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MAPK Activation and Cell Viability after H_2O_2 Stimulation in Cultured Feline Ileal Smooth Muscle Cells

Hyun Ju Song, Ji Hoon Jeong, Dong Kyu Lee, Tai Sang Lee, Young Sil Min, and Uy Dong Sohn

Department of Pharmacology, College of Pharmacy, Chung Ang University, Seoul 156–756, Korea

Recent data have shown the importance of oxidative stresses in the pathogenesis of inflammatory bowel disease, crohn's disease and ulcerative colitis. H_2O_2, reactive oxygen species (ROS) donor, has been reported to act as a signaling molecule involved in a variety of cellular functions such as apoptosis and proliferation. In the present study, we investigated viability of cultured ileal smooth muscle cells (ISMC) after stimulation with H_2O_2. Trypan blue method revealed that the cell viability of ISMC treated with 1 mM H_2O_2 was not different from that of controls at up to 2 h time point, while treatment of ISMC with 1 mM H_2O_2 for 48 h finally induced significant decrease in the cell viability. Therefore, we evaluated whether H_2O_2 was capable of ERKs activation in ISMC for the short-term exposure and examined whether tyrosine kinase was involved in the process of ERK activation by H_2O_2 in ISMC. We also investigated the effects of H_2O_2 on activation of SAPK/JNK and p38 MAP kinase in ISMC. Thus, ISMC were cultured and exposed to H_2O_2, and western blot analysis was performed with phospho-specific MAP kinase antibodies. Robust activation of ERK occurred within 30 min of 1 mM H_2O_2 treatment. H_2O_2-induced ERK activation was attenuated by a tyrosine kinase inhibitor, genistein, indicating that tyrosine kinase was probably involved in the ERK activation by H_2O_2. H_2O_2 was a moderate activator of SAPK/JNK, while p38 MAP kinase was not activated by H_2O_2. We suggest that ERK activation induced by short-term H_2O_2 treatment plays a critical role in cellular protection in the early stage of response to oxidative stress. The present study suggests the necessity of identification of MAPK signaling pathways affected by ROS, since it could ultimately elucidate cellular consequences involved in initiation and perpetuation of intestinal tissue damage in the diseases such as crohn's disease and ulcerative colitis, resulted from excessive ROS.

Key Words: H_2O_2, ERKs, SAPK/JNK, Cell viability, Ileal smooth muscle cells

INTRODUCTION

Intestinal inflammation is accompanied by excessive production of reactive oxygen species (ROS) such as H_2O_2, $O_2^{\cdot -}$, $OH^{\cdot}$ and singlet oxygen in inflammatory bowel disease, crohn's disease and ulcerative colitis (Rhee, 1999; Kruidenier et al, 2003a). Although the intestinal mucosa contains an extensive system to eliminate ROS, the levels and the balance between the most important antioxidants are seriously impaired within the intestinal mucosa in inflammatory bowel disease (Kruidenier & Verspaget, 1998; Kruidenier et al, 2003b). In addition, chronic exposure to ROS is thought to play a important role in the etiology of numerous disease processes including gastrointestinal tract disorders, cancer and degenerative alterations that occur with aging (Guyton & Kensler, 1993; Kruidenier & Verspaget, 2002).

H_2O_2 is a small, diffusible, and ubiquitous molecule that can rapidly be synthesized destroyed, in response to external stimuli such as irradiations (X-rays, UV), environmental pollutants, or inflammatory systems. Even in the absence of extracellular stimulation, H_2O_2 is generated by superoxide dismutase from superoxide anions ($O2^{\cdot -}$), which is constantly produced by metabolic reactions in aerobic organisms. Due to its relentless production with the nature to indiscriminately damage many cellular components, H_2O_2 is generally considered to be a cytotoxic byproduct.

However, it has been reported that H_2O_2 stimulates multiple signaling pathways related to smooth muscle contraction (Jin & Rhoades, 1997; Sotnikova, 1998), cell growth/proliferation and DNA synthesis in different types of cells (Rao & Berk, 1992; Baas & Berk, 1995; Fiorani et al, 1995), as well as apoptosis (Li et al, 1997), indicating that H_2O_2 acts as an important signaling molecule. In addition, it has been reported that intracellularly generated H_2O_2 produces its effects through the activation of tyrosine kinase or MAP kinase in several types of cells (Sundaresan et al, 1995; Guyton et al, 1996). Three major MAP kinase signaling cascades have also been evaluated in pancreatic acinar cells in response to H_2O_2 and other physiologic stimuli (Dabrowski et al, 2000).

Corresponding to: Uy Dong Sohn, Department of Pharmacology, College of Pharmacy, Chung Ang University, Seoul 156-756, Republic of Korea. (Tel) 02-820-5614, (Fax) 02-826-8752, (E-mail) udsohn@cau.ac.kr

ABBREVIATIONS: ERK, extracellular signal-regulated protein kinases; FBS, Fetal bovine serum; H_2O_2, Hydrogen peroxide; ISMC, Ileal Smooth Muscle Cells; MAPK, Mitogen-activated protein kinase; ROS, Reactive Oxygen Species; SAPK/JNK, stress-activated protein kinase/Jun-N-terminal kinase.

In the present study, we firstly undertook a comparative analysis of cell viability after stimulation with H_2O_2 in cultured ileal smooth muscle cells (ISMC). The cell viability assay showed no difference between H_2O_2 -treated cells and the control cells during the short period of time, while long-term exposure to H_2O_2 led to cell death. Therefore, we evaluated whether H_2O_2 was capable of ERKs activation in ISMC, and also examined the possibility that the observed increase in ERK activity after H_2O_2 exposure might be mediated by activation of tyrosine kinases. We also investigated the effects of H_2O_2 on activation of SAPK/JNK and p38 MAP kinase in ISMC, since SAPK/JNK and p38 MAP kinase, two other subtypes of MAP kinases, mediate signals in response to stress and cytokines, respectively. Our data showed that H_2O_2 activated SAPK/JNK in a concentration-dependent manner. However, p38 MAP kinase was not activated by H_2O_2. The findings suggest that short-term oxidative stress induced by H_2O_2 activates the signal transduction of cell mitogenic effects, which are thought to be a protective response against oxidant injury in ISMC, as well as mediates the signals involved in cellular response to stress.

METHODS

Cultures of ileal smooth muscle cell

Adult cats of either sex, weighing between 2.0 and 3.0 kg, were anesthetized with ketamine (50 mg/ml/kg), and the abdomen was then opened with a midline incision. The ileum was excised and cleaned of fat tissue. Three-centimeter ileal segments were slipped over a glass rod, and the serosa and the longitudinal muscle layer were separated from the circular muscle layer by tangential stroking of the mesenteric attachment as described by Bitar et al (Bitar & Makhlouf, 1982) The ileal segments were then opened and cut into small pieces. The circular muscle layer was sliced off into 0.5 mm thick with a Stadie Riggs tissue slicer (Thomas Scientific Apparatus, Philadelphia, PA, the last slices containing mucosa and submucosa were discarded. The sliced tissue was then placed into DMEM supplemented with 50% FBS containing 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 μg/ml amphotericin B and incubated in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. On the following day, fresh DMEM containing 10% FBS was added. Ten days later, the tissue explants were removed, and the medium was changed with fresh DMEM containing 10% FBS. After reaching confluence, cells were detached with 1% trypsin in HBSS with bicarbonate. Cells were then counted, seeded at 1×10^6 cells/ml on 100-mm culture dishes, and maintained in DMEM containing 10% FBS. The medium was changed every 48 h until the cells reached confluence. Experiments were performed on cells of passage 4.

Cell viability assays

Trypan blue exclusion was used as a marker of cell viability. Under the same experimental conditions, the growth-arrested cells were treated with 1 mM H_2O_2 for the indicated time periods. At each time after incubation, cells were washed with HBSS and detached with 1% trypsin in PBS. Cells were stained with trypan blue solution and then counted by using phase contrast microscope (model ULWSD 0.30 Olympus, Japan), haemacytometer, and a counter. This experiment had to be performed within 5 min after staining not to damage cells.

Stimulation of MAP kinases

When the cells reached confluence, they were serum starved by incubation in DMEM containing 0.1% FBS for 48 h to arrest cell growth and silence gene activity. The growth-arrested cells were then stimulated either with H_2O_2 for indicated time periods or at indicated concentrations. In addition, cells were stimulated with 1 mM H_2O_2 for 30 min in the presence or absence of different inhibitors. After incubation, the cells were washed twice with ice-cold PBS and harvested in 5 mM EDTA-PBS. The harvested cells were then maintained on ice for 10 min and centrifuged for at 1300 rpm 5 min. The pellets were washed with ice-cold PBS and recentrifuged. The obtained pellets were homogenized in a homogenizing buffer, composed of 20 mM Tris (hydroxymethyl) aminomethane, 0.5 mM EDTA, 0.5 mM EGTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 mM β-mercaptoethanol (pH 7.5). The homogenates were then sonicated (3s, 5x) and centrifuged at 12500 rpm for 7 min at 4°C to remove cellular debris, and the supernatants were collected.

Protein determination

The protein concentration of the supernatant in each reaction vial was spectrophotometrically measured, using the Bio-Rad assay (Bio-Rad Chemical Devision, Richmond, California). Absorption was monitored at 595 nm.

Western blot analysis of MAP kinase activation

Equal amounts of protein from each sample were resolved on a 10% SDS-polyacrylamide gel by electrophoresis. Rainbow prestained molecular mass markers (Amersham, Arlington Heights, IL) were also run in an adjacent lane to permit molecular mass determination. The separated proteins were transferred to 0.45 μm nitrocellulose membrane in transfer buffer containing 25 mM Tris (pH 8.3), 192 mM glycine, and 20% (v/v) methanol, using power supply [Power Pac 1000 (Bio-Rad, Melville, NY)] the membranes were incubated in a PBS buffer containing 5% non-fat dry milk for 1 h at room temperature to block nonspecific binding. After washing three times in PBS, the blots were incubated with 1 : 1000 phospho-specific p44/p42 MAP kinase (Tyr-202/Tyr-204) antibody, phospho- SAPK/JNK antibody, or p38 MAP kinase antibody (purchased from Cell Signaling) in a PBS solution containing 0.1% BSA at 4°C overnight. The membranes were washed by PBS containing 0.05% tween 20 and then incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL; PerkinElmer Life Sciences, Boston, MA) (Sohn et al, 1993). The same blot was subsequently stripped and reproved with p44/42 MAP kinase antibody, SAPK/JNK antibody, or p38 MAP kinase antibody. Developed films from ECL were scanned and analyzed densitometrically by using Scion Image. Fold inductions in MAP kinases were calculated as the ratios of phosphorylated MAP kinases to total MAP kinases. Most of other reagents were purchased from Sigma Chemical Co. (St Louis, MO).

Data analysis

The data are expressed as means±SEM of n separate experiments, and the statistical differences between means were determined by Student's t test, with $P<0.05$ considered significant.

RESULTS

Cell viability assay

The importance of oxidative stresses in the pathogenesis of inflammatory bowel disease, crohn's disease and ulcerative colitis has been reported (Guyton & Kensler, 1993; Kruidenier & Verspaget, 2002). In the present study, we investigated the viability of cultured ISMC treated with 1 mM H_2O_2, using trypan blue exclusion as a marker of cell viability (Fig. 1).

Cells of passage 3 were seeded at a density of 1×10^6/100-mm culture dishes. The cells were maintained in 10% FBS containing DMEM, and the medium was changed every 48 h. After reaching confluence, cells in half of the culture dishes were growth-arrested with DMEM supplemented with 0.1% FBS for 48 h. The plates were then treated with 1 mM H_2O_2 for the indicated time periods, while the controls were not treated with any stimulant. At each time after incubation, cells were washed with HBSS and detached with 1% trypsin in PBS. Cells were stained with trypan blue solution and then counted by using haemacytometer, phase contrast microscope and a counter. This experiment had to be performed within 5 min after staining, so that not to damage the cells.

The other cells were growth-continued with DMEM supplemented with 10% FBS for 48 h in order to be compared, after reaching confluence with cells of passage 4. The plates were then treated with 1 mM H_2O_2 for the indicated time periods, while the controls were not treated with any stimulant. At each time after incubation, cells were counted by the same process as mentioned above.

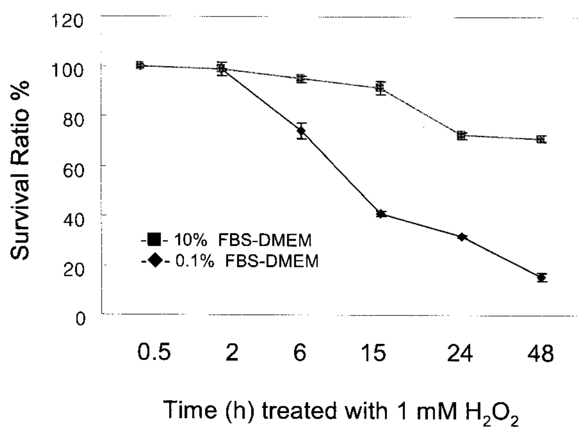

Fig. 1. Cell viability assay. Cell viability was expressed as ratios of the survival rates of H_2O_2-stimulated cells to that of control cells in 0.1% FBS-DMEM or in 10% FBS-DMEM. Each value is mean ±SEM of four individual experiments performed in duplicate, respectively, for the indicated time periods, using trypan blue exclusion methods.

Each survival rate at each time was calculated for total number of cells incubated in 10% FBS-DMEM or 0.1% FBS-DMEM. The cell viability was then expressed as ratios of the survival rates of H_2O_2-stimulated cells to that of control cells in 0.1% FBS-DMEM or in 10% FBS-DMEM.

There was no difference in trypan blue exclusion between the control cells (100±0%) and cells treated with 1 mM H_2O_2 for 120 min (98.8±1.2%, n=4). In addition, under the conditions, total numbers of the control and H_2O_2-treated cells were not different ($25.2\pm1.9\times10^5$ versus $24.9\pm0.4\times10^5$ cells, n=4 respectively). However, the treatment of ISMC with 1 mM H_2O_2 for 48 h finally induced significant decrease in the cell viability. The difference between the survival ratios of cells in 10% FBS-DMEM and in 0.1% FBS-DMEM can be explained by the fact that various growth factors and antioxidant enzymes in 10% FBS may play a critical role in cell survival following cell damage induced by H_2O_2.

H_2O_2 activates ERKs in ISMC in a concentration- and a time- dependent manner

Whether H_2O_2 was capable of ERKs activation in ISMC for the short-term exposure was evaluated, since as shown in cell viability assay of Fig. 1, there was no harmful effect on cells up to 2 h time point. Firstly, the growth-arrested ISMC were exposed to 10^{-6}, 10^{-5}, 10^{-4} and 10^{-3} M H_2O_2 for 30 min. As seen in Fig. 2A, western blot analysis showed a concentration-dependent increase of the activated forms of both ERK_1 and ERK_2. Those two distinct bands were identified with the phospho-specific MAP kinase antibody,

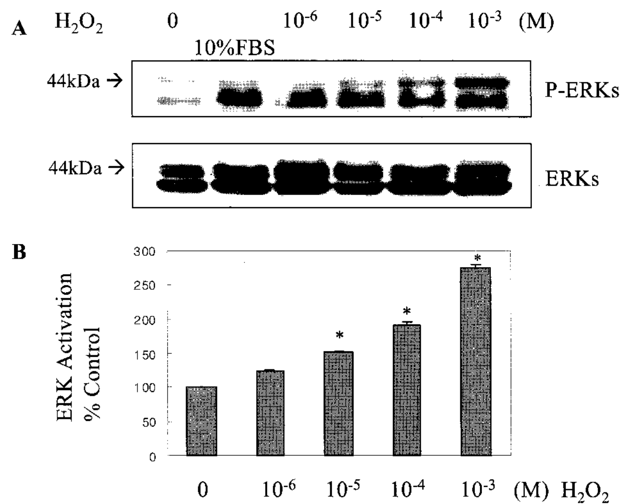

Fig. 2. H_2O_2 activates ERKs in a concentration- dependent manner in ISMC. (A) Western blot analysis, showing phosphorylated 44-kDa and 42-kDa ERKs detected with anti-phospho-p44/42 MAP kinase antibody. Growth-arrested ISMC were stimulated with H_2O_2 at indicated concentrations or with 10% FBS-DMEM for 30 min. The same blot was stripped and probed with anti-p44/42 MAP kinase antibody to show equal loading of proteins. (B) The integrated density of both bands (p44 and p42 MAP kinases) was quantified and shown in the diagram, indicating that H_2O_2 activates ERKs in a concentration- dependent manner. Each value is mean±SEM of three individual experiments performed in duplicate. *$P<0.05$ compared with controls.

since the increase in density of protein at positions corresponding to 42 kDa and 44 kDa indicated increased phosphorylation of ERKs at their tyrosine residues, therefore, their activation. Under the same experimental conditions, 10% FBS-DMEM activated ERKs by 2.5-fold over basal levels, while exposure of ISMC to 10^{-3} M H_2O_2 for 30 min significantly increased ERKs phosphorylation by 2.8 ± 0.1 times over the level of control samples, which consisted of ISMC incubated without any stimulant (Fig. 2B).

Fig. 3 presents the time-course of H_2O_2-induced ERK activation in ISMC. Incubation of ISMC with 1 mM H_2O_2 caused a time-dependent increase in the activation of ERKs: ERKs phosphorylation rapidly reached the maximal levels at 30 min, showing only slight activation at 15 min, and then slowly declined.

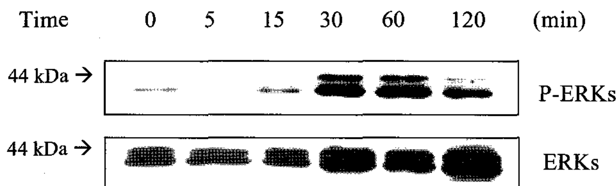

Fig. 3. Time course of H_2O_2-induced ERK activation. Growth-arrested ISMC were incubated with 10^{-3} M H_2O_2 for the indicated time periods. Western blot analysis shows that treatment of ISMC with H_2O_2 activated ERKs in a time-dependent manner.

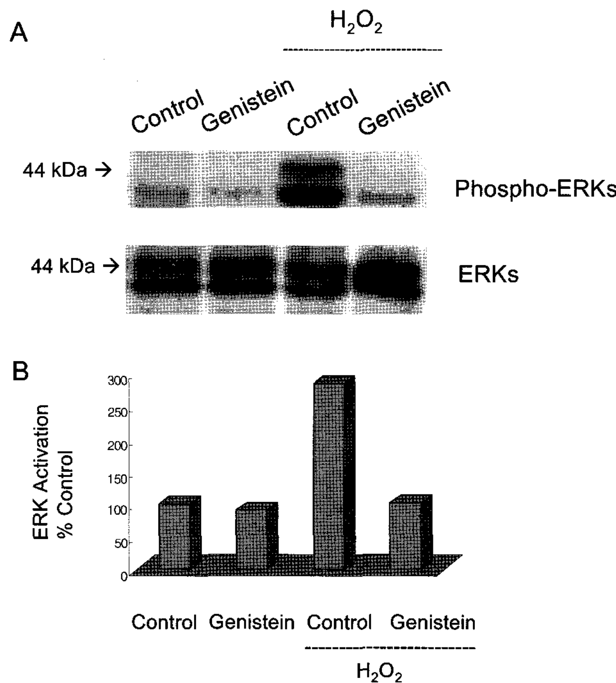

Fig. 4. Effects of the tyrosine kinase inhibitor on H_2O_2-induced ERK activation. (A) Growth-arrested ISMC were pretreated with 90 μM genistein for 45 min prior to 1 mM H_2O_2 stimulation for 30 min. Genistein pretreatment significantly reduced ERK activation by H_2O_2. The same blot was stripped and probed with anti-p44/42 MAP kinase antibody to show equal loading of proteins. (B) The integrated density of both bands (p44 and p42 MAP kinases), corresponding to active MAP kinase, was quantified and shown in the diagram.

Tyrosine kinase may be involved in H_2O_2-induced ERK activation in ISMC

Fig. 4 shows the effect of tyrosine kinase inhibitor, genistein, on H_2O_2-induced ERK activation. We examined the possibility that the observed increase in ERK activation after treatment with H_2O_2 might be mediated by H_2O_2-induced activation of tyrosine kinases, since several studies have shown that H_2O_2 causes activation of tyrosine kinases and regulates protein tyrosine phosphorylation (Abe et al, 1994; Whisler et al, 1995; Zhang et al, 1998). The concentration of H_2O_2 and time of treatment for ISMC stimulation were chosen, based on the concentration-response and time-course data, shown in Fig. 2 and 3 as well as data obtained by others with different types of cell cultures (Abe et al, 1996; Guyton et al, 1996; Kamata & Hirata, 1999).

To test the involvement of tyrosine kinase, we performed experiments both in the presence and absence of tyrosine-kinase inhibitor genistein. Pretreatment of ISMC with 90 μM genistein for 45 min prior to 1 mM H_2O_2 stimulation for 30 min resulted in a significant reduction in H_2O_2-induced ERK activation (Fig. 4), indicating that tyrosine kinases may be involved in the ERK activation by H_2O_2.

H_2O_2 activates SAPK/JNK in a concentration-dependent manner

The ability of H_2O_2 to activate SAPK/JNK was also evaluated in cultured ISMC, since SAPK/JNK mediates

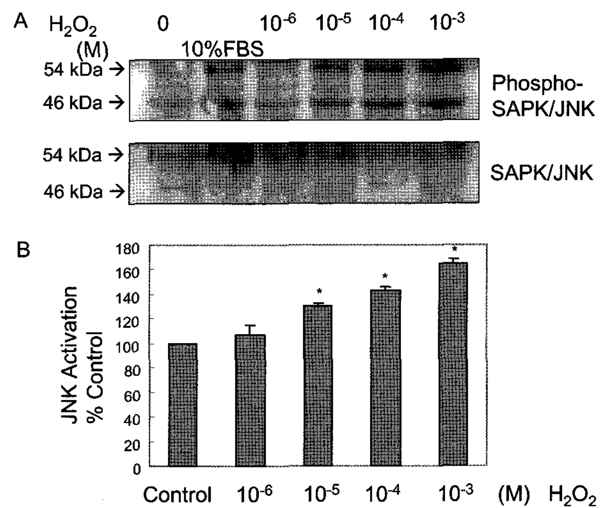

Fig. 5. H_2O_2 activates SAPK/JNK in a concentration-dependent manner. (A) Growth-arrested ISMC were stimulated with H_2O_2 at indicated concentrations or with 10% FBS-DMEM for 30 min. Western blot analysis shows phosphorylated 54-kDa SAPK and 46-kDa JNK detected with anti-phospho-SAPK/JNK antibody. The same blot was probed separately with anti-SAPK/JNK antibody. (B) The density of active-JNK band was quantified and shown in the diagram. Each value is mean±SEM of two individual experiments performed in duplicate. $^*P < 0.05\%$ compared with controls.

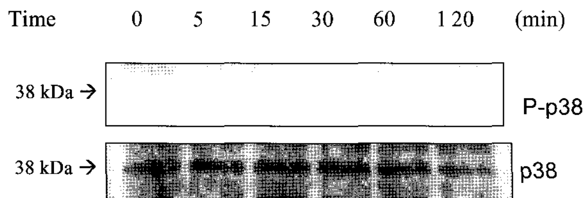

Fig. 6. Effect of H_2O_2 on p38 MAP kinase activation. Western blot analysis shows that phosphorylated p38 MAP kinase was not detected with anti-phospho p38 MAP kinase antibody. Growth-arrested cells were incubated with 10^{-3} M H_2O_2 for the indicated time periods. The immunoblots shown are representative of four independent experiments.

signals in response to stress. Cells treated with 10^{-6}, 10^{-5}, 10^{-4} and 10^{-3} M H_2O_2 for 30 min showed a concentration-dependent increase in the activated forms of both SAPK and JNK, which were detected with phospho-specific SAPK/JNK antibody (Fig. 5A). Under the same experimental conditions, 10% FBS stimulated JNK phosphorylation by 1.6-fold over basal levels. Incubation of ISMC with H_2O_2 caused a moderate increase in the density of two bands of 54 kDa and 46 kDa, which correspond to SAPK and JNK, respectively. Exposure of cells to 10^{-3} M H_2O_2 for 30 min increased JNK phosphorylation by 1.6 ± 0.1 times over the level of control (Fig. 5B).

Effect of H_2O_2 on p38 MAP kinase in ISMC

Recent data have shown that intestinal inflammation was accompanied by excessive production of ROS in inflammatory bowel disease (Rhee, 1999; Kruidenier et al, 2003a), and that p38 MAP kinase was activated in pancreatic acini by H_2O_2 and menadione (Dabrowski et al, 2000). Therefore, the effect of H_2O_2 on p38 MAP kinase was also examined in cultured ISMC. In four independent experiments, however, there was no significant change observed in p38 MAP kinase activation by 1 mM H_2O_2 during the time course studied, as detected with phospho-specific p38 MAP kinase antibody (Fig. 6).

DISCUSSION

In the present study, cell viability of the ISMC treated with 1 mM H_2O_2, examined by using trypan blue methods, showed no difference from that of controls up to 2h time point, however, treatment of ISMC with 1 mM H_2O_2 for 48 h finally induced significant decrease in the cell viability. In addition, we demonstrated that short-term treatment of ISMC with H_2O_2 caused a significant increase in the density of protein at positions corresponding to 42 kDa and 44 kDa, indicating increased phosphorylation of ERKs. Convincing evidence to show the opposing effects of H_2O_2 on cell growth by stimulating proliferation (Guyton et al, 1996; Goldkorn et al, 1998) and triggering apoptosis (Li et al, 1997) has been presented. These dual effects might depend on the duration of H_2O_2 treatment, the concentration of H_2O_2, and the types of cells. Our data imply that short-term stimulation with H_2O_2 activates the signaling pathways of cell mitogenic responses.

Also, the involvement of tyrosine kinase in the process of ERK activation by H_2O_2 in ISMC was investigated. Tyrosine kinase activation is one of the most early events in response to a variety of growth stimulators. Inhibition of tyrosine kinase not only blocks mitogen-stimulated growth response, but also leads to cell apoptosis, suggesting tyrosine kinase plays a key role in the balance between cell growth and death (Knebel et al, 1996). By experiments with nonspecific tyrosine kinase inhibitor genistein, our data showed that the mechanism of H_2O_2-induced ERK activation probably includes activation of tyrosine kinases. These findings are consistent with previous observations that H_2O_2 increases tyrosine phosphorylation of proteins in different cell types (Abe et al, 1994; Fialkow et al, 1994; Whisler et al, 1995).

In addition, we showed that SAPK/JNK were concentration-activated in response to H_2O_2 in ISMC dependently. Compared with its activation of ERKs, H_2O_2 activated SAPK/JNK to a moderate degree. This observation was not unexpected, since SAPK/JNK mediate signals in response to various stresses (Treisman, 1996). Our findings are in agreement with previous observations that SAPK/JNK were activated by H_2O_2 in different cell types, including rat aortic smooth muscle cells (Guyton et al, 1996) and pulmonary arterial smooth muscle cells (Zhang et al, 1998). However, functional importance and mechanisms of activation of SAPK/JNK by H_2O_2 remain to be determined.

Interestingly, our results showed that p38 MAP kinase was not activated at any time period when ISMC were stimulated with 1 mM H_2O_2 for 5, 15, 30, 60, and 120 min. These observations are different from the findings of the previous studies, which showed that p38 MAP kinase activity was increased by H_2O_2 stimulation in aortic smooth muscle cells (Guyton et al, 1996) and pulmonary arterial smooth cells (Zhang et al, 1998). One of that possible explanations for such discrepancy is that H_2O_2- induced MAP kinase activation is a cell-specific response. There has been a report in which H_2O_2 stimulation (1 μM to 2 mM, 1 to 60 min) did not increase MAP kinase activity, although it stimulated aortic smooth muscle cell growth (Baas & Berk, 1995). In addition, Guyton et al reported that H_2O_2 stimulation under the same experimental conditions induced ERK activation with various extents in different types of cells. Experimental conditions, such as culture medium, FBS concentration, and treatment buffer could affect H_2O_2-induced MAP kinase activation. Another explanation is that superoxide-generating systems in ISMC may differ from those in other types of cells. Recent report has suggested that superoxide generated from different sources such as cyclooxygenase, xanthine oxidase, and NADPH oxidase, may have distinct signal transduction pathways and mediate different cellular responses (Lander, 1997).

In summary, our investigations demonstrated that short-term stimulation with H_2O_2 in ISMC has no effect on cell viability, while long-term exposure to H_2O_2 leads to cell death. We focused on the cellular consequences by the short-term stimulation with H_2O_2. Our data imply that H_2O_2 produces its effects through the activation of ERK and SAPK/JNK in ISMC, and that the mechanisms by which H_2O_2 activates ERKs may include activation of tyrosine kinases. These findings suggest that ERK activation induced by short-term treatment with H_2O_2 plays a critical role in controlling cellular protection in the early stage of response to oxidative stress. The present study implicates the necessity of identification of MAPK signaling pathways affected by ROS, since it might ultimately lead to the

development of rational and molecular therapeutics for the diseases, such as crohn's disease and ulcerative colitis which result from excessive ROS.

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