Differential role of glutaredoxin and thioredoxin in metabolic oxidative stress-induced activation of apoptosis signal-regulating kinase 1

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Redox-sensing molecules such as thioredoxin (TRX) and glutaredoxin (GRX) bind to apoptosis signal-regulating kinase 1 (ASK1) and suppress its activation. Glucose deprivation disrupted the interaction between TRX/GRX and ASK1 and subsequently activated the ASK1–stress-activated protein kinase/extracellular-signal-regulated kinase kinase–c-Jun N-terminal kinase 1 (JNK1) signal-transduction pathway. L-Buthionine-(*S*,*R*)-sulphoximine, which decreases intracellular glutathione content, enhanced glucose deprivation-induced activation of JNK1 by promoting the dissociation of TRX, but not GRX, from ASK1. Treatment of cells with exogenous glutathione disulphide ester resulted in the dissociation of GRX, but not TRX, from ASK1 and the subsequent activation of JNK1. Nonetheless, overexpression of calatase, an H_2O_2 scavenger, inhibited JNK1 activation and cytotoxicity as well as the dissociation of TRX and GRX from ASK1 during combined glucose deprivation and L-buthionine-(*S*,*R*)-sulphoximine treatment. Taken together, glucose deprivation-induced metabolic oxidative stress may activate ASK1 through two different pathways: glutathione-dependent GRX–ASK1 and glutathione-independent TRX–ASK1 pathways.

Key words: apoptosis signal-regulating kinase 1, glucose deprivation, glutaredoxin, glutathione, metabolic oxidative stress, thioredoxin.

INTRODUCTION

We have observed previously that glucose deprivation increases the intracellular levels of hydroperoxide and glutathione disulphide (GSSG) [1,2]. The increased steady-state levels of hydroperoxide activate signal-transduction pathways, such as stress-activated protein kinase (SAPK), and subsequently promote expression of genes associated with stress responses as well as cytotoxicity [1,3,4]. However, a fundamental question, which remains unanswered, is how metabolic oxidative stress triggers signal-transduction pathways in mammalian cells. Recent studies have revealed that a conserved family of activator protein-1 (AP-1)-like factors (named yAP-1) are redox-sensing molecules in yeast [5]. H_2O_2 activates yAP-1 through disulphide formation of two cysteine residues, which is essential for yAP-1 activation after oxidative stress [6]. In *Escherichia coli*, SoxR and OxyR are reduction–oxidation (redox) status sensors and are transcription factors that activate antioxidant genes in response to superoxide anion and H_2O_2 [7]. SoxR is activated by superoxide anion-generating agents or by nitric oxide through two consecutive steps of gene activation [8,9]. Two forms of SoxR have been characterized: Fe-SoxR contains non-haem iron and apo-SoxR is devoid of iron and other metals [10]. Both forms of SoxR bind to the target *in vivo*, namely the soxS promoter. However, only Fe-SoxR stimulated transcription initiation at soxS [11]. The Fe-SoxR is a homodimer, and each monomer has a redox-active [2Fe-2S] cluster. The oxidation state of [2Fe-2S] centre controls its activity as a transcription activator independent of its DNA-binding ability [12,13]. Unlike SoxR, OxyR does not contain metal ions and other prosthetic groups in the redox-active centre. Instead, OxyR molecules form an intramolecular disulphide bond between cysteine residues when the environment becomes oxidizing. Formation of an intramolecular disulphide bond between residues Cys^{199} and Cys^{208} leads to a conformational change, which activates the OxyR transcription factor [7]. Similar molecules such as the thiol-disulphide oxidoreductase family of proteins [thioredoxin (TRX), glutaredoxin (GRX) and protein disulphide-isomerase] are found in eukaryotic cells. Several studies show that these molecules contain two redox-active half-cysteine residues, -Cys-Gly-Pro-Cys- or -Cys-Pro-Tyr-Cys-, in an active centre [14,15].

In the present study, we hypothesized that GRX and TRX are sensor molecules that recognize metabolic oxidative stress and co-operatively regulate the activation of apoptosis signal-regulating kinase 1 (ASK1) in mammalian cells. ASK1 is a mitogen-activated protein kinase (MAPK) kinase kinase that can activate both SAPKs [via activation of MAPK kinase 4/7 (MKK4/7)] and p38 MAPKs (via activation of MKK3/6) [16]. Previous studies have revealed that TRX is a negative regulator of ASK1 [17]. It binds directly to the N-terminal portion of ASK1 and inhibits ASK1 kinase activity [17]. Our recent studies have shown that GRX also directly binds to ASK1 and inhibits ASK1 activation [18]. However, unlike TRX, GRX binds to the C-terminal portion of ASK1 [18]. Our studies have also shown that the interaction between GRX and ASK1 is dependent on the redox status of GRX during glucose deprivation. GRX may recognize metabolic oxidative stress through catalytic reactions with GSSG [18]. As mentioned previously, GRX contains two redox-active half-cysteine residues, Thr-Cys-Pro-Tyr-Cys-Arg in an active centre [19]. However, unlike TRX, GRX is highly selective for glutathione-containing mixed disulphides, and its catalytic cycle involves a covalent glutathionyl-enzyme

Abbreviations used: AP-1, activator protein-1; ASK1, apoptosis signal-regulating kinase 1; BSO, L-buthionine-(*S*,*R*)-sulphoximine; Daxx, death-associated protein; γ-GGT, γ-glutamyl transpeptidase; GRX, glutaredoxin; GSSG, glutathione disulphide; GST, glutathione S-transferase; HA, haemagglutinin; JNK1, c-Jun N-terminal kinase 1; MAPK, mitogen-activated protein kinase; MEK, MAPK/extracellular-signal-regulated kinase kinase; MKK4/7, MAPK kinase 4/7; MOI, multiplicity of infection; ROS, reactive oxygen species; SAPK, stress-activated protein kinase; SEK1, SAPK/extracellular-signal-regulated kinase kinase; TRAF2, tumour necrosis factor receptor-associated factor 2; TRX, thioredoxin.

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disulphide intermediate (GRX-SSG) rather than an intramolecular disulphide at its active site [20]. GRX may recognize glucose deprivation-induced metabolic oxidative stress through catalytic reactions with GSSG and form GRX-SSG. Subsequently, GRX is interconverted between GRX-SSG and an intramolecular disulphide form of GRX (GRX-[S-S]) by side reaction [20]. The oxidized GRX, GRX-SSG or GRX-[S-S], dissociates from ASK1. Unlike GRX, TRX may directly recognize H_2O_2 [21] or oxidized proteins. The dissociation of GRX and TRX from ASK1 may result in the activation of ASK1.

The tripeptide L-γ-glutamyl-L-cysteinyl-glycine or GSH, an ubiquitous thiol, acts as a major non-enzymic antioxidant and plays a major role in maintaining the intracellular redox state [22–24]. It is well known that L-buthionine-(*S*,*R*)-sulphoximine (BSO), a specific inhibitor of γ-glutamylcysteine synthetase, reduces the intracellular GSH content by inhibiting the first step and rate-limiting step of GSH synthesis [25,26]. In the present study, we employed BSO to investigate a possible involvement of glutathione in the regulation of the interaction between GRX/TRX and ASK1. Our studies demonstrate that BSO inhibits glucose deprivation-induced dissociation of GRX, but not TRX, from ASK1. Interestingly, BSO enhances glucose deprivation-induced c-Jun N-terminal kinase (JNK) activation. In contrast, treatment of the cells with exogenous GSSG ester caused dissociation of GRX, but not TRX, from ASK1 as well as activation of JNK1. However, overexpression of catalase prevents dissociation of GRX and TRX from ASK1 as well as ASK1 activation during glucose deprivation. Taken together, the dissociation of both regulators from ASK1 is not required for ASK1 activation. These results suggest that release of GRX and TRX from ASK1 occurs via different mechanisms and release of either one of them is sufficient for ASK1 activation.

EXPERIMENTAL

Materials

Media, serum, Protein A–agarose and lipofectAMINE™ reagent were purchased from Life Technologies (Gathersburg, MD, U.S.A.). Rat anti-haemagglutinin (HA) (clone 3F10) and mouse anti-HA antibody (clone 12CA5) were obtained from Roche (Mannheim, Germany). Glutathione–Sepharose 4B and enhanced chemiluminescence reagent were purchased from Amersham Pharmacia Biotech (Arlington Heights, IL, U.S.A.). Rabbit anti-catalase antibody and Protein G–agarose were obtained from Calbiochem (San Diego, CA, U.S.A.). Goat anti-GRX and anti-TRX antibodies were obtained from American Diagnostica (Greenwich, CT, U.S.A.). Monoclonal mouse anti-actin antibody was purchased from ICN (Costa Mesa, CA, U.S.A.). Rabbit anti-ACTIVE JNK antibody was purchased from Promega (Madison, WI, U.S.A.). Anti-His_5 mouse IgG1 and anti-ASK1 antibody were obtained from Qiagen (Valencia, CA, U.S.A.) and Alexis Corp. (Lausen, Switzerland) respectively. Anti-γ-glutamyl transpeptidase (GGT) antibody was a gift from Dr A. A. Stark (Tel-Aviv University, Israel). All other reagents were purchased from Sigma–Aldrich (St. Louis, MO, U.S.A.).

Methods

Cell culture and treatment with glucose deprivation

Human prostate adenocarcinoma cells (DU-145) were cultured in Dulbecco's modified Eagle's medium with 10 % (v/v) foetal bovine serum 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 PBS and then exposed to glucose-free Dulbecco's modified Eagle's medium containing 10 % dialysed foetal bovine serum.

Treatment with BSO

Cells were replaced with a fresh medium containing BSO. Sham group also had a complete medium change solely excluding BSO.

Vector construction

pcDNA3-His-GRX was made by inserting *Eco*RI fragment from pQE-GRX (a gift from Dr J. J. Mieyal, Case Western Reserve University, Cleveland, OH, U.S.A.) into *Eco*RI-cut pcDNA3. An N-terminally His-tagged 324 bp human TRX gene was isolated from pcDNA-His-TRX (a gift from Dr J. Yodoi, Kyoto University, Japan) digestion with *Eco*RI and cloned into the *Eco*RI site of pAdlox shuttle vector [27]. pcDNA3-His-GRX was made by inserting *Eco*RI fragment from pQE-GRX into *Eco*RI-cut pcDNA3. pAdlox-His-GRX was made by inserting *Hin*dIII–*Xba*I fragment from pcDNA3-His-GRX into *Hin*dIII–*Xba*I-cut pAdlox shuttle vector containing N-terminal fused form with hexahistidine (His_6) tag. pcDNA3-HA-ASK1 was kindly provided by Dr H. 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 vectors

The adenovirus construct containing the transgene for human catalase was kindly provided by Dr Beverly Davidson (Gene Transfer Vector Core, University of Iowa, Iowa City, IA, U.S.A.). All other recombinant adenoviruses were constructed by employing the *Cre-lox* recombination system [27]. The selective cell line CRE8 has a β-actin-based expression cassette driving a Cre recombinase gene with an N-terminal nuclear localization signal stably integrated into HEK-293 cells. Transfections were done using lipofectAMINE™. Cells (5×10^5) 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/GRX 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, analysed and purified. The insertion of HA–ASK1 or His–GRX/His–TRX into adenovirus was confirmed by Western-blot analysis after infection of corresponding recombinant adenovirus into DU-145 cells. Modifications of the native parts of the proteins did not occur by tagging HA or His at their N-termini.

In vivo binding of ASK1 and GRX

To examine the interaction between ASK1 and TRX or GRX, adenovirus of HA-tagged ASK1 (Ad.HA–ASK1) at an MOI (multiplicity of infection, i.e. number of viral particles per cell) of 10 and His-tagged TRX or GRX (Ad.His–TRX or Ad.His–GRX) at an MOI of 30 were co-infected into DU-145 cells in

10 cm 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 PMSF, 80 μM aprotinin and 2 mM leupeptin, and the lysates were incubated with 2 μg of anti-His_5 mouse IgG1 for 2 h. For immunoprecipitation with HA–ASK1, the lysates were incubated with 0.5 μg of rat anti-HA for 2 h. After the addition of Protein G–agarose, the lysates were incubated for an additional 2 h. The beads were washed three times with the lysis buffer, separated by SDS/PAGE, and immunoblotted with rat anti-HA or mouse anti-His_5 or mouse anti-HA antibodies. The proteins were detected with the enhanced chemiluminescence reaction. To confirm the interaction between endogenous ASK1 and GRX or TRX, cell lysates were immunoprecipitated with 2 μg of anti-GRX or anti-TRX antibodies followed by immunoblotting with anti-ASK1, anti-GRX or anti-TRX antibody. To examine the effect of BSO on glucose deprivation-induced dissociation of TRX or GRX from ASK1, Ad.HA–ASK1-co-infected DU-145 cells and Ad.His–TRX- or Ad.His–GRX-co-infected DU-145 cells were exposed to glucose-free medium after BSO pretreatment for 24 h.

In vitro kinase assay

The plasmid containing glutathione S-transferase (GST)–human JNK1 for bacterial fusion protein was constructed in pGEX-4T-1 by inserting *Hin*dIII–*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. GST–SEK1 (where SEK1 represents SAPK/extracellular-signal-regulated kinase kinase) was purified from ten 100 mm plates of HEK-293 cells transfected with the pEBG/SEK1 (kindly provided by J. M. Kyriakis, Massachusetts General Hospital, Charlestown, MA, U.S.A.), and the purification step was performed as described previously [28]. DU-145 cells were infected with Ad.HA–ASK1 at an MOI of 10 followed by treatment with BSO for 24 h. After 48 h 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. Cell extracts were clarified by centrifugation, and the supernatants immunoprecipitated with mouse anti-HA antibody and Protein A–agarose. 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 were washed once with the kinase buffer solution, and then they were subjected to kinase assays. To measure immunocomplex activity, 0.2 μg of GST–SEK1 is first incubated with the immunocomplexes 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 is subsequently incubated 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 analysed by rabbit anti-ACTIVE JNK antibody. To determine the amount of ASK1 protein in the same sample, the upper part of the SDS/PAGE (>116 kDa) was cut and immunoblotted with the rat anti-HA antibody.

Preparation of GSSG ester

GSSG ester was prepared from 10 mM GSH ethyl ester (Sigma–Aldrich) by treatment with 100 mM H_2O_2 for 1 h followed by treatment with 10 units/ml catalase for 1 h [29]. Previous studies have shown that H_2O_2 treatment converts the GSH ester into the GSSG ester with a yield in excess of 90 % [29].

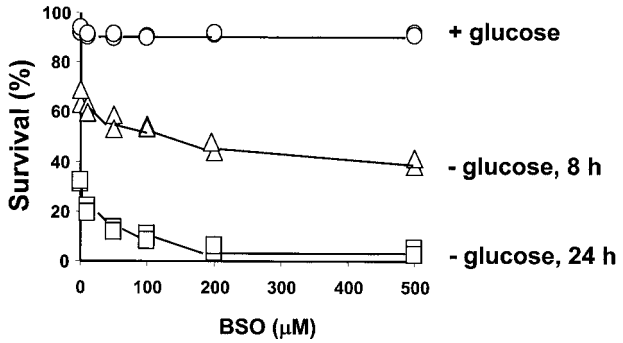

Figure 1 Effect of BSO on glucose deprivation-induced cytotoxicity

DU-145 cells were treated with various concentrations of BSO (0–500 μM) for 24 h and then exposed to glucose-free medium for 8 h (–glucose) or 24 h (–glucose). Cell survival was determined using the Trypan Blue exclusion assay. The data are a compilation of two separate experiments. +Glucose, cells were treated only with BSO.

Immunoblot analysis

Cell lysates were subjected to SDS/PAGE (12 % gel) under reducing conditions, and the proteins in the gels were transferred on to a PVDF membrane. The membranes were incubated with 7 % (w/v) skimmed milk in PBST [PBS containing 0.1 % (v/v) Tween 20] and then reacted with primary antibodies. 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 following the manufacturer's instructions.

RESULTS

BSO enhances glucose deprivation-induced cytotoxicity

The effect of BSO on glucose deprivation-induced cytotoxicity was assessed in DU-145 cells. Cells were treated with various concentrations of BSO for 24 h, followed by glucose deprivation for 8 or 24 h. Figure 1 shows that little or no cytotoxicity was observed with BSO alone up to 500 μM. In contrast, glucose deprivation-induced cell death was enhanced by BSO. For example, the survival of the cells decreased from 66 to 46 % or 32 to 5 % by 8 or 24 h of incubation respectively in glucose-free medium containing 200 μM BSO, which reduces the intracellular glutathione content by 99 % [18]. BSO-enhanced cytotoxicity was concentration-dependent (Figure 1).

BSO promotes glucose deprivation-induced JNK activation

We have observed previously that glucose deprivation activates JNK1 and overexpression of its dominant-negative mutant inhibits metabolic oxidative stress-induced cytotoxicity [3]. These results suggest that JNK-signalling pathways control the expression of proteins contributing to cell death mediated by metabolic oxidative stress during glucose deprivation. We further examined whether combined BSO and glucose deprivation enhance JNK1 activation, which may result in enhancement of glucose deprivation-induced cytotoxicity. Western blots using an

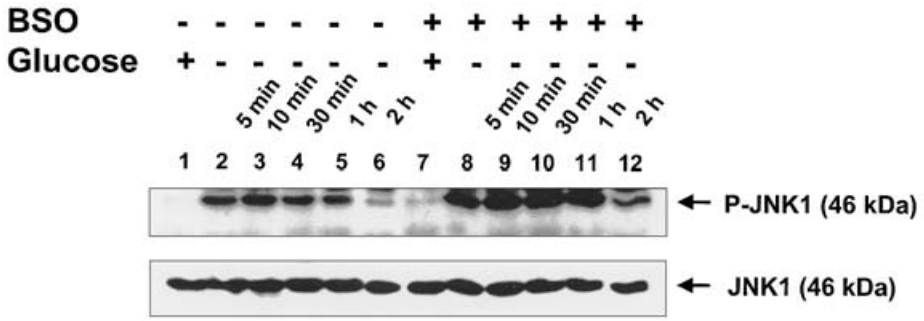

Figure 2 Effect of BSO on glucose deprivation-induced JNK1 activation

DU-145 cells were treated with or without 200 μM BSO for 24 h and then exposed to glucose-free medium for various time periods (0–2 h). Cell lysates containing equal amounts of protein (20 μg) were separated by SDS/PAGE and immunoblotted with anti-phospho-JNK or anti-JNK1 antibody.

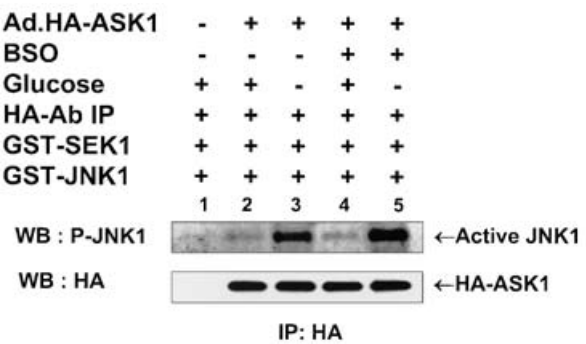

Figure 3 Effect of BSO on glucose deprivation-induced ASK1 activation

DU-145 cells were infected with adenoviral vectors containing HA-tagged ASK1 (Ad.HA–ASK1) at an MOI of 10. After 24 h incubation, 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. The immunocomplex was incubated with GST–SEK1 followed by GST–JNK1. Active JNK1 was detected with anti-phospho-JNK antibody. ASK1 was detected with anti-HA antibody. Lane 1, uninfected control cells. IP, immunoprecipitation.

antibody specific for the active form of JNK1 (anti-ACTIVE JNK1 antibody) showed that glucose deprivation-induced JNK1 activation was observed within 5 min and its activation was maintained for more than 2 h (Figure 2). Figure 2 also shows that BSO enhanced glucose deprivation-induced JNK activation. These results were similar to our previous reports using MCF-7/ADR cells [3].

Glucose deprivation in combination with BSO enhances ASK1 activity

Previous studies have shown that ASK1 activates SEK1-JNK- and MKK3/MKK6-p38-signalling cascades [16,17,30]. To examine whether the enhanced JNK/p38 activation by treatment with BSO in combination with glucose deprivation is mediated through the activation of ASK1, DU-145 cells were infected with Ad.HA–ASK1 at an MOI of 10 and then treated with 200 μM BSO for 24 h followed by 1 h of exposure to glucose-free medium. Cell lysates were immunoprecipitated with anti-HA antibody. ASK1 enzyme activity was measured by an immunocomplex kinase assay using GST–SEK1 and GST–JNK1 as sequential substrates. Activated JNK1 was detected by anti-ACTIVE JNK antibody. Results from *in vitro* kinase assay show that glucose deprivation activated JNK1 (Figure 3, lane 3) and its activation was enhanced by treatment with BSO (Figure 3, lane 5). These results indicate that glucose deprivation-induced JNK1 activation is mediated through the ASK1–SEK1–JNK1 signal-transduction pathway. BSO facilitates this signal-transduction pathway by promoting ASK1 activity.

Effect of BSO on glucose deprivation-induced dissociation of GRX or TRX from ASK1

Previous studies have shown that GRX and TRX bind to the C- and N-terminal portions of ASK1 respectively [17,18]. We examined whether GRX or TRX dissociates from ASK1 during glucose deprivation and whether GSSG plays any regulatory role in the interaction between them. Results from immunoprecipitation followed by immunoblotting show that TRX and GRX dissociated from ASK1 during glucose deprivation (Figures 4A–4C, lane 3). BSO inhibited dissociation of GRX, but not TRX, from ASK1 (Figures 4A–4C, lane 5). These results suggest that glutathione plays a regulatory role in the interaction between GRX, but not TRX, and ASK1. These results also indicate that TRX and GRX sense metabolic oxidative stress through different pathways. To examine whether endogenous ASK1 and GRX or TRX interact, cells were lysed and immunoprecipitated with anti-GRX or anti-TRX antibody followed by Western blotting with anti-ASK1, anti-GRX or anti-TRX antibody. The presence of endogenous GRX, TRX and ASK1 protein was detected (Figure 4D, lower panel). Figure 4(D) shows that indeed the endogenous factors interact. Figure 4(D) also shows that endogenous GRX as well as TRX dissociated from endogenous ASK1 during glucose deprivation (Figure 4D, upper panel, lane 2 versus lane 3).

Effect of BSO on H_2O_2-induced dissociation of GRX or TRX from ASK1

Previous studies have shown that glucose deprivation-induced metabolic oxidative stress results from an increase in reactive oxygen species (ROS), such as superoxide anion or H_2O_2 [1]. Superoxide anion is known to be rapidly dismutated to H_2O_2 by superoxide dismutase, and then H_2O_2 is transformed to water by catalase or glutathione peroxidase in the presence of GSH. To examine whether H_2O_2 is responsible for the dissociation of GRX or TRX from ASK1, DU-145 cells were treated with 5 mM H_2O_2 and the immunocomplex analysis was performed. As shown in Figure 5, GRX and TRX dissociated from ASK1 on treatment with H_2O_2 (Figure 5A, lanes 3 and 7; Figure 5B, lane 3). Interestingly, BSO inhibited dissociation of GRX, but

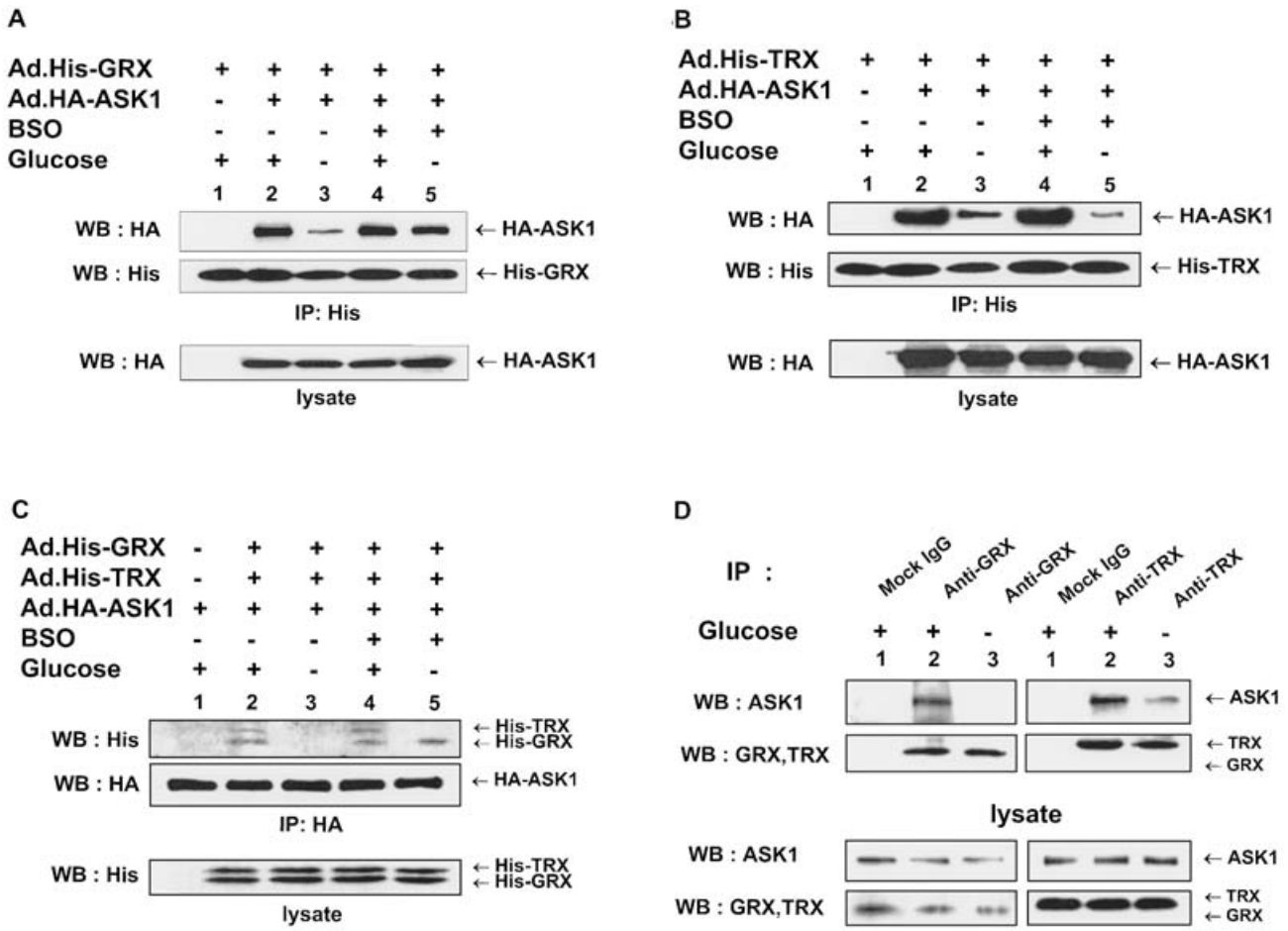

Figure 4 Effect of BSO on glucose deprivation-induced dissociation of GRX or TRX from ASK1

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) and/or His-tagged TRX (Ad.His–TRX) at an MOI of 30. After 24 h incubation, cells were treated with (+) or without (–) 200 μM BSO for 24 h and then exposed to glucose-free medium for 1 h. (**A**, **B**) Lysates were immunoprecipitated with anti-His antibody, and immunoblotted with anti-HA (upper panel) or anti-His antibody (lower panel). (**C**) Lysates were immunoprecipitated with anti-HA antibody, and immunoblotted with anti-His (upper panel) or anti-HA antibody (lower panel). (**D**) To confirm the interaction between endogenous ASK1 and GRX or TRX, uninfected cells were exposed to complete medium or glucose-free medium for 1 h, and cell lysates were immunoprecipitated with 2 μg of anti-GRX, anti-TRX or anti-mouse antibody, and then immunoblotted with anti-ASK1, anti-GRX or anti-TRX antibody (upper panels). Endogenous level of ASK1, GRX or TRX was examined by Western-blot analysis with anti-ASK1, anti-GRX or anti-TRX antibody (lower panels). IP, immunoprecipitation.

not TRX, from ASK1 during treatment with H_2O_2 (Figure 5A, lanes 4 and 8; Figure 5B, lane 5). These results suggest that dissociation of GRX is dependent on the presence of glutathione, probably GSSG. Unlike GRX, TRX may directly sense H_2O_2 or other oxidized molecules.

GSSG-mediated JNK activation

To examine the involvement of GSSG in the activation of the ASK1–MEK–MAPK signal-transduction pathway (where MEK is MAPK/extracellular-signal-regulated kinase kinase), we treated cells with exogenous GSSG ester. Before this experiment, we measured the cellular level of GGT. GGT is an 87 kDa type II transmembrane ectoenzyme. It is a heterodimer composed of 65 and 22 kDa subunits. Most of the molecule is extracellular, and its role is to cleave glutamyl groups from extracellular GSH or GSSG. Thus it is necessary to examine whether DU-145 cells have GGT activity. If they do, then exogenous glutathione derivatives are likely to be degraded rapidly. Figure 6 shows that human prostate cancer cell lines (DU-145, LNCap and PC-3) do not contain any detectable GGT.

To examine the effect of GSSG, Ad.HA–ASK1- and Ad.His–GRX- or Ad.His–TRX-infected DU-145 cells were treated with 1 mM GSSG ester for 1 h. Cells were harvested and immunoprecipitated with anti-His antibody. Figure 7(A) shows that treatment with GSSG ester led to dissociation of GRX, but not TRX, from ASK1. Interestingly, the maximum reduction in the binding of GRX to ASK1 was approx. 50 % (Figure 7A, lane 3 versus lane 2). This relatively inefficient inhibition may have resulted from the short treatment time (i.e. bolus of GSSG ester). Activation of JNK1 was also observed in these cells (Figure 7B). These results show that GSSG is responsible for dissociation of GRX, but not TRX, from ASK1 and subsequently JNK1 activation.

Overexpression of catalase suppresses glucose deprivation-induced JNK1 activation as well as cytotoxicity

Catalase, a tetrameric Fe (III)-haem protein, is found in the cytosol and serves as an efficient scavenger of H_2O_2. It has been reported that catalase becomes important when glutathione is depleted. These results suggest that catalase can serve as a

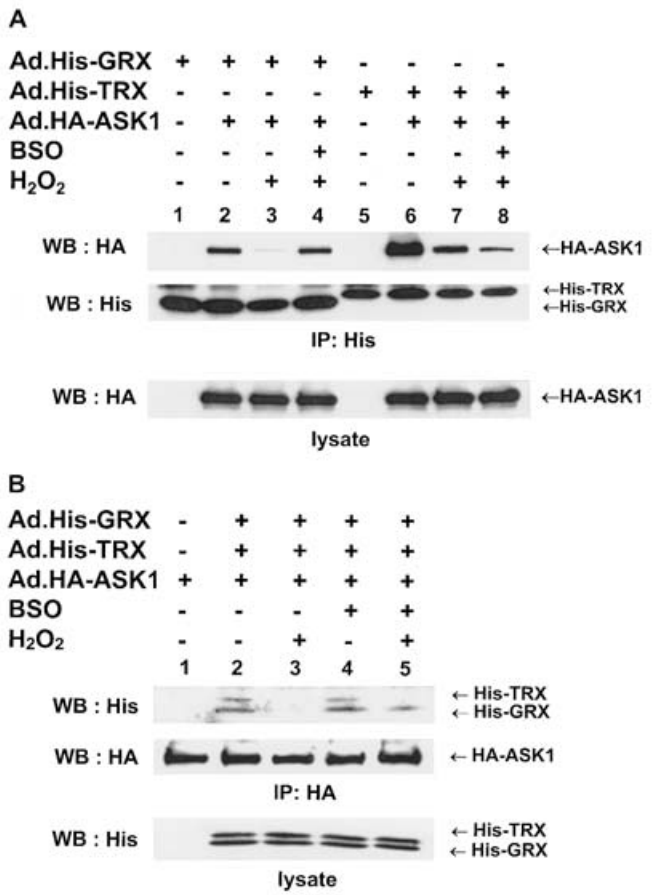

Figure 5 Effect of BSO on H_2O_2-induced dissociation of GRX or TRX from ASK1

DU-145 cells were co-infected with Ad.His–GRX at an MOI of 30, Ad.His–TRX at an MOI of 30 and Ad.HA–ASK1 at an MOI of 10. After 24 h incubation, cells were treated with (+) or without (–) 200 μM BSO for 24 h and then exposed to 5 mM H_2O_2 for 1 h. (**A**) Lysates were immunoprecipitated with anti-His antibody, and immunoblotted with anti-HA (upper panel) or anti-His antibody (lower panel). (**B**) Lysates were immunoprecipitated with anti-HA antibody, and immunoblotted with anti-His (upper panel) or anti-HA antibody (lower panel). IP, immunoprecipitation.

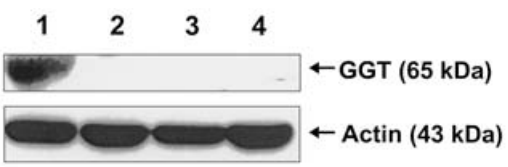

Figure 6 Immunoblot detection of GGT in HEK-293 cells and various human prostate cancer cells (DU-145, LNCap and PC-3)

Lysates containing equal amounts of protein (20 μg) were separated by SDS/PAGE and immunoblotted with anti-GGT or anti-actin antibody. Lane 1, HEK-293 cells; lane 2, DU-145 cells; lane 3, LNCap cells; lane 4, PC-3 cells.

secondary defence when the glutathione system becomes limiting [22]. To examine whether glucose deprivation-induced JNK activation would be suppressed by overexpression of catalase, DU-145 cells were infected with adenoviral vectors containing catalase at an MOI of 5. Figure 8 shows that overexpression of catalase inhibited glucose deprivation-induced JNK1 activation (Figure 8A, lane 2 versus lane 6) as well as cytotoxicity (Figure 8B, histogram 2 versus 6). Figure 8 also shows that BSO enhanced glucose deprivation-induced JNK1 activation as well as cytotoxicity (Figure 8A, lane 4; Figure 8B, histogram 4). This enhancement was suppressed by overexpression of catalase (Figure 8A, lane 8; Figure 8B, histogram 8). The protective effect of catalase was dependent on MOIs (Figure 9). JNK1 activation and cytotoxicity were effectively inhibited by increasing catalase expression (Figures 9A and 9B). Effect of catalase on the time course of JNK1 activation during glucose deprivation can be seen in Figure 10. Overexpression of catalase inhibited JNK1 activation early as well as late time period during glucose deprivation.

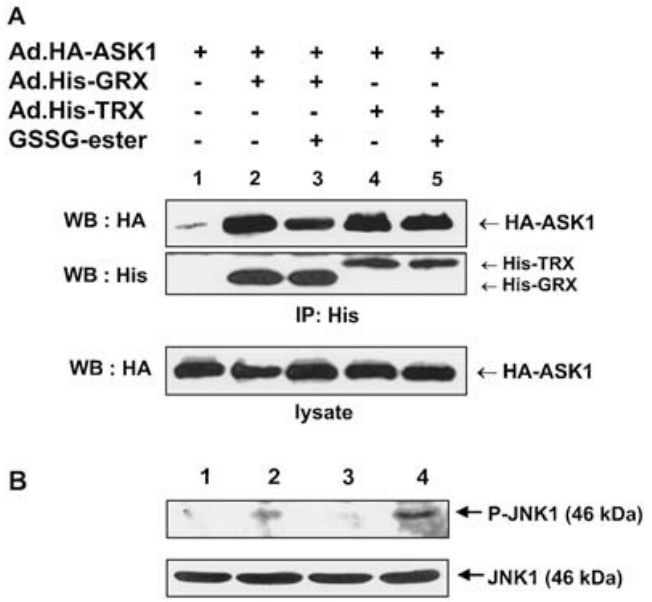

Figure 7 GSSG induces dissociation of GRX, but not TRX, from ASK1 and activates JNK1

(**A**) DU-145 cells were co-infected with Ad.HA–ASK1 at an MOI of 10, and Ad.His–GRX at an MOI of 30 or Ad.His–TRX at an MOI of 30. After 24 h incubation, cells were treated with medium containing GSSG ester (lanes 3 and 5) or mock medium (lanes 2 and 4) for 1 h. Cell lysates were divided into two portions. One portion was immunoprecipitated with anti-His antibody, and then immunoblotted with anti-HA (HA–ASK1) or anti-His antibody (His–GRX or His–TRX). The other portion was immunoblotted with anti-HA antibody. (**B**) DU-145 cells were treated with GSH ester (lane 2), H_2O_2 + catalase (lane 3) or GSSG ester (lane 4) for 1 h. Lysates containing equal amounts of protein (20 μg) were separated by SDS/PAGE and immunoblotted with anti-phospho-JNK (upper panel) or anti-JNK1 (lower panel) antibody. Lane 1, untreated control cells. IP, immunoprecipitation.

Overexpression of catalase inhibits dissociation of GRX and TRX from ASK1 during glucose deprivation

We extended our studies to investigate whether overexpression of catalase inhibits dissociation of GRX and TRX from ASK1 during glucose deprivation. Figures 11(A) and 11(B) show that GRX and TRX dissociated from ASK1 during glucose deprivation (Figures 11A and 11B, lane 3). Their dissociations were prevented by catalase overexpression (Figures 11A and 11B, lane 8). As shown previously, dissociation of GRX, but not TRX, from ASK1 was inhibited by treatment with BSO (Figures 11A and 11B, lane 5). Interestingly, overexpression of catalase suppressed dissociation of TRX from ASK1 during BSO pretreatment followed by glucose deprivation (Figure 11B, lane 10).

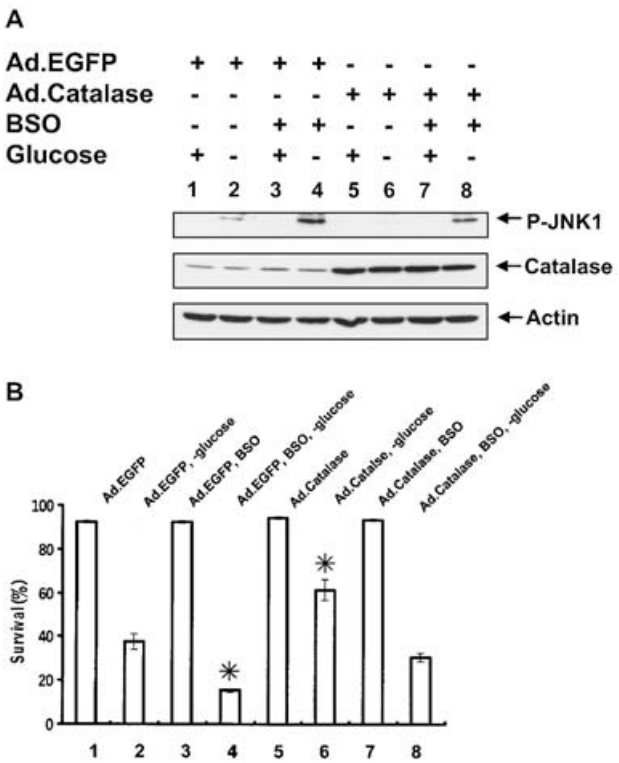

Figure 8 Effect of catalase overexpression on glucose deprivation-induced JNK1 activation in BSO-treated or -untreated cells

DU-145 cells were infected with adenoviral vectors containing green fluorescent protein (Ad.EGFP) or catalase (Ad.Catalase) at an MOI of 5. After 24 h incubation, cells were treated with (+) or without (–) 200 μM BSO for 24 h. (**A**) Cells were exposed to glucose-free medium for 1 h and harvested. Cell lysates containing equal amounts of protein (20 μg) were separated by SDS/PAGE and immunoblotted with anti-phospho-JNK, anti-catalase or anti-actin antibody. (**B**) Cells were exposed to glucose-free medium for 24 h and then survival was determined by the Trypan Blue exclusion assay. Asterisks indicate values different from Ad.EGFP, –glucose control (paired t-test, $P < 0.05$) and error bars represent means $\pm$ standard deviation for three separate experiments.

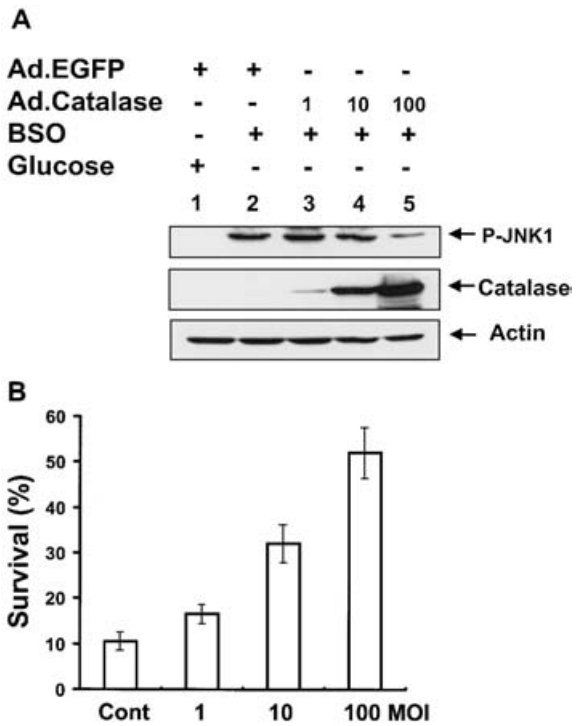

Figure 9 Effect of catalase overexpression on glucose deprivation-induced JNK activation in BSO-treated or -untreated cells

DU-145 cells were infected with Ad.EGFP at an MOI of 100 or Ad.Catalase at various MOIs (1–100). After 24 h incubation, cells were treated with (+) or without (–) 200 μM BSO for 24 h. (**A**) Cells were exposed to glucose-free medium for 10 min and harvested. Cell lysates containing equal amounts of protein (20 μg) were separated by SDS/PAGE and immunoblotted with anti-phospho-JNK, anti-catalase or anti-actin antibody. (**B**) Cells were exposed to glucose-free medium for 24 h and then survival was determined by the Trypan Blue-exclusion assay. Error bars represent means $\pm$ S.D. for three separate experiments.

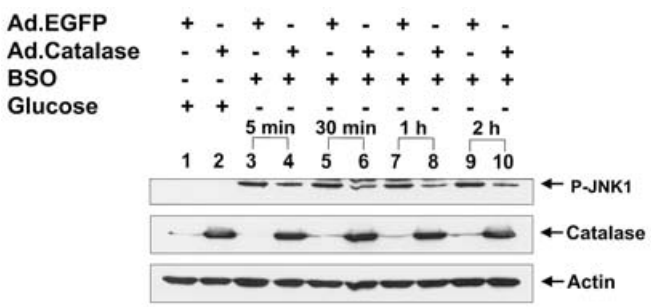

Figure 10 Effect of catalase overexpression on glucose deprivation-induced JNK activation in BSO-pretreated DU-145 cells

Cells were infected with either Ad.EGFP or Ad.Catalase at an MOI of 100. After 24 h incubation, cells were treated with (+) or without (–) 200 μM BSO for 24 h. Cells were exposed to glucose-free medium for various time periods (5 min to 2 h). Cell lysates containing equal amounts of protein (20 μg) were separated by SDS/PAGE and immunoblotted with anti-phospho-JNK, anti-catalase or anti-actin antibody.

Glucose deprivation-induced ASK1 activation

Our results are summarized in Figure 12. They indicate that glucose deprivation promotes the level of ROS. The elevation of ROS may activate ASK1 through two different pathways (glutathione-dependent GRX–ASK1 pathway and glutathione-independent TRX–ASK1 pathway). BSO inhibits the GRX–ASK1 pathway, but not the TRX–ASK1 pathway.

DISCUSSION

Several conclusions can be drawn after considering the results presented. BSO, which effectively reduces the intracellular glutathione content, enhances glucose deprivation-induced cytotoxicity as well as JNK1 activation. This is probably due to promotion of dissociation of TRX, but not GRX, from ASK1. Nonetheless, catalase, an efficient scavenger of H_2O_2, suppresses the effect of BSO on glucose deprivation-induced cytotoxicity, on JNK1 activation and on dissociation of TRX from ASK1. Our results suggest a differential role of GRX and TRX in glucose deprivation-induced JNK1 activation.

We have reported previously that glucose deprivation results in an increase in steady-state levels of intracellular GSSG (3–10-fold) and an increase in intracellular hydroperoxide level (2–4-fold) as determined by increased fluorescence of an oxidation-sensitive probe [1,2,31]. The cells also appear to respond to glucose deprivation-induced metabolic oxidative stress by attempting to increase the synthesis of glutathione [1,2]. However, in the absence of substrates necessary for the regeneration of

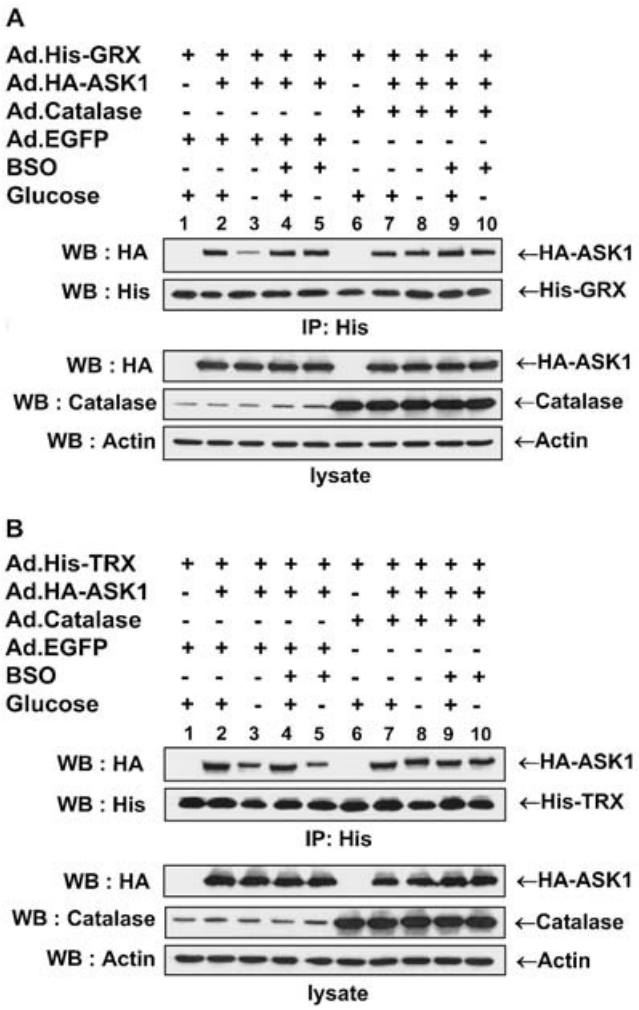

Figure 11 Effect of catalase overexpression on glucose deprivation-induced dissociation of GRX (A) or TRX (B) from ASK1

DU-145 cells were co-infected with Ad.Catalase/Ad.EGFP at an MOI of 100, Ad.HA–ASK1 at an MOI of 10 and Ad.His–GRX/Ad.His–TRX at an MOI of 30. 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-His antibody, and immunoblotted with anti-HA or anti-His antibody. Lysates were also immunoblotted with anti-catalase or anti-actin antibody. IP, immunoprecipitation.

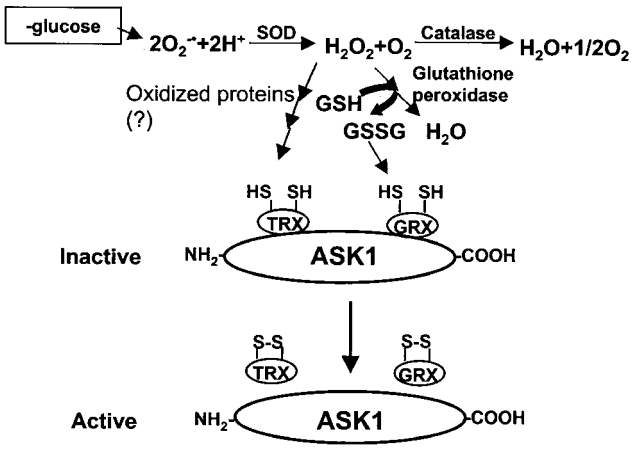

Figure 12 A model for the mechanism of glucose deprivation-induced ASK1 activation

NADPH, glutathione cannot be maintained in the reduced state [31]. In this study, we observed that the depletion of intracellular glutathione by treatment with BSO inhibits dissociation of GRX, but not TRX, from ASK1. These results are consistent with our previous studies [18]. GRX, unlike TRX, probably interacts with GSSG and forms a covalent glutathionyl-enzyme-disulphide intermediate (GRX-SSG) and subsequently an intramolecular disulphide form of GRX (GRX-[S-S]). The glutathionylated GRX may dissociate from ASK1, leading to activation of the ASK1–MEK–MAPK signal-transduction pathway. Figure 7 indeed shows that treatment of the cells with GSSG ester resulted in dissociation of GRX from ASK1 and activation of the ASK1–MEK–MAPK signal-transduction pathway. We believe that this effect was due to a proportion of the exogenous GSSG ester entering the cells and directly interacting with GRX bound to ASK1. However, at the present time, we cannot rule out the possibility that GSSG ester has effects at the cell surface, where it may glutathionylate and/or oxidize vicinal dithiols of cell-surface proteins, subsequently activating the ASK1–MEK–MAPK system. Obviously, further studies at the biochemical level are necessary to understand the role of GSSG in the GRX–ASK1 pathway. Unlike GRX, TRX may not react with glutathione-containing mixed disulphides. TRX may directly sense H_2O_2 [32,33]. These results suggest that glucose deprivation-induced signals can be intermediated through the glutathione-dependent GRX–ASK1 pathway and the glutathione-independent pathway (Figure 12). Interestingly, BSO alone does not lead to cytotoxicity, ASK1/JNK activation and TRX/GRX dissociation (Figures 1–5). These results suggest that depletion of intracellular glutathione alone is not sufficient for inducing apoptosis. These results are consistent with previous reports [26,34,35]. In fact, most cells appear to be capable of tolerating a decrease in GSH levels up to approx. 90 % without adverse consequences [23]. Our results also show that overexpression of catalase inhibits glucose deprivation-induced JNK1 activation, cytotoxicity and dissociation of GRX and TRX from ASK1 (Figures 8–11). Overexpression of catalase also suppresses the promoting effect of BSO on glucose deprivation. This is probably because catalase serves as a secondary defence against ROS-induced cytotoxicity when glutathione is depleted [22].

The results of the present study show that BSO enhances glucose deprivation-induced JNK1 activation and cytotoxicity. At the present time, we can only speculate on the mechanism for effects of BSO on glucose deprivation-induced ASK1 activation. Previous studies have shown that the kinase activity of ASK1 is stimulated by tumour necrosis factor or Fas ligand via tumour necrosis factor receptor-associated factor 2 (TRAF2) or death-associated protein (Daxx) respectively [36,37]. It is well known that TRAF2 can interact with the N- and C-terminal portions of ASK1. The interaction of ASK1 and TRAF2 enhances ASK1 homo-oligomerization and subsequently activates ASK1 [36]. Similar to TRAF2, Daxx binds to ASK1 and activates its kinase activity [37]. However, unlike TRAF2, Daxx translocates from the nucleus to the cytoplasm and interacts with the death domain of Fas and ASK1 [38–40]. Previous studies also show that ASK1 proapoptotic activity is antagonized by association of 14-3-3 proteins [41]. 14-3-3 binds to Ser^{967} of ASK1 and disruption of the ASK1–14-3-3 interaction facilitates ASK1 activation [41]. We postulate that depletion of glutathione by BSO increases the intracellular level of ROS during glucose deprivation. The

elevated level of ROS stimulates non-receptor-associated signal transduction and results in promotion of the TRAF2/Daxx–ASK1 interaction or disruption of the 14-3-3–ASK1 interaction. Although we are far from understanding how BSO enhances glucose deprivation-induced JNK1 activation and cytotoxicity, we postulate that TRAF2, Daxx or 14-3-3 is probably involved in the ASK1 activation processes and BSO may affect the involvement of these molecules. We believe that this model will provide a framework for future studies.

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