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Inhibition of activated ERK1/2 and JNKs improves vascular function in mouse aortae in the absence of nitric oxide

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ABSTRACT

Activation of mitogen-activated protein kinases (MAPKs) is important for vascular contraction. Decreased nitric oxide availability combined with activation of MAPKs contributes to an increase in vascular tone. In this study, we have determined the involvement of extracellular signal-regulated kinases1/2 (ERK1/2) and c-Jun N-terminal kinases (JNKs) in reactivity of mouse aortae in the absence of nitric oxide. Additionally, we have examined the contribution of these kinases to endothelium-dependent and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-induced contractions. Precontracted aortic rings were treated with MAPK/ERK kinase1/2 (MEK1/2) inhibitor U0126 or JNKs inhibitor SP600125 to determine reactivity after inhibition of nitric oxide synthase using organ bath chambers. Additionally, rings were pretreated with or without these inhibitors to assess $PGF_{2\alpha}$- and acetylcholine-induced, endothelium-dependent contractions. Specificity of the inhibitors was evaluated in each aortic ring by determining the phosphorylation levels of ERK1/2 and c-Jun using Bio-Plex™ phosphoprotein detection kit. In the absence of nitric oxide both inhibitors caused relaxation, and the dilator response was increased by 2.5-fold using SP600125 in comparison with U0126. Transient endothelium-dependent contractions were blocked by U0126, whereas SP600125 strongly attenuated sustained $PGF_{2\alpha}$-induced contractions. U0126 inhibited only phosphorylation of ERK1/2, while SP600125 at higher concentrations not only inhibited phosphorylation of c-Jun but also ERK1/2 phosphorylation. In conclusion, the present study demonstrates that in aortae inhibition of activated ERK1/2 and JNKs mediates vascular relaxation, even in the absence of nitric oxide. Activation of ERK1/2 contributes predominantly to transient endothelium-dependent contractions while JNKs, possibly synergistically with ERK1/2, leads to sustained $PGF_{2\alpha}$-induced contractions.

1. Introduction

Contractile and dilator factors regulate vascular tone. Cardiovascular risk factors such as obesity and diabetes reduce the availability of nitric oxide, a potent vasodilator, and impair vascular function (Stepp, 2006). Reduction in nitric oxide availability activates mechanisms leading to endothelium-dependent contractions (Vanhoutte, 2009). These transient contractions are induced by endothelium-derived contractile factors (EDCFs) which are rapidly degraded into inactive metabolites (Gluais et al., 2005). Acetylcholine, arachidonic acid and ATP stimulate production of these factors in the endothelial cell layer which diffuse and activate adjacent smooth muscle cells (Vanhoutte and Tang, 2008). In contrast to EDCFs, $PGF_{2\alpha}$ induces sustained contractions involving phosphorylation of myosin regulatory light chain, a central molecule in the contractile apparatus of smooth muscle cells (Seto and Sasaki, 1990). Deletion of F-prostanoid receptor which is activated by $PGF_{2\alpha}$ has been shown to attenuate hypertension and atherosclerosis development (Yu et al., 2009).

Mitogen-activated protein kinases (MAPKs) are intracellular signalling molecules propagating signals of extracellular stimuli by consecutive phosphorylation events at tyrosine and threonine residues (Raman et al., 2007). Prominent members include extracellular signal-regulated kinases1/2 (ERK1/2), p38 MAPKs, and c-Jun N-terminal kinases (JNKs) (Roux and Blenis, 2004). MAPK/ERK kinase1/2 (MEK1/2) are upstream activators of ERK1/2 and regulate multiple cellular processes (Kavurma and Khachigian, 2003; Muslin, 2008). In a parallel pathway, MEK4 and MEK7 activate JNKs (Raman et al., 2007) which in turn phosphorylate the transcription factor c-Jun controlling cellular functions such as migration (Ioroi et al., 2003). These kinases not only regulate central cellular processes such as proliferation and migration (Mu et al., 2009; Wei et al., 2010) but also contribute to vascular

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reactivity (D'Angelo and Adam, 2002; Ding et al., 2007; Touyz et al., 2002; Zhou et al., 2010).

In the present study, we investigated in mouse aortae whether inhibition of MAPKs affect existing contraction by inducing relaxation even in the absence of nitric oxide. Furthermore, we examined whether MAPKs (ERK1/2 and JNKs) participate in transient endothelium-dependent and sustained $PGF_{2\alpha}$-induced contractions.

2. Materials and methods

2.1. Animals

C57BL/6J mice (Füllinsdorf, Switzerland) were used for the experiments at the age of 6–9 months. Housing was provided in the animal facility of the Centre of Clinical Research of the University Hospital of Zurich with a 12 h:12 h light–dark cycle. The animals received standard chow and tap water *ad libitum*. Mice were anesthetized intraperitoneally (xylazine: 100 mg/kg body weight; ketamine: 23 mg/kg body weight; and acepromazine: 3 mg/kg body weight) and exsanguinated via cardiac puncture. All animal experiments were performed in accordance to Swiss federal animal regulations and were approved by the local authorities (Kommission für Tierversuche des Kantons Zürich, Switzerland). The experiments conform to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2. Vascular function analysis

After sacrifice, thoracic aorta was dissected, placed in cold (4 °C) Krebs–Ringer bicarbonate solution (Mundy et al., 2007), and cleaned of adhering connective tissue and fat. Aorta was cut into equal-sized rings (2.5 mm of length), and care was taken not to damage the endothelium during preparation. Rings were mounted on tungsten wires (100 μm in diameter) and placed vertically in water-jacketed organ chambers containing 10 ml of Krebs buffer maintained at 37 °C and pH 7.4 with constant gassing (95% O_2 and 5% CO_2). One end of each wire was fixed to a stainless steel rod, and the other end was connected to force transducers (Hugo Sachs Electronik, March-Hugstetten, Germany). Rings were stretched in a stepwise manner till a resting tension of 3.5 g was achieved. Stretched rings were allowed to equilibrate for 30 min before repeatedly exposing with KCl (100 mM) until a stable contractile response was reached (Bhattacharya et al., 2008; Mundy et al., 2007).

To block nitric oxide synthase selected aortic rings were treated with N^G-nitro-L-arginine methyl ester (L-NAME) (Bhattacharya et al., 2008). Rings were subjected to one of the following experimental protocols.

Protocol 1 Vascular rings were pretreated with L-NAME (300 μM) for 30 min, and then exposed to phenylephrine (0.1–100 nM) until a contractile response of 60% of KCl was attained. Upon reaching a stable contractile plateau, rings were treated either with U0126 (0.1–10 μM), an inhibitor of MEK1/2 (Favata et al., 1998), or SP600125 (0.1–10 μM), an inhibitor of JNKs (Bennett et al., 2001), and vasoreactivity was recorded for 30 min. Control rings were treated with 0.1% DMSO, which was used to dilute the inhibitors. The half maximal inhibitory concentrations (IC_{50}) of U0126 for MEK1 is 70 nM (Favata et al., 1998) and of SP600125 for JNK1, 2 and 3 are 40–90 nM (Bennett et al., 2001).

Protocol 2 Aortic rings were treated with or without L-NAME (300 μM) for 10 min, before the addition of U0126 (10 μM), SP600125 (10 μM) or DMSO (0.1%). After 20 min, rings were treated with $PGF_{2\alpha}$ (1 μM) and upon attaining a stable contractile response, increasing concentrations of acetylcholine were added to either elicit endothelium-dependent contractions (in the presence of L-NAME) or relaxation (in the absence of L-NAME).

2.3. Phospho-protein detection

The relative phosphorylation levels of ERK1/2 and c-Jun proteins were determined using the Bio-Plex™ phospho-protein detection kit (Bio-Plex, Bio-Rad, Hercules CA). The assay principle is based on a multiplex sandwich bead immunoassay (Chergui et al., 2009). Each well in the microtiter plate was coated with antibodies against phosphorylated ERK1/2 (Thr202/Tyr204, Thr185/Tyr187) and phosphorylated c-Jun (Ser63), which were coupled to fluorescent beads. Equal sized (2.5 mm) aortic rings used in the organ chamber experiments were subjected to protein extraction, following the manufacturer's protocol. Protein determination was performed using Amido Black assay (Schaffner and Weissmann, 1973). The level of phosphorylation in aortic lysate was measured in duplicates following the guidelines in the Bio-Plex™ phospho-protein manual. The phosphorylation levels of proteins correspond to mean fluorescence intensity (MFI).

2.4. Drugs and solutions

L-NAME (N^G-nitro-L-arginine methyl ester), U0126 (1,4-diamino-2, 3-dicyano-1,4-bis[2-aminophenylthio]butadiene) and SP600125 (anthra[1,9-cd]pyrazol-6(2H)-one) were from Alexis Corp (Lausanne, Switzerland). All other chemicals were from Sigma Chemicals Co. (Buchs, Switzerland).

2.5. Statistical analyses

Data are given as means ± standard error of the mean (S.E.M.), *n* represents the number of mice per group. Concentration- or time-dependent curves were analyzed using ANOVA for repeated measurements with Bonferroni correction for multiple comparisons. Normally distributed values were analyzed by two-group comparisons using the unpaired Student's *t*-test. Relaxation to inhibitors and acetylcholine was normalized to precontraction with phenylephrine and $PGF_{2\alpha}$, respectively. $PGF_{2\alpha}$-induced and endothelium-dependent contractions were normalized to KCl-induced contractions. A P value <0.05 was considered significant.

3. Results

3.1. Nitric oxide-independent relaxation and phospho-protein detection

To investigate whether inhibition of activated ERK1/2 or JNKs in nitric oxide-depleted condition allows relaxation, phenylephrine-contracted aortic rings were treated with increasing concentrations (0.1, 1, and 10 μM) of U0126 or SP600125 in the presence of L-NAME (Fig. 1A, D). Treatment with U0126 at 1 μM or 10 μM caused relaxation in a concentration- and time-dependent manner (Fig. 1A), and maximal relaxation was detected after 30 min at 10 μM (27.8 ± 2.4%; $P<0.05$; Fig. 1A). Vascular reactivity remained unaffected after treatment with DMSO (0.1%) or with low concentration of U0126 (0.1 μM; Fig. 1A). Treatment with SP600125 (10 μM) relaxed aortic rings more strongly (65.2 ± 3.6%) as compared with 1 μM (13.3 ± 2.2) after 30 min of incubation (Fig. 1D). Vascular response was unaffected upon treatment with SP600125 at low concentration (0.1 μM; Fig. 1D). At high concentrations (10 μM), SP600125 allowed aortic rings to relax 2.5-fold greater as compared with U0126 (65.2 ± 3.6% vs. 27.8 ± 2.4%; $P<0.05$; Fig. 1A, D).

To analyse the specificity and inhibition by U0126 and SP600125, phosphorylation of ERK1/2 and c-Jun were measured in the aortic rings used for vascular function experiments. Treatment with phenylephrine

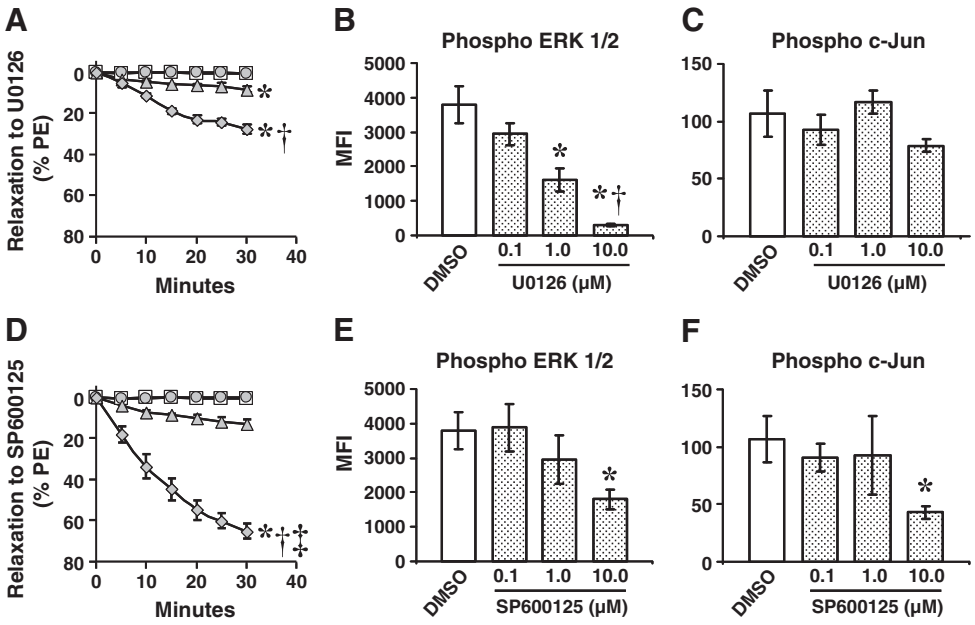

Fig. 1. Inhibition of activated MAPKs induces relaxation. Nitric oxide-depleted aortic rings precontracted with phenylephrine were treated either with U0126 (A) or SP600125 (D) at 0.1 μM (grey circle), 1 μM (grey triangle) or 10 μM (grey rhombus) and relaxation was recorded for 30 min. Control rings were treated with DMSO (0.1%; open square). Relaxation was calculated as a percentage of precontraction with phenylephrine (% PE). n = 4–9 mice/group, * $P<0.05$ vs. DMSO; † $P<0.05$ vs. U0126 (1 μM); ‡ $P<0.05$ vs. U0126 (10 μM). Phosphorylation levels of ERK1/2 and c-Jun were determined in aortic rings in duplicates after treatment with U0126 (B and C) or SP600125 (E and F) and presented as mean fluorescent intensity (MFI). n = 3–6 mice/group. * $P<0.05$ vs. DMSO (0.1%); † $P<0.05$ vs. U0126 (1 μM).

induced a strong ERK1/2 phosphorylation in the absence of inhibitors, and subsequent treatment with U0126 at 1 μM and 10 μM significantly reduced phosphorylation by 58% and 92%, respectively (Fig. 1B). Treatment with U0126 had no effect on phosphorylation of c-Jun (Fig. 1C). However, treatment with SP600125 (10 μM) significantly decreased phosphorylation of c-Jun by 60%, and in line with the relaxation experiments no effect was seen at 0.1 μM and 1 μM (Fig. 1F). Importantly, treatment with SP600125 (10 μM) inhibited ERK1/2 phosphorylation by 52%, while at lower concentrations (0.1 and 1 μM) it had no effect (Fig. 1E).

3.2. Contractions induced by $PGF_{2\alpha}$

Depletion of nitric oxide by adding L-NAME resulted in a 3-fold greater $PGF_{2\alpha}$-induced contraction compared to saline (65.7 ± 8% vs. 21.0 ± 7%; $P<0.05$; Table 1). In the absence of L-NAME, $PGF_{2\alpha}$-induced contraction was reduced by 2-fold using U0126 (10 ± 3%; Table 1) and by 6-fold using SP600125 (3.4 ± 1%; $P<0.05$), respectively. A very similar inhibitory effect was observed in the presence of L-NAME, as $PGF_{2\alpha}$-induced contraction was reduced by approximately 1.8-fold using U0126 (36.6 ± 9%; $P<0.05$) and by 4-fold using SP600125 (16.2 ± 7%; $P<0.05$).

Table 1
Effects of MAPK inhibitors on $PGF_{2\alpha}$-induced contractions in the presence or absence of nitric oxide.

Treatment	DMSO	SP600125	U0126
Saline	21.03 ± 7.3	3.35 ± 0.9[a]	9.98 ± 2.6
L-NAME	65.68 ± 7.6[a]	16.22 ± 6.6[b]	36.63 ± 8.9[b]

Aortic rings were treated with DMSO (0.1%), SP600125 (10 μM) or U0126 (10 μM) followed by contraction with $PGF_{2\alpha}$ (1 μM) in the absence or presence of L-NAME (300 μM). The contractions (in %) were normalized to KCl-induced contractions (n = 4–10 mice/group, [a] $P<0.05$ vs. DMSO and saline; [b] $P<0.05$ vs. DMSO in the presence of L-NAME).

3.3. Acetylcholine-induced relaxation/contraction and phospho-protein detection

Since the experiments in the present study were performed using arterial rings with endothelium, integrity of the endothelial layer was randomly tested by addition of acetylcholine to $PGF_{2\alpha}$-contracted rings to induce endothelium-dependent relaxation. Acetylcholine-induced relaxant response was 85.3 ± 3% in the absence of MAPK inhibitors (Fig. 2). In the presence of SP600125 (10 μM), the relaxant response was increased to 97.1 ± 5% ($P<0.05$) while with U0126 (10 μM) the relaxation remained unchanged (90.6 ± 4%; Fig. 2).

To induce endothelium-dependent contractions, rings were pre-contracted with $PGF_{2\alpha}$ since without precontraction endothelium-dependent contractions induced by acetylcholine were negligible (data not shown). High concentrations of acetylcholine (≥0.3 μM) elicited a contractile response after nitric oxide inhibition and in the absence of a MAPK inhibitor (30.3 ± 2.4%; Fig. 3A). Treatment with U0126 (10 μM) completely blocked the contractions (0.5 ± 0.5%;

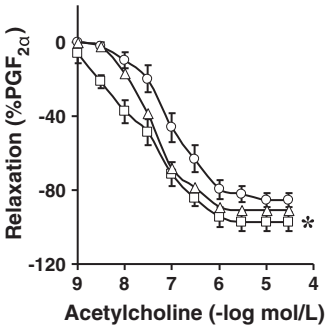

Fig. 2. Role of MAPK in endothelium-dependent relaxation. Aortic rings precontracted with $PGF_{2\alpha}$ were exposed to acetylcholine (1 nM–30 μM) in the presence of U0126 (10 μM; triangle) or SP600125 (10 μM; square). Control rings were treated with DMSO (0.1%; circle). Acetylcholine-induced, endothelium-dependent relaxations were calculated as percentage of $PGF_{2\alpha}$-induced contraction. n = 4–5 mice/group, * $P<0.05$ vs. DMSO.

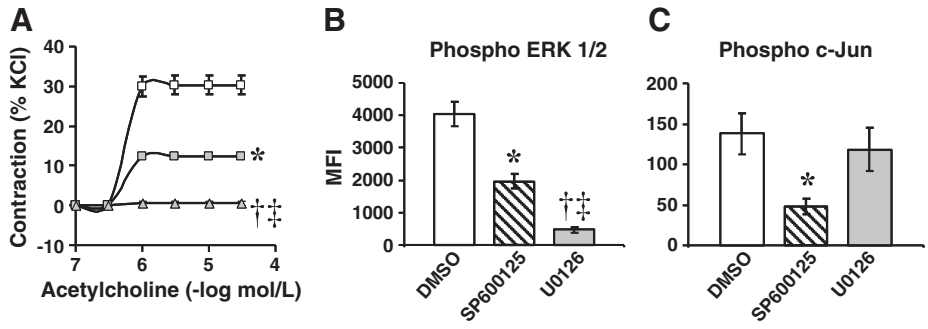

Fig. 3. Role of MAPK in endothelium-dependent contraction. (A) Endothelium-dependent contractions were determined in the presence of acetylcholine (0.1–30 μM) in nitric oxide-depleted aortic rings contracted with $PGF_{2\alpha}$. Rings were preincubated either with U0126 (10 μM; grey triangle) or SP600125 (10 μM; grey square), and control rings were treated with DMSO (0.1%; open square). Contractions were calculated as a percentage of KCl-induced contractions. n = 3–7 mice/group, * and † $P<0.05$ vs. DMSO; ‡ $P<0.05$ vs. SP600125. Phosphorylation levels of ERK1/2 (B) and c-Jun (C) were determined in aortic rings in duplicates after treatment with SP600125 and U0126 and presented as mean fluorescent intensity (MFI). n = 4–6 mice/group. * and † $P<0.05$ vs. DMSO; ‡ $P<0.05$ vs. SP600125.

$P<0.05$) and SP600125 (10 μM) reduced the contractions by 60% (12.5 ± 0.7%; $P<0.05$; Fig. 3A).

To analyse the inhibitory effect and specificity of U0126 and SP600125 in endothelium-dependent contractions, the extent of phosphorylation of ERK1/2 and c-Jun was determined in aortic rings that had been used for vascular function experiments. Contraction to $PGF_{2\alpha}$ followed by acetylcholine treatment induced a strong ERK1/2 phosphorylation in the absence of an inhibitor. Treatment with SP600125 (10 μM) reduced ERK1/2 phosphorylation by 50% ($P<0.05$; Fig. 3B) and with U0126 (10 μM) ERK1/2 phosphorylation was reduced by 88% ($P<0.05$; Fig. 3B). On the other hand, SP600125 (10 μM; $P<0.05$; Fig. 3C) reduced phosphorylation of c-Jun by 65% and U0126 (10 μM) had no effect (Fig. 3C).

4. Discussion

In the present study, we demonstrated that activated ERK1/2 and JNKs differentially contribute to vascular contractions, and inhibition of these activated MAPKs in contracted aortic rings induces a nitric oxide-independent dilator response. In addition, this is the first study to demonstrate that transient endothelium-dependent contractions depend essentially on activation of ERK1/2, whereas sustained $PGF_{2\alpha}$-induced contractions depend on activation of JNKs and ERKs.

The presence of relaxation after inhibition of ERK1/2 and JNKs even in the absence of nitric oxide is an important finding since unequivocal evidence indicates that basal nitric oxide contributes to vasodilation, primarily by antagonizing the effects of vasoconstrictor pathways (Michel and Vanhoutte, 2010). Indeed, inhibition of nitric oxide is associated with heightened arterial contraction, and increased ERK1/2 phosphorylation (Girardot et al., 2003). Our data showing a 3-fold increase of $PGF_{2\alpha}$-induced contraction after nitric oxide synthase inhibition are in line with these observations. Agonist-induced ERK1/2 phosphorylation is increased after inhibition of endogenous nitric oxide, and endothelin-1- and angiotensin II-induced phosphorylation of ERK1/2 and c-Jun is attenuated by nitric oxide (Bouallegue et al., 2007; Wang et al., 1998). This suggests that nitric oxide counteracts the agonist-induced MAPK activation and subsequent vascular contraction. Reduced bioavailability of nitric oxide has been associated with endothelial dysfunction, which in part is characterized by a defect in dilator response (Williams et al., 2002). In this regard, relaxant response after treatment with MAPK inhibitors (U0126 and SP600125) is relevant since it occurred in the absence of nitric oxide. In arterial rings with denuded endothelium, agonist-induced contractions are attenuated by inhibition of ERK1/2 or JNKs activation (Ishihata et al., 2002; Lee et al., 2006; Yu et al., 2005). These data are consistent with our findings, which show reduced $PGF_{2\alpha}$-induced contraction after ERK1/2 and/or JNK inhibition and suggest that inhibition of these MAPKs attenuate contractions even in the absence of nitric oxide. In addition, our results provide evidence that these kinases also contribute to maintaining the contractile response as inhibition of these MAPKs switched the contractile to a dilator response. Interestingly, and in accordance with the previous results (Minutoli et al., 2004), SP600125 inhibited not only JNKs but also ERK1/2 phosphorylation. Recent analysis of the role of JNKs in the regulation of vascular tone showed that in rat aorta KCl- and norepinephrine-induced contractions were attenuated to a greater extent using SP600125 rather than L-JNKI1, a cell permeable specific peptide inhibitor for JNKs (Zhou et al., 2010). Together these data suggest that inhibition of phosphorylation of ERKs and JNKs using SP600125 (10 μM) participate synergistically in relaxation of the vascular smooth muscle seen in the present study. However, it remains unclear whether SP600125 directly inhibits phosphorylation of ERK1/2 or whether a crosstalk between JNKs and ERK1/2 is responsible for inhibition of ERK1/2 activation (Bennett et al., 2001; Dong and Bode, 2003; Fabian et al., 2005).

We next determined the contribution of ERK1/2 and JNKs in transient versus sustained contractions in aortic rings. Transient, endothelium-dependent contractions are elicited in the underlying smooth muscle by endothelial prostanoids termed as endothelium-derived contracting factors (EDCFs) (Vanhoutte, 2009; Vanhoutte and Tang, 2008). Release of EDCFs is increased by aging, hypertension, diabetes and obesity (Vanhoutte, 2009; Vanhoutte and Tang, 2008). The endothelial enzymes cyclooxygenases (COX) -1 and -2 catalyze the conversion of arachidonic acid to EDCFs (Gluais et al., 2005). The isoform of COX involved in the generation of EDCFs, depends on the animal model. In mice, COX-1 is essential for endothelium-dependent contractions (Tang et al., 2005b), while COX-2 is the main isoform in hamsters (Wong et al., 2009). It is well established that the release and the action of EDCFs are negatively regulated by nitric oxide (Auch-Schwelk et al., 1992; Yang et al., 2002). Confirming previous studies (Vanhoutte and Tang, 2008), we show that acetylcholine-induced, endothelium-dependent contractions or relaxations depend on the absence or presence of nitric oxide, respectively. It should be mentioned, that acetylcholine-induced relaxation was not assessed in every experimental ring to avoid a significant reduction in acetylcholine-induced, endothelium-dependent contractions (Tang et al., 2005a). The detection of endothelium-dependent contractions in our study is a further indication that the endothelium was intact as in endothelium-denuded vessels these contractions are absent (Katusic et al., 1987; Zhou et al., 2005). Interestingly, inhibition of ERK1/2 activation abolished these contractions indicating a crucial role for these kinases in the induction of these contractions. In animal models

of hypertension, activated ERK1/2 was associated with an increase in spontaneous contractile tone (Ding et al., 2007) and the inhibition of ERK1/2 activation prevented the development of hypertension in angiotensin II-infused rats (Laplante et al., 2003). Moreover, endothelium-dependent contractions are activated in hypertension (Vanhoutte et al., 2005). Taken together, it can be speculated that the beneficial effect of ERK1/2 inhibition on hypertension is partly attributed to the blockade of endothelium-dependent contractions.

In contrast to transient endothelium-dependent contractions, $PGF_{2\alpha}$ induces a sustained contraction and is implicated in increasing blood pressure and development of atherosclerosis (Yu et al., 2009). Prostaglandin $F_{2\alpha}$ induces contractions via two parallel pathways. The first involves a Ca^{2+}-dependent activation of myosin light chain kinase and inhibition of myosin light chain kinase phosphatase by Rho kinase (Ito et al., 2003). Moreover, $PGF_{2\alpha}$-induced contractions are dependent on mono- and diphosphorylation of the myosin light chain of which monophosphorylation is persistent for up to 40 min (Seto and Sasaki, 1990). This observation may explain the sustained character of this contraction which is mediated by Rho kinase-dependent and independent mechanisms (Ito et al., 2003). The second pathway involves MAPK-mediated phosphorylation of the actin binding protein caldesmon to allow for the actin-myosin interaction important to induce contraction (Horowitz et al., 1996). JNKs contribute to norepinephrine-induced contraction by phosphorylation of caldesmon in rat aortic smooth muscle strips deprived of endothelium (Lee et al., 2006). In the present study, we show indirectly using inhibitors that $PGF_{2\alpha}$ induces phosphorylation of JNKs and ERK1/2 suggesting that both kinases synergistically contribute to the contractile response. It is tempting to speculate that JNKs and ERK1/2 directly participate in $PGF_{2\alpha}$-induced contractile pathways by affecting caldesmon phosphorylation levels.

In conclusion, our data demonstrate that activated ERK1/2 and JNKs differentially participate in eliciting transient and sustained contractions. Thus, attenuation of contraction and induction of relaxation using MAPK inhibitors may result in an improvement of vascular tone even in conditions with decreased nitric oxide availability.

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