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Hydrogen Peroxide-induced ERK Activation in Cultured Feline Ileal Smooth Muscle Cells

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H_2O_2-induced ERK Activation in ISMC

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ABBREVIATION: DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediamine tetraacetic acid; EGTA, ethylene glycol-bis-(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid; ERK, extracellular signal-regulated protein kinases; FBS, Fetal bovine serum; HBSS, Hank's Balanced Salt Solution-Modified; H_2O_2, Hydrogen peroxide; ISMC, Ileal Smooth Muscle Cells; JNK, Jun-N-terminal kinases; MAPK, Mitogen-activated protein kinase; PBS, phosphate-buffered saline; PKC, Protein kinase C; PMA, phorbol-12-myristate-13-acetate; ROS, Reactive Oxygen Species; SAPK, stress-activated protein kinase; SDS, sodium dodecyl sulfate

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ABSTRACT

H_2O_2 has been shown to act as a signaling molecule involved in many cellular functions such as apoptosis and proliferation. In the present study, we characterized the effects of H_2O_2 on activation of MAP kinases and examined the factors involved in the process of ERK activation by H_2O_2 in ileal smooth muscle cells (ISMC). ISMC were cultured and exposed to H_2O_2. Western blot analysis was performed with phospho-specific MAP kinase antibodies. Potent activation of ERK and moderate activation of SAPK/JNK occurred within 30min of 1mM H_2O_2 treatment. However, p38 MAP kinase was not activated by H_2O_2. The activation of ERK by H_2O_2 was reduced by MEK inhibitor PD98059, Ras inhibitor FTS, removal of extracellular Ca^{2+}, depletion of the intracellular Ca^{2+} pool by thapsigargin, or pretreatment of ISMC with the calmodulin antagonist W-7. Also, H_2O_2-induced ERK activation was attenuated by a receptor tyrosine kinase inhibitor, tyrphostin 51, but not by downregulation of protein kinase C (PKC) with phorbol-12-myristate-13-acetate (PMA) or by a PKC inhibitor, GF109203X. Growth factor receptor antagonist suramin pretreatment inhibited H_2O_2-induced ERK activation, highlighting a role for growth factor receptors in this activation. Further, the ERK activation by H_2O_2 was blocked by pretreatment with either *N*-acetyl-cysteine, *o*-phenanthroline, or mannitol, indicating that metal-catalyzed free radical formation may mediate the initiation of signal transduction by H_2O_2. These data suggest that short-term stimulation with H_2O_2 activates the signaling pathways of cell mitogenic effects which are thought to be a protective response against intestinal oxidative stress.

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Hydrogen peroxide (H_2O_2) is generated by superoxide dismutase from superoxide anions ($O_2^{\cdot-}$) constantly produced by metabolic reactions in aerobic organisms. Also, it is inevitably produced when cells are exposed to extracellular stimuli such as irradiations (X-rays, UV), or environmental pollutants, inflammatory systems. H_2O_2 is generally regarded as an unwanted toxic byproduct since it is easily converted to hydroxyl radicals ($OH^{\bullet}$) which cause damage to many cellular components indiscriminately. Indeed, intestinal inflammation is accompanied by excessive production of reactive oxygen species (ROS) in inflammatory bowel disease such as crohn's disease and ulcerative colitis (Rhee, 1999; Kruidenier et al., 2003). Although cells have clearly evolved multiple defenses for elimination of such as H_2O_2, $O_2^{\cdot-}$, $OH^{\bullet}$ and singlet oxygen, chronic exposure to ROS leads to the degenerative alterations that occur with aging and in the etiology of numerous disease processes including gastrointestinal tract disorders and cancer (Guyton and Kensler, 1993; Kruidenier and Verspaget, 2002).

In contrast with the wide belief that H_2O_2 has only harmful functions due to its relentless production with the damaging nature, recent data have shown that H_2O_2 stimulates cell growth/proliferation and DNA synthesis in different types of cells, indicating that H_2O_2 act as an important signaling molecule (Rao and Berk, 1992; Fiorani et al., 1995). Exposure of cells to exogenous H_2O_2 has been used to investigate multiple signaling pathways related to smooth muscle contraction (Jin and Rhoades, 1997) and proliferation,(Baas and Berk, 1995) as well as apoptosis (Li et al., 1997)

The activation and phosphorylation of extracellular signal-regulated protein kinases (ERKs), one subtype of MAP kinases, is a pivotal step in the signal transduction cascade regulating cell proliferation by extracellular stimuli such as epidermal growth factors (EGF) and platelet-derived growth factor (PDGF). Two major isoforms of ERK, p44 (ERK_1) and p42 (ERK_2), have been identified**.** In contrast, p38 MAP kinase and p46 to p54 MAP kinases (SAPK/JNK), two other subtypes of MAP kinases, mediate signals in response to cytokines and stress. It has been suggested that a major pathway involved in ERK_1 and ERK_2 stimulation in various types of cells requires the sequential activation of Ras, Raf, and MEK (Herlaar and Brown, 1999).

It has been reported that intracellularly generated H_2O_2 stimulates tyrosine kinase or MAP kinase activities in several types of cells (Sundaresan et al., 1995; Guyton et al., 1996). Also, three major MAP kinase signaling cascades have been evaluated in pancreatic acinar cells in response to H_2O_2, menadione and other physiologic stimuli (Dabrowski et al., 2000).

In the present study, exposure to exogenous H_2O_2 at physiological levels was used to characterize the effects of H_2O_2 on activation of MAP kinases including ERK_1/ERK_2, SAPK/JNK, and p38 MAP kinase in cultured ileal smooth muscle cells (ISMC). The results clearly indicate that H_2O_2 is very potent activator of the ERKs in

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ISMC. We also investigated the involvement of various factors in the process of ERK activation by H_2O_2 in ISMC, including the roles of protein kinase C (PKC), Ca^{2+}/calmodulin, receptor tyrosine kinase, MEK, and Ras, as well as effects of several antioxidants and a growth factor receptor antagonist in H_2O_2-induced ERK activation. 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.

Materials and Methods

Cultures of Ileal smooth muscle cells. Adult cats of either sex weighing between 2.0 and 3.0 kg were anesthetized with ketamine (50mg/ml/kg) and then the abdomen was opened with a midline incision. The ileum was excised, 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 and 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℃. On the following day, fresh DMEM containing 10% FBS was added. 10 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 X 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.

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 5 min at 1300 rpm. The pellets were washed with ice-cold PBS and recentrifuged. The obtained pellets were homogenized in a homogenizing buffer composed of 20 mM

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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). Samples homogenates were then sonicated (3s, 5x) and centrifuged for 7 min at 12500 rpm 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 measured spectrophotometrically using the Bio-Rad assay (Bio-Rad Chemical Devision, Richmond, California). The absorption was monitored at a wavelength of 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℃ overnight. The membranes were washed using 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 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).

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 using phase contrast microscope (model ULWSD 0.30 Olympus, Japan), haemacytometer, and a counter. This experiment should be performed within 5 min after staining not to damage cells.

Data Analysis. The data are expressed as the means ± S.E.M. of n separate experiments and the statistical

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differences between means were determined by Student's *t* test, with $p<0.05$ considered significant.

Results

Characterization of H_2O_2-induced MAP kinase activation in ISMC. To characterize the response of the cultured cells to various doses of ROS donor H_2O_2, the growth-arrested ISMC were exposed for 30 min with 10^{-6}, 10^{-5}, 10^{-4} to 10^{-3}M H_2O_2. In Figure 1A, Western blot analysis showed a concentration-dependent increase in the activated forms of both ERK_1 and ERK_2. Those two distinct bands were identified with the phospho-specific MAP kinase antibody 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, and therefore their activation. Under the same experimental conditions, 10% FBS-DMEM activated ERKs by 2.5-fold over basal levels. Exposure of cells to 10^{-3} M H_2O_2 for 30 min significantly increased p44/42 MAP kinase phosphorylation by 2.8 $\pm$ 0.1 times over the level of control samples, which consisted of ISMC incubated without any stimulant (Fig. 1B).

Figure 2A 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. MAP kinase phosphorylation rapidly reached the maximal levels at 30 min showing only slight activation at 15 min and then slowly declined. Compared with control cells, cells exposed with 1 mM H_2O_2 for 30 min showed a 2.5 $\pm$ 0.1 fold increase in ERK_1 and ERK_2 activation (Fig. 2B).

In most of the later experiments, therefore, ISMC were stimulated with exogenous H_2O_2 at a final concentration of 1 mM for 30 min according to the concentration-response and time-course data. To investigate any harmful effect of this concentration- and time- treatment of H_2O_2 on cells, cell viability between H_2O_2-stimulated and untreated cells was compared by using trypan blue methods. There was no difference between the percentages of control cells that excluded trypan blue (100 $\pm$ 0%) and cells treated with 1mM H_2O_2 for 120 min (98.8 $\pm$ 1.2%, n = 4) that excluded it. In addition, under the treatment conditions, the total numbers of control and H_2O_2-treated cells were no different. (25.2 $\pm$1.9 x 10^5 versus 24.9$\pm$0.4 x 10^5 cells, n=4 respectively)

The ability of H_2O_2 to activate SAPK/JNK was also evaluated in ISMC. Cells treated with 10^{-6}, 10^{-5}, 10^{-4} to 10^{-3}M H_2O_2 for 30min showed a concentration-dependent increase in the activated forms of both SAPK and JNK, which were detected with phospho-specific SAPK/JNK antibody (data not shown). Incubation of ISMC with 10^{-3}M H_2O_2 for 30 min caused a moderate increase by 1.6 $\pm$ 0.1 times over the level of control in the density of two bands of 54 kDa and 46 kDa corresponding to SAPK and JNK.

Figure 3A shows a time course of H_2O_2-induced SAPK/JNK activation. Even though the time-dependent

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increase in the activated forms of SAPK/JNK was similar to the activation of p44/42 MAP kinases by H_2O_2 with maximal activation observed at 30 min, the activation of SAPK/JNK by H_2O_2 had a slight different characterization in time course. We found that incubation of ISMC with 1 mM H_2O_2 caused relatively rapid activation of JNK. An increase in the density of the band corresponding to active JNK was noticed at just 5 min. Thereafter the JNK phosphorylation has been gradually increased, reaching a maximum at 30 min. Interestingly, the activation of JNK quickly declined, reaching close to the basal values at 60 min after H_2O_2 challenge (Fig. 3B).

It has been recently reported 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, there was, however, no significant change 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. 3C).

MEK mediates the ERK activation by H_2O_2 in ISMC. It has been known that in several different signaling cascades leading to ERK activation, the mitogen-activated/ERK-activating kinase (MEK), a dual specific protein kinase, phosphorylates ERK_1 and ERK_2. To examine if MEK is involved in H_2O_2-induced ERK activation, a specific MEK inhibitor was used in this study. Growth-arrested cells were treated with 10 μM 2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD98059) (Lee et al., 2002; Shin et al., 2002) for 40 min prior to 1 mM H_2O_2 stimulation for 30 min. Preincubation of PD98059 decreased the ERK activation by H_2O_2 in cultured ISMC (Figure 4). The data suggest that MEK is an upstream regulator of MAP kinase and the ability of H_2O_2 to stimulate ERK activation is mediated by MEK in ISMC.

PKC may not be involved in H2O2-induced ERKs activation. Several studies have suggested that MAP kinase activation is PKC dependent (Seger and Krebs, 1995; Dabrowski et al., 1996; Zhang et al., 1998), and that H_2O_2 stimulates both cytosolic and membrane-associated PKC activities (Stauble et al., 1994). Therefore, we evaluated whether inhibition of PKC has any effect on the activation of MAP kinase by H_2O_2 in ISMC. Growth-arrested cells were pretreated with 100 nM PMA for 24 h to downregulate PKC, and were then stimulated with 1 mM H_2O_2 or 100 nM PMA for 30 min to activate PKC since phorbol esters are known as potent activators of PKC as treated for the short time (Zhang et al., 1998). While downregulation of PKC caused about 90% inhibition of acute PMA-induced ERK activation, pretreatment of cells with PMA did not alter basal levels of ERK activation, as shown Figure 5A.

To confirm that PKC is not involved in H_2O_2-induced ERK activation in ISMC, the effects of 3-[1-(Dimethylaminopropyl)indol-3-yl]-4-(indol-3-yl)maleimide hydrochloride (GF109203X) (Dabrowski et al.,

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1997; Dabrowski et al., 2000), a relatively specific PKC inhibitor, were also examined. Pretreatment of ISMC with 20 μM GF109203X for 40 min had no effect on ERK activation by H_2O_2 (Fig. 5B). These results imply that the signaling pathway of H_2O_2-induced ERK activation appears to be PKC independent.

Calcium and Calmodulin play a role in H_2O_2-induced ERK activation. It has been reported that H_2O_2 induced increases in intracellular Ca^{2+} concentration and Ca^{2+} release from skeletal muscle sarcoplasmic reticulum (Favero et al., 1995). Also, Ca^{2+} influx and Ca^{2+}/calmodulin have been shown to be involved in activation of MAP kinases.(Chao et al., 1992) Accordingly, we examined the possible involvement of Ca^{2+} in H_2O_2-induced ERKs activation in ISMC. First, to investigate the effects of extracellular Ca^{2+} on H_2O_2-induced ERK activation, growth-arrested cells were treated with 1 mM H_2O_2 for 30 min in the Ca^{2+}- free incubation buffer containing 2 mM EGTA (Zhang et al., 1998). As shown in Figure 6A, the ability of H_2O_2 to stimulate the activation of ERKs was abolished. Secondly, we evaluated the effects of thapsigargin, a selective inhibitor of the sarcoplasmic reticulum Ca^{2+}-ATPase, on the ERK activation by H_2O_2. It has been reported that treatment of cells with thapsigargin effectively depletes the inositol-1,4,5-triphosphate (IP_3)-releasable pool of intracellular Ca^{2+} (Sagara et al., 1992). In this study, pretreatment with 1 μM thapsigargin in the presence of extracellular Ca^{2+} for 2 h inhibited H_2O_2 –induced ERK activation by 37%.

We also investigated the effects of calmodulin on the phosphorylation of ERKs in response to H_2O_2 stimulation. Growth-arrested ISMC were pretreated with the calmodulin antagonists 50 μM N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) (Zhang et al., 1998) for 1 h prior to incubation with 1 mM H_2O_2 for 30 min. Pretreatment of ISMC with W-7 caused a significant decrease in ERK activation by H_2O_2 (Fig. 6B). The basal level of ERK activity was not affected by W-7 treatment in ISMC. Taken together, the data suggest that Ca^{2+}/Calmodulin as well as Ca^{2+} influx and the release of Ca^{2+} from intracellular stores seems to play a prominent role in the amplification of ERK stimulation by H_2O_2.

Growth factor receptor and metal-catalyzed free radical formation mediates the initiation of signal transduction by H_2O_2. It has recently been reported that H_2O_2 stimulated tyrosine kinase activity and caused receptor tyrosine phosphorylation, suggesting that H_2O_2 –induced mitogenic response is, in part, mediated through PDGF receptor in aortic smooth muscle cells (Jin et al., 2000). Among two classic tyrosine kinases: receptor and nonreceptor tyrosine kinases, receptor tyrosine kinases virtually are the growth factor receptors located in the inner side of the cytoplasmic membrane and subjected to dimerization and autophosphorylation upon activation. Therefore, we evaluated the effects of tyrphostin 51 on H_2O_2 –induced ERK activation. Tyrphostin, a member of the genistein family of tyrosine kinase inhibitor, is specific to receptor tyrosine kinases

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(Majumdar et al., 1996). Pretreatment of ISMC with 90 μM tyrphostin 51 for 45 min prior to the addition of 1 mM H_2O_2 for 30 min significantly reduced ERK activation by H_2O_2 (Fig. 7A). The data imply that H_2O_2 –induced ERK activation is partly mediated through growth factor receptor.

Therefore, we assumed the possible involvement of growth factor receptors in the initiation of ERK activation by H_2O_2. The effects of suramin on H_2O_2 –induced ERK activation in ISMC were examined, since suramin has been known to block the binding of ligands to several growth factor receptors, and to inhibit ERK_2 activation by epidermal growth factor or by UVC irradiation.(Sachsenmaier et al., 1994) As shown in Figure 7B, pretreatment of ISMC with 0.4 mM suramin for 45 min before the direct addition of 1 mM H_2O_2 blocked ERK activation. The inhibition of H_2O_2 –induced ERK activation by 0.4 mM suramin is greater than that by 0.3mM suramin pretreatment.

H_2O_2 has been shown to produce its effect through the activation of MAP kinase since catalase or *N*-acetyl-cysteine block MAP kinase activation.(Sundaresan et al., 1995; Guyton et al., 1996) We therefore explored the effects of several antioxidants on ERK activation by H_2O_2 in ISMC, as shown in Figure 8. Firstly, pretreatment of ISMC with the glutathione precursor *N*-acetyl-cysteine (20mM) blocked H_2O_2 –induced ERK activation. The data suggest that oxidative stress initiates ERK activation by H_2O_2. Secondly, mannitol (100 mM), a free radical scavenger with specificity for hydroxyl radical (Guyton et al., 1996; Chuang et al., 2000), also abolished the ability of H_2O_2 to activate ERKs. Thirdly, the iron chelator *o*-phenanthroline (100μM) (Guyton et al., 1996; Regan et al., 2001) also significantly reduced H_2O_2 –induced ERK activation. This implies that metal-catalyzed reactions are required for ERK activation by H_2O_2. In fact, the major mechanism of hydroxyl radicals ($OH^{\bullet}$) generation inside cells is from H_2O_2 via Fenton reaction ($H_2O_2 + Fe^{2+}$ or $Cu^{+} \rightarrow OH^{-} + OH^{\bullet} + Fe^{3+}$ or Cu^{2+}). Besides, Fe^{2+} or Cu^{+} can be readily regenerated by cellular electron donors such as reduced nicotinamide adenine dinucleotide phosphate (NADPH), ascorbate, and various thiol compounds (Stadtman and Berlett, 1998). Accordingly, these data indicate that H_2O_2 can partly convert via metal-catalyzed reactions to other oxygen-derived free radical species including hydroxyl radical, and the oxidation by this free radical initiates signal transduction inducing to ERK activation. In addition, the findings provide that the ERK activation is mediated by H_2O_2 rather than by nonspecific stimulation under the experimental conditions since cells treated with an antioxidant alone did not show any change in ERK phosphorylation compared with controls.

H_2O_2-induced ERK activation may occur through Ras-dependent pathways. The membrane-associated small (21 kDa) G protein Ras is important in activation of MEK/ERK cascades by many types of stimuli. However, MAP kinase activation can also occur through Ras-independent pathways.(Kolch et al., 1993)

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Therefore, we examined the inhibitory effects of S-farnesylthiosalicylic acid (FTS) (Gana-Weisz et al., 1997) on H_2O_2-induced ERK activation in ISMC to check whether Ras is a component of the signaling transduction leading to ERK activation. Growth-arrested ISMC were pretreated with 50 μM FTS for 24 h prior to incubation with 1 mM H_2O_2 for 30 min. Significant inhibition of ERK activation in ISMC by FTS was observed. The FTS did not however cause a decrease in the total amount of ERKs (Figure 9). The data implies an involvement of Ras in mediating H_2O_2-induced ERK activation.

Discussion

In this study, we demonstrate that short-term treatment of ISMC with H_2O_2 causes a significant increase in the density of protein at positions corresponding to 42 kDa and 44 kDa, indicating the increased phosphorylation of ERKs. Cell viability in the ISMC treated with 1 mM H_2O_2 showed no difference from that of controls at up to 2h time point, while treatment of ISMC with 1 mM H_2O_2 for 48 h finally induces the significant decrease in the cell viability. Our data indicate that short-term stimulation with H_2O_2 activates the signaling pathways of cell mitogenic responses. Convincing evidence has been presented 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).These dual effects might depend on the time of H_2O_2 treatment, the concentration of H_2O_2, and the types of cells**.** We also showed that moderate activation of SAPK/JNK occurred within 30min of 1 mM H_2O_2 treatment in ISMC in a time-dependent manner. Our findings are consistent with previous observations that ERK and SAPK/JNK were activated by H_2O_2 in different cell types including rat aortic smooth muscle cells (Guyton et al., 1996), pulmonary arterial smooth muscle cells (Zhang et al., 1998), and bovine tracheal myocytes (Abe et al., 1994). On the contrary, 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 and Berk, 1995).

Our results, interestingly, provided that p38 MAP kinase was not activated at any time period when ISMC were stimulated with 1mM H_2O_2 for 5, 15, 30, 60, 120min. These observations are different from those findings of the previous studies since 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). The first possible explanation for such discrepancy is that H_2O_2-induced MAP kinase activation is a cell-specific response. 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,

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and treatment buffer could affect H_2O_2-induced MAP kinase activation (Baas and Berk, 1995; Guyton et al., 1996). 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, NADPH oxidase, etc may have distinct signal transduction pathways and mediate different cellular responses (Lander, 1997). We also observed that ERK activation in response to H_2O_2 is weaker in ISMC at higher passage numbers. Although, the mechanisms underlying this observation are not fully understood, it seems to be deterioration of cell protective mechanism against oxidant injury with aging as a broad range of view since ERK has been shown to play a critical role in cell survival.

PKC is present in the cell cytoplasm and, upon agonist stimulation, rapidly translocates to the particulate or membrane fraction observed by Western blot analysis (Sohn et al., 1997a; Sohn et al., 1997b). Our findings suggest that H_2O_2-induced ERK activation may not be mediated through PKC-dependent mechanisms. These are consistent with recent reports that, in pancreatic acinar cells, ROS activates MAP kinases through a PKC-independent mechanism (Dabrowski et al., 2000). On the other hand, in some studies, activation of the MAP signaling cascade was PKC-dependent (Seger and Krebs, 1995; Dabrowski et al., 1996). Interestingly, there have been several reports suggesting that H_2O_2-induced activation of MAP kinases was mediated by both PKC-dependent and –independent mechanisms (Abe et al., 1994; Zhang et al., 1998). Possible interpretation for the discrepancy of these observations is that H_2O_2 may be able to utilize multiple pathways to produce mitogenic effects depending on cell types. Also, H_2O_2 may cause MAP kinase activation by stimulating PKC isoforms that are insensitive to either GF109203X or PMA treatment.

Our investigation provides evidence that H_2O_2-induced ERK activation appears to be mediated by Ca^{2+} influx, Ca^{2+} -release from intracellular stores, and Ca^{2+}/Calmodulin. The data imply that the Ca^{2+} influx triggers the intracellular Ca^{2+} –channel opening responsible for releasing Ca^{2+} from the intracellular stores, because the removal of extracellular Ca^{2+} abolished H_2O_2 effects on ERK activation close to the basal level. The process of Ca^{2+} -induced Ca^{2+} release is important in regulating the way in which cells mobilize Ca^{2+} from their internal stores. The most important diffusible messenger for activating the intracellular Ca^{2+} stores is Ca^{2+} itself. The data suggest that the release of Ca^{2+} from intracellular stores is also critical to the amplification of ERK stimulation by H_2O_2, since depletion of the intracellular Ca^{2+} pool by thapsigargin in the presence of extracellular Ca^{2+} reduced ERK activation by 37%. These findings are consistent with the previous study that Ca^{2+} influx and Ca^{2+}/calmodulin were involved in H_2O_2-induced activation of MAP kinases in pulmonary artery smooth muscle cells (Zhang et al., 1998).

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Activation of tyrosine kinase is one of 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). H_2O_2 has also been shown to activate receptor tyrosine kinases, such as fibroblast growth factor receptors and EGF receptors (Rao, 1997; Goldkorn et al., 1998), as well as several nonreceptor tyrosine kinases, such as $p56^{lck}$ and $p72^{syk}$ (Schieven et al., 1993; Hardwick and Sefton, 1995). Our data imply that the activation of growth factor receptor may play a role in H_2O_2-induced ERK activation, since H_2O_2-induced ERK activation was inhibited by receptor tyrosine kinase inhibitor, tyrphostin 51. In the future study, the data need to be validated by dominant negative approaches such as DN EGF receptor. It has been known that mitogen-stimulated signal cascade leading to ERK activation is initiated through the interaction of peptide growth factors with their receptors (Guyton et al., 1996). Many growth factor receptors have cysteine-rich motifs, the oxidation of which can simulate ligand binding (Heldin, 1995). Through receptor activation mediated by sulfhydryl oxidation, H_2O_2 has been postulated to mimic the actions of insulin and other receptor-binding proteins (Schieven et al., 1994). Our results show that suramin blocked H_2O_2-induced ERK activation, suggesting that oxidation of such cell surface receptors may mediate signal initiation by H_2O_2. We confirmed the sulfhydryl reactivity of the oxidant signal generated from H_2O_2, since pretreatment with the glutathione precursor *NAC* abolished the ability of H_2O_2 to activate ERK. Free radical species generated from H_2O_2 may directly oxidize and thereby activate cell surface receptors, although the oxidative modification of other molecules may also function in the regulation of ERKs by H_2O_2. Our findings suggest that free radicals or other redox mechanisms may constitute a pivotal component of the signaling cascade to ERK activation normally utilized by growth factors and other stimuli.

Ras proteins function as intermediates in signaling pathways activated by a variety of cell-surface receptors involved in cell growth and differentiation (Ullrich and Schlessinger, 1990; Fields et al., 1996). Ras has been known to activate several signal transduction pathways including the Raf-1/MEK/ERK cascade and the MEK kinase/JNK cascade (Herlaar and Brown, 1999). To transduce growth signals, Ras must be anchored to the inner surface of the plasma membrane. FTS might affect Ras membrane association and interfere specifically with Ras functions, thereby interfering with Ras-dependent signals associated with cell growth and proliferation. It has been demonstrated that inhibition of the Ras-dependent Raf-1 MAP kinase cascade is achieved by FTS (Gana-Weisz et al., 1997). They suggest that FTS affect various types of Ras proteins, unlike specific farnesyltransferase inhibitors. Our study also showed that pretreatment of ISMC with FTS significantly inhibited H_2O_2 -induced ERK activation in ISMC. The data implies that Ras appears to be involved in mediating

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H_2O_2-induced ERK activation. Such Ras involvement has been proposed by Guyton and colleagues since H_2O_2-induced ERK activation was abolished in PC12 cells by inducible or constitutive expression of the dominant negative Ras-N-17 allele (Guyton et al., 1996).

In summary, we focused on the cellular consequences of modulating the ERK signaling cascade induced by H_2O_2 in ISMC. Our data imply that H_2O_2 undergoes metal-catalyzed conversion to a hydroxyl-radical-like species and that oxidation by this free radical initiates signal transduction leading to ERK activation. In these signaling pathways to ERK activation by H_2O_2, receptor tyrosine kinase, Ca^{2+}- and calmodulin-dependent kinase, Ras and MEK are probably involved. The present study provides insight into mechanisms of MAP kinase regulation by H_2O_2, and suggests that ERK signaling pathways play a critical role in controlling cellular protection in the early stage in response to oxidative stress.

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Footnotes

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Figure Legends

Fig. 1. H_2O_2 activates ERKs in a concentration-dependent manner. **(A)** Western blot analysis showing that phosphorylated 44-kDa and 42-kDa ERKs were 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), corresponding to active MAP kinase, was quantified and shown in the diagram. Each value is the mean $\pm$ SE of three individual experiments performed in duplicate. *p<0.05 compared with controls.

Fig. 2. Time course of H_2O_2-induced ERK activation. **(A)** Western blot analysis showing phosphorylated ERK_1 and ERK_2. Growth-arrested cells were incubated with 10^{-3} M H_2O_2 for the indicated time periods. Treatment of ISMC with H_2O_2 activated ERKs in a time-dependent manner. **(B)** The integrated density of both bands (p44 and p42 MAP kinases), corresponding to active MAP kinases, was quantified and shown in the diagram. Each value is the mean $\pm$ SE of two individual experiments performed in duplicate. *p<0.05 compared with controls.

Fig. 3. (A) Time course of H_2O_2-induced SAPK/JNK activation. Western blot analysis shows that phosphorylated SAPK and JNK were detected with anti-phospho SAPK/JNK antibody. Growth-arrested cells were incubated with 10^{-3} M H_2O_2 for the indicated time periods. Treatment of ISMC with H_2O_2 activated SAPK/JNK in a time-dependent manner. **(B)** The density of active-JNK band was quantified and shown in the diagram as a percentage of the average value at time 0. Each value is the mean $\pm$ SE of two individual experiments performed in duplicate. *p<0.05 compared with controls. **(C)** Western blot analysis showing 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.

Fig. 4. Effects of MEK inhibitor PD98059 on H_2O_2-induced ERK activation. Growth-arrested cells were treated with 10μM PD98059 for 40 min prior to 10^{-3} M H_2O_2 stimulation for 30 min. PD98059 pretreatment decreased H_2O_2 effect on ERK activation.

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Fig. 5. PKC may not be involved in H_2O_2-induced ERK activation. **(A)** Effects of PKC downregulation on H_2O_2-induced ERK activation. Cells were or were not pretreated with 100nM PMA for 24 h, and were then exposed to H_2O_2 (10^{-3} M) or PMA (100 nM) for 30 min. PKC downregulation had no effect on ERK activation by H_2O_2. **(B)** Effects of PKC inhibitor GF109203X on H_2O_2-induced ERK activation. Growth-arrested ISMC were or were not pretreated with 20 μM GF109203X for 40 min, and were then stimulated with PMA (100 nM) or H_2O_2 (10^{-3} M) for 30 min. Pretreatment of cells with GF109203X had no effect on ERK activation by H_2O_2.

Fig. 6. (A) Effects of thapsigargin pretreatment on H_2O_2-induced ERK activation. Growth-arrested ISMC were or were not pretreated with 1 μM thapsigargin (TG) for 2 h prior to stimulation with 10^{-3} M H_2O_2 for 30 min in the presence or absence of extracellular Ca^{2+}. Removal of extracellular Ca^{2+} or thapsigargin pretreatment resulted in a decrease on ERK activation by H_2O_2. **(B)** Effects of calmodulin antagonist on H_2O_2-induced ERK activation. Growth-arrested ISMC were pretreated with 50μM W-7 for 1 h prior to incubation with 10^{-3} M H_2O_2 for 30 min. Treatment of cells with W-7 caused a decrease in ERK activation by H_2O_2.

Fig. 7. (A) Effects of the receptor tyrosine kinase inhibitor on H_2O_2-induced ERK activation. Growth-arrested ISMC were pretreated with 90 μM tyrphostin 51 for 45 min prior to 10^{-3} M H_2O_2 stimulation for 30 min. The receptor tyrosine kinase inhibitor tyrphostin 51 pretreatment reduced ERK activation by H_2O_2. **(B)** Effects of suramin on H_2O_2 -induced ERK activation. Growth-arrested ISMC were treated with suramin for 45 min prior to 10^{-3} M H_2O_2 stimulation for 30 min. Suramin inhibited ERK activation by H_2O_2.

Fig. 8. Role of free radicals in H_2O_2-induced ERK activation. **(A)** Growth-arrested ISMC were pretreated with *N*-acetyl-cysteine (20 mM), or mannitol (100 mM), *o*-phenanthroline (100 μM) for 2 h prior to 10^{-3} M H_2O_2 stimulation for 30 min. Treatment of ISMC with free radical inhibitors decreased ERK activation by H_2O_2. The inhibitors alone did not activate ERKs. **(B)** The integrated density of both bands (p44 and p42 MAP kinases), corresponding to active MAP kinases, was quantified and shown in the diagram. Each value is the mean $\pm$ SE of three individual experiments performed in duplicate. * and # $p<0.05$ compared with controls and with H_2O_2-treated cells, respectively.

Fig. 9. Effects of Ras Antagonist FTS on H_2O_2-induced ERK activation. Growth-arrested ISMC were pretreated with 50μM S-farnesythiosalicylic acid for 24 h prior to 10^{-3} M H_2O_2 stimulation for 30 min. Treatment of ISMC

with Ras antagonist FTS decreased ERK activation by H_2O_2.

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Figure 1

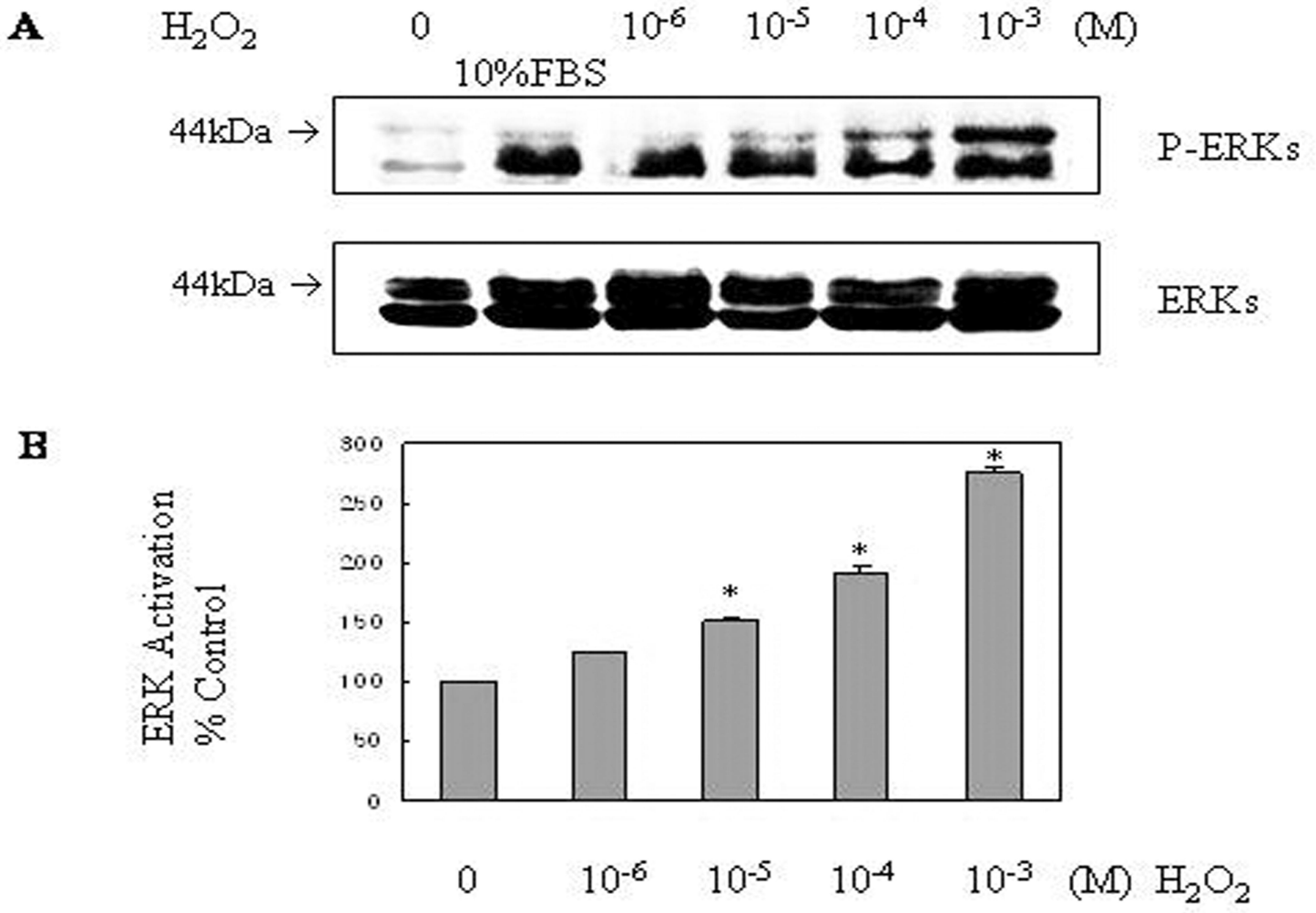

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Figure 2

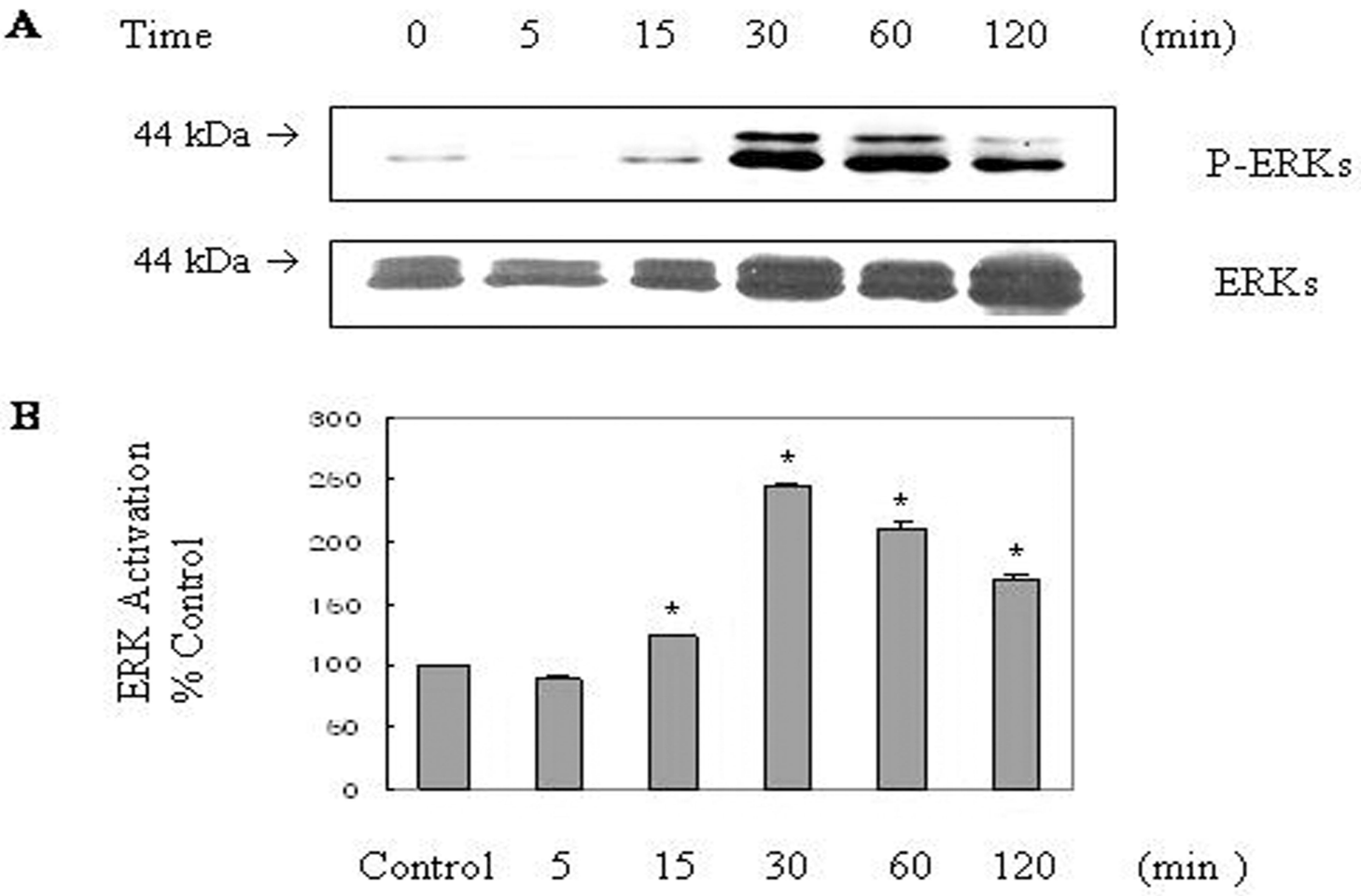

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Figure 3

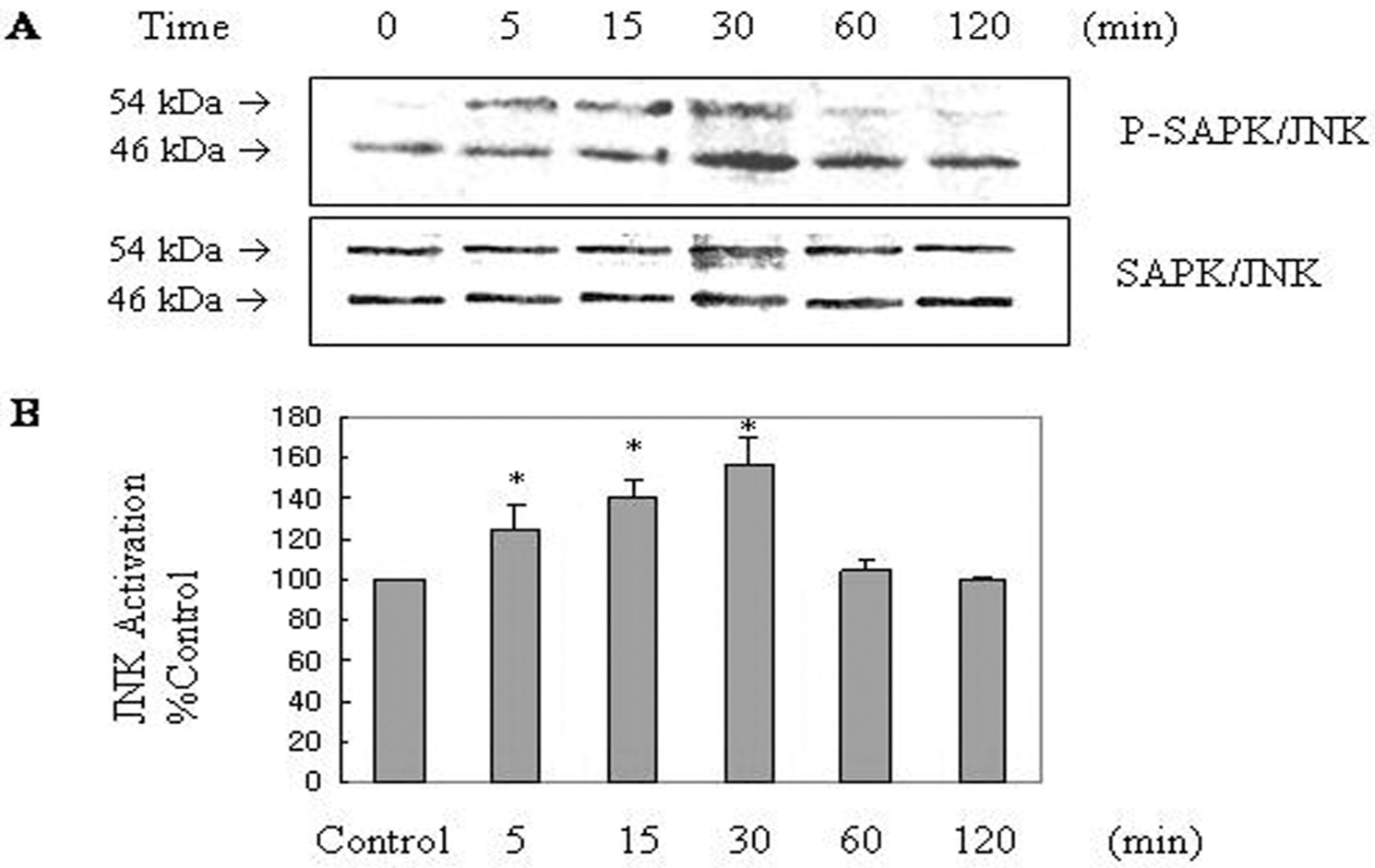

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Figure 4

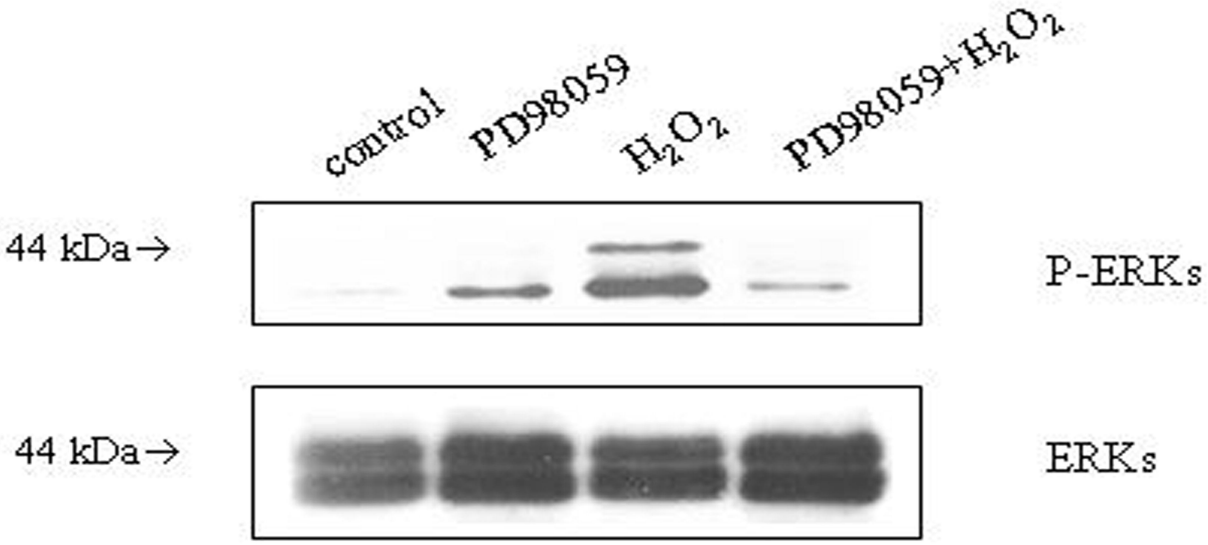

Figure 5

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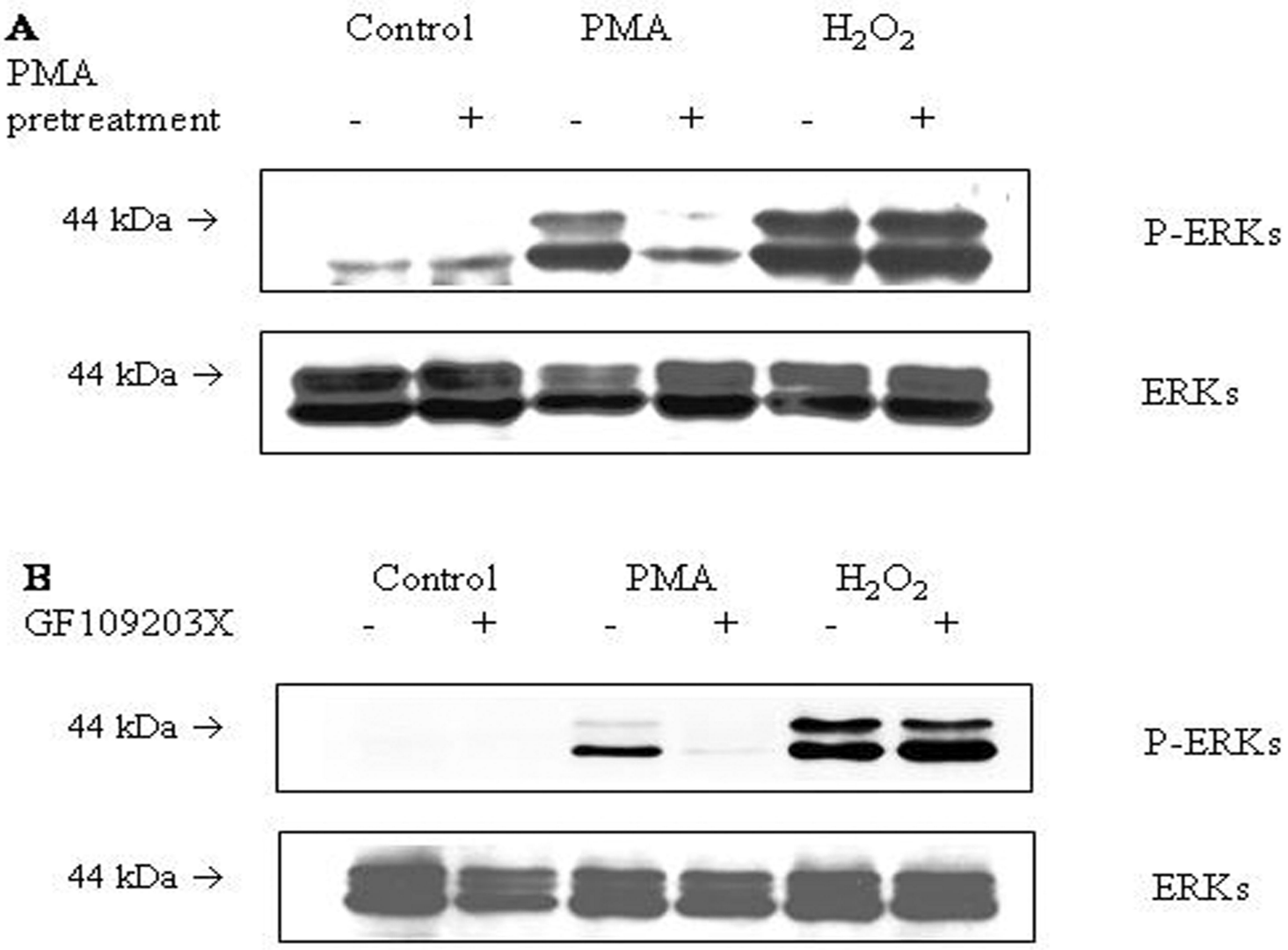

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Figure 6

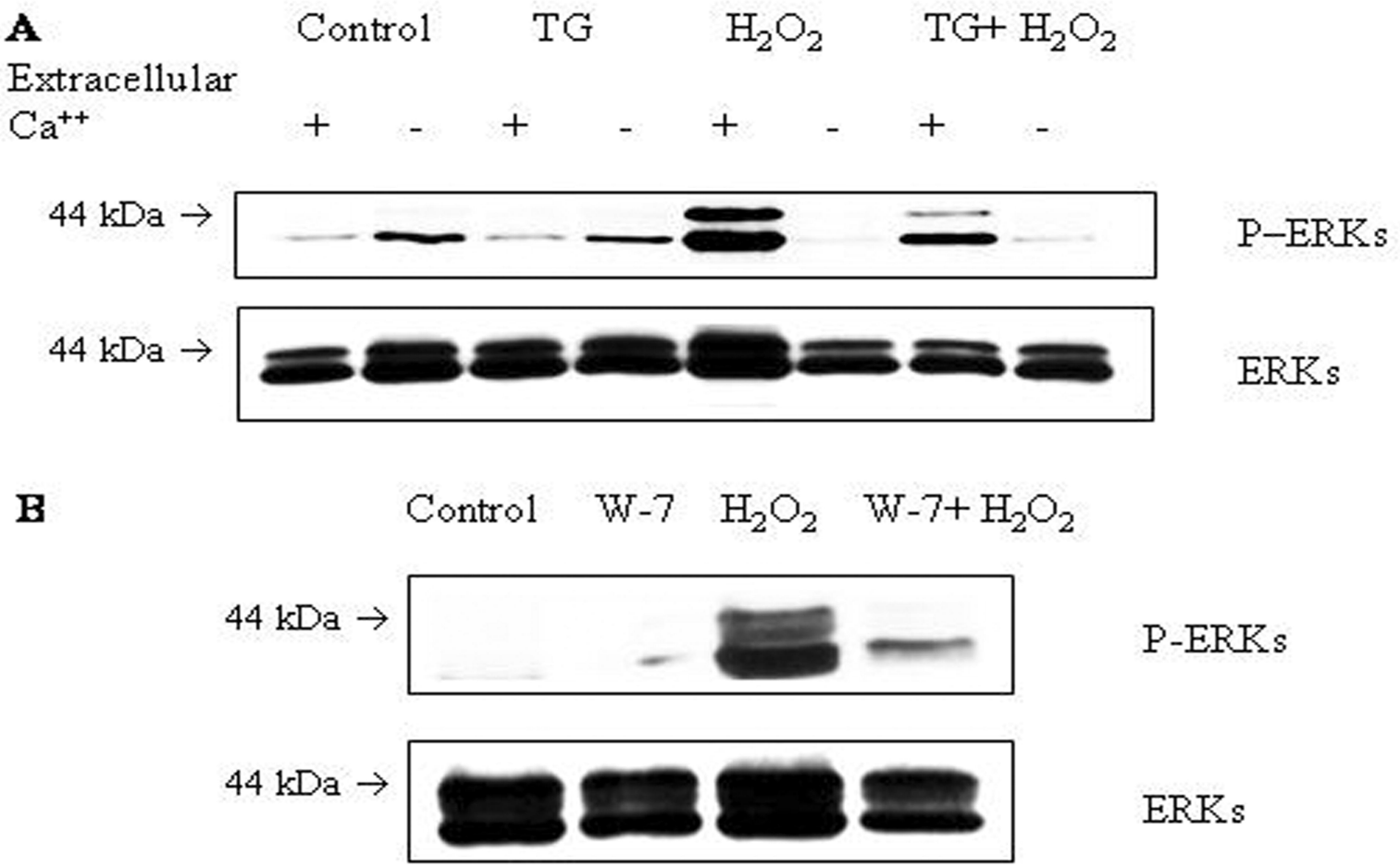

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Figure 7

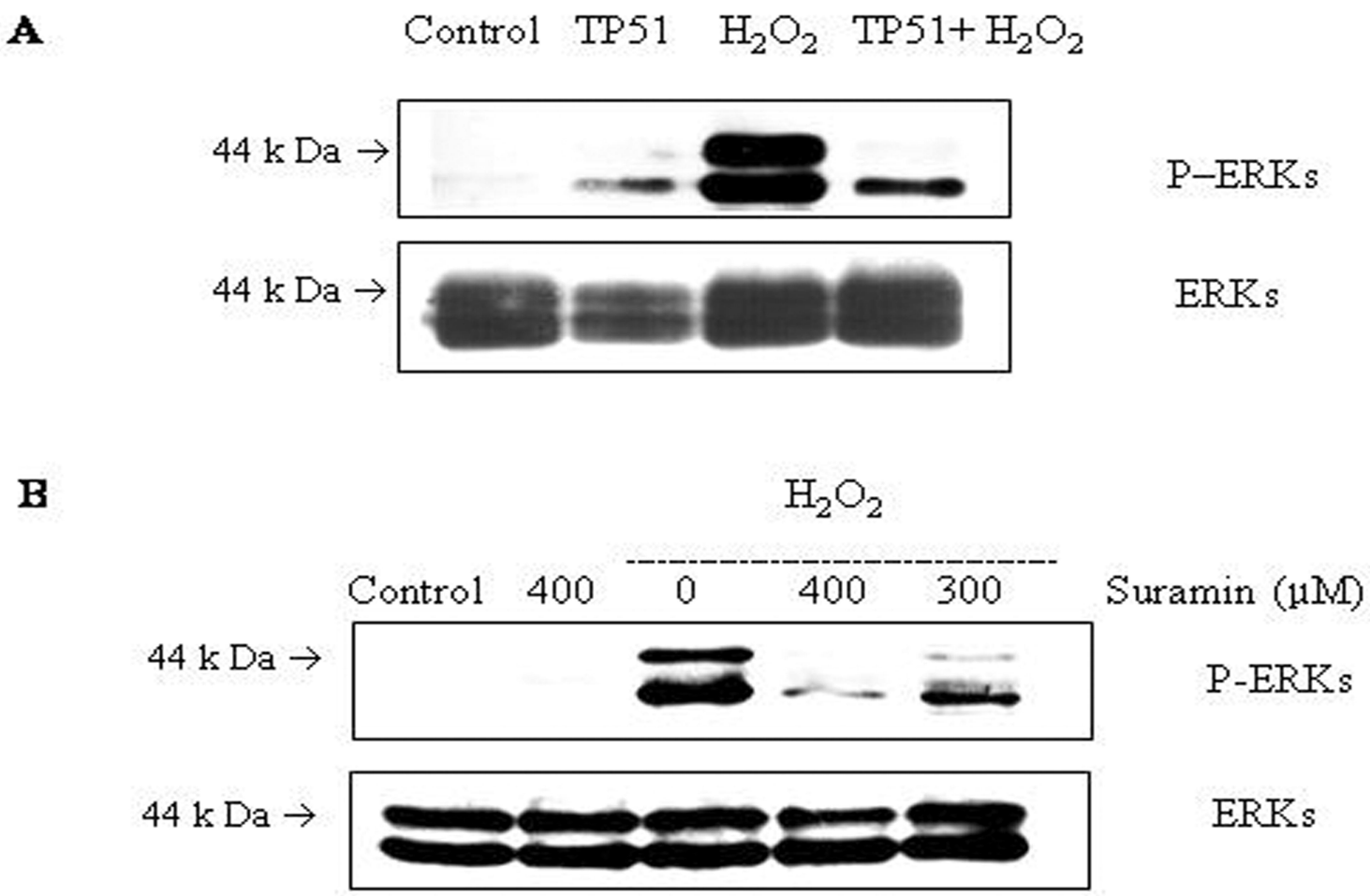

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Figure 8

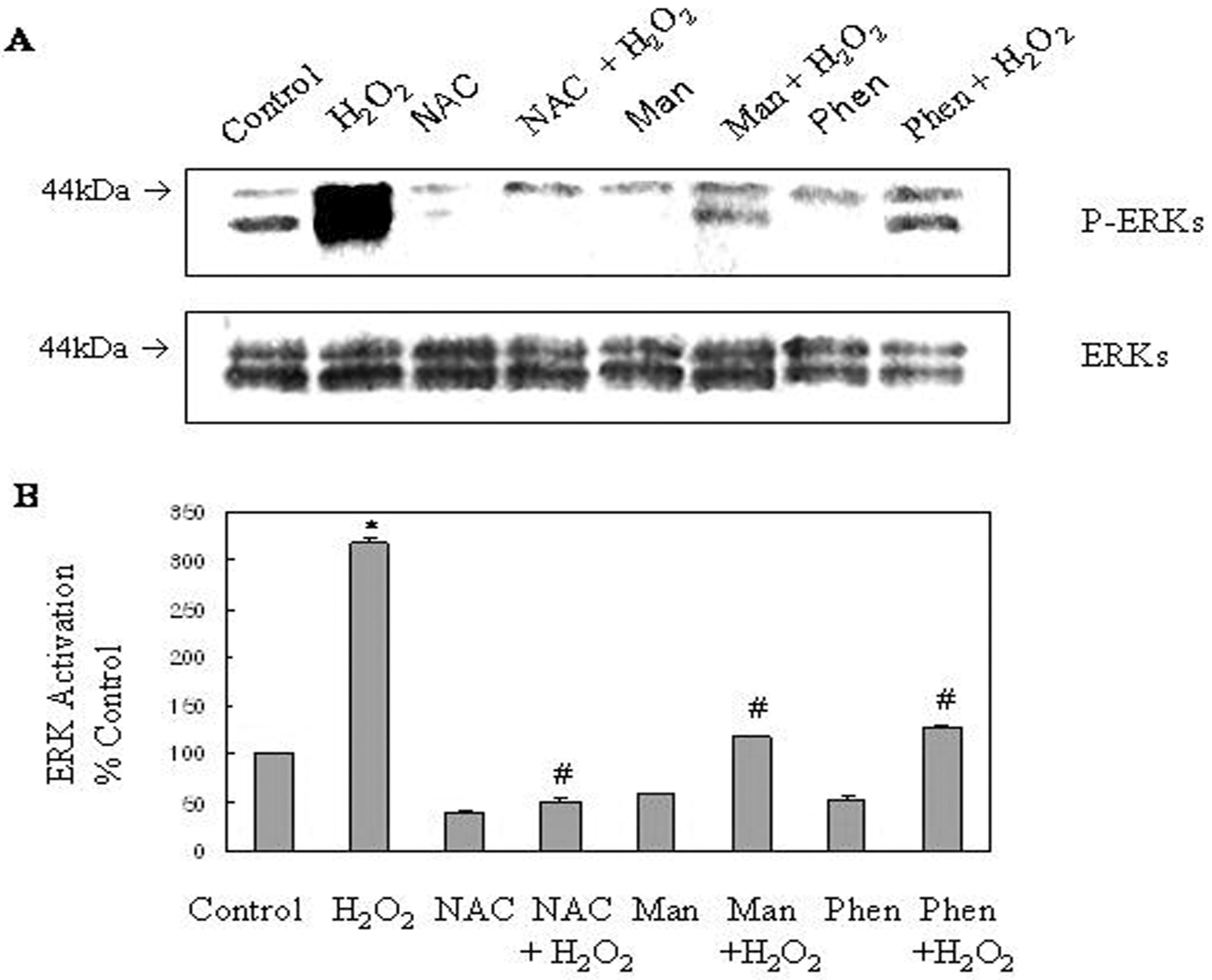

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Figure 9