Modulation of PGF$_{2\alpha}$- and hypoxia-induced contraction of rat intrapulmonary artery by p38 MAPK inhibition: a nitric oxide-dependent mechanism


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We have previously shown that the sustained phase of HPV is endothelium dependent and largely dependent on activity of endothelium-derived vasodilators NO and prostacyclin, also involves an interaction with thin filament regulatory proteins via phosphorylation of heat shock protein 20 (HSP20) (11, 31).

Using a relatively high single concentration of the inhibitor 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4′-pyridyl)-1H-imidazole (SB-202190), structurally similar to SB-203580, Karamsetty et al. (18) presented evidence that p38 MAPK plays a major role in the sustained phase of the hypoxic constrictor response in main PA. It is well known that for hypoxic pulmonary vasoconstriction (HPV) to occur in isolated PA the presence of a subthreshold concentration of a contractile agonist is required. For this purpose, Karamsetty et al. (18) used phenylephrine, which causes a strong sustained contraction in main PA. They also found that SB-202190 suppressed this phenylephrine-induced contraction. Furthermore, although they suggested that hypoxia itself directly led to activation of p38 MAPK, the influence of other factors such as activity of endothelium-derived vasodilators, as well as potential nonselective actions of the inhibitor, were not examined.

We have previously shown that the sustained phase of HPV is endothelium dependent and largely dependent on activity of Rho-activated kinase (ROCK) (32), but there is some evidence to suggest a degree of interaction between the ROCK and p38 MAPK pathways (29). In light of the observations of Karam-
were continually gassed with 95% air-5% CO2 during this procedure. Distribution to the fluorescence signal from the endothelium (8, 20). IPA challenge. We induced hypoxia by gassing with 1% O2-95% N2-5% CO2. Similar loading protocols have previously shown that subsequent changes in [Ca2+]i were stable for 70 min; stability was not affected by any treatment used (data not shown). Once tension had reached a plateau (~10 min), we assessed the effects of MAPK inhibitors by cumulative-concentration response studies, allowing the response to stabilize between successive applications of agent.

Estimation of [Ca2+]i. IPA were loaded with the Ca2+-sensitive fluorophore fura PE3, via incubation of the vessels with the acetoxy-methyl ester fura PE3-AM (4 M) for 2 h at room temperature. Similar loading protocols have previously shown that subsequent measurements are of smooth muscle [Ca2+]i, with negligible contribution to the fluorescence signal from the endothelium (8, 20). IPA were continually gassed with 95% air-5% CO2 during this procedure. After loading, the arteries were washed with PSS, the temperature increased to 37°C, and the myograph was transferred to an inverted fluorescence microscope (Nikon Diaphot, Nikon UK). We assessed changes in [Ca2+]i by calculating the ratio of the light emitted through 340/380 nm (Cairn spectrophotometer; Cairn Research, Newnham, Kent, UK).

Hypoxic protocol. As we have previously shown, a small degree of agonist-induced tone is required to facilitate the hypoxic response in rat IPA and mesenteric artery (23). The vessels were therefore exposed to sufficient PGF2α (3–6 μM) to induce a constriction of ~15% of that to KPSS for 20 min before and during the hypoxic challenge. We induced hypoxia by gassing with 1% O2-95% N2-5% CO2 for 45 min, after which time the vessels were reoxygenated and washed with PSS. In some experiments, oxygen tension in the myograph chamber was continuously monitored via a dissolved oxygen meter (Diamond General oxygen electrode, Ann Arbor, MI; Strathkelvin oxygen meter, Glasgow, UK). During the hypoxic challenge the chamber PO2 was typically 18–20 mmHg, compared with the control PO2 of 135–145 mmHg.

α-Toxin permeabilization of IPA. Isometric tension was recorded in α-toxin-permeabilized arteries, as described previously (8, 20). Briefly, IPA were mounted on a myograph as above but bathed in PIPES-buffered solution (pH 7.1), gassed with 100% air rather than 5% CO2, and incubated at 26°C rather than 37°C. To prevent involvement of intracellular Ca2+ stores, 10 μM cyclopiazonic acid was present throughout. We regulated pCa by adjusting the ratio of K2EGTA to CaEGTA. Permeabilization was performed with 60 μg/ml α-toxin in pCa 6.5. After reequilibration with Ca2+-free relaxing solution, a submaximal constriction was elicited by raising pCa to 7.0–6.9. Once this contraction reached a plateau, PGF2α, GTP (1 μM), and/or sodium nitroprusside (SNP) were added. Higher concentrations of agonists are commonly required in permeabilized compared with intact preparations, and in this case we used 100 μM PGF2α.

Western blotting. Isolated IPA were incubated with 20 μM PGE2 for 5 min (controls) or preincubated for 10 min with 2 μM SB-203580 or SB-202474 and then treated with PGE2 for a further 5 min. We then snap froze them by immersing them in liquid nitrogen and homogenizing them in SDS sample buffer containing protease and phosphatase inhibitors (Sigma) using a Wheaton microtissue grinder. Samples were centrifuged at 6,000 rpm for 1 min and loaded onto 4–12% NUPAGE Bis-Tris gels, electrophoretically separated (200 V for 1 h), and transferred to nitrocellulose membranes in 25 mM Tris, 192 mM glycine, and 20% methanol using a mini Trans-blot unit (100 V for 1 h at 4°C). The membranes were washed in Tris-buffered saline (TBS; 20 mM Tris-HCl, pH 7.5, and 500 mM NaCl) and blocked with 5% skim milk in TBS for 1 h at room temperature. The membranes were then probed with phospho-p38 MAPK and phospho-HSP27 antibodies (Cell Signaling) at 1:1,000 dilution in 1% milk in TBS containing 0.1% Tween (TBS-Tween) overnight at 4°C. The membranes were washed 6 × 10 min in TBS-Tween and probed with goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (1:10,000 dilution) for 1 h at room temperature and then exposed to West Femto chemiluminescent substrate (Pierce Biotechnology). Band intensity was quantified using ImageJ software. To analyze total protein, the membranes were stripped for 1 h at room temperature and reprobed with pan-p38 MAPK and pan-HSP27 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). The bands were visualized using West Pico chemiluminescent substrate (Pierce Biotechnology) as described above.

cGMP assay. Human umbilical vein endothelial cells (HUVECs) were isolated from fresh umbilical veins and grown to confluence in M-199 containing 20% fetal calf serum. Once confluent, cells were equilibrated for 10 min in HEPES-buffered saline, pH 7.4, containing 100 μM L-arginine [to provide substrate for endothelial nitric oxide synthase (eNOS)] and 500 μM 3-isobutyl-1-methylxanthine (to inhibit all phosphodiesterase activity), were preincubated with 2 μM SB-203580 or left free of inhibitor (15 min), and then were treated with 20 μM PGE2 for a further 5 min at 37°C. ATP (100 μM) was used as a positive control. Cells were then immediately lysed with ice-cold 0.5 M HCl, and the lysate was collected for assessment of cGMP content. This provided a measurement of both NO production and guanylyl cyclase activity (34), using an ACE competitive enzyme immunoassay kit (Cayman Chemical) (30).

Data analysis. Changes in [Ca2+]i are represented in terms of the change in the fluorescence PE3 ratio at 340/380 nm. Mean changes are expressed as a percentage of the maximum ratio change seen during the final 80 mM KPSS challenge during the run-up procedure (R340/380). Although not linearly related to [Ca2+]i, this provides a reliable qualitative index of changes in [Ca2+]i. Values for IC50 and maximum response were derived by nonlinear curve fitting of concentration-response data (SigmaPlot; SPSS, Chicago, IL). Results are expressed as means ± SE, and means were compared by ANOVA for repeated measures or paired or unpaired Student’s t-test as appropriate (SigmaStat, Jandel). A difference was deemed significant if P < 0.05.
RESULTS

Relaxation of PGF\(_{2\alpha}\)-contracted IPA by MAPK inhibitors. To study the role of p38 MAPK in PGF\(_{2\alpha}\)- and hypoxia-induced contractions, we first established the range of concentrations over which SB-203580 is likely to be acting specifically via inhibition of p38 MAPK activity by constructing relaxation concentration-response curves to SB-203580 and SB-202190, as well as to 4-ethyl-2-(p-methoxyphenyl)-5-(4'-pyridyl)-1H-imidazole (SB-202474, a structural analog of SB-203580 that is inactive against p38 MAPK) (24). We also examined the effect of the MAPKK (MEK) blocker PD-98059. PGF\(_{2\alpha}\) (20 \(\mu\)M, \(\sim EC_{70}\)) induced constriction of intact IPA was inhibited by SB-203580 with an IC\(_{50}\) of 1.6 \(\pm\) 0.3 \(\mu\)M and maximum effect of 80 \(\pm\) 5\% \((n = 12)\), whereas SB-202474 was \(\sim 30\)-fold less potent (Fig. 1A). SB-203580 had only a minor inhibitory effect on KPSS-induced constriction (Fig. 1A), suggesting that relaxation of PGF\(_{2\alpha}\)-induced constriction was unlikely to be due to inhibition of voltage-gated Ca\(^{2+}\) channels. SB-202190 also relaxed PGF\(_{2\alpha}\)-contracted IPA, with an IC\(_{50}\) of 1.2 \(\pm\) 0.2 \(\mu\)M \((n = 8)\), whereas the MEK inhibitor PD-98059 had only a minor effect at relatively high concentrations (Fig. 1B). These results implicate a role for p38 MAPK activation, but not for p42/44 MAPK, in PGF\(_{2\alpha}\)-induced constriction of IPA.

Inhibition of PGF\(_{2\alpha}\)-induced p38 MAPK and HSP27 phosphorylation by SB-203580. As shown in Fig. 2A, treatment of IPA with PGF\(_{2\alpha}\) (20 \(\mu\)M) caused a significant increase in phosphorylation of both p38 MAPK (Fig. 2B) and its downstream effector HSP27 (Fig. 2C). The data presented in Fig. 1A suggest that the selective action of SB-203580 against p38 MAPK reaches a maximum at \(<2\) \(\mu\)M, if it can be assumed that its nonselective effects are similar to those of the inactive analog SB-202474. Consistent with this assumption, the PGF\(_{2\alpha}\)-induced increase in p38 MAPK phosphorylation was abolished by 2 \(\mu\)M SB-203580 but unaffected by SB-202474 (Fig. 2B). Similar results were obtained for HSP27 phosphorylation (Fig. 2C). SB-203580 did not significantly reduce the levels of phospho-p38 MAPK or phospho-HSP27 in unstimulated IPA. In light of these results, in most subsequent experiments where only a single concentration of inhibitor was used, we chose 2 \(\mu\)M.

Effect of p38 MAPK inhibition on HPV. As we have previously reported for rat small IPA (23, 32), application of hypoxia to IPA precontracted with PGF\(_{2\alpha}\) a “pretone” level equivalent to 10–15\% of an 80 mM KPSS contraction caused a transient vasoconstriction superimposed on a sustained vasoconstriction (Fig. 3A). In the presence of 2 \(\mu\)M SB-203580, wherein the concentration of PGF\(_{2\alpha}\) was raised to compensate for blockade of pretone by the p38 MAPK inhibitor, both phases of HPV were depressed (% block: first phase, 47.0 \(\pm\) 11.7, \(P < 0.01\); second phase, 41.2 \(\pm\) 5.8, \(P < 0.01\), \(n = 8\)) (Fig. 3A). Furthermore, the specificity of this action to p38 MAPK blockade was confirmed by the lack of effect of the inactive analog SB-202474 (2 \(\mu\)M) on either phase of HPV (Fig. 3B).

Although the results of the above experiments are consistent with those of Karamsetty et al. (18) in that they showed attenuation of HPV by SB-203580, they also revealed that PGF\(_{2\alpha}\)-induced contraction was also inhibited, and to a similar extent as HPV. To understand the mechanisms by which p38 MAPK inhibition suppresses contraction, we first explored in more depth the effects of SB-203580 on the PGF\(_{2\alpha}\)-induced contraction.

Effects of p38 MAPK inhibition on PGF\(_{2\alpha}\)-induced rises in [Ca\(^{2+}\)]\(_i\). To determine whether SB-203580 was causing relaxation of PGF\(_{2\alpha}\)-induced vasoconstriction by suppressing the elevation of [Ca\(^{2+}\)]\(_i\), we studied the effect of SB-203580 on [Ca\(^{2+}\)]\(_i\), measured simultaneously with tension in fura PE3-loaded IPA. SB-203580 fluoresces under UV illumination; to circumvent this, SB-203580 was applied before addition of PGF\(_{2\alpha}\) so that its effect on baseline fluorescence could stabilize first. Under these conditions, despite significantly inhibiting

![Fig. 1. Relaxant effects of MAPK inhibitors on PGF\(_{2\alpha}\)-induced constriction of intact intrapulmonary arteries (IPA). A: concentration-dependent relaxation of PGF\(_{2\alpha}\) (20 \(\mu\)M)-contracted intact IPA by SB-203580 \(\bullet\), \(n = 12\) and SB-202474 \(\downarrow\), \(n = 10\), and of 80 mM KPSS (KPSS)-contracted intact IPA by SB-203580 \(\square\), \(n = 5\). B: concentration-dependent relaxation of PGF\(_{2\alpha}\) (20 \(\mu\)M)-contracted intact IPA by SB-202190 \(\blacksquare\), \(n = 8\) and PD-98059 \(\downarrow\), \(n = 4\). Symbols are means \(\pm\) SE.](http://ajplung.physiology.org/10.1152/ajplung.00354.2004)
tension, SB-203580, even at 4 μM, did not alter the [Ca^{2+}]_{i} response to PGF_{2α} (n = 5, Fig. 4). Neither tension nor [Ca^{2+}]_{i} responses to KPSS were altered by SB-203580 (n = 2, data not shown).

**Fig. 2.** Western blot analysis of phosphorylation of p38 MAPK and heat shock protein (HSP) 27 in intact IPA. A: representative blots of intact IPA [unstimulated, PGF_{2α} (20 μM), SB-203580 (2 μM), or PGF_{2α} + SB-203580] with anti-phospho-p38 MAPK, anti-p38 MAPK, anti-phospho-HSP27, or anti-HSP27. Total p38 MAPK and HSP27 levels were unaffected by the various treatments. B and C: cumulative data showing the quantification of phosphorylation of p38 MAPK and HSP27. *P < 0.01 vs. control, n = 16 (B); †P < 0.05 vs. control, n = 21 (C); ‡P < 0.05 vs. PGF_{2α} treatment, n = 5 (B); ††P < 0.05 vs. PGF_{2α} treatment, n = 8 (C). SB-202474 had no significant effect on PGF_{2α}-induced phosphorylation of p38 MAPK (n = 7) (B) or HSP27 (n = 8) (C).

**Fig. 3.** Effects of p38 MAPK inhibition on hypoxic pulmonary vasoconstriction (HPV). A: control, ●; SB-203580 (2 μM), ○; SB-203580 caused significant inhibition of both the transient (P < 0.01, n = 8) and sustained phase of HPV (P < 0.01, ANOVA). B: in contrast, the inactive analog SB-202474 (2 μM) had no significant effect on either phase of HPV (control, ■; SB-202474, □, n = 6). Symbols are means ± SE.

**Effects of p38 MAPK inhibition on PGF_{2α}-induced Ca^{2+} sensitization.** PGF_{2α} induces contraction partly via enhancing the sensitivity of the smooth muscle to [Ca^{2+}], (Ca^{2+} sensitization) (4, 21, 27, 28, 35, 38). To evaluate whether SB-203580
caused relaxation of PGF2α-induced vasoconstriction of IPA by suppressing Ca²⁺ sensitization, we examined whether SB-203580 relaxed the contraction elicited by PGF2α (100 μM) in α-toxin-permeabilized IPA clamped at pCa ~6.9. SB-203580, even at 10 μM, was not able to cause significant relaxation of IPA under these conditions (n = 8, not significant; but see below and Fig. 9).

Because preliminary experiments showed that endothelium-dependent relaxation is inoperative following α-toxin-permeabilization in this preparation, the above experiments do not rule out the possibility that inhibition of p38 MAPK might suppress contraction in intact arteries by enhancing the release or effect of endothelial vasodilators that could affect Ca²⁺ sensitization. To explore this possibility, we examined whether SB-203580-induced relaxation was affected by removal of the endothelium.

**Effects of endothelial removal and inhibition of NOS.** As shown in Fig. 5A, endothelial denudation markedly suppressed relaxation of PGF2α-evoked contractions by SB-203580, with a large rightward shift in the SB-203580 concentration-response relationship (IC₅₀: 14 ± 3 μM, n = 10, P < 0.001 compared with intact IPA). However, it had no effect on relaxation to the inactive analog SB-202474, and in the absence of the endothelium, the concentration-response relationships for SB-203580 and SB-202474 were the same (Fig. 5A).
These results suggest that the relaxation to low concentrations of SB-203580, which are more likely to cause selective block of p38 MAPK activation, was endothelium dependent. In support of this conclusion, the relaxation to SB-203580 over a similar concentration range was also suppressed by the eNOS inhibitor Nω-nitro-L-arginine methyl ester (L-NAME, 1 mM), albeit to a smaller extent (IC50: 8.7 ± 1.7 μM, n = 13, P < 0.001 compared with control; Fig. 5B). Inhibition of cyclooxygenase with indomethacin (1 μM) also caused a small but significant increase in IC50 of the SB-203580-induced relaxation (IC50: 4.7 ± 1.0 μM, n = 11, P < 0.05 compared with control; Fig. 5B); indomethacin alone did not alter the response to PGF2α. To confirm the involvement of NO and the cGMP/PKG pathway in SB-203580-induced relaxation, we also examined the response to SB-203580 in the presence of the PKG inhibitor KT-5823 (1 μM). KT-5823 reduced 2 μM SB-203580-induced relaxation to a similar extent as L-NAME [control (SB-203580 alone): 44.8 ± 3.6% relaxation, n = 12; +L-NAME, 25.4 ± 2.5%, P < 0.01 vs. control, n = 13; +KT-5823, 25.9 ± 3.8%, P < 0.001 vs. control, n = 8].

Because these results suggested that the vasorelaxant effect of p38 MAPK inhibition was largely due to enhancement of the release or effect of endothelium-derived NO, we reexamined the suppression of HPV by SB-203580 following inhibition of eNOS with 1 mM L-NAME. L-NAME alone had no significant effect on HPV, as we have previously reported in this preparation [Ref. 23, and see discussion in Aaronson et al. (1)]. However, in the presence of L-NAME, SB-203580 was without effect on either phase of HPV (Fig. 6), strongly suggesting that its suppressive action on HPV, as on PGF2α-induced contraction of endothelium-denuded IPA, in the absence and presence of PGF2α, had no significant effect on the increase in cGMP induced by PGF2α. *P < 0.01 compared with control (untreated); n = 8–10.

Effects of p38 MAPK inhibition on cGMP production in endothelial cells. To determine whether p38 MAPK inhibition might be altering NO release or guanylyl cyclase activity, we measured cGMP production as an indirect measure of NO production in HUVECs. As shown in Fig. 7, cell lysate cGMP concentration was significantly enhanced (approximately two- to threefold) by treatment with 20 μM PGF2α. However, preincubation with 2 μM SB-203580 before PGF2α application had no additional effect on cGMP concentration (Fig. 7).

Effects of p38 MAPK inhibition on sensitivity of endothelium-denuded IPA to an NO donor. Because SB-203580 did not affect production of cGMP, at least in HUVECs, we next examined whether p38 MAPK inhibition might be potentiating the effects of NO on the vascular smooth muscle (VSM). To do this, we studied the effects of the NO donor S-nitroso-N-acetylpenicillamine (SNAP) on PGF2α-induced contraction of endothelium-denuded IPA, in the absence and presence of PGF2α.
SB-203580 or SB-202474. As shown in Fig. 8, 2 μM SB-203580 significantly enhanced the SNAP-induced relaxation, causing both a leftward shift in the IC50 [control (SNAP alone): 0.28 ± 0.11 μM, n = 8; +SB-203580, 0.12 ± 0.03 μM, n = 8, P < 0.05] and an enhancement of the apparent maximum relaxation (control: 56.0 ± 8.4%; +SB-203580, 82.8 ± 6.0%, P < 0.05). The same concentration of SB-202474 on the other hand did not significantly alter the response to SNAP (IC50, 0.58 ± 0.13 μM; maximum relaxation, 48.2 ± 10.6%, n = 8), indicating that these actions of SB-203580 were likely to be specific to p38 MAPK inhibition.

We further investigated which of the actions of NO thought to be involved in relaxation of VSM were being influenced by p38 MAPK. We first examined the potential influence of p38 MAPK inhibition on SNAP-induced suppression of both contraction and [Ca2+]i responses to the stable thromboxane A2 analog U-46619 (100 nM) in endothelium-denuded IPA. U-46619 produces [Ca2+]i responses via the prostanoid TP receptor, which is also stimulated by PGF2α. We used U-46619 in preference to PGF2α, because our recent experiments (Snetkov V, Knodle G, Baxter L, Ward J, and Aaronson P, unpublished observations) have shown that the latter also causes a rise in [Ca2+]i mediated via FP receptors, but which is not coupled to contraction. Thus by using U-46619 any effects of SB-203580 on [Ca2+]i would be more likely to influence contraction. Despite enhancing SNAP-induced relaxation [control (SNAP alone): 63.5 ± 5.7%, +SB-203580: 80.9 ± 3.8%, P < 0.05, n = 4], 2 μM SB-203580 did not potentiate the associated SNAP-mediated suppression of the rise in [Ca2+]i, induced by U-46619 (control: 67.7 ± 9.2% R340/380, +SB-203580: 56.8 ± 8.2%, n = 4, not significant).

These results suggested that SB-203580 was not enhancing the response to NO by potentiating its ability to reduce [Ca2+]i. Alternatively, p38 MAPK inhibition might be enhancing NO-mediated Ca2+ desensitization. Because endothelial release of NO was not operative following α-toxin permeabilization, we studied this possible effect by applying SB-203580 (10 μM) to permeabilized IPA contracted with 100 μM PGF2α at pCa 6.9, in the presence of the NO donor SNP (100 μM) to generate an ambient level of NO sufficient to cause a partial relaxation (not shown). In contrast to its lack of effect when applied in the absence of SNP (Fig. 9, A and D), SB-203580 in the presence of SNP caused a significant relaxation (Fig. 9, B and D).

Fig. 8. Effects of SB-203580 on S-nitroso-N-acetylpenicillamine (SNAP)-induced relaxation of endothelium-denuded IPA contracted with 20 μM PGF2α. Control, ○ (n = 8); 2 μM SB-203580 (●, n = 8); 2 μM SB-202474, ■ (n = 8). Symbols = means ± SE.

Fig. 9. Effects of p38 MAPK inhibition on PGF2α-induced contraction in α-toxin-permeabilized IPA. A–C: example traces of contractions to 100 μM PGF2α in pCa 6.9 and 1 μM GTP. Alone, 10 μM SB-203580 had little effect (A), but in the presence of 100 μM sodium nitroprusside (SNP) it caused a significant relaxation (B). 10 μM SB-202474 had little effect even in the presence of SNP (C). D: summarized data for 6–8 IPA; neither the response to SB-203580 alone nor SB-202474 in the presence of SNP was significantly different from zero, whereas that to SB-203580 in the presence of SNP was significantly different from both SB-203580 alone and SB-202474 in the presence of SNP (p < 0.01, 1-way ANOVA).
Conversely, SB-202474 at this concentration had no effect (Fig. 9, C and D).

**DISCUSSION**

The aim of this study was to clarify the role of p38 MAPK in agonist- and hypoxia-induced contraction of rat small IPA. Our results suggest that the contribution of p38 MAPK activation to smooth muscle contraction elicited by either PGF2α or hypoxia is endothelium dependent and occurs primarily via suppression of the actions of endothelium-derived vasodilators, particularly NO. Inhibition of p38 MAPK would therefore relieve this suppression and result in vasorelaxation.

We have shown that both SB-203580 and SB-202190 inhibited PGF2α-induced constriction with IC50 close to 1 μM, whereas SB-202474 (a structural analog of SB-203580 and SB-202190 that is ineffective as a blocker of p38 MAPK) only caused significant relaxation at concentrations >10 μM. In addition, SB-203580, but not SB-202474 (both at 2 μM), abolished PGF2α-induced phosphorylation of both p38 MAPK and of its downstream effector protein HSP27. SB-203580 is the most extensively used p38 MAPK inhibitor and has been shown to inhibit contractions induced by several agonists in several vascular beds (4, 21, 27, 28, 35, 38). Importantly, in each of these studies, the agonist(s) causing SB-203580-inhibitable constriction also caused SB-203580-sensitive phosphorylation of p38 MAPK and/or HSP27. These observations are consistent with the hypothesis that agonist-induced p38 MAPK activation acts via HSP27 phosphorylation to cause constriction, as originally suggested for bombesin-stimulated contraction of rabbit rectosigmoid smooth muscle (3).

The selectivities of SB-203580 and SB-202190 have been extensively investigated, and in an in vitro assay system (7) both drugs caused near complete block of p38-α and -β MAPK activity at 10 μM without significantly affecting the activities of 22 other kinases, including MAPKAPK-1/2, ROCK-II, PKC-α, ERK-2, MKK-1, and JNK-1α. In cell-based systems, the IC50 for SB-203580 against p38 MAPK is ~0.6 μM (e.g., Ref. 6), similar to that obtained in the present study (1.6 μM). SB-203580 activates phospholipase A2 (10), but this does not explain our results since SB-202190 has no effect on phospholipase A2 and yet still inhibited PGF2α-induced constriction with a potency similar to SB-203580. SB-203580 is also reported to inhibit cyclooxygenase (COX-1 and COX-2) in platelets with an IC50 close to 2 μM (5). However, this is unlikely to be a major factor here, because PD-98059 also inhibits COX with equal potency (5) but was a poor blocker of PGF2α-induced constriction. MAPK inhibitors also inhibit phosphodiesterase activity in rat pinealocytes (15). However, these effects only occur at concentrations >1 μM, and SB-202474 was markedly more effective than SB-203580. This effect, if it occurs in IPA, is therefore unlikely to explain our observed effects of SB-203580 at ~2 μM, although it may explain the relaxation induced by SB-202474 at 3, 10, and 30 μM and the residual relaxation to higher SB-203580 concentrations after endothelial denudation.

Considering all the above and the fact that SB-203580 but not SB-202474 abolished PGF2α-induced p38 MAPK and HSP27 phosphorylation, we find it reasonable to suggest that at the concentrations used in this study, SB-203580 and SB-202190 are selective for p38 MAPK in this preparation.

To our knowledge this is the first study to address the role of the vascular endothelium in the effect of SB-203580 on constriction. Removal of the endothelium caused a >10-fold rightward shift in the concentration-response curve to SB-203580, such that it matched that of the inactive analog SB-202474. Preincubation with l-NAME also caused a significant rightward shift in the SB-203580 concentration-response curve, and a similar effect was observed with the PKG inhibitor KT-5823. Treatment with the COX inhibitor indomethacin had a similar, though much smaller, effect. This supports the concept that the bulk of SB-203580-induced relaxation was endothelium dependent.

In light of the observed dependence of SB-203580-mediated relaxation on the presence of an intact endothelium and on NO, we hypothesized that p38 MAPK inhibition might be either affecting the release of NO from the endothelium or altering the sensitivity of the underlying smooth muscle to NO. We evaluated the first of these possibilities by measuring the effects of SB-203580 and SB-202474 on cGMP production in primary cultured HUVECs. This particular method is useful because the endothelial cell acts as both producer of NO and sensor of NO production via an autocrine action on cGMP production. It was not surprising that stimulation with PGF2α alone resulted in increased production since the stimulation of NO production by contractile agonists is well documented (e.g., see Ref. 19) and indeed specifically with PGF2α in IPA (16). However, the combination of SB-203580 and PGF2α caused a rise in cGMP similar to that induced by PGF2α alone, suggesting that under the conditions used in our experiments SB-203580 was not affecting the release of NO or its ability to stimulate guanylate cyclase. On the other hand, SB-203580 did alter the apparent sensitivity of endothelium-denuded IPA to exogenous NO, as provided by the NO donor SNAP. Furthermore, this action is likely to be specific to p38 MAPK inhibition since SB-202474 was without effect. We infer from this that p38 MAPK is contributing to PGF2α-induced contraction by interfering with the underlying relaxing actions of NO.

We also attempted to determine at which point the p38 MAPK pathway was acting to inhibit NO/cGMP-mediated vasorelaxation. NO relaxes smooth muscle partly by increasing the pumping of Ca2+ out of cells and into the sarcoplasmic reticulum, as well as via the opening of K+ channels and hyperpolarization, all of which are reflected by a drop in [Ca2+]i. However, the combination of SB-203580 and PGF2α caused a rise in [Ca2+]i, nor did it influence the SNAP-induced reversal of the U-46619-mediated rise in [Ca2+]i. This implied that it must be acting by decreasing the sensitivity of the contractile apparatus to Ca2+. Indeed, for as has been suggested, the p38 MAPK/HSP27 axis is enhancing agonist-induced contraction via regulation of actin polymerization, cross-bridge cycling, or membrane attachment (2, 12), this should be largely independent of [Ca2+]i. Indeed, a role for p38 MAPK in mediating agonist-induced contraction via Ca2+ sensitization has already been suggested by Yamboiev et al. (38). They found that ET-1-induced contraction in α-toxin-permeabilized canine MA was inhibited by ~30% by 10 μM SB-203580. They also provided more direct evidence for the involvement of HSP27 in VSM by demonstrating that an antibody to HSP27 inhibited contraction to ET-1 by ~20% in saponin-permeabilized canine PA. Massett et al. (26) have similarly observed that although pressure-induced myo-
genic tone was partially blocked by SB-203580, smooth muscle [Ca$^{2+}$]$_i$ was unaffected. It has also been suggested that HSP27 may play an important role in sustained constriction of smooth muscle because it mediates association between RhoA and ROCK and thus maintains activation of ROCK-mediated Ca$^{2+}$ sensitization (29). However, in the present study we were only able to elicit a relaxation to SB-203580 in permeabilized arteries, when 100 µM SNP was added to the buffer.

Taking into account the endothelium dependency of the relaxation to SB-203580 in intact IPA, we infer from this that the endothelium either was damaged during the α-toxin permeabilization or was unable to release NO because [Ca$^{2+}$]$_i$ was clamped to pCa 6.9. Indeed, preliminary experiments showed that permeabilized IPA did not relax to acetylcholine and did not contract to l-NAME as do intact PGF$_{2\alpha}$-constricted IPA. In any case, the restoration in permeabilized IPA of SB-203580-mediated, but not SB-204274-mediated, relaxation in the presence of an NO donor confirmed our conclusion that p38 MAPK activity is contributing to PGF$_{2\alpha}$-induced contraction via an inhibition of an underlying NO-mediated relaxation and, in addition, that this occurs via a pathway acting upon the sensitivity of the smooth muscle to [Ca$^{2+}$]$_i$. Consistent with this concept, it has been suggested that cyclic nucleotide-mediated relaxation via activation of PKGs involves phosphorylation of HSP20 (31) and that upon stimulation of the p38 MAPK pathway an increased phosphorylation of HSP20 (32, 37). Karamsetty et al. (18) suggested that activation of p38 MAPK may play a major role in the sustained phase of HPV, which we have previously suggested is dependent on the endothelium and ROCK-mediated Ca$^{2+}$ sensitization (32, 37). Karamsetty et al. (18) showed that hypoxia increased p38 MAPK phosphorylation in rat main PA and that both this and the sustained phase of HPV were suppressed by 10 µM SB-202190. Our results with SB-203580 on HPV in rat small IPA were broadly similar, although in our study the effect on the transient phase was larger and that on the sustained phase smaller. The ineffectiveness of SB-204274 does indeed suggest that this effect is mediated via specific inhibition of p38 MAPK. However, blockade of eNOS with l-NAME abolished the effect of SB-203580, strongly suggesting that its suppressive action on HPV, like that on PGF$_{2\alpha}$-induced vasoconstriction, was largely mediated by a permissive enhancement of the actions of NO on the smooth muscle rather than any effect on the mechanisms of HPV per se.

**Conclusions.** We investigated the role of p38 MAPK in PGF$_{2\alpha}$-induced vasoconstriction and HPV of rat small IPA. SB-203580 and SB-202190 caused relaxation of PGF$_{2\alpha}$-contracted IPA at low concentrations, at which they are most likely to exert a selective action on p38 MAPK, via an endothelium- and largely NO-dependent mechanism. Our data imply that activation of p38 MAPK inhibits the actions of NO and possibly other endothelium-derived vasodilators on the smooth muscle, and this inhibition involves a pathway that regulates the sensitivity of the contractile apparatus to intracellular Ca$^{2+}$. Our results do not support a direct or specific role for p38 MAPK in sustained HPV, though its putative suppressive action on NO-mediated relaxation would enhance HPV. We speculate instead that p38 MAPK-dependent suppression of NO mediated vasodilatation could provide a physiological brake for the action of NO during agonist-induced vasoconstriction and HPV, thereby allowing greater development of tension. Considering the importance of both endothelial-derived NO and ROCK-mediated Ca$^{2+}$ sensitization for the regulation of pulmonary vascular resistance in health and disease, and their known interaction (36), this novel influence of p38 MAPK could potentially be of therapeutic importance.

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