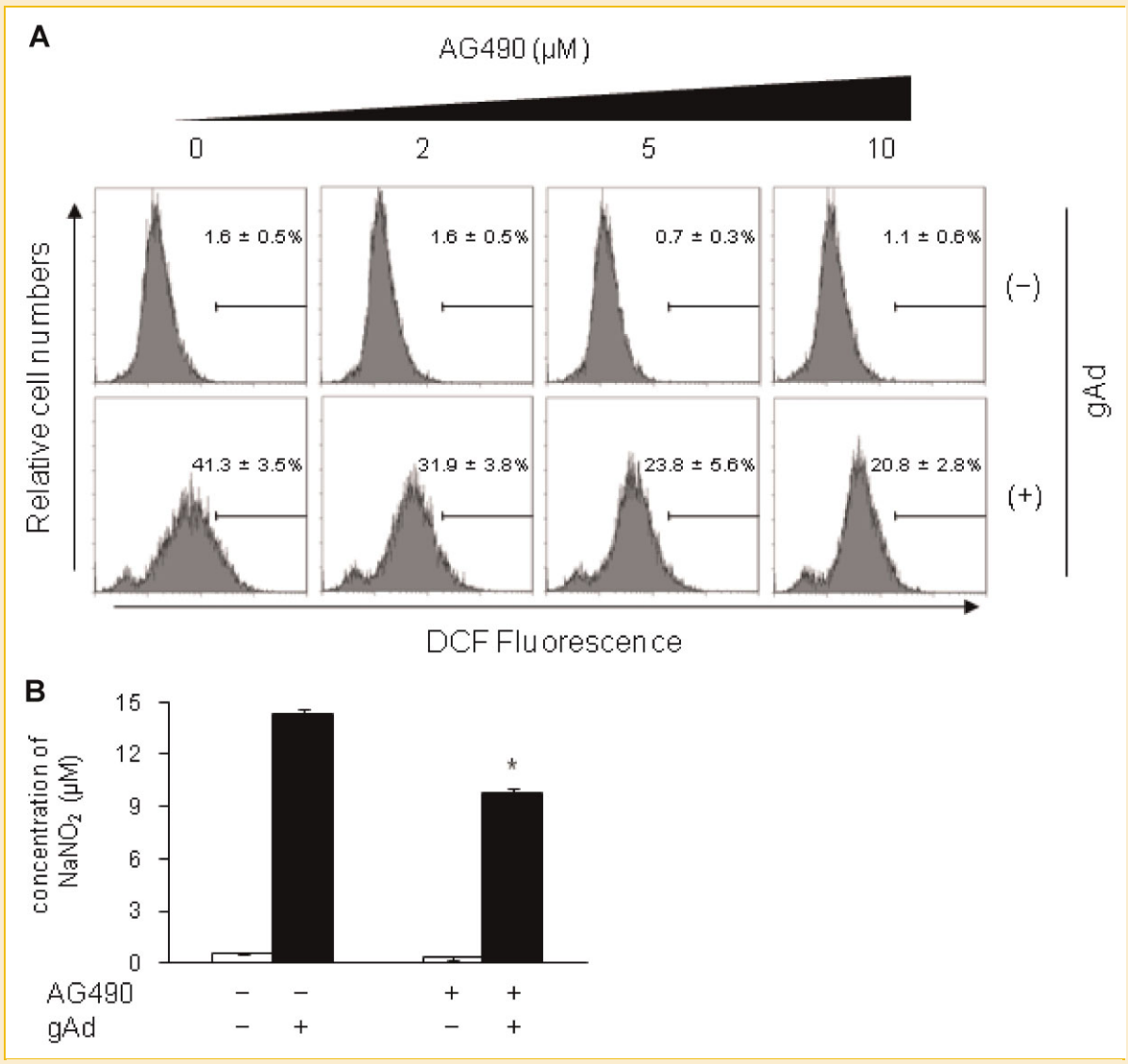

Fig. 2. Inhibitory effect of AG490 on ROS and NO generation in gAd-treated RAW 264 cells. RAW 264 cells were stimulated by 10 μg/ml gAd for 24 h. A: Dose-dependent effects of AG490 on gAd evoked intracellular ROS generation in RAW 264 cells. DCF-positive cell populations indicated intracellular ROS was measured by flow cytometric analysis as described in the Methods Section. Values are the means ± SD from three independent experiments. B: Effects of AG490 on NO release in gAd-treated RAW 264 cells. Values are the means ± SD from three independent experiments. Error bars indicate the SD. *$P < 0.01$ compared with untreated cells.

with AG490, a JAK2-specific inhibitor (Fig. 1B). These results suggest that gAd stimulation activates the JAK-STAT signaling pathway in RAW 264 cells.

ADIPONECTIN-INDUCED ROS AND NO GENERATION IS DEPENDENT ON JAK ACTIVITY

We previously reported that gAd stimulation induces ROS and NO generation in RAW 264 cells [Akifusa et al., 2008]. To assess the inhibitory effect of AG490 on gAd-evoked ROS generation, the intracellular DCF fluorescence-positive population was observed by flow cytometry. AG490 pretreatment also reduced the gAd-induced ROS generation detected by DCF fluorescence in a dose-dependent manner (Fig. 2A). After the administration of 10 μg/ml gAd, the percentage of the DCF-positive population that were AG490-pretreated cells (20.8 ± 2.8%) was decreased by approximately half, compared with the percentage of the population that did not receive the AG490 pretreatment (41.3 ± 3.5%). Similarly, AG490 pretreatment suppressed NO release from gAd-treated RAW 264 cells in a dose-dependent manner (Fig. 2B). The amount of gAd-induced NO release in 10 μM AG490-pretreated cells (9.7 ± 0.3 μM $NaNO_2$) was significantly lower than the NO amount in cells that were not administered AG490 (14.3 ± 0.3 μM $NaNO_2$).

To confirm the role of Jak-2 in gAd-induced ROS and NO generation, we further examined the effect of RNA interference of Jak-2 on the ROS and NO generation in gAd-treated RAW 264 cells. After transfection with Jak-2-specific siRNA or control siRNA, the gAd-induced intracellular and mitochondrial ROS were assessed

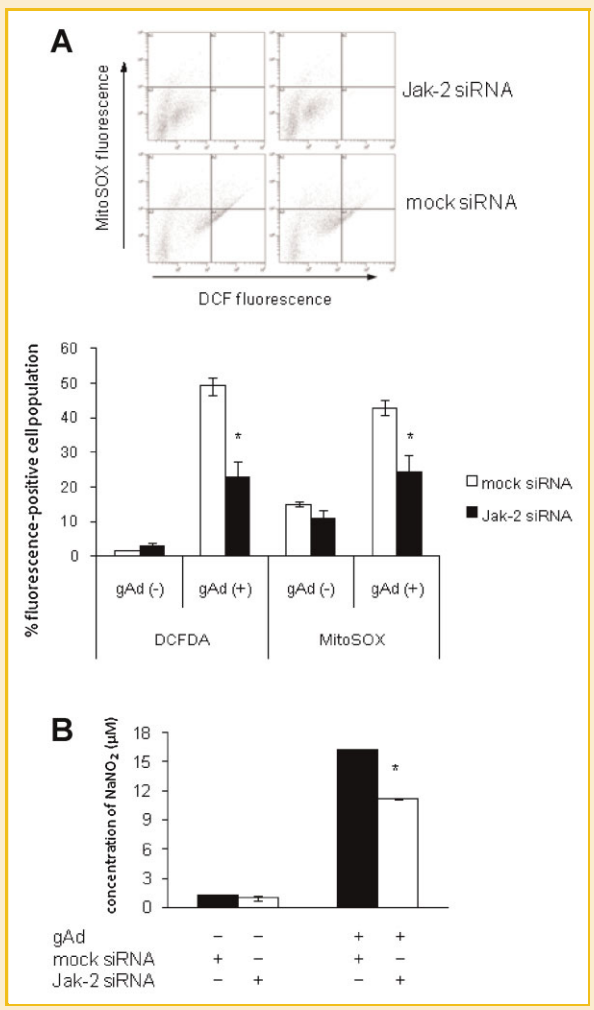

Fig. 3. Effect of Jak-2-specific siRNA on gAd-induced ROS and NO generation in RAW 264 cells. Cells transfected with mock or Jak-2-specific siRNA were treated with 10 μg/ml gAd for 24 h. A: Effect of Jak-2 siRNA on gAd-induced ROS generation DCF-positive and MitoSOX-positive cell populations indicate intracellular and mitochondrial ROS, respectively, and were measured by flow cytometric analysis as described in the Methods Section. The population ratio for each A2 + A3 (DCF-positive) and A1 + A2 (MitoSOX-positive) plotted area was calculated. The values represent the means ± SD of three independent experiments. $^*P < 0.01$ compared with mock siRNA. B: Effect of Jak-2 siRNA on gAd-induced NO generation. The amount of released NO was measured by the Griess method as described in the Methods Section. Values are the means ± SD (n = 3). $^*P < 0.01$ compared with mock control stimulated by gAd.

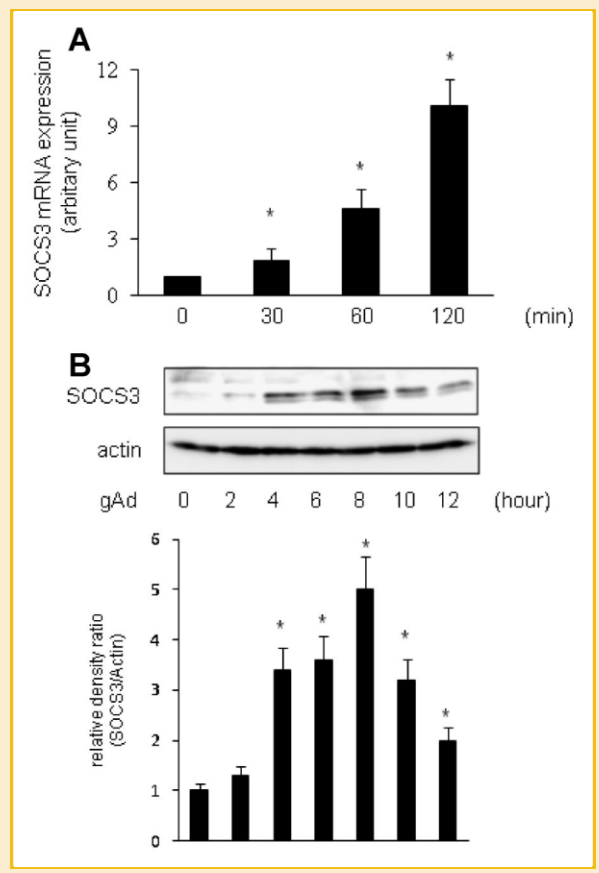

Fig. 4. Effect of gAd stimulation on SOCS3 in RAW 264 cells. A: Effect of gAd stimulation on the expression of SOCS3 mRNA. Cells were incubated with gAd for the times indicated. Values are the means ± SD (n = 3). B: Effect of gAd treatment on the SOCS3 protein level. Cells were exposed to gAd for the lengths of time indicated. Levels of SOCS3 and actin protein were analyzed by Western blotting. Quantified data of protein levels indicates in lower panel. The relative density ratio at 0 h was set at a value of 1.0. The values represent the mean ± standard deviation (SD) of three independent experiments ($^*P < 0.05$).

by the fluorescence of DCFDA and MitoSOX, respectively. The population of DCFDA- and MitoSOX-positive cells transfected with Jak-2-specific siRNA were markedly reduced (22.9 ± 3.4 and 24.4 ± 3.7, respectively), compared with cells transfected with control siRNA (49.2 ± 4.3 and 43.0 ± 5.2, respectively) in the presence of 10 μg/ml gAd (Fig. 3A). As shown in Figure 3B, the amount of gAd-induced NO release in Jak-2-specific siRNA-transfected cells (11.2 ± 1.0 μM $NaNO_2$) was significantly lower than the NO amount in control siRNA-transfected cells that were not administered AG490 (16.2 ± 1.3 μM $NaNO_2$).

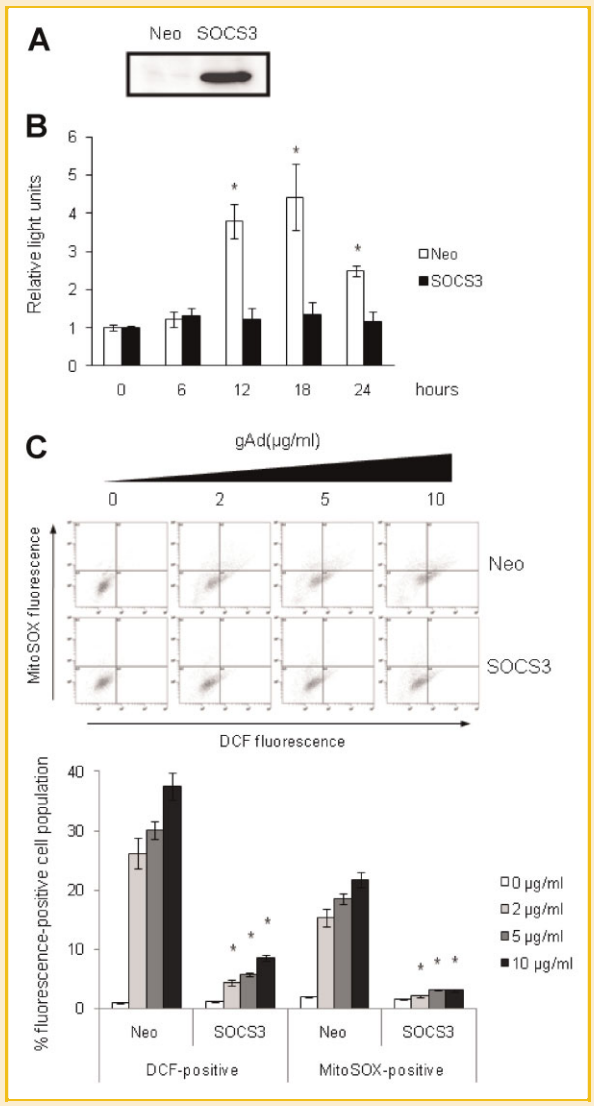

Fig. 5. Effect of SOCS3 overexpression on gAd-induced ROS generations in RAW 264 cells. A: SOCS3 protein level of each tranfectant detected by Western blotting. B: Intracellular ROS generation in gAd-treated SOCS3 and Neo transfectants. Each value represents the mean ± SD from three independent experiments. Each DCF fluorescence intensity of cell extract was detected as shown in Methods Section. The values represent the means ± SD of three independent experiments. $^{*}P < 0.01$ compared with Neo transfectants in each time point. C: DCF-positive and MitoSOX-positive cell populations indicate intracellular and mitochondrial ROS, respectively, and were measured by flow cytometric analysis as described in the Methods Section. The population ratio for each A2 + A3 (DCF-positive) and A1 + A2 (MitoSOX-positive) plotted area was calculated. The values represent the means ± SD of three independent experiments. $^{*}P < 0.01$ compared with Neo transfectants in each gAd concentration.

ADIPONECTIN INDUCES SOCS3 EXPRESSION

To further investigate the gAd-induced JAK-STAT signaling pathway, we assessed the gene expression level of SOCS3, an endogenous negative feedback agent of JAK-STAT signaling, in RAW 264 cells. Stimulation by gAd increased SOCS3 mRNA levels in a time-dependent manner: 4.6-fold at 60 min and 10.0-fold at 120 min after gAd treatment compared with the level at 0 min (Fig. 4A). The SOCS3 protein level in RAW 264 cells peaked at 8 h and then slowly decreased, but still remained until 12 h after gAd treatment (Fig. 4B).

SOCS3 OVEREXPRESSION SUPPRESSES ADIPONECTIN-INDUCED ROS AND NO GENERATION

To investigate the effect of SOCS3 on gAd-induced ROS and NO generation, we compared SOCS3-overexpressing transfectants with Neo vector control transfectants. As shown in Figure 5A, the SOCS3-transfectant stably expressed SOCS3 protein, but not Neo transfectant. The gAd stimulated ROS formation in Neo transfectants occurred in a time-dependent manner; peaked at 4.4 ± 0.9-fold after 18 h, and then gradually decreased (Fig. 5B). In contrast, there was no significant change in ROS formation in gAd-treated SOCS3 transfectants. Next, we examined intracellular and mitochondrial ROS generation as detected by DCF and MitoSOX fluorescence,

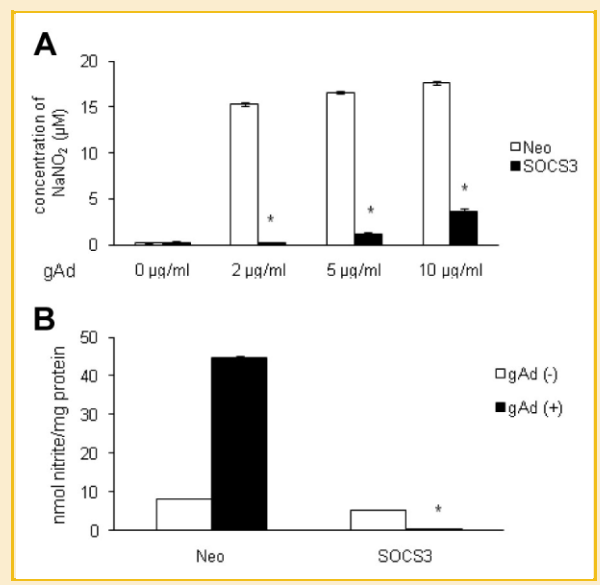

Fig. 6. Effect of SOCS3 overexpression on NO production in gAd-stimulated RAW 264 cells. A: SOCS3-expressing and control cells were treated with 10 μg/ml gAd for 24 h. The amount of released NO was measured by the Griess method as described in the Methods Section. Values are the means ± SD (n = 3). $^{*}P < 0.01$ compared with Neo transfectants. B: NOS activity in gAd-treated Neo- and SOCS3-transformed RAW 264 cells. Values are the means ± SD (n = 3). $^{*}P < 0.01$ compared with Neo transfectants.

respectively, in gAd-treated RAW 264 cells. As shown in the upper panel of Figure 5C, gAd stimulation in Neo transfectants increased the number of DCF-positive (sum of populations in A2 + A4 plotted area) and MitoSOX-positive (sum of A1 + A2 populations) cells. The intracellular and mitochondrial ROS increased in a dose-dependent manner, and the percentages of DCF- and MitoSOX-positive cells were 37.5 ± 2.1% and 21.8 ± 1.1%, respectively, in 10 μg/ml gAd-treated Neo transfectants. In contrast, the percentages of DCF- and

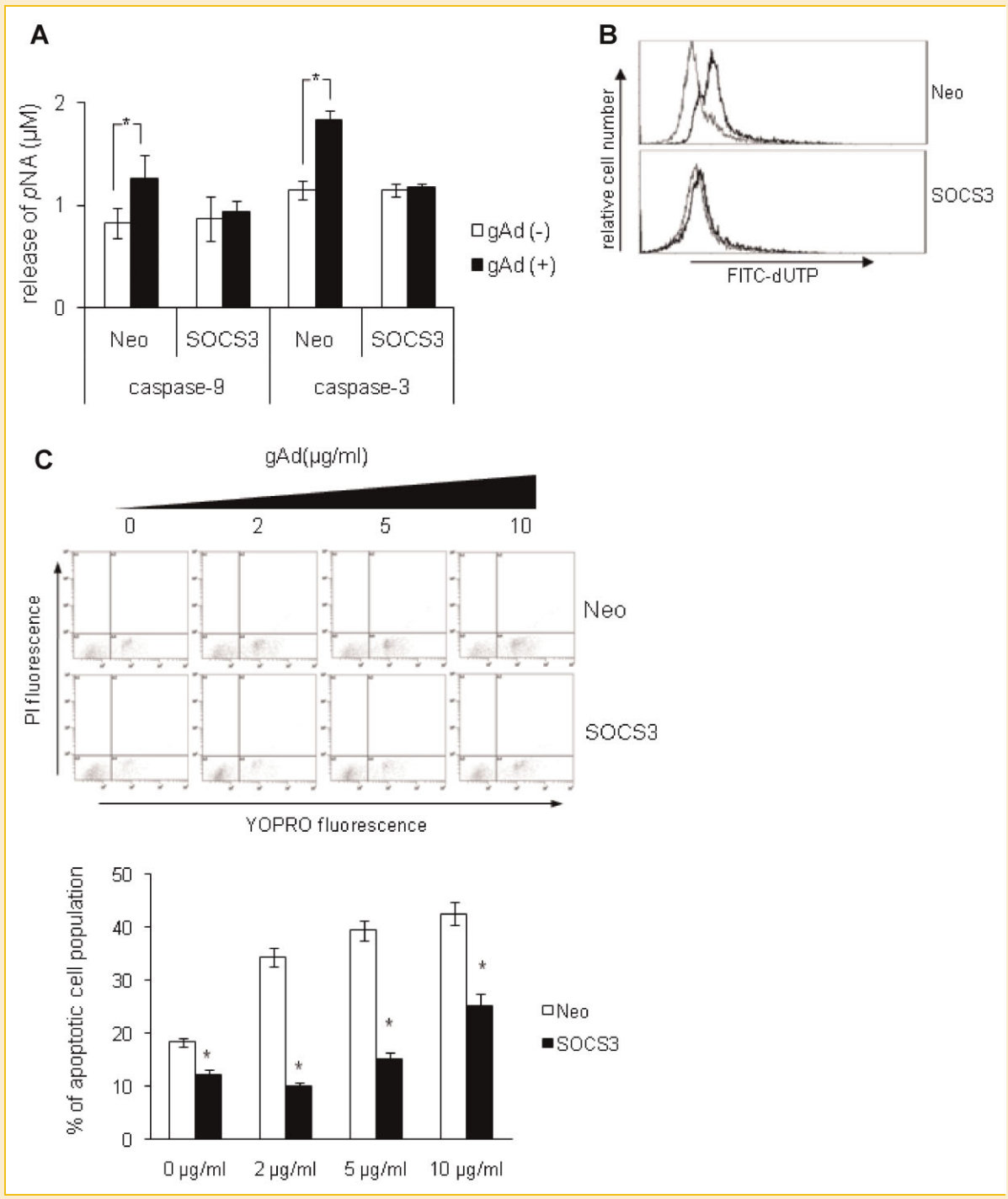

Fig. 7. Effect of SOCS3-overexpression on gAd-induced apoptosis in RAW 264 cells. A: Activities of caspase-3, and -9 in gAd-treated cells. Cells were lysed and the caspase activities were measured by incubation of lysates from gAd-treated cells with *p*NA-labeled specific substrate for caspase-3, and -9 as described in Methods Section. Values are mean ± SD (n = 3). $^{**}P < 0.01$ as compared to cells untreated with gAd. B: Analysis of DNA fragmentation estimated by TUNEL assay, indicated in Methods Section, was detected by flow cytometory. Cells were treated with 10 μg/ml gAd for 24 h, and fixed for TUNEL assay. Thin lines indicate the cells with medium alone and bold lines indicate the gAd-treated cells. C: YO-PRO-positive/PI-negative staining cell population (A3 plotted area) indicated early apoptotic cell were measured by flow cytometric analysis.

MitoSOX-positive cells in the gAd-treated SOCS transfectants were 8.6 ± 2.1% and 3.1 ± 1.0%, respectively. Next, we examined the impact of SOCS3 overexpression on gAd-evoked NO release. The amount of released NO was significantly lower in gAd-treated SOCS3 transfectants (3.7 ± 0.3 μM $NaNO_2$) than gAd-treated Neo transfectants (17.6 ± 0.2 μM $NaNO_2$; Fig. 6A). As shown in Figure 6B, gAd treatment markedly enhanced NOS activity (44.7 ± 0.8 nmol nitrite/mg protein) in Neo transfectants compared with medium alone (8.2 ± 0.1 nmol nitrite/mg protein). In contrast, gAd treatment did not induce the activation of NOS activity in gAd-treated SOCS3 transfectants (0.2 ± 0.1 nmol nitrite/mg protein).

SOCS3 OVEREXPRESSION SUPPRESSES ADIPONECTIN-INDUCED APOPTOSIS

We examined whether SOCS3 overexpression affected gAd-induced apoptosis in RAW 264 cells. As shown in Figure 7A, gAd treatment markedly enhanced caspase-9 and -3 activities (1.3 ± 0.2 and 1.8 ± 0.1 μM *p*NA, respectively) in Neo transfectants compared with medium alone (0.8 ± 0.1 and 1.2 ± 0.1 μM *p*NA, respectively). There was no significant difference in caspase-9 and -3 activities between gAd-treated SOCS3 transfectants (0.9 ± 0.2 and 1.2 ± 0.1 μM *p*NA, respectively) and medium-treated control SOCS3 transfectants (0.9 ± 0.2 and 1.1 ± 0.1 μM *p*NA, respectively). TUNEL assay revealed that Neo transfectant after a 24-h treatment with 10 μg/ml gAd showed DNA degradation typical of apoptosis, but not in SOCS3 transfectants (Fig. 7B). Although gAd administration clearly induced apoptosis in a dose-dependent manner in Neo control transfectants, SOCS3 overexpression partially reduced the gAd-evoked apoptotic population (Fig. 7C). After stimulation with gAd at 10 μg/ml, there was a significant reduction in the population of apoptotic cells stained by YO-PRO in SOCS3 transfectants (25.1 ± 6.3%) compared with Neo transfectants (42.6 ± 5.2%).

DISCUSSION

The results of the present study demonstrate that gAd stimulation activates the JAK-STAT signal transduction pathway and that inhibiting JAK activity with AG490 reduces gAd-evoked ROS and NO generation in RAW 264 cells. SOCS3 overexpression clearly reduced mitochondrial and intracellular ROS generation, and NO release, followed by apoptosis in gAd-treated RAW 264 cells. SOCS3 plays important roles in IL-6, G-CSF, leptin, and chemokine receptor signaling [Croker et al., 2004; Howard et al., 2004; Le et al., 2007]. SOCS proteins are crucial in balancing the beneficial effects of cytokines with the potentially harmful effects of overshooting activity [Dalpke et al., 2008]. SOCSs induced by IL-1β, which continuously rises during chronic inflammation, block insulin signaling in pancreatic beta cells [Emanuelli et al., 2004]. Based on this evidence, persistent SOCS1 and SOCS3 expression is implicated in the etiology of insulin resistance, obesity, and metabolic syndrome [Emanuelli et al., 2004; Liu et al., 2008]. These conflicting findings concerning the physiological/pathogenic role of SOCS may be caused by a tissue-specific signal transduction pathway. A recent report demonstrated the significant depression of SOCS3 expression in lymphocytes from type 2 diabetes patients, suggesting that SOCS3 regulates an important checkpoint to prevent inappropriate activation of lymphocyte subtypes or secretion of cytokines that underlie the pathogenic mechanisms of type 2 diabetes [Gokulakrishnan et al., 2009]. In our findings, gAd induced SOCS3 expression in RAW 264 cells, suggesting a negative feedback to prevent excessive immunomodulation by adiponectin stimulation in macrophages.

The JAK-2 protein tyrosine kinase-specific inhibitor AG490 inhibits STAT-3 phosphorylation [Abe and Berk, 1999]. The JAK/STAT signaling pathway plays pleiotropic roles in physiological and pathological cellular actions such as embryonic development, regulation of self-renewing tissues, and carcinogenesis [Caceres-Cortes, 2008]. By inhibiting JAK-2 protein tyrosine kinase activity, AG490 acts as a potent anti-carcinoma reagent, triggering tumor cell apoptosis in Sezary syndrome, a leukemic variant of cutaneous T cell lymphoma [Caceres-Cortes, 2008]. SOCS3 overexpression significantly suppressed gAd-evoked apoptosis in RAW 264 cells (Fig. 7). Multiple lines of evidence have demonstrated that SOCS3 overexpression protects against apoptosis induced by various stimulants, including LPS, concanavalin, IL-1β, and interferon-γ, which supports our findings [Karlsen et al., 2001; Jo et al., 2005]. Taken together, these results suggest that gAd-evoked apoptosis following ROS and NO generation may conjugate alterative intracellular signaling pathways, which may be inhibited by endogenous SOCS3.

A recent report demonstrated that gAd induces IL-6 production mediated by AdipoR1, which leads to stimulation of the JAK/STAT signaling pathway in adult mouse cardiac fibroblasts [Liao et al., 2009]. In contrast, another study demonstrated that adiponectin treatment induces STAT-3 phosphorylation, but prevents further STAT-3 activation upon addition of IL-6 in human macrophages [Folco et al., 2009]. In the present study, phosphorylated STAT-3 was detected at 10 min, suggesting a rapid and direct action induced by gAd stimulation. In addition, our previous study revealed that the IL-6 transcriptional level had not increased at 30 min after gAd administration, as assessed by DNA microarray [Kamio et al., 2008]. Taken together, it is suggested that stimulation of gAd may directly activate the JAK/STAT signaling in RAW 264 cells.

AdipoR1 and AdipoR2 appear to be integral membrane proteins and are topologically opposite to all other reported G protein-coupled receptors [Yamauchi et al., 2003]. The suppression of AdipoR1 with small interfering RNA (siRNA) reduces the increase in fatty-acid oxidation induced by gAd, suggesting that gAd utilizes AdipoR1 as its specific receptor, with high affinity [Yamauchi et al., 2003]. Injection of adiponectin into the hypothalamus increases JAK2 and SOCS3 phosphorylation levels via AdipoR1 signaling [Coope et al., 2008]. In the present study, we found no evidence of binding activity between AdipoR1/AdipoR2 and SOCS3 or JAK2 using immunoprecipitation (data not shown). Signaling via AdipoR requires the endosomal adaptor protein APPL1 [Deepa and Dong, 2009]. APPL1 mediates Akt/PI3 signaling and AMPK activation via the LKB1 transducing pathway [Zhou et al., 2009]. Future work will include an investigation of the relationship between JAK/STAT signaling and APPL1/AdipoR.

In conclusion, gAd-provoked ROS/NO generation was modulated by the JAK/STAT signal transduction pathway, and gAd treatment induced transient SOCS3 expression in RAW 264 cells. Although the molecular machinery is still unknown, SOCS3 overexpression suppressed not only ROS/NO generation but also apoptosis in gAd-treated cells. These findings suggest that SOCS3 may put the gAd-activated macrophage to immunological rights to negatively modulate JAK/STAT activity.

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