MAPK and PKC activity are not required for H$_2$O$_2$-induced arterial muscle contraction


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Hydrogen peroxide concentrations within the range shown to cause contraction have been reported in vivo in the pathogenesis of a variety of conditions. Patients with exacerbated chronic obstructive pulmonary disease (COPD) (10) and asthmatic children (18) exhale micromolar concentrations of hydrogen peroxide. Non-survivors of adult respiratory distress syndrome (ARDS) were reported to have higher levels of oxidative stress than do survivors (44). Vasconstriction due to increased hydrogen peroxide levels likely contributes to the impaired perfusion reported in these pathological conditions. Thus it has become important to address the possibility that oxidant stimulation of the vascular muscle could play a role in pulmonary disease such as COPD, ARDS, and asthma.

The possibility that oxidants such as hydrogen peroxide may act as normal physiological signals was introduced in 1989 (23). Evidence from both plant and animal cells suggest that hydrogen peroxide may act as an intracellular second messenger (37). Recently, oxidants were shown to be released by vascular smooth muscle cells in response to angiotensin II stimulation, whereas catalase loading of the cells inhibited the response to angiotensin II (41), suggesting a signal transduction cascade that requires hydrogen peroxide as a second messenger. Therefore, it is important to elucidate the cellular mechanism of arterial smooth muscle tension development in response to hydrogen peroxide.

Smooth muscle contraction is normally thought to be regulated by Ca$^{2+}$ and involves reversible phosphorylation of the 20-kDa regulatory light chain subunit of myosin, MLC$_{20}$, by the Ca$^{2+}$/calmodulin-dependent myosin light chain kinase (MLCK) (5). However, hydrogen peroxide was reported to contract rabbit carotid artery (14) and rat pulmonary artery, even in calcium-free solution and in the absence of endothelium (29). Studies on permeabilized arterial muscle showed that ion fluxes across smooth muscle cell membranes are not required for the hydrogen peroxide-induced contraction.
traction (27). Furthermore, the same investigation showed that hydrogen peroxide causes vascular smooth muscle contraction even as myosin light chain phosphorylation declines to near-zero levels. These results suggest that an alternative regulatory mechanism must be involved.

Artificial activation of PKC by phorbol esters leads to smooth muscle contraction without a significant increase in intracellular free Ca\(^{2+}\) (36). Studies of particular relevance have shown that oxidants stimulate PKC (30, 39). H-7, a PKC inhibitor, reduces the contractile response to H\(_2\)O\(_2\) (17, 31). However, PKC inhibitors are nonspecific. H-7 is known to inhibit PKA, PKG, and MLCK as well as PKC (21).

Oxidants are known to activate mitogen-activated protein kinase (MAPK) in vascular smooth muscle (7, 20, 28) and in other cell systems (1, 12, 34). Reports suggest that MAPK may phosphorylate caldesmon to disinhibit actin/myosin interactions in vascular smooth muscle (2, 8, 19, 42). H-caldesmon is a known substrate for MAPK in smooth muscle cells in vivo (4, 38, 42). MAPK activity and h-caldesmon phosphorylation correlate with phorbol ester-induced contractile force (36).

The purpose of the current study was to identify steps in the H\(_2\)O\(_2\)-induced alternative signal transduction cascade of active tension development in vascular smooth muscle. Specifically, the hypothesis that PKC activation and MAPK phosphorylation are necessary and sufficient for the contractile response of arterial muscle to hydrogen peroxide was addressed. The time courses of PKC activity, MAPK phosphorylation, and force development during H\(_2\)O\(_2\)-induced contractions were measured. In addition, specific kinase activity blockers were used to determine cause and effect relationships between the biochemical parameters and contractile force.

**METHODS**

Second generation porcine pulmonary arteries ranging from 2–4 mm in diameter were isolated from young castrated male pigs and placed in cold Krebs-Henseleit buffer solution (KHB, in mM: 115 NaCl, 25 NaHCO\(_3\), 1.38 NaH\(_2\)PO\(_4\), 2.51 KCl, 2.46 MgSO\(_4\), 1.91 CaCl\(_2\), and 5.56 dextrose). The endothelium was removed mechanically by dragging the luminal surface along a lightly sanded surgical steel post. Helical strips, cut with dimensions of 12 mm in length and 2 mm in width, ranged from about 4–18 mg in mass due to variation in arterial wall thickness. Strips were attached to strain-gauge force transducers (Grass FT.03c) in muscle baths of KHB bubbled with 95% O\(_2\)-5% CO\(_2\). The strips were stretched to optimal resting length by adjusting the passive tension to the mean optimal resting tension (R\(_{Po}\)) of 3.8 g for maximum force development determined in earlier experiments. The strips were allowed to equilibrate for 1 h at R\(_{Po}\). The arterial muscle strips were maximally contracted with 120 mM KCl to determine maximum force development (P\(_{Po}\)). After the contractions had come to plateaus, the baths were washed out and fresh KHB was added. After complete relaxation of about 1 h duration, strips were stimulated with 120 mM KCl, 10 \(\mu\)M phorbol myristic acetate (PMA), or 1 mM H\(_2\)O\(_2\), a concentration found to produce consistent and significant force development within 5 min and reported to occur physiologically. Wash-out following contraction in response to 1 mM H\(_2\)O\(_2\) resulted in relaxation with no damage to the muscle as indicated by subsequent contractile responses to KCl or norepinephrine stimulation. Some experiments were performed in the absence of calcium (0 calcium KHB, mM: 115 NaCl, 25 NaHCO\(_3\), 1.38 NaH\(_2\)PO\(_4\), 2.51 KCl, 2.46 MgSO\(_4\), 0 CaCl\(_2\), 0.1 EGTA, and 5.56 dextrose). Another series of experiments was performed using an MAPK kinase [MAPK/extracellular signal-regulated kinase (MEK)] inhibitor, 50 \(\mu\)M PD-98059. Tissue was freeze clamped with tongs cooled to the temperature of liquid N\(_2\) (−195.79°C) at varying time points during 120 mM KCl and 1 mM H\(_2\)O\(_2\)-induced contractions for determination of myosin and MAPK phosphorylation. The frozen tissue was stored at −70°C until processed. In some cases, 50 \(\mu\)M PD-98059 in dimethyl sulfoxide vehicle was added to block MEK 10 min prior to stimulation. The PD-98059 blocker slowly precipitated out of solution so that only data acquired during the initial 15 min of each experiment were useful.

PhosphoPlus MAPK antibody (New England Biolabs) was used to measure relative changes in tyrosine/threonine phosphorylation of MAPK. Protein was loaded identically onto two gradient (4–20%) CAP gels (ICN), electrophoresed simultaneously, and then transferred to Immobilon-P followed by Western blot technique. One membrane was treated with an antibody that recognizes both phosphorylated and nonphosphorylated MAPK, whereas the other membrane was treated with an antibody specific for tyrosine phosphorylated (activated) MAPK. *Lanes 1* and *2* of each gel contained control proteins: dephosphorylated MAPK in *lane 1* and phosphorylated MAPK in *lane 2*. Control proteins included only the p42 MAPK isoform. Since the physiological range of MAPK phosphorylation is narrower than that of the control proteins, *lanes 9* and *10* on each gel contained samples that gave the minimum (negligible) MAPK phosphorylation levels (i.e., PD-
98059 exposed samples) and samples that gave the highest MAPK phosphorylation levels in response to any of the agonists used (PMA exposed samples), respectively. Negative and positive control protein densitometric measures were used to normalize phosphorylation levels of the experimental samples for each blot. The linearity of the assay was confirmed by applying various amounts of protein determined with a Bio-Rad protein assay. Optimal readings within the physiological range were obtained for 10 mg of protein per lane. Relative changes for comparison of MAPK phosphorylation were determined by simultaneously electrophoresing samples from tissue freeze clamped at various time points or with different agonists on the same gel. Relative increases or decreases in phosphorylated MAPK were expressed as a percentage of phosphorylated to total MAPK (MAPK-P/MAPK) and normalized to the positive and negative control ratios for each gel.

PKC activity was measured in freeze-clamped contracted PASM according to the method of Henrich and Pickett (15) slightly modified by Zhao and Davis (43). Freeze-clamped arterial muscle strips (20–40 mg tissue) were added to wells containing 300 μl assay/lysis buffer [0.137 M NaCl, 5.4 mM KCl, 0.3 mM Na2HPO4, 0.4 mM K2HPO4, 1 mg/ml glucose, 20 mM HEPES, 10 mM MgCl2, 25 mM β-glycerophosphate, 5 mg/ml digitonin, 100 μM [α-32P]-ATP (4,000–6,000 cpm/mmol), 2.5 mM CaCl2, and 5 mM EGTA, pH 7.2] containing 50 μM Ac-MB-(4–14) (GIBCO BRL protein kinase C substrate myelin basic protein peptide, GIBCO no. 13127-014). Negative control assays were performed on one-half of each tissue sample using CaCl2-free solution with 5 mM EGTA, adding 100 μM PKC-(19–36) (GIBCO BRL protein kinase C inhibitor peptide, GIBCO no. 13122-015) in place of the CaCl2. After a 30-min incubation period, 50 μl from each well was spotted onto phosphocellulose disks and washed with 1% aqueous H2PO4, and 32P incorporation was measured in a scintillation counter. Background counts measured in the presence of the PKC inhibitor peptide represent phosphorylation due to kinases other than PKC. These values were subtracted from the total counts. Counts were then normalized to tissue weight and expressed as mean values for each agonist at the various time points. For comparisons of PKC activity, samples were processed simultaneously to minimize error resulting from variations in 32P specific activity.

Results are presented as means ± SE. Statistically significant differences were established when P < 0.05 with Student’s t-test for any two mean values and with ANOVA followed by Newman-Keuls test for multiple means.

RESULTS

The present study demonstrates that hydrogen peroxide directly contracts isolated porcine pulmonary arterial muscle as shown in Fig. 1. The H2O2-induced contractile response is Ca2+-independent, as shown by obtaining similar data to that in Fig. 1 but in a zero Ca2+-EGTA solution.

The contractile responses of porcine pulmonary arterial muscle to KCl, PMA, and H2O2 in the presence and absence of 50 μM PD-98059, an MEK inhibitor, are compared in Fig. 2. MEK is an MAPK kinase and is responsible for phosphorylating and activating MAPK. PD-98059 that was added 10 min before the contractile agonists had no effect on the contractile response to any of these agonists. Changes in relative amounts of tyrosine-phosphorylated MAPK compared with total MAPK were measured by gradient CAP-gel SDS-PAGE followed by Western blot analyses. Radiograms are shown in Fig. 3. MAPK phosphorylation was reduced below resting levels in the presence of 50 μM PD-98059, even though the magnitude of the isometric force developed was unaltered by PD-98059 for all agonists used.

Mean data in Fig. 4 show a significant decrease in MAPK phosphorylation in the presence of PD-98059 for tissue frozen at 2 min during contraction to KCl or at 5 min for the more slowly developing contraction to H2O2.
H₂O₂ (P < 0.001). Figure 5 shows that, in general, MAPK tyrosine phosphorylation levels increase together with force development. However, MAPK phosphorylation actually dropped briefly below resting levels early in the time course of contraction in response to PMA or H₂O₂.

Figure 6 shows that the MAPK phosphorylation/force relationship was greatest for PMA, intermediate for H₂O₂, and least for KCl stimulation of porcine pulmonary arterial muscle. The MEK inhibitor, PD-98059, substantially diminished MAPK phosphorylation (Fig. 4) while having no effect on force (Fig. 2). The latter result indicates that in no case (i.e., for any agonist used) was force production dependent on MAPK phosphorylation.

PKC activity increased during initiation of force development in response to KCl and PMA stimulation of pulmonary arterial muscle. In contrast, PKC activity decreased below resting levels as tension developed in PASM in response to hydrogen peroxide (Fig. 7).

DISCUSSION

The contractile response of vascular smooth muscle to hydrogen peroxide is independent of the endothelium (18, 32). Since a rise in intracellular Ca²⁺ and myosin light chain phosphorylation are not involved in hydrogen peroxide-induced vasoconstriction (14, 17, 27), alternative signaling pathways were explored. MAPK was a logical candidate, but blocking MAPK phosphorylation with the MEK inhibitor, PD-98059, had no effect on force development. Clearly, MAPK phosphorylation is either a concurrent event or a consequence of contractile activation, rather than a contractile regulatory mechanism. The fact that the force/MAPK phosphorylation relationship is different for PMA, H₂O₂, and KCl stimulation suggests that MAPK phosphorylation is also not a simple response to the amount of force generated. Therefore, valid speculations might include the following: 1) the three agonists utilized activate the signal transduction pathways for both contractile function and for other physiological functions that might require MAPK such as growth or synthesis simultaneously, and 2) cytoskeletal rearrangements that are known to occur during contractile

**Fig. 3.** Radiograms of Western blots of phosphorylated MAPK and total MAPK during contraction. Antibodies were specific for phosphorylated MAPK (doublets in the a rows) or for total MAPK (doublets in the b rows). The MAPK (both isoforms, ERK1 and ERK2) was extracted from porcine pulmonary arterial muscle fast-frozen at rest and at various time points during a contractile response in the presence and absence of the MEK inhibitor, PD-98059. A: arterial muscle fast-frozen at rest and at various time points during contractile response to 1 mM H₂O₂ (lanes 3–6, A) or in the presence of 50 μM PD-98059 at rest and 5 min into a contractile response to H₂O₂ (lanes 7 and 8, A). B: arterial muscle fast-frozen at rest and at various time points during a contractile response to 120 mM KCl stimulation (lanes 3–6, B) or in the presence of 50 μM PD-98059 at rest and at 2 min into a contractile response to KCl (lanes 7 and 8, B). Unphosphorylated recombinant ERK2 (C, lane 1, A and B) and maximally phosphorylated ERK2 (P, lane 2, panels 1 and 2) were used as antibody standards. Phosphorylated MAPK extracted from 10⁻⁸ M PMA-stimulated porcine pulmonary arterial muscle fast-frozen 30 min into a contraction was used as a positive control (lane 9, A and B) for normalization to derive mean values.

**Fig. 4.** MAPK phosphorylation in porcine pulmonary arterial muscle in the presence and absence of MEK inhibitor. Muscle was frozen under slack conditions, at rest but stretched to optimal length for maximum tension development, at 5 min into a contractile response to 1 mM H₂O₂, and at 2 min into a contractile response to 120 mM KCl, all in the presence (hatched bar) or absence (open bar) of 50 μM PD-98059 (MEK inhibitor). Muscle stretched to optimal length (RP₀) had higher levels of MAPK phosphorylation than slack muscle. Indeed, MAPK phosphorylation levels at these time points during force development in response to H₂O₂ and KCl were not significantly above resting levels for muscle stretched to optimal length. The MEK inhibitor significantly reduced MAPK phosphorylation in all cases. *P < 0.001 compared with resting muscle stretched to optimal length for maximum. *P < 0.001 in the presence vs. absence of PD-98059. MAPK-P, phosphorylated MAPK.
apparatus activity in smooth muscles (22, 35) could conceivably result in activation of strain-sensitive signaling kinases that are not actually involved in the contractile function.

PKC is a possible signaling candidate for an alternative regulatory pathway that could mediate H₂O₂-induced contractions, since phorbol esters like PMA that activate PKC also cause arterial muscle contraction independent of increases in intracellular Ca²⁺ and myosin light chain phosphorylation (3, 36). PKC has been implicated in putative smooth muscle cell signal transduction cascades that activate muscle contraction by way of thin filament-linked proteins such as caldesmon and calponin (4, 39). PKC activity has also been linked to intracellular hydrogen peroxide release by the blood vessel wall (24, 40, 44). One study reported a calphostin C-sensitive increase in hydrogen peroxide release from endothelial cells during reoxygenation following hypoxia (44). On the other hand, oxidants may activate PKC. Gopalakrishna and Anderson (11) showed that selective oxidative modification of the regulatory domain of PKC results in increased kinase activity in the absence of Ca²⁺. A study in isolated pulmonary arterial muscle showed that H-7, another PKC inhibitor, reduced the active contractile response to phorbol esters and to oxidants (17). Calphostin C inhibits the regulatory domain of PKC, whereas H-7 is known to act at the catalytic site (32). However, the specificity of kinase inhibitors in intact tissue cannot be clearly established. Therefore, in the current study, PKC activation in hydrogen peroxide-induced contractile responses was determined directly. Surprisingly, PKC activation actually declined during force development in response to hydrogen peroxide. Thus the mechanism for regulation of the contractile response to hydrogen peroxide still remains to be elucidated.

Direct oxidation of contractile proteins is a possible mechanism for the contractile response to hydrogen peroxide that remains to be explored. In retrospect, previous reports suggest that oxidation may directly activate actomyosin interactions. In 1970, Sparrow et al. (33) extracted smooth muscle contractile proteins to demonstrate the calcium concentration dependence for smooth muscle actomyosin ATPase activity. Dithiothreitol (DTT), a thiol-reducing agent, was required in the storage media, or the calcium ion dependence of the ATPase activity was lost (33). Since DTT keeps sulfhydryl groups in the reduced state, this early study suggested that oxidation of contractile or regulatory protein sulfhydryl groups might activate smooth muscle contractile activity in the absence of calcium. Two studies in the 1980s confirmed these results; Chandra et al. (9) and Ngai and Walsh (25) both detected increased calcium-independent actin-activated ATPase

Fig. 5. Time courses of MAPK phosphorylation during contraction of porcine pulmonary arterial muscle smooth muscle in response to 120 mM KCl (n = 5; A), 10⁻⁶ M PMA (n = 7; B), and 1 mM H₂O₂ (n = 10; C). The trend is for MAPK phosphorylation to increase in correlation with force.

Fig. 6. The MAPK phosphorylation/force relationship in porcine pulmonary arterial muscle during contraction in response to PMA, H₂O₂, or KCl. The MAPK phosphorylation/force relationship depends on the agonist used. The correlation between MAPK phosphorylation and contractile force is lost in the presence of PD-98059.
of dephosphorylated myosin due to slow oxidation. The calcium and phosphorylation dependence of the ATPase activation was protected by DTT or storage under nitrogen. On the other hand, oxidation of myosin or regulatory proteins such as caldesmon might result in increased internal loading and impedance of the molecular motor, as suggested by studies of the effects of oxidants and antioxidants in motility assays (13, 16). However, as these authors point out, extrapolation of results from test tube studies to the intact tissue and/or in vivo conditions must be done with caution. Exactly which proteins might be oxidized or reduced in the cell in response to extracellular oxidant exposure is largely unknown.

In summary, the vascular contractile response to H$_2$O$_2$ involves a cellular mechanism different from the signal transduction cascades described for other receptor-activated contractions in that it does not involve the usual Ca$^{2+}$ and MLC$_{20}$ phosphorylation signals, is independent of ion fluxes across smooth muscle cell membranes, and does not require PKC or MAPK activation. Other mechanisms such as direct contractile protein oxidation or cytoskeletal element reorganization remain to be explored. Understanding the cellular mechanism of H$_2$O$_2$-induced vascular smooth muscle contraction will increase the understanding of smooth muscle contractile regulation in general and should lead to therapeutic intervention in oxidant-mediated cardiovascular pathologies.

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REFERENCES

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