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Catalase, but not MnSOD, Inhibits Glucose Deprivation-Activated ASK1-MEK-MAPK Signal Transduction Pathway and Prevents Relocalization of Daxx: Hydrogen Peroxide as a Major Second Messenger of Metabolic Oxidative Stress

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Abstract Overexpression of catalase, but not manganese superoxide dismutase (MnSOD), inhibited glucose deprivation-induced cytotoxicity and c-Jun N-terminal kinase 1 (JNK1) activation in human prostate adenocarcinoma DU-145 cells. Suppression of JNK1 activation by catalase overexpression resulted from inhibition of apoptosis signal-regulating kinase 1 (ASK1) activation by preventing dissociation of thioredoxin (TRX) from ASK1. Overexpression of catalase also inhibited relocalization of Daxx from the nucleus to the cytoplasm as well as association of Daxx with ASK1 during glucose deprivation. Taken together, hydrogen peroxide (H_2O_2) rather than superoxide anion ($O_2^{\bullet -}$) acts as a second messenger of metabolic oxidative stress to activate the ASK1-MAPK/extracellular signal-regulated kinase (ERK) kinase (MEK)-mitogen-activated protein kinase (MAPK) signal transduction pathway. J. Cell. Biochem. 90: 304–314, 2003. © 2003 Wiley-Liss, Inc.

Key words: hydrogen peroxide; manganese superoxide dismutase; glucose deprivation; Daxx; metabolic oxidative stress; thioredoxin

Apoptosis signal-regulating kinase 1 (ASK1) is a mitogen-activated protein kinase kinase kinase (MAPKKK) family member, and it is activated in response to various cytotoxic stresses including tumor necrosis factor (TNF), Fas ligand, and reactive oxygen species (ROS) [Ichijo et al., 1997; Chang et al., 1998; Saitoh et al., 1998]. The activated ASK1 subsequently activates c-Jun N-terminal kinase (JNK) and p38 pathways, and induces apoptosis in various cells through the mitochondria-dependent caspase activation [Chakraborti and Chakraborti, 1998; Wang et al., 1998; Hatai et al., 2000; Benhar et al., 2001; Du et al., 2001; Tobiume et al., 2001]. Studies with ASK1 knock out mice clearly demonstrated that TNF-induced apoptosis requires ROS-dependent activation of ASK1-JNK/p38 pathways [Tobiume et al., 2001]. We previously observed that glucose deprivation increases the intracellular levels of hydroperoxide (i.e., superoxide anion ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2)) and causes cytotoxicity [Lee et al., 1998; Blackburn et al., 1999]. The increased steady-state levels of

Abbreviations used: ROS, reactive oxygen species; MnSOD, manganese superoxide dismutase; H_2O_2, hydrogen peroxide; $O_2^{\bullet -}$, superoxide anion; JNK, c-Jun N-terminal kinase; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle medium; SDS, sodium dodecyl sulphate; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; PVDF, polyvinylidine difluoride; ASK1, apoptosis signal-regulating kinase 1; SEK1, stress-activated protein kinase/extracellular signal-regulated kinase 1; MEK, MAPK/ERK kinase; TRX, thioredoxin; HA, Hemagglutinin; BSO, L-buthionine-(S,R)-sulfoximine; GSH, glutathione.

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hydroperoxide activate the ASK1-(MAPK/extracellular signal-regulated kinase (ERK) kinase) (MEK)-MAPK signal transduction pathway [Song et al., 2002] and inhibition of the ASK1-MEK-MAPK pathways prevent cytotoxicity [Lee et al., 2000, 2002]. However, a fundamental question that remains unanswered is, which particular ROS is responsible for activating the ASK1-MEK-MAPK signal transduction pathway?

Superoxide dismutase (SOD) is a family of antioxidant enzymes that converts harmful superoxide radicals into H_2O_2 and oxygen molecules. H_2O_2 is subsequently detoxified to harmless water and oxygen molecules by catalase present in the peroxisomes, or by glutathione (GSH) peroxidase present in the mitochondria and the cytosol [Fridovich, 1997; Manna et al., 1998; Wenk et al., 1999]. It has previously been reported that overexpression of manganese superoxide dismutase (MnSOD) in human breast tumor MCF-7 cells blocks TNF-induced cytotoxicity and activation of caspase-3, NF-κB, AP-1, JNK, and MEK [Manna et al., 1998], suggesting that superoxide radicals are involved in their activation. Also, antimycin (a respiratory chain inhibitor)-mediated cytotoxicity was inhibited by overexpression of MnSOD. This finding suggests that a mitochondria-derived free radical ($O_2^{\bullet -}$) plays a critical role in cell death pathways [Kiningham et al., 1999]. However, Wenk et al. [1999] reported that enhanced MnSOD activity causes an unbalanced H_2O_2 overproduction and subsequently results in AP-1 mediated connective tissue degradation. In this study, we investigated whether $O_2^{\bullet -}$ or H_2O_2 is responsible for activating the ASK1-MEK-MAPK signal transduction pathway during glucose deprivation.

MATERIALS AND METHODS

Cell Culture and Glucose Deprivation

Human prostate adenocarcinoma (DU-145) cells were cultured in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (HyClone, Logan, UT), and 26 mM sodium bicarbonate for monolayer cell culture. The cells were maintained in a humidified atmosphere containing 5% CO_2 and air at 37°C. Cells were rinsed three times with phosphate-buffered saline (PBS) and then exposed to glucose-free DMEM containing 10% dialyzed fetal bovine serum (Gibco BRL, Gaithersburg, MD).

Shuttle Vector Construction

A His-tagged 324 bp thioredoxin (*TRX*) gene was isolated from pcDNA-His-TRX by digesting with EcoR I and cloned into the EcoR I site of pAdlox shuttle vector [Hardy et al., 1997]. pFlag-CMV2-Daxx was kindly provided by Dr. Horikoshi (Mallinckrodt Institute of Radiology in Washington University, Saint Louis, Missouri). pAdlox-Flag-Daxx was made by inserting Spe I/BamH I fragment from pFlag-CMV2-Daxx into Spe I/BamH I-cut pAdlox shuttle vector. pcDNA3-Hemagglutinin (HA)-ASK1 was kindly provided by Dr. Ichijo (Tokyo Medical and Dental University, Tokyo, Japan). pAdlox-HA-ASK1 was made by inserting Spe I/Xba I fragment from pcDNA3-HA-ASK1 into Xba I-cut pAdlox. The complete shuttle vector was co-transfected into CRE8 cells with Ψ5 viral genomic DNA for homologous recombination as described below.

Adenoviral Vector Construction

All recombinant adenoviruses were constructed by employing the Cre-lox recombination system [Hardy et al., 1997]. The selective cell line CRE8 has an β-actin-based expression cassette driving a Cre recombinase gene with an N-terminal nuclear localization signal stably integrated into 293 cells. Transfections were done using Lipofectamine Reagent (Gibco BRL). The 5×10^5 cells were split into a 6-well plate 1 day before transfection. For the production of recombinant adenovirus, 2 μg of Sfi I/Apa I-digested Adlox/ASK1 fragment or Sfi I/Apa I-digested Adlox/TRX or Sfi I-digested Adlox/Daxx and 2 μg of Ψ5 viral genomic DNA were co-transfected into CRE8 cells. The recombinant adenoviruses were generated by intermolecular homologous recombination between the shuttle vector and Ψ5 viral DNA. A new virus has an intact packaging site and carries a recombinant gene. Plaques were harvested, analyzed, and purified. The insertion of HA-ASK1 or His-TRX or Flag-Daxx to adenovirus was confirmed by Western blot analysis, after infection of corresponding recombinant adenovirus into DU145 cells.

In Vivo Binding of ASK1 and TRX or Daxx

To examine the interaction between ASK1 and TRX or Daxx, adenovirus of HA-tagged ASK1 (Ad.HA-ASK1) at an MOI of 10 and His-tagged TRX (Ad.His-TRX) at an MOI 30 or Daxx

(Ad.Flag-Daxx) at an MOI of 10 were co-infected into DU145 cells in 100-mm culture plates. For immunoprecipitation with His-TRX, cells were lysed in buffer containing 150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 10 mM EDTA, 1% Triton X-100, 1% deoxycholate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 80 μM aprotinin, 2 mM leupeptin, and the lysates were incubated with 2 μg of anti-penta His mouse IgG1 (Qiagen, Valencia, CA) for 2 h. For immunoprecipitation with HA-ASK1, the lysates were incubated with 0.5 μg of rat anti-HA (clone 3F10, Roche, Mannheim, Germany) for 2 h. After the addition of protein G agarose (Santa Cruz Biotechnology, Santa Cruz, CA), the lysates were incubated an additional 2 h. The beads were washed three times with lysis buffer, separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), and immunoblotted with rat anti-HA, mouse anti-penta His (Qiagen), mouse anti-HA (clone 12CA5, Roche) antibodies or mouse anti-Flag (Sigma-Aldrich, St. Louis, Mo). The proteins were detected with the enhanced chemiluminescence (ECL) reaction (Amersham Pharmacia Biotech, Arlington Heights, IL).

In Vitro Kinase Assay

The plasmid containing GST-human JNK1 for bacterial fusion protein was constructed in pGEX-4T-1 by inserting Hind III/Xba I fragment followed by Klenow treatment from pcDNA3-JNK1. The expression of GST-JNK1 protein was confirmed by Western blot analysis and purified by using glutathione Sepharose 4B (Amersham Pharmacia Biotech). GST-stress-activated protein kinase/extracellular signal-regulated kinase 1 (SEK1), was purified from 10 100-mm plates of 293 cells transfected with pEBG/SEK1 (kindly provided by J.M. Kyriakis, Massachusetts General Hospital, Charlestown, MA), and the purification step was performed as described previously [Yuasa et al., 1998]. DU145 cells were infected with Ad.HA-ASK1 at an MOI of 10. After 48 h of infection, cells were lysed in a buffer solution containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EGTA, 10 mM NaF, 1% Triton X-100, 0.5% deoxycholate, 1 mM DTT, 1 mM sodium orthovanadate, 1mM PMSF, and protein inhibitor cocktail solution (Sigma-Aldrich). Cell extracts were clarified by centrifugation, and the supernatants immunoprecipitated with mouse anti-HA antibody (12CA5, Roche) and protein A-agarose (Gibco BRL). The beads were washed twice with a solution containing 150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 5 mM EGTA, 0.5 mM DTT, 1 mM sodium orthovanadate, and 1 mM PMSF, and washed once with the kinase buffer solution, and subjected to kinase assays. To measure immune complex activity, 0.2 μg of GST-SEK1 is first incubated with the immune complexes for 10 min at 30°C in a final volume of 25 μl of a solution containing 20 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, and 100 μM ATP, and subsequently with 1 μg of GST-JNK1 for 10 min at 30°C. Thereafter, the activated complex is subjected to SDS–PAGE and the phosphorylated JNK is analyzed by rabbit anti-ACTIVE JNK antibody (Promega, Madison, WI). To determine the amount of ASK1 protein in the same sample, the upper part of the SDS–PAGE (>116 kD) was cut out and immunoblotted with the rat anti-HA (Roche, rat IgG_1) antibody.

Immunoblot Analysis

Cell lysates were subjected to electrophoresis on 12% polyacrylamide gels containing SDS under reducing conditions, and the proteins in the gels were transferred onto a polyvinylidine difluoride (PVDF) membrane. The membranes were incubated with 7% (w/v) skim milk in PBST (PBS containing 0.1% Tween 20, v/v) and then reacted with primary antibodies. Polyclonal rabbit anti-ACTIVE JNK was obtained from Promega. Monoclonal mouse anti-actin antibody was purchased from ICN (Costa Mesa, CA). After washing three times with PBST, the membranes were incubated with horseradish peroxidase-conjugated anti-IgG. Proteins in the membranes were then visualized using the enhanced ECL reagent (Amersham Pharmacia Biotech) as recommended by the manufacturer.

Immunofluorescence

Cellular localization of Flag-Daxx and HA-ASK1 were investigated using fluorescence microscopy. The cells were plated onto Lab-Tek chamber slide (Nalge Nunc, Naperville, IL), infected with Ad/Flag-Daxx or Ad/HA-ASK1 at an MOI of 10. After 48 h of infection, cells were fixed in 100% cold methanol for 10 min at −20°C. After washing twice with cold PBS, the cells were blocked in 1% BSA + 10% rabbit or goat serum (depending on the source of 2nd Ab) for 1 h at room temperature (RT). They are then incubated with anti-Flag (clone M2; mouse) or anti-HA (clone 3F10; rat) antibodies

containing 1% BSA + 10% rabbit or goat serum for 1 h at RT, followed by three washes with cold PBS. Samples of Flag-Daxx or HA-ASK1 were incubated for 1 h FITC-conjugated rabbit anti mouse IgG or Rhodamine-conjugated goat anti rat IgG, respectively. After washing three times with cold PBS, and the slides were mounted in 90% glycerol.

RESULTS

Overexpression of Catalase, but not MnSOD, Inhibits Glucose Deprivation-Induced Cytotoxicity and JNK1 Activation

We have previously observed that glucose deprivation induces cytotoxicity, probably by increasing the rate of intracellular prooxidant production [Lee et al., 1998]. In this study, we examined whether ROS ($O_2^{\bullet -}$ or H_2O_2) are involved in metabolic oxidative stress-induced cytotoxicity. DU-145 cells were infected with adenoviral vectors containing MnSOD, catalase, or enhanced green fluorescent protein (EGFP). Figure 1 shows that glucose deprivation-induced cytotoxicity was not prevented by overexpression of EGFP or MnSOD. In contrast, overexpression of catalase, either alone or in combination with MnSOD, significantly inhibited glucose deprivation-induced cytotoxicity. Expression of MnSOD, catalase, or MnSOD + catalase was measured in Figure 2. We previously reported that glucose deprivation-induced JNK1 activation is responsible for cell death [Lee et al., 2000]. Figure 2 shows that overexpression of catalase, but not MnSOD, inhibited glucose deprivation-induced JNK1 activation. These results indicate that H_2O_2 is mainly responsible for glucose deprivation-induced cytotoxicity.

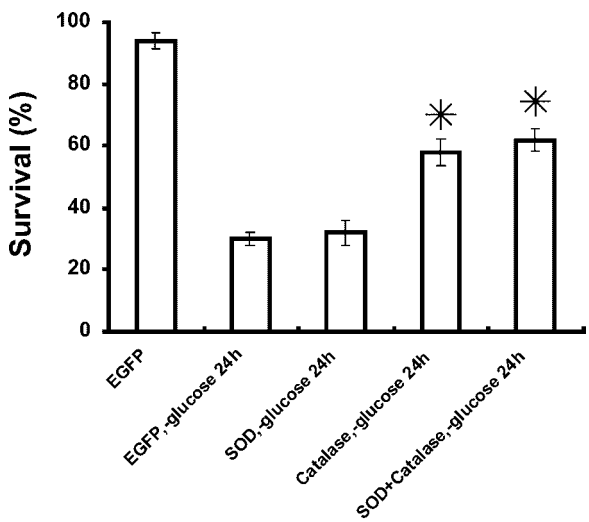


Fig. 1. Effect of manganese superoxide dismutase (MnSOD) or catalase overexpression on glucose deprivation-induced cytotoxicity. DU-145 cells were infected with adenoviral vectors containing EGFP (Ad.EGFP), MnSOD (Ad.MnSOD), or catalase (Ad.Catalase) at an MOI of 100. After 48 h incubation, cells were exposed to glucose-free medium for 24 h. Cell survival was determined by the Trypan blue exclusion assay. Asterisks indicate values are different from EGFP, glucose 24 h (pairs *t*-test, $P < 0.05$). Error bars represent ±SD from three separate experiments.

We further examined whether catalase suppresses JNK1 activation by inhibiting the ASK1-mediated signaling pathway. Our recent studies demonstrated that ASK1 is activated during glucose deprivation [Song et al., 2002]. ASK1 enzyme activity was measured using an immune complex kinase assay with GST-SEK1 and GST-JNK1 serving as sequential substrates. Data from an immune complex kinase assay confirmed that JNK1 activation is mediated through the ASK1-SEK1-JNK1 signal transduction pathway during glucose deprivation (lane 3 in Fig. 3). Overexpression of catalase, either alone or in combination with MnSOD, inhibited glucose deprivation-induced JNK1 activation by preventing ASK1 activation (lanes 5 and 6 in Fig. 3).

Next, we examined whether overexpression of catalase during glucose deprivation inhibits ASK1 activation by inhibiting dissociation of TRX, a negative regulator of ASK1, from ASK1. Figure 4 shows that TRX binds to ASK1 (lane 2 in Fig. 4). TRX dissociated from ASK1 during glucose deprivation (lane 3 in Fig. 4). Overexpression of catalase, but not MnSOD, inhibited dissociation of TRX from ASK1 during glucose deprivation (lane 7 vs. lane 5 in Fig. 4). These results and literature [Saitoh et al., 1998] suggested that H_2O_2 is detected by TRX and dissociation of TRX from ASK1 activates the ASK1-SEK1-JNK1 signal transduction pathway during glucose deprivation.

Interaction Between Daxx and ASK1 During Glucose Deprivation

Previous studies have shown that Daxx, a Fas-binding protein, binds to ASK1, thereby activating the ASK1 kinase [Yang et al., 1997; Ko et al., 2001]. We investigated whether glucose deprivation induces interaction between Daxx and ASK1, and whether overexpression of catalase inhibits such an interaction. Figure 5 shows that Daxx associated with

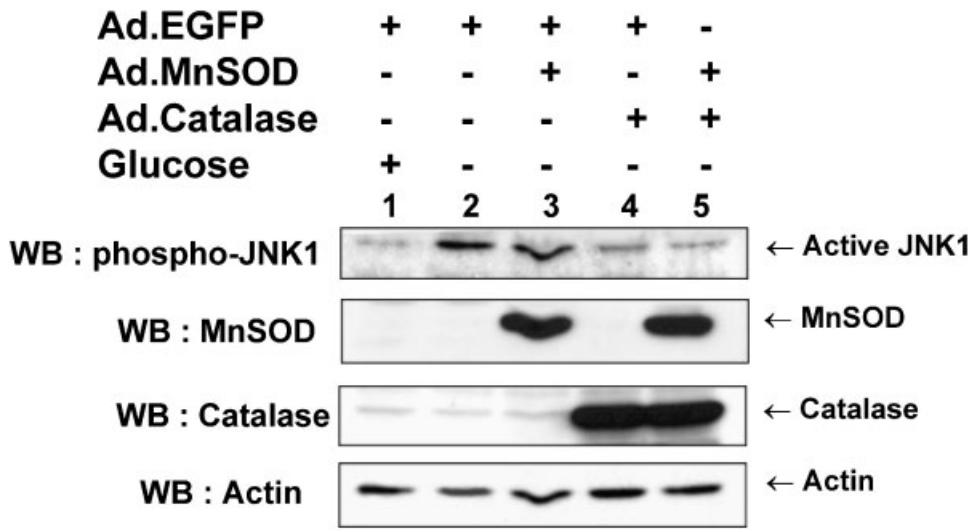


Fig. 2. Effect of MnSOD or catalase overexpression on glucose deprivation-induced c-Jun N-terminal kinase 1 (JNK1) activation. DU145 cells were infected with Ad.EGFP, Ad.MnSOD, or Ad.Catalase at an MOI of 100. After 48 h incubation, cells were exposed to glucose-free medium for 1 h. Cell lysates containing equal amounts of protein (20 μg) were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotted with anti-phospho JNK1 antibody, anti-MnSOD antibody, anti-catalase antibody, or anti-actin antibody. WB, Western blot.

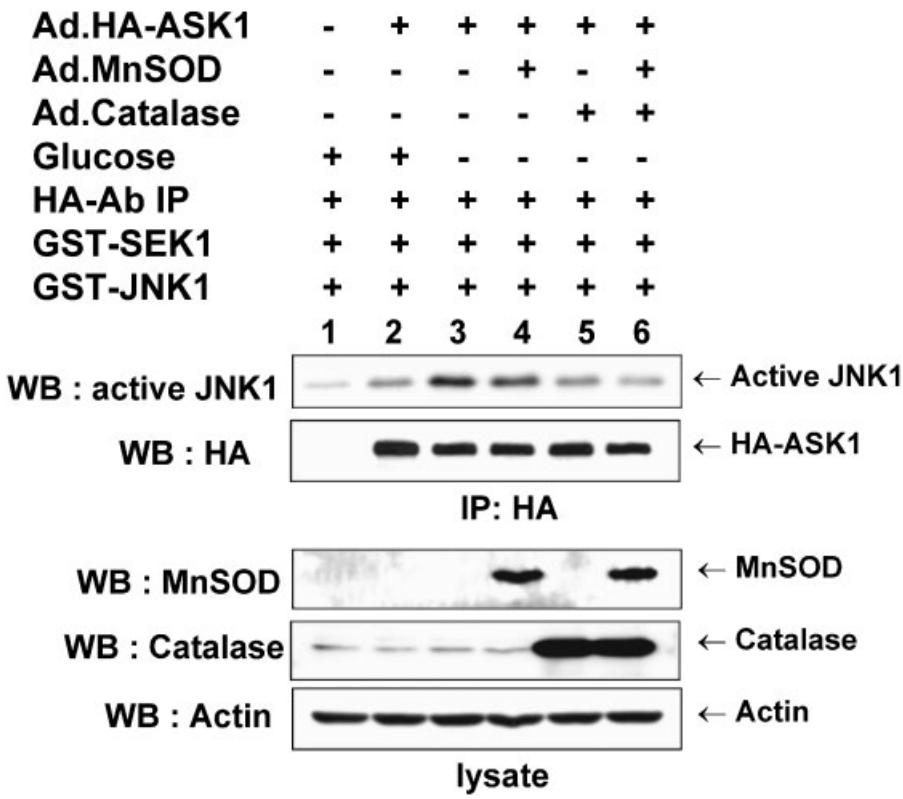


Fig. 3. Effect of MnSOD or catalase overexpression on glucose deprivation-induced apoptosis signal-regulating kinase 1 (ASK1) activation. DU-145 cells were co-infected with adenoviral vectors containing Hemagglutinin (HA)-tagged ASK1 (Ad.HA-ASK1) at an MOI of 10 and Ad.EGFP, Ad.MnSOD, or Ad.Catalase at an MOI of 100. After 48 h incubation, cells were exposed to glucose-free medium for 1 h. Lysates were divided and immunoprecipitated with anti-HA antibody (**upper panels**) or immunoblotted with anti-MnSOD, anti-catalase, or anti-actin antibody (**lower panels**). The immune complex was incubated with GST-stress-activated protein kinase/extracellular signal-regulated kinase 1 (SEK1) followed by GST-JNK1. Active JNK1 was detected with anti-phospho JNK1 antibody. ASK1 was detected with anti-HA antibody.

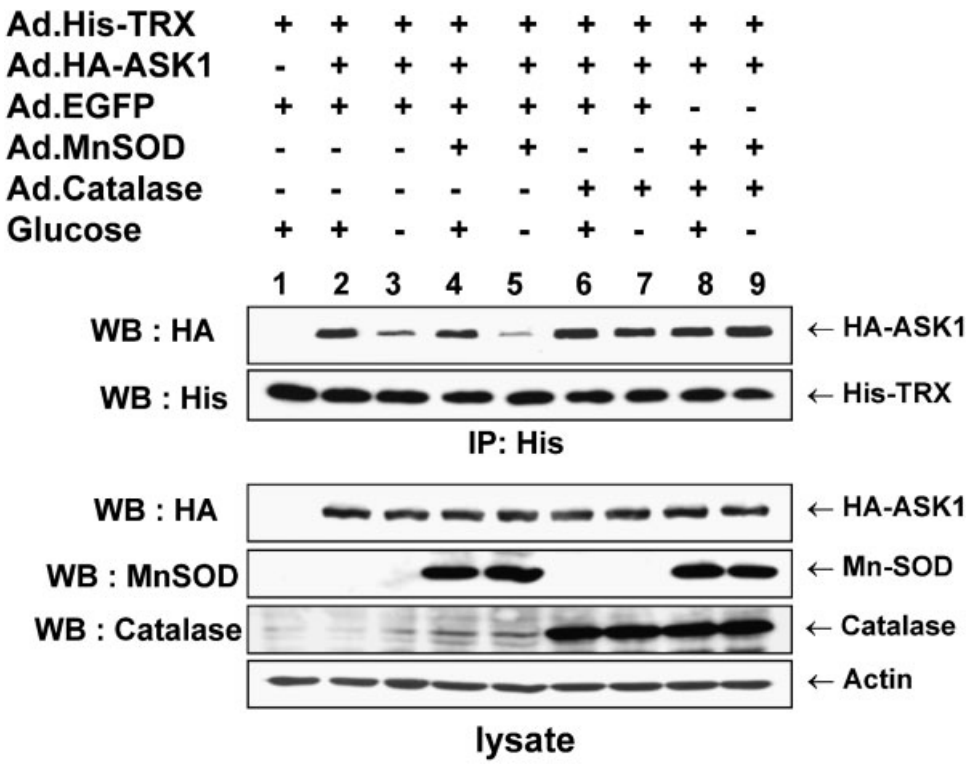


Fig. 4. Effect of MnSOD or catalase overexpression on glucose deprivation-induced dissociation of thioredoxin (TRX) from ASK1. DU-145 cells were co-infected with adenoviral vectors containing His-tagged TRX (Ad.His-TRX) at an MOI of 30, Ad.HA-ASK1 at an MOI of 10, and Ad.EGFP/Ad.MnSOD/Ad.Catalse at an MOI of 100. After 48 h incubation, cells were exposed to glucose-free medium for 1 h. Lysates were divided. One fraction of lysates was immunoprecipitated with anti-His antibody, and then immunoblotted with anti-HA antibody or anti-His antibody (**upper panels**). The other fraction of lysates was immunoblotted with anti-HA, anti-MnSOD, anti-catalase, or anti-actin antibody (**lower panels**).

ASK1 during oxidative stress (H_2O_2 or glucose deprivation) (lanes 3–6 in Fig. 5A or lane 3 in Fig. 5B). Overexpression of catalase, but not MnSOD, inhibited Daxx binding to ASK1 during glucose deprivation (lane 5 vs. lane 4 in Fig. 5B). Previous studies have shown that Daxx is mainly located in the nucleus. In contrast, ASK1 is localized in the cytoplasm [Ko et al., 2001]. Figure 6 shows that Daxx was relocalized to the cytoplasm during oxidative stress (glucose deprivation or H_2O_2 treatment). Unlike Daxx, ASK1 did not change its intracellular location. Importantly, overexpression of catalase, but not MnSOD, prevented the relocalization of Daxx from the nucleus to the cytoplasm during glucose deprivation (Fig. 6). Thus, relocalization of Daxx to the cytoplasm represents a potential point of regulation of the ASK1-SEK1-JNK1 pathway.

Several researchers have shown that GSH is a nonenzymatic peroxide scavenger. To examine a possible role of GSH in glucose deprivation-induced association of Daxx with ASK1, DU-145 cells were co-infected with Ad.Flag-Daxx, Ad.HA-ASK1, and Ad.Catalase, and then treated with 200 μM L-buthionine-(S,R)-sulfoximine (BSO), a GSH synthesis inhibitor for 24 h. Our previous studies showed that BSO reduced the intracellular GSH content by 99% [Song et al., 2002]. Figure 7A shows that BSO increased the interaction between Daxx and ASK1, while decreasing the association of TRX from ASK1 during glucose deprivation. Overexpression of catalase effectively suppressed the association of Daxx with ASK1 during glucose deprivation with or without pretreatment with BSO (Fig. 7B). These results suggest that catalase still serves as a secondary defense against ROS-induced Daxx association with ASK1 when GSH is depleted.

DISCUSSION

The main goal of this study was to identify the primary mediator by which glucose deprivation activates redox-sensitive pathways. $O_2^{\bullet -}$ and

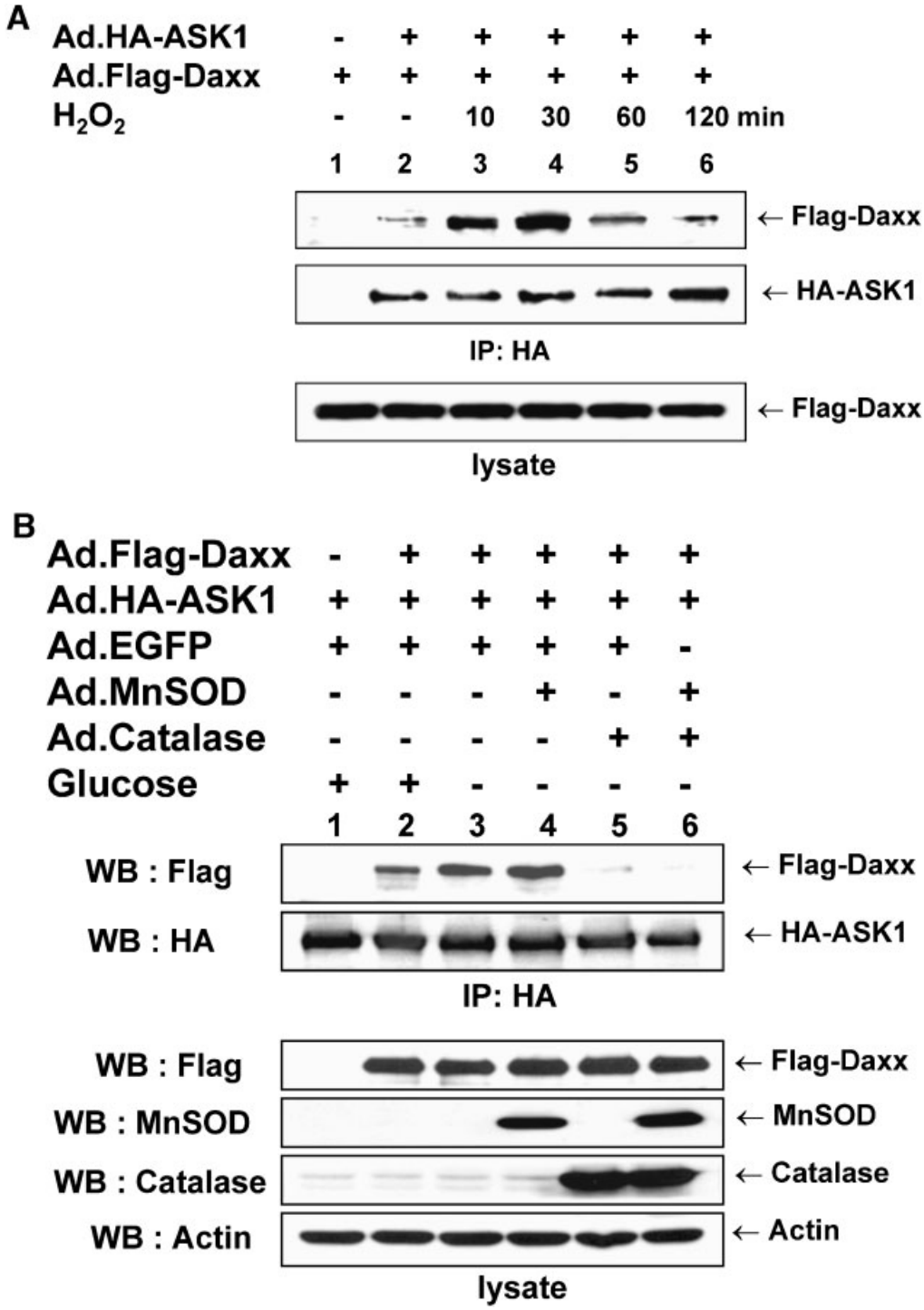


Fig. 5. Hydrogen peroxide (H_2O_2)/glucose deprivation-induced association of Daxx with ASK1. DU-145 cells were co-infected with Ad.Flag-Daxx at an MOI of 10, Ad.HA-ASK1 at an MOI of 10, or Ad.MnSOD/Ad.Catalase/Ad.EGFP at an MOI of 100. After 48 h incubation, cells were exposed to 500 μM H_2O_2 for various times (**panel A**) or glucose-free medium for 1 h (**panel B**). Lysates were immunoprecipitated with anti-HA antibody, and immunoblotted with anti-Flag or anti-HA antibody (**upper panels**). The presence of Flag-Daxx, MnSOD, catalase, or actin in the lysates was verified by immunoblotting with anti-Flag, anti-MnSOD, anti-catalase, or anti-actin antibody (**lower panels**).

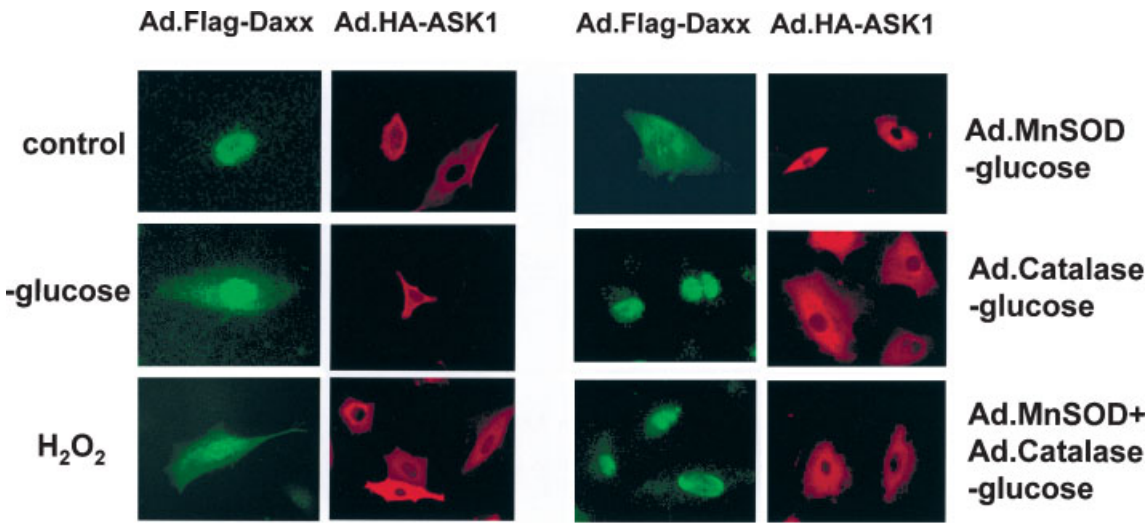
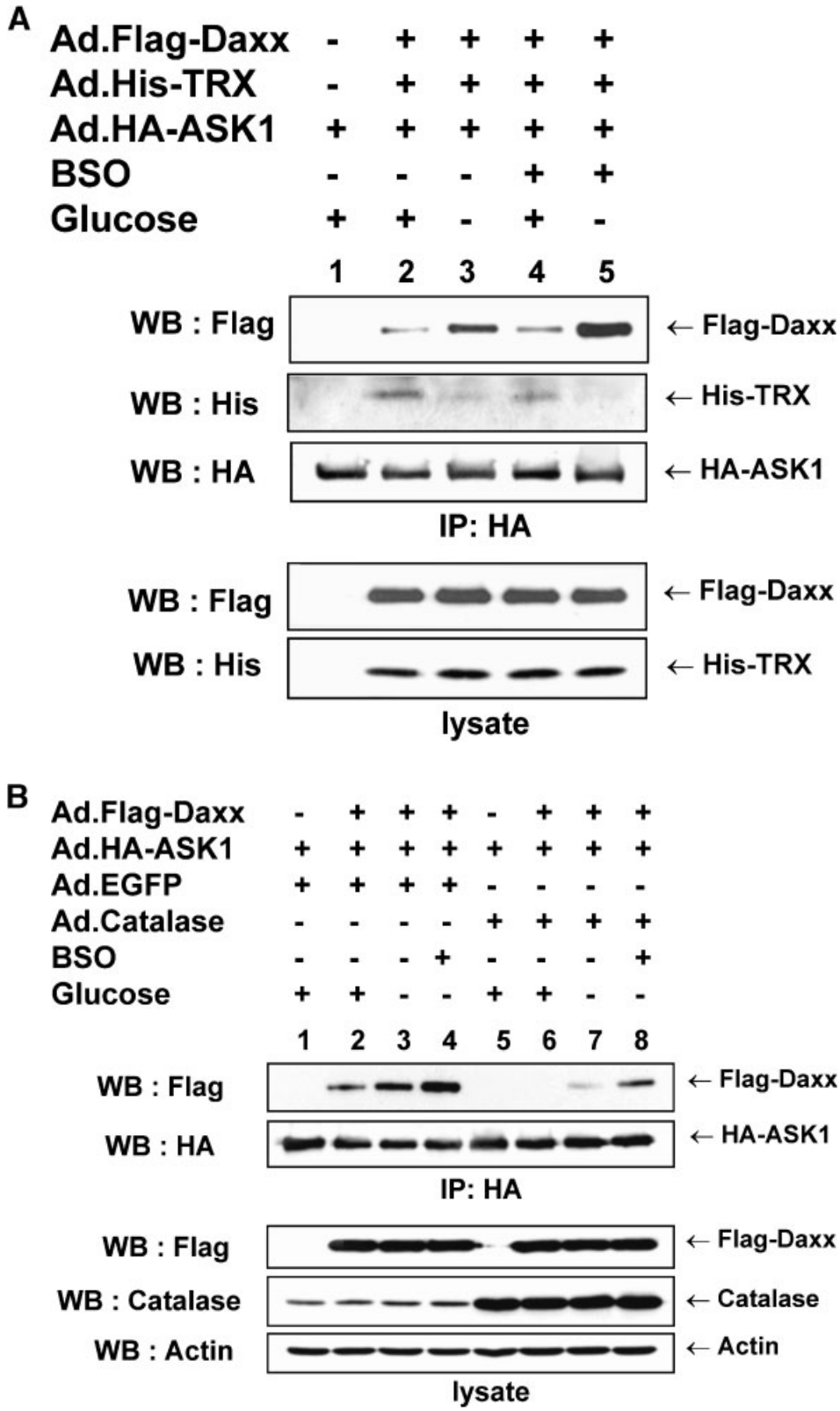


Fig. 6. Effect of MnSOD or catalase overexpression on relocalization of Daxx during glucose deprivation or H_2O_2 treatment in DU-145 cells. Cells were infected with Ad.HA-ASK1 at an MOI of 10 or Ad.Flag-Daxx at an MOI of 10 in combination with Ad.MnSOD/Ad.Catalase at an MOI of 100. After 48 h incubation, cells were exposed to glucose-free medium for 1 h or H_2O_2 (500 μM) for 30 min. The subcellular localization of Daxx or ASK1 was determined by immunofluorescent staining with anti-Flag or anti-HA antibody.

H_2O_2 produced as by-products of mitochondrial respiration should be scavenged by MnSOD, catalase, and GSH peroxidase. However, glucose deprivation decreases intracellular NADPH and pyruvate, followed by a decrease in reduced GSH, an important nonenzymatic peroxide scavenger [Spitz et al., 2000]. Although SOD is well known as an essential enzyme that eliminates $O_2^{\bullet-}$ and thus protects cells from damage induced by free radicals [Fridovich, 1995; Huang et al., 2000], the inhibition of tumor cell growth can be attributed to the increase in the steady-state levels of H_2O_2 as a result of the increased dismutating activity of MnSOD [Kim et al., 2001]. Our studies clearly demonstrate that overexpression of catalase, but not MnSOD, inhibits glucose deprivation-induced cytotoxicity as well as activation of the ASK1-SEK1-JNK1 signal transduction pathway. These results suggest that H_2O_2 rather than $O_2^{\bullet-}$ is the mediator of cytotoxicity and activator of signal transduction during glucose deprivation.

Our studies use ROS generated by glucose deprivation to elucidate the molecular pathway utilized by ROS signal transduction, which is somewhat more relevant to the pathophysiological state than using a bolus of oxidant such as H_2O_2 (Fig. 5A), because it makes use of endogenous ROS generated at physiological rates. This difference may explain why Daxx associated with ASK1 within a relatively short period during H_2O_2 treatment in comparison to glucose deprivation (Fig. 5A vs. Fig. 5B).

Although Daxx is known to mediate the recruitment of ASK1 to Fas after Fas ligation for Fas-mediated apoptosis [Chang et al., 1998], it is mainly localized in the nucleus [Kim et al., 2001]. In contrast to Daxx, ASK1 is mainly located in the cytoplasm [Ko et al., 2001]. Interestingly, Figure 6 shows that the cytoplasmic staining of ASK1 appears non-uniform, suggesting a cytoskeletal or membranous distribution. Obviously, we need further studies on the subcellular localization of ASK1 and possible alterations during glucose deprivation. As shown in Figures 5 and 7, Daxx associated with ASK1 and glucose deprivation elevated the association of Daxx with ASK1. These results suggest that Daxx is relocalized from the nucleus to the cytoplasm during glucose deprivation. Figure 6 shows that relocalization of Daxx indeed occurs during glucose deprivation. However, it still remains to be determined exactly how Daxx is relocalized from the nucleus to the cytoplasm during glucose deprivation. At the present time, we can only speculate on the mechanism(s) for the relocalization of Daxx during glucose deprivation. We observed that Daxx is phosphorylated during glucose deprivation (unpublished data). We postulate that glucose deprivation-activated JNK1 may directly or indirectly be involved in Daxx phosphorylation. Recent studies have shown that


A
Ad.Flag-Daxx - + + + +
Ad.His-TRX - + + + +
Ad.HA-ASK1 + + + + +
BSO - - - + +
Glucose + + - + -
1 2 3 4 5
WB : Flag
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WB : His
← His-TRX
WB : HA
← HA-ASK1
IP: HA
WB : Flag
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Ad.Flag-Daxx - + + + - + + +
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WB : Flag
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Fig. 7.

homeodomain-interacting protein kinase 1 (HIPK1) interacts with Daxx and relocalizes Daxx [Ecsedy et al., 2003]. HIPK1 also phosphorylates Daxx on Ser 669, and phosphorylation of this site diminishes the function of Daxx as a transcriptional repressor [Ecsedy et al., 2003]. Thus, it is possible that JNK1 activates HIPK1 and subsequently relocalizes Daxx. Obviously, further studies at the biochemical level are necessary to understand the possible involvement of JNK1-HIPK1 in the relocalization of Daxx. Nigg [1997] illustrated that the biological function of nuclear localization signals is regulated by phosphorylation/dephosphorylation. Thus, we hypothesize that phosphorylation of Daxx results in conformational changes, exposing the nuclear export signal (NES) which is then recognized by an export receptor. Interaction between the NES of Daxx and the export receptor may thus be the critical step in redirecting nuclear Daxx to the cytoplasm. We present this possibility as a framework for future experiments.

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Fig. 7. Effect of L-buthionine-(S,R)-sulfoximine (BSO) on glucose deprivation-induced association of Daxx with ASK1. **Panel A**: DU-145 cells were co-infected with Ad.HA-ASK1 at an MOI of 10, Ad.His-TRX at an MOI of 30, and Ad.Flag-Daxx at an MOI of 10. After 24 h incubation, cells were treated with (+) or without (−) 200 μM BSO for 24 h. Cells were exposed to complete medium (+) or glucose-free medium (−). Cell lysates were divided. One fraction of lysates was immunoprecipitated with anti-HA antibody, and immunoblotted with anti-Flag, anti-His, or anti-HA antibody (**upper panels**). The other faction of lysates was immunoblotted with anti-His or anti-Flag antibody to verify the presence of His-TRX or Flag-Daxx (**lower panels**). **Panel B**: DU-145 cells were co-infected with Ad.Flag-Daxx at an MOI of 10, Ad.HA-ASK1 at an MOI of 10, or Ad.MnSOD/Ad.Catalase/Ad.EGFP at an MOI of 100. After 24 h infection, cells were treated with (+) or without (−) 200 μM BSO for 24 h and then exposed to glucose-free medium for 1 h. Lysates were immunoprecipitated with anti-HA antibody, and immunoblotted with anti-Flag or anti-HA antibody (upper panels). The presence of Flag-Daxx, catalase, or actin in the same lysates was verified by immunoblotting with anti-Flag, anti-catalase, or anti-actin antibody (lower panels).

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