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


Department of Physiology and Biophysics, Indiana University School of Medicine, Indianapolis, Indiana 46202-5120; Department of Biochemistry, Queen’s University, Kingston, Ontario, Canada K7L 3N6; and Department of Internal Medicine, Pulmonary Division, University of Cincinnati, Cincinnati, Ohio 45267-0564

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Pelaez, N. J., S. L. Osterhaus, A. S. Mak, Y. Zhao, H. W. Davis, and C. S. Packer. MAPK and PKC activity are not required for H$_2$O$_2$-induced arterial muscle contraction. Am J Physiol Heart Circ Physiol 279: H1194–H1200, 2000.—H$_2$O$_2$-induced pulmonary arterial smooth muscle (PASM) contractions are independent of Ca$^{2+}$ and myosin light chain phosphorylation. The purpose of this study was to determine whether mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK) 1 and ERK2, or protein kinase C (PKC) activation is required for H$_2$O$_2$-induced contraction. Porcine PASM strips were stimulated with 1 mM H$_2$O$_2$, 120 mM KCl, or 10 μM phorbol myristic acetate and freeze clamped at various times during the contractions. Changes in relative amounts of tyrosine/threonine phosphorylated MAPK compared with total MAPK were measured. MAPK tyrosine phosphorylation levels increased in correlation with tension development. However, 50 μM PD-98059, a MAPK/ERK kinase-MAPK kinase blocker, reduced MAPK phosphorylation below resting levels, even though the magnitude of the isometric tension development was unaltered. Freeze-clamped PASM strips were placed in a PKC activity assay buffer containing $^{32}$P and CaCl$_2$ to measure the total myelin basic protein phosphorylation. The data show that: 1) the time courses of PKC activity and force produced in response to H$_2$O$_2$ do not correlate, and 2) MAPK activation may be a concurrent event with, or a consequence of, tension development in response to a variety of agonists but is not responsible for contractions to H$_2$O$_2$, high K$^+$, or phorbol esters.

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). Vasoconstriction 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 con-
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\textsubscript{3}, 1.38 NaH\textsubscript{2}PO\textsubscript{4}, 2.51 KCl, 2.46 MgSO\textsubscript{4}, 1.91 CaCl\textsubscript{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\textsubscript{2}-5% CO\textsubscript{2}. The strips were stretched to optimal resting length by adjusting the passive tension to the mean optimal resting tension (R_{P0}) of 3.8 g for maximum force development determined in earlier experiments. The strips were allowed to equilibrate for 1 h at R_{P0}. The arterial muscle strips were maximally contracted with 120 mM KCl to determine maximum force development (P_{max}). 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 myristate acetate (PMA), or 1 mM H\textsubscript{2}O\textsubscript{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\textsubscript{2}O\textsubscript{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\textsubscript{3}, 1.38 NaH\textsubscript{2}PO\textsubscript{4}, 2.51 KCl, 2.46 MgSO\textsubscript{4}, 0 CaCl\textsubscript{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\textsubscript{2} (−195.79°C) at varying time points during 120 mM KCl and 1 mM H\textsubscript{2}O\textsubscript{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-
The present study demonstrates that hydrogen peroxide directly contracts isolated porcine pulmonary arterial muscle as shown in Fig. 1. The H$_2$O$_2$-induced contractile response is Ca$^{2+}$-independent, as shown by obtaining similar data to that in Fig. 1 but in a zero Ca$^{2+}$-EGTA solution.

The contractile responses of porcine pulmonary arterial muscle to KCl, PMA, and H$_2$O$_2$ 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 H$_2$O$_2$. 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 are presented as means ± SE.
H_2O_2 (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 H_2O_2.

Figure 6 shows that the MAPK phosphorylation/force relationship was greatest for PMA, intermediate for H_2O_2, and least for KCl stimulation of porcine pulmonary arterial muscle. 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, H_2O_2, 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

**DISCUSSION**

The contractile response of vascular smooth muscle to hydrogen peroxide is independent of the endothelium (18, 32). Since a rise in intracellular Ca^{2+} 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, H_2O_2, 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
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 H$_2$O$_2$-induced contractions, since phorbol esters like PMA that activate PKC also cause arterial muscle contraction independent of increases in intracellular Ca$^{2+}$ 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 Ca$^{2+}$. 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$^{-8}$ M PMA ($n = 7$; B), and 1 mM H$_2$O$_2$ ($n = 10$; 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, H$_2$O$_2$, 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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