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*Clin Cancer Res* 2005;11:7607-7613.

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## Signal Pathway of Hypoxia-Inducible Factor-1 $\alpha$ Phosphorylation and its Interaction with von Hippel-Lindau Tumor Suppressor Protein During Ischemia in MiaPaCa-2 Pancreatic Cancer Cells

Seok J. Kwon, Jae J. Song, and Yong J. Lee

**Abstract** **Purpose and Experimental Design:** Previously, we observed that the activation of p38 mitogen-activated protein kinase (MAPK) and *c-Jun* NH<sub>2</sub>-terminal kinase (JNK1) is mediated through the activation of apoptosis signal-regulating kinase 1 (ASK1) as a result of the reactive oxygen species-mediated dissociation of glutaredoxin and thioredoxin from ASK1. In this study, we examined whether p38 MAPK and JNK1 are involved in the accumulation of hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) during ischemia. Human pancreatic cancer MiaPaCa-2 cells were exposed to low glucose (0.1 mmol/L) with hypoxia (0.1% O<sub>2</sub>). **Results and Conclusions:** During ischemia, p38 MAPK and JNK1 were activated in MiaPaCa-2 pancreatic cancer cells. The activated p38 MAPK, but not JNK1, phosphorylated HIF-1 $\alpha$ . Data from *in vivo* binding assay of von Hippel-Lindau tumor suppressor protein with HIF-1 $\alpha$  suggests that the p38-mediated phosphorylation of HIF-1 $\alpha$  contributed to the inhibition of HIF-1 $\alpha$  and von Hippel-Lindau tumor suppressor protein interaction during ischemia. SB203580, a specific inhibitor of p38 MAPK, inhibited HIF-1 $\alpha$  accumulation during ischemia, probably resulting from the ubiquitination and degradation of HIF-1 $\alpha$ .

Pancreatic cancers are highly aggressive, and because there is no effective therapy for this cancer, one of the major causes of cancer death. Pancreatic cancer cells survive and proliferate even in the severe hypoxia and nutrient deprivation resulting from the poor blood supply in a tumor (1). When the tumor cells are exposed to hypoxic stress, hypoxia-inducible factor-1 (HIF-1) plays an important role in cancer angiogenesis and anaerobic metabolism (2). A previous study reported that pancreatic cancer cells with constitutive expression of HIF-1 $\alpha$  were more resistant to apoptosis induced by hypoxia and glucose deprivation than those without constitutive expression of HIF-1 $\alpha$  (3). Thus far, the role of oxygen in the regulation of intracellular levels of HIF-1 $\alpha$  has been well studied (4). Under normoxic conditions, HIF-1 $\alpha$  is rapidly degraded by the proteasome. This proteolytic regulation is mediated by hydroxylation of HIF-1 $\alpha$  protein on proline residues 402 and 504 by specific HIF-prolyl hydroxylases in the presence of iron and oxygen (5–7). The hydroxylated HIF-1 $\alpha$  protein then interacts with the von Hippel-Lindau tumor suppressor protein (pVHL),

which functions as an E3 ubiquitin ligase. Under hypoxic conditions, HIF-1 $\alpha$  is not hydroxylated, resulting in the prevention of its interaction with pVHL and its subsequent ubiquitination and degradation. In addition to studies on hypoxia-induced stabilization of HIF-1 $\alpha$ , there are several reports relating to up-regulation of HIF-1 $\alpha$  through its phosphorylation by the following signal pathways: phosphoinositide-3-kinase/AKT signaling, mitogen-activated protein kinase (MAPK) signaling, and the signal pathway mediated by reactive oxygen species (ROS; refs. 8–11). However, the exact regulatory mechanism of HIF-1 $\alpha$  phosphorylation remains unknown.

In the present study, we focused on the HIF-1 $\alpha$  phosphorylation pathway during ischemia (hypoxia and low glucose) in pancreatic cancer cells, MiaPaCa-2. Previous reports showed that either hypoxia or glucose deprivation could increase ROS, resulting in the activation of p38 and *c-Jun* NH<sub>2</sub>-terminal kinase (JNK) in various human cells (12–15). Several researchers reported that ROS is sensed through thioredoxin and glutaredoxin (14, 16). Thioredoxin and glutaredoxin, which act as physiologic inhibitors of apoptosis signal-regulating kinase-1 (ASK1) by association, dissociate from ASK1 and result in the activation of the ASK1-MEK-MAPK signal transduction pathway (14, 16). Another report revealed that ROS in the form of hydrogen peroxide stabilizes HIF-1 $\alpha$ , and that this response was abolished by overexpression of catalase, a H<sub>2</sub>O<sub>2</sub> scavenger (11). Taken together, we hypothesized that ischemia elevates the intracellular level of ROS and activates MAPK signals, resulting in the phosphorylation of HIF-1 $\alpha$  and accumulation of HIF-1 $\alpha$ . Our data reveal that phosphorylation of HIF-1 $\alpha$ , which is mediated through activation of p38 MAPK, but not JNK, prevents its interaction with pVHL during ischemia.

**Authors' Affiliation:** Departments of Surgery and Pharmacology, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania  
Received 5/4/05; revised 6/20/05; accepted 8/9/05.

**Grant support:** National Cancer Institute grants CA95191 and CA96989, Elsa U. Pardee Foundation, the Pittsburgh Foundation, and Department of Defense prostate program fund (PC020530).

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**Requests for reprints:** Yong J. Lee, Department of Surgery, University of Pittsburgh, Hillman Cancer Center, Room 1.19, 5117 Centre Avenue, Pittsburgh, PA 15213. Phone: 412-623-3268; Fax: 412-623-1415; E-mail: leeyj@msx.upmc.edu.

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doi:10.1158/1078-0432.CCR-05-0981

## Materials and Methods

**Cell culture and ischemia.** Human pancreatic cancer MiaPaCa-2 cells were maintained in DMEM (Life Technologies, Gaithersburg, MD) including 10% fetal bovine serum (HyClone, Logan, UT). Cells were cultured at 37°C in a humidified atmosphere and 5% CO<sub>2</sub> in air. Mycoplasma contamination was tested periodically. Prior to experiments, cells were grown to ~80% confluence in 60 or 100 mm tissue culture dishes and washed thrice with PBS solution. Low glucose condition was achieved by exposing cells to the DMEM containing 0.1 mmol/L glucose and 10% dialyzed fetal bovine serum (Life Technologies). For hypoxia treatment, Petri dishes containing cells were incubated in a hypoxic chamber (Forma Scientific, Marietta, OH) with a 94.9:5:0.1 mixture of N<sub>2</sub>/CO<sub>2</sub>/O<sub>2</sub>. It is difficult to accurately measure oxygen concentration in unstirred systems above monolayers. Thus, we incubated stirred medium in the hypoxic chamber overnight before use. Petri dishes containing cells were put on a shaker for 15 minutes after the medium was replaced in the hypoxic chamber. The oxygen level was monitored by an oxygen meter which was equipped with OXELP oxygen electrode (World Precision Instruments, Sarasota, FL). This electrode has a 2-mm diameter tip and can accurately measure dissolved oxygen concentrations (0.1-100%) with a resolution of 0.1 ppm. The oxygen concentration at 0.1% was maintained. Ischemia was achieved by combining low glucose (0.1 mmol/L) with hypoxia (0.1% O<sub>2</sub>). Cell lysates were immunoblotted with anti-p38 MAPK (Cell Signaling, Beverly, MA), anti-phospho-p38 MAPK (Cell Signaling), anti-JNK1 (Santa Cruz, Santa Cruz, CA), anti-ACTIVE-JNK (Promega, Madison, WI), anti-phospho-*c-Jun* (Cell Signaling), anti-activating transcription factor-2 (ATF-2; Cell Signaling), or anti-actin antibody (Sigma, St. Louis, MO). Pretreatment of cells with p38 inhibitor SB203580 (5-20 μmol/L; EMD Biosciences, La Jolla, CA) and JNK inhibitor SP600125 (5-20 μmol/L; EMD Biosciences) was done for 30 minutes before exposing cells to ischemia as well as during ischemia for 4 hours.

**Adenovirus vectors and plasmids.** Adenoviral vector containing HA-tagged ASK1 (Ad.HA-ASK1), His-tagged glutaredoxin (Ad.His-glutaredoxin), or His-tagged thioredoxin (Ad.His-thioredoxin) was constructed as described previously (14). In brief, all recombinant adenoviruses were constructed by employing the *Cre-lox* recombination system (17). The selective cell line, CRE8, has a β-actin-based expression cassette driving a *Cre* recombinase gene with an NH<sub>2</sub>-terminal nuclear localization signal stably integrated into 293 cells. Cells (5 × 10<sup>5</sup>) were plated into a six-well plate 1 day before transfection. For the production of recombinant adenovirus, CRE8 cells were cotransfected with shuttle vector and ψ5 viral genomic DNA by using LipofectAMINE Reagent (Invitrogen, Carlsbad, CA). The recombinant adenoviruses were generated by intermolecular homologous recombination between the shuttle vector and ψ5 viral DNA. The new virus has an intact packaging site and carries a recombinant gene. Plaques were harvested, analyzed, and purified. The insertion of HA-ASK1, His-glutaredoxin, or His-thioredoxin to adenovirus was confirmed by Western blot analysis after infection of the corresponding recombinant adenovirus into MiaPaCa-2 cells. The adenovirus containing human catalase was kindly provided by B. Davidson (University of Iowa). The pcDNA3-HA-HIF-1α was kindly donated by F.S. Lee (University of Pennsylvania School of Medicine). The pcDNA3-FLAG-p38α, FLAG-p38β, FLAG-p38δ, and FLAG-p38γ were gifts from R.J. Davis (University of Massachusetts Medical School). The pRc/cytomegalovirus-HA-pVHL was kindly obtained from W. G. Kaelin and M. O'Hea (Harvard Medical School).

**Western blot analysis.** Cell lysates were subjected to electrophoresis on 12% SDS-PAGE unless otherwise indicated and transferred to a polyvinylidene difluoride membrane. The membranes were incubated with 8% (w/v) skim milk in PBS containing 0.1% (v/v) Tween 20 for blocking, and then reacted with specific primary antibodies. Secondary antibodies conjugated with horseradish peroxidase were used and

immunoreactive proteins were detected with the enhanced chemiluminescence reagent (Amersham, Arlington Heights, IL).

**Binding of apoptosis signal-regulating kinase 1 with glutaredoxin or thioredoxin.** To examine the interaction between ASK1 and glutaredoxin or thioredoxin, Ad.HA-ASK1 at a multiplicity of infection (MOI) of 5 and Ad.His-glutaredoxin or Ad.His-thioredoxin at an MOI of 30 were coinfecting into MiaPaCa-2 cells in 100-mm diameter culture plates. For immunoprecipitation, cells were lysed with lysis buffer containing 150 mmol/L NaCl, 20 mmol/L Tris-HCl (pH 7.5), 10 mmol/L EDTA, 1% Triton X-100, 1% deoxycholate, 1 mmol/L phenylmethylsulfonyl fluoride, 80 μmol/L aprotinin, and 2 mmol/L leupeptin, and the lysates were incubated with 2 μg of anti-penta-His mouse IgG<sub>1</sub> (Qiagen, Valencia, CA) for 10 hours. After the addition of protein G-agarose (Santa Cruz), the lysates were incubated for an additional 2 hours. The beads were washed thrice with the lysis buffer, the proteins bound to the bead were separated by SDS-PAGE, and immunoblotted with rat anti-HA (clone 3F10, Roche Diagnostics, Mannheim, Germany) or mouse anti-penta-His (Qiagen). The proteins were detected using the enhanced chemiluminescence reagent (Amersham).

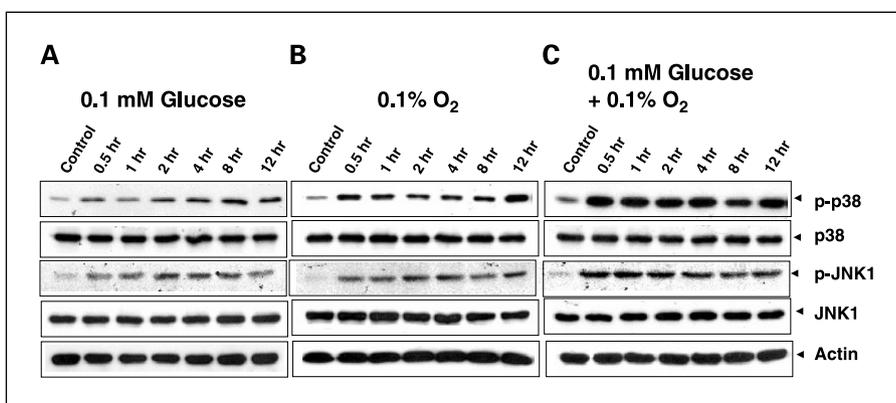
**In vitro kinase assay.** MiaPaCa-2 pancreatic cancer cells were transiently transfected with pcDNA3-FLAG-p38 MAPK isoforms using LipofectAMINE reagent and cells were incubated in DMEM with 10% fetal bovine serum at 37°C for 48 hours to fully express the FLAG-p38 MAPK isoforms. Cells were then exposed to normoxia or ischemia for 1 hour and FLAG-tagged p38 MAPK isoforms were obtained by immunoprecipitation as described above. HA-HIF-1α was translated *in vitro* using the TNT-coupled reticulocyte lysate system (Promega). The HA-HIF-1α translation was done in the presence of L-methionine. The 5 μL of translation mixture (for each condition) were immunoprecipitated using anti-HA antibody and used as substrate. The kinase assay was done in a buffer containing 25 mmol/L Tris-HCl (pH 7.5), 5 mmol/L β-glycerophosphate, 2 mmol/L DTT, 0.1 mmol/L Na<sub>3</sub>VO<sub>4</sub>, 10 mmol/L MgCl<sub>2</sub>, 20 μmol/L ATP, and 5 μCi of [<sup>32</sup>P]ATP for 30 minutes at 30°C. Proteins were then resolved in SDS-PAGE (8%) and analyzed by autoradiography and Western blotting.

**In vivo binding of hypoxia-inducible factor-1α with von Hippel-Lindau tumor suppressor protein.** Vector containing FLAG-tagged pVHL (pRc/cytomegalovirus-HA-pVHL) was transiently transfected into MiaPaCa-2 cells in 100 mm culture plates and incubated for 44 hours. The MiaPaCa-2 cells were then exposed to normoxia, ischemia, or H<sub>2</sub>O<sub>2</sub> for 4 hours in the presence or absence of 10 μmol/L MG-132 (EMD Biosciences) to prevent HIF-1α from proteasomal degradation. The FLAG-tagged pVHL was immunoprecipitated from the cell lysates as described above. The HIF-1α protein bound to FLAG-pVHL was separated by SDS-PAGE, and immunoblotted with anti-HIF-1α antibody (BD Biosciences, Palo Alto, CA). Proteins in the membranes were then visualized using the enhanced chemiluminescence reagent (Amersham).

## Results

**Activation of p38 and c-Jun NH<sub>2</sub>-terminal kinase during low glucose, hypoxia, or ischemia.** We previously reported that glucose deprivation elevates the intracellular levels of ROS such as superoxide anion and hydrogen peroxide, and leads to the activation of ASK1-MEK-MAPK signal transduction through the dissociation of thioredoxin and glutaredoxin from ASK1 (14). As mentioned previously, the tumor microenvironment is characterized by low oxygen tensions as well as nutrient deprivation due to insufficient vascular delivery of oxygen and nutrients (18). Thus, we hypothesized that hypoxia and ischemia, which combines the effects of nutrient deprivation and low oxygen tension, would also activate the ASK1-MEK-MAPK signal transduction pathway (19, 20). To test this

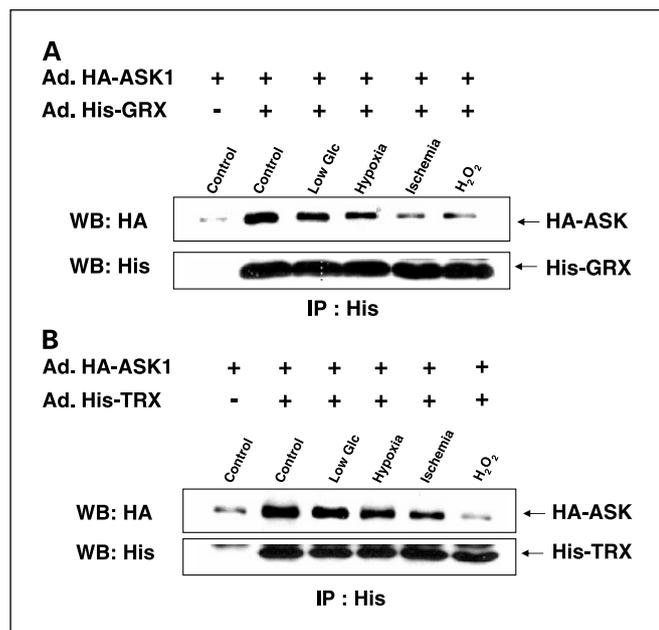
**Fig. 1.** Low glucose, hypoxia, or ischemia-induced p38 MAPK and JNK1 activation. MiaPaCa-2 cells were exposed to low glucose (0.1 mmol/L glucose), hypoxia (0.1% O<sub>2</sub>), or ischemia (0.1 mmol/L glucose and 0.1% O<sub>2</sub>) for various times (0.5-12 hours). Cell lysates were immunoblotted with anti-phospho-p38 MAPK, anti-p38 MAPK, anti-ACTIVE-JNK, anti-JNK1, or anti-actin antibody.



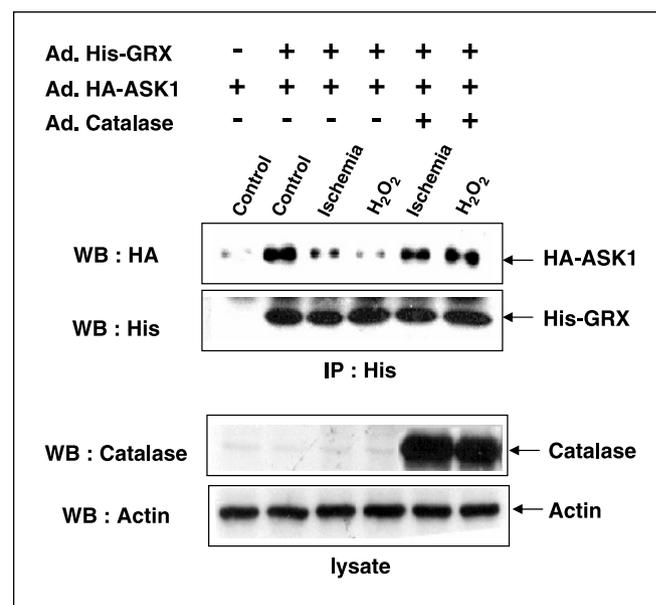
hypothesis, MiaPaCa-2 pancreatic cancer cells were exposed to the following conditions—low glucose (0.1 mmol/L), hypoxia (0.1% O<sub>2</sub>), or ischemia (0.1 mmol/L glucose in 0.1% O<sub>2</sub>). Figure 1 shows that the activation of JNK1 and p38 MAPK occurred at low glucose, hypoxia, and ischemia. Notably, the activation of p38 MAPK was more prominent under ischemia than under low glucose or hypoxia. Data from densitometer analysis show that phosphorylation of p38 MAPK under ischemia was ~2-fold or 1.9-fold higher than that under low glucose or hypoxia, respectively.

*Effect of low glucose, hypoxia, or ischemia on the interaction of glutaredoxin with apoptosis signal-regulating kinase 1.* Previous studies have shown that glucose deprivation increases the intracellular level of ROS such as H<sub>2</sub>O<sub>2</sub>

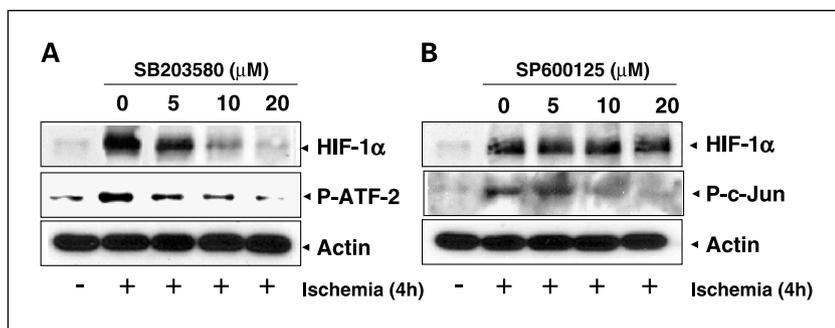
(19, 20). This is probably due to the disruption of H<sub>2</sub>O<sub>2</sub> scavenging systems such as the glutathione peroxidase/glutathione reductase system (19). Increases in steady state levels of H<sub>2</sub>O<sub>2</sub> and glutathione disulfide cause thioredoxin and glutaredoxin to dissociate from ASK1 (14, 16, 21) and subsequently activate the ASK1-MEK-MAPK signal transduction pathway (16, 19–22). Schumacker's group (11, 23) reported that hypoxia increases mitochondrial ROS generation at complex III. In light of these findings, we hypothesized that the activation of ASK1 occurs through the ROS-induced dissociation of glutaredoxin and/or thioredoxin from ASK1 during hypoxia as well as ischemia. To investigate this possibility, MiaPaCa-2 cells were coinfecting with Ad.HA-ASK1 and Ad.HA-glutaredoxin, or Ad.His-thioredoxin and then exposed to low glucose, hypoxia, ischemia, or H<sub>2</sub>O<sub>2</sub>. The H<sub>2</sub>O<sub>2</sub> condition was



**Fig. 2.** Effect of low glucose, hypoxia, or ischemia on the interaction between ASK1 and glutaredoxin (A) or thioredoxin (B) in MiaPaCa-2 cells. Cells were coinfecting with adenoviral vectors containing HA-tagged ASK1 (Ad.HA-ASK1, 5 MOI) and His-tagged glutaredoxin (Ad.His-glutaredoxin, 30 MOI) or His-tagged thioredoxin (Ad. His-thioredoxin, 30 MOI). After 24 hours of incubation, cells were exposed to low glucose (0.1 mmol/L), hypoxia (0.1% O<sub>2</sub>), ischemia (0.1 mmol/L, 0.1% O<sub>2</sub>), or H<sub>2</sub>O<sub>2</sub> (500  $\mu$ mol/L) for 30 minutes. Cell lysates were immunoprecipitated with 2  $\mu$ g of anti-His antibody, and then immunoblotted with anti-HA or anti-His antibody.



**Fig. 3.** Effect of catalase on ischemia or H<sub>2</sub>O<sub>2</sub>-induced dissociation of glutaredoxin from ASK1. MiaPaCa-2 cells were coinfecting with Ad. His-glutaredoxin at an MOI of 30, Ad.HA-ASK1 at an MOI of 5, and Ad.catalase at an MOI of 150. After 24 hours of incubation, cells were exposed to complete medium under normoxia (control), ischemia (0.1 mmol/L, 0.1% O<sub>2</sub>), or H<sub>2</sub>O<sub>2</sub> (500  $\mu$ mol/L) for 30 minutes. Cell lysates were divided into two fractions. One fraction of lysates was immunoprecipitated with anti-His antibody, and then immunoblotted with anti-HA antibody or anti-His antibody. The other fraction of lysates was immunoblotted with anti-catalase antibody or anti-actin antibody.



**Fig. 4.** Effect of p38 MAPK and JNK inhibitors on the HIF-1 $\alpha$  accumulation during ischemia in MiaPaCa-2 cells. SB203580 (A) or SP600125 (B), at the indicated concentrations, were added 30 minutes prior to ischemia as well as during ischemia for 4 hours. Cell lysates were immunoblotted with anti-HIF-1 $\alpha$ , anti-phospho-ATF-2, anti-phospho-c-Jun, or anti-actin antibody.

included to confirm that an increase in the level of ROS is sufficient to cause dissociation of glutaredoxin and/or thioredoxin from ASK1 irrespective of the initiating condition up the line. Figure 2 shows that glutaredoxin and thioredoxin dissociated from ASK1 under these conditions. Interestingly, the dissociation of glutaredoxin and thioredoxin from ASK1 was greater under ischemic conditions compared with low glucose or hypoxic conditions.

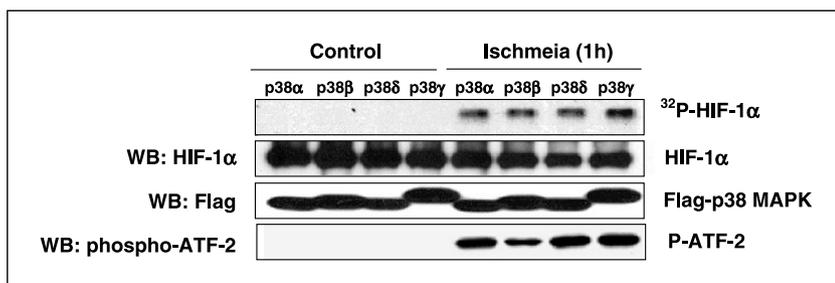
**Effect of catalase on the interaction of glutaredoxin with apoptosis signal-regulating kinase 1 during hypoxia or ischemia.** Catalase, a scavenger of H<sub>2</sub>O<sub>2</sub>, was overexpressed to confirm ROS-mediated dissociation of glutaredoxin from ASK1 during ischemia. Figure 3 shows that the dissociation of glutaredoxin from ASK1 under ischemic conditions or during treatment with H<sub>2</sub>O<sub>2</sub> was prevented by catalase overexpression. These data also suggest that the activation of JNK1 and p38 MAPK was promoted by the ROS-mediated activation of ASK1 during ischemia in MiaPaCa-2 cells (Figs. 2 and 3; ref. 14).

**Effects of c-Jun NH<sub>2</sub>-terminal kinase and p38 mitogen-activated protein kinase inhibitors on the accumulation of hypoxia-inducible factor-1 $\alpha$  during ischemia.** To determine whether the JNK1 and/or p38 MAPK signaling pathways are required for HIF-1 $\alpha$  accumulation, MiaPaCa2 cells were treated with SP600125, an inhibitor of JNK1, or SB203580, an inhibitor of p38 MAPK, during ischemia. Figure 4 shows that under ischemic conditions, SP600125 did not have any inhibitory effect on the HIF-1 $\alpha$  level, whereas SB203580 caused a dose-dependent inhibition of HIF-1 $\alpha$  level in MiaPaCa-2 cells. The specificity of these inhibitors were confirmed by inhibiting phosphorylation of ATF-2, a substrate for p38 MAPK, and c-Jun, a substrate for JNK1, during ischemia (Fig. 4A and B). These results suggest that the activation of p38 MAPK is required for HIF-1 $\alpha$  stabilization.

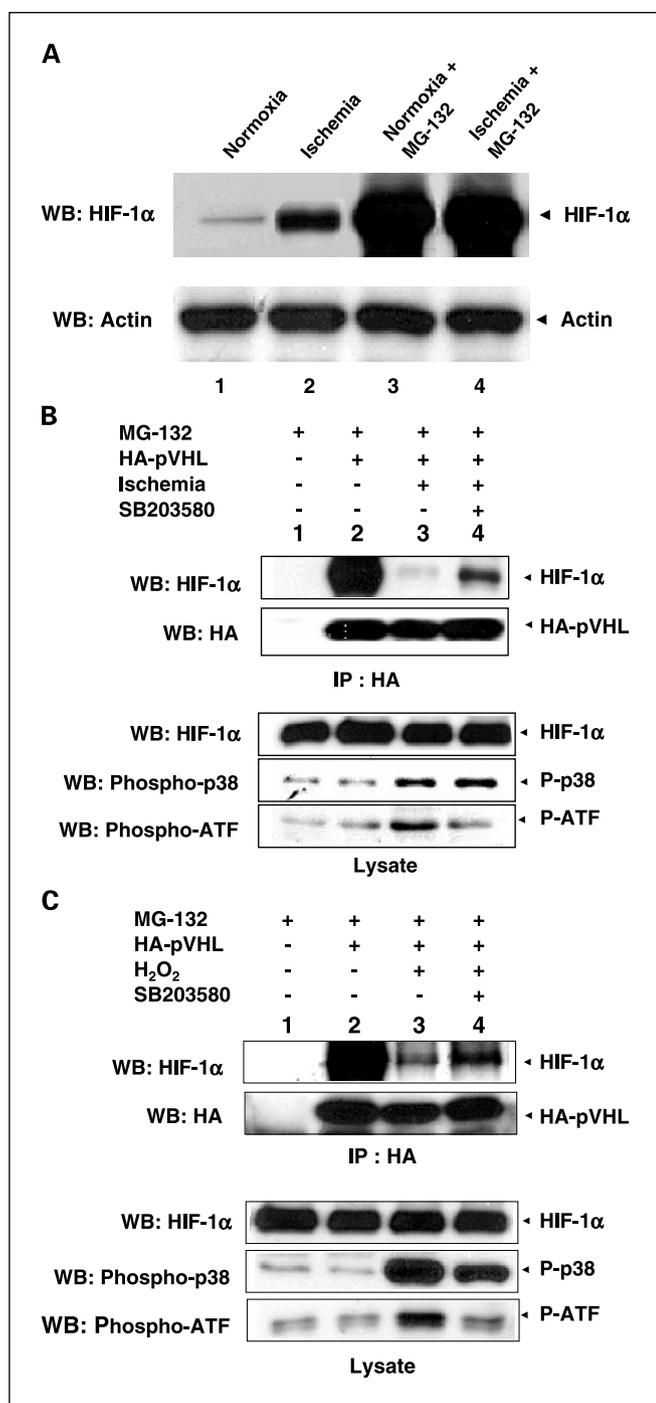
**Phosphorylation of hypoxia-inducible factor-1 $\alpha$  with p38 mitogen-activated protein kinase isoforms in vitro.** We further

sought to identify which p38 isoform(s) is(are) capable of phosphorylating HIF-1 $\alpha$ . HIF-1 $\alpha$  was translated *in vitro* using TNT-coupled reticulocyte lysate system and incubated with the inactive p38 MAPK isoforms or the active p38 MAPK isoforms, which were obtained from no-stressed or ischemic-stressed MiaPaCa-2 cells, respectively. Figure 5 shows that the active p38 MAPK, but not the inactive p38 MAPK, directly phosphorylated HIF-1 $\alpha$  as well as ATF-2 *in vitro* regardless of p38 MAPK isoforms.

**Interaction between phosphorylated hypoxia-inducible factor-1 $\alpha$  and von Hippel-Lindau tumor suppressor protein during ischemia.** Several MAPKs have been reported to cause the phosphorylation of HIF-1 $\alpha$ , which results in the activation and stabilization of HIF-1 $\alpha$  (9). The p38 MAPK among several MAPKs is required for stabilization of HIF-1 $\alpha$  induced by Cr(VI) and sodium arsenite (24, 25). In addition, the fusion protein with glutathione S-transferase and trans-activation domain of HIF-1 $\alpha$  is directly phosphorylated *in vitro* by activated p38 $\alpha$  and p38 $\gamma$  MAPK resulting in the stimulation of transactivating activity of HIF-1 $\alpha$  (26). However, the relation between HIF-1 $\alpha$  phosphorylation and its stability during ischemia remains unclear. We hypothesized that during ischemia, the interaction of HIF-1 $\alpha$  with pVHL, which mediates the ubiquitination and degradation of HIF-1 $\alpha$  (4, 27), would be changed when HIF-1 $\alpha$  is phosphorylated. To investigate this possibility, it was necessary to inhibit the proteosomal degradation of HIF-1 $\alpha$  during normoxia as well as hypoxia. MG-132, a proteasome inhibitor, was used for preventing the proteosomal degradation of HIF-1 $\alpha$ . Figure 6A shows that the level of HIF-1 $\alpha$  was increased during ischemia, as expected. Interestingly, the level of HIF-1 $\alpha$  was markedly increased in the presence of MG-132 in both the normoxic and ischemic conditions. These results suggest two possibilities; one is that pVHL-independent ubiquitination of HIF-1 $\alpha$  may exist because HIF-1 $\alpha$  was still degraded even under ischemic conditions. The other is that



**Fig. 5.** Phosphorylation of HIF-1 $\alpha$  by p38 MAPK isoforms. *In vitro* translated HA-HIF-1 $\alpha$  protein was obtained with the use of the TNT-coupled reticulocyte lysate system. The translation mixture was immunoprecipitated using an anti-HA antibody and incubated with [ $\gamma$ -<sup>32</sup>P]ATP in the presence of p38 MAPK protein, which was obtained from pcDNA3-FLAG-p38 $\alpha$ -, FLAG-p38 $\beta$ -, FLAG-p38 $\delta$ -, or FLAG-p38 $\gamma$ -transfected MiaPaCa-2 cells under normoxia or ischemia for 30 minutes at 30°C. Samples were analyzed by SDS-PAGE (8% gel) and then autoradiographed. The kinase activity of p38 MAPK was confirmed by using ATF-2 as a substrate. Samples were immunoblotted with anti-phospho-ATF-2 antibody.

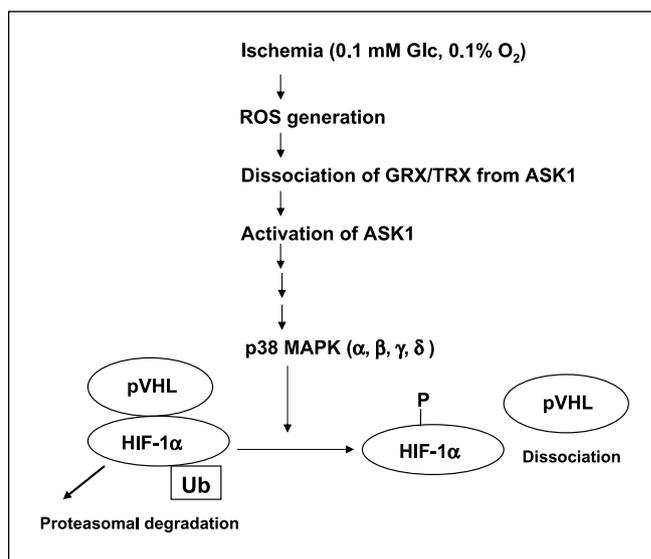


**Fig. 6.** *In vivo* binding assay of HIF-1 $\alpha$  with pVHL. MiaPaCa-2 cells were transiently transfected with pRc/cytomegalovirus-HA-pVHL and incubated for 44 hours. **A**, cells were treated with/without 10  $\mu$ mol/L MG-132 during normoxia or ischemia. **B**, cells were exposed to normoxia or ischemia with MG-132 for 4 hours. One fraction of lysates was immunoprecipitated with anti-HA antibody and then immunoblotted with anti-HA or anti-HIF-1 $\alpha$  antibody. The other fraction of lysates was immunoblotted with anti-HIF-1 $\alpha$  anti-phospho-p38, or anti-phospho-ATF-2 antibody. **C**, cells were exposed to 500  $\mu$ mol/L H<sub>2</sub>O<sub>2</sub> in the presence of MG-132 with or without pretreatment with SB203580. Cell lysates were analyzed as described in (B).

the binding of pVHL and HIF-1 $\alpha$  might exist in ischemia, but could be weaker, resulting in partial degradation of HIF-1 $\alpha$ . These possibilities were examined by comparing the HIF-1 $\alpha$ -pVHL interaction with the phospho-HIF-1 $\alpha$ -pVHL interaction.

We transiently expressed pVHL in MiaPaCa-2 cells and treated cells with MG-132 in the presence or absence of SB203580 under normoxia or ischemia (Fig. 6B). Under normoxic conditions, the HIF-1 $\alpha$  strongly associated with pVHL (lane 2), because the specific prolyl residues (Pro<sup>402</sup> and Pro<sup>564</sup>) of HIF-1 $\alpha$  are hydroxylated by prolyl hydroxylases under normoxia (4). However, during ischemia, the prolyl hydroxylation of HIF-1 $\alpha$  is suppressed (27) and the phosphorylation of HIF-1 $\alpha$  occurs (9). Indeed, Fig. 6B shows that HIF-1 $\alpha$  almost completely dissociated from pVHL during ischemia (lane 3). In contrast, the interaction between HIF-1 $\alpha$  and pVHL still occurred in the presence of SB203580, which inhibited the phosphorylation of HIF-1 $\alpha$  by p38 MAPK. These results suggest that the phosphorylation of HIF-1 $\alpha$  by p38 MAPK contributed to the inhibition of HIF-1 $\alpha$ -pVHL interaction during ischemia. Next, we further examined the role of phosphorylation of HIF-1 $\alpha$  in the interaction between HIF-1 $\alpha$  and pVHL under normoxic conditions. Cells were treated with H<sub>2</sub>O<sub>2</sub>, which is known to activate p38 MAPK (19), in the presence of MG-132 with or without pretreatment with SB203580. Under these conditions, proteosomal degradation of HIF-1 $\alpha$  was prevented with MG-132 (lane 2, Fig. 6C). Figure 6C shows that the binding affinity of HIF-1 $\alpha$  with pVHL decreased significantly in the presence of H<sub>2</sub>O<sub>2</sub>, even under normoxia. These results suggest that the phosphorylation of HIF-1 $\alpha$  was sufficient to decrease the binding of HIF-1 $\alpha$  with pVHL regardless of the hydroxylation of HIF-1 $\alpha$  during normoxia. However, when the activity of p38 MAPK was inhibited by treating with SB203580, the binding affinity of HIF-1 $\alpha$  with pVHL was partially restored (lane 4, Fig. 6C).

**Model for the signal pathway of hypoxia-inducible factor-1 $\alpha$  phosphorylation and its role in the interaction between hypoxia-inducible factor-1 $\alpha$  with von Hippel-Lindau tumor suppressor protein during ischemia.** We present a schematic signal model based on our results (Fig. 7). The model illustrates that ischemia-mediated ROS generation activates ASK1 due to the dissociation of glutaredoxin and thioredoxin from ASK1,



**Fig. 7.** A schematic model for the signal pathway of HIF-1 $\alpha$  phosphorylation and its role in interaction between HIF-1 $\alpha$  with pVHL in MiaPaCa-2 cells during ischemia.

resulting in the activation of p38 MAPK and the consequent phosphorylation of HIF-1 $\alpha$ . As a result of these sequential events, pVHL dissociates from HIF-1 $\alpha$  and accumulation of HIF-1 $\alpha$  occurs.

## Discussion

Ischemia produces decreased levels of energy substrates (O<sub>2</sub> and glucose) in human cells (28). Decreasing either one of these triggers the generation of ROS from mitochondria (11, 29). In addition, Schumacker's group has observed that ROS (H<sub>2</sub>O<sub>2</sub>) are sufficient for HIF-1 $\alpha$  stabilization during normoxia, and overexpression of catalase, which eliminates ROS, abolishes HIF-1 $\alpha$  stabilization (11, 30). Verma's group has reported that low glucose induces HIF-1 $\alpha$  in human glioma cells during normoxia (31). We investigated the involvement of MAPK signal pathways in stabilizing HIF-1 $\alpha$  via ROS generation under both low glucose and hypoxia.

In the present studies, we investigated several topics. First, we examined how the activation of JNK1 and p38 MAPK occurs as a result of the ROS-mediated activation of ASK1. Our data reveal that both glutaredoxin and thioredoxin dissociated from ASK1 through the generation of ROS under ischemic conditions, resulting in the activation of ASK1 followed by the activation of JNK1 and p38 MAPK (Figs. 1–3). Dissociation of glutaredoxin and thioredoxin from ASK1 occurred more prominently under ischemic conditions than under either hypoxic or low glucose conditions alone. Data from densitometer analysis show that interaction between thioredoxin/glutaredoxin and ASK1 was reduced by 9% to 25%, 20% to 36%, or 31% to 71% under low glucose, hypoxia, or ischemia, respectively.

Second, because the stabilization and activation of HIF-1 $\alpha$  by phosphorylation through the MAPK signaling pathway has already been demonstrated (9, 10), we investigated whether either JNK1 or p38 plays an important role in stabilizing HIF-1 $\alpha$ . Our experiments with the p38 inhibitor SB203580 completely abolished the accumulation of HIF-1 $\alpha$  under ischemia, whereas the JNK inhibitor SP600125 did not affect ischemia-induced HIF-1 $\alpha$  accumulation (Fig. 4). These observations are consistent with the previous report explaining that ROS-mediated activation of p38 MAPK plays an important role in stabilizing HIF-1 $\alpha$  (24). In addition, we investigated *in vitro* phosphorylation of HA-HIF-1 $\alpha$  translated from the TNT system using the p38 isoforms activated from ischemic stress (Fig. 5). Our data illustrate, for the first time, that the full length of HA-HIF-1 $\alpha$  is directly phosphorylated by the active p38 MAPK regardless of isoforms. Similar results were observed with the fragment of HIF-1 $\alpha$ . However, Gutkind et al. (26) reported that active p38 $\alpha$  and p38 $\gamma$  were able to phosphorylate the fusion protein of glutathione S-transferase with HIF-1 $\alpha$  regulatory

region (531-826), but that active p38 $\delta$  and JNK were unable to phosphorylate this fusion protein. Thus, the relation between HIF-1 $\alpha$  phosphorylation and HIF-1 $\alpha$  stabilization still needs to be further studied.

Third, for the first time, we investigated whether the HIF-1 $\alpha$  phosphorylation induced by ROS generation under ischemia affects the stability of HIF-1 $\alpha$ . We compared the binding affinity of pVHL with either phospho-HIF-1 $\alpha$  or HIF-1 $\alpha$  during ischemia. We know that this activation of the MAPK signaling system results in the stabilization of HIF-1 $\alpha$  (11) and that HIF-1 $\alpha$  stability is strongly related with the pVHL binding of its oxygen-dependent degradation domain through specific recognition of hydroxylated Pro<sup>402</sup> or Pro<sup>564</sup> (4, 5). On the other hand, our data clearly shows that the binding affinity of pVHL with phospho-HIF-1 $\alpha$  was lower than that of pVHL with HIF-1 $\alpha$  during ischemia (Fig. 6B). The inhibition of HIF-1 $\alpha$  phosphorylation by active p38 MAPK with SB203580 caused an increase in the binding of pVHL with HIF-1 $\alpha$ , revealing that the phosphorylation of HIF-1 $\alpha$  plays an important role in the inhibition of pVHL-HIF-1 $\alpha$  binding during ischemia (Fig. 6B). These results suggest that both reduction of prolyl hydroxylation and phosphorylation of HIF-1 $\alpha$  prevent the pVHL binding with HIF-1 $\alpha$  during ischemia, resulting in the prevention of proteosomal degradation. Nevertheless, significant amounts of HIF-1 $\alpha$  protein seemed to degrade even under ischemia, because the HIF-1 $\alpha$  level under ischemia was lower than that under proteosomal inhibition with MG-132. These results suggest that pVHL-independent ubiquitination of HIF-1 $\alpha$  occurs during ischemia (Fig. 6A). These observations support the previous report relating to pVHL-independent ubiquitination of HIF-1 $\alpha$  even though the exact mechanism still remains unknown (32, 33). Under normoxic conditions, the interaction between pVHL and phospho-HIF-1 $\alpha$  was observed after inducing the p38 MAPK-mediated phosphorylation of HIF-1 $\alpha$  by H<sub>2</sub>O<sub>2</sub> (22). In this case, although both HIF-1 $\alpha$  and phospho-HIF-1 $\alpha$  are hydroxylated by prolyl hydroxylases, the binding affinity of phospho-HIF-1 $\alpha$  was significantly decreased compared with HIF-1 $\alpha$ . These results suggest that phosphorylation of HIF-1 $\alpha$  results in the inhibition of pVHL binding irrespective of the hydroxylation of HIF-1 $\alpha$  (Fig. 6C). Interestingly, the interaction between pVHL with HIF-1 $\alpha$  was partially restored by treatment with SB203580, an inhibitor of p38 MAPK (Fig. 6C). These results suggest that another MAPK and/or protein kinase B-mediated phosphorylation of HIF-1 $\alpha$  plays an important role in the interaction between pVHL and HIF-1 $\alpha$  (8, 9) and that multiphosphorylation sites in HIF-1 $\alpha$  exist (34). Obviously, further studies are necessary to understand the role of phosphorylation of HIF-1 $\alpha$  in the stabilization of HIF-1 $\alpha$ . Our model will provide a framework for future studies.

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