Stretch Induces Mitogen-Activated Protein Kinase Activation and Myogenic Tone Through 2 Distinct Pathways

Laurent Loufrani, Stéphanie Lehoux, Alain Tedgui, Bernard I. Lévy, Daniel Henrion

Abstract—The aim of this study was to evaluate the involvement of the mitogen-activated protein kinase (ERK1/2) pathway in response to stretch in a blood vessel developing myogenic tone on stretch. Indeed, in resistance arteries and veins, the main effect of pressure is to induce a maintained vasoconstrictor (myogenic) tone. Isolated segments of rabbit facial vein were mounted in organ baths and submitted to isometric stretch. In this experimental model, myogenic tone was absent when the bath temperature was 33°C. ERK1/2 activity was determined in each isolated segment by an in-gel kinase assay. Wall tension and ERK1/2 activity were measured in the same samples in the presence (39°C) or in the absence of myogenic tone (33°C). At 39°C, a 5-mN wall tension induced myogenic tone (5.7 ± 1.8 mN) and an increase in ERK1/2 activity (282 ± 52% versus unstretched vessels, \( P < 0.05 \)). At 33°C, in the absence of myogenic tone, ERK1/2 activity was similarly increased by stretch (254 ± 35% versus unstretched vessels). The calcium-dependent and -independent protein kinase C (PKC) blocker Ro-31-8220 (5 × 10^{-7} mol/L), but not the calcium-dependent PKC blocker Go-6976 (10^{-6} mol/L), inhibited myogenic tone. However, ERK1/2 activity was not affected by either PKC blocker. Genistein (10^{-7} mol/L), a general tyrosine kinase inhibitor, but not herbimycin A (5 × 10^{-7} mol/L), a cSrc-family tyrosine kinase inhibitor, suppressed stretch-induced ERK1/2 activation (\( P < 0.05 \)) without affecting myogenic tone. Nifedipine (10^{-6} mol/L), a voltage-dependent calcium entry inhibitor, and ryanodine (10^{-6} mol/L), which depletes calcium stores, both inhibited ERK1/2 activity (113 ± 12% and 121 ± 7%, respectively; \( P < 0.05 \)) without affecting myogenic tone. The mitogen-activated protein kinase kinase inhibitor PD 98059 (5 × 10^{-6} mol/L) also inhibited ERK1/2 activation without affecting myogenic tone. The present results suggest that stretching the rabbit facial vein induced 2 distinct pathways, one leading to myogenic tone (via a non–calcium-dependent PKC activation) and one leading to ERK1/2 activation through a calcium-dependent pathway involving tyrosine kinase. (Arterioscler Thromb Vasc Biol. 1999;19:2878-2883.)

Key Words: protein kinase C • stretch • extracellular signal–related kinase • mitogen-activated protein kinase • rabbits

Pressure in resistance arteries and veins is responsible for a sustained contraction, which is mainly of myogenic origin. Myogenic tone is a major phenomenon in the regulation of regional blood flow. Although resistance vessels represent the majority of the arterial tree, their sizes limit the possibility to perform biochemical measurements needed to identify the signaling pathways involved in the response to pressure. In these vessels, myogenic tone is decreased by protein kinase C (PKC)² and phospholipase C inhibitors. In isolated cells, stretch leads to the opening of stretch-activated cationic channels⁴ and to the activation of phosphoinositide turnover,⁵ PKC,⁶ and calcium/calmodulin-dependent kinases.⁷ In addition, although calcium entry is required for the development of myogenic tone,⁸ the amount of calcium needed is much smaller than necessary for agonist-induced tone.⁹ Activation by stretch of the different transduction pathways leads to immediate cellular responses, such as secretion of bioactive substances,¹⁰ activation of smooth muscle cell contraction,¹¹ and cytoskeletal rearrangement.¹² Finally, a recent study has shown that pressure-induced (myogenic) tone in rat resistance cerebral arteries was sensitive to the tyrosine kinase inhibitor herbimycin A.¹³ The mitogen-activated protein (MAP) kinase pathway plays an important role in the signal transduction in vascular smooth muscle and endothelial cells. Indeed, extracellular signal–related kinases (ERKs) 1 and 2 are involved in the mechanotransduction of flow in endothelial cells¹⁴ and of pressure in the vascular smooth muscle.¹⁵,¹⁶ We have previously reported that in a model of aortic organ culture, ERK1/2 was activated in vascular smooth muscle cells in response to elevated intraluminal pressure.¹⁶ However, there is no information concerning the activation of ERK1/2 in blood vessels developing myogenic tone. The rabbit facial vein (RFV) develops a myogenic tone similar to that observed in resistance arteries.³,¹⁷ Furthermore, myogenic tone is abolished in the RFV that is exposed to low
temperatures (33°C). Therefore, we used this experimental model to test the effect of vascular wall stretch on ERK1/2 activity in the presence (39°C) or the absence (33°C) of myogenic tone and to determine whether ERK1/2 activation is involved or affected by the development of myogenic tone.11,18

Methods

Rabbit Facial Vein

Buccal segments of RFV were isolated as previously described.11 Ring segments (3 mm long) of RFV were mounted between parallel stainless steel wires in 5-mL organ baths. One wire was attached to a fixed support; the other was connected to a moveable holder supporting a tension transducer, so that isometric force could be recorded. Data were collected by a Biopac data acquisition system (Biopac MP 100). The vein segments were maintained at 39°C in a physiological salt solution with the following composition (in mmol/L): NaCl 135.0, NaHCO3 15.0, KCl 4.6, CaCl2 1.5, MgSO4 1.2, glucose 11.0, and HEPES 10.0, pH 7.4. P02 was maintained at a value of 160 mm Hg; P02, at a value of 37 mm Hg. After a 30-minute equilibration period, the segments were subjected to a 5.0-nm force (stretch), which is optimal for the development of myogenic tone.11 The procedure followed in the care and euthanasia of the study animals was in accordance with the European Community standards on the care and use of laboratory animals (authorization No. 00577).

Experimental Protocol

ERK1/2 activity was determined as previously described,15 and RFV segments were submitted to one of the following protocols. Each sample of vein was divided in 2 segments. One of the 2 segments was used as a control vessel,16 and the other was mounted in the myograph for force measurement. ERK1/2 activity was measured in both segments. The activity of each sample that had been mounted in a myograph and submitted to an experimental protocol was calculated as a percentage of the activity found in the corresponding control unstretched segment.16

At the end of each experimental protocol, the vein segment was rapidly removed from the organ bath, frozen in liquid nitrogen, and subsequently stored at −80°C until determination of ERK1/2 activity.

In a first series of experiments, RFV segments were stretched to an optimal tension (5 mN) and allowed to stabilize for 30 minutes in the absence (33°C, n = 8) or in the presence (39°C, n = 11) of myogenic tone. In a second series of experiments, RFV segments were stretched at 39°C. Veins were then exposed during 30 minutes to one of the following agents: the PKC activator phorbol dibutyrate (PDBu, 10−6 mol/L, n = 11), the calcium-dependent PKC inhibitor Go-6976 (10−6 mol/L, n = 9), the nonselective PKC inhibitor Ro-31-8220 (5×10−7 mol/L, n = 8), or the nonselective and nonspecific kinase inhibitor staurosporine (10−7 mol/L, n = 7).

In a third series of experiments, the involvement of tyrosine kinase and ERK1/2 was assessed in RFV segments with or without myogenic tone. The tyrosine kinase inhibitors genistein (10−7 mol/L, n = 9, 30 minutes) and herbimycin A (5×10−7 mol/L, n = 7, 30 minutes) were used separately. Specific inhibition of MAP kinase kinase activity was obtained by using PD 98059 (5×10−6 mol/L, n = 6, 30 minutes).

In a fourth series of experiments, the involvement of calcium in myogenic tone and ERK1/2 activity was assessed in the presence of stretch, with or without myogenic tone. Vein segments were submitted to a calcium-free physiological salt solution containing EGTA (2 mmol/L, n = 9), to nifedipine (10−6 mol/L, n = 6), or to ryanodine (10−5 mol/L, n = 6).

In each protocol, the pharmacological agent was left for 30 minutes in the organ bath before stretching the segment. Myogenic tone was monitored continuously from the onset of each experiment.

In-Gel ERK1/2 Assays

Kinase assays in myelin basic protein (MBP)-containing gels were performed as described previously.16 Laemmli sample buffer (70 µL) was added to 100 µL aliquots of vein extracts, and samples were loaded on a 9% SDS-polyacrylamide gel containing 0.5 mg/mL MBP. After electrophoresis, SDS was removed from the gel by washing with 3 changes at 100 mL each of 20% 2-propanol in 50 mmol/L Tris-HCl (pH 8.0) for 15 minutes and then with 100 mL of 50 mmol/L Tris-HCl (pH 8.0) containing 5 mmol/L 2-mercaptoethanol for 30 minutes at room temperature. Gels were further treated with 50 mL of 6 mol/L guanidine-HCl in 50 mmol/L Tris-HCl (pH 8.0) at room temperature for 1 hour, followed by 5 changes of 50 mmol/L Tris-HCl (pH 8.0) containing 0.04% Tween 20 and 5 mmol/L 2-mercaptoethanol at room temperature for 15 minutes each. Gels were left in the same buffer at +4°C overnight. Afterward, gels were immersed in 10 mL of 40 mmol/L HEPES (pH 8.0) containing 2 mmol/L dithiothreitol and 10 mmol/L MgCl2, for 1 hour at 25°C. Phosphorylation of MBP was carried out by incubating the gels with 50 μCi [γ−32P]ATP at 25°C for 3 hours in 10 mL of 40 mmol/L HEPES (pH 8.0) containing 2 mmol/L dithiothreitol, 10 mmol/L MgCl2, 0.5 mmol/L EGTA, and 40 μmol/L ATP. After incubation, the gels were washed with 10% acetic acid and 1% sodium pyrophosphate until the radioactivity of the washing solution became negligible. The washed gels were dried and then subjected to autoradiography.

Materials

[γ−32P]ATP (6000 Ci/mmol) was obtained from Amersham (Les Ulis). Gel electrophoresis reagents came from Bio-Rad; staurosporine, from LC Laboratories Europe; and Ro-31-8220 and Go-6976, from France-Biochem. The MAP kinase kinase inhibitor (PD 98059) was purchased from New England Biolabs. All other reagents were obtained from Sigma Chemical Co.

Data Analysis

All experiments were performed at least 5 times, and results are expressed as mean±SE. A 1-way ANOVA was constructed with data of ERK1/2 activity to test the effects of time and pressure. Comparisons were performed by use of the Tukey test. Statistical significance was accepted for values of P<0.05.

Results

Figure 1 shows a typical recording obtained in an RFV segment. In RFV segments, stretch (5 mN) at 39°C induced myogenic tone (5.7±0.6 mN, n = 11). Decreasing the temperature from 39°C to 33°C totally suppressed myogenic tone. There was no difference between 33°C and 0-calcium solution at 33°C containing sodium nitroprusside (10−4 mol/L) and EGTA (10−7 mol/L). At 39°C in the presence of PDBu (10−4 mol/L), myogenic tone increased significantly (10.7±1.8 mN), whereas the PKC inhibitor Ro-31-8220 (5×10−7 mol/L) totally inhibited myogenic tone. ERK1/2 activity was determined in RFV segments submitted to stretch for 30 minutes (Figure 2). In vein segments
The role of PKC in stretch-induced ERK1/2 activity and myogenic tone was assessed by using 2 inhibitors (Figure 5). Genistein (10⁻⁷ mol/L), a broad tyrosine kinase inhibitor, abolished ERK1/2 activity (121.8±50.2%, n=9, stretched vessels), whereas herbimycin A (5×10⁻⁷ mol/L), a c-src family tyrosine kinase inhibitor, did not affect ERK1/2 activity (276±12.3%, n=7 stretched vessels). Neither genistein nor herbimycin A significantly affected myogenic tone. The specific MAP kinase kinase inhibitor, PD 98059 (5×10⁻⁶ mol/L), significantly decreased ERK1/2 activity at 39°C (33.2±8.4% decrease in stretched vessels [n=6] versus unstretched vessels) without affecting myogenic tone. Similarly, PD 98059 decreased ERK1/2 activity at 33°C (29.4±6.4% decrease in stretched vessels [n=6] versus unstretched vessels).

Both the voltage-dependent calcium channel inhibitor nifedipine (10⁻⁶ mol/L) and ryanodine (10⁻⁶ mol/L), which depletes calcium stores, inhibited ERK1/2 activity (Figure 6) (113±12.4%, n=6, and 121±7.4%, n=6, respectively) without affecting myogenic tone (Figure 6). On the other hand, in vessels bathed in a 0-calcium physiological salt solution+EGTA (2 mMol/L), both myogenic tone and ERK1/2 activity were abolished.

**Figure 1.** Typical recording of wall tension in an RFV segment isolated in a myograph. In the top panel, myogenic tone (MT) was abolished by lowering bath temperature to 33°C or by the presence of sodium nitroprusside (SNP, 10⁻⁴ mol/L) and EGTA (10⁻³ mol/L) in a solution without calcium. In the middle panel, the RFV was stimulated by PDBu (10⁻² mol/L) in a solution without calcium. In the bottom panel, the inhibitory effect of Ro-31-8220 (5×10⁻⁷ mol/L) on MT.

without stretch, the basal ERK1/2 activity was considered to be 100%. The time course of stretch-induced activation of MAP kinase activation was as follows: 282±52% (n=11) at 30 minutes, 268±38% after 40 minutes (n=5), 259.4±47% after 60 minutes (n=5), 230.3±41% (n=5) after 2.5 hours, and 169.2±23% (n=5) after 5.5 hours.

In the absence of myogenic tone (33°C), stretch induced an increase in ERK1/2 activity (254±33%, n=8; P<0.05 versus unstretched RFV) (Figure 3), providing evidence that ERK1/2 is not activated by myogenic tone. In RFV segments maintained at 39°C (myogenic tone present), stretch increased ERK1/2 activity to an extent similar to that at 33°C (282±52%, n=11; P<0.05 versus unstretched RFV) (Figure 3).

The role of PKC in stretch-induced ERK1/2 activity and myogenic tone was assessed by using inhibitors or activators of PKC at 39°C in the presence of myogenic tone (Figure 4). On the other hand, Ro-31-8220 (5×10⁻⁷ mol/L), a specific inhibitor of all PKCs, induced a total blockade of myogenic tone but did not affect ERK1/2 activity (264±47% versus unstretched control veins, n=9). The selective calcium-dependent PKC inhibitor Go-6976 (10⁻⁴ mol/L) had a significant effect neither on myogenic tone (4.58±0.57 versus 5.7±0.59 mN in control conditions, n=8) nor on ERK1/2 activity (225±48% versus unstretched control veins, n=8). Only staurosporine, a nonspecific kinase inhibitor (10⁻⁷ mol/L), abolished both myogenic tone and ERK1/2 activity. Activation of PKC with PDBu (10⁻⁶ mol/L) increased myogenic tone (10.7±1.8 mN, n=11; P<0.05) and ERK1/2 activity (570±129%, n=11; P<0.05). Downregulation of PKCs with PDBu (10⁻⁶ mol/L, 24 hours) did not significantly affect myogenic tone (4.6±1 versus 5.7±1.8 mN in control conditions) but partially decreased ERK1/2 activity (157±14%). These inhibitors had the same effect on ERK1/2 activity at 33°C as at 39°C (data not shown).
The main finding of the present study is that acute stretch in an RFV triggers 2 independent pathways, one leading to the development of myogenic tone and one leading to the activation of ERK1/2.

Although myogenic tone is mainly studied in resistance arteries, it also occurs in veins. As pointed out by Monos, only recently have veins been considered “an active component of the cardiovascular system.” In veins, myogenic tone is an active regulator of systemic venous capacity. Myogenic tone in the RFV is very similar to that in resistance arteries, but it is temperature sensitive. This unique feature was used to measure ERK1/2 activation on stimulation by stretch in the presence or in the absence of myogenic tone.

The implication of calcium in myogenic tone has been previously shown in arteries and veins. The degree of involvement of voltage-activated channels is dependent on the type of vessel studied. Voltage-activated channels play a major role in cerebral arteries, whereas they are only partly involved in coronary arteries and do not play a role in the RFV (present study). A role for PKC activation in myogenic tone has been previously suggested in studies in which myogenic tone was inhibited by pharmacological agents in arteries and veins. Nevertheless, in these studies, the selectivity of the PKC inhibitors was rather weak. Using recent and more selective PKC inhibitors, we could show that calcium-independent PKC(s) are involved in myogenic tone in the RFV. This observation is compatible with previous studies showing that myogenic tone in this vessel depends on a low increase in intracellular calcium, suggesting that myogenic tone might be related to a PKC-dependent sensitization of the contractile apparatus to calcium in the RFV and in resistance arteries. We have previously shown that myogenic tone in the RFV is related to a much lower ratio of calcium influx to force than other forms of tone, such as agonist- or KCl-induced tone. Nevertheless, further investi-
The dissociation between force development on stretch (myogenic tone) and stretch-induced ERK1/2 activation is in agreement with recent studies that have reported a similar dissociation between wall force and ERK1/2 activation in swine carotid arteries stimulated by histamine and phorbol ester and in the rat aorta in response to angiotensin II. Thus, 2 distinct pathways were activated by stretch. One led to myogenic tone through a PKC-dependent pathway, and one led to ERK1/2 activation through a calcium-dependent and PKC-independent pathway.

The level of ERK1/2 activation was similar at 33°C and 39°C, and the inactivation of ERK1/2 by PD 98059, a specific MAP kinase inhibitor, was total at both temperatures. Thus, we can exclude a specific effect of temperature on ERK1/2 activation.

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