Pressure-Induced Activation of Extracellular Signal-Regulated Kinase 1/2 in Small Arteries

Yvonne E.G. Eskildsen-Helmond, Michael J. Mulvany

Abstract—Extracellular signal-regulated kinase 1/2 (ERK1/2) may play a central signaling role in vascular remodeling. We investigated a possible combined role for the renin-angiotensin system and platelet-derived growth factor β-receptor (PDGF-β-R) in pressure-induced ERK1/2 activation in intact rat mesenteric small arteries. In an organ culture model, vessels were pressurized (70 mm Hg) for 1 hour plus a 5-minute intervention period. The intervention was either a rise in intraluminal pressure (up to 140 mm Hg) or challenge with angiotensin II (Ang II, 0.1 μmol/L) or PDGF-BB (30 μg/L). ERK1/2 activation was determined by Western blotting as formation of phosphorylated ERK1/2. All interventions caused ERK1/2 activation that was inhibited by the MEK inhibitor PD98059. The response to pressure was inhibited by an ACE inhibitor (perindoprilat), an Ang II receptor type 1 (R-AT1) antagonist (candesartan), and tyrosine kinase inhibitors (genistein, herbimycin A). An R-AT2 antagonist (PD123319) had no significant effect. Both a PDGF-receptor tyrosine kinase inhibitor (RPR101511A) and a neutralizing PDGF-β-R antibody (AF385) inhibited the activation of ERK1/2 caused by PDGF-BB, Ang II, and pressure. That the latter interventions could indeed inhibit the PDGF-β-R was supported by experiments with unmounted vessels in which PDGF-β-R activation was measured by Western blot; both PDGF-BB and Ang II–mediated PDGF-β-R activation were inhibited by RPR101511A and AF385. Immunohistochemistry showed that ERK1/2 and PDGF-β-R was located in the adventitia, tunica media, and intima. The results suggest that pressure in rat mesenteric small arteries causes acute activation of ERK1/2 through pathways involving Ang II and PDGF-β-R. (Hypertension. 2003;41:891-897.)

Key Words: platelet-derived growth factor • kinase • mesenteric arteries • rats • angiotensin-converting enzyme

Extracellular signal-regulated kinase 1/2 (ERK1/2) plays a central signaling role in many cellular processes in involving signals originating from many receptors, including growth factor and angiotensin II (Ang II) receptors. Furthermore, ERK1/2 is thought to play an essential role in the mechanism of vascular remodeling involving regulation of protooncogene expression (c-fos), protein expression, growth, and proliferation.

Vascular remodeling is involved in several diseases, and remodeling of the resistance vasculature plays a crucial role in the pathogenesis of essential hypertension. In essential hypertension, the resistance vessels show eutrophic remodeling, which is a reduction in the relaxed lumen and increase in medial:lumen ratio but unchanged media cross-sectional area. The remodeling is thought to be largely an adaptive response to the raised pressure, but pressure-independent mechanisms related to long-term infusion of non-pressor doses of Ang II have been demonstrated. Consistent with this, many antihypertensive treatments, in particular with ACE inhibitors, reverse the remodeling.

Although the morphological characteristics of resistance artery remodeling in hypertension are relatively well defined, the cellular mechanisms remain unclear. Much of the work to identify these has been performed in cell cultures, in which interpretation is complicated by factors such as change of phenotype and absence of normal wall stress. Other evidence has been obtained from larger arteries, such as cannulated rabbit aorta, in which ERK1/2 was activated synergistically by pressure and Ang II. The possible relevance of these findings to smaller vessels has now been investigated in intact resistance vessels by ourselves and others, in which the effect of Ang II on ERK1/2 activity was found to be Src tyrosine kinase and protein kinase C (PKC)-dependent, to be mediated through the Ang II receptor type 1 (R-AT1), and to be potentiated by pressure.

Recent evidence, mainly in cell culture, has demonstrated an important role for the platelet-derived growth factor-β-receptor (PDGF-β-R) and epidermal growth factor (EGF) receptor in Ang II–induced activation of ERK1/2. Furthermore, under in vivo conditions, the PDGF-receptor (PDGF-R) in rat aorta was activated by hypertension, whereas in cannulated rabbit aorta, raised pressure caused synthesis of Ang II. These findings raise the possibility that pressure-induced ERK1/2 activation is mediated through the combined action of autocrine production of Ang II and the PDGF-R and that this could be of relevance to the control of...
blood pressure. The purpose of the present investigation was to test this hypothesis in rat mesenteric small arteries, vessels small enough to be considered resistance vessels.20

Methods
Preparation and Interventions
Mesenteric small arteries (200 to 300 μm diameter, 4 to 8 mm long) were dissected from 12-week-old male Wistar rats (Møllegaard, Skensved, Denmark, treated in accordance with Danish regulations) and mounted in a 2-chamber pressure myograph allowing control of intravascular pressure. Any side branches were sutured to avoid flow during the experiments. In some cases, where indicated, vessels were not mounted but were otherwise processed as for the mounted vessels. Vessels were incubated in Hanks balanced salt solution (BioWhittaker Europe), high penicillin (113200 U/L), and streptomycin (0.113 g/L) for 30 minutes, sutured on glass pipettes, and equilibrated in 6 mL of sterile serum-free Dulbecco’s Modified Eagle Medium (DMEM) in an incubator containing 5% CO₂ at 37°C, with internal pressure 70 mm Hg for 1 hour. Vessels were then exposed to a 5-minute intervention consisting of either raising the internal pressure (to 105 mm Hg or 140 mm Hg) or adding Ang II (0.1 μmol/L) or PDGF-BB (1 μmol/L, 10 μg/mL, or 30 μg/mL) to the sulfatide. Where indicated, PD98059 (2-(2-amino-3-(methoxyphenyl)-ox-anaphtalen-4-one, MEK inhibitor, 10 μmol/L), candesartan (R-AT1 inhibitor, 10 μmol/L), PD123319 (Ang II receptor type 2, R-AT2, inhibitor, 10 μmol/L), herbimycin A (c-Src family tyrosine kinase inhibitor, 1 μmol/L), genistein (general tyrosine kinase inhibitor, 1 μmol/L), AF385 (PDGF-β-R extracellular domain, human polyclonal antibody, 1 mg/mL), RPR101511A (PDGF receptor tyrosine kinase inhibitor, 1 μmol/L), or perindoprilat (ACE inhibitor, 1 μmol/L or 10 μmol/L) was present throughout the equilibration and intervention periods. Control vessels were held at 70 mm Hg during the 1-hour equilibration period and the 5-minute intervention period without drugs. In another series, vessels were exposed to PD98059 throughout the equilibration period plus a further 5 minutes at 70 mm Hg to determine if the procedure itself was causing activation of ERK1/2. At the end of the intervention period, vessels were snap-frozen in liquid nitrogen and kept until further use at −80°C. For the immunohistochemistry experiments, vessels were kept in serum-free DMEM and fixed in 4% formaldehyde after snap-freezing.

Tissue Extraction
Frozen vessel segments were pulverized and resuspended in ice-cold lysis buffer (25 μL, 20 mmol/L Tris/HCl, pH 7.5, 5 mmol/L EGTA, 150 mmol/L NaCl, 20 mmol/L glycerophosphate, 10 mmol/L NaF, 1 mmol/L Na₂VO₃, 1% Triton X-100, 0.1% Tween-20, 1 mg/L aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride, 0.5 mmol/L N-p-tosyl-L-phenylalanine chloromethyl ketone, 0.5 mmol/L N-acetyl-p-tosyl-L-lysine chloromethyl ketone). Artery extracts were incubated for 15 minutes on ice and then centrifuged (3600 rpm, 15 minutes, 4°C). The detergent-soluble supernatant fractions were retained, and for 15 minutes, 14 aliquots, 14 μL of sample buffer (for 100 mL: 60 mL 62.5 mmol/L Tris/HCl, 10 mL glycerol [99%]), 20 mL 10% SDS, 10 mL B-mercaptoethanol, 0.1% bromophenol blue) was added; samples were heated (95 to 100°C, 3 minutes) and cooled before loading on gel. Proteins were separated (Mini gel Protein II system, Bio-Rad, 200 V, 35 minutes; 350 mL 25 mmol/L Tris, 192 mmol/L glycine, 0.1% SDS) on a 10% resolving gel and 4% stacking gel (Tris-HCl Ready Gel, Bio-Rad) and then transferred (35 V, overnight, 4°C using 800 mL 25 mmol/L Tris, 192 mmol/L glycine, 0.1% methanol) to PVDF blotted membranes (Immunoblot PVDF membrane, Bio-Rad). Membranes were then washed in TBS-T (10 mmol/L Tris/buffer, pH 7.5, 0.1 mol/L NaCl, 1 mmol/L EDTA, 0.1% Tween 20) and blocked for 1 hour (5% fat-free dry milk in TBS-T). After washing, they were incubated (overnight, 4°C) with primary antibody against p-ERK1/2 (1:250) in fresh blocking solution, washed again, and incubated with horse radish peroxidase (HRP)-conjugated secondary Zymax antibody (2 hours, RT, 1:4000). Membranes were washed, and p-ERK1/2 bands (44 and 42 kDa) were visualized by means of the ECL Plus Chemiluminescence kit (Amersham). Storm 860 (Molecular Dynamics) Imaging System and Image Quant software were used for quantification. After correction for protein content, phosphorylated ERK1/2 of stimulated vessels was expressed relative to control vessels processed in parallel as described above.

With the use of the same protocol, phosphorylated (activated) PDGF-β-R was visualized on Western blot (with a primary antibody against p-PDGF-β-R, 1:250, Santa Cruz) (band, 180 kDa) in un-mounted control, Ang II–stimulated vessels, and PDGF-stimulated vessels.

Immunohistochemistry of p-ERK1/2 and p-PDGFR-β-R
To analyze the localization of nonphosphorylated ERK1/2 (non–p-ERK1/2) and p-ERK1/2 and of nonphosphorylated and phosphorylated PDGF-β-R (non-p-PDGF-β-R, p-PDGF-β-R) in the rat mesenteric small arteries, these were prepared and mounted as described before for control conditions, snap-frozen, and fixed in 4% formaldehyde. Two vessels (one stained, one control without primary antibody) from each of 5 animals were prepared for immunohistochemistry.

After fixation, vessels were embedded in paraffin and longitudinal sections (3 μm) were cut. Sections were deparaffinized in xylene by using 2 changes for 5 minutes each, hydrated using graded alcohols (99%, 96%, and 70%), and washed in deionized H₂O for 1 minute. Sections were then incubated in 3% hydrogen peroxide in deionized H₂O for 10 minutes to block endogenous peroxidase activity and washed twice in PBS (5 minutes). Slides were heat-treated in a microwave twice for 5 minutes in 10 mmol/L sodium citrate buffer, pH 6.0, cooled in the buffer, and incubated in pepsin (1%, 15 minutes) and rinsed twice (5 minutes) in PBS (BioWhittaker Europe).

The specimens were incubated overnight at 4°C in primary antibody against non–p-PDGF-β-R (1:200, AF385 R&D Systems), p-PDGF-β-R (1:200), p-ERK1/2 (1:200), or non–p-ERK1/2 (1:500). Slides were washed in PBS twice for 5 minutes and then incubated in biotinylated secondary antibody for 20 minutes and washed twice. HRP-streptavidin complex was applied (20 minutes), washed twice in PBS, and HRP substrate mix (including 3,3 di-aminobenzidine, DAKO) was added for development (5 minutes). Slides were rinsed in tap water for 1 minute, incubated in Mayer’s hematoxylin (1 minute), rinsed in tap water for 5 minutes, dehydrated (70%, 96%, and twice 99%), and mounted for light microscopy (2 slides per artery). Each artery was assessed along its entire length.

Drugs
PD123319 was supplied by Research Biochemicals International; PD98059 by New England Biolabs; genistein, herbimycin A, and Ang II by Sigma; DMEM by Life Technology; ERK1/2 (sc-153), p-ERK (sc-7383) and p-PDGFR (sc-12907) antibodies by Santa Cruz Biotechnology Inc; PDGF-BB (520-BB-050) and AF385-antibody (AF385) by R&D Systems; secondary Zymax grade antibody (AF385) by Zymed Laboratories Inc; and immunohistochemistry substrates by DAKO. Candesartan, perindoprilat, and RPR101511A were kind gifts of AstraZeneca (London, UK), Servier Laboratories (Paris, France), and Rhone-Poulenc Rorer (Strasbourg, France), respectively. PD123319 and AF385 were dissolved in water. Other drugs were dissolved in dimethyl sulfoxide, with final concentrations not exceeding 0.01%, a concentration low enough not to have effects in this preparation.21
Results expressed as mean±SEM. Differences between groups were analyzed by 1-way ANOVA and, if appropriate, t tests after Bonferroni correction. Probability values <0.05 were considered significant.

Results

Effect of Intraluminal Pressure and Ang II on ERK1/2 Activation

After 60-minute equilibration (at 70 mm Hg), vessels were stimulated by a rise in intraluminal pressure, 105 mm Hg or 140 mm Hg or Ang II (0.1 μmol/L) for 5 minutes, and in all cases an increase in p-ERK1/2 (ERK1/2 activation) was seen (Figure 1). The presence of the MEK inhibitor PD98059 (10 μmol/L) during equilibration and the intervention inhibited the ERK1/2 activation: for 105 mm Hg, 140 mm Hg, and Ang II vessels, ERK1/2 activation was, respectively, 78±14%, 99±14%, and 88±6% of control (n=5). In other experiments, vessels were exposed to PD98059 during both equilibration and a further 5 minutes at continued 70 mm Hg; the ERK1/2 activity of these vessels did not differ from that of control vessels (ERK1/2 activation of PD98059 vessels was 97±8%, n=14, of control), suggesting that the procedure itself was not activating ERK1/2.

Inhibition of Pressure-Induced Activation of ERK1/2

As shown in Figure 2A, the pressure-induced (140 mm Hg) p-ERK1/2 expression (ERK1/2 activity) in rat mesenteric small arteries: A, R-AT1 antagonist candesartan (cand, 10 μmol/L) and tyrosine kinase inhibitors genistein (gen, 1 μmol/L) and herbimycin A (herb, 1 μmol/L); B, ACE inhibitor perindoprilat (per, 1 μmol/L and 10 μmol/L); C, PDGF-R tyrosine kinase inhibitor RPR101511A (RPR, 10 nmol/L, 100 nmol/L, 1 μmol/L) in rat mesenteric small arteries (n=5). Data are expressed (mean±SEM) as percentage of p-ERK1/2 expression in control vessels held at 70 mm Hg for 5 minutes in DMEM. *P<0.05 vs control.
Mechanical Activation of ERK1/2

Initial studies of the effect of mechanical stress on cellular signaling pathways in vascular smooth muscle cells (VSMC) were mainly performed on cell cultures grown on flexible substrates subjected to cyclic stretch (1 Hz). As reviewed by Li and Xu,22 these show that cyclic stretch causes within 10 minutes activation of PDGF-R and accompanying activation of ERK1/2,17,23 The activation of ERK1/2 in VSMC by mechanical loading is mediated through β1-integrins,24 and the mechanotransduction mechanism appears to require the presence of intact actin filaments.25 The cell culture results thus indicate that mechanical stress is able to activate ERK1/2 through mechanisms including PDGF-R and integrins.

As demonstrated in the present study, there is now good evidence that these cell culture experiments mimic results obtained with intact vessels both in aorta14,15 and in small arteries.4,16 Furthermore, other recent evidence shows that in other cell types subjected to cyclic stretch or other forms of mechanical loading, there is increased phosphorylation of the β-R.26-28 This increase is associated with increased phosphorylation of ERK1/2,27,29,30 suggesting that the β-R is activated by similar mechanisms to those described above.

Discussion

The major new finding of the present study is that pressure-induced activation of ERK1/2 in intact rat mesenteric small arteries requires the activation of both the renin-angiotensin system and the PDGF-β-R. The study has therefore allowed assessment of mechanisms relevant for structural changes in vessels that contribute to the peripheral resistance and thus to the development of hypertension.
small arteries, activation of Src tyrosine kinase is an early event, peaking within 1 minute of pressure elevation and before activation of focal adhesion kinase, and the present results confirm a role of Src tyrosine kinase in that herbimycin A was able to inhibit the rapid pressure-induced ERK1/2 activation. Importantly, our finding that an ACE inhibitor, an AT1-R antagonist, and a PDGF-R antibody inhibit the transient pressure-induced activation of ERK1/2, suggest that the renin-angiotensin system and the PDGF-R play crucial roles in the response of these intact resistance vessels to acute hypertension.

Role of the Renin-Angiotensin System in Pressure-Mediated ERK1/2 Activation

In intact rabbit aorta, increased pressure causes release of angiotensin. Moreover, in those experiments in which pressure-induced fibronectin expression was being determined, the fibronectin expression was inhibited by inhibition of the renin-angiotensin system. Thus, even though the angiotensin release was measured over a 3-day period, this suggested that mechanical loading activated the local renin-angiotensin system, as in rat aorta. In a balloon-injury model, activation of ERK1/2 was inhibited by an AT1 receptor antagonist 5 minutes after injury, supporting the concept that Ang II mediates mechanical activation of ERK1/2 also in the short-term. Our results indicating that pressure-induced ERK1/2 activation can be inhibited by ACE-inhibition or by blockade of the AT1 receptor support both these findings and indicate that pressure itself leads to autocrine production of Ang II, which then acts on the AT1 receptor. It remains to be established how mechanical loading can cause such rapid activation of the renin-angiotensin system. Any released Ang II can cause activation of ERK1/2 through multiple pathways. Thus, activation of the AT1 receptor can, through the PLC-IP3 pathway, cause a rise in cytosolic Ca2+, with resulting stimulation of ERK1/2 through Ca2+-dependent processes, such as PYK2. AT1 receptor activation also causes, through the PLC-DAG pathway, production of PKC, in which PKC-ζ can activate ERK1/2.
Distribution of ERK1/2 and PDGF-ßR

<table>
<thead>
<tr>
<th>Layer</th>
<th>Non–p-ERK</th>
<th>p-ERK</th>
<th>PDGF-ßR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adventitia</td>
<td>2.4±0.2</td>
<td>1.1±0.1</td>
<td>1.1±0.1</td>
</tr>
<tr>
<td>Media</td>
<td>3.0±0.0</td>
<td>0.4±0.2</td>
<td>1.3±0.2</td>
</tr>
<tr>
<td>Endothelium</td>
<td>3.9±0.1</td>
<td>0.6±0.2</td>
<td>1.3±0.2</td>
</tr>
</tbody>
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Semi-quantitative analysis of immunohistochemical results, as shown in Figure 6. Table shows the mean ± SEM of distribution within adventitia, media and endothelium of nonphosphorylated ERK1/2 (non–p-ERK), phosphorylated ERK1/2 (p-ERK), and nonphosphorylated PDGF-ß receptor (PDGF-ßR) in arteries incubated in DMEM (n=5, see Methods). The following scale was used: 0, no staining; 1, traces of staining; 2, discrete areas of staining; 3, weak staining seen throughout preparation; and 4, strong staining throughout preparation. Evaluation is based on material from 5 animals, with full study of 2 longitudinal (2 mm) sections per animal. In all cases, corresponding sections were also made without primary antibody, and none of these showed staining.

Platelet-Derived Growth Factor-R

PDGF-R is found in both an α-form and β-form, in which the α-form can bind PDGF-AA, -AB and -BB, but the β-form binds only PDGF-BB with high affinity.1 In our experiments, we used PDGF-BB to activate PDGF-R, and since the activation of ERK1/2 was completely blocked by AF385, a polyclonal antibody against PDGF-β-R extracellular domain, it appears that it is this form that predominates in our preparation, as is generally found in vascular preparations.32,33 Recently, consistent with earlier results,34 it has been found in rat aortic VSMC that PDGF-R can be activated by Ang II independent of PDGF through transactivation,17 as also shown for the epidermal growth factor (EGF) receptor.18 However, in the in vivo situation, Ang II causes activation of PDGF-R but not of the EGF receptor.19 Similarly, in mesangial cells, angiotensin activates ERK1/2 through a ligand-independent activation of the PDGF-R, not of the EGF receptor.24

Our data showing that Ang II is able to phosphorylate PDGF-β-R support the concept of Ang II-mediated transactivation.17 Furthermore, our finding that this phosphorylation was inhibited both by AF385, the PDGF-β-R antibody, and by RPR101511A, the PDGF-R tyrosine kinase inhibitor, supports the effectiveness of these 2 modes of PDGF-R inhibition.31 As regards AF385, this antibody was developed on the basis of ability to block the biological activity of soluble PDGF-β-R in the presence of PDGF-BB (R&D Systems, Oxon, specification, www.rndsystems.com), but the mechanism is still unclear. As regards RPR101511A, in which the previous evidence for its specificity was based on the effects of the drug on isolated receptors and on receptors in VSMC cultures, these are the first data to demonstrate its effectiveness in an intact vascular preparation. The evidence thus indicates that PDGF-β-R plays an important role in mediating the responses not only of PDGF but also of Ang II in this preparation.

Our finding that RPR101511A and AF385 inhibited the response of ERK1/2 to pressure shows that the PDGF-β-R may play a role in mediating pressure-mediated activation of ERK1/2. Taken together with our other findings, this suggests 2 possibilities. First, raised intravascular pressure may lead to activation of the renin-angiotensin system and the release of Ang II, which, through the AT1 receptor, then causes transactivation of the PDGF-β-R and hence through the Ras-Raf pathway, activation of ERK1/2. An alternative scheme is that AT1 and PDGF-R are normally in a state of partial activity and that a combination of some degree of activity of these receptors together with mechanical loading may be needed for activation of the Ras-Raf cascade, leading to ERK1/2 activation. Support for the first, linear scheme comes from the findings that individual inhibition of any one of the components (ACE, AT1 receptor, PDGF receptor) inhibits the ERK1/2 activation. Support for the second scheme comes from the synergism previously reported for Ang II and pressure-induced activation of ERK1/2 in these vessels.16 To distinguish between these 2 possibilities, it would be important in future experiments to determine whether pressure induces activation of the PDGF-β-R. It would also be important to establish how Src tyrosine kinases are involved, based on the present findings that herbimycin A inhibited the pressure-induced activation of ERK1/2 activation, as previously found for Ang II–induced activation of ERK1/2.16 Finally, future experiments should address the question of how the mechanical loading causes activation of the renin-angiotensin system, but it can be speculated that integrins are involved.36,37

Potential Limitations

A potential difficulty in interpretation of the results is that the small-artery preparation contains multiple cell types. Indeed, the immunohistochemistry results showed that ERK1/2 and the PDGF receptors are distributed throughout the preparation, not only in the VSMC within the tunica media. Therefore, although the media is quantitatively the largest part of the vessel, important contributions from the other cell types cannot be excluded. A further limitation is the degree of mechanical manipulation required to dissect the vessels, to mount them on the myograph, and to tie off any side branches. The effect of this manipulation on ERK1/2 activity is unknown, but as indicated in Results section, the degree of phosphorylation of ERK1/2 was not affected if the MEK inhibitor PD98059 was present throughout the 60-minute equilibration period and 5-minute intervention period compared with control vessels. This suggests that the procedure has not caused a large increase in ERK1/2 activity and that the measured ERK1/2 phosphorylation in the control vessels represents a minimum level. Furthermore, the result suggests that the inhibition of ERK1/2 activity shown by the various agonists is due to their inhibition of the effect of the intervention (pressure, Ang II, PDGF) rather than to an effect on the basal level of ERK1/2 activity. Finally, it must be recognized that the pressure stimuli used here (140 mm Hg) is higher than would be experienced in vivo, even during systole in hypertensive animals.38

Perspectives

Activation of ERK1/2 is likely to be involved with growth mechanisms and thus may be expected to be related to hypertrophic remodeling. Such hypertrophic remodeling of rat small arteries has been seen both with the Ang II infusion model19 and in a model in which pressure was varied.40 The present results are therefore likely to be of relevance for those forms of hypertension and point to ways in which the hypertrophic remodeling of the resistance vasculature could be inhibited, for example, inhibition of the PDGF receptor.
However, in essential hypertension, the form of remodeling is eutrophic, unaccompanied by growth. It will therefore be of interest to determine if ERK1/2 activation also plays a role in this form of remodeling, a form that has now been reproduced in vitro. The present results thus provide a basis for understanding and comparing the different forms of remodeling. In the longer term, the results should point to rational ways for correcting the abnormal structure of the resistance vasculature observed in hypertension.

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