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Molecules and Cells

Activation of JNK and c-Jun Is Involved in Glucose Oxidase-Mediated Cell Death of Human Lymphoma Cells

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Mitogen-activated protein kinases (MAPK) affect the activation of activator protein-1 (AP-1), which plays an important role in regulating a range of cellular processes. However, the roles of these signaling factors on hydrogen peroxide (H_2O_2)-induced cell death are unclear. This study examined the effects of H_2O_2 on the activation of MAPK and AP-1 by exposing the cells to H_2O_2 generated by either glucose oxidase or a bolus addition. Exposing BJAB or Jurkat cells to H_2O_2 affected the activities of MAPK differently according to the method of H_2O_2 exposure. H_2O_2 increased the AP-1-DNA binding activity in these cells, where continuously generated H_2O_2 led to an increase in mainly the c-Fos, FosB and c-Jun proteins. The c-Jun-NH_2-terminal kinase (JNK)-mediated activation of c-Jun was shown to be related to the H_2O_2-induced cell death. However, the suppression of H_2O_2-induced oxidative stress by either JNK inhibitor or c-Jun specific antisense transfection was temporary in the cells exposed to glucose oxidase but not to a bolus H_2O_2. This was associated with the disruption of death signaling according to the severe and prolonged depletion of reduced glutathione. Overall, these results suggest that H_2O_2 may decide differently the mode of cell death by affecting the intracellular redox state of thiol-containing antioxidants, and this depends more closely on the duration exposed to H_2O_2 than the concentration of this agent.

INTRODUCTION

Hydrogen peroxide (H_2O_2) induces cell death by either apoptosis or necrosis (Chiarugi, 2008; Sancho et al., 2006). The dual modes of cell death by H_2O_2 are dependent on the concentration of this agent and the type of cells being studied. However, it is more likely that the cell death modes in H_2O_2-exposed cells are strongly affected by the ways of H_2O_2 exposure, i.e., continuous generation vs. the direct addition of H_2O_2 (Barbouti et al., 2002). It has been demonstrated that exposure of cells to a bolus H_2O_2 leads to a typical apoptotic process through caspase-dependent pathway, whereas the addition of glucose oxidase (to generate H_2O_2 continuously) suppresses the process and converts to caspase-independent cell death (Barbouti et al., 2007). This anti-apoptotic action of H_2O_2 in glucose oxidase-exposed cells is believed to be associated with the rapid and dramatic decrease in adenosine triphosphate (ATP) accompanied by excessive DNA damage and subsequent poly (ADP-ribose) polymerase (PARP) activation (Barbouti et al., 2007; Cregan et al., 2004; Kang et al., 2004; Li and Osborne, 2008; Lin and Yang, 2008). Our recent findings also showed that continuously generated H_2O_2 induces caspase-independent, apoptosis-inducing factor (AIF)-mediated pyknotic cell death in lymphoma cells (Son et al., 2009).

On the other hand, it was suggested that H_2O_2 triggers the activation of protein kinases and modulates the expression of many redox sensitive genes (Sen and Packer, 1996). Among the kinases, mitogen-activated protein kinases (MAPK), including c-Jun NH_2-terminal kinase (JNK), extracellular signal-regulated kinase (ERK) and p38 kinase, play critical roles in modulating apoptosis signaling in response to oxidative stress (Chang and Karin, 2001). MAPK also have roles as upstream effectors of activator protein-1 (AP-1), which regulates a broad range of cellular events (Jacobs-Helber et al., 1998; Kook et al., 2008). Thus, it is likely that the phosphorylation of MAPK and subsequent activation of AP-1 are associated with H_2O_2-induced apoptosis. Indeed, exposure of retinal pigment epithelial cells to H_2O_2 induced apoptosis through a Racl/JNK/p38 signaling cascade (Ho et al., 2006). H_2O_2 also causes apoptosis by activating AP-1-mediated signaling in oligodendrocytes (Vollgraf et al., 1999) or by up-regulating both JNK and p38 in testicular germ cells (Maheshwari et al., 2009). However, the roles of MAPK in H_2O_2-induced apoptosis appear to differ according to the types of cells examined because the activation of ERK, not JNK/p38, is essential for mediating the H_2O_2-induced apoptosis of osteoblasts (Park et al., 2005). Moreover, the cellular mechanisms by which H_2O_2 affects the activation of MAPK and AP-1 according to the method of exposure to this agent,

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i.e., to continuous generation vs. direct addition, are unclear.

In this study, we examined whether the activation of MAPK and AP-1 is actually involved in H_2O_2-induced cell death using human lymphoma cells. This is because actively replicating lymphocytes are particularly sensitive to ROS because their membranes are rich in poly-unsaturated fatty acids (Goppelt-Strube and Resch, 1987). We also compared the cellular effects of H_2O_2 on these factors using two H_2O_2 generating systems, glucose oxidase and bolus H_2O_2. In addition, we investigated the possible roles of Jun family proteins on H_2O_2-induced cell death and determined the intracellular status of antioxidant defense enzymes.

MATERIALS AND METHODS

Chemicals

Pharmacological inhibitors of ERK (PD98059), p38 (SB203580), and JNK (SP600125) were purchased from TOCRIS (Missouri). Unless specified otherwise, all chemicals were obtained from Sigma Chemical Co. (USA). All reagents were prepared immediately before use.

Cell culture and treatment

Human lymphoma BJAB and Jurkat cells were cultured in RPMI-1640 medium supplemented with antibiotics (Gibco™, USA) and 10% fetal bovine serum (FBS; HyClone, USA). Cultures were placed into a fresh batch of RPMI-1640 medium supplemented with 0.5% FBS immediately before the cells were exposed to H_2O_2 in the presence or absence of each MAPK inhibitor. When glucose oxidase is used to generate H_2O_2, the enzyme can produce H_2O_2 continuously at low levels, e.g. a 10 mU/ml of glucose oxidase generates 1 to 2.4 μM H_2O_2/min up to 24 h depending on the incubation time (Lee et al., 2006). In contrast, H_2O_2 by a bolus addition is removed quickly, and its concentration in the culture media is not apparent within 60 min after addition (Barbouti et al., 2002).

Measurement of cytotoxicity and succinate dehydrogenase (SDH) activity

The cytotoxicity was determined by staining aliquots of cells with 0.4% trypan blue; approximately 100 cells were counted for each treatment. Cell death was calculated as follows: % cytotoxicity = (total cells-viable cells)/total cells) × 100%. In addition, 3-(4,5-dimethylthiazol-2yl-)-2,5-diphenyl tetrazolium bromide (MTT) was used to evaluate SDH activity of cells. In this assay, only living cells take up the yellow MTT, which is then converted to a dark blue product by an enzyme, SDH, located in mitochondria. Therefore, the concentration of the blue product is proportional to the SDH activity reducing MTT, which is closely associated with cell viability. After various times (0-12 h) of H_2O_2 exposure, 10 μl of a MTT solution (5 mg/ml in PBS as stock solution) was added into each well, and the cells were incubated for a further 4 h at 37°C. After adding acidic isopropanol (70 μl) to each well, the absorbance of the plates was read at 570 nm using a SpectraCount™ ELISA reader (Packard, Instrument Co., USA).

Western blot analysis

Cell lysates were made in a buffer (50 mM Tris-Cl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% NP 40, 0.25% Na-deoxycholate, and 1 μg/ml of each of aprotinin, leupeptin and pepstatin), and the protein content was quantified according to the Bradford method (1976). Equal amount (30 μg) of proteins per each sample was separated by 12% SDS-PAGE and blotted onto PVDF membranes. The blots were treated with PBS containing 5% skim milk for 1 h, probed with primary antibodies at room temperature for 2 h, and incubated with secondary antibodies for 1 h. Finally, the blots were developed with enhanced chemiluminescence (Amersham Pharmacia Biotech, UK) immediately before exposure to X-ray film (Eastman-Kodak, USA). In this study, polyclonal antibodies specific to JNK, ERK, p38, FosB, c-Fos, Fra1, Fra2, JunB, c-Jun, and JunD, and monoclonal antibodies specific to β-actin, p-ERK, p-JNK, p-p38, p-c-Jun, and Ref-1 were obtained from Santa Cruz Biotechnology (USA). Monoclonal antibody against α-tubulin was purchased from BD Bioscience Pharmingen (USA).

Electrophoretic mobility shift assay (EMSA)

Nuclear proteins prepared as described elsewhere (Maulik et al., 1998) were used in the DNA binding reactions, which were carried out for 30 min at room temperature with 10-15 μg of the protein in 20 μl of a buffer [1 μg/ml BSA, 0.5 μg/μl poly (dI-dC), 5% glycerol, 1 mM DTT, 1 mM PMSF, 10 mM Tris-Cl (pH 7.5), 50 mM NaCl, 30,000 cpm of (α-^{32}P) dCTP-labeled oligonucleotides, and the Klenow fragment of DNA polymerase]. The samples were separated on 6% polyacrylamide gels, which were then dried and exposed to X-ray film (Eastman Kodak Co., USA) for 12-48 h at -70°C. The oligonucleotide primer sequences used for EMSA specific to AP-1 were: 5′-AAG GGA TCC GGC TGA CTC ATC ACT AG-3′ and 3′-CTA GGC CGA CTG AGT AGT GAT CGG AA-5′.

Transient transfection

The c-Jun and JunD anti-sense sequences were chosen as the 15-mer sequences containing the AUG translation initiation codon, which was synthesized by Cosmo Co. Ltd. (Korea). The sequences are follows: 5′-CAT CTT TGC AGT CAT-3′ for c-Jun, 5′-GAA GGG CGT TTC CAT-3′ for JunD, and 5′-ATG ACT GCA AAG ATG-3′ for control oligonucleotides, respectively. Each transfection was performed using Lipofectamine™ 2000 Transfection Reagent (Invitrogen) according to the manufacturer's protocol. At 24 h, the cells were exposed to glucose oxidase and then processed for further analyses.

Enzyme assays

At various times, BJAB cells were rinsed in ice-cold PBS and destroyed by repeated freezing and thawing in a 50 mM phosphate buffer (pH 7.8) containing 0.1% Triton X-100 and 0.1 mM EDTA. The supernatants were then centrifuged at 15,000 × *g* at 4°C for 20 min and used as the cellular extracts. The content of glutathione (GSH) and the activities of superoxide dismutase (SOD) and catalase (CAT) were determined as described elsewhere (Son et al., 2004).

Statistical analysis

All the data are expressed as a mean ± standard deviation (SD). One-way analysis of variance (ANOVA) (SPSS version 16.0 software) followed by a Scheffe's test was used to determine significant differences between the groups. A value of $p < 0.05$ was considered significant.

RESULTS

Effect of H_2O_2 on MAPK activation

The current study showed that H_2O_2 regulates the activities of the three MAPKs differently. Exposure of Jurkat cells to continuously generated H_2O_2 induced the activation of both JNK and p38 MAPK in a dose- (Fig. 1A) and time-dependent manner (Fig. 1B). The level of p-JNK and p-p38 increased approximately 2.4-fold and 1.7-fold, respectively, after 6 h exposure to

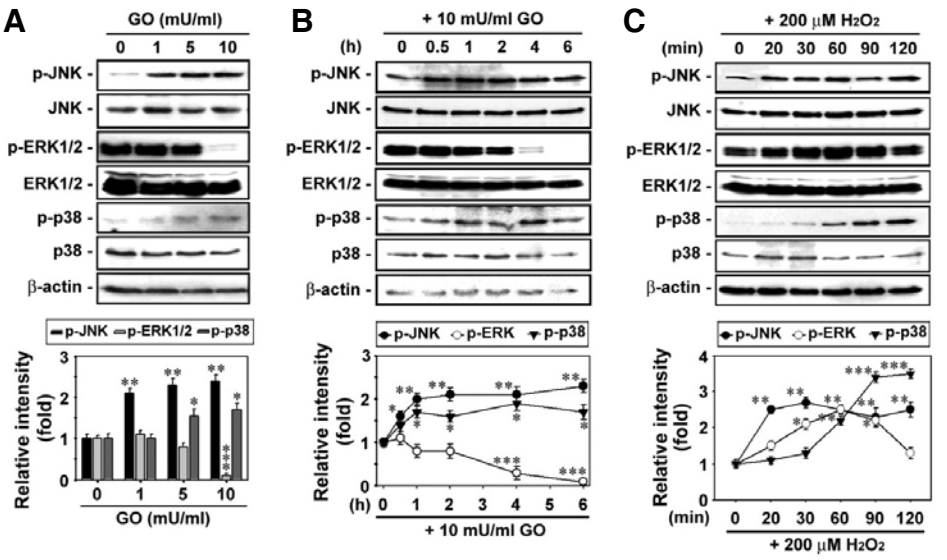

Fig. 1. Concentration- and time-dependent changes in MAPK activation in H_2O_2-exposed cells. Jurkat cells (2×10^6 cells/ml) were exposed to various concentrations (0-10 mU/ml) of glucose oxidase (GO) for 6 h (A) or 10 mU/ml GO for the indicated times (0-6 h) (B). (C) The cells were also exposed to a 200 μM bolus of H_2O_2 for various times (0-120 min). After exposure, the levels of MAPK proteins were analyzed by western blotting and a representative data was shown. The data from triplicate experiments were quantified by densitometry. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ vs. the untreated control cells.

10 mU/ml glucose oxidase compared to control levels. In contrast, the cellular level of p-ERK decreased apparently after exposure to 10 mU/ml glucose oxidase, which began in the cells from 4 h after exposure. There were no significant changes in whole protein levels of these MAPKs. This activation of MAPK was also similar to the case where BJAB cells were exposed to the enzyme (data not shown).

In order to determine the effects of H_2O_2 on MAPK activation according to the method to generate this agent, Jurkat cells were exposed to a bolus 200 μM H_2O_2 for intervals ranging from 0 to 120 min (Fig. 1C). Similar to the cells exposed to glucose oxidase, the bolus H_2O_2 addition induced a sustained activation of JNK and p38. p-JNK and p-p38 levels increased from 20 min and 60 min after exposure, and reached the maximum level of approximately 2.5-fold and 3.5-fold, respectively, compared to the control levels. Unlike exposure to glucose oxidase, ERK was activated by bolus H_2O_2, in that the increase in p-ERK began from 30 min, was maintained for up to 90 min, and returned to the basal levels at 2 h after exposure. However, there were no bolus H_2O_2-induced increases in MAPK phosphorylation observed 6 h after exposure (results not shown).

H_2O_2 induces DNA binding activation of AP-1

Treating lymphoma cells with glucose oxidase for 6 h activated AP-1 binding to its target DNA elements, even when these cells were exposed to 1 mU/ml of the enzyme (Fig. 2A). A bolus 200 μM H_2O_2 also induced the DNA-binding activity of AP-1, in which the activity was stimulated from 30 min, peaked at 2 h, and was maintained for up to 4 h after exposure to H_2O_2 (Fig. 2B). In contrast, exposing the cells to higher concentrations of bolus H_2O_2 (up to 1 mM) did not further augment the AP-1 activity. The pattern of AP-1 activation was similar to the case that Jurkat cells were exposed to the bolus H_2O_2 (data not shown).

The mechanism for how H_2O_2 affects the induction of Jun and Fos proteins was examined by Western blot analysis using the same nuclear proteins those used in EMSA. Exposure of BJAB or Jurkat cells to glucose oxidase resulted in a nuclear increase in c-Fos, FosB and c-Jun proteins in a dose- and time-dependent manner but not Fra1, Fra2 and JunD (Figs. 2C and 2D). In contrast, the nuclear level of JunB protein was decreased after exposure to 10 mU/ml glucose oxidase for 6 h. The nuclear levels of AP-1 family proteins were also affected individually by bolus H_2O_2, where the increases in c-Fos, c-Jun, JunB, and JunD were observed (data not shown).

Regulatory effects of MAPK inhibitors on H_2O_2-induced cell death

We next examined whether MAPK activation is required for H_2O_2-induced cell death. The concentration of MAPK inhibitors used in this study was 20 μM. At this dose, inhibitors did not cause any significant cytotoxicity during the experiments (data not shown). When cells were exposed to 10 mU/ml glucose oxidase for 12 h, the level of SDH activity was only 38% of that observed in the controls (Fig. 3, upper panel). Pretreating the cells with a JNK inhibitor (SP600125) or p38 inhibitor (SB203580) for 2 h inhibited the H_2O_2-mediated decrease in SDH activity. This result is similar to the case where the cells were incubated with 200 μM H_2O_2 in the presence of these inhibitors for 12 h. Interestingly, ERK inhibitor (PD98059) facilitated the H_2O_2-mediated depletion of SDH activity in the cells exposed to H_2O_2 generated from glucose oxidase but not in the cells exposed to H_2O_2 by a bolus addition. When cells were exposed to these H_2O_2 generating systems for 24 h, the protective effect of JNK or p38 MAPK inhibitor was shown only in the cells exposed to the bolus H_2O_2 (Fig. 3, below panel).

JNK activation is associated with the H_2O_2-induced cell death

Among the Jun family proteins, H_2O_2 increased the nuclear induction of c-Jun the most (Fig. 2), and the activation of JNK and p38 appeared to play important role in H_2O_2-mediated decrease of cell viability (Fig. 3). The effects of their specific inhibitors on c-Jun phosphorylation were examined using the cellular proteins prepared from BJAB cells at 2 h after H_2O_2 exposure. Inhibitors of JNK but not p38 MAPK blocked the level of p-c-Jun that had augmented in H_2O_2-exposed cells (Fig. 4A). The ERK inhibitor did not affect the level of cellular p-c-Jun (data not shown). In order to confirm the role of JNK on the H_2O_2-induced decrease in SDH activity, the cells were exposed to 10 mU/ml glucose oxidase for various times (0 to 24 h) in the presence of 20 μM SP600125 (Fig. 4B). JNK inhibitor prevented the H_2O_2-mediated decrease in SDH activity, whereas the prevention was temporary because significant inhibition was observed only until 12 h after H_2O_2 exposure. This was similar to the result obtained from the Jurkat cells exposed to

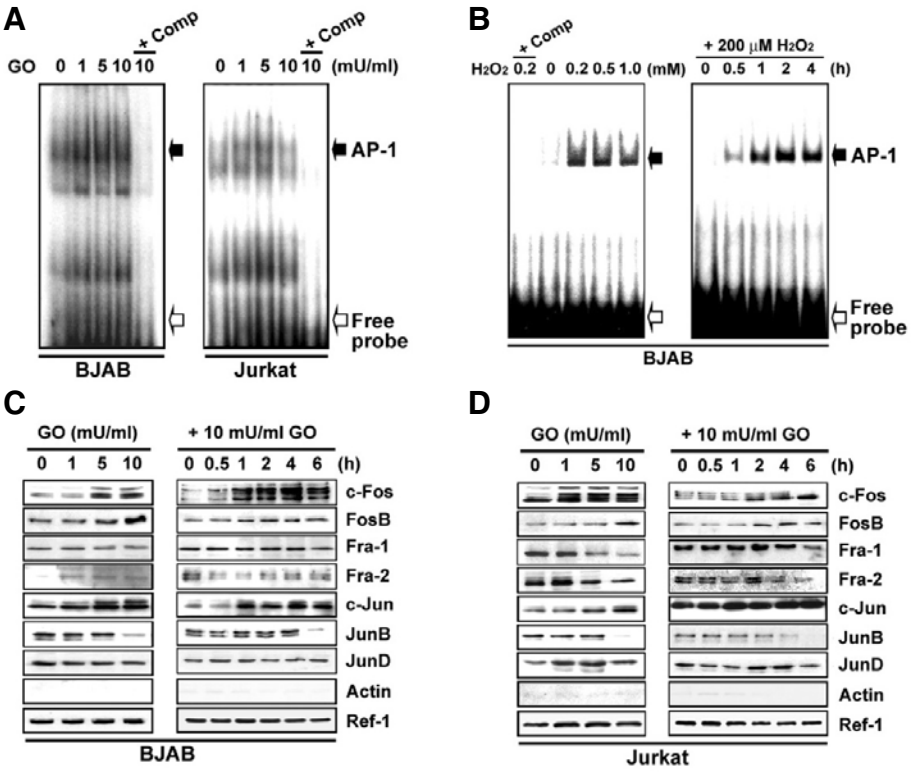

Fig. 2. H_2O_2 induces DNA binding activation of AP-1 in human lymphoma cells. AP-1 DNA-binding activity was analyzed by EMSA using nuclear extracts prepared from BJAB or Jurkat cells that had been treated for 6 h with various GO concentrations (0-10 mU/ml) (A) or with the indicated doses of bolus H_2O_2 for various times (0-4 h) (B). (C, D) Nuclear proteins obtained from glucose oxidase-treated cells were also subjected to immunoblot analysis using antibodies specific to Jun and Fos subfamily proteins. A representative result from triplicate experiments is shown.

the enzyme at the same conditions (data not shown).

Blockage of c-Jun attenuates the H_2O_2-induced cell death

The effects of transfection with the c-Jun or JunD anti-sense oligonucleotides were determined to better understand the functional role of AP-1 in the H_2O_2-mediated cell death. In this experiment, the transfected cells were exposed to H_2O_2 after 18 h of transfection because the decrease in c-Jun and JunD expression was apparent at 12 h after transfection (Fig. 5A). Down-regulation of c-Jun by transfection inhibited the cytotoxicity caused either by glucose oxidase or by a bolus H_2O_2 in BJAB cells (Fig. 5B). In contrast, blockage of JunD expression did not offer any protection against H_2O_2-induced cytotoxicity. The suppression of H_2O_2-mediated cell death by c-Jun antisense transfection was also time-dependent, in that the protection was not observed at 18 h after H_2O_2 exposure (Fig. 5C). These results were in parallel with the case where Jurkat cells were exposed to the enzyme or bolus H_2O_2 (data not shown). However, there was still significant protection against cell death when the transfected cells were exposed to a bolus H_2O_2 for 24 h compared to that of the non-transfected cells (data not shown).

Continuously generated H_2O_2 leads to the prolonged and apparent decrease in intracellular GSH level

To determine how H_2O_2 alters the antioxidant defense systems of the lymphoma cells according to continuous generation vs. direct addition, a time-course study of intracellular antioxidant enzyme contents in BJAB cells was performed (Fig. 6). The content of reduced GSH was gradually decreased according to the times exposed to glucose oxidase. When the cells were exposed to 10 mU/ml glucose oxidase for 3, 6 and 12 h, the GSH level fell to 33.4, 24.7, and 14.3 nmol/mg protein, respectively, compared to control (52 nmol/mg protein). Treating the cells with the bolus H_2O_2 (200 μM) decreased the reduced

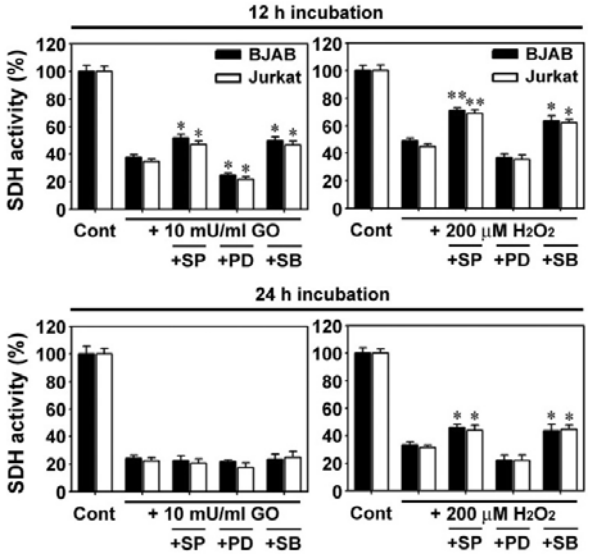

Fig. 3. Differential effects of MAPK inhibitors on viability of H_2O_2-exposed cells. BJAB and Jurkat cells were exposed to 10 mU/ml GO or 200 μM bolus H_2O_2 for 12 h and 24 h in the presence or absence of the MAPK inhibitors, SP600125 (for JNK), PD98059 (for ERK), or SB203580 (for p38). These cells were analyzed for their SDH activity at the indicated times of H_2O_2 exposure. $^*p < 0.05$ and $^{**}p < 0.01$ vs. the GO or bolus H_2O_2 exposure alone.

GSH content in an acute and temporal manner. The GSH level recovered quickly after 1 h of the exposure to bolus H_2O_2 and then the level became similar to that of control value. However, there were no significant changes in the activities of SOD and CAT according to the methods of exposure to H_2O_2 during the

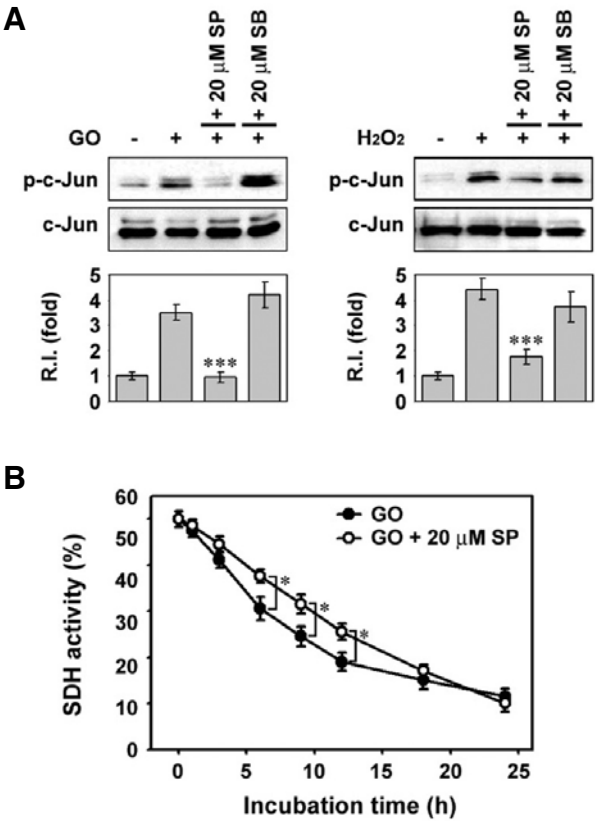

Fig. 4. Inhibition of JNK reduces the level of p-c-Jun and increases cell viability in H_2O_2-exposed cells. (A) BJAB cells were exposed to 10 mU/ml GO or 200 μM bolus H_2O_2 for 2 h in the presence of 20 μM JNK or p38 inhibitor, and the cellular levels of p-c-Jun and c-Jun were determined by Western blot analysis. ***$p < 0.001$ vs. the untreated control cells. (B) The cells were treated with 10 mU/ml GO with and without 20 μM JNK inhibitor. The SDH activity was measured at various exposure times (0-24 h). *$p < 0.05$ represents a significant difference between the experiments.

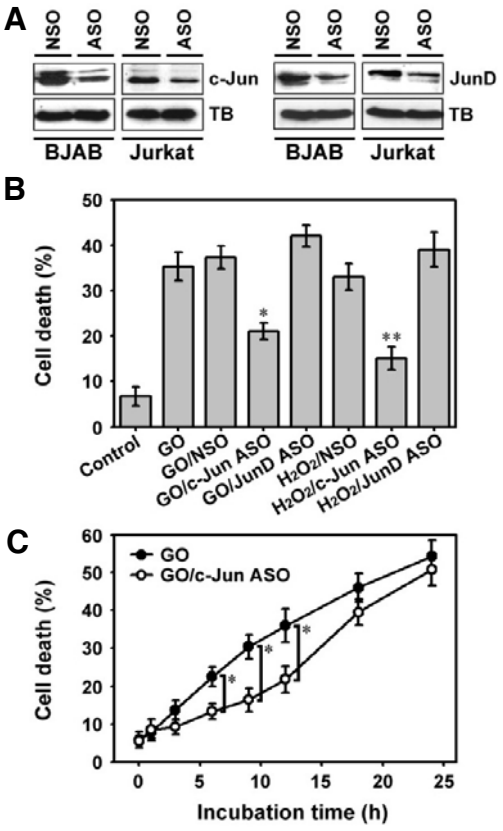

Fig. 5. Blockage of c-Jun significantly protects cells against the H_2O_2-induced cytotoxicity. (A) BJAB and Jurkat cells were transfected with the nonsense and anti-sense c-Jun or JunD oligonucleotides, and after 12 h of transfection, cellular levels of Jun proteins were determined by Western blotting. (B) After 18 h of the transfection, BJAB cells were exposed to 10 mU/ml GO or 200 μM bolus H_2O_2 for 12 h, and then the cytotoxicity was measured by trypan blue exclusion assay. *$p < 0.05$ and **$p < 0.01$ vs. GO treatment alone. (C) Control and c-Jun-transfected BJAB cells were exposed to 10 mU/ml GO for various times (0-24 h) and then processed for the analysis of cell death. *$p < 0.05$ represents a significant difference between the experiments. NSO, nonsense oligonucleotides; ASO, anti-sense oligonucleotides; TB, tubulin.

experimental periods, even though the cells had the same treatment of glucose oxidase or bolus H_2O_2.

DISCUSSION

It is obvious that H_2O_2 affects the activity of MAPK proteins, whereas their regulatory roles in H_2O_2-induced cell death are differ according to the types of cells employed. For example, H_2O_2 exposure increases JNK and p38 activity in human retinal pigment epithelial cells (Ho et al., 2006). JNK is involved in H_2O_2-mediated apoptosis in HeLa cells and mesangial cells, whereas p38 has no effect on the cell viability in these cells (Ishikawa and Kitamura, 2000; Wang et al., 1998). ERK also mediates both the cell survival and apoptosis signaling pathways in H_2O_2-exposed cells (Ishikawa and Kitamura, 2000; Park et al., 2005; Xia et al., 1995). In parallel with these reports, our results support the different roles of MAPK in H_2O_2-exposed cells according to the types of cells studied and the duration, i.e., the transient, delayed or sustained activation of MAPK.

This study shows that AP-1 is activated in both the cells exposed to glucose oxidase and a bolus H_2O_2. AP-1 could mediate both the positive and negative signals to cells in response to chemical stress, UV irradiation, and growth factor withdrawal (Gass and Herdegen, 1995; Hess et al., 2004). This dual action is thought to be dependent on a combination of AP-1 subproteins, although the precise mechanisms by which a combination determines either cell survival or death are not completely understood (Hilfiker-Kleiner et al., 2005; Jacobs-Helber et al., 1998; Suzuki et al., 1991; Yoshioka et al., 1995). Our current study indicates that continuously generated-H_2O_2 induces the AP-1 complex, which consists mainly of homodimer of c-Jun and/or heterodimers between c-Jun and c-Fos or FosB. This is in part similar to a previous report showing that the direct addition of H_2O_2 increases nuclear induction of c-Jun, c-Fos, JunD, and JunB in oligodendrocytes (Vollgraf et al., 1999). The inhibition of c-Jun phosphorylation by JNK inhibitor and the blockage of c-Jun by its antisense transfection attenuated the H_2O_2-mediated cell death. These results led us to suggest that Jun proteins play important roles in determining the fate of cells exposed to oxidative stress. This study also reveals that p38 MAPK is associated with the H_2O_2-induced cell death, but additional experiments on its cellular mechanism will be needed.

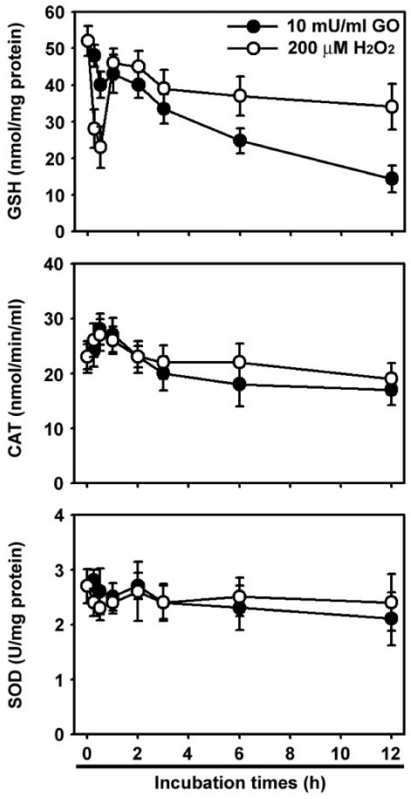

Fig. 6. Effects of H_2O_2 on intracellular antioxidant enzymes. BJAB cells were exposed to 10 mU/ml glucose oxidase or 200 μM of bolus H_2O_2 for various times (0-12 h). Enzymatic activities of intracellular reduced GSH, CAT, and SOD were determined and the results are expressed as the mean ± SD of three separate experiments.

Taken together, the sustained activation of JNK and subsequent c-Jun phosphorylation is one of the main apoptotic signaling elicited by H_2O_2 at the initial stages of exposure in both the H_2O_2-generating systems.

The most interesting area of this study was the observation showing that MAPK inhibitors and c-Jun specific transfection differently inhibited the H_2O_2-mediated cell death depending on the methods of exposure to this agent. Unlike the case where cells were exposed to a bolus H_2O_2, the cells subjected to glucose oxidase showed a temporal protection against oxidative stress after treatment with pharmacological inhibitors of JNK or c-Jun antisense transfection. With this regard, it is postulated that a different status of intracellular energy according to the H_2O_2-producing systems involves the different protection. The typical apoptosis process mediated by caspase cascades has energy-requiring steps, and thus a rapid and dramatic reduction of intracellular ATP prevents caspase activation and PARP cleavage (Cregan et al., 2004; Kang et al., 2004; Sancho et al., 2006). We previously reported that an almost complete loss of ATP occurred in cells exposed to H_2O_2 generated by glucose oxidase, not by a bolus H_2O_2, within 12 h of the exposure (Son et al., 2009). This suggests that an excessive reduction of intracellular energy in the cells exposed to glucose oxidase prevents the cascade activation of cellular death effectors, by which the MAPK inhibitors or c-Jun antisense transfection does not influence the H_2O_2-mediated cell death in the late stages of exposure.

Otherwise, intracellular GSH functions as either a substrate or a cofactor of protective enzymes and as an efficient radical scavenger (Jeong et al., 2004; Meister and Anderson, 1983). The reduced GSH level is related to the induction of apoptosis through protease activation and changes in gene expression as well as the regulation of the intracellular redox state. Significant and sustained decrease in GSH content could disrupt most cellular signaling pathways, eventually leading to pyknosis or necrosis in H_2O_2-exposed cells (Han et al., 2006). This study showed that the decrease of intracellular GSH level was apparent in the cells exposed to glucose oxidase rather than to bolus H_2O_2. In contrast, the activity of SOD and CAT was not affected either by exposing cells to H_2O_2 generated from glucose oxidase or a bolus addition. These results indicate that a temporal inhibition of H_2O_2-mediated cell death by blocking JNK- or Jun-mediated signaling is associated with the disruption of apoptosis-related signaling according to the severe and prolonged depletion of GSH. It is worthy to consider that continuously generated H_2O_2 induces mainly a caspase-independent, AIF-mediated pykonotic death without typical apoptotic characters (Son et al., 2009). Collectively, it is likely that H_2O_2 decides the mode of cell death, i.e., apoptosis, pyknosis or necrosis, by affecting cellular ATP levels as well as by changing cellular redox state of thiol-containing antioxidants. It is also important to consider that glucose oxidase produces H_2O_2 continuously at micromolar levels, whereas H_2O_2 by a bolus addition is rapidly removed (half-life about 8 min) (Barbouti et al., 2002; 2007). In addition, the continuous presence of H_2O_2 inhibited the activation of caspases in Jurkat cells exposed to the bolus H_2O_2, even when 100 ng/ml glucose oxidase (able to generate about 2.0 μM H_2O_2/min) was added into the cultures (Barbouti et al., 2007). Therefore we believe that the timing of H_2O_2 exposure than the concentration of this agent employed affected more sensitively the mode of cell death.

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