Activation of p38 Mitogen-Activated Protein Kinases by Endothelin and Noradrenaline in Small Arteries, Regulation by Calcium Influx and Tyrosine Kinases, and Their Role in Contraction

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Abstract—Small-artery responses to vasoconstrictor agonists are important for vascular function. To investigate the signaling pathways involved in contraction, we studied the activation and regulation of p38 mitogen-activated protein kinases (p38MAPKs) and heat shock protein (HSP) kinase by endothelin and noradrenaline in rat mesenteric arteries. Both vasoconstrictors activated p38α and/or p38β but not p38γ or p38δ, leading to increased HSP kinase activity. p38MAPK activation by noradrenaline was maximum between 2 and 10 minutes and was wholly dependent on calcium influx but insensitive to the tyrosine kinase inhibitor herbimycin A. In contrast, endothelin induced a biphasic response, with activation at 2 and 10 minutes. The early activity was wholly dependent on calcium influx and inhibited by herbimycin A. The later activity was only 50% calcium dependent, was insensitive to herbimycin A, but was 50% inhibited by genistein, a nonselective tyrosine kinase inhibitor. With both agonists, p38MAPK activity returned to basal by 30 minutes. SB203580, a p38MAPK inhibitor, blocked agonist-induced HSP kinase activity, and herbimycin A inhibited activation by endothelin but not by noradrenaline. In addition, SB203580 inhibited noradrenaline-induced contraction but had little effect on contraction to endothelin. These data show that vasoconstrictors use different upstream activators of p38MAPK in vascular tissue and that the p38MAPK pathway is selectively implicated in the contractile response to noradrenaline in small arteries. (Arterioscler Thromb Vasc Biol. 2001;21:1921-1927.)

Key Words: vasoconstrictors ■ vascular smooth muscle ■ heat shock proteins ■ signal transduction

Vascular tone is an important determinant of peripheral resistance and blood pressure, and abnormalities in small-artery contractility contribute to pathological states such as vasospasm and hypertension. The major mechanism of smooth muscle contraction is an increase in cytoplasmic calcium and phosphorylation of the regulatory light chains of myosin. However, there is considerable evidence indicating that vasoconstrictors activate multiple ancillary pathways that modulate the contractile response (see reviews). Among the many pathways activated, protein kinase C, Rho family G proteins, nonreceptor tyrosine kinases, and extracellular signal–regulated kinases (ERK1/2) have been shown to play a role in smooth muscle contraction. Recently, stress-activated protein kinases have also been implicated in sustained contraction through regulation of the phosphorylation of heat shock protein (HSP)27.

HSP27 belongs to a family of small HSPs that includes HSP20, myotonic dystrophy kinase–binding proteins, and crystallins. Increased phosphorylation of HSP27 has been reported in response to a variety of vasoconstrictors in smooth muscle, and inhibition of HSP27 phosphorylation or interference with its function reduces contraction. HSPs may also play a role in vascular diseases such as hypertension. For instance, stress-induced hypertension in rats increased the expression and phosphorylation of HSP27 in vascular tissue as did surgical stress. HSP27 phosphorylation is catalyzed by mitogen-activated protein kinase (MAPK)-activated protein kinase 2 (MAPKAPK2), which is, in turn, a substrate for stress-activated protein kinases, p38MAPKs.

p38MAPKs are members of the MAPK superfamily. Four p38MAPKs have been cloned, α (SAPK1), β (SAPK2), γ (SAPK3), and δ (SAPK4), of which the most extensively studied are p38α and p38β; this is due in part to the availability of SB203580, a specific inhibitor of their kinase activity. p38MAPKs are activated in response to cellular stress, ischemia/reperfusion, and also G protein–coupled receptors (GPCRs). Upstream regulators of p38MAPKs include Src family tyrosine kinases, p21-activated kinases, and small G proteins of the Rho family. p38MAPKs are directly activated by phosphorylation on Thr180 and Tyr182 by kinases MKK3 and MKK6.

Activation of p38MAPKs by GPCR agonists in smooth muscle is implicated in arachidonic acid release.
phosphorylation, and possibly contraction. In addition, there is evidence that growth factors and cytokines regulate smooth muscle cell migration through the activation of p38MAPK and the phosphorylation of HSP27. These studies point to an important functional role for p38MAPKs in smooth muscle; however, comparatively little is known regarding the mechanisms that control the activation of this pathway. In response to vasopressin, protein kinase C appears to be upstream from p38MAPKs, whereas angiotensin II uses a redox-sensitive mechanism. Also, tyrosine kinases are implicated possibly through GPCR agonist transactivation of the epidermal growth factor receptor or Src family tyrosine kinases. However, these studies have been conducted with smooth muscle cells, and the mechanisms involved in intact tissues have not yet been fully characterized.

We have investigated the role of nonreceptor tyrosine kinases and calcium in p38MAPK activation by GPCRs. Our results show that in rat mesenteric small arteries, noradrenaline (NA) and endothelin-1 (ET-1) activate p38MAPKα and/or p38MAPKβ, leading to increased HSP27 kinase activity. However, these agonists use different mechanisms such that NA-induced p38MAPK activity is independent of Src tyrosine kinase activity and wholly dependent on calcium influx, whereas the response to ET-1 is partially dependent on a herbinycin A–sensitive tyrosine kinase and calcium influx. In addition, we show that the p38MAPK pathway is selectively implicated in the contractile response to NA.

### Methods

#### Preparation of Small Arteries

Adult female Sprague-Dawley rats (200 to 300 g, Charles River UK Ltd, Kent, England) were used for all experiments. All procedures performed were carried out by trained personnel in accordance with institutional guidelines and the UK Animals (Scientific Procedures) Act of 1986. The mesentery was excised and kept in ice-cold physiological salt solution until dissection. Mesenteric small arteries (<400-μm internal diameter) were cleaned of adjoining fat and connective tissues and equilibrated in culture medium (medium 199) before the addition of 100 nmol/L ET-1. DMSO (0.01%), 1 mmol/L MgCl₂, 1 mmol/L EGTA, 0.1 mmol/L NaF, 1 mmol/L vanadate, 4 mmol/L dithiothreitol, 40 μCi [γ-32P]ATP, and 10 μCi [γ-32P]ATP, pH 7.2) in a final volume of 60 μL for 20 minutes at 30°C. The reaction was terminated by the addition of 0.5 mL ice-cold PBS (pH 7.0). The beads were washed twice with ice-cold PBS, resuspended in SDS sample buffer, boiled for 5 minutes, and processed as described for MAPKAPK2.

#### Tissue Extracts for Western Blotting and p38MAPK In Vitro Kinase Assays

Small arteries were homogenized in ice-cold lysis buffer, and solubilized proteins were stored at −80°C. Preliminary experiments showed no difference in kinase activity between fresh and frozen samples (data not shown). p38MAPK activity in the extracts (40 μg protein quantified by the Bradford assay) was determined by using glutathione S-transferase (GST)-MAPKAPK2 fusion protein bound to glutathione-Sepharose beads as a substrate. A control incubation without small-artery homogenate was included in each assay to measure MAPKAPK2 autophosphorylation. The reaction products were resolved on 10% SDS-PAGE and stained with Coomassie blue to confirm equivalent substrate loading. The gel was imaged, and radioactivity incorporated into GST-MAPKAPK2 was counted directly. Background signal due to MAPKAPK2 autophosphorylation constituted <13% of the total kinase activity and was corrected by subtraction from the sample counts. The data were expressed as fold change from control. Forty micrograms of the extract was used in the assay; to the remainder of the sample, SDS sample buffer (diluted 1:5 [vol/vol]) was added. The samples were boiled for 5 minutes and stored at −20°C.

### Srp Kinase Assay

Immunoprecipitates were prepared from small-artery homogenates by using anti-–v-Src antibody (Clone 327, Oncogene Research Products). Srp kinase activity in the immunoprecipitates was determined in 48 μL of kinase reaction buffer containing 0.25 mg/mL acid-denatured enolase, 20 μmol/L ATP, and 18 μCi [γ-32P]ATP at 30°C for 20 minutes. Reactions were stopped by the addition of SDS sample buffer and boiling. The incorporation of 32P into enolase was measured by direct counting after separation of the reaction products by SDS-PAGE and transfer to the nitrocellulose membrane. The presence of equivalent amounts of Src in the immunoprecipitates was confirmed by immunoblotting with the use of anti-c–Src (sc-18, Santa Cruz). The data were expressed as percent change from control.

#### HSP27 Kinase Activity Assay

Tissue extracts (40 μg protein) were incubated with GST-HSP27 fusion protein bound to glutathione-Sepharose beads in kinase buffer (25 mmol/L MOPS, 25 mmol/L β-glycerophosphate, 15 mmol/L MgCl₂, 1 mmol/L EGTA, 0.1 mmol/L NaF, 1 mmol/L vanadate, 4 mmol/L dithiothreitol, 40 μCi [γ-32P]ATP, and 10 μCi [γ-32P]ATP, pH 7.2) in a final volume of 60 μL for 20 minutes at 30°C. The reaction was terminated by the addition of 0.5 mL ice-cold PBS (pH 7.0). The beads were washed twice with ice-cold PBS, resuspended in SDS sample buffer, boiled for 5 minutes, and processed as described for MAPKAPK2.

### Immunodetection

Western blot analysis of tissue extracts (20 μg protein) was performed as previously described.

### Small-Artery Contraction

Contrast response was determined from segments of small artery (~320-μm internal diameter) by pressure myography, as described previously. Vessel segments were incubated with vehicle, 0.01% dimethyl sulfoxide (DMSO), 1 μmol/L SB203580, or 1 μmol/L SB202474 for 15 minutes before the recording of cumulative concentration-response curves to ET-1 (0.03 to 300 nmol/L) and NA (0.1 to 15 μmol/L). Lumen diameter was measured 2 minutes after the addition of the agonist, immediately before the addition of the next concentration. NA concentration-response curves in the presence of vehicle and inhibitor were obtained from a single vessel segment; for ET-1, individual segments were used for each treatment. In a second set of experiments, the contractile response to ET-1 in the absence of extracellular calcium was recorded. Vessel segments were incubated in calcium-free HEPES buffer containing 1 mmol/L EGTA for 15 minutes and stimulated twice with 15 μmol/L NA for 2 minutes to empty the intracellular calcium stores before the addition of 100 mmol/L ET-1, DMSO (0.01%), 1 μmol/L SB203580, or 1 μmol/L SB202474 was included throughout the protocol. The contractile response to ET-1 was recorded over a 10-minute period.

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### Statistical Analysis

Comparisons between groups were made by Student t test or ANOVA with a Bonferroni correction for multiple comparisons; GraphPad Prism software was used. A value of P<0.05 was considered statistically significant.

### Materials

Many of the reagents used were obtained from sources previously described. A gift from Prof C. Marshall (Chester Beatty Laboratories, Institute of Cancer Research, London, UK), and HSP27, a gift from Prof Saklatvala (Kennedy Institute Rheumatology Division, London, UK), were expressed as GST fusion proteins in Escherichia coli and purified by using glutathione Sepharose 4B beads according to the manufacturer’s protocol (Pharmacia Biotech). The radioactivity in gels and on membranes was counted directly by using an Instant imager (Packard).

Polyclonal p38MAPK and phospho-p38MAPK...
Vasoconstrictor Hormones Activate p38MAPK

Using antibodies that recognize activated p38MAPK when phosphorylated on Thr180 and Tyr182, we detected increased phosphorylation of p38α and/or p38β after stimulation with 100 nmol/L ET-1 and 15 μmol/L NA (Figure 1B and 1C). There was no detectable change in SAPK3 or SAPK4 phosphorylation (data not shown). To further investigate the activation of p38MAPK, we used an in vitro pull-down assay with GST-MAPKAPK2 as a substrate that preferentially phosphorylates (data not shown). To confirm the specificity of this assay, we used immunoblot analysis of GST-MAPKAPK2 pull downs prepared from nontreated and 100 nmol/L ET-1-stimulated vessels. The results represent 3 separate experiments.

Figure 1. Immunodetection of p38MAPKs in rat mesenteric small arteries: activation by ET-1 and NA. A, Small-artery homogenates were analyzed with antibodies against p38MAPKα/β, p38γ/SAPK3, and p38δ/SAPK4. B and C, Small arteries were stimulated with 100 nmol/L ET-1 or 15 μmol/L NA and analyzed with anti–phospho-p38MAPKα/β antibody (top blots). Membranes were stripped and probed with anti-p38MAPKα/β (bottom blots), confirming equivalent loadings. D, Immunodetection of p38MAPKα/β in GST-MAPKAPK2 pull downs from nonstimulated artery homogenates showed very low levels of phosphorylation (Figure 1D). The molecular mass of SAPK3 (p38γ) and SAPK4 (p38δ) was ≈39 kDa.

Results

Rat Mesenteric Small Arteries Express Multiple p38MAPK Isoforms

Immunoochemical analysis showed the presence of p38α (RK/SAPK2a) and/or p38β (SAPK2b), p38γ (SAPK3), and p38δ (SAPK4) in small-artery extracts (Figure 1A). The p38α/β band had an apparent molecular mass of 41 kDa, in agreement with canine smooth muscle p38MAPK. The molecular mass of SAPK3 (p38γ) and SAPK4 (p38δ) was ≈39 kDa.

Activation of p38MAPK by Vasoconstrictors Is Dependent on Extracellular Calcium

Elevation of intracellular calcium is a major component of vasoconstrictor signaling. Therefore, we incubated vessels in calcium-free medium (0 mmol/L calcium/1 mmol/L EGTA-HEPES buffer) for 10 minutes before agonist stimulation. This treatment completely blocked p38MAPK activity induced by NA (Figure 2B). An early but statistically insignificant increase in activity was observed (Figure 2B), possibly reflecting incomplete removal of calcium or release from intracellular stores. In contrast, only the initial phase of p38MAPK activity in response to ET-1 was completely blocked by the removal of extracellular calcium, whereas the second phase was reduced by ≈50% (Figure 2A).

Activation of p38MAPK by Vasoconstrictors Is Dependent on Tyrosine Kinase Activity

In mesenteric small arteries, ET-1 induces a rapid increase in protein tyrosine phosphorylation that is blocked by tyrosine kinase inhibitors. Accordingly, we investigated whether the tyrosine kinase inhibitor genistein affected the response to ET-1. Fifteen minutes of preincubation with 100 μmol/L genistein inhibited p38MAPK activation by 100 μmol/L ET-1 (10 minutes). Daidzein, a negative analogue of genistein, slightly reduced basal p38MAPK activity but was without effect on ET-1–stimulated activity (p38MAPK activity as fold change from control, where control = 1: ET-1 2.11±0.29-
fold \( P < 0.05 \) compared with control by Student \( t \) test, genistein 1.15-fold, genistein \( P < 0.05 \) compared with control by Student \( t \) test, and ET-1 1.62-fold, daidzein 0.77-fold \( P < 0.05 \) compared with control by Student \( t \) test; mean \( \pm \) SEM, \( n = 3 \).

### Activation of Src Family Tyrosine Kinases by ET-1 and NA

Src tyrosine kinases are implicated in p38MAPK activation by GPCRs. As shown in Figure 3A and 3B, ET-1 (100 nmol/L) and NA (15 \( \mu \)mol/L) induced a rapid (detectable at 30 to 45 seconds) and transient (reduced below basal at 5 minutes) increase in Src kinase activity. Removal of extracellular calcium prevented Src activation by NA but not by ET-1 (Figure 3C).

### Effect of Herbimycin A on NA-Induced and ET-1-Induced p38MAPK Activity

The rapid activation of Src by NA and ET-1 suggested that these tyrosine kinases lie upstream from p38MAPK. Incubation with herbimycin A, an Src-selective inhibitor, blocked the first phase of p38MAPK activation in response to ET-1 but was without effect on the second phase (Figure 4A). In contrast, herbimycin A had no effect on the response to NA (Figure 4B). Herbimycin A completely inhibited ET-1-induced and NA-induced Src kinase activity (Figure 3C), confirming Src inhibition in our preparation. These results implicate a herbimycin A-sensitive tyrosine kinase in ET-1-induced but not NA-induced p38MAPK activation.

### Activation of HSP27 Kinase Activity by NA and ET-1

To determine whether HSP phosphorylation may occur in response to p38MAPK activation, we assayed for endogenous

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**Figure 3.** Regulation of Src tyrosine kinases. A and B, Small arteries were stimulated with 100 nmol/L ET-1 (A) and 15 \( \mu \)mol/L NA (B) for various times up to 5 minutes. Src immunoprecipitates were prepared, and kinase activity was measured as described in Methods. C, Small arteries were stimulated with 100 nmol/L ET-1 (40 seconds) or 15 \( \mu \)mol/L NA (30 seconds) in the presence of 1.26 mmol/L calcium (open bars), 0 mmol/L calcium/1 mmol/L EGTA (hatched bars), or 1 \( \mu \)mol/L herbimycin A (solid bars). The data are mean \( \pm \)SEM (\( n = 5 \) [A], \( n = 4 \) [B and C]) expressed as percent change from control. *\( P < 0.05 \) by Student \( t \) test.

**Figure 4.** Effect of herbimycin A on p38MAPK activity. Small arteries were untreated (open squares with solid line) or treated with 1 \( \mu \)mol/L herbimycin A (open triangles with dotted line) for 15 minutes before stimulation with 100 nmol/L ET-1 (A) and 15 \( \mu \)mol/L NA (B) for various times up to 20 minutes. p38MAPK activity was measured as described in Methods. The data are mean \( \pm \)SEM (\( n = 3 \) [A], \( n = 6 \) [B]) expressed as fold change from control, where control=1. *\( P < 0.05 \) by ANOVA and Bonferroni post hoc test.

**Figure 5.** Regulation of HSP27 kinase activity. Small arteries were treated with 0.01% DMSO (open bars), 1 \( \mu \)mol/L SB203580 (hatched bars), or 1 \( \mu \)mol/L SB202474 (solid bars) in panel A or 0.01% DMSO (open bars) and 1 \( \mu \)mol/L herbimycin A (solid bars) in panel B for 15 minutes before stimulation with 100 nmol/L ET-1 or 15 \( \mu \)mol/L NA for 2 minutes. Endogenous HSP27 kinase activity was measured as described in Methods. The data are mean \( \pm \)SEM (\( n = 3 \) [A], \( n = 5 \) [B]) expressed as fold change from control, where control=1. *\( P < 0.05 \) compared with vehicle control, and \( P < 0.05 \) compared with NA, by Student \( t \) test (A). *\( P < 0.05 \) compared with ET-1, by Student \( t \) test (B).
Role of p38MAPK Activation in Small-Artery Contraction

We have shown previously that tyrosine kinase activity is important for the tonic phase of ET-1–induced contraction in small arteries.30 Furthermore, our present observations that p38MAPK activation in response to agonists is dependent on tyrosine kinase activity and calcium influx suggests that this pathway may be involved in contraction. Accordingly, we studied the effect of p38MAPK inhibition on the contractile response of segments of rat mesenteric artery to NA and ET-1. In the presence of SB203580 (1 µmol/L), there was a significant rightward shift of the concentration response curve to NA (Figure 6A), an increase in the EC_{50} (for log EC_{50}, NA+0.1% DMSO 1.61±0.26 µmol/L and NA+1 µmol/L SB203580 2.77±0.25, P<0.05; mean±SEM, n=5) and a reduction of maximum contraction (Figure 6A), implicating p38MAPK activity in NA-induced contraction. SB202474 was without effect (Figure 6B). The ET-1 concentration response curve was also shifted rightward in the presence of SB203580 and SB202474, but there was no reduction in maximum contraction (Figure 7A). Additionally, we determined whether either compound affected ET-1–stimulated contraction in the absence of extracellular calcium. ET-1 induced a small and sustained contraction in the absence of extracellular calcium, which was inhibited by SB202474 but not SB203580 (Figure 7B). These data show that the effect of SB202474 on ET-1–induced contraction is unlikely to be due to p38MAPK inhibition. That conclusion is supported by the fact that SB202474 does not directly inhibit p38MAPK activity in the in vitro kinase assay (for GST-MAPKAPK2 phosphorylation with use of 40 µg rat mesenteric artery homogenate, 0.01% DMSO 20±9 cpm, 1 µmol/L SB202474 35±15 cpm, and 1 µmol/L SB203580 0 cpm; mean±SEM, n=3) or inhibit basal or ET-1–induced HSP27 kinase activity (Figure 5A).

Discussion

We have demonstrated that NA and ET-1 activate p38MAPKα and/or p38MAPKβ in rat mesenteric arteries, leading to an increase in HSP kinase activity. Inhibition of this response with SB203580 inhibited NA-induced contraction but had little effect on the contractile response to ET-1. In addition, the mechanisms of p38MAPK activation differed between agonists, with NA having a requirement for calcium but not herbimycin A–sensitive tyrosine kinases and with ET-1 being partially dependent on extracellular calcium and tyrosine kinases.
Identification of 4 p38MAPK isoforms in rat mesenteric small arteries confirms and extends the observations of Hedges et al., who identified p38α in canine smooth muscle. The present study shows GPCR activation of p38α and/or p38β isoforms in smooth muscle, with no detectable activation of the SAPK3/p38γ or SAPK4/p38δ isoforms. Activation of p38α and/or p38β by NA and ET-1 occurred within 2 minutes and returned to baseline by 20 to 30 minutes. Similar rapid activation of p38MAPK by GPCR agonists has been observed in smooth muscle cells. However, the biphasic activation (by ET-1) observed in the present study has not been reported previously.

Removal of extracellular calcium completely inhibited NA activation of p38MAPK (Figure 2B), showing that this was a calcium-dependent response. In addition, NA activated Src tyrosine kinases in a rapid and calcium-dependent manner, suggesting that Src may be upstream from p38MAPK. However, inhibition of Src kinase with herbimycin A did not affect NA-induced p38MAPK activity, showing that this tyrosine kinase is not involved. There is evidence that calcium may regulate MAPK activity through a calmodulin-dependent protein kinase cascade. For instance, in PC12 and vascular smooth muscle cells, calmodulin is necessary for ERK activation by membrane depolarization and angiotensin II, respectively, and constitutively active calmodulin-kinase IV increased p38MAPK activity when expressed in PC12 cells. Further studies are required to show whether NA is acting through such a mechanism in smooth muscle.

ET-1 appeared to activate p38MAPK by at least 2 mechanisms: calcium-dependent and tyrosine kinase–dependent pathways. Removal of extracellular calcium completely inhibited the first phase of ET-1–induced p38MAPK activity and partially inhibited the second phase (Figure 2A). Similar to NA, ET rapidly activated Src, but this activation was independent of extracellular calcium; therefore, increased Src activity in the absence of calcium was not sufficient to activate p38MAPK. Herbimycin A inhibited Src and the early phase of p38MAPK activity in response to ET-1, suggesting that Src activity was required for a calcium-dependent step in the activation sequence. A possible candidate is the calcium-dependent cytosolic tyrosine kinase PYK2 (also known as RAFTK and CADTK). Recently, it has been shown in mesangial cells that Src activity is necessary for PYK2 and subsequent p38MAPK activation in response to ET-1. Furthermore, in rat intact small arteries, NA and ET-1 induce rapid tyrosine phosphorylation of PYK2 (J. and V. Ohanian, unpublished data, 2001), suggesting that PYK2 may be involved in GPCR activation of p38MAPK in smooth muscle. However, our data do not exclude the possibility that a herbimycin A–sensitive tyrosine kinase other than Src is involved in the early phase of ET-1–stimulated p38MAPK activity. The second phase of the response was partially dependent on calcium and tyrosine kinase activity, although not a herbimycin A–sensitive kinase. These data again implicate PYK2, although further experiments are required to confirm the involvement of this kinase.

Inhibition of p38MAPK activity with SB203580 reduced the sensitivity and the maximum contractile response of small-artery segments to NA. This observation is in agreement with a recent report in canine pulmonary artery showing that SB203580 inhibited phenylephrine-induced contraction.

In contrast, SB203580 had only a minor effect on ET-1–induced contraction in the presence of calcium, and it was unclear whether this was due to p38MAPK inhibition or nonrelated effects of pyridinyl imidazoles, because SB202474, a negative analogue, inhibited ET-1–induced contraction with equal potency despite having no effect on endogenous HSP27 kinase activity or NA-induced contraction. These data demonstrate that the p38MAPK pathway is selectively involved in NA responses, although the mechanism by which p38MAPK might regulate contraction is not known. Recent evidence suggests that small HSPs, downstream effectors of p38MAPKs, are involved in smooth muscle contraction (HSP27) and relaxation (HSP20). HSP27 is strongly expressed in smooth muscle, and increased phosphorylation has been associated with smooth muscle contraction. The mechanism(s) by which HSP27 modulates contractility is not clear but may involve effects on the actin cytoskeleton, because increased HSP27 phosphorylation has been shown to affect actin microfilament dynamics and because changes in the association of HSP27 with actin have been observed during sustained smooth muscle contraction. In addition, there is evidence that phosphorylation of HSP20 is important for cyclic nucleotide–dependent smooth muscle relaxation and that phosphorylated HSP27 inhibits this mechanism, suggesting that the balance between these 2 HSPs may regulate the level of contraction. We detected in rat small-artery homogenates endogenous HSP27 kinase activity that was stimulated by NA and ET-1. The activation was dependent on p38MAPK activity, as shown by inhibition of the response by SB203580 and the differential sensitivity to herbimycin A. These observations suggest that HSP27 phosphorylation could occur in response to NA and ET-1 in intact small arteries. However, although SB203580 inhibited the increase in HSP27 kinase activity in response to both agonists, it inhibited only NA-induced contraction, suggesting that the role of p38MAPK in NA-induced contraction may be through a different mechanism (eg, regulation of calcium influx, as reported for bradykinin). Furthermore, these data show that HSP27 kinase activity and, presumably, HSP27 phosphorylation are not essential for small-artery contraction.

In summary, we have shown that calcium-dependent tyrosine kinase activity appears to be a major regulator of p38MAPK activation in intact small arteries, although ET-1 is able to activate additional mechanisms that are not dependent on calcium but are mediated by tyrosine kinases. Finally, we have shown that the p38MAPK pathway is selectively involved in the NA-induced constriction of small arteries.

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References


39. Ohanian et al, p38MAPKs in Vascular Smooth Muscle Contraction
Activation of p38 Mitogen-Activated Protein Kinases by Endothelin and Noradrenaline in Small Arteries, Regulation by Calcium Influx and Tyrosine Kinases, and Their Role in Contraction

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Tissue extracts for western blotting and p38MAPK in vitro kinase assays.

Small arteries were homogenised in ice-cold homogenisation buffer (0.15mol/L NaCl, 50mmol/L Tris pH 7.2, 1% deoxycholate, 1% Triton-X-100, 0.1% SDS, 5% glycerol and 1mmol/L dithiothreitol) containing the following protease and phosphatase inhibitors; 2µg/ml pepstatin A, 0.5µg/ml leupeptin, 1mmol/L phenylmethylsulfonyl fluoride, 50µmol/L vanadate, 20mmol/L β-glycerophosphate and 10mmol/L p-nitrophenol phosphate. The homogenates were placed on a rotary mixer for 30 minutes at 4°C and centrifuged at 12,000xg for 5 minutes at 4°C. The supernatant was removed, an aliquot was taken for protein estimation (BioRad) and the remainder stored at –80°C. Preliminary experiments showed no difference in kinase activity between fresh and frozen samples (data not shown). p38MAPK activity in the extracts was determined using the solid state in vitro kinase assay of Westwick and Brenner \(^1\). Briefly, 40µg protein was incubated with GST-MAPKAPK2 fusion protein bound to glutathione-sepharose beads for 3 hours at 4°C. A control incubation without small artery homogenate was included in each assay to measure MAPKAPK2 autophosphorylation. The beads were precipitated by centrifugation at 12,000xg for 15 seconds and washed 3 times in HEPES Balanced Buffer (HBB) containing 20mmol/L HEPES pH 7.7, 50mmol/L NaCl, 0.1mmol/L EDTA, 2.5mmol/L MgCl\(_2\), 0.05% Triton X-100, 1mmol/L dithiothreitol, 2µg/ml pepstatin A, 0.5µg/ml leupeptin, 50µmol/L vanadate, 20mmol/L β-glycerophosphate and 10mmol/L p-nitrophenol phosphate, followed by 2 washes in kinase buffer containing 15mmol/L HEPES pH 7.7, 2mmol/L EGTA, 10mmol/L MgCl\(_2\), 2mmol/L dithiothreitol, 0.1% Triton X-100, 50µmol/L vanadate, 20mmol/L β-glycerophosphate and 10mmol/L p-nitrophenol phosphate. The beads were incubated with 35µl of kinase buffer containing 40µmol/L ATP and 5µCi \(^{32}\)P-γ-ATP for 20 min at 30°C. The reaction was terminated by washing the beads twice in ice-cold HBB, SDS
sample buffer was added to the beads which were boiled for 5 min. The reaction products were resolved on 10% SDS-PAGE and stained with Coomassie Blue to confirm equivalent substrate loading. The gel was imaged and radioactivity incorporated into GST-MAPKAPK2 counted directly. Background signal due to MAPKAPK2 autophosphorylation comprised <13% of the total kinase activity and was corrected by subtraction from the sample counts. The data were expressed as fold change from control. 40µg of extract was used in the assay, to the remainder of the sample SDS sample buffer (diluted 1:5 [vol/vol]) was added. The samples were boiled for 5 minutes and stored at –20°C.

**Src kinase assay.**

Small artery homogenates were precleared by incubation for 30 minutes at 4°C with Protein A/Protein G sepharose followed by centrifugation at 12,000xg for 5 minutes. Aliquots of the supernatants containing 80µg protein were incubated for 2 hours at 4°C with 1µg anti-v-src antibody (Clone 327, Oncogene Research Products), Protein A/Protein G plus sepharose was added and the incubation was left for a further 30 minutes. Immunoprecipitates were collected by centrifugation and washed 3 times with ice-cold homogenisation buffer and once with kinase reaction buffer (50mmol/L MOPS, pH 7.0, 10mmol/L MgCl₂, 0.05mmol/L β-glycerophosphate). Reactions were carried out in 48µl of kinase reaction buffer containing 0.25mg/ml acid-denatured enolase, 20µmol/L ATP and 18µCi [³²P]-γATP at 30°C for 20 minutes. Reactions were stopped by the addition of SDS sample buffer and boiling. The incorporation of ³²P into enolase was measured by direct counting following separation of the reaction products by SDS-PAGE and transfer to nitrocellulose membrane. The presence of equivalent amounts of Src in the immunoprecipitates was confirmed by immunoblotting using anti-c-src (sc-18, Santa Cruz). The data were expressed as percent change from control.
**HSP27 kinase activity assay**

Intact small arteries were incubated for 15 minutes with 0.01% DMSO (vehicle), 1µmol/L SB203580 (p38MAPK inhibitor) or 1µmol/L SB202474 (negative control) before stimulation with 15µmol/L NA or 100nmol/L ET-1 for 2 minutes and tissue extracts were prepared as described above. 40µg protein was incubated with GST-HSP27 fusion protein bound to glutathione-sepharose beads in kinase buffer (25mmol/L MOPS, 25mmol/L β-glycerophosphate, 15mmol/L MgCl₂, 1mmol/L EGTA, 0.1 mmol/L NaF, 1mmol/L vanadate, 4mmol/L dithiothreitol, 40µmol/L ATP, 10µCi ³²P-γ-ATP pH 7.2) in a final volume of 60µl for 20 minutes at 30°C. The reaction was terminated by the addition of 0.5ml ice-cold phosphate buffered saline (PBS pH7.0). The beads were washed twice with ice-cold PBS, resuspended in SDS sample buffer and boiled for 5 minutes. The reaction products were separated by SDS-PAGE, stained with Coomassie Blue to confirm equivalent substrate loading and the radioactivity incorporated into GST-HSP27 counted directly.

**Immunodetection.**

Western blot analysis of tissue extracts (20µg protein) was performed as previously described²;³.

**Small artery contraction.**

Small artery contractile responses were determined by pressure myography. Segments of small artery (~320µm i.d.) were cannulated and mounted in a Living Systems (Vermont, USA) pressure myograph as described previously⁴. The artery segment was set to an intraluminal pressure of 50mmHg, an approximation of *in vivo* pressure and left to stabilise for 30 minutes. Culture media M199 gassed with 5% CO₂ in air was used for all incubations, and vessel diameter was monitored using a Video Dimension Analyser (Living Systems, Vermont). Data was collected and analysed
using a computerised data acquisition system (Windaq). Intraluminal diameter was used as an index of the contractile response. Vessel segments were incubated with vehicle 0.01% DMSO, 1µmol/L SB203580 or 1µmol/L SB202474 for 15 minutes before recording of cumulative concentration response curves to ET-1 (0.03-300nmol/L) and NA (0.1-15µmol/L). Lumen diameter was measured 2 minutes following addition of agonist, immediately prior to addition of the next concentration. NA concentration response curves in the presence of vehicle and inhibitor were obtained from a single vessel segment, for ET-1 individual segments were used for each treatment. In a second set of experiments the contractile response to ET-1 in the absence of extracellular calcium was recorded. Vessel segments were incubated in calcium free HEPES buffer containing 1mmol/L EGTA for 15 minutes, stimulated twice with NA 15µmol/L for 2 minutes to empty intracellular calcium stores before addition of ET-1 100nmol/L. 0.01% DMSO, 1µmol/L SB203580 or 1µmol/L SB202474 were included throughout the protocol. The contractile response to ET-1 was recorded over a 10 minute period.

REFERENCES

