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Distinct Roles of Ca^{2+}, Calmodulin, and Protein Kinase C in H_2O_2-Induced Activation of ERK1/2, p38 MAPK, and Protein Kinase B Signaling in Vascular Smooth Muscle Cells

ANTOINE BLANC, NIHAR R. PANDEY, and ASHOK K. SRIVASTAVA

ABSTRACT

We have shown earlier that extracellular signal-regulated kinases 1 and 2 (ERK1/2) and protein kinase B (PKB), two key mediators of growth-promoting and proliferative responses, are activated by hydrogen peroxide (H_2O_2) in A10 vascular smooth muscle cells (VSMC). In the present studies, using a series of pharmacological inhibitors, we explored the upstream mechanisms responsible for their activation in response to H_2O_2. H_2O_2 treatment of VSMC stimulated ERK1/2, p38 mitogen-activated protein kinase (MAPK), and PKB phosphorylation in a dose- and time-dependent fashion. BAPTA-AM and EGTA, chelators of intracellular and extracellular Ca^{2+}, respectively, inhibited H_2O_2-stimulated ERK1/2, p38 MAPK, and PKB phosphorylation. Fluphenazine, an antagonist of the Ca^{2+}-binding protein calmodulin, also suppressed the enhanced phosphorylation of ERK1/2, p38 MAPK, and PKB. In contrast, the protein kinase C (PKC) inhibitors Gö 6983 and Rö 31-8220 attenuated H_2O_2-induced ERK1/2 phosphorylation, but had no effect on p38 MAPK and PKB phosphorylation. Taken together, these data demonstrate that the activation of Ca^{2+}/calmodulin-dependent pathways represents a key component mediating the stimulatory action of H_2O_2 on ERK1/2, p38 MAPK, and PKB phosphorylation. On the other hand, PKC appears to be an upstream modulator of the increased ERK1/2 phosphorylation, but not of p38 MAPK and PKB in response to H_2O_2 in VSMC. *Antioxid. Redox Signal.* 6, 353–366.

INTRODUCTION

INCREASED PRODUCTION OF REACTIVE OXYGEN SPECIES (ROS) has been suggested to contribute to the pathogenesis of conditions such as atherosclerosis, cancer, diabetes, and cardiac abnormalities (22, 23, 59). The main site of ROS generation is believed to be the mitochondria, which convert ~1–2% of consumed molecular oxygen to superoxide anion (O_2^-) (13). O_2^- dismutation by superoxide dismutase produces hydrogen peroxide (H_2O_2), which is finally converted to H_2O by catalase or other ROS scavenging systems (12, 59). H_2O_2 generation has been shown to be essential in triggering the cellular responses of several growth factors, peptide hormones, and cytokines (9, 31, 39–41, 62). ROS have also been demonstrated to serve as important mediators of ischemic preconditioning of cardiac system injury (8, 24). The precise mechanism by which ROS generation contributes to this response remains poorly characterized, but recent work has suggested an important role of mitogen-activated protein kinases (MAPKs) in the process (20, 42).

MAPKs are serine/threonine protein kinases that are activated in response to various external stimuli, including growth factors, hormones, and stress. MAPKs have been classified into several subfamilies: extracellular signal-regulated kinases 1 and 2 (ERK1/2), p38 MAPK, c-Jun NH_2-terminal kinase/stress-activated protein kinase (JNK/SAPK), ERK3/4, and ERK 5

Centre de recherche, Centre hospitalier de l'Université de Montréal (CHUM)—Hôtel-Dieu and Department of Medicine, Université de Montréal, Montréal, Québec, Canada.

(for review, see 68). MAPKs are activated by dual phosphorylation on both tyrosine and threonine residues by dual specificity protein kinases known as MAPKK or MEK (mitogen extracellular signal-regulated kinase kinase) (58). In addition to MAPK, another serine/threonine kinase, protein kinase B (PKB), also known as Akt (a product of akt protooncogene) (11), has also been shown to play a key role in a myriad of physiological processes, including cell growth, gene expression, cell survival and death, protein synthesis, and glucose transport (21). Three isozymic forms of PKB have been identified, and all of them are activated by dual phosphorylation on threonine (Thr^{308}) and serine (Ser^{473}) (6). Phosphorylation of PKB/Akt in Thr^{308} is catalyzed by phosphatidylinositol 3,4,5-trisphosphate (PIP3)-dependent protein kinase 1 (PDK-1) (7). The protein kinase responsible for catalyzing Ser^{473} phosphorylation remains elusive, but is tentatively referred to as PDK-2 (7). PIP3 is generated in a reaction catalyzed by phosphatidylinositol 3-kinase (PI3-K).

PI3-Ks are heterodimeric enzymes composed of an 85-kDa (p85) regulatory subunit and a 110-kDa (p110) catalytic subunit. The p85 subunit contains the src homology-2 (SH-2) domain and is able to interact with phosphorylated tyrosine residues on receptor or other docking proteins, leading to the stimulation of the 110-kDa catalytic subunit. The catalytic subunit catalyzes the phosphorylation of phosphatidylinositol (PI), PI 4-phosphate, and PI 4,5-bisphosphate in the 3′ position of the inositol ring to generate PI 3-phosphate, PI 3,4-bisphosphate, and PI 3,4,5-trisphosphate, respectively (26). PI3-Ks are activated in response to growth factors and other agents, including vasoactive peptides (26, 36).

H_2O_2 has been demonstrated to activate both MAPKs and PKB signaling systems in a variety of cell types, including vascular smooth muscle cells (VSMC) (10, 60, 65, 66) as well as cardiomyocytes (5, 17). However, the upstream mechanism responsible for H_2O_2-induced MAPK and PKB activation remains largely unclear. Ca^{2+}-dependent pathways are known to play a critical role in mediating the effects of vasoactive peptides such as angiotensin II (AII) (15, 64), which in turn leads to H_2O_2 generation (31). In addition, activation of protein kinase C (PKC) has been implicated in mediating some of the responses of vasoactive peptides and H_2O_2 (3). PKC is a serine/ threonine protein kinase comprised of at least 11 isozymic forms (38, 48, 49). These isozymic forms have been classified as classical, novel, and atypical. Classical PKCs (α, βI, βII, and γ) are activated by Ca^{2+}, diacylglycerol (DAG), phosphatidylserine (PS), and the tumor promoter, phorbol 12-myristate 13-acetate. Novel PKCs (δ, ε, η, μ, and ο) are activated by DAG, PS, and unsaturated fatty acids, whereas atypical PKCs (ζ, λ, and τ) are insensitive to DAG, but are activated by PS and phosphatidylinositides (for reviews, see 38, 48, 49). Recent studies have shown that H_2O_2 treatment activated several isoforms of PKC by tyrosine phosphorylation (35). In addition, both PKC-dependent and -independent pathways have been implicated in the mechanism leading to enhanced ERK1/2 and p38 MAPK phosphorylation in response to various agonists, including H_2O_2 in several cell types (18, 19, 51, 53, 63, 69). However, the possible role of PKC in PKB phosphorylation remains uncharacterized. The present studies were designed to investigate the potential involvement of Ca^{2+}, calmodulin (CaM), and PKC in H_2O_2-induced ERK1/2, p38 MAPK, and PKB activation in A10 VSMC.

MATERIALS AND METHODS

Materials

Phospho-specific antibodies to ERK1/2 (Thr^{202}/Tyr^{204}), p38 MAPK (Thr^{180}/Tyr^{182}), and PKB (Ser^{473}) and total ERK1/2 and total p38 MAPK antibodies were obtained from New England Biolabs (Beverly, MA, U.S.A.). Total PKB antibody was purchased from Santa Cruz Biosciences (Santa Cruz, CA, U.S.A.). EGTA, BAPTA-AM [1,2-bis(2-aminophenoxy) ethane-*N,N,N′,N′*-tetraacetic acid tetrakis(acetoxymethylester)], fluphenazine, Rö 31-8220 and Gö 6983 were procured from Calbiochem (San Diego, CA, U.S.A.), and H_2O_2 from Anachemia Canada Inc. (Montreal, Quebec, Canada). All other chemicals were from either Sigma Chemical Co. (St. Louis, MO, U.S.A.) or other commercial sources (50).

Cell culture

A10 VSMC were maintained in Dulbecco's modified Eagle medium containing 10% fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 μg/ml amphotericin B in a humidified atmosphere of 5% CO_2 exchange at 37°C (60). The cells were passaged twice a week by harvesting with trypsin/EDTA from Invitrogen Corp. (Grand Island, NY, U.S.A.).

Stimulation of VSMC

VSMC were grown to 80–90% confluence in 60-mm culture plates and incubated in serum-free medium at least 20 h prior to the experiments. On the day of the experiment, they were stimulated in serum-free Dulbecco's modified Eagle medium with the indicated concentrations of H_2O_2 for the indicated time periods in the absence or presence of various pharmacological agents. After incubation, the cells were rapidly washed three times with ice-cold Krebs/calcium buffer (119 m*M* NaCl, 4.7 m*M* KCl, 1.25 m*M* $CaCl_2$, 1.2 m*M* $MgCl_2$, 1.2 m*M* KH_2PO_4, 1.2 m*M* $MgSO_4 \cdot 7H_2O$, and 25 m*M* HEPES, pH 7.4) and frozen immediately in liquid nitrogen. They were then scraped on ice in 100 μl of lysis buffer [25 m*M* Tris-HCl, pH 7.4, 25 m*M* NaCl, 10 m*M* $Na_4P_2O_7$, 10 m*M* NaF, 2 m*M* EGTA, 2 m*M* EDTA, 2% (vol/vol) Triton X-100, 0.1% (vol/vol) sodium dodecyl sulfate (SDS), 1 m*M* orthovanadate, 2 m*M* benzamidine, 1 m*M* phenylmethylsulfonylfluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin] and clarified by centrifugation at 14,000 *g* for 10 min at 4°C.

ERK1/2, p38 MAPK, and PKB detection by immunoblotting

Clarified lysates containing equal amounts of protein, as determined by Bradford's method (14), were separated on 12% SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidine difluoride membranes. The membranes were blocked for 1 h with phosphate-buffered saline–Tween 20 (PBS-T) containing 5% bovine serum albumin at room temperature and washed three times with PBS-T at room temperature. The blots were incubated overnight with anti-phospho-specific ERK1/2, p38 MAPK, or PKB antibodies (1:2,000, 1:1,000, and 1:1,000 dilution, respectively) in 5% bovine serum

albumin in PBS-T buffer at 4°C followed by three washings with PBS-T buffer at room temperature. After incubation with secondary antibodies (horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibody at 1:2,000 dilution), the signal was detected by the enhanced chemiluminescence method (ECL kit from Amersham Biosciences, Little Chalfont, Buckinghamshire, U.K.). The same blots were subsequently reprobed with anti-ERK1/2, anti-p38 MAPK, or anti-PKB antibodies

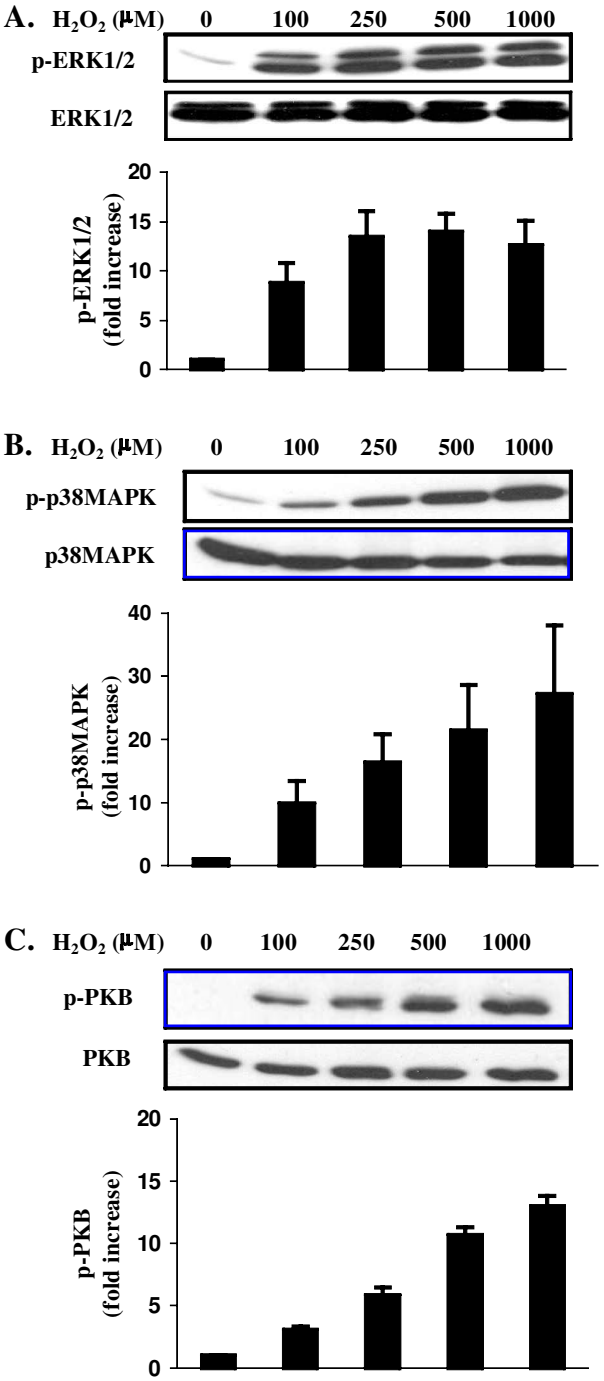

FIG. 1. H_2O_2-induced dose responses of ERK1/2, p38 MAPK, and PKB phosphorylation in A10 VSMC. Serum-starved quiescent A10 VSMC were treated with the indicated H_2O_2 concentrations for 10 min. Cell lysates were prepared, and equal amount of protein was separated on 12% SDS-PAGE. ERK1/2, p38 MAPK, and PKB phosphorylation was detected by immunoblot analysis with phospho-specific ERK1/2, p38 MAPK, and PKB antibodies (**top panels** of each section). Blots were also analyzed for total ERK1/2, p38 MAPK, and PKB as shown in **middle panels** of each section. **Bottom panels** represent average data quantified by densitometric scanning of immunoblots from top panels expressed as a fold increase in phosphorylation above control, which was arbitrarily set at 1. (**A**) ERK1/2 phosphorylation. (**B**) p38 MAPK phosphorylation. (**C**) PKB phosphorylation. Values are the means ± SE of at least three independent experiments.

to detect the total amount of these proteins. The band intensities were quantified by densitometry of immunoblots using the NIH Image software program.

Data analysis

The data presented here are means ± SE of at least three individual experiments. Statistical significance was determined with paired or unpaired one-tailed Student's *t* test.

RESULTS

Characterization of H_2O_2-induced ERK1/2, p38 MAPK, and PKB phosphorylation

The effect of H_2O_2 on ERK1/2, p38 MAPK, and PKB activation was kinetically characterized with respect to dose and time of incubation. ERK1/2, p38 MAPK, and PKB activation was studied by following their phosphorylation status using phospho-specific antibodies. H_2O_2 treatment of A10 VSMC caused a dose-dependent increase in ERK1/2 (Fig. 1A), p38 MAPK (Fig. 1B), and PKB (Fig. 1C) phosphorylation. Increments in the phosphorylation of all three kinases were detected at the lowest H_2O_2 concentration used (Fig. 1). However, the increase in ERK1/2 phosphorylation appeared to level off between 250 and 500 μ*M* H_2O_2 (Fig. 1A), whereas p38 MAPK and PKB phosphorylation peaked at ~1,000 μ*M* (Fig. 1B and C).

Time-course analysis using 250 μ*M* H_2O_2 revealed a rapid rise in the ERK1/2, p38 MAPK, as well as PKB phosphorylation, which was detectable at 5 min, the earliest time point tested (Fig. 2). However, the time course of this increase was distinct for each of the kinases. ERK1/2 phosphorylation, which peaked at ~10 min, persisted for up to 30 min and then declined to submaximal levels within 120 min (Fig. 2A). p38 MAPK phosphorylation was biphasic, peaking at 10 min of treatment and declining to almost 40% of maximal phosphorylation within 30 min. However, the continued presence of H_2O_2 for 60–120 min fully restored p38 MAPK phosphorylation to the peak observed at 10 min (Fig. 2B). In contrast to the effect of H_2O_2 on ERK1/2 and p38 MAPK, the PKB phosphorylation in response to H_2O_2 was transient. It peaked at 10 min and then slowly declined to just above the baseline by 120 min (Fig. 2C).

Role of Ca^{2+} in H_2O_2-induced ERK1/2, p38 MAPK, and PKB phosphorylation

Recent studies have demonstrated that H_2O_2 can influence Ca^{2+} homeostasis in many cell types, including VSMC (55), and as Ca^{2+} is believed to mediate some of the signaling events triggered by H_2O_2 (52), we assessed its role in H_2O_2-induced ERK1/2, p38 MAPK, and PKB phosphorylation. Pretreatment of VSMC with different concentrations of EGTA, a chelator of extracellular Ca^{2+}, resulted in dose-dependent inhibition of the H_2O_2-induced ERK1/2 (Fig. 3A), p38 MAPK (Fig. 3B), and PKB (Fig. 3C) phosphorylation. As low a dose as 2.5 m*M* EGTA caused >60% inhibition (Fig. 3) of this response, with further suppression of ERK1/2 and PKB to ~80% by 5 m*M* EGTA (Fig. 3A and C). Like EGTA, VSMC pretreatment with BAPTA-AM, a chelator of intracellular Ca^{2+}, attenuated H_2O_2-stimulated phosphorylation of all three kinases in a dose-dependent fashion (Fig. 4). The inhibitory effect of BAPTA-AM was more pronounced on p38 MAPK and PKB than on ERK1/2. At 25 μ*M*, BAPTA-AM reduced ERK1/2 phosphorylation by 20%, whereas >60% suppression of H_2O_2-induced p38 MAPK and PKB phosphorylation was observed at this concentration (Fig. 4B and C). However, a higher concentration of this Ca^{2+} chelator decreased the phosphorylation of all three kinases by >70% in response to H_2O_2 (Fig. 4). The data clearly indicated a key role of both extracellular and intracellular Ca^{2+} in mediating the H_2O_2 effect on the kinases.

Role of CaM in H_2O_2-induced ERK1/2, p38 MAPK, and PKB phosphorylation

As the Ca^{2+}-binding protein CaM is known to mediate many of the responses of Ca^{2+} (43, 44), and it has been shown to modulate the AII-induced effects on ERK1/2 activation (28), we investigated if CaM contributed to H_2O_2-induced enhanced ERK1/2, p38 MAPK, and PKB phosphorylation in VSMC. Pretreatment of VSMC with fluphenazine, a selective irreversible antagonist of CaM, caused a dose-dependent inhibition of H_2O_2-induced ERK1/2, p38 MAPK, and PKB phosphorylation (Fig. 5). At 25 μ*M*, fluphenazine inhibited H_2O_2-stimulated phosphorylation of all three kinases by ~80%. Increasing its concentration to 50 μ*M* resulted in virtually complete blockade of the H_2O_2 response.

Role of PKC in H_2O_2-induced ERK1/2, p38 MAPK, and PKB phosphorylation

A possible involvement of PKC-dependent pathways in H_2O_2-induced phosphorylation of ERK1/2, p38 MAPK, and PKB in A10 VSMC was examined by using two broad-spectrum pharmacological inhibitors of PKC, Gö 6983 (32) and Rö 31-8220 (61). Pretreatment with Gö 6983 caused dose-dependent inhibition in H_2O_2-induced ERK1/2 phosphorylation (Fig. 6A). At 1 μ*M*, it elicited ~40% inhibition of ERK1/2 phosphorylation, which was synergized to ~80% by increasing the inhibitor dose to 10 μ*M* (Fig. 6A). In contrast to the inhibitory effect on ERK1/2 phosphorylation, Gö 6983 did not attenuate the stimulatory influence of H_2O_2 on p38 MAPK or PKB phosphorylation (Fig. 6B and C). In fact, it potentiated the effect of H_2O_2 on p38 MAPK phosphorylation (Fig. 6B). Like Gö 6983, Rö 31-8220 attenuated the ERK1/2 phosphorylation induced by H_2O_2 (Fig. 7A), but had no impact on p38 MAPK or PKB phosphorylation (Fig. 7B and C).

DISCUSSION

In these studies, we have established that H_2O_2 treatment of A10 VSMC causes a rapid and concurrent increase of ERK1/2, p38 MAPK, and PKB phosphorylation in a dose- and time-dependent fashion. Although the stimulatory effect of H_2O_2 on ERK1/2, p38 MAPK, and PKB phosphorylation has been reported earlier in VSMC, to the best of our knowledge, our experiments are the first to demonstrate simultaneous and concurrent enhancement in the phosphorylation of these key

signaling kinases by H_2O_2 in A10 VSMC. By using pharmacological chelators of both intracellular and extracellular Ca^{2+}, we showed an absolute requirement of Ca^{2+} for H_2O_2-induced effects on the phosphorylation of three signaling molecules. We also found that the Ca^{2+}-binding protein CaM was essential to trigger the stimulatory response to H_2O_2 in the above signaling intermediates. H_2O_2-induced changes in Ca^{2+} levels have been observed in many cell types, including cardiomyocytes (27) and VSMC (30). Moreover, Ca^{2+} involvement in mediating ERK1/2 phosphorylation in response

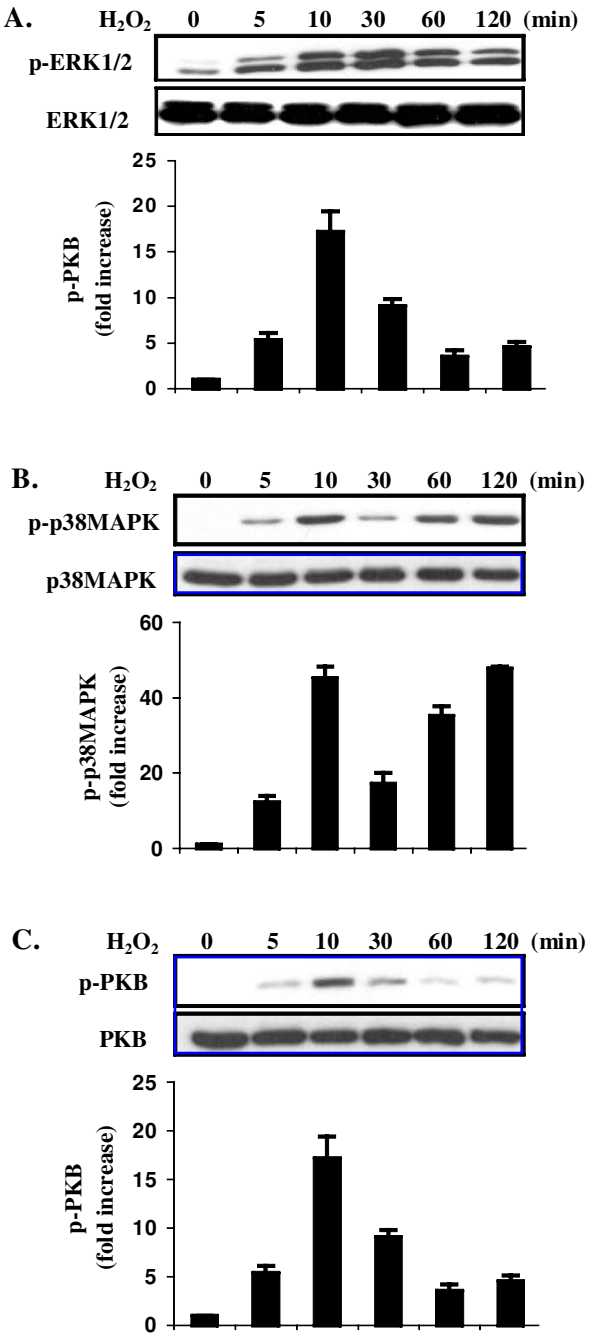

FIG. 2. Time course of H_2O_2-induced ERK1/2, p38 MAPK, and PKB phosphorylation in A10 VSMC. Serum-starved quiescent A10 VSMC were treated with 250 μM H_2O_2 for the indicated times. Cell lysates were prepared, and equal amount of protein was separated on 12% SDS-PAGE. ERK1/2, p38 MAPK, and PKB phosphorylation was detected by immunoblot analysis with phospho-specific ERK1/2, p38 MAPK, and PKB antibodies (**top panels** of each section). Blots were also analyzed for total ERK1/2, p38 MAPK, and PKB as shown in **middle panels** of each section. **Bottom panels** represent average data quantified by densitometric scanning of immunoblots from top panels expressed as a fold increase in phosphorylation above control, which was arbitrarily set at 1. (**A**) ERK1/2 phosphorylation. (**B**) p38 MAPK phosphorylation. (**C**) PKB phosphorylation. Values are the means ± SE of at least three independent experiments.

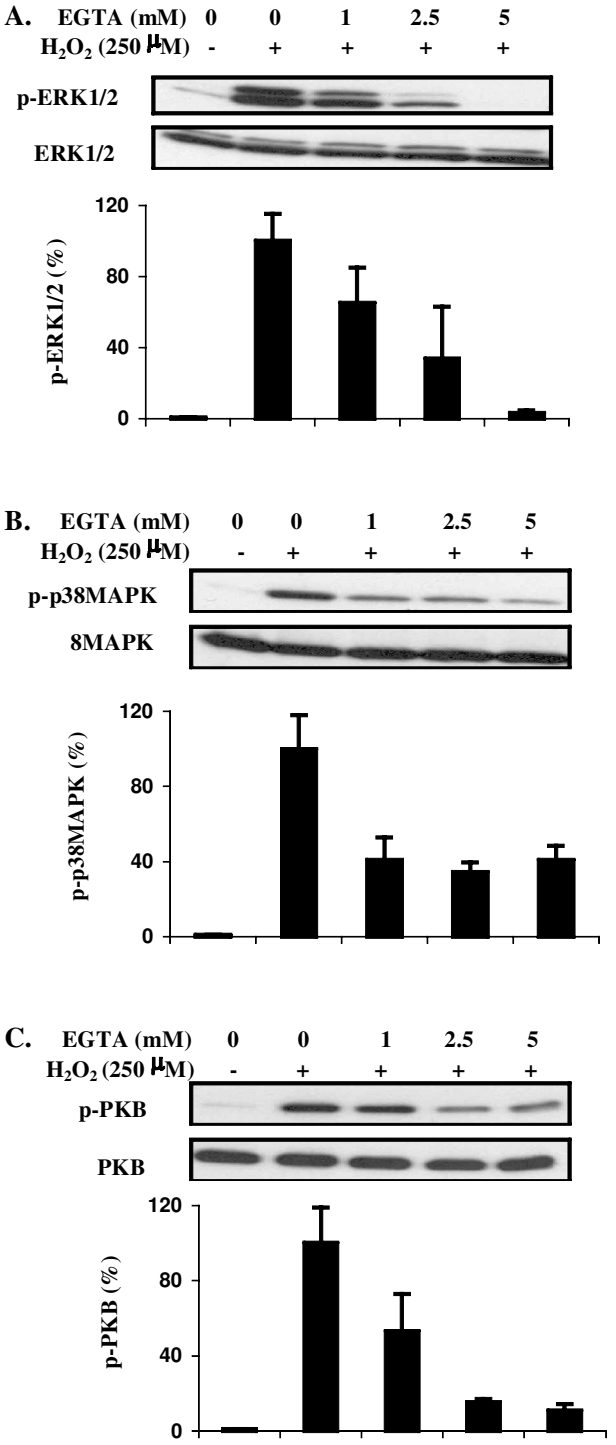

FIG. 3. Dose-dependent effect of the extracellular Ca^{2+} chelator EGTA on H_2O_2-induced ERK1/2, p38 MAPK, and PKB phosphorylation in A10 VSMC. Serum-starved quiescent A10 VSMC were treated with or without the indicated EGTA concentrations for 30 min followed by 250 μM H_2O_2 for 10 min. Cell lysates were prepared, and equal amount of protein was separated on 12% SDS-PAGE. ERK1/2, p38 MAPK, and PKB phosphorylation was detected by immunoblot analysis with phospho-specific ERK1/2, p38 MAPK, and PKB antibodies (**top panels** of each section). Blots were also analyzed for total ERK1/2, p38 MAPK, and PKB as shown in **middle panels** of each section. **Bottom panels** represent average data quantified by densitometric scanning of immunoblots from top panels, expressed as % phosphorylation where H_2O_2-induced phosphorylation (without inhibitor) was arbitrarily set at 100%. (**A**) ERK1/2 phosphorylation. (**B**) p38 MAPK phosphorylation. (**C**) PKB phosphorylation. Values are the means ± SE of at least three independent experiments.

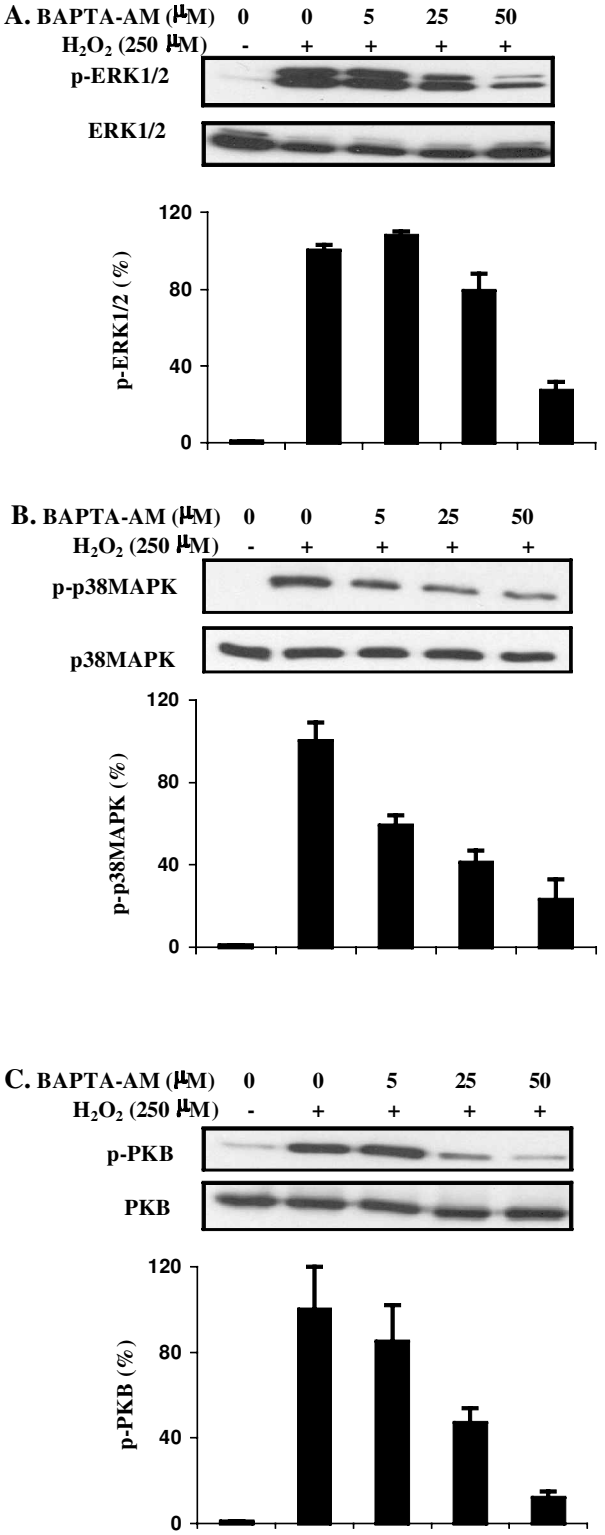

FIG. 4. Dose-dependent effect of the intracellular Ca^{2+} chelator BAPTA-AM on H_2O_2-induced ERK1/2, p38 MAPK, and PKB phosphorylation in A10 VSMC. Serum-starved quiescent A10 VSMC were treated with or without the indicated BATA-AM concentrations for 30 min followed by 250 μ*M* H_2O_2 for 10 min. Cell lysates were prepared, and equal amount of protein was separated on 12% SDS-PAGE. ERK1/2, p38 MAPK, and PKB phosphorylation was detected by immunoblot analysis with phospho-specific ERK1/2, p38 MAPK, and PKB antibodies (**top panels** of each section). Blots were also analyzed for total ERK1/2, p38 MAPK, and PKB as shown in **middle panels** of each section. **Bottom panels** represent average data quantified by densitometric scanning of immunoblots from top panels, expressed as % phosphorylation where H_2O_2-induced phosphorylation (without inhibitor) was arbitrarily set at 100%. (**A**) ERK1/2 phosphorylation. (**B**) p38 MAPK phosphorylation. (**C**) PKB phosphorylation. Values are the means ± SE of at least three independent experiments.

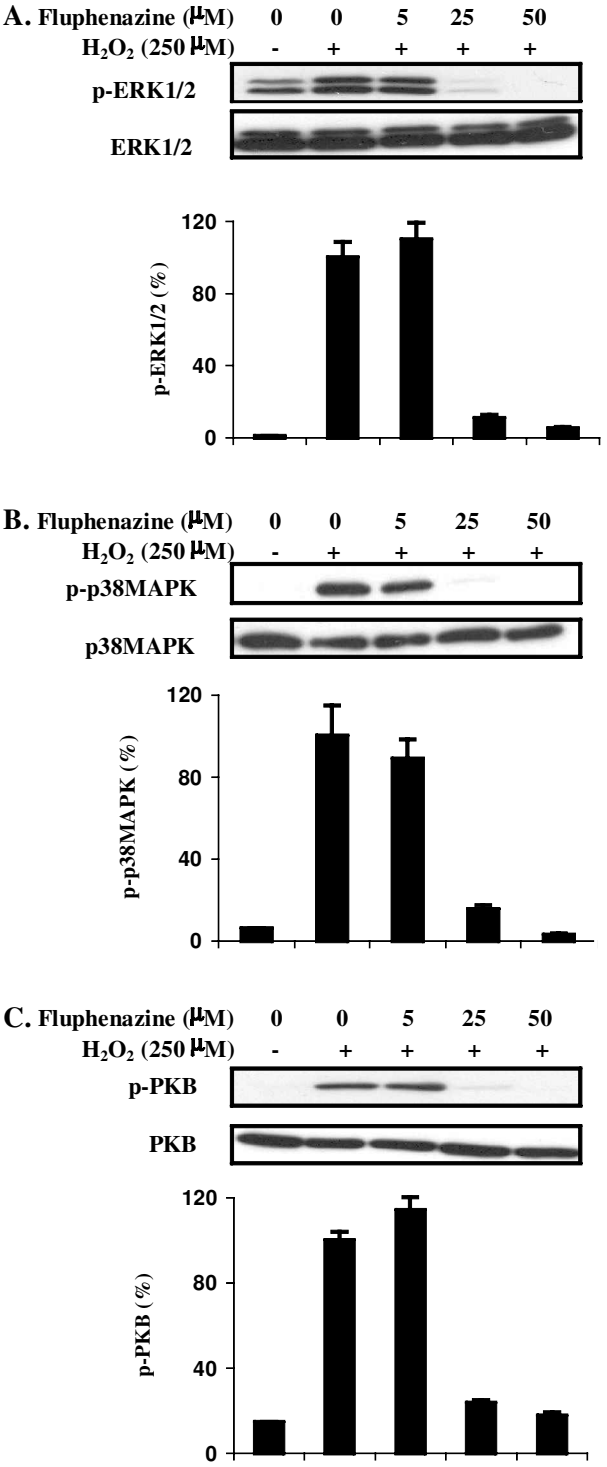

FIG. 5. Dose-dependent effect of fluphenazine, a CaM antagonist, on H_2O_2-induced ERK1/2, p38 MAPK, and PKB phosphorylation in A10 VSMC. Serum-starved quiescent A10 VSMC were treated with or without the indicated fluphenazine concentrations for 30 min followed by 250 μ*M* H_2O_2 for 10 min. Cell lysates were prepared, and equal amount of protein was separated on 12% SDS-PAGE. ERK1/2, p38 MAPK, and PKB phosphorylation was detected by immunoblot analysis with phospho-specific ERK1/2, p38 MAPK, and PKB antibodies (**top panels** of each section). Blots were also analyzed for total ERK1/2, p38 MAPK, and PKB as shown in **middle panels** of each section. **Bottom panels** represent average data quantified by densitometric scanning of immunoblots from top panels, expressed as % phosphorylation where H_2O_2-induced phosphorylation (without inhibitor) was arbitrarily set at 100%. (**A**) ERK1/2 phosphorylation. (**B**) p38 MAPK phosphorylation. (**C**) PKB phosphorylation. Values are the means ± SE of at least three independent experiments.

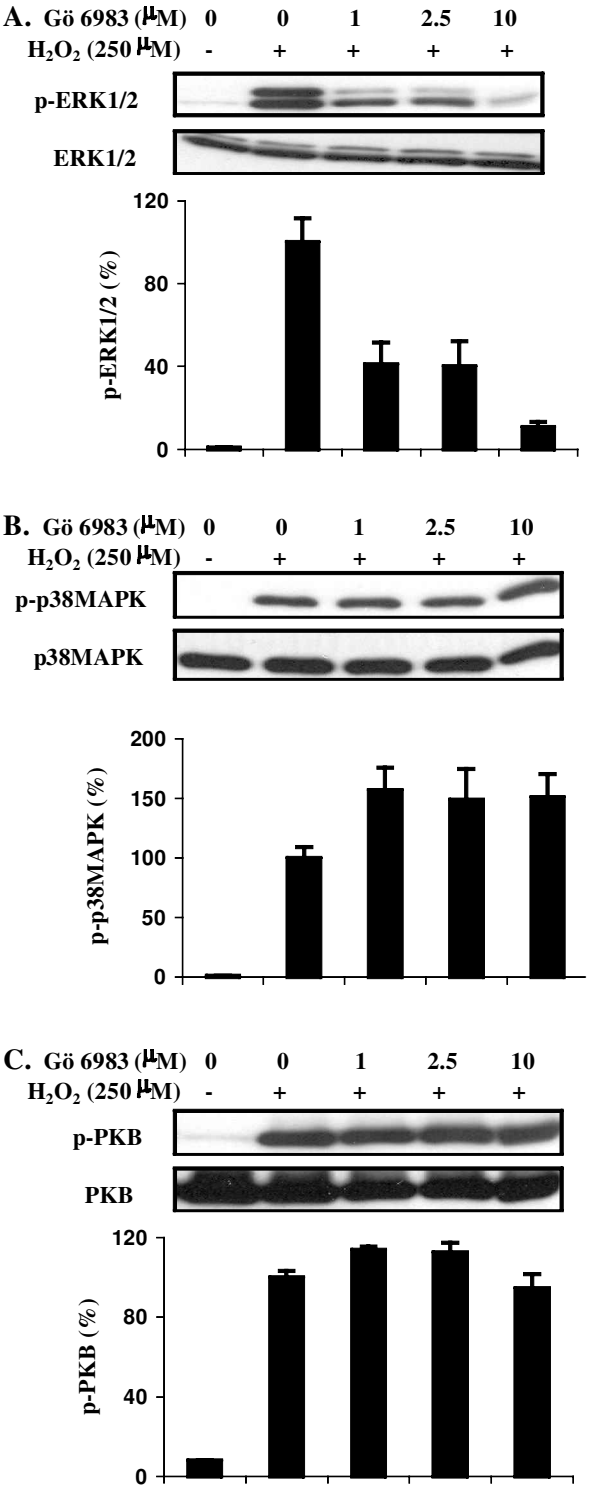

FIG. 6. Dose-dependent effect of the PKC inhibitor Gö 6983 on H_2O_2-induced ERK1/2, p38 MAPK, and PKB phosphorylation in A10 VSMC. Serum-starved quiescent A10 VSMC were treated with or without the indicated Gö 6983 concentrations for 30 min followed by 250 μ*M* H_2O_2 for 10 min. Cell lysates were prepared, and equal amount of protein was separated on 12% SDS-PAGE. ERK1/2, p38 MAPK, and PKB phosphorylation was detected by immunoblot analysis with phospho-specific ERK1/2, p38 MAPK, and PKB antibodies (**top panels** of each section). Blots were also analyzed for total ERK1/2, p38 MAPK, and PKB as shown in **middle panels** of each section. **Bottom panels** represent average data quantified by densitometric scanning of immunoblots from top panels, expressed as % phosphorylation where H_2O_2-induced phosphorylation (without inhibitor) was arbitrarily set at 100%. (**A**) ERK1/2 phosphorylation. (**B**) p38 MAPK phosphorylation. (**C**) PKB phosphorylation. Values are the means ± SE of at least three independent experiments.

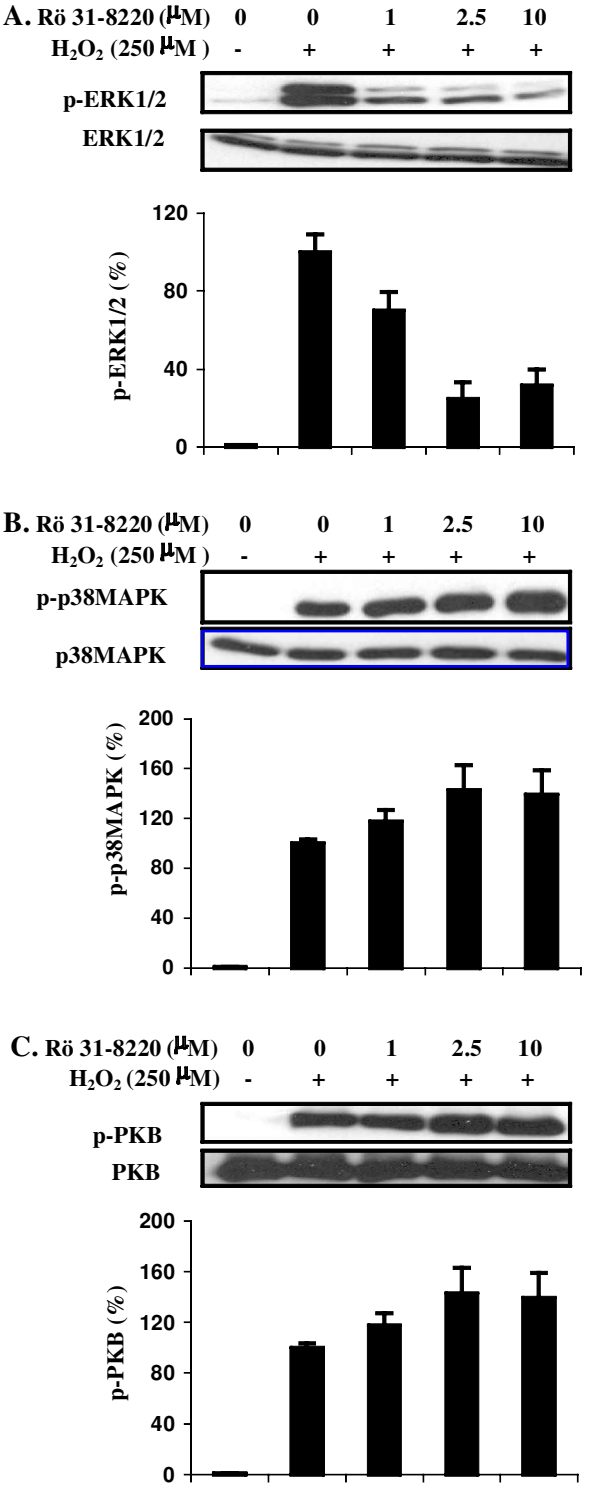

FIG. 7. Dose-dependent effect of the PKC inhibitor Rö 31-8220 on H_2O_2-induced ERK1/2, p38 MAPK, and PKB phosphorylation in A10 VSMC. Serum-starved quiescent A10 VSMC were treated with or without the indicated Rö 31-8220 concentrations for 30 min followed by 250 μ*M* H_2O_2 for 10 min. Cell lysates were prepared, and equal amount of protein was separated on 12% SDS-PAGE. ERK1/2, p38 MAPK, and PKB phosphorylation was detected by immunoblot analysis with phospho-specific ERK1/2, p38 MAPK, and PKB antibodies (**top panels** of each section). Blots were also analyzed for total ERK1/2, p38 MAPK, and PKB as shown in **middle panels** of each section. **Bottom panels** represent average data quantified by densitometric scanning of immunoblots from top panels, expressed as % phosphorylation where H_2O_2-induced phosphorylation (without inhibitor) was arbitrarily set at 100%. (**A**) ERK1/2 phosphorylation. (**B**) p38 MAPK phosphorylation. (**C**) PKB phosphorylation. Values are the means ± SE of at least three independent experiments.

to various agonists (57) and H_2O_2 (46) has been reported. As Ca^{2+} exerts some of its effects through CaM, it is possible that Ca^{2+}/CaM, through the activation of Ca^{2+}/CaM-dependent protein kinase II (CaM kinase II), may trigger the stimulatory action of H_2O_2 on ERK1/2, p38 MAPK, and PKB phosphorylation in A10 VSMC. This notion is supported by the recent studies in which KN-93, a selective inhibitor of CaM kinase II, attenuated AII-induced ERK1/2 phosphorylation (4). Furthermore, PYK-2, a proline-rich nonreceptor tyrosine kinase, has been implicated as an upstream modulator of AII-induced ERK1/2 and PKB activation (56). Interestingly, like AII, H_2O_2 was recently shown to stimulate PYK-2 phosphorylation and activation in VSMC (25). As PYK-2 phosphorylation in response to ionomycin, a Ca^{2+} ionophore, is dependent on CaM kinase II (28), it is suggested that CaM kinase II may play a key role in H_2O_2-induced ERK1/2, p38 MAPK, and PKB phosphorylation observed in the present studies. However, whether H_2O_2 activates CaM kinase II in A10 VSMC remains to be seen.

Our findings that broad-spectrum PKC inhibitors blocked H_2O_2-stimulated ERK1/2 activation in A10 VSMC indicate an upstream role of PKC in this process and are in agreement with the studies of Abe *et al.* (3) and Zhang *et al.* (69), who have demonstrated a requirement of PKC in H_2O_2-induced ERK1/2 phosphorylation/activation in pulmonary arterial tracheal smooth muscle cells. In contrast to these studies, however, the PKC inhibitor calphostin C had no effect on H_2O_2-stimulated ERK activity in neonatal cardiocytes (5). Thus, it is possible that H_2O_2-mediated responses are modulated in a cell-specific manner, and both PKC-independent and -dependent pathways might be implicated in this response. The fact that PKC inhibition did not decrease p38 MAPK and PKB phosphorylation indicates that there is a divergence of signaling at the level of PKC, and that PKC activation is critical only for the stimulation of the ERK1/2 pathway. PKC activation by H_2O_2 may be achieved by two mechanisms: directly by H_2O_2-induced tyrosine phosphorylation (35), and indirectly by tyrosine phosphorylation of phospholipase C-γ-1. Tyrosine-phosphorylated and activated phospholipase C-γ-1 hydrolyzes PI 4,5-bisphosphate, generating two second messengers, DAG and inositol 1,4,5-trisphosphate. DAG activates some isoenzymic forms of PKC and inositol 1,4,5-trisphosphate releases Ca^{2+} from intracellular stores (67).

The underlying mechanisms responsible for MAPKs or PKB activation in response to H_2O_2 in VSMC have not been fully characterized. However, there are reports demonstrating increased transactivation of epidermal growth factor (54) and platelet-derived growth factor-receptor protein tyrosine kinase (PTK) (34), as well as the Src family of PTKs, such as c-src and fyn in cells treated with H_2O_2 (1, 2). H_2O_2 has also been shown to inhibit the activities of protein tyrosine phosphatases (PTPases) such as PTP-1B (37), and SHP-2 (45). PTPase inhibition is accomplished by oxidation of catalytically essential cysteine residue in the active site of PTPase (33). Inhibition of cellular PTPases shifts the equilibrium of the phosphorylation–dephosphorylation cycle by promoting a burst in PTK activity, resulting in an increase in the tyrosyl phosphorylation of substrate proteins. These tyrosyl phosphorylated proteins promote the assembly of signaling modules responsible for activating various components of the MAPKs and PI3-K signaling pathway. The activated MAPKs phosphorylate downstream regulatory target proteins such as $p90^{rsk}$ and several transcription factors, leading to increased protein synthesis and gene expression (12). Similarly, PIP3-induced downstream targets of PKB, such as mTOR, $p70^{s6k}$, glycogen synthase kinase-3 (GSK-3), forkhead transcription factor (FKHR), BAD, caspase, and some forms of PKC, are critical effectors of cellular growth responses, including cell-cycle entry, cell division, growth, gene expression, and survival (16, 36). Thus, H_2O_2-induced inhibition of PTPase may serve as a triggering mechanism to activate these signaling events (12).

In summary, our studies show that H_2O_2 stimulates ERK1/2, p38 MAPK, and PKB signaling pathways in A10 VSMC in a Ca^{2+}- and CaM-dependent manner. In contrast, PKC appears to mediate only ERK1/2 activation in these cells. The activation of this pathway may potentially contribute to ROS-induced effects on VSMC growth, hypertrophy, and survival.

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ABBREVIATIONS

AII, angiotensin II; BAPTA-AM, 1,2-bis(2-aminophenoxy) ethane-*N,N,N′,N′*-tetraacetic acid tetrakis(acetoxymethylester); CaM, calmodulin; CaM kinase II, Ca^{2+}/calmodulin-dependent protein kinase II; DAG, diacylglycerol; ERK1/2, extracellular signal-regulated kinase 1 and 2; H_2O_2, hydrogen peroxide; MAPK, mitogen-activated protein kinase; PAGE, polyacrylamide gel electrophoresis; PBS-T, phosphate-buffered saline–Tween 20; PDK-1,2, phosphoinositide-dependent protein kinase 1 and 2; PI, phosphatidylinositol; PI3-K, phosphatidylinositol 3-kinase; PIP3, phosphatidylinositol 3,4,5-trisphosphate; PKB, protein kinase B; PKC, protein kinase C; PS, phosphatidylserine; PTK, protein tyrosine kinase; PTPase, protein tyrosine phosphatase; PYK-2, proline-rich nonreceptor tyrosine kinase 2; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; VSMC, vascular smooth muscle cell(s).

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Address reprint requests to:
Ashok K. Srivastava, Ph.D.
Centre de recherche
CHUM–Hôtel-Dieu (7-135)
3840, rue St-Urbain
Montréal (Québec) H2W 1T7, Canada.

E-mail: ashok.srivastava@umontreal.ca

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