Adaptive concentrations of hydrogen peroxide suppress cell death by blocking the activation of SAPK/JNK pathway

Do Kyun Kim[1], Eun Sook Cho[1], Je Kyung Seong[2] and Hong-Duck Um[1,3,*]

[1]Laboratory of Cell Biology, Yonsei Medical Research Center, Yonsei University College of Medicine, CPO Box 8044, Seoul 120-752, Korea
[2]Department of Laboratory Animal Science, Yonsei Medical Research Center, Yonsei University College of Medicine, CPO Box 8044, Seoul 120-752, Korea
[3]Brain Korea 21 Center for Medical Sciences, Yonsei University College of Medicine, CPO Box 8044, Seoul 120-752, Korea
*Author for correspondence (e-mail: hdum@yumc.yonsei.ac.kr)

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SUMMARY

Low levels of H_2O_2 can induce cellular resistance to subsequent higher levels of H_2O_2. By using human U937 leukemia cells, it was previously shown that such an adaptive response can be induced without increasing the cellular capacity to degrade H_2O_2, thus conferring on the cells a cross-resistance to other stimuli such as serum withdrawal and C_2-ceramide. In this study, it was found that stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) acts as a common mediator of the cell death induced by high H_2O_2 concentrations, serum withdrawal and C_2-ceramide. Although SAPK/JNK activation by H_2O_2 was mediated by two upstream mitogen-activated protein kinase (MAPK) kinases MKK4 and MKK7, only MKK7 played such a role in serum withdrawal and C_2-ceramide. Interestingly, all these lethal stimuli failed to activate SAPK/JNK and its upstream kinases in the cells that were pretreated with low adaptive concentrations of H_2O_2. By contrast, the phosphorylation levels of extracellular signal-regulated kinase and p38 MAPK were not significantly influenced by this H_2O_2 pretreatment. Inducing the SAPK/JNK-suppressing effect of H_2O_2 required a time lag, which correlated with the time lag required for the induction of the adaptive response. Overall, the results suggest that H_2O_2 adaptation confers on cells a resistance to multiple stimuli by specifically blocking their ability to activate the SAPK/JNK pathways.

Key words: Adaptation, SAPK/JNK, Hydrogen peroxide, Oxidative stress, Cell death

INTRODUCTION

Although oxidative stress is generally considered to be cytotoxic, it can be beneficial to cell viability under certain circumstances. For example, when cells are first exposed to low H_2O_2 concentrations, they develop a resistance to subsequent challenges with high concentrations of the same agent that would otherwise be lethal (Farr and Kogoma, 1991; Davies et al., 1995; Wiese et al., 1995; Lee and Um, 1999). As this adaptive response requires a time lag (Wiese et al., 1995; Lee and Um, 1999), it is widely accepted that this protective effect of H_2O_2 is induced by an accumulation of H_2O_2-responsive proteins. Given that H_2O_2 can elevate the cellular levels and activities of H_2O_2-degrading enzymes such as glutathione peroxidase and catalase (Shull et al., 1991; Lu et al., 1993; Lee and Um, 1999), it is believed that cells adapt to H_2O_2, at least in part, by enhancing their capacity to degrade H_2O_2.

In recent years, evidence has been accumulating that this adaptation may not always depend on H_2O_2-degrading enzymes. It has been shown that H_2O_2 induced the synthesis of 20-25 new proteins in vascular endothelial and fibroblast cells (Lu et al., 1993; Wiese et al., 1995). Although the nature of these proteins is largely unknown, the induction of numerous proteins by H_2O_2 implies a system of H_2O_2 adaptation involving multiple mechanisms. Consistent with this possibility, H_2O_2 pretreatment was found to confer on human U937 leukemia cells a cross-resistance to other lethal stimuli such as serum withdrawal and C_2-ceramide (Lee and Um, 1999). Interestingly, it was observed that C_2-ceramide, in the absence of such pretreatment, killed U937 cells in a manner independent of H_2O_2. A lethal concentration of C_2-ceramide did not elevate the cellular levels of H_2O_2 in U937 cells, and C_2-ceramide-induced cell death was not suppressed by the addition of antioxidants (Lee and Um, 1999). Therefore, the H_2O_2-induced resistance of U937 cells to C_2-ceramide cannot be explained by the enhanced cellular capacity to degrade H_2O_2. Moreover, H_2O_2 was able to adapt U937 cells without signs of enhanced antioxidant capacity, such as an increase in protein levels and activities of the H_2O_2-degrading enzymes or an enhancement in the cellular capacity to degrade H_2O_2 (Lee and Um, 1999). A similar observation was also reported using hamster fibroblast cells (Wiese et al., 1995). Taken together, these observations suggests that H_2O_2 can impart cells with a survival advantage in a manner independent of H_2O_2-degrading activity. In the case of U937 cells, this alternative mechanism was selectively induced when the cells were

exposed to 0.05 mM H_2O_2, whereas an increase in H_2O_2-degrading activity required 0.25 mM H_2O_2 (Lee and Um, 1999). Therefore, the former mechanism appears to be more sensitive to H_2O_2 than the latter, at least in U937 cells. On the basis of these observations, it was suggested that relatively low H_2O_2 concentrations protect the cells by blocking the lethal signaling triggered by subsequent stimuli. It was the purpose of this study to investigate this hypothesis using U937 cells. A prerequisite of this investigation was to define the cell death pathways induced by H_2O_2, serum withdrawal and C_2-ceramide, because H_2O_2 adaptation controls the degree of lethality of all of these stimuli. The data presented in this report indicates that stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK), a member of the mitogen-activated protein kinase (MAPK) family, acts as a common mediator of U937 cell death induced by high H_2O_2 concentrations, serum withdrawal and C_2-ceramide. Moreover, evidence is provided showing that low H_2O_2 concentrations induce a protective response against all these lethal stimuli by specifically blocking their ability to activate SAPK and its upstream kinases. The mechanism of this interesting phenomenon is discussed.

MATERIALS AND METHODS

Materials

U937 cells were obtained from the American Type Culture Collection (Rockville, MD). All the antibodies used in this study were raised against human antigens. The anti-MKK4/SEK1 (anti-MAPK kinase 4) and anti-MKK7/SKK4 (anti-MAPK kinase 7) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and the anti-SAPK was obtained from PharMingen/Transduction Laboratories (San Diego, CA). All other antibodies were purchased from New England Biolabs (Beverly, MA). New England Biolabs and Upstate Biotechnology (Lake Placid, NY) supplied the recombinant c-Jun and SAPK proteins. The phosphorylated heat and acid-stable protein (PHAS)-1 was purchased from Stratagene (La Jolla, CA).

Cell culture, DNA transfection and treatments

U937 cells were cultured in a RPMI 1640 medium supplemented with 10% heat-inactivated FBS and gentamicin (50 μg/ml). Cells at a concentration of 3×10^5/ml were exposed to H_2O_2 (1 mM), C_2-ceramide (0.06 mM), or washed in PBS and then cultured in a serum-depleted medium. Where indicated, the cells were pretreated with 0.05 mM H_2O_2 for set periods before receiving the lethal treatments. For DNA transfection, the dominant negative mutants of MKK4 (Sanchez et al., 1994) and MKK7 (Moriguchi et al., 1997) were cloned into the pcDNA vector and delivered into the U937 cells by electroporation. The transfected cells were selected by using 1 mg/ml of G418 sulfate, after which they received the indicated treatments.

Analysis of cellular viability

The treated and untreated control cells received propidium iodide (PI) (5 μg/ml) followed by flow cytometry analysis to simultaneously monitor the PI uptake (FL-2 channel) and cell size (forward light scatter). The cells that displayed both a normal size and a low permeability to PI were understood to be viable cells, as previously defined (Mangan et al., 1991). All other populations were understood to be dead.

Western blot analysis

The cells were lysed in a solution containing 70 mM β-glycerophosphate (pH 7.2), 0.1 mM sodium orthovanadate, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 0.5% Triton X-100 and protease inhibitors (2 μg/ml aprotinin, 2 μg/ml leupeptin and 100 μg/ml PMSF). After the removal of the cell debris by centrifugation at 13,000 ***g*** for 15 minutes, equal amounts of proteins (50 μg) were separated by 12% SDS-PAGE. The proteins were then electrotransferred to Immobilon membranes (Millipore, Bedford, MA), which were subsequently blotted using the indicated antibodies and visualized by chemiluminescence (ECL; Amersham, Arlington Heights, IL).

In vitro kinase assay

The cells were lysed in a Hepes buffer (50 mM, pH 7.4) containing 100 mM NaCl, 10% glycerol, 1% NP-40, 1 mM EDTA, 20 mM β-glycerophosphate, 1 mM NaF, 1 mM *p*-nitrophenyl phosphate, 1 mM sodium orthovanadate and the protease inhibitors listed above. The lysates were clarified by centrifugation at 13,000 ***g*** for 15 minutes. Immunoprecipitation was performed by using 400 μg of the lysate proteins and the indicated antibodies. The precipitates were resolved in 20 μl of a kinase buffer containing 20 mM Hepes (pH 7.4), 10 mM $MgCl_2$, 20 mM β-glycerophosphate, 10 mM NaF, 1 mM DTT, 0.5 mM sodium orthovanadate, 50 μM ATP, and 10 μCi [γ-^{32}P]ATP. The kinase reactions were initiated by adding 2 μg of the specified substrates to the solution. After a certain incubation time, the reaction was stopped by adding a boiled sample buffer, and the proteins were then separated by 12% SDS-PAGE. The gels were dried, and a PhosphoImager using Tina 2.0 software visualized the radioactive bands.

RESULTS

Both MKK4 and MKK7 mediate the SAPK activation and cell death induced by H_2O_2

SAPK has emerged as a mediator of the cell death induced by H_2O_2 (Verheij et al., 1996) and many other cytotoxic agents (Cross et al., 2000). As with other members of the MAPK family, such as the extracellular signal-regulated kinase (ERK) and the p38 MAPK, SAPK is activated by its phosphorylation (Whitmarsh and Davis, 1996). Therefore, to confirm the ability of H_2O_2 to activate SAPK under the experimental conditions of this study, U937 cells were exposed to lethal H_2O_2 concentrations (1 mM). After various incubation times, the SAPK phosphorylation levels were monitored by western blot analysis using an antibody specific to the phosphorylated forms of SAPK. This H_2O_2 treatment resulted in an increase in the phosphorylation levels of two SAPK isoforms, p54 and p46 (Fig. 1A). This effect was most clear when the cells were exposed to H_2O_2 for 15-30 minutes. Under these conditions, the SAPK protein levels did not change significantly, suggesting that the enhanced SAPK phosphorylation levels reflected its activation. This was confirmed by an in vitro kinase assay using c-Jun as a substrate (Fig. 1B). Although it was reported that H_2O_2 can activate ERK and p38 MAPK under other experimental conditions (Guyton et al., 1996; Wesselborg et al., 1997), the phosphorylation levels of these MAPKs were not significantly enhanced after up to 30 minutes of H_2O_2 treatment (Fig. 1A). Similar results were obtained when the ERK and p38 MAPK activities were analyzed using PHAS-1 as a substrate (Wang et al., 2000; Zhang et al., 2000) (Fig. 1B). All these results suggest that 1 mM H_2O_2 specifically, or at least most efficiently, activates SAPK in U937 cells.

SAPK can be phosphorylated/activated by two upstream MAPK kinases, MKK4 (Sanchez et al., 1994) and MKK7

(Moriguchi et al., 1997; Tournier et al., 1997). To determine which one was involved in the H_2O_2-induced activation of SAPK, the activities of MKK4 and MKK7 were analyzed using SAPK as a substrate. Treatment with H_2O_2 resulted in increased activity of both MKK4 and MKK7 (Fig. 1B), which occurred more rapidly than SAPK activation, being evident as early as 5 minutes after H_2O_2 treatment. Therefore, both MKK4 and MKK7 appeared to act as mediators of the H_2O_2-induced SAPK activation. To confirm this, the U937 cells were stably transfected with a pcDNA vector containing the dominant negative mutant of either MKK4 (U937/MKK4) or MKK7 (U937/MKK7). Cells receiving the empty vector (U937/pcDNA) were used as a control. As in the case of untransfected U937 cells, 1 mM H_2O_2 efficiently activated SAPK in U937/pcDNA cells (Fig. 1C). However, this activation was greatly reduced in both U937/MKK4 and U937/MKK7 cells. Therefore, it was clear that both MKK4 and MKK7 mediate H_2O_2-induced SAPK activation.

The expression of MKK4 mutants has been reported to suppress H_2O_2-induced death in U937 cells (Verheij et al., 1996). To investigate whether this can also be achieved using the MKK7 mutant, the transfectants were exposed to various H_2O_2 concentrations, and their viability was compared using flow cytometry. Unlike the untransfected U937 cells that were marginally susceptible to 0.1 mM H_2O_2 (Lee and Um, 1999), the exposure of U937/pcDNA cells to the same H_2O_2 concentration for 48 hours resulted in a cell death of more than 60% (Fig. 1D). Therefore, the susceptibility of U937 cells to H_2O_2 appeared to be enhanced during the transfection procedure, as reported previously (Kim et al., 2001). Increasing the concentration of H_2O_2 to 0.25 mM killed almost all of the U937/pcDNA cells. However, the cell death was dramatically reduced when the U937/MKK7 cells were exposed to the same H_2O_2 concentrations. A direct comparison of U937/MKK7 and U937/MKK4 cells revealed that the MKK7 mutant was slightly more protective than the MKK4 mutant. These results suggested that both MKK4 and MKK7 act as major mediators of H_2O_2-induced death in U937 cells.

The MKK7, but not MKK4, pathway is shared by serum withdrawal and C_2-ceramide

The role of the SAPK pathways in the cell death induced by serum withdrawal and C_2-ceramide was investigated. Both of these stimuli activated SAPK and MKK7 (Fig. 2A), but interestingly MKK4 was not stimulated (Fig. 2B). Activation of SAPK, induced by either serum withdrawal or C_2-ceramide, was consistently reduced only by mutant MKK7 expression (Fig. 2A), but not by mutant MKK4 (Fig. 2B). Moreover, although the MKK7 mutant efficiently reduced the cell death induced by serum withdrawal and C_2-ceramide, such protection was not detected using the MKK4 mutant (Fig. 2C). This finding contrasts with a previous report showing that the expression of MKK4 mutant rescued U937 cells from C_2-ceramide (Verheij et al., 1996). Although the reason for this discrepancy is not clear, our data suggests that serum withdrawal and C_2-ceramide selectively utilize MKK7 to activate SAPK and kill U937 cells, at least under the experimental conditions of this study. Considering these results and the above findings together, the route leading to SAPK activation appears to vary in a single cell type depending on the types of stimuli. This has also been reported in other studies (Moriguchi et al., 1997).

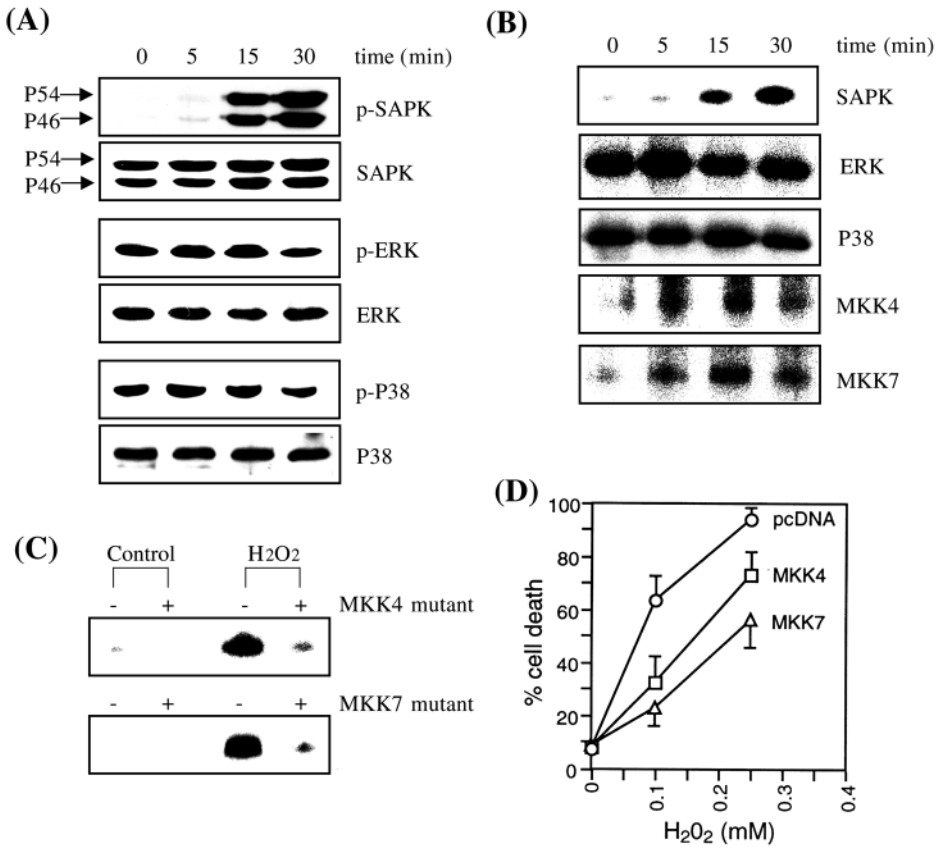

Fig. 1. Both MKK4 and MKK7 mediate H_2O_2-induced SAPK activation and cell death. (A) U937 cells were exposed to 1 mM H_2O_2 for the indicated periods. The cells were lysed, and the SAPK, ERK and p38 MAPK phosphorylation levels were analyzed by western blotting using antibodies specific to the phosphorylated forms of each MAPK. The protein levels of these MAPKs were also probed using their specific antibodies. (B) The SAPK, ERK, p38 MAPK, MKK4 and MKK7 activities in the lysates were analyzed by in vitro kinase assay. The substrates were recombinant c-Jun protein for SAPK, PHAS-1 for ERK and p38 MAPK, and SAPK protein for MKK4 and MKK7. (C) The U937 cells were stably transfected with the pcDNA vector containing the dominant negative mutant of either MKK4 or MKK7. These transfectants and the cells that received the empty control vector were exposed to 1 mM H_2O_2 for 30 minutes. Levels of SAPK activity in the treated and untreated control cells were compared by in vitro kinase assay. (D) The transfectants were treated with the indicated H_2O_2 concentrations for 48 hours and the cellular viability was analyzed by flow cytometry.

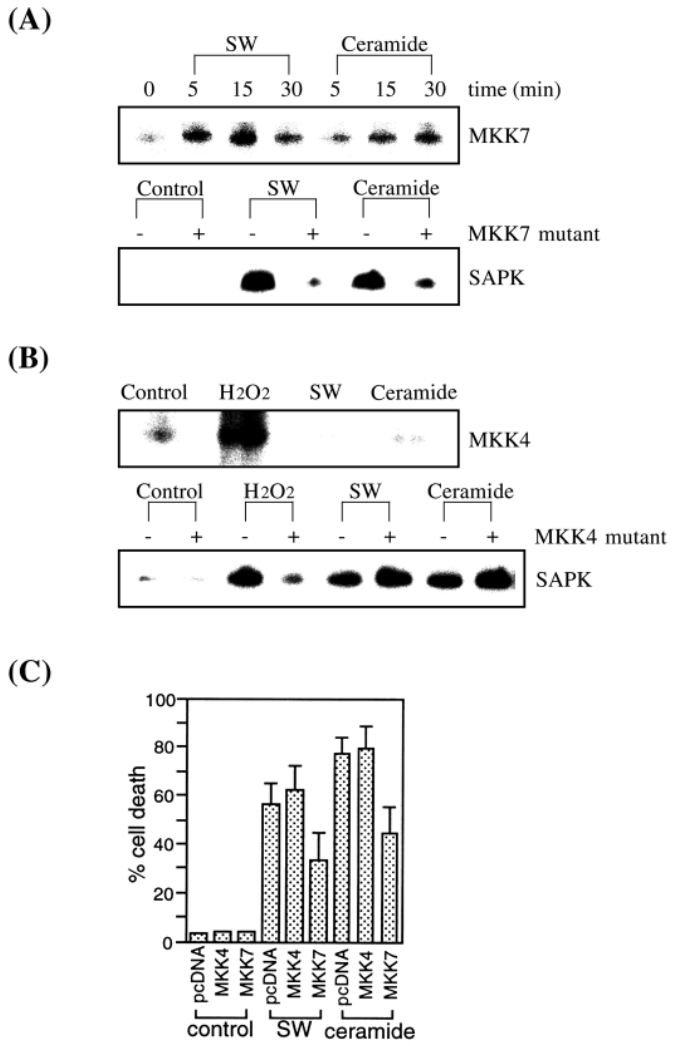

Fig. 2. MKK7, but not MKK4, is involved in the SAPK activation and cell death induced by serum withdrawal (SW) and C_2-ceramide. (A) The U937 cells were exposed to a serum-depleted medium or 0.06 mM C_2-ceramide. At the end of the indicated incubation periods, the MKK7 activity was analyzed (top). U937/pcDNA and U937/MKK7 cells received the same treatments for 30 minutes. SAPK activities in the treated and untreated cells were compared (bottom). (B) U937 cells were treated with the indicated stimuli for 15 minutes, and MKK4 activity was measured (top). U937/pcDNA and U937/MKK4 cells received the indicated treatments for 30 minutes, and the SAPK activity was compared (bottom). (C) The U937/pcDNA, U937/MKK4 and U937/MKK7 cells were treated with either the serum-depleted medium or C_2-ceramide for 48 hours. The viability of the treated and untreated cells was compared by flow cytometry.

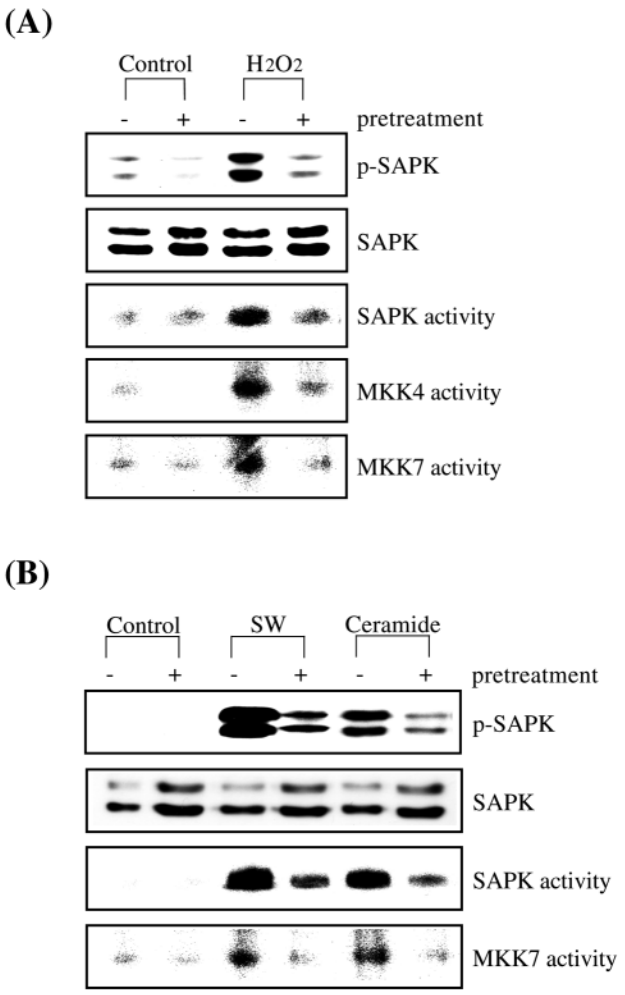

Fig. 3. H_2O_2 pretreatment reduces the activation of both the MKK4 and MKK7 pathways induced by diverse stimuli. (A) U937 cells were incubated in the presence or absence of 0.05 mM H_2O_2 for 24 hours, followed by a challenge with 1 mM H_2O_2 for 30 minutes. The SAPK phosphorylation and protein levels (top two panels) and its activity (third panel) were compared. Alternatively, the activities of MKK4 and MKK7 were analyzed 15 minutes after the challenge (bottom two panels). (B) The preincubated cells received the indicated challenges for 30 minutes. The SAPK phosphorylation and protein levels (top two panels), and the SAPK and MKK7 activities (bottom two panels) were analyzed.

H_2O_2 pretreatment blocks the activation of SAPK pathway induced by diverse stimuli

Because the SAPK pathway was shown to be crucial for the lethal actions of H_2O_2, serum withdrawal, and C_2-ceramide, it was of interest to investigate whether H_2O_2 pretreatment protects the cells from those stimuli by interfering with their ability to activate the SAPK pathway. To address this question, U937 cells were incubated in the presence or absence of 0.05 mM H_2O_2 for 24 hours, an optimal condition for inducing the antioxidant-independent adaptation (Lee and Um, 1999). The pretreated and untreated control cells were then challenged with 1 mM H_2O_2 for 30 minutes, and analyzed for the levels of SAPK phopshorylation/activity. Pretreatment with H_2O_2 itself did not significantly influence the levels of SAPK phopshorylation/activity as analyzed from 5 minutes (data not shown) up to 24 hours. However, it did dramatically reduce the challenge-induced enhancement of SAPK phosphorylation/activity (Fig. 3A). By contrast, H_2O_2 pretreatment did not significantly influence phosphorylation levels of ERK and p38 MAPK, regardless of the challenge (data not shown). This data suggested that H_2O_2-induced SAPK activation was specifically downregulated in the cells that experienced sublethal concentrations of H_2O_2. To determine whether the pretreated cells also have a defect in MKK4 and MKK7 activation, the ability of H_2O_2 to activate MKK4 and MKK7 in the pretreated

and untreated cells was compared. As shown in Fig. 3A, the H_2O_2-induced activation of both MKK4 and MKK7 was dramatically reduced by H_2O_2 pretreatment. Therefore, the cells that have previously experienced low concentrations of H_2O_2 lose their ability to efficiently activate the MKK4 and MKK7 pathways in response to subsequent high H_2O_2 concentrations.

To investigate whether H_2O_2 pretreatment similarly influences the SAPK activation induced by other stimuli, the pretreated and untreated cells received a challenge with either serum withdrawal or C_2-ceramide. As shown in Fig. 3B, H_2O_2 pretreatment efficiently reduced the enhancement of SAPK phosphorylation/activity induced by both serum withdrawal and C_2-ceramide. Moreover, the ability of these stimuli to activate MKK7 was also attenuated by pretreatment. These findings indicate that H_2O_2 pretreatment interferes with the SAPK pathway activation induced by diverse stimuli.

Time course of the inhibitory effect

H_2O_2 requires a time lag to induce cellular resistance to death stimuli (Wiese et al., 1995; Lee and Um, 1999). In the case of U937 cells, pretreatment with 0.05 mM H_2O_2 for 4 hours was not sufficient for inducing such resistance. This resistance was initially detected 8 hours after the pretreatment, and was further enhanced as the pretreatment was extended up to 24 hours (Lee and Um, 1999). To investigate whether a similar time lag was also required to inhibit SAPK activation, U937 cells were pre-exposed to 0.05 mM H_2O_2 for various time periods, challenged with 1 mM H_2O_2 for 15 minutes and then analyzed for MKK4 and MKK7 activity. Alternatively, SAPK activity was analyzed 30 minutes after the challenge. Pretreatment itself did not significantly influence the activities of all those kinases under any of the tested conditions. Interestingly, the challenge-induced activation of MKK4, MKK7 and SAPK were not significantly suppressed in the cells that had been pretreated for 4 hours (Fig. 4). Pretreatment for 8 hours resulted in a dramatic suppression of MKK7, but not MKK4, activation. Therefore, the MKK4 and MKK7 pathways appear to be differentially regulated by H_2O_2 pretreatment. Pretreatment for 8 hours also displayed some suppressive effect on SAPK activation, suggesting that the selective MKK7 suppression can influence SAPK activation. When the cells were pretreated for 24 hours, the challenge-induced activation of all of MKK4, MKK7, and SAPK were almost completely blocked. These results revealed that the time course required for the inhibition of SAPK activation co-relates exceedingly well with that observed for the induction of cellular resistance to H_2O_2. This strongly supports the suggestion that the suppression of SAPK activation is a mechanism whereby H_2O_2 induces this protection.

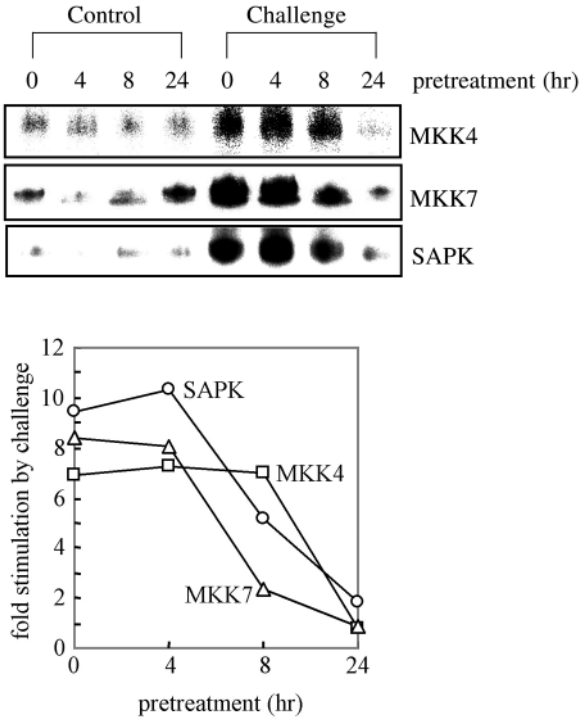

Fig. 4. H_2O_2 requires a time lag to induce the SAPK-suppressing effect. The U937 cells were pre-exposed to 0.05 mM H_2O_2 for the indicated periods, followed by a challenge with 1 mM H_2O_2 either for 15 minutes for the analysis of the MKK4 and MKK7 activities, or for 30 minutes for SAPK (top). The intensity of each band was quantified by using Tina 2.0 software. The challenge-induced fold stimulation of each kinase was determined over its basal level obtained from the cells that did not receive H_2O_2 in the whole incubation (bottom). The values are the mean of three separate experiments. The standard deviations were routinely less than 15% of the means. Circle, SAPK; square, MKK4; triangle, MKK7.

DISCUSSION

This study showed that H_2O_2 can induce an adaptive response by specifically suppressing the activation of SAPK and its upstream kinases. As H_2O_2 also exhibits such an effect against serum withdrawal and C_2-ceramide, the suppression of the SAPK pathways appears to be a mechanism whereby H_2O_2-adapted cells withstand diverse stimuli. Given that the H_2O_2 concentration employed for the pretreatment does not enhance cellular antioxidant capacity (Lee and Um, 1999), the suppression of the SAPK pathways is believed to be due to a blockage of the pathways rather than a degradation of the challenged H_2O_2. This belief is further supported by the suppression of SAPK pathways induced by C_2-ceramide, which acts on U937 cells in a H_2O_2-independent manner (Lee and Um, 1999). Although cellular adaptation to oxidative stress has long been observed, to our knowledge this is the first case demonstrating that the response can modulate a specific signaling pathway. Our findings also suggest that oxidative stress can regulate the SAPK pathway in two opposing manners depending on its intensity. Although relatively high-intensity stress rapidly activates the pathway, lower-intensity stress induces a blockage of the pathway. This latter mechanism may have applications in certain types of cancer cells that constitutively generate reactive oxygen intermediates to levels higher than their normal counterparts, and display a resistance to oxidative cytolysis and chemotherapy (O'Donnell-Tormey et al., 1985; Yagoda, 1989; Toyokuni, 1995).

The time lag required for inducing the SAPK-suppressing response implies that H_2O_2 induces macromolecules that can inhibit the activation of the SAPK pathways. Given that the MKK4- and MKK7-suppressing responses are induced by

different kinetics, multiple factors may be involved in suppressing the SAPK pathways. Although the identity of such factors is currently unclear, several anti-death proteins such as Bcl-2, Bcl-X_L and heat-shock proteins have been eliminated as potential candidates (Lee and Um, 1999). Because SAPK is activated by its phosphorylation, we have investigated the possibility that H_2O_2 pretreatment (0.05 mM) induces phosphatases such as MKP-1 and MKP-2 that can dephosphorylate SAPK (Chu et al., 1996; Sanchez-Perez et al., 2000). However, no evidence from western blot analysis was found (data not shown). Although it has been reported that the overexpression of $p21^{WAF1/CIP1/Sdi1}$, an inhibitor of cyclin-dependent kinases, can attenuate UV-induced SAPK activation (Shim et al., 1996), the adaptive concentrations (0.05 mM) of H_2O_2 in this study did not significantly alter the cellular levels of p21 (data not shown). Exposure of the U937 cells to 0.05 mM H_2O_2 consistently showed no significant influence on their cycling distribution. Therefore, the adaptation induced by 0.05 mM H_2O_2 does not appear to depend on the p21 level or the cellular cycling status. The authors have recently reported that nuclear factor κB (NF-κB) is a mediator of the U937 cell adaptation induced by 0.05 mM H_2O_2 (Kim et al., 2001). However, the promotion of H_2O_2-induced NF-κB activation by the overexpression of I-κB kinase α (IKKα) (Kim et al., 2001) did not enhance the SAPK-suppressing activity of H_2O_2 (data not shown). This observation suggests that NF-κB is not involved in the SAPK-suppressing pathway induced by H_2O_2. Therefore, the antioxidant-independent adaptation appears to be induced by at least two different mechanisms: one mediated by NF-κB and the other that suppresses SAPK activation. The authors are currently attempting to establish experimental conditions for proteomics to enable them to identify a cellular factor involved in these phenomena.

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