Intraluminal pressure stimulates MAPK phosphorylation in arterioles: temporal dissociation from myogenic contractile response

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Intraluminal pressure stimulates MAPK phosphorylation in arterioles: temporal dissociation from myogenic contractile response. Am J Physiol Heart Circ Physiol 285: H1764–H1773, 2003. First published June 12, 2003; 10.1152/ajpheart.00468.2003.—Members of the MAPK family of enzymes, p42/44 and p38, have been implicated in both the regulation of contractile function and growth responses in vascular smooth muscle. We determined whether such kinases are activated during the arteriolar myogenic response after increases in intraluminal pressure. Particular emphasis was placed on temporal aspects of activation to determine whether such phosphorylation events parallel the known time course for myogenic contraction. Experiments used single cannulated arterioles isolated from the cremaster muscle of rats with some vessels loaded with the fluorescent Ca$^{2+}$ sensitive dye fura 2 (2 μM). The p42/44 inhibitor PD-98059 (50 μM) caused vasodilation but did not prevent pressure-induced myogenic constriction. The vasodilator response was accompanied by decreased smooth muscle intracellular Ca$^{2+}$. Western blotting revealed a significant increase in the level of phosphorylation of p42/44 15 min after the application of a 30- to 100-mmHg pressure step. Phosphorylation of p42/44 was a late event that appeared to be temporally dissociated from contraction, which was complete within 1–5 min. EGF (80 nM) caused marked phosphorylation of p42/44 but only acted as a weak vasoconstrictor. The p38 inhibitor SB-203580 (10 μM) did not alter baseline diameter, nor did it prevent myogenic vasoconstriction. Consistent with these observations, SB-203580 did not cause a measurable change in intracellular Ca$^{2+}$. The results demonstrate activation of the p42/44 class of MAPK resulting from increased transmural pressure. Such activation is, however, dissociated from the acute pressure-induced vasoconstrictor response in terms of time course and may represent the activation of compensatory, but parallel, pathways, including those related to growth and remodeling.

myogenic response; arteriolar tone; mechanotransduction; mitogen-activated protein kinase; vasoconstriction

SKELETAL MUSCLE ARTERIOLES respond to an increase in intraluminal pressure with vasoconstriction, a phenomenon termed the myogenic response (28). Studies have shown that this contractile response is largely dependent on Ca$^{2+}$ influx into vascular smooth muscle cells with subsequent activation of Ca$^{2+}$/calmodulin/myosin light chain kinase and phosphorylation of myosin regulatory light chains (for reviews, see Refs. 9, 22, 26, and 46). In addition to this pathway, it is evident that the mechanical stimulus provided by the pressure increase initiates a large number of signaling mechanisms that may, or may not, be directly related to the contractile response (40, 41, 50, 55). Thus, in some arterial vessels, it is likely that pressure increases lead to adaptation or remodeling events that are not related to contraction per se. Consistent with this, mechanical forces activate a variety of pathways, including those leading to cellular events occurring over rapid, intermediate, and prolonged time frames (for example, Refs. 29 and 35).

Vascular smooth muscle is known to contain a number of proteins that are regulated by tyrosine phosphorylation (for example, Refs. 12, 15, and 27). Such tyrosine phosphorylation events regulate diverse responses including contraction and remodeling (1, 11, 12, 14, 20, 25, 33, 35). For example, we (25) have recently reported that tyrosine phosphorylation-dependent pathways mediate constrictor responses and indexes of early remodeling in arterioles subjected to prolonged norepinephrine exposure. Specifically in relation to mechanotransduction pathways, it has been hypothesized that that extracellular matrix binding through integrins activates a series of proteins (e.g., c-Src and focal adhesion kinase) that are regulated, in part, by tyrosine phosphorylation (10, 57). In addition, the activity of downstream kinases (for example, p42/44 MAPK, also referred to as extracellular signal-regulated protein kinases (ERK1 and -2) and p38 MAPK) is similarly regulated by tyrosine phosphorylation (for a review, see Ref. 47). These enzymes are of considerable interest as they are implicated in both the regulation of contractile processes and transcriptional events (for example, Ref. 53). Phosphorylation of p42/44 MAPK has been shown to occur after loading or stretch of conduit artery smooth muscle (1, 18). Targets of activated p42/44 MAPK include the proteins calponin and caldesmon, which have been implicated in thin filament-based regulation of smooth muscle contraction (11, 23). Similarly, p38 MAPK through activation of heat shock protein (HSP)27 may also regulate con-
traction (19, 32, 56). Further support for a role for the various MAPKs in mechanotransduction has also been provided by studies of cultured vascular smooth muscle cells (33, 53) and nonvascular smooth muscle preparations, such as the bladder and trachea (20, 30, 44).

In this study, we aimed to determine the involvement of selected MAPKs during increases in transmural pressure and specifically the arteriolar myogenic contractile response. With the use of cannulated arteriolar preparations, the effects of pharmacological inhibitors (PD-98059 and SB-203580) on myogenic reactivity were examined with simultaneous measurements of pressure-induced phosphorylation of p42/44 MAPK by electrophoresis and Western blotting. This approach was designed to extend our previous studies (41, 50) examining pressure-induced changes in global arteriolar wall tyrosine phosphorylation by examining specific phosphorylated species and reducing the reliance on broad-spectrum inhibitors, which may suffer from nonselectivity/specificity. Furthermore, emphasis was placed on temporal aspects of activation to determine whether such phosphorylation events parallel the known time course for myogenic contraction (59, 60). This latter point is of particular significance to the interpretation of data from studies where tyrosine phosphorylation (including phosphorylation of p42/44 MAPK) has been measured at single time points (36).

MATERIALS AND METHODS

Animals

The studies used male Sprague-Dawley rats weighing between 200 and 350 g. Before experiments, rats were housed in pairs in a dedicated animal facility with a 12:12-h light-dark cycle. During this period, rats were allowed free access to a standard rat chow and drinking water. All procedures were approved by the Animal Experimentation and Ethics Committees at RMIT University.

Isolated Arteriole Preparation

Rats were anesthetized with pentothal sodium (100 mg/kg), after which the right and/or left cremaster muscles were exteriorized, excised from the animal, and placed in a cooled (4°C) chamber containing dissection buffer (3 mM MOPS, 145 mM NaCl, 5 mM KCl, 2.5 mM CaCl2, 1 mM MgSO4, 1 mM Na2HPO4, 0.02 mM EDTA, 2 mM pyruvate, 5 mM glucose, and 1% albumin) (13). Segments of the main intramuscular arteriole were dissected from the muscle as previously described (39, 59). Individual vessel segments were then cannulated with glass micropipettes, secured using 10-0 monofilament silk sutures, and mounted in a custom-designed tissue chamber (volume: 7 ml). The cannulated arterioles were continually superfused (2–4 ml/min) with physiological salt solution containing (in mM) 111 NaCl, 25.7 NaHCO3, 4.9 KCl, 2.5 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, 11.5 glucose, and 10 HEPES. Vessel segments were gradually pressurized to 70 mmHg and warmed to 34°C (in vivo temperature of rat cremaster muscle) during a 60-min equilibration period. During this period, vessels were checked for pressure leaks and allowed to develop spontaneous basal tone. Vessel length was adjusted before the development of spontaneous tone by increasing the segment length such that pressure steps to 120 mmHg did not cause a lateral bowing of the vessel. This approach allowed consistency between preparations with respect to this parameter. The vessel preparation was positioned on the stage of an inverted microscope equipped with a video-based imaging/photometry system. Measurements of vessel diameter (in μm) were made using an electronic video caliper.

In experiments requiring measurement of changes in intracellular Ca2+ concentration ([Ca2+]i), vessels were incubated (60 min at room temperature) with 2 μM fura 2-AM (Molecular Probes; Eugene, OR) in buffer containing 0.5% DMSO and 0.01% pluronic F-127. Only the abbluminal surface of the vessel segment was exposed to the fura 2-AM solution to restrict dye-loading to the vascular smooth muscle layer (31). The dye-loading procedure was followed by a 30-min washout period with physiologic salt solution. Fura 2-AM-loaded vessels were exposed to epi-illumination (75-W xenon source) with light of alternating excitation wavelengths (340 and 380 nm) using a computer-controlled filter wheel. Images of fluorescence emission at 510 nm were acquired using an image intensifier (Videoscope International; Washington, DC) and a charge-coupled device (Hamamatsu; Bridgewater, NJ). Intracellular Ca2+ concentration ([Ca2+]i) was calculated from fura 2 fluorescence intensity using a ratio (340/380 ratio) to allow quantitative estimates of [Ca2+]i. Measurements of vessel diameter (in μm) were made using an electronic video caliper.

Electrophoretic Measurement of MAPK Phosphorylation

Vessel segments were dissected and cannulated as described above with the exception that longer preparations (two to three times that used for general mechanical studies) were typically prepared, which required the ligation of side branches. This enabled phosphorylation measurements to be performed on single vessel segments and removed the need for pooling vessel samples. After the specific protocols (see Experimental Protocols) were performed, vessel segments were snap frozen while cannulated and pressurized using dry ice-acetone-cooled tongs. While frozen (under dry ice-acetone), the segments of vessels distal to the surgical ties were removed using microfine spring scissors. Vessel segments were homogenized in buffer containing 25 mM Tris-HCl (pH 6.8), 1% SDS, 5 mM EGTA, 50 mM NaF, 1 mM sodium vanadate, 10% glycerol, 10 μg/ml leupetin, 1 μg/ml apro- nin, and 17.4 μg/ml PMSP. Volume was then adjusted in buffer containing 25 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 10 mM DTTO, 2% β-mercaptoethanol, and 0.01% bromophenol blue. Samples were then subjected to polyacrylamide (10%) gel electrophoresis and Western blot transfer using a semidry blotting system (Bio-Rad). Membranes were then probed with appropriate rabbit primary antibodies (anti-p42/44 IgG, 1:2,000; anti-phospho-p42/44 IgG, 1:10,000; New England Biolab), followed by an anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (1:4,000; New England Biolab). Proteins were then visualized using enhanced chemiluminescence (Perkin-Elmer) and Hyper ECL photographic film (Amersham Pharmacia Biotech).

Differences in band intensity were determined using NIH Image. To provide a quantitative index of p42/44 MAPK phosphorylation, band intensities obtained for phospho-MAPK and total MAPK were normalized to an internal standard of rat aorta homogenate that was included in each electrophoretic separation. The normalized phospho-MAPK...
signal was then expressed as a ratio against the normalized total MAPK signal. This procedure was utilized to account for variations in sample loading.

As a positive control, a separate set of arterioles was exposed to the known activator (14) of p42/44 MAP kinase, EGF (50 ng/ml), at 30 mmHg for 10 min before being frozen. As a control for between-gel variability, a pooled sample of homogenized aorta was included in each electrophoretic run (see above). The effectiveness of the inhibitor SB-203580 was demonstrated by its ability to inhibit H2O2 (500 μM)-induced phosphorylation of p38 in cultured endothelial cells. Western blots were performed as above using anti-p38 IgG (1:1,000) and anti-phospho-p38 (1:1,000) antibodies (New England Biolab).

Experimental Protocols

Effect of inhibitors on myogenic tone and reactivity. Arterioles were subjected to intraluminal pressures over the range of 30–150 mmHg, during which time luminal diameter was continuously monitored and recorded. Each pressure level was maintained for a minimum of 5 min. After collection of baseline data, vessels were superfused with physiological salt solution containing either the MEK inhibitor PD-98059 (50 μM) or the p38 MAPK inhibitor SB-203580 for 20 min (2, 16). Pressure-diameter responses were then repeated in the presence of the inhibitor. Vessels were then superfused with 0 mM Ca2+ physiological salt solution containing 2 mM EGTA, and pressure-diameter responses were recorded under passive conditions.

In a further set of cannulated arterioles, the effects of PD-98059 (50 μM) and SB-203580 (10 μM) on arteriolar smooth muscle intracellular Ca2+ were examined. The effect of each inhibitor was studied at pressures of 50, 70, and 120 mmHg. As in the above experiments, passive responses were obtained after superfusion with 0 mM Ca2+ physiological salt solution containing 2 mM EGTA.

Indomethacin and adenosine controls. In separate sets of arterioles, pressure-diameter responses were performed (as in Effect of inhibitors on myogenic tone and reactivity) in the presence of either indomethacin (28 μM) or a concentration of adenosine (1–10 μM) aimed at causing dilation comparable with that caused by the higher concentration of PD-98059 (50 μM). These studies were performed as controls for 1) reported inhibitory effects of PD-98059 and SB-203580 on cyclooxygenase-1 and -2 (6, 43) and 2) nonspecific effects of vasodilatation on myogenic responsiveness.

MAPK phosphorylation in response to intraluminal pressure and EGF stimulation. After cannulation and equilibration, arterioles were set to an intraluminal pressure of 30 mmHg for 15 min. Intraluminal pressure was then either maintained at 30 mmHg or step increased to 100 mmHg for 1, 5, 15, or 60 min, at which time the vessel segments were snap frozen using liquid N2-cooled tongs and placed in acetone-dry ice (as described in Electrophoretic Measurement of MAPK Phosphorylation). An additional set of vessels exposed to EGF (80 nM) at 30 mmHg (15 min) was collected in a similar manner. Vessel homogenates were then stored at −80°C until electrophoretic protein separation and Western blot transfer for p42/44 MAPK was performed.

Statistics

Results are presented as means ± SE. Comparisons of the means and SEs between two groups were performed using Student’s t-test. Multiple comparisons were performed using ANOVA, followed by the paired least square difference test. Significance was assumed at P < 0.05.

Drugs and Chemicals

Unless stated, all chemicals and reagents were purchased from Sigma (St. Louis, MO). PD-98059 (Bio-Mol) was prepared as a 7.48 mM stock in DMSO and stored in aliquots at −20°C. Subsequent dilutions were made in Krebs buffer solution. SB-203580 (Calbiochem) was prepared as a 5 mM stock in DMSO and stored as aliquots at −20°C. Further dilutions were made in Krebs buffer solution. Indomethacin (ICN; Seven Hills, North South Wales, Australia) was prepared daily by dissolving 34.75 mg in 10 ml (30 mM) of 0.1 M Na2CO3. Indomethacin was then diluted to 28 μM in Krebs buffer for use. Adenosine was prepared as a 100 mM solution in Krebs buffer and diluted as required.

RESULTS

The experiments used 76 arterioles with passive diameters (measured in 0 mM Ca2+/2 mM EGTA buffer) of 144.9 ± 1.6 μm.

Effect of MAPK Inhibitors on Myogenic Tone and Reactivity

The MEK inhibitor PD-98059 caused significant dilation at a concentration of 50 μM (Fig. 1), whereas diameter was not significantly altered at 20 μM. Despite reducing the level of baseline myogenic tone, arterioles continued to exhibit pressure-dependent myogenic vasoconstriction as evidenced by a negative slope in the pressure-diameter relationship (Fig. 1).

Consistent with a dilator effect of PD-98059 (50 μM), the MEK inhibitor caused a significant reduction in intracellular Ca2+ in arterioles loaded with fura 2 (Fig. 2). Fura 2-loaded vessels similarly showed a small, but significant, reduction in fura 3 fluorescence, consistent with a dilator effect of PD-98059.

![Fig. 1. Effect of the MEK inhibitor PD-983509 (PD; 50 μM) on steady-state arteriolar (n = 6) diameter over the pressure range of 50–150 mmHg. A concentration of 20 μM was also examined (data not shown). Data are expressed relative to the diameter obtained under passive conditions (in the absence of extracellular Ca2+) at 70 mmHg. Vessel diameters were 70.1 ± 10.1 μm (70 mmHg) before 50 μM PD and 93.8 ± 14.9 μm after the addition of the inhibitor. Results are presented as means ± SE. *P < 0.05 compared with the control at the same pressure (by ANOVA).](http://ajpheart.physiology.org/)

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Adenosine was titrated (4.143 ± 1.55 μM) to give a dilation comparable with that seen in the presence of PD-98059. As shown for PD-98059, adenosine-treated vessels exhibited acute myogenic responsiveness despite the vasodilator effect (Fig. 4).

**Electrophoretic Determination of MAPK Phosphorylation in Response to Intraluminal Pressure and EGF Stimulation**

**p42/p44 MAPK phosphorylation.** Initial studies examined EGF-induced phosphorylation of p42/p44 MAPK to demonstrate that changes in phosphorylation could be detected in arterioles. EGF was chosen as a positive control due to its previously reported ability to cause phosphorylation of p42/p44 MAPK (14). Vessels maintained at an intraluminal pressure of 30 mmHg showed an increase in phosphorylation from 84 ± 14 to 483 ± 96 (relative band intensity) in response to EGF (80 nM) (Fig. 5). These arterioles did not show a significant constrictor response to this concentration of EGF (65.6 ± 2.5% of passive diameter, n = 5; control 69.7 ± 12.8% of passive diameter, P > 0.05). An additional three arterioles maintained at an increased intraluminal pressure (70 mmHg) and subjected to EGF concentrations over the range of 10–100 nM showed some evidence of vasoconstriction (52.3 ± 3.3% of passive diameter at baseline and 44.9 ± 2.5 of passive diameter in the presence of 100 nM EGF, n = 3).

![Figure 2](http://ajpheart.physiology.org/)

**Effect of Indomethacin and Adenosine on Myogenic Tone and Reactivity**

Baseline diameter and myogenic responsiveness were unaffected by pretreatment of vessels with the cyclooxygenase inhibitor indomethacin (28 μM, n = 4; Fig. 4). Similarly, indomethacin pretreatment had no effect on the contractile response to an acute pressure step from 30 to 100 mmHg (n = 3, data not shown). This is consistent with a lack of contribution of cyclooxygenase-dependent products in acute myogenic contraction or the effects of PD-98059 detailed above.

![Figure 3](http://ajpheart.physiology.org/)
In contrast, the \( \beta \)-adrenoceptor agonist phenylephrine (10 M) constricted the arterioles (maintained at the same intraluminal pressure) to a peak value of 28.9% of passive diameter \( (n = 3) \).

To examine MAPK phosphorylation in response to an acute myogenic stimulus, arterioles were subjected to a pressure step from 30 to 100 mmHg. Temporal patterns of p42/44 phosphorylation after the pressure step were examined to allow comparisons with previous studies \( (59, 60) \) determining the role of Ca\(^{2+}\)-induced myosin light chain phosphorylation in myogenic constriction. Grouped MAPK phosphorylation and corresponding vasomotor responses are shown in Fig. 6. After an initial pressure-induced distension, vasoconstriction was evident within the first minute and reached a steady state within 5 min, which was typical of previous studies in this preparation \( (59) \). Significant \( (P < 0.05) \) phosphorylation of p42/44 was not observed until 15 min into the time course \( (245 \pm 82 \text{ relative band intensity at } 100 \text{ mmHg after } 15 \text{ min compared with } 98 \pm 14 \text{ under steady-state conditions at } 30 \text{ mmHg}) \). This phosphorylation appeared transient as it was not detected at 60 min \( (\text{relative band intensity } 103 \pm 10) \) despite the vasoconstrictor response being maintained for this period.

To demonstrate that the phosphorylation was related to the upstream activation of MEK, vessels exposed to the 30- to 100-mmHg pressure step for 15 min were studied in the presence of PD-98059 (50 M). The MEK inhibitor totally prevented the pressure-induced phosphorylation and further reduced levels below baseline \( (\text{Fig. 7}) \). As in previous studies, PD-98059 caused a small, but significant, dilation but did not prevent the pressure-induced vasoconstriction, demonstrating a dissociation between the phosphorylation event and the mechanical response. An example trace of the diameter response to the 30- to 100-mmHg pressure step in the absence and presence of PD 98059 \( (50 \text{ M}) \) is shown in Fig. 8.

To examine Ca\(^{2+}\) dependency and dissociate p42/44 MAPK phosphorylation from the vasomotor response, the effect of superfusion with 0 mM Ca\(^{2+}\)-2 mM EGTA buffer was examined. Ca\(^{2+}\) removal from the superfusate did not significantly decrease phosphorylation levels at either 30 mmHg or 15 min after the application of Ca\(^{2+}\) removal.
of a pressure step to 100 mmHg. At 30 mmHg, MAPK phosphorylation was $84/1006$ (relative band intensity) in the presence of extracellular $Ca^{2+}$ and $100/1006$ in its absence ($P < 0.05$), whereas at 100 mmHg phosphorylation was $245/1006$ in the presence of extracellular $Ca^{2+}$ and $180/1006$ in its absence ($P < 0.05$). After superfusion with 0 mM $Ca^{2+}$-2 mM EGTA buffer, vessels behaved passively to the increase in intraluminal pressure.

Effectiveness of SB-203580 on p38 MAPK phosphorylation in endothelial cells. As a positive control for p38 MAPK phosphorylation, and to demonstrate the effectiveness of SB-203580, cultured bovine aortic endothelial cells were exposed to $H_2O_2$ ($500/9262$ M) for 5 min in six-well tissue culture plates. Cells were homogenized in homogenizing buffer and subject to electrophoresis/Western blotting as described in MATERIALS AND METHODS. Cells exposed to $H_2O_2$ showed an increase in p38 MAPK phosphorylation to $1.42/1006$ (arbitrary intensity units) compared with virtually undetectable levels under control conditions. $H_2O_2$-induced phosphorylation of p38 MAPK was significantly ($P < 0.05$) attenuated ($0.56/1006$) by preincubation of cells with SB-203580 ($10/9262$ M).

DISCUSSION

In this study, we measured pressure-induced phosphorylation of p42/44 MAPK in functional, cannulated arterioles. Of importance, the data reveal that phosphorylation of MAPKs is not critical to the development of myogenic responsiveness because the MEK inhibitor PD-98059 prevented pressure-induced MAPK phosphorylation without abolishing acute myogenic responsiveness. Moreover, peak MAPK phosphorylation, and hence activation, occurred considerably later than the establishment of steady-state myogenic constriction. Additional support for a lack of a direct involve-ment of p42/44 MAPK in contraction per se was provided by the observation that EGF caused marked phosphorylation of the kinase while acting as a relatively weak vasoconstrictor. No evidence was obtained for a role for p38 MAPK in steady-state tone or control of global intracellular $Ca^{2+}$ levels. As such, the data support and extend our earlier studies using broad-spectrum tyrosine phosphorylation inhibitors (tyrphostin A47 and genistein), where it was concluded that tyrosine phosphorylation events are not fundamental to pressure-induced vasoconstriction (41, 50). Importantly, the present experiments examined phosphorylation of specific signaling molecules activated by a change in intraluminal pressure and further avoided issues related to nonspecificity/selectivity often associated with the use of small-molecular-weight kinase inhibitors. In this regard, agents such as genistein have been reported to inhibit $Ca^{2+}$ entry via mechanisms not involving tyrosine phosphorylation (45, 54).

Despite the above conclusion, it is evident that p42/44 MAPK inhibition did cause a degree of vasodilatation and a reduction in intracellular $Ca^{2+}$ levels. However, it appears that this likely represents a non-specific effect on arteriolar myogenic tone rather than identifying a key role for MAPK activation in acute pressure-induced constriction. Analogous to the effects of the kinase inhibitors were the effects of adenosine (at a concentration titrated to match the dilation caused by PD-98059), which similarly caused a rightward shift in the arteriolar pressure-diameter relation-

Fig. 7. Effect of PD on pressure-induced phosphorylation of p42/44 MAPK in arterioles ($n = 5–6$). Inset: typical example of Western blot analysis. Phosphorylation is expressed in terms of relative band intensity. Group data are presented as means ± SE. *$P < 0.05$.

Fig. 8. Example tracings illustrating the lack of effect of PD on the constrictor response to an acute pressure step (30–100 mmHg). A: basal; B: PD; C: 0 mM $Ca^{2+}$. Tracings are representative of 6 experiments.
ship. Similar conclusions were drawn by Lagaud et al. (31) in studies of rat middle cerebral arteries, where they reported nonspecific inhibition of myogenic tone by PD-98059. As PD-98059 inhibited tone induced by KCl, vasopressin, and increasing concentrations of extracellular Ca\(^{2+}\) (0.4–1.6 mM), these authors suggested a nonspecific effect on Ca\(^{2+}\) entry. Consistent with this, the present study demonstrated a PD-98059-induced decrease in intracellular Ca\(^{2+}\). A further series of control experiments in the present study involved examining the effects of direct cyclooxygenase inhibition on in vitro arteriolar myogenic responsiveness. These were included as the MAPK inhibitors (PD-98059 and SB-203580) have been reported to lead to inhibition of this pathway and therefore have the potential to alter the production of vasoactive prostanoids (6, 43). The present results indicate that PD-98059, despite causing a decrease in the basal level of tone, did not influence myogenic constriction through such a mechanism.

It is evident that the events associated with an increase in arteriolar intraluminal pressure activate a number of signaling pathways within vascular smooth muscle cells. What remains uncertain, however, is whether all such events relate only to contractile activation or whether there is simultaneous activation of pathways that are specific for compensatory events including those involved in hypertrophic and/or hyperplastic responses. An attractive hypothesis is that in response to an increase in pressure, both contractile and compensatory growth pathways are initially activated in an effort to oppose increased wall tension/stress, with the latter being inhibited when effective constriction occurs. This conclusion is supported by the data of Allen et al. (4) in mesenteric resistance vessels, which indicated that pressure-induced expression of the mRNA for the protooncogene c-myc was attenuated in arteries showing myogenic reactivity compared with those demonstrating passive behavior. Similarly, in the present study, removal of extracellular Ca\(^{2+}\) did not prevent p42/44 MAPK phosphorylation, whereas contractile activity was inactivated.

An additional observation that supports the proposition that MAPK phosphorylation is activated by increased pressure/wall tension but independent from myogenic constriction is the dissociation between the respective time courses for contraction and phosphorylation. MAPK phosphorylation was found to be significantly increased only some 15 min after the increase in pressure, whereas myogenic contraction reached steady state within 5 min. Supporting this, our previous studies have shown that myosin light chain phosphorylation, a key event in smooth muscle contraction, reaches a maximum within 30 s of the pressure increase (60), whereas global tyrosine phosphorylation was found to increase over a 60-min time period after the application of an acute pressure step (41). A similar dissociation between MAPK and contractile activation was reported by Gorenne et al. (21) in studies of the histamine-stimulated swine carotid artery and Ratz (48) in studies where the rabbit femoral artery was exposed to phenylephrine. Furthermore, both studies demonstrated that, whereas PD-98059 prevented the increase in MAPK phosphorylation, contractile responsiveness was maintained.

There are few studies in the literature specifically examining the role of the various MAPKs in the contractile responsiveness of intact and functional arterioles. Loufrani et al. (34) examined the involvement of MAPK in temperature-dependent myogenic tone exhibited by the rabbit facial vein. This vessel, while exhibiting a myogenic or stretch-dependent tone similarly to arterioles, appears to differ in that a component of this tone develops independent of a requirement for Ca\(^{2+}\). Regardless of this difference, p42/44 MAPK activation was not a determinant of the developed myogenic tone, and the authors thus concluded that stretch activates two independent pathways. Matrougui et al. (37, 38) examined the effect of angiotensin II in pressurized mesenteric arteries on p42/44 MAPK phosphorylation and activity by in gel kinase assays and Western blotting. While these authors demonstrated a synergistic effect between pressure and angiotensin in increasing MAPK activation, the relationship with acute vascular reactivity was not detailed. In particular, the degree of myogenic tone was not provided. Differences in the extent of pressure-induced myogenic constriction between preparations is an important consideration as the magnitude of the contractile response will impact on variables such as wall tension and therefore on stimuli that may lead to the activation of pathways such as those involving p42/44 MAPK.

In a more recent study (36) of myogenically reactive rat gracilis muscle arterioles, Massett et al. suggested a role for p42/44 MAPK activation in wall tension-dependent vasoconstriction. This was based, in part, on demonstration of protein phosphorylation by electrophoresis and Western blotting. However, as these studies were performed at a single time point after pressurization (30 min), such data lack the temporal resolution necessary to conclusively link the activation of MAPK to acute myogenic constriction. Thus, as outlined above, MAPK phosphorylation in the present study did not parallel the known time course for Ca\(^{2+}\)-induced myosin light chain phosphorylation and myogenic constriction (59, 60).

The results of the present study do not discount a role for events upstream of MAPK activation in modulating myogenic reactivity. For example, elements of the integrin-focal adhesion-c-Src axis, which can lie upstream of pressure-induced p42/44 MAPK activation (52, 55), have been implicated in modulation of ion channel activity and possibly Ca\(^{2+}\) sensitivity of the contractile proteins. In this regard, integrins and Src have been shown to modulate the activity of voltage-gated Ca\(^{2+}\) channels (10, 57), and the tyrosine phosphatase inhibitor pervanadate causes contraction of myogenically active cremaster muscle arterioles by a mechanism involving Ca\(^{2+}\) sensitization (41). Furthermore, D'Angelo and Adam (8) have suggested that
p42/44 MAPK activation exerts an effect through myosin light chain phosphorylation (as opposed to phosphorylation of a thin filament protein such as caldesmon) to modulate force. While this would be consistent with the observed action of PD-98059 to decrease intracellular Ca²⁺, it would not necessarily be consistent with lack of effect of the inhibitor on pressure-dependent vasoconstriction.

An additional consideration is that interactions may occur between factors activating tyrosine phosphorylation-dependent pathways and myogenic signaling. Thus, in the present study, EGF was seen to be ineffective as a contractile agent in experimental hypertension (17) and to potentiate the constrictor response to vasoconstrictors such as angiotensin II (49).

A number of studies have implicated mechanical forces in the control of vascular smooth muscle phenotype. For example, Birukov et al. (5) reported that a contractile agent at 30-mmHg intraluminal pressure, whereas it caused a modest contraction at 70 mmHg. Supporting this, previous groups have shown EGF to be a more potent contractile agent in experiments with lack of effect of the inhibitor on pressure-dependent vasoconstriction.

Thus, in the present study, EGF was seen to be ineffective as a contractile agent at 30-mmHg intraluminal pressure. This study was supported by grants from the National Health and Medical Research Council of Australia, the National Heart Foundation of Australia, and the Edward Dunlop Foundation.

**DISCLOSURES**

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**REFERENCES**

MAPK PHOSPHORYLATION IN PRESSURIZED ARTERIOLES

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