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ARTICLES

Effect of Glucose Concentration on Activation of the ASK1–SEK1–JNK1 Signal Transduction Pathway

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Abstract Recently, acute total glucose deprivation has been shown to cause activation of ASK1–MEK–MAPK signal transduction and dissociation of glutaredoxin (GRX) from apoptosis signal-regulating kinase 1 (ASK1). In this study, we investigated whether clinically relevant concentrations (0.01–0.1 mM) of glucose promote ASK1 activation. We observed that a prominent activation of JNK1 occurred at a glucose concentration less than or equal to 0.01 mM. Similar to JNK1 activation, we also observed that low glucose-induced ASK1 activation, dissociation of GRX and thioredoxin (TRX) from ASK1, dimerization of ASK1, and association of Daxx and TRAF2 with ASK1 significantly occurred at a glucose concentration less than or equal to 0.01 mM. J. Cell. Biochem. 89: 653–662, 2003. © 2003 Wiley-Liss, Inc.

Key words: glucose deprivation; glutaredoxin; thioredoxin; ASK1; SEK1; C-Jun N-terminal kinase; TRAF2; Daxx

It is well known that severe architectural and functional abnormalities are commonly observed in the capillary network that develops during tumor growth [Vaupel et al., 1989]. Tumor venules are tortuous, elongated, and often dilated. The host vessels per unit tumor mass do not increase in number, thus leading to a reduction of the available exchange area for oxygen, nutrients, hormones, growth factors, and waste products [Vaupel et al., 1989]. These abnormalities cause insufficient blood supply and development of a pathophysiological tumor micro-environment. Previous studies with the micropore chamber sampling procedure [Gullino and Grantham, 1961] and tumor-isolated preparations [Gullino et al., 1964] reveal differences in the constituents of serum (vascular compartment) compared to interstitial fluid (interstitial compartment). Vascular and interstitial compartments are two major compartments of the extracellular space of solid tumors. Several researchers have shown that major differences between them are glucose concentrations, oxygen tensions, extracellular pH, and lactate concentrations. However, there are little or no changes in the levels of protein, amino acids, and salts [Gullino, 1975]. The tumor micro-environment is characterized by low oxygen tensions (hypoxia) [Vaupel et al., 1991], low glucose concentrations [Gullino, 1975], high lactate concentrations [Schwickert et al., 1995; Walenta et al., 1997], and low extracellular pH [Wike-Hooley et al., 1984]. For example, the micropore sampling procedure shows that the interstitial compartment or vascular compartment contains 0.03 or 9.5 mM glucose, respectively [Gullino, 1975].

Among these tumor micro-environmental factors, we have previously investigated the effect of glucose-deprivation on various types of tumor cell lines [Lee et al., 1998, 2000; Spitz et al., 2000]. During glucose deprivation, the fact that steady state levels of intracellular hydroperoxides appear to increase immediately [Lee et al., 1998; Blackburn et al., 1999] suggests

Abbreviations used: JNK1, c-Jun N-terminal kinase; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle medium; SDS, sodium dodecyl sulphate; SAPK, stress-activated protein kinase; PVDF, polyvinylidine difluoride; ASK1, apoptosis signal-regulating kinase 1; SEK1, stress-activated protein kinase/extracellular signal regulated–kinase kinase; GRX, glutaredoxin; TRX, thioredoxin; GSSG, glutathione disulfide; HA, hemagglutinin; His, histidine.; MOI, multiplicity of infection; ROS, reactive oxygen species.

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that hydroperoxides are produced by ongoing metabolic processes. It also suggests that the metabolic decomposition of these hydroperoxides is compromised by the removal of glucose probably via a decrease in intracellular pools of NADPH and pyruvate. We postulated that mitochondrial electron transport chain activity could result in increases in steady state levels of peroxidants (i.e., H_2O_2) due to decreases in peroxide scavenging by pyruvate and the glutathione/glutathione peroxidase/glutathione reductase system during glucose deprivation. Our previous studies indeed reveal that glucose deprivation results in increased steady state levels of intracellular oxidized glutathione (GSSG) (3- to 10-fold), and hydroperoxide (two- to fourfold), as determined by increased fluorescence of an oxidation sensitive probe [Lee et al., 1998, 2000; Blackburn et al., 1999].

Our recent studies have shown that the accumulation of oxidized glutathione (GSSG) is sensed through glutaredoxin (GRX) and triggers the ASK1–MEK–MAPK signal transduction pathway during glucose deprivation [Song et al., 2002]. GRX appears to act as a physiological inhibitor of ASK1 by associating with the C-terminal portion of ASK1 and inhibiting ASK1 kinase activity. Previous studies have shown that GRX contains two redox-active cysteine residues, Thr–Cys^{22}–Pro–Tyr–Cys^{25}–Arg in an active catalytic center [Chrestensen et al., 1995]. Cys^{22} is probably involved in forming a covalent glutathionyl GRX disulfide intermediate form (GRX-SSG) during glucose deprivation. Subsequently, GRS-SSG may be converted to the intramolecular disulfide form of GRX-(S-S). The oxidized form of GRX, GRX-(S-S), probably dissociates from ASK1. Similar to GRX, TRX also contains a redox-active disulfide/dithiol active site within structure –Cys–Gly–Pro–Cys–, which can be reversibly oxidized and reduced. It is, therefore, not only GRX but also TRX possibly plays a role in the regulation of ASK1 activity.

One of the drawbacks in our previous studies is the lack of relevance to pathophysiological condition. Acute total deprivation of glucose has little pathophysiological counterpart in tumors. If the ASK1–MEK–MAPK signal transduction pathway is only induced at extremely low glucose concentrations then it is of little relevance to the situation in tumors. Thus, in this study, we investigated the effect of glucose concentration on activation of the ASK1–SEK1–JNK1 signal transduction pathway. Our data demonstrated that 0.01 mM glucose or less activated the ASK1–SEK1–JNK1 signal transduction.

MATERIALS AND METHODS

Cell Culture and Treatment With 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, Gathersburg, MD).

Vector Construction

A His-tagged 324 bp 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]. pcDNA3–His–GRX was made by inserting the *EcoR I* fragment from pQE–GRX into *EcoR I*-cut pcDNA3. pAdlox–His–GRX was made by inserting the *Hind* III/*Xba* I fragment from pcDNA3–His–GRX into *Hind* III/*Xba* I-cut pAdlox shuttle vector containing N-terminal fused form with hexahistidine (6Xhis) tag. pcDNA3–HA–ASK1 was kindly provided by Dr. Ichijo (Tokyo Medical and Dental University, Tokyo, Japan). pAdlox–HA–ASK1 was made by inserting the *Spe* I/*Xba* I fragment from pcDNA3–HA–ASK1 into *Xba* I-cut pAdlox. pcDNA3–myc–ASK1 was made by inserting the PCR product of ASK1 to pCDNA3–myc. Sense primer was 5′-ATTATACGTATAGCACGGAGGCGGACGAGGG-3′ introducing a SnaB I site and antisense primer was 5′-CGCGTCTAGATCAAGTCTGTTT-GTTTCGAAAGTCAATG-3′ introducing a Xba I site for inserting into pcDNA3–myc (BamH I → Klenow → Xba I). pAdlox–myc–ASK1 was made by inserting the *Spe* I/*Xba* I fragment from pcDNA3–myc–ASK1 into *Spe* I/*Xba* I-cut pAdlox. pFlag–CMV2–Daxx was kindly provided by Dr. Horikoshi (Mallinckrodt Institute of Radiology in Washington University, Saint Louis, MO). pAdlox–Flag–Daxx was made by inserting the Spe I/BamH I fragment from pFlag–CMV2–Daxx into Spe I/BamH-cut pAdlox shuttle

vector. pcDNA3–HA–ASK1 was kindly provided by Dr. Ichijo (Tokyo Medical and Dental University, Tokyo, Japan). pAdlox–HA–ASK1 was made by inserting the *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 Vectors

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 by using Lipofectamine Reagent (Gibco BRL). A total of 5×10^5 cells were split into a 6 well plate one 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/GRX 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–GRX (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 GRX (or TRX) or Daxx (or TRAF2)

To examine the interaction between ASK1 and GRX (or TRX) or Daxx (or TRAF2), adenovirus of HA-tagged ASK1 (Ad.HA–ASK1) at an MOI of 10 and His-tagged GRX or TRX (Ad.His–GRX or TRX) or Daxx (Ad.Flag–Daxx) at an MOI of 30 were co-infected into DU145 cells in 100-mm culture plates. In the case of TRAF2, adenovirus of HA-tagged ASK1 at an MOI of 10 and 5 μg of pFlagCMV2/TRAF2 were co-infected/transfected into DU145 cells in 100-mm culture plates. For immunoprecipitation, 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. In case of 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 (Calbiochem, Darmstadt, Germany), the lysates were incubated for an additional 2 h. The beads were washed three times with the lysis buffer, separated by SDS–polyacrylamide gel electrophoresis (PAGE), and immunoblotted with rat anti-HA or mouse anti-penta His (Qiagen) or mouse anti-HA (clone 12CA5, Roche) antibodies or mouse anti-Flag (Sigma-Aldrich, St. Louis, MO). The proteins were detected with the enhanced chemiluminescence 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–SEK1 was purified from ten 100-mm plates of 293 cells transfected with the 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 followed by treatment with BSO for 24 h. 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 sodium orthovanadate, 1 mM PMSF, and protein inhibitor cocktail solution (Sigma Chemical Co., St. Louis, MO). 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, 1 mM sodium orthovanadate, and 1 mM PMSF, and washed once with the kinase buffer solution, and then they were 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 kDa) 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) skin 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 chemiluminescence (ECL) reagent (Amersham, Arlington Heights, IL) as recommended by the manufacturer.

RESULTS

Effect of Various Concentrations of Glucose on JNK Activation

We have previously observed that glucose deprivation activates c-Jun N-terminal kinase 1 (JNK1). We further examined the dose-response for activation of JNK1 by decreased glucose concentration in DU-145 cells. As mentioned previously, interstitial fluids contain 0.03 mM glucose [Gullino, 1975]. It is, therefore, more likely that some glucose would be present in the tumor micro-environment. Figure 1 shows that glucose deprivation-induced activation of JNK1 was dependent upon glucose concentrations. Western blots using an antibody specific for the active form of JNK (anti-ACTIVE JNK antibody) showed that a prominent activation of JNK1 occurred at a glucose concentration less than or equal to 0.01 mM. We also observed a minimal activation of JNK1 as high as 0.1 mM.

Effect of Various Concentrations of Glucose on ASK1 Activity

Previous studies have shown that apoptosis signaling kinase-1 (ASK1) activates the SEK1–JNK and the MKK3/MKK6-p38 signaling cascades [Ichijo et al., 1997; Saitoh et al., 1998; Tobiume et al., 2001]. To examine whether the glucose dose dependent-JNK activation is mediated through the activation of ASK1, DU-145 cells were infected with Ad.HA–ASK1 at an MOI of 10. Cell lysates were immunoprecipitated with anti-HA-antibody. ASK1 enzyme activity was measured by an immune complex kinase assay using glutathione S-transferase (GST)–SEK1 and GST–JNK1 as sequential substrates. Activated JNK1 was detected by anti-ACTIVE JNK antibody. Data from in vitro kinase assay show that glucose deprivation-induced ASK1 activation was dependent upon glucose concentration. We observed that a significant activation of ASK1 occurred at a glucose concentration less than or equal to 0.01 mM (Fig. 2).

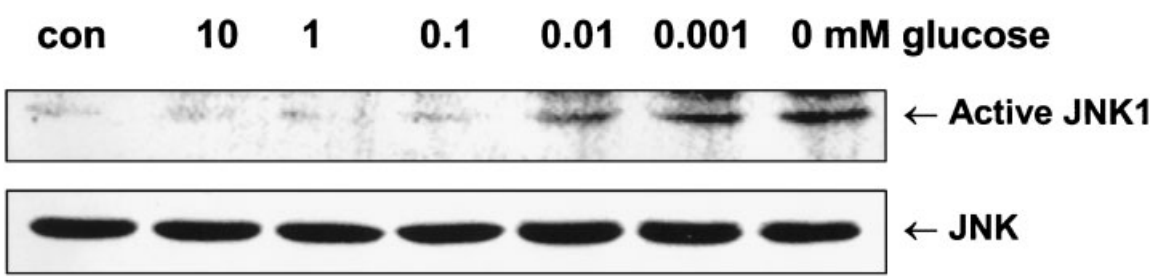

Fig. 1. Effect of various concentrations of glucose on JNK1 activation. DU-145 cells were exposed to low glucose medium (0–10 mM) for 1 h. Cell lysates containing equal amounts of protein (20 μg) were separated by SDS–PAGE and immunoblotted with anti-phospho JNK antibody or anti-JNK antibody.

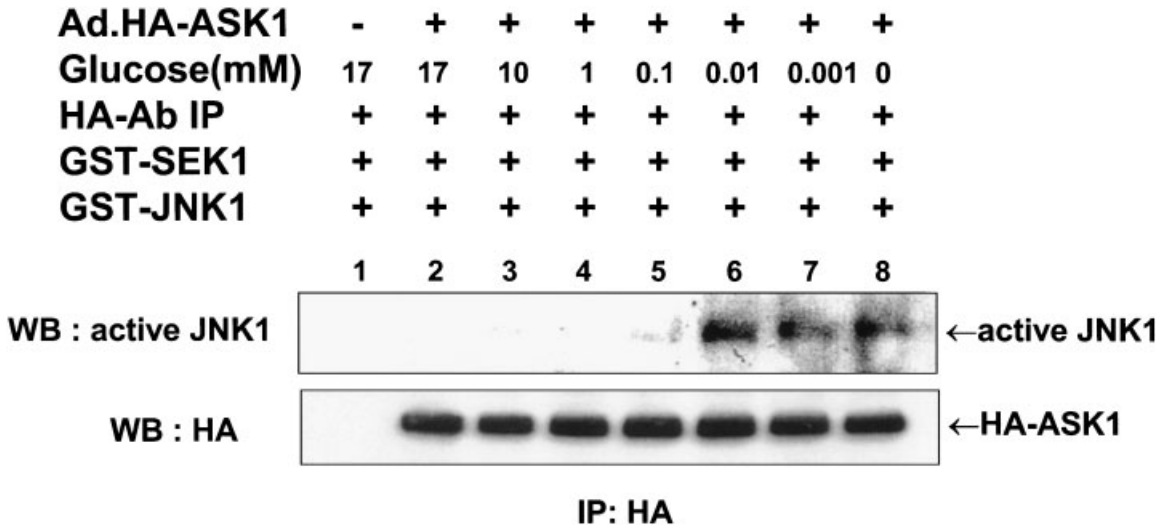

Fig. 2. Effect of various concentrations of glucose on ASK1 activation. DU-145 cells were infected with adenoviral vectors containing HA-tagged ASK1 (Ad.HA–ASK1) at an MOI of 10. After 48 h incubation, cells were exposed to low glucose medium (0–10 mM) for 1 h. Lysates were immunoprecipitated with anti-HA antibody. The immune complex was incubated with GST–SEK1 followed by GST–JNK1. Active JNK1 or ASK1 was detected with anti-phospho JNK antibody, or anti-HA antibody, respectively. **Lane 1**: uninfected control cells.

Effect of Various Concentrations of Glucose on Dissociation of GRX or TRX from ASK1

We previously reported that GRX binds to ASK1 and negatively inhibits the activation of ASK1 [Song et al., 2002]. We hypothesized that another thiol-containing signaling molecule, TRX is involved in the regulation of ASK1 activation in a similar fashion to GRX during glucose deprivation. To test this hypothesis, DU145 cells were co-infected with adenoviral vectors containing His-tagged GRX/TRX (Ad. His–GRX/TRX) and HA-tagged ASK1 (Ad.HA–ASK1). After 24 h infection, cells were exposed to low glucose medium (0–10 mM), cell lysates were prepared and immonoprecipitated with anti-His antibody followed by immunoblotting with anti-HA and anti-His. Figure 3 shows that dissociation of GRX/TRX from ASK1 occurred at a glucose concentration less than or equal to 0.01 mM.

Effect of Various Concentrations of Glucose on Dimerization of ASK1

Previous studies reveal that ROS such as H_2O_2 activates ASK1 via dimerization of ASK1 [Gotoh and Cooper, 1998]. To examine whether glucose deprivation, which is known to generate metabolic oxidative stress, induces ASK1 dimerization, DU-145 cells were co-infected with adenoviral vectors containing myc- or Ha-tagged ASK1. As shown in Figure 4, glucose deprivation induced dimerization of ASK1. Glucose deprivation-induced dimerization of ASK1 was dependent upon glucose concentrations. We observed that a significant dimerization of ASK1 occurred at a glucose concentration less than or equal to 0.01 mM (Fig. 4). We also observed a minimal dimerization of ASK1 as high as 1 mM glucose.

Effect of Various Concentrations of Glucose on Association of Daxx or TRAF2 with ASK1

Daxx, one of the ASK1-binding molecule which is known to be an adaptor of Fas, recruits and activates ASK1 [Yang et al., 1997; Chang et al., 1998; Charette et al., 2000]. In contrast to TRX and GRX, it acts as an activator of ASK1 through binding to ASK1 [Chang et al., 1998]. We examined whether Daxx binds to ASK1 during glucose deprivation. Figure 5 shows that Daxx associated with ASK1 during glucose deprivation and association of ASK1 with Daxx prominently occurred less than or equal to 0.01 mM of glucose concentration (Fig. 5A). We also investigated association of TNF receptor-associated factor 2 (TRAF2), another ASK1-binding molecule [Nishitoh et al., 1998; Liu et al., 2000] with ASK1 during glucose deprivation. TRAF2 is known to be an enhancer of

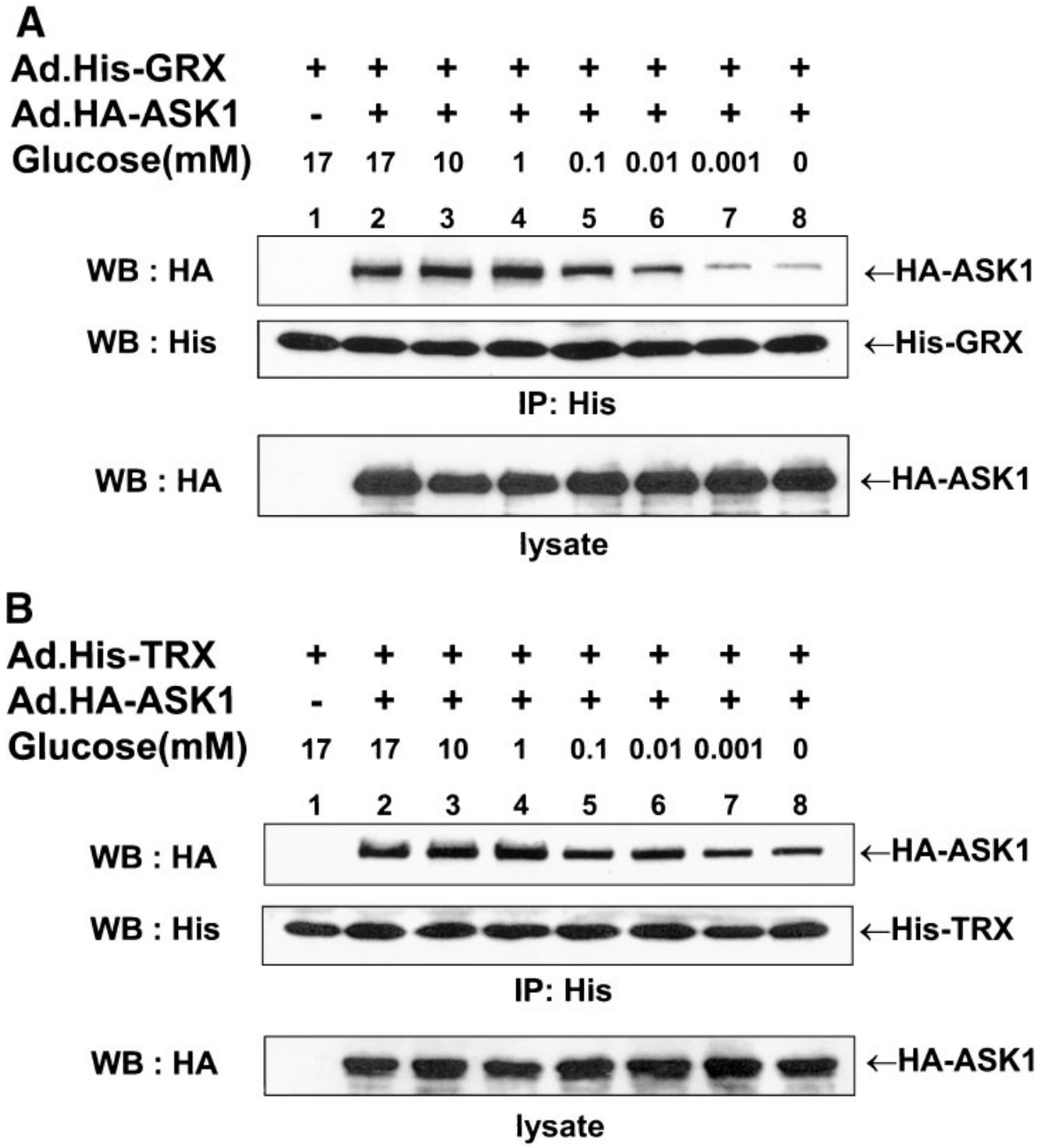

Fig. 3. Effect of various concentrations of glucose on dissociation of GRX or TRX from ASK1. **Panel A**: DU-145 cells were co-infected with Ad.HA–ASK1 at an MOI of 10 and adenoviral vectors containing His-tagged GRX (Ad.His–GRX) at an MOI of 30. After 48 h incubation, cells were exposed to low glucose medium (0–10 mM) for 1 h. Lysates were immunoprecipitated with anti-His antibody, and immunoblotted with anti-HA antibody or anti-His antibody (**upper**). The presence of HA–ASK1 in the lysates was verified by immunoblotting with anti-HA antibody (**lower**). **Panel B**: DU-145 cells were co-infected with Ad.HA–ASK1 at an MOI of 10 and adenoviral vectors containing His-tagged TRX (Ad.His–TRX) at an MOI of 30. After 48 h incubation, cells were exposed to low glucose medium (0–10 mM) for 1 h. Lysates were immunoprecipitated with anti-His antibody, and immunoblotted with anti-HA antibody or anti-His antibody (**upper**). The presence of HA–ASK1 in the lysates was verified by immunoblotting with anti-HA antibody (**lower**).

ASK1 homo-oligomerization for the JNK/SAPK activation. As like Daxx, association of ASK1 with TRAF2 occurred during glucose deprivation and significant association between them was observed when glucose concentration was less than or equal to 0.01 mM (Fig. 5B). We also observed a minimal association of Daxx or TRAF2 with ASK1 as high as 0.1 mM glucose.

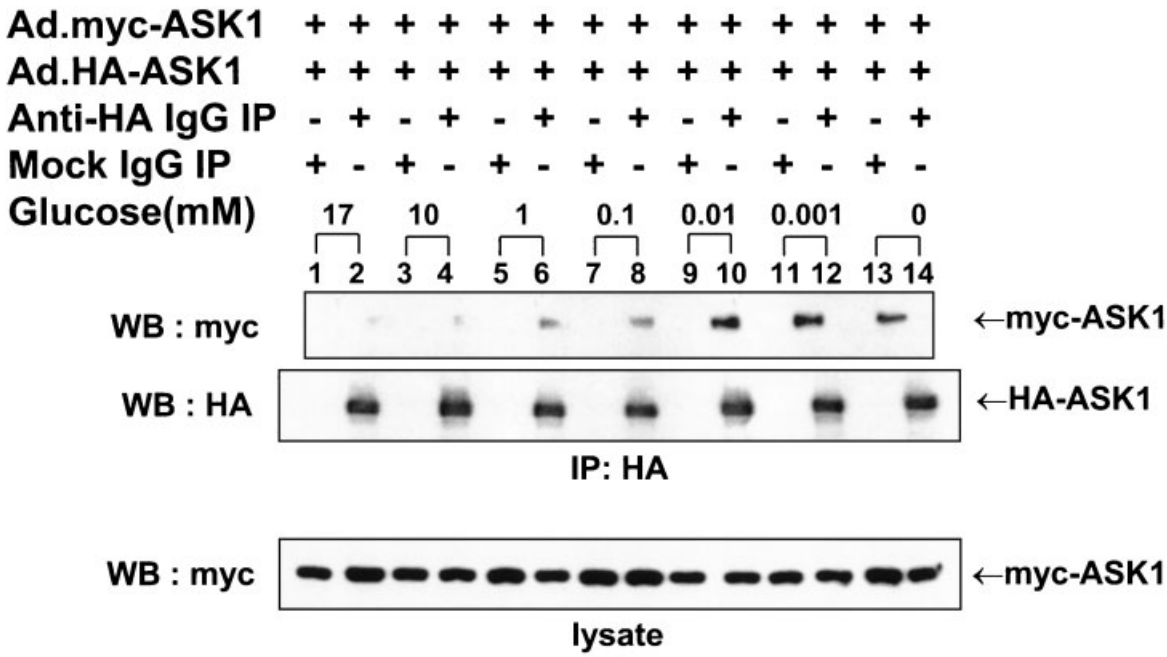

Fig. 4. Effect of various concentrations of glucose on dimerization of ASK1. DU-145 cells were co-infected with Ad.HA–ASK1 at an MOI of 10 and adenoviral vectors containing myc-tagged ASK1 (Ad.myc–ASK1) at an MOI of 10. After 48 h incubation, cells were exposed to low glucose medium (0–10 mM) for 1 h. Lysates were immunoprecipitated with anti-HA antibody, and immunoblotted with anti-myc antibody or anti-HA antibody (**upper**). The presence of myc–ASK1 in the lysates was verified by immunoblotting with anti-myc antibody (**lower**).

DISCUSSION

It has been noted that cells that have undergone neoplastic transformation (cancer cells) demonstrate altered metabolism when compared to untransformed (normal) cells [Dang and Semenza, 1999]. In general, it has been noticed that cancer cells exhibit increased glycolysis and pentose phosphate cycle activity, while demonstrating only slightly reduced rates of respiration [Weber, 1977a,b; Lee et al., 1998; Blackburn et al., 1999]. During glucose deprivation in these cancer cells, which have required increased amount of glucose for the metabolic demand for ATP, increased the intracellular level of superoxide and hydrogen peroxide due to decrease in the production of scavengers such as NADPH or pyruvate [Lee et al., 1998; Spitz et al., 2000]. It is well known that the removal of glucose from human tumor cells preferentially causes cytotoxicity as well as activation of signaling related to MAPK kinase compared to normal cells [Lee et al., 1998; Spitz et al., 2000]. These findings resulted in production of reactive oxygen species such as superoxide and hydrogen peroxide during glucose deprivation, so called metabolic oxidative stress. Nonetheless, the major shortcoming with that study is the fact that total deprivation of glucose does not reflect a true tumor environment. There are very few, if any, cells in tumors that are totally deprived of glucose, or even at a relatively low concentrations. In this study, we observed that the ASK1–SEK1–JNK1 signal transduction pathway is prominently activated at a glucose concentration less than or equal to 0.01 mM. We also observed a minimal activation of JNK1 as high as 0.1 mM. This concentration of glucose is close to that in the tumor interstitial compartment which is about 0.6 mg/100 ml (0.03 mM) [Gullino, 1975]. Recent studies from Walenta et al. [2002] demonstrated pronounced heterogeneity in the intratumoral distribution of glucose in comparison to normal tissue. The existence of steep glucose gradients was observed in the tumor tissue in comparison to the normal tissue. A glucose content of less than 0.5 μmol/g was detected in the tumor tissue by quantitative bioluminescence and single photon imaging assays. Glucose was imaged

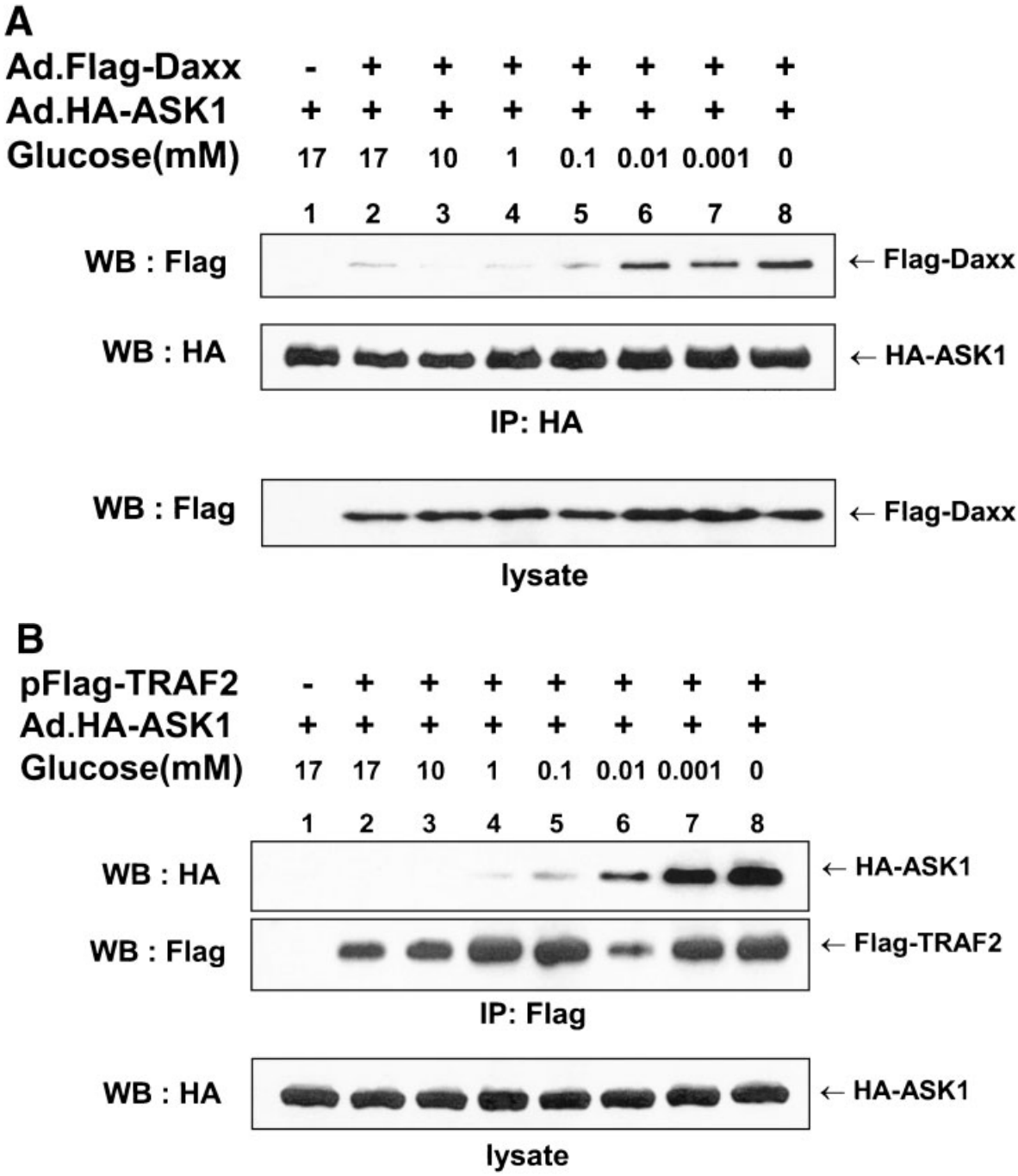

Fig. 5. Effect of various concentrations of glucose on association of Daxx or TRAF2 with ASK1. **Panel A**: DU-145 cells were co-infected with Ad.HA–ASK1 at an MOI of 10 and adenoviral vectors containing Flag-tagged Daxx (Ad.Flag–Daxx) at an MOI of 30. After 48 h incubation, cells were exposed to low glucose medium (0–10 mM) for 1 h. Lysates were immunoprecipitated with anti-HA antibody, and immunoblotted with anti-Flag antibody or anti-HA antibody (**upper**). The presence of Flag–Daxx in the lysates was verified by immunoblotting with anti-Flag antibody (**lower**). **Panel B**: DU-145 cells were transfected first with 5 μg of Flag-tagged TRAF2 (pFlag–TRAF-2) and infected with Ad.HA–ASK1 at an MOI of 10. After 48 h incubation, cells were exposed to low glucose medium (0–10 mM) for 1 h. Lysates were immunoprecipitated with anti-HA antibody, and immunoblotted with anti-HA antibody or anti-Flag antibody (**upper**). The presence of HA–ASK1 in the lysates was verified by immunoblotting with anti-HA antibody (**lower**).

quantitatively and within microscopic dimensions in cryosections from shock frozen biological specimens using enzyme reactions and light emission by luciferases [Mueller-Klieser et al., 1988]. A drawback of this technique is that the minimum detectable tissue content of glucose is 0.1–0.5 μmol/g.

In this study we used ROS generated by glucose deprivation under oxygenated conditions as a tool, not to investigate a pathophysiological state, but to elucidate the molecular pathway utilized by ROS signal transduction. This is, indeed, a novel approach. It is also a very relevant approach; in fact, more relevant than using a bolus of oxidant such as H_2O_2 because it makes use of endogenous ROS generated at physiological rates. However, cells in tumors that are at low glucose concentrations are also deprived of oxygen. Thus our observations provide a framework for future studies. Obviously, our next step will be to investigate the ASK1–MEK–MAPK activation at low glucose concentrations and low oxygen tensions.

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