Original Article

Impact of Mitochondrial Reactive Oxygen Species and Apoptosis Signal–Regulating Kinase 1 on Insulin Signaling

Koujiro Imoto,[1] Daisuke Kukidome,[1] Takeshi Nishikawa,[1] Takako Matsuhisa,[1] Kazuhiro Sonoda,[1] Kazuo Fujisawa,[1] Miyuki Yano,[1] Hiroyuki Motoshima,[1] Tetsuya Taguchi,[1] Kaku Tsuruzoe,[1] Takeshi Matsumura,[1] Hidenori Ichijo,[2] and Eiichi Araki[1]

Tumor necrosis factor (TNF)-α inhibits insulin action; however, the precise mechanisms are unknown. It was reported that TNF-α could increase mitochondrial reactive oxygen species (ROS) production, and apoptosis signal–regulating kinase 1 (ASK1) was reported to be required for TNF-α–induced apoptosis. Here, we examined roles of mitochondrial ROS and ASK1 in TNF-α–induced impaired insulin signaling in cultured human hepatoma (Huh7) cells. Using reduced MitoTracker Red probe, we confirmed that TNF-α increased mitochondrial ROS production, which was suppressed by overexpression of either uncoupling protein-1 (UCP)-1 or manganese superoxide dismutase (Mn-SOD). TNF-α significantly activated ASK1, increased serine phosphorylation of insulin receptor substrate (IRS)-1, and decreased insulin-stimulated tyrosine phosphorylation of IRS-1 and serine phosphorylation of Akt, and all of these effects were inhibited by overexpression of either UCP-1 or MnSOD. Similar to TNF-α, overexpression of wild-type ASK1 increased serine phosphorylation of IRS-1 and decreased insulin-stimulated tyrosine phosphorylation of IRS-1, whereas overexpression of dominant-negative ASK1 ameliorated these TNF-α–induced events. In addition, TNF-α activated c-jun NH_2-terminal kinases (JNKs), and this observation was partially inhibited by overexpression of UCP-1, MnSOD, or dominant-negative ASK1. These results suggest that TNF-α increases mitochondrial ROS and activates ASK1 in Huh7 cells and that these TNF-α–induced phenomena contribute, at least in part, to impaired insulin signaling. *Diabetes* 55:1197–1204, 2006

From the [1]Department of Metabolic Medicine, Faculty of Medical and Pharmaceutical Sciences, Kumamoto University, Kumamoto, Japan; and the [2]Laboratory of Cell Signaling and Core Research for Evolutional Science and Technology, Graduate School of Pharmaceutical Sciences, University of Tokyo, Tokyo, Japan.

Address correspondence and reprint requests to Takeshi Nishikawa, Department of Metabolic Medicine, Faculty of Medical and Pharmaceutical Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto 860-8556, Japan. E-mail: takeshi@kaiju.medic.kumamoto-u.ac.jp.

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K.I. and D.K. contributed equally to this work.

ASK1, apoptosis signal–regulating kinase 1; ASK1-KM, ASK1 dominant-negative mutant; ASK1-WT, ASK1 wild type; DMEM, Dulbecco's modified Eagle's medium; IRS, insulin receptor substrate; JNK, c-jun NH_2-terminal kinase; MnSOD, manganese superoxide dismutase; ROS, reactive oxygen species; TNF, tumor necrosis factor; Trx, thioredoxin; UCP, uncoupling protein.

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Tyrosine phosphorylation of the insulin receptor substrates (IRSs) including IRS-1 and IRS-2 through the tyrosine kinase of the insulin receptor is an early key event of the insulin signal transduction (1–3). Impaired tyrosine phosphorylation of IRS-1 has been reported to be involved in various status of insulin resistance in vivo. In recent years, it has been suggested that elevation of tumor necrosis factor (TNF)-α from hypertrophic adipocytes may play a role in causing impaired insulin action (4–8). Administration of TNF-α to rats modifies lipid and protein metabolism in the liver, adipose tissue, and skeletal muscle (9,10) and impairs several actions of insulin in these tissues, resulting in decreased peripheral glucose disposal and increased hepatic glucose output (11). In addition, the absence of TNF-α and two of its receptors was reported to improve insulin action in both diet-induced and *ob/ob* obese mice (12,13). At the molecular level, TNF-α has consistently been shown to induce the activation of c-jun NH_2-terminal kinases (JNKs) (14,15), increase serine phosphorylation of IRS-1, and subsequently decrease insulin-stimulated tyrosine phosphorylation of IRS-1 (14,16). However, the mechanisms by which TNF-α inhibits insulin action have not been completely elucidated.

Previously, we demonstrated that hyperglycemia increased mitochondrial reactive oxygen species (ROS) production, which could represent a key event in the development of diabetes complications (17,18). Similarly, TNF-α has been reported to increase mitochondrial ROS production in tumor cells (19,20), hepatocytes (21), and endothelial cells (22). Since micromolar concentrations of hydrogen peroxide were reported to inhibit insulin-stimulated tyrosine phosphorylation of IRS-1 (23) and α-lipoic acid, a novel antioxidant, increased insulin-stimulated glucose uptake in muscles (24–26), and decreased hepatic glucose output (27), oxidative stress may be a common mediator of insulin resistance.

On the other hand, apoptosis signal–regulating kinase 1 (ASK1) was recently identified as a mitogen-activated protein kinase kinase kinase. ASK1 activates the JNK and p38 signaling pathways and is required for TNF-α–induced apoptosis (28). In addition, thioredoxin (Trx), which regulates the cellular reduction/oxidation (redox) status, was reported to bind directly to the NH_2-terminal region of ASK1 (29,30). Treatment with hydrogen peroxide was reported to induce dissociation of Trx from ASK1, thereby

activating ASK1 by inducing oligomerization and the subsequent phosphorylation of a critical threonine residue within the activation loop of ASK1 (29,31,32). Therefore, TNF-α may increase mitochondrial ROS production and activate ASK1 in insulin target tissues, and this integrating paradigm may cause insulin resistance in diabetes and obesity.

In the present study, in order to clarify the impact of mitochondrial ROS production and ASK1 activation on the pathogenesis of insulin resistance, we investigated whether TNF-α could increase mitochondrial ROS production and activate ASK1 using cultured human hepatoma (Huh7) cells. In addition, we evaluated the effects of overexpression of either uncoupling protein (UCP)-1 or manganese superoxide dismutase (MnSOD), which normalize mitochondrial ROS production, on the TNF-α–induced ASK1 activation, JNK activation, increase in serine phosphorylation of IRS-1, and decrease in insulin-stimulated tyrosine phosphorylation of IRS-1. Furthermore, to clarify the role of ASK1 in insulin signaling, we examined the effects of ASK1 wild type (ASK1-WT) and an ASK1 dominant-negative mutant (ASK1-KM) on TNF-α–induced insulin resistance.

RESEARCH DESIGN AND METHODS

Human insulin and recombinant human TNF-α were purchased from Eli Lilly Japan (Hyogo, Japan) and Strathmann Biotec (Hamburg, Germany), respectively. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were obtained from Sigma (St. Louis, MO) and Moregate Biotech (Bulimba, Queensland, Australia), respectively. Antibodies against phosphotyrosine (4G10), IRS-1, and IRS-1 Ser^{307} (IRS1-pS307) were purchased from Upstate Biotechnology (Lake Placid, NY). Antibodies against phospho–stress-activated protein kinase/JNK (Thr183/Tyr185), stress-activated protein kinase/JNK, and phospho-Akt (Ser ^{473}) were obtained from Cell Signaling Technology (Beverly, MA). SP600125, a pharmacological inhibitor of JNK, was obtained from Merck Biosciences (Darmstadt, Germany).

Cell culture. Human Huh7 hepatocarcinoma cells (JCRB0403) were obtained from the Health Science Research Resources Bank (Osaka, Japan) and maintained in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified 5% CO_2 environment.

Adenoviral vectors. Rat UCP-1 and human MnSOD adenoviral vectors were kindly provided by Dr. M. Brownlee (Albert Einstein College of Medicine, Bronx, NY) (33). ASK1-WT and ASK1-KM adenovirus vectors were prepared as previously described (29).

TNF-α treatment and adenovirus-mediated gene transfer of Huh7 cells. Huh7 cells were grown to confluence in 10-cm dishes or six-well plates, incubated overnight in serum-free DMEM, and then treated with recombinant human TNF-α (25 ng/ml) for 0–3 h. After incubation for a further 60 min, the cells were exposed to insulin (100 nmol/l) for 3 min. For adenovirus-mediated gene transfer, confluent cells were infected with the recombinant adenovirus vectors at a multiplicity of infection of 50 plaque-forming units per cell for 1 h and then cultured in DMEM supplemented with 5% fetal bovine serum for 24 h. Cells were serum-starved overnight and subjected to the assays described below.

Immunoprecipitation and Western blot analysis. Immunoprecipitation and Western blot analysis were performed as previously described (34). Briefly, immunoprecipitation was conducted by incubating whole-cell lysates with an antibody for 3–4 h at 4°C. For Western blot analysis, samples were denatured in SDS-PAGE sample buffer for 5 min at 97°C, separated in an SDS-polyacrylamide (7.5–10%) gel, and then transferred to a polyvinylidine fluoride membrane. After blocking with Tris-buffered saline (10 nmol/l Tris-HCl, pH 7.4, 150 nmol/l NaCl) containing 0.05% Tween-20 and 5% nonfat dry milk, the membranes were incubated with the indicated primary antibodies in Tris-buffered saline with Tween 20 containing 5% BSA. Immunodetection was accomplished by incubating the membranes with horseradish peroxidase–conjugated secondary antibodies in Tris-buffered saline with Tween 20 containing 5% nonfat dry milk for 1 h. Visualization was performed with a BM Chemiluminescence Blotting Substrate Kit (Roche Diagnostics, Mannheim, Germany).

Measurement of intracellular ROS. Intracellular ROS production was detected using 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA; Molecular Probes, Eugene, OR) as previously described (18). Briefly, cells were treated with TNF-α (25 ng/ml) for 1 h under each condition, and then H_2DCF-DA was added at a final concentration of 5 μmol/l. After incubation for a further 30 min at 37°C, the cells were analyzed using a Fluoroskan Ascent FL (Labsystems Oy, Helsinki, Finland) and the Ascent Software (Labsystems Oy). The ROS concentrations were determined from a standard curve of H_2O_2 (5–100 μmol/l) and expressed as a percentage of the ROS production in serum-free DMEM.

Detection of mitochondrial ROS. Mitochondrial ROS production was detected using MitoTracker Red CM-H_2 × Ros (Molecular Probes). The reduced probe does not fluoresce until it enters an actively respiring cell, where it is oxidized to a fluorescent probe that becomes selectively sequestered in the mitochondria (35). Cells were treated with TNF-α (25 ng/ml) for 30 min under each condition, and then MitoTracker Red was added at a final concentration of 1 μmol/l. After incubation for a further 30 min, the cells were washed twice with Hanks' balanced salt solution (Nissui Pharmaceutical, Tokyo, Japan) and observed under a confocal laser-scanning microscope (model FV500; Olympus, Tokyo, Japan).

In vitro kinase assay. ASK1 activity assays were performed as described previously using a GST-MKK4 fusion protein as the substrate (36). Briefly, 500-μg aliquots of cell lysates were immunoprecipitated with 4 μg of an anti-ASK1 antibody (H-300; Santa Cruz Biotechnology, Santa Cruz, CA). Immunoprecipitates were mixed with 1 μg GST-MKK4 (Upstate Biotechnology) suspended in kinase buffer (20 nmol/l HEPES, pH 7.6, 20 nmol/l $MgCl_2$, 25 nmol/l β-glycerophosphate, 100 nmol/l sodium orthovanadate, 2 nmol/l dithiothreitol, and 20 nmol/l ATP) containing 1 μl (10 μCi) of [γ-^{32}P]ATP and incubated at 30°C for 30 min. The reaction was terminated by the addition of Laemmli sample buffer, and the phosphorylated GST-MKK4 was visualized by autoradiography.

Detection of apoptosis. Apoptosis was evaluated by both detection of DNA fragmentation (37) and measurement of the DNA content by incorporation of propidium iodide (38). Briefly, for detection of DNA fragmentation, cells were treated with TNF-α (25 ng/ml) for 1 h and lysed in 200 μl of PBS. The lysates were collected from the culture plates, and total DNA was isolated using a DNA extraction kit (Invitrogen, Carlsbad, CA). DNA fragmentation was subsequently analyzed by 1.6% agarose gel electrophoresis in the presence of ethidium bromide.

For measurement of the DNA content, cells were treated with TNF-α (25 ng/ml) for 1 h. The adherent cells were then washed with PBS and collected by trypsinization with 0.05% trypsin/EDTA. The cells were suspended in PBS containing 0.2% Triton X and 0.02% RNase and then incubated with 50 μg/ml propidium iodide at 37°C in the dark. Data acquisition and analysis were performed using a FACScan flow cytometer and the accompanying CellQuest software (BD Biosciences, San Jose, CA).

Statistical analysis. Data were expressed as the means ± SE. Statistical significances were analyzed by one-way ANOVA followed by the Bonferroni test for differences between two groups. $P < 0.05$ denoted the presence of a statistically significant difference. All analyses were performed using Statview Software (SAS Institute, Cary, NC).

RESULTS

Effects of TNF-α on insulin-stimulated tyrosine phosphorylation of IRS-1 and phosphorylation of IRS-1 Ser^{307} in Huh7 cells. To elucidate whether TNF-α could impair insulin signaling in Huh7 cells, we first examined the effect of TNF-α on insulin-stimulated tyrosine phosphorylation of IRS-1. As shown in Fig. 1*A*, stimulation of Huh7 cells with 100 nmol/l insulin for 3 min resulted in a significant increase in the tyrosine phosphorylation of IRS-1 compared with the basal condition (218.2 ± 43.2% of the basal level, $P < 0.05$). On the other hand, incubation of cells with 25 ng/ml TNF-α for 3 h before the insulin stimulation inhibited the insulin-stimulated tyrosine phosphorylation of IRS-1 (61.4 ± 7.1% of insulin alone, $P < 0.05$). During these experiments, the amount of IRS-1 protein remained unaltered.

Since phosphorylation of Ser^{307} in IRS-1 has been reported to suppress insulin signaling by inhibiting insulin-stimulated tyrosine phosphorylation of IRS-1, IRS-1 Ser ^{307} phosphorylation was evaluated by Western blot analysis with anti–IRS-1 Ser^{307}–phosphospecific antibodies. As shown in Fig. 1*B*, TNF-α–induced IRS-1 Ser^{307} phosphorylation was observed at 60 min after the TNF-α stimula-

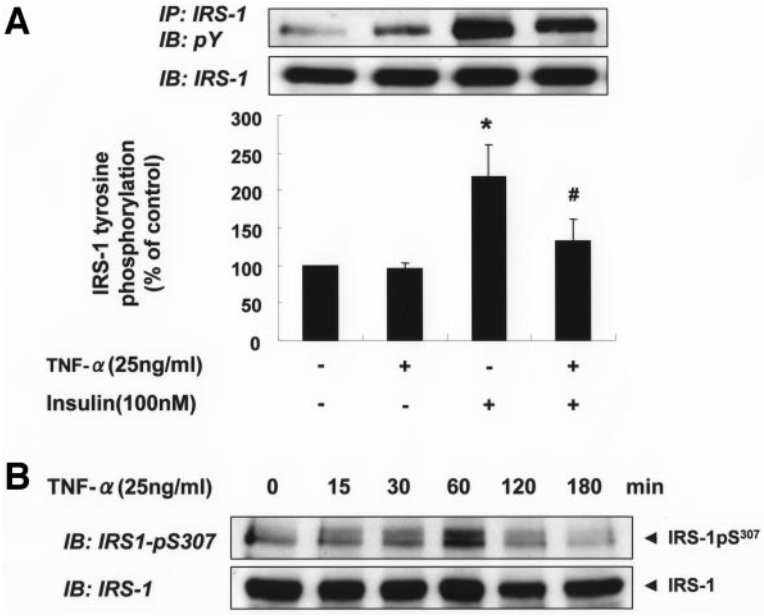

FIG. 1. Effects of TNF-α on insulin-stimulated tyrosine phosphorylation of IRS-1 and phosphorylation of IRS-1 Ser^{307}. Cells were treated with the indicated conditions. *A*: After immunoprecipitation, tyrosine phosphorylation of IRS-1 was determined by immunoblotting. The results are expressed as relative values (means ± SE) to the cells without TNF-α and insulin (n = 4). *P < 0.05 vs. cells without TNF-α and insulin; #P < 0.05 vs. cells with insulin only. *B*: Serine phosphorylation of IRS-1 was determined by immunoblotting. Representative results of four independent experiments are shown.

tion. During these experiments, the amounts of IRS-1 remained unaltered.

Effects of TNF-α on mitochondrial ROS production. To evaluate whether TNF-α could increase intracellular and mitochondrial ROS production in Huh7 cells, we loaded the cells with the fluorescent probes H_2DCF-DA and MitoTracker Red CM-H_2 × Ros, respectively. As shown in Fig. 2, H_2DCF-DA–associated fluorescence, representing intracellular ROS production, was significantly increased after incubation with 25 ng/ml of TNF-α for 1 h (474.5 ± 85.3% of the control adenovirus alone, $P < 0.05$). Furthermore, this TNF-α–induced ROS overproduction was suppressed by overexpression of MnSOD or UCP-1 (166.9 ± 9.9%, $P < 0.05$, and 134.9 ± 11.2%, $P < 0.05$, of the control adenovirus alone, respectively).

As shown in Fig. 3*A–D*, MitoTracker Red CM-H_2 × Ros fluorescence, representing mitochondrial ROS production, was increased after incubation with 25 ng/ml of TNF-α for 15 min, and this increase was sustained for up to 180 min. Furthermore, this TNF-α–induced fluorescence was suppressed by overexpression of UCP-1 or MnSOD (Fig. 3*E–J*).

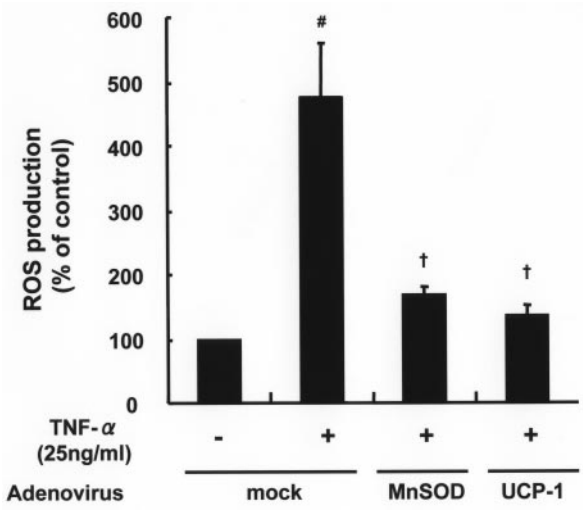

FIG. 2. Effects of TNF-α on intracellular ROS production. Cells were treated with the indicated conditions. Intracellular ROS production was quantified using the fluorescent probe H_2DCF-DA (n = 6). The results are expressed as relative values (means ± SE) to the control adenovirus. #P < 0.05 vs. mock; †P < 0.05 vs. cells infected with control adenovirus plus TNF-α.

Effects of normalization of mitochondrial ROS production on the TNF-α–induced decrease in insulin-stimulated tyrosine phosphorylation of IRS-1 and increase in phosphorylation of IRS-1 Ser^{307}. To determine the roles of mitochondrial ROS in impaired insulin signaling, we examined the effects of overexpression of either MnSOD or UCP-1 on insulin-stimulated tyrosine phosphorylation of IRS-1. As shown in Fig. 4*A*, preincubation with TNF-α suppressed the insulin-stimulated tyrosine phosphorylation of IRS-1 in Huh7 cells infected with the control adenovirus (mock) but not in cells infected with adenoviruses expressing MnSOD or UCP-1. Similarly, TNF-α increased Ser^{307} phosphorylation of IRS-1 (192.3 ± 8.7% of mock), and overexpression of either MnSOD or UCP-1 suppressed the TNF-α–induced Ser^{307} phosphorylation of IRS-1 (92.4 ± 8.6% and 100.3 ± 12.3% of mock, respectively) (Fig. 4*B*). In these experiments, IRS-1 protein contents remained unaltered.

Effect of TNF-α and mitochondrial ROS on ASK1 activation. To determine whether TNF-α could activate ASK1 in Huh7 cells, ASK1 activity was evaluated using an in vitro kinase assay with GST-MKK4 as the substrate. As shown in Fig. 5*A*, the ASK1 activity in Huh7 cells was increased as early as 15 min after treatment with TNF-α compared with the basal condition, and the maximal activation occurred at 60 min after the TNF-α stimulation.

To clarify the correlation between mitochondrial ROS and ASK1 activation, the effects of overexpression of either MnSOD or UCP-1 on TNF-α–induced activation of ASK1 were examined. As shown in Fig. 5*B*, incubation with TNF-α increased the ASK1 activity in Huh7 cells infected with the control adenovirus (mock) (155.3 ± 13.6% of mock in the absence of TNF-α, $P < 0.05$), while overexpression of either MnSOD or UCP-1 inhibited this effect (84.5 ± 6.7%, $P < 0.05$, and 77.4 ± 11.5%, $P < 0.05$, of mock in the absence of TNF-α, respectively).

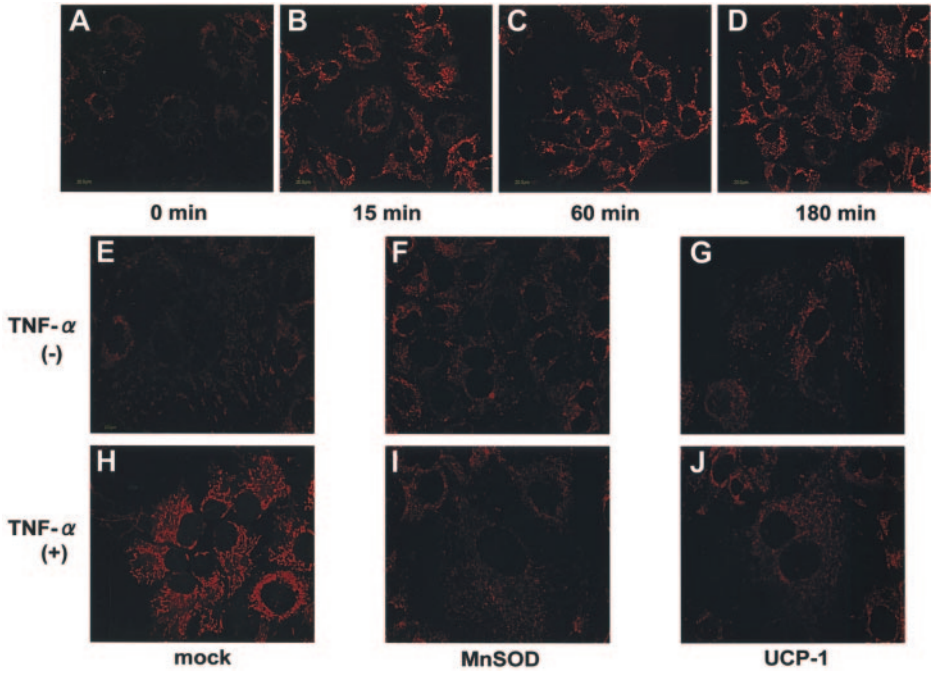

FIG. 3. Effects of TNF-α on mitochondrial ROS production. *A–D*: Cells were incubated with TNF-α for the indicated times. *E–J*: Cells were treated with the indicated conditions. Mitochondrial ROS production was detected with MitoTracker Red CM-H2 × Ros. Representative results of four independent experiments are shown.

Interaction between ASK1 activation and impaired insulin signaling. To clarify the role of ASK1 activation in insulin resistance, we examined the ASK1 activities and IRS-1 tyrosine phosphorylation in Huh7 cells infected with a control adenovirus (mock) or adenovirus expressing ASK1-WT or ASK1-KM. As shown in Fig. 6*A*, treatment with TNF-α increased the ASK1 activity in Huh7 cells (mock). Overexpression of ASK1-WT exhibited marked activation of ASK1, even without TNF-α stimulation. In contrast, overexpression of ASK1-KM diminished the TNF-α–induced activation of ASK1. As shown in Fig. 6*B*, overexpression of ASK1-WT decreased the insulin-stimulated tyrosine phosphorylation of IRS-1, similar to the incubation with TNF-α. However, overexpression of ASK1-KM diminished the TNF-α–induced inhibition of insulin-stimulated tyrosine phosphorylation of IRS-1.

As shown in Fig. 6*C*, overexpression of ASK1-WT increased the Ser^{307} phosphorylation of IRS-1 (140.3 ± 8.7% of mock) in either the absence or presence of TNF-α. In contrast, overexpression of ASK1-KM reduced the TNF-α–induced Ser^{307} phosphorylation of IRS-1 (106.4 ± 14.2% of ASK1-KM in the absence of TNF-α). In these experiments, the IRS-1 protein contents remained unaltered.

As shown in Fig. 6*D*, the effect of overexpression of either MnSOD or ASK-KM on serine phosphorylation of Akt, which is one of the downstream signals of IRS-1, was examined. Similar to the results of tyrosine phosphorylation of IRS-1, TNF-α decreased insulin-induced serine phosphorylation of Akt. In addition, overexpression of either MnSOD or ASK1-KM diminished this TNF-α–induced inactivation of Akt.

Although ASK1 is reported to be involved in apoptosis of the cells, cell apoptosis, evaluated by both detection of DNA fragmentation and measurement of the DNA content by incorporation of propidium iodide, did not increase when Huh7 cells infected with a control adenovirus (mock) or an adenovirus expressing ASK1-WT were incubated with 25 ng/ml TNF-α for 1 h (data not shown).

Effects of normalization of mitochondrial ROS production on the TNF-α–induced JNK activation. Since TNF-α was reported to inhibit insulin action, in part, by activating JNK, we examined the relationships among mitochondrial ROS production, ASK1 activation, and JNK activation. As shown in Fig. 7*A*, preincubation with TNF-α increased the phosphorylation of both the 54- and 46-kDa forms of JNK (202 ± 55.8% and 203.7 ± 34.7% of mock,

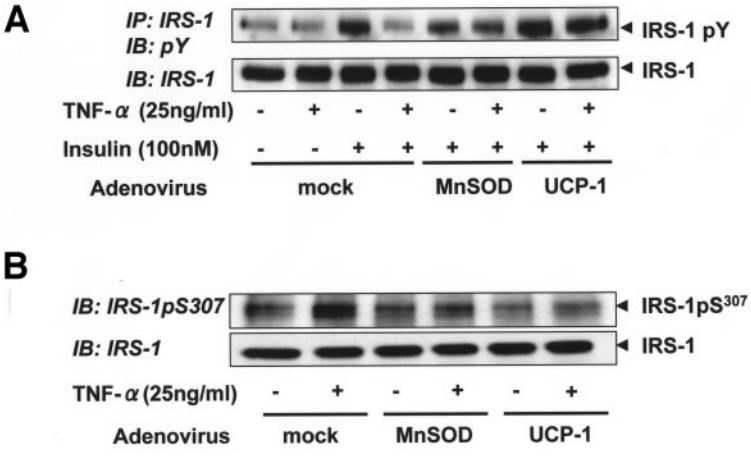

FIG. 4. Effects of MnSOD or UCP-1 overexpression on the TNF-α–induced insulin resistance. Cells were treated with the indicated conditions. Tyrosine (*A*) and serine (*B*) phosphorylation of IRS-1 was determined by immunoblotting. Representative results of four independent experiments are shown.

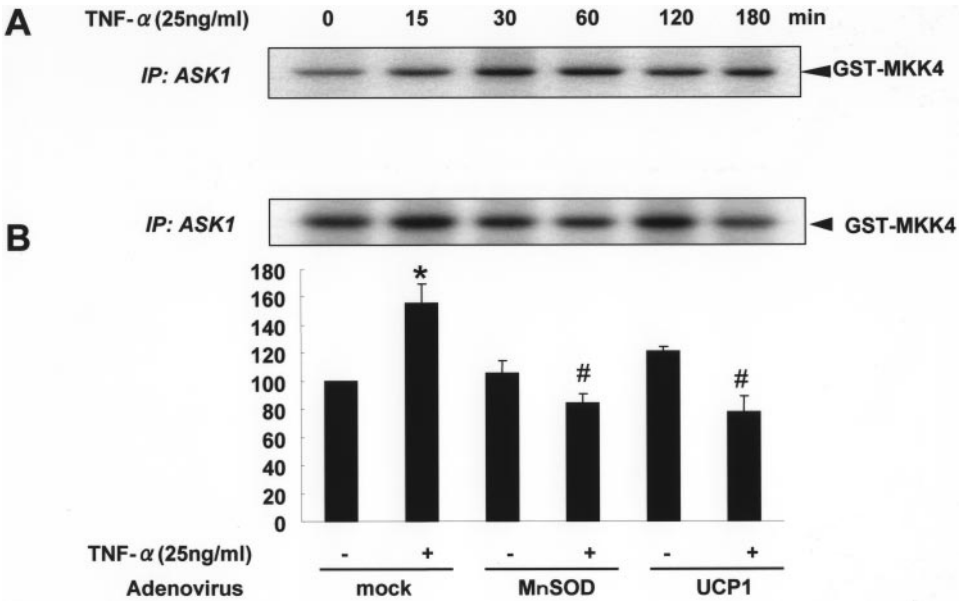

FIG. 5. Effect of TNF-α and MnSOD or UCP-1 overexpression on ASK1 activity. *A*: Cells were treated with TNF-α for the indicated times. *B*: After the TNF-α stimulation, the ASK1 activity was detected using an in vitro kinase assay. The results are expressed as relative values (means ± SE) to the cells infected with the control adenovirus (mock) without TNF-α ($n = 4$). *$P < 0.05$ vs. mock without TNF-α; #$P < 0.05$ vs. mock with TNF-α.

respectively). Furthermore, overexpression of either MnSOD or UCP-1 partially suppressed the TNF-α–induced phosphorylation of the 54- and 46-kDa forms of JNK (p54: 59.0 ± 17.0% and 79.1 ± 14.2% of mock, respectively; p46: 72.1 ± 17.4% and 55.3 ± 6.4% of mock with TNF-α, respectively).

As shown in Fig. 7*B*, overexpression of ASK1-WT increased the phosphorylation of the 54- and 46-kDa forms of JNK in either the absence or presence of TNF-α (p54: 192.3 ± 28.6% and 344.5 ± 101.1% of mock, respectively; p46: 176.3 ± 33.1% and 304.8 ± 32.4% of mock, respectively). In contrast, overexpression of ASK1-KM did not significantly increased the phosphorylation of JNK by TNF-α (p54: 110.2 ± 22.5% and p46: 118.8 ± 23.1% of ASK1-KM without TNF-α, respectively). Although it was not statistically significant, overexpression of ASK1-KM tended to decrease the TNF-α–induced phosphorylation of JNK compared with mock with TNF-α (p54: 174.6 ± 62.5% and p46: 178.3 ± 53.1% of mock without TNF-α, respectively).

As shown in Fig. 7*C*, we evaluated the effect of SP600125, a pharmacological inhibitor of JNK, on Ser^{307} phosphorylation of IRS-1. SP600125 partially, but significantly, inhibited TNF-α– or ASK1-induced Ser^{307} phosphorylation of IRS-1, suggesting that JNKs, at least in part, connect TNF-α–induced activation of ASK1 with Ser^{307} phosphorylation of IRS-1.

As shown in Fig. 7*D*, we performed Western blot analysis using specific antibody against ASK1 after immunoprecipitaion with specific IRS-1 antibody. No ASK1 band

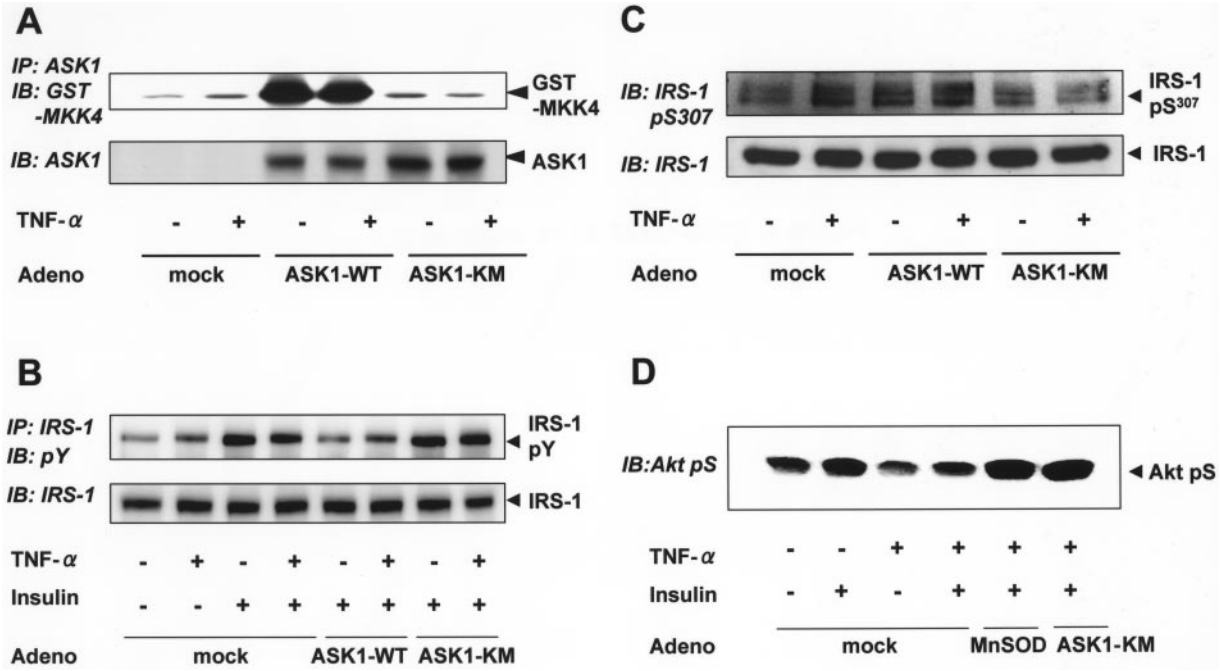

FIG. 6. Effects of ASK1 activation on insulin resistance. Cells were treated with the indicated conditions. *A*: The ASK1 activity was detected using an in vitro kinase assay. Tyrosine (*B*) and serine (*C*) phosphorylation of IRS-1 and serine phosphorylation of Akt (*D*) was determined by immunoblotting. Representative results of four independent experiments are shown.

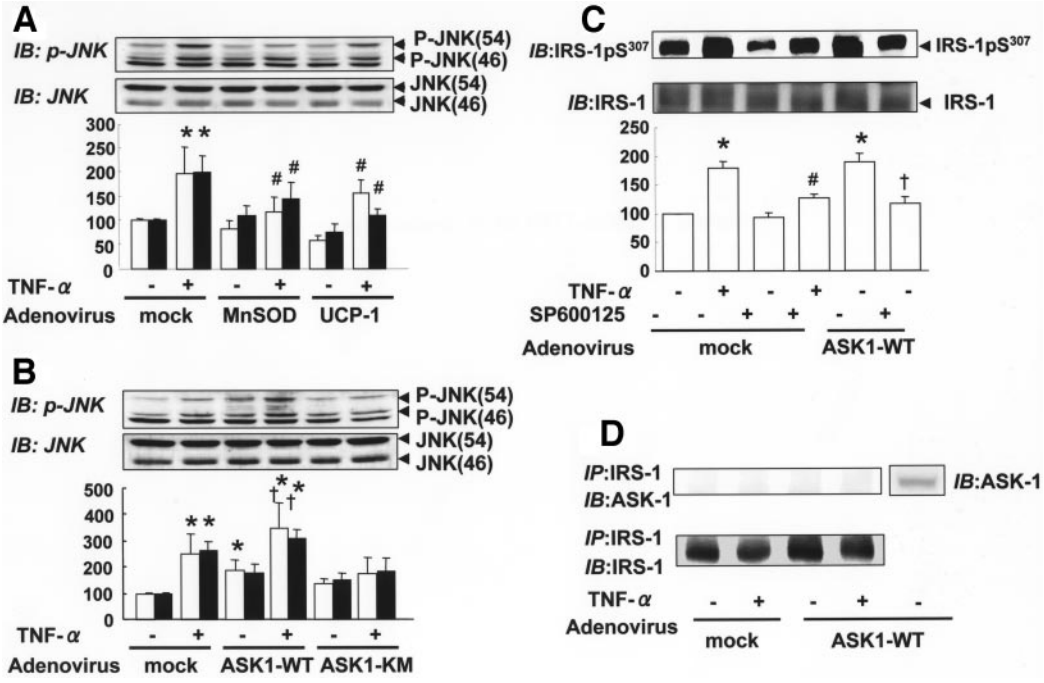

FIG. 7. Relationship among mitochondrial ROS production, ASK1 activation, and JNK activation. Cells were treated with the indicated conditions. The phosphorylation of JNK (*A* and *B*) and serine phosphorylation of IRS-1 (*C*) was determined by immunoblotting. The results are expressed as relative values (means ± SE) to the cells infected with the control adenovirus (mock) without TNF-α (n = 4). *P < 0.05 vs. mock without TNF-α; #P < 0.05 vs. mock with TNF-α; †P < 0.05 vs. cells infected with ASK1-WT without TNF-α or SP600125. *D*: Western blot analysis using specific antibody against ASK1 was determined after immunoprecipitaion with specific IRS-1 antibody. Representative results of three independent experiments are shown.

was detected in any condition, which suggested that ASK1 did not bind to IRS-1 with or without TNF-α stimulation, indicating that ASK1 could not directly phosphorylate IRS-1 on Ser^{307}. In these experiments, the JNK and IRS-1 protein contents remained unaltered.

DISCUSSION

Previously, we have shown that hyperglycemia can increase ROS production by the mitochondrial electron transport chain in bovine endothelial cells (17) and human mesangial cells (18) and that these mitochondrial ROS may play important roles in the pathogenesis of diabetes complications. On the other hand, TNF-α, which may play a role in the development of insulin resistance in vivo, was reported to increase mitochondrial ROS production in tumor cells (19,20), hepatocytes (21), and endothelial cells (22). In this study, we examined whether an increase in mitochondrial ROS production also associates with the pathogenesis of TNF-α–induced insulin resistance in hepatoma cells.

MnSOD, which is localized in mitochondria, is known to be a key enzyme that protects the cells against oxidative stress. On the other hand, UCPs, which are inner mitochondrial membrane anion transporters, allow protons to leak back into the mitochondrial matrix, thereby decreasing the potential energy available for ADP phsphorylation and ROS generation. The fluorescence of both H_2DCF-DA and reduced MitoTracker Red increased when the cells were incubated with TNF-α, and this increased fluorescence was reduced by overexpression of either UCP-1 or MnSOD. In addition, we confirmed that overexpression of either UCP-1 or MnSOD restored the TNF-α–induced decrease in insulin-stimulated tyrosine phosphorylation of IRS-1 and serine phosphorylation of Akt, activation of JNK, and increase in serine phosphorylation of IRS-1, all of which have been considered molecular bases for TNF-α–induced insulin resistance (14–16). The fluorescence of H_2DCF-DA indicates intracellular ROS production (18). However, the increased fluorescence induced by TNF-α may represent mitochondrial ROS production, since overexpression of either MnSOD or UCP-1 suppressed TNF-α–induced intracellular ROS production (17,18). Furthermore, MitoTracker Red CM-H_2 × Ros specifically detects mitochondrial ROS, since the probe specifically accumulates inside mitochondria and is predominantly oxidized by reactions involving hydrogen peroxide production (35). Hence, mitochondria could, at least in part, be a source of the TNF-α–induced ROS production in Huh7 cells, and this study is the first to provide evidence that mitochondrial ROS may contribute to the pathogenesis of TNF-α–induced insulin resistance. It remains unclear whether mitochondria are the only source of the TNF-α–induced ROS production, since TNF-α has been reported to increase ROS production in a TNF receptor–associated factor 2–dependent manner (39). Furthermore, the mechanisms by which TNF-α increases mitochondrial ROS production are also unclear, although TNF-α was reported to increase mitochondrial ROS production via a ceramide-dependent signaling pathway in cultured rat hepatocytes (21) and human umbilical vein endothelial cells (22). Further studies are required to clarify the roles of TNF receptor–associated factor 2 and ceramide in TNF-α–induced mitochondrial ROS production.

The present study also provides evidence that TNF-α can activate ASK1, which has been reported to activate the JNK signaling pathways and that this effect was inhibited by overexpression of either UCP-1 or MnSOD. In addition, overexpression of ASK1-WT activated JNK, increased serine phosphorylation of IRS-1, and decreased insulin-stimulated tyrosine phosphorylation of IRS-1, similar to incubation with TNF-α. On the other hand, overexpression of ASK1-KM restored the TNF-α–induced decrease in insulin-stimulated tyrosine phosphorylation of IRS-1 and serine phosphorylation of Akt, activation of JNK, and increase in serine phosphorylation of IRS-1. Although it was reported that TNF-α and hydrogen peroxide can remove Trx from Trx-ASK1 complexes, leading to ASK1 activation and the subsequent ASK1-dependent signaling cascade (29), it remains unclear whether TNF-α–induced activation of ASK1 is primarily mediated by intracellular

redox changes, including the production of ROS. Our results further suggest that mitochondrial ROS production by TNF-α is one of the key mediators of ASK1 activation and that activation of ASK1 connects mitochondrial ROS production with JNK activation. To the best of our knowledge, this is also the first study to demonstrate a role of ASK1 in the pathogenesis of TNF-α–induced insulin resistance. Recently, ASK1 was reported to be localized in both the cytoplasm and mitochondria where it binds to cytosolic Trx (Trx1) and mitochondrial Trx (Trx2), respectively (40–42). Given its charged nature, superoxide does not readily cross membranes (43), and, therefore, mitochondrial ROS may remove Trx2 from Trx2-ASK1 complexes in mitochondria, thereby leading to selective ASK1 activation. Further studies are required to clarify the details of the mechanisms by which TNF-α and mitochondrial ROS activate ASK1 in insulin-sensitive cells.

We demonstrated here that the impact of mitochondrial ROS production and ASK1 activation in TNF-α–induced impaired insulin action. However, the effect of overexpression of UCP-1 or ASK1-KM on TNF-α–induced JNK activation was limited. TNF-α was reported to induce serine phosphorylation of IRS-1 through the induction of several other kinases. Activations of protein kinase Cζ (44,45) and inhibitor of κB kinase (46) have been shown to be involved in serine phosphorylation of IRS-1. Especially, it has recently been demonstrated that mice, which selectively expressed constitutively active inhibitor of κB kinase β in hepatocytes, exhibited a type 2 diabetes phenotype, characterized by hyperglycemia and insulin resistance (47). In addition, we previously reported that normalization of mitochondrial ROS production by overexpression of UCP-1 or MnSOD prevented glucose-induced activation of protein kinase C and activation of nuclear factor κB in bovine vascular endothelial cells (17). Therefore, mitochondrial ROS may directly activate protein kinase Cζ or inhibitor of κB kinase without JNK activation, thereby leading to serine phosphorylation of IRS-1. On the other hand, the hexosamine pathway was also reported to play a role in fat-induced insulin resistance (48). Furthermore, activation of the hexosamine pathway has recently been shown to increase serine phosphorylation of IRS-1 and decrease insulin-stimulated tyrosine phosphorylation of IRS-1 in rat insulinoma cells (49). Since hyperglycemia was reported to induce a decrease in glyceraldehyde-3-phosphate dehydrogenase activity in bovine endothelial cells via increased production of mitochondrial ROS and increased activation of the hexosamine pathway (33), mitochondrial ROS may activate the hexosamine pathway in Huh7 cells, thus leading to serine phosphorylation of IRS-1. Although our present study could not clarify which of these pathways is the most important in the pathogenesis of mitochondrial ROS-induced insulin resistance, it clearly demonstrated that mitochondrial ROS is important, at least in part, for TNF-α–induced insulin resistance.

In conclusion, our current study demonstrates that TNF-α increases mitochondrial ROS production, which results in ASK1 activation. In addition, normalization of mitochondrial ROS production or suppression of ASK1 activity restored the TNF-α–induced decrease in insulin-stimulated tyrosine phosphorylation of IRS-1, activation of JNK, and increase in serine phosphorylation of IRS-1, all of which could be involved in the molecular basis of TNF-α–induced insulin resistance. These chain reactions may contribute, at least in part, to TNF-α–induced insulin resistance in vivo. Since impaired insulin action is critical for the development of type 2 diabetes, we emphasize that mitochondrial ROS may be a key factor not only in diabetic vascular complications but also in the development of type 2 diabetes. This integrating paradigm could provide a new conceptual framework for further research and therapies for the treatment of type 2 diabetes.

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