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Transforming Growth Factor-β1 Impairs Endothelin-1-Mediated Contraction of Brain Vessels by Inducing Mitogen-Activated Protein (MAP) Kinase Phosphatase-1 and Inhibiting p38 MAP Kinase

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ABSTRACT

Brain levels of transforming growth factor-β1 (TGF-β1) are increased in Alzheimer's disease and have been implicated in the associated cerebrovascular pathology. We recently reported that transgenic mice that overexpress TGF-β1 (TGF$^+$ mice) display, with aging, selectively reduced endothelin-1 (ET-1)-mediated contractions. Because ET-1 is a key regulator of cerebrovascular tone and homeostasis, we investigated how increased levels of TGF-β1 could selectively alter this contractile response. We found that ETA receptors, via activation of p38 mitogen-activated protein (MAP) kinase, mediate the ET-1-induced contraction in mouse cerebral arteries, a response significantly decreased in aged TGF$^+$ mice (−39%; $p < 0.01$) despite unaltered ETA receptor levels or affinity. In cerebrovascular smooth muscle cell cultures, long-term treatment with TGF-β1 significantly decreased (>50%; $p < 0.05$) the ET-1-induced activation of the p38 MAPK/27-kDa heat shock protein (HSP27) signaling pathway. This occurred with no effect upstream to p38 MAP kinase but with the concomitant induction of mitogen-activated protein kinase phosphatase-1 (MKP-1) expression. Inhibition of MKP-1 expression with Ro-31-8220 or suppression of MKP-1 expression by short interfering RNA restored the ET-1-mediated p38 MAP kinase response. These results disclose a new role for long-term increases of TGF-β1 in modulating cerebrovascular tone by dampening ET-1-mediated activation of the p38 MAPK/HSP27 signaling pathway. Such changes in ET-1-mediated signaling may help maintain vascular wall homeostasis by compensating for the diminished dilatory function induced by TGF-β1 and amyloid-β; brain levels of these two molecules are increased in patients with Alzheimer's disease.

Although a diagnosis of Alzheimer's disease (AD) can only be established upon neuropathological confirmation of neurofibrillary tangles, neuronal loss, and amyloid-β (Aβ) peptide deposition in brain parenchyma and blood vessels, recent studies suggest that pathologic cerebrovascular lesions are an early marker of the disease, compatible with impaired cerebral blood flow in cognitively asymptomatic patients with AD (for review, Iadecola, 2004). Together with aging, coronary artery diseases, hypertension, atherosclerosis, and diabetes have been identified as the main risk factors for sporadic AD (Roher et al., 2003; de la Torre, 2004; Gorelick, 2004). These pathologic conditions affect both the structure of the blood vessels and their responsiveness to vasomotor stimuli and, as in AD (Grammas and Ovase, 2002), have been associated with increased levels of transforming growth factor-β1 (TGF-β1) (Blobe et al., 2000; August and Suthanthiran, 2006). Moreover, TGF-β1 is increased in the brains of patients who have experienced ischemic stroke (Krupinski et al., 1996), another risk factor for AD (Kalaria, 2000).

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ABBREVIATIONS: AD, Alzheimer's disease; Aβ, amyloid-β; TGF-β1, transforming growth factor-β1; ET-1, endothelin-1; ETA, endothelin receptor A; ETB, endothelin receptor B; MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase or p44/42 MAPK (ERK1/2); PE, phenylephrine; U-0126, 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene; BQ-123, cyclo(L-Leu-D-Trp-D-Asp-L-Pro-D-Val); BQ-788, *N*-cis-2,6-dimethylpiperidinocarbonyl-L-γMeLeu-D-Trp(COOMe)-D-Nle-Ona; Ro-31-8220, 3-[1-(3-(amidinothio) propyl-1H-indol-3-yl)]-3-(1-methyl-1H-indol-3-yl) maleimide (bisindolylmaleimide IX); SB-203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole; GF 109203X, 3-[1-[3-(dimethylaminopropyl]-1*H*-indol-3-yl]-4-(1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione monohydrochloride; MKP-1, mitogen-activated protein kinase phosphatase-1; COX, cyclooxygenase; FITC, fluorescein isothiocyanate; WT, wild-type; MCA, middle cerebral artery; 5-HT, 5-hydroxytryptamine; MEK, ERK MAPK kinase; MKK3/6, p38 MAPK kinase; PKC, protein kinase C; SMC, smooth muscle cell; siRNA, small interfering RNA.

In animal models of AD, reactive oxygen species mediate the Aβ-induced deficits in cerebrovascular dilatory responses, these being readily restored by antioxidants (Iadecola et al., 1999; Tong et al., 2005). In contrast, in transgenic mice that overexpress TGF-β1 (TGF^+ mice) that best recapitulate the structural pathologic cerebrovascular lesions seen in AD (Wyss-Coray et al., 2000), impaired dilations or constrictions are progressive, insensitive to antioxidants, and selective for specific vasoactive mediators (Tong et al., 2005). Indeed, the decreased contractile response of brain arteries to endothelin-1 (ET-1) occurs despite preserved contractions to serotonin (5-HT), suggesting that the increased thickness of the vessel wall displayed by TGF^+ mice (Wyss-Coray et al., 2000; Tong et al., 2005) cannot explain the selectively altered vascular reactivity observed with aging. Rather, akin to systemic arteries from hypertensive rats (Kim et al., 2004), the selective deficit in the ET-1-mediated contraction could be attributed to alterations in its signaling pathway. ET-l elicits contraction of cerebral arteries primarily by activating smooth-muscle ETA receptors (Zubkov et al., 2000). However, ETB receptors exist in both smooth muscle and endothelial cells and have been linked to contraction and dilatation, respectively (Tirapelli et al., 2005), and they are also involved in ET-1 clearance (Szok et al., 2001; Sanchez et al., 2002). ET-1 receptors activate various signaling pathways and, particularly, different families of mitogen-activated protein kinases (MAPKs), such as the extracellular signal-regulated kinase (ERK1/2 or p44/p42), c-Jun NH2-terminal protein kinase or stress-activated protein kinase, and p38 MAPK (Yue et al., 2000; Zubkov et al., 2000).

Because ET-1 is a key regulator of cerebrovascular tone and homeostasis, we investigated whether long-term increases in TGF-β1 levels, as seen in AD brains, could selectively hamper the ET-1-mediated contraction by alteration in its signal transduction pathway. We found that TGF-β1 inhibited ET-1-induced activation of the p38 MAPK/27-kDa heat shock protein (HSP27) pathway via expression of the transcriptionally regulated mitogen-activated protein kinase phosphatase-1 (MKP-1). These data show, for the first time, that persistently increased TGF-β1 directly affects the signaling pathway underlying the ET-1-mediated cerebral contraction. Such an effect could account for the impaired vasoconstrictive response to ET-1 in aged TGF^+ mice and may represent a means to compensate for the lessened dilatory function.

Materials and Methods

Reagents, siRNA, and Antibodies. Porcine TGF-β1 was from R&D Systems (Minneapolis, MN); 5-HT, phenylephrine (PE), U-0126, and $A\beta_{1-40}$ were from Sigma (St. Louis, MO); and ET-1, ETA (BQ-123) and ETB (BQ-788) receptor antagonists were from American Peptide (Vista, CA). Ro-31-8220 and SB-203580 were from Calbiochem (San Diego, CA), and GF 109203X was from Tocris Bioscience (Ellisville, MO). Short-interfering RNA (Stealth siRNA) constructs homologous to rat MKP-1 or control (scrambled) siRNAs were from Invitrogen (Burlington, ON, Canada). Antibodies were, rabbit anti-p38, anti-phospho $p38^{Thr180/Tyr182}$ (pp38), anti-p44/42 MAPK (ERK1/2), anti-phospho $ERK1/2^{Thr202/Tyr204}$ (pERK), anti-MKK3, anti-phospho $MKK3/6^{Ser189/207}$ (Cell Signaling Technologies, Danvers, MA), anti-MKP-1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-ETA and anti-ETB receptors (Alomone Labs, Jerusalem, Israel) or anti-cyclooxygenase-2 (COX-2; Cayman Chemical, Ann Arbor, MI) for Western blot or goat anti-COX-2 (Santa Cruz Biotechnology) for immunocytochemistry), mouse anti-cyclooxygenase-1 (COX-1, Cayman Chemical), anti-smooth muscle actin (NeoMarkers, Fremont, CA, or Sigma for FITC-conjugate), or anti-β-actin (Sigma). Biotinylated secondary antibodies and the avidin-biotinylated enzyme complex kit were from Vector Labs (Burlingame, CA) and FITC, Cy2-, or Cy3-conjugated antibodies were from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). Cell culture materials were from Invitrogen and Sigma.

Animals and Vascular Tissues. Aged (≥18 months, body weight ~40 ± 10 g) heterozygous transgenic TGF^+ mice and wild-type (WT) littermate controls on a C57BL/6 background were used (Wyss-Coray et al., 2000; Tong et al., 2005). Cerebrovascular reactivity was measured in aged transgenic and WT littermates (n = 20/group) and signaling pathways tested in aged WT littermates (n = 4). For vascular reactivity studies, the first segments of the middle cerebral artery (MCA) or, where indicated, the posterior cerebral artery were isolated from WT and TGF^+ mice sacrificed by cervical dislocation and used for online videomicroscopy analysis of diameter changes (Tong et al., 2005). Remaining vessels of the circle of Willis and their branches were immediately dissected under a microscope, frozen (dry ice), and stored (−80°C) until Western blot analysis. Experiments were approved by the Institute's Animal Ethics Committee and abided to the Canadian Council for Animal Care.

Vascular Reactivity Studies. MCA segments (~2 mm long; average intraluminal diameter, ~50 μm) were cannulated on a glass micropipette (~40-μm diameter) at one end, sealed to another glass micropipette on the other end, and filled with an oxygenated (95%) Krebs' solution, as detailed previously (Tong et al., 2005). Vessels were then pressurized (60 mmHg), superfused with Krebs, and allowed to stabilize and acquire basal tone (45–60 min). Then, increasing concentrations of ET-1 (10^{-10} to 3×10^{-7} M), 5-HT, or PE (10^{-9} to 10^{-5} or 10^{-4}M) were applied extraluminally, and changes in intraluminal diameters from basal tone were measured online using a closed*circuit video system coupled to a video caliper (Imagen Instrumentation, Trenton, NJ). In some vessels (n = 5/group), ETA or ETB receptors were blocked with their selective antagonists BQ-123 or BQ-788 (10^{-7} M, 30-min preincubation and in each ET-1 solution) (Ishikawa et al., 1994). Intracellular signaling pathways were assessed in vessels preincubated (1 h) with a maximal effective yet nontoxic dose of SB-203580 (50 μM, a p38 MAPK inhibitor) or U-0126 (10 μM, a ERK1/2 MAP kinase or MEK inhibitor) (Davies et al., 2000) as determined in vessels from 6-month old WT mice using 10 to 50 μM SB-203580 (66 and 71% inhibition at 25 and 50 μM, respectively) or 0.1 to 50 μM U-0126 (36 and 49% inhibition at 1 and 10 μM, respectively, with toxicity and complete loss of vascular reactivity at 25 and 50 μM). Vessels were then rinsed in fresh Krebs' solution (10 min) and exposed to ET-1 or PE, two agonists reported to mediate constriction via activation of these signaling pathways in cerebral or peripheral arteries (Zubkov et al., 2000; Zhao et al., 2003; Kim et al., 2004).

Cell Culture. Primary cultures of rat or, when indicated, 4- or 18 month-old WT mouse smooth muscle cells (SMC) were generated from brain intracortical microvessels. After dissociation of the microvessels with type IV collagenase (1 mg/ml, 6 min at 37°C), cells were seeded onto 0.5% gelatin-coated culture plates containing 64% medium M199, 30% fetal bovine serum, peptone (0.05%), D-glucose (1%), BME Amino Acid (1×), BME Vitamin (1%), and antibiotics. After 2 to 3 weeks in culture, >85% of cells stained positively for smooth muscle α-actin. For activation of ERK and p38 MAPK pathways, cells were pretreated with TGF-β1 (3 ng/ml, 72 h) (Grammas and Ovase, 2002) in serum-free medium and then in serum-free medium alone (2 h) before stimulation with ET-1 or PE (1×10^{-7} or 10^{-4} M, 10 or 15 min). Some cultures were similarly treated with $A\beta_{1-40}$ (1 μM, 72 h) (Niwa et al., 2000) before ET-1 stimulation. Inhibition of p38 MAPK or pERK activation was performed on cells kept overnight in serum-free condition and pretreated (1 h) with SB-203580 (25 μM) or U-0126 (10 μM) before stimulation with ET-1

or PE. The contribution of MKP-1 or protein kinase C (PKC) in TGF-β1 regulation of p38MAPK/HSP27 activity was assessed in cells pretreated with TGF-β1 (48 h) and with both TGF-β1 and different concentrations of Ro-31-8220 or GF 109203X for another 24 h, followed by 2-h incubation in serum-free medium before ET-1 stimulation.

MKP-1 siRNA Transfection. Primary cultures of rat brain smooth muscle cells in primary plating medium (with antibiotics) were seeded onto 30-mm diameter dishes (2 days), and then changed to antibiotic-free medium. On the third day, siRNA was transfected using Lipofectamine 2000 reagent according to the manufacturer's instructions (Invitrogen). After 8 to 14 h, transfection reagents were washed, and the cells were stimulated with TGF-β1 (3 ng/ml) for 24 h in medium containing 3% bovine serum and then for an additional 36 h in serum-free medium before stimulation with ET-1 as above. RNA oligonucleotides were as follows: forward, 5′-GAG UAC UAG UGU GCC UGA CAG UGC A-3′; reverse, 5′-CCA GCU GCU GCA AUU UGA GUC CCA A-3′. Control siRNA were: forward, 5′-GAG GAU CGU GUG GUG UCC GAC AUA UGC A-3′; reverse, 5′-UUG CGG UCA GAA ACC GUU ACG AUG G-3′.

Western Blot. Western blot analysis in vessels from WT and TGF$^+$ mice were performed as described previously (Tong et al., 2005). For SMC cultures, cells were lysed (20 mM Tris-Cl, pH 7.4, 150 mM NaCl, 0.1% Nonidet P-40, 1% glycerol, 0.2 mM sodium vanadate, and protease inhibitor mixture from Roche diagnostic) and proteins similarly prepared. Membranes (~20 μg of protein) were probed with antibodies using β-actin as a control for loading, proteins detected with ECL (GE Healthcare, Baie d'Urfé, QC, Canada), quantified by densitometry, and compared by one-way ANOVA or Student's t tests.

Immunofluorescence in Brain Microvascular SMC Cultures. SMC cultured onto coverslips (1–2 weeks) were treated with TGF-β1 (3 ng/ml, 72 h) (COX-2 experiments) or with TGF-β1 (48 h) and then with both TGF-β1 and Ro-31-8220 (4 μM) for 24 h (MKP-1 experiments), or were transfected as above. Pharmacologically treated or siRNA-transfected cells were fixed (30 min, 4% paraformaldehyde), permeabilized (0.3% Triton X-100, 10 min), and incubated (1 h) with primary antibodies (COX-2, MKP-1 or smooth muscle actin) detected with FITC, CY2- or CY3-conjugated secondary antibodies, and viewed under epifluorescence microscopy.

Statistical Analyses. Agonist-induced concentration-dependent or maximal (EA_{max}) contractions (percentage change from baseline diameter) and potency (pD_2 values or $-\log$ of EC_{50}), expressed as mean ± S.E.M, were compared by one-way ANOVA followed by Newman-Keuls or Dunnett's (COX-2 experiments) post hoc comparison test. ET-1 receptor antagonist potency was expressed as pK_B (competitive antagonism) or pD2′ (noncompetitive antagonism when a significant decrease in ET-1 EAmax was obtained by Student's t test) values. Statistical analyses were performed with GraphPad Prism 4 (GraphPad Software, San Diego, CA); $p < 0.05$ was considered significant.

Results

ET-1-Induced Contractions Were Impaired in Brain Vessels from Old TGF$^+$ Mice. In agreement with our previous findings (Tong et al., 2005), contractions elicited by ET-1 were significantly decreased in aged TGF$^+$ mice compared with WT littermate control mice (Fig. 1A). The response was maximally affected at 3×10^{-7} M ET-1 (−39%, $p < 0.001$), and this impairment was selective as contractions induced by 5-HT or PE were unaltered (Fig. 1A). The decreased ET-1-mediated contractions occurred with no change in ET-1 potency (pEC_{50} values of 8.52 ± 0.16 and 8.52 ± 0.27 in TGF$^+$ and WT mice, respectively) or in cerebrovascular ETA receptor levels (Fig. 1B), suggesting that mechanisms other than agonist-induced ETA receptor desensitization or down-regulation are involved. In contrast, ETB receptor protein levels were slightly but significantly increased (+27%; $p < 0.05$) (Fig. 1B). Because ETB receptors can mediate endothelium-dependent dilation (Szok et al., 2001; Tirapelli et al., 2005), we tested whether their increased levels could account for the diminished ET-1 contractile response. This possibility was discarded because blockade of ETB receptors with the selective ETB receptor antagonist BQ-788 did not restore ET-1-mediated contractions (Fig. 1C). Rather, BQ-788 slightly but significantly ($p < 0.05$) reduced ET-1 EA_{max} in WT control mice or shifted the ET-1 concentration-response curve in TGF$^+$ mice (Fig. 1C), with potencies that corresponded to its affinity at ETA receptors (respective pD2′ and pK_B values of 6.6 and 7.3) (Ishikawa et al., 1994). Moreover, as expected for an ETA receptor-mediated response, the selective ETA receptor antagonist BQ-123 potently inhibited the ET-1 concentration-response curves in both WT and TGF$^+$ mice ($p < 0.05$), with respective pK_B values of 8.1 and 7.8 (shown here in TGF$^+$ mice; Fig. 1C).

ET1-Induced Vasoconstriction Was Mediated by p38 MAPK Signal Transduction Pathway. The contribution of p38 MAPK and ERK pathways in the ET-1- or PE-mediated contraction was tested in cerebral arteries from aged WT mice treated with a maximal effective dose of respective inhibitor of p38 MAPK (SB-203580, 50 μM) or MEK (U-0126, 10 μM). The ET-1-mediated contraction was potently de-

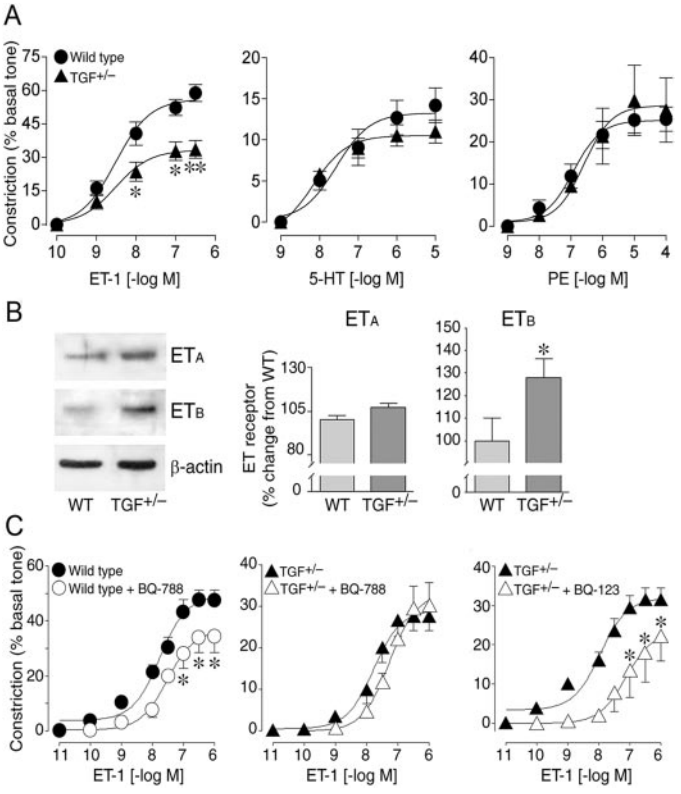

Fig. 1. A, ET-1-induced contractions were decreased in MCA segments from 18-month-old TGF$^+$ mice (▲, $n = 9$) compared with wild-type (WT) littermate control mice (●, $n = 9$). In contrast, the contractions induced by 5-HT ($n = 6$) and PE ($n = 3$–4) were unaltered. B, in pial vessels from TGF$^+$ mice ETB, but not ETA, receptor levels were increased compared with WT littermate control mice. C, respective blockade of ETB receptors with BQ-788 (10^{-7}M) or ETA receptors with BQ-123 (10^{-7}M, shown here in PCAs from TGF$^+$ mice) antagonized the ET-1-induced contractions in WT and TGF$^+$ mice, with potencies corresponding to their affinities at ETA receptors (see text). *, $p < 0.05$, **, $p < 0.01$.

creased (−66%; $p < 0.001$) by inhibition of p38 MAPK, whereas inhibition of MEK/ERK1/2 pathway exerted a more modest effect (−32%, $p < 0.05$), both without any change in ET-1 potency (Fig. 2A). In brain microvascular SMC cultures, ET-1 induced activation of both p38 MAPK and ERK, and these were significantly inhibited by pretreatment with their respective inhibitors (Fig. 2, B and C). In contrast, p38 MAPK or MEK inhibition had minimal effect on the contraction elicited by PE in mouse cerebral arteries despite effective blockade of the PE-induced activation of p38 and ERK pathways in SMC cultures (Fig. 2, D and E). These results indicated that ET-1 and PE both activate p38 MAPK and ERK signaling pathways in brain vascular SMC and that activation of p38 MAPK mediates the bulk of the ET-1-induced contraction in mouse cerebral arteries with a smaller contribution from the ERK pathway; these two pathways are of lesser importance in the PE-mediated cerebral contraction.

Long-Term TGF-β1 Treatment Selectively Blocked ET-1-Induced p38 MAPK/HSP27 Activation. To test whether long-term increase in TGF-β1 could alter ET-1 signaling pathways, we treated brain microvascular SMC with TGF-β1 and assessed phosphorylation of ERK and p38 MAPK. COX-1 and COX-2 protein levels were also measured because these proteins are, respectively, constitutively expressed and induced in these cells (Rodriguez et al., 2006), thus allowing COX-2 to be used as a marker of the cells' response to TGF-β1. TGF-β1 induced COX-2 expression, a response increased at 24 h and significantly maintained over the 72-h exposure period; COX-2 was detected primarily in the nucleus and, to a smaller extent, in the cytoplasm (Fig. 3A). TGF-β1 stimulated phosphorylation of both p38 MAPK and ERK; the former developed progressively over a 1-h period and returned to prestimulated levels by 24 h, whereas the latter was rapid and brief (peak at ~10 min), returning to baseline within 1 h (Fig. 3B). All subsequent experiments were performed at 72 h when cells still exhibited significant TGF-β1-induced COX-2 increase, whereas ERK and p38 MAPK pathways had returned to basal levels. Under these conditions, TGF-β1 inhibited (by ~50%, $p < 0.05$) the ET-1-induced activation of p38 MAPK (37% increase in phospho-p38 MAPK levels; $p < 0.05$) but had no significant effect on ET-1-induced ERK phosphorylation (Figs. 4, A and B), as also found in SMC cultures from young or aged mice (data not shown). Moreover, TGF-β1 impaired (−65%; $p < 0.05$) the ET-1-induced phosphorylation of HSP27 (Fig. 4A). In contrast, $A\beta_{1-40}$ peptide, at a concentration previously shown to affect cerebrovascular reactivity (Niwa et al., 2000) and above that found in AD brains, did not induce COX-2 expression or alter the stimulatory effect of ET-1 on p38 MAPK (Fig. 4C).

Inhibition of Downstream ET-1 Transduction Pathway at the p38 Phosphorylation Step. To decipher the mechanisms involved in the inhibition of ET-1-induced p38 MAKP activation by TGF-β1, we assessed the protein levels of ETA and ETB receptors, and ET-1-induced activation of p38 MAPK kinase (MKK3/6) directly upstream to the p38 MAPK. These were not altered in SMC exposed to TGF-β1 (Fig. 5), indicating inhibition of the ET-1 transduction pathway by TGF-β1 directly at the p38 phosphorylation step. Because MKP-1 is a transcriptionally regulated phosphatase that tightly regulates MAPK activities (see *Discussion*), we hypothesized that long-term exposure to high levels of TGF-β1 could induce MKP-1 in brain microvascular SMC that would subsequently inhibit p38 MAPK activity. In non-treated SMCs, MKP-1 expression was very low. However, in cells exposed to TGF-β1 and stimulated or not with ET-1, MKP-1 protein levels were significantly increased (~36%, $p < 0.001$)(Fig. 6), indicating that TGF-β1 mediated the MKP-1 induction. Moreover, the inhibitory effect of TGF-β1 on ET-1-induced p38 MAPK activation occurred concomitantly with MKP-1 up-regulation and relapsed when MKP-1 expression was prevented by Ro-31-8220 (Figs. 6, A–C). In

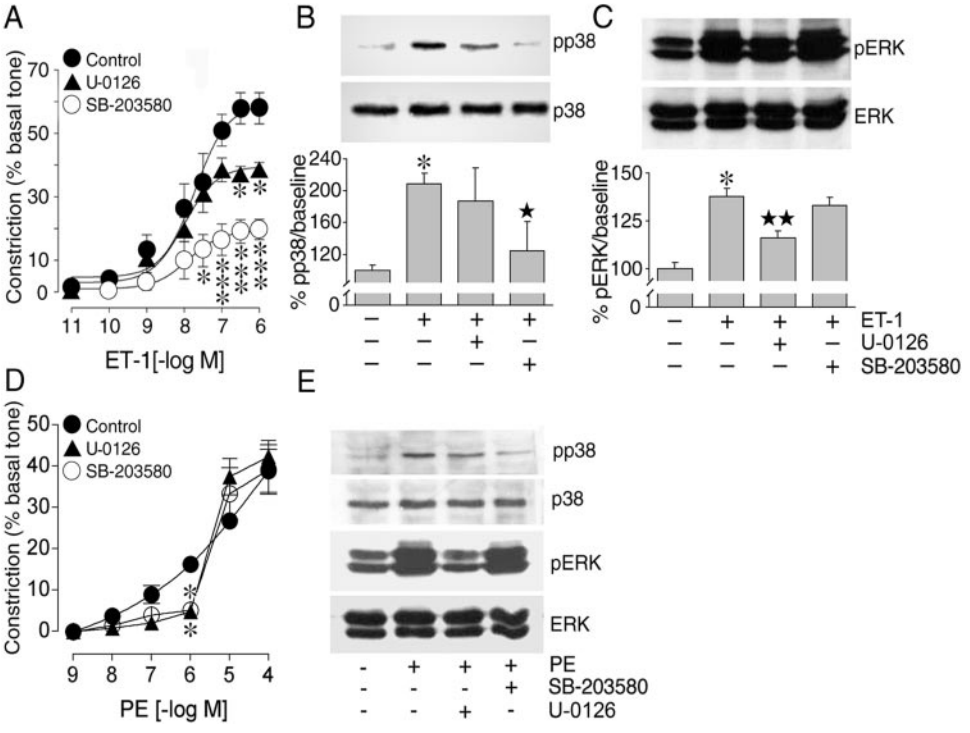

Fig. 2. Concentration-response curves to ET-1 (A) or PE (D) in WT mice under control conditions (●), after inhibition of MEK with U-0126 (▲, 10 μM), or after inhibition of p38 MAPK with SB-203580 (○, 50 μM). Decreases in the ET-1- or PE-induced contractions occurred without change in ET-1 (pEC_{50}, 7.71 ± 0.13, 8.0 ± 0.13, and 7.90 ± 0.10, respectively) or PE (pEC_{50}, of 5.69 ± 0.19, 5.43 ± 0.15, and 5.40 ± 0.21) potency. *, $p < 0.05$; ***, $p < 0.001$. Phosphorylation of p38 (pp38) (B) or ERK (pERK) (C) induced by ET-1 in cerebrovascular SMC was affected by inhibition of p38 MAPK (SB-203580, 25 μM) or MEK (U-0126, 10 μM), as determined by Western blotting and densitometry analysis using p38 and ERK as baseline ($n = 3$ independent experiments). *, $p < 0.05$ compared with baseline (show the stimulatory effect of ET-1), and ⋆, $p < 0.05$; ⋆⋆, $p < 0.01$, compared with the ET-1 effect. E, likewise, the PE-induced phosphorylation of p38 MAPK and ERK (pp38 or pERK) was reduced by their respective inhibition with SB-203580 and U-0126, as shown from a representative Western blot from three different experiments.

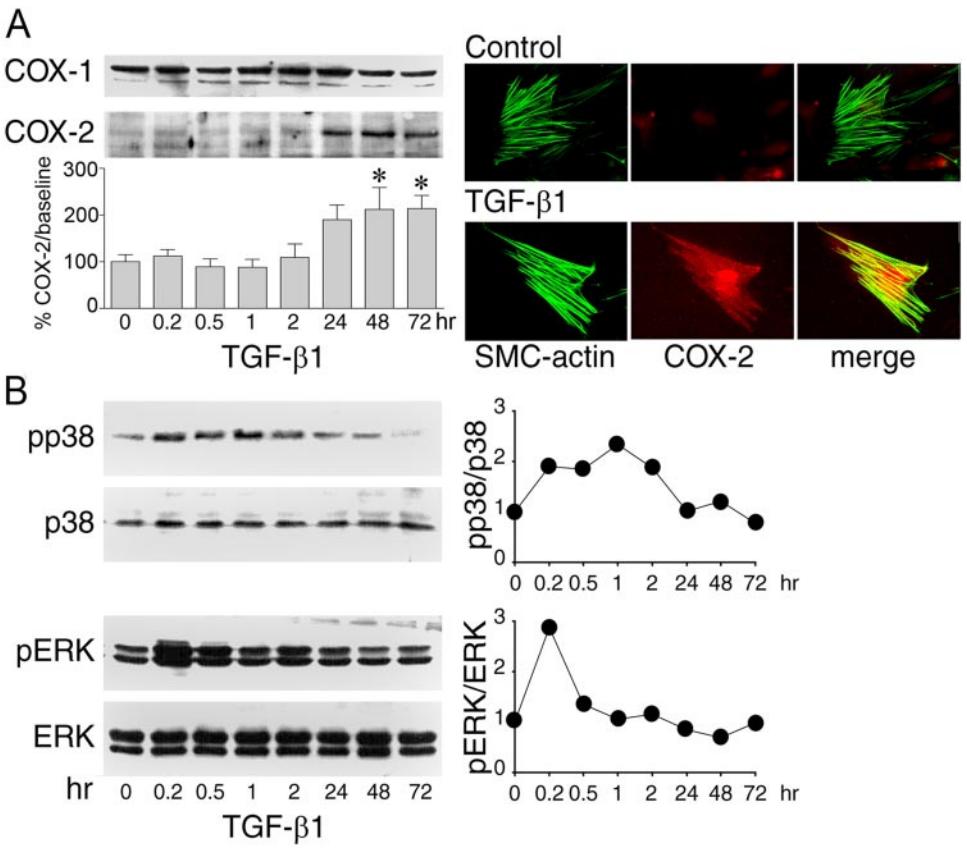

Fig. 3. A, treatment of SMC with TGF-β1 induced expression of COX-2 but not COX-1. COX-2 was increased, albeit nonsignificantly, by 24 h and remained upregulated for the 72 h of the experiment (*, $p < 0.05$; ANOVA followed by a Dunnett's post hoc comparison test), as shown in both the nucleus and cytoplasm of cells treated for 72 h and double-immunostained for SMC-actin (green, showing actin filaments) and COX-2 (red) (right). Values are mean ± S.E.M. of densitometry readings ($n = 3$ independent experiments). B, TGF-β1 induced phosphorylation of p38 and ERK with different time courses. Activation of p38 MAPK (pp38) was progressive over a 1-h period and returned to prestimulated levels by 24 h, whereas ERK phosphorylation (pERK) was rapid and brief, and levels were back to prestimulated values by 1 h. Controls for COX-1 and COX-2 loading are p38 and ERK because they are from the same gels.

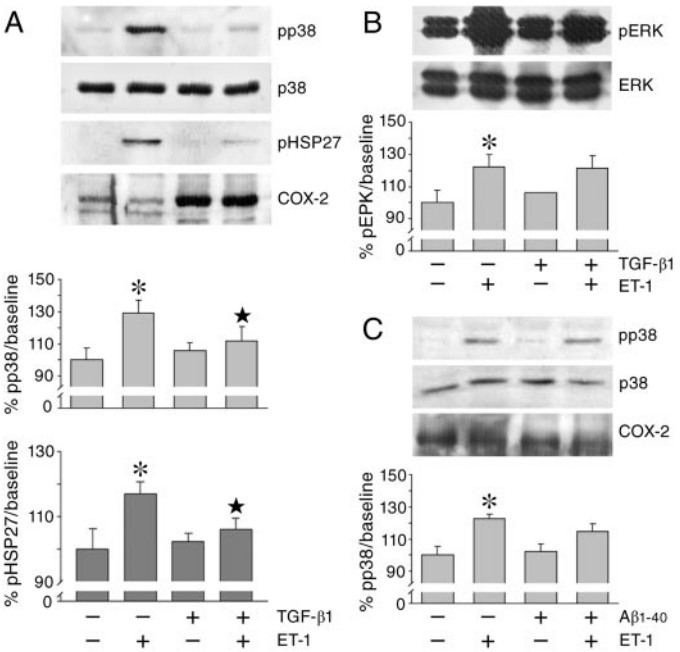

Fig. 4. TGF-β1 selectively affects p38 MAPK/HSP27 (A) but not ERK (B) activation. Stimulation of cerebrovascular SMC with ET-1 induced phosphorylation of the p38 MAPK/HSP27 and ERK pathways (*, $p < 0.05$), but treatment with TGF-β1 before ET-1 stimulation selectively decreased the ET-1-mediated p38 MAPK/HSP27 activation (⋆, $p < 0.05$, compared with the ET-1 effect). COX-2 expression confirmed that SMC were activated upon exposure to TGF-β1. C, in contrast, $A\beta_{1-40}$ treatment had no inhibitory effect of ET-1-induced p38 MAPK activation. Values are mean ± S.E.M. of densitometry readings ($n = 3$–6 experiments).

contrast, the negative effect of TGF-β1 on the ET-1-induced activation of the p38 MAPK/HSP27 pathway was not restored in cells treated with the selective PKC inhibitor GF 109203X (Harkin et al., 1998) (Fig. 6D), indicating that Ro-31-8220 did not act through PKC inhibition (Beltman et al., 1996).

To confirm these pharmacological data, we then inhibited gene expression of endogenous MKP-1 by siRNA. Specific MKP-1 siRNA treatment reduced the expression of MKP-1 protein induced by TGF-β1 by 67.2% ($p < 0.01$), as also evidenced by the drastic decrease in SMC cells immunopositive for MKP-1 after siRNA transfection (Figs. 7, A and B). Down-regulation of MKP-1 expression by siRNA resulted in the normalization of p38 MAPK activation, further demonstrating that TGF-β1-induced MKP-1 expression negatively regulated p38 MAPK activity (Fig. 7A). In contrast, transfection with control siRNA had no effect on the TGF-β1-induced alterations in MKP-1 levels or p38 MAPK activation in response to ET-1 (Fig. 7A).

Discussion

Our results disclose a new role for persistently increased levels of TGF-β1 in brain vessels in modulating their responsiveness to selective vasomotor stimuli. In particular, we show that long-term treatment with TGF-β1 promotes MKP-1 expression in cerebrovascular SMC, which results in decreased ETA receptor-induced activation of the p38 MAPK/HSP27 pathway that, for the most part, mediates the ET-1-induced contraction of cerebral arteries. These data further suggest that selectively impaired ET-1-mediated cerebrovascular contractions induced in vivo by increased TGF-β1 can

be instigated, at least in part, by direct interaction with ET-1 signaling pathway.

ETA versus ETB Receptors in the ET-1-Mediated Contraction in Mouse Brain Vessels. We showed that ETA receptors mediated the ET-1-induced contraction in mouse cerebral arteries, as evidenced by the potent antagonism of the selective ETA receptor antagonist BQ-123, the affinity of which (pK_B, 8.1) corresponded exquisitely well to that reported at ETA receptors (pIC_{50}, 7.6–8.0) (Ishikawa et al., 1994). In contrast, the potency of the selective ETB receptor antagonist BQ-788 (pD2′, 6.6) corresponded to its affinity at ETA receptors (pIC_{50}, 5.9–6.5) (Ishikawa et al., 1994) further supporting ETA receptors as the prime mediators of the contractile response (Zubkov et al., 2000). In TGF^+ mice, BQ-788 was slightly more potent (pA_2, 7.3) in blocking the ET-1-induced contraction compared with wild-type control mice. Although far from the affinity of BQ-788 at ETB receptors (pIC_{50}, ~9.0) (Ishikawa et al., 1994), this shift toward a higher potency in TGF^+ mice may suggest a larger contribution of ETB receptors in the contractile response, as seen in other pathologic conditions, such as brain ischemia (Stenman et al., 2002), whereby ETB receptors change from a relaxant to a contractile phenotype. Yet the failure of ETB receptor antagonism to restore the lessened ET-1 contractions in TGF^+ mice, notwithstanding increased ETB receptors, indicated that the latter, akin to recent studies in rat coronary arteries (Tirapelli et al., 2005), does not mediate a sizeable dilatation in mouse cerebral arteries and hence does not account for the impaired ET-1-induced contraction.

It is noteworthy that, similar to TGF^+ mice, transgenic mice that overexpress endothelial ET-1 display reduced contractile responses to ET-1, and increased ETB but not ETA receptor expression (Amiri et al., 2004). In contrast, ETB receptor expression is down-regulated in ET-1-deficitent astrocytes (Ho et al., 2001), suggesting that the levels of ET-1 regulate the expression of ETB receptors known to mediate its uptake and clearance (Sanchez et al., 2002). Together these observations suggest that up-regulation of vascular ETB receptors in TGF^+ mice is likely to result from increased levels of ET-1 after its induction by TGF-β1 (Rodríguez-Pascual et al., 2003), which could be implicated in the initiation and maintenance of the vascular fibrosis seen in these mice, the latter being normally associated with activation of the MEK/ERK pathway (Yue et al., 2000; Kapoun et al., 2006).

P38 MAPK Activation Mediated the ET-1-Induced Contraction. Our findings show that the cerebrovascular response of contractile ETA receptors is altered in TGF^+ mice, independently from changes in receptor levels or agonist potency. Moreover, our results show, for the first time in brain vessels, that the ET-1-induced contraction involves primarily activation of the p38 MAPK/HSP27 pathway. Similar to our findings, inhibition of p38 MAPK but not ERK signaling reduced the ETA receptor-mediated contraction of pulmonary artery, even though ET-1 induced phosphorylation of both ERK and p38 MAPK (Yamboliev et al., 2000). In addition, in aortic smooth muscle of deoxycorticosterone acetate-salt hypertensive rats (Kim et al., 2004) or spontaneously hypertensive rats (Kwon et al., 2004), the ET-1-mediated contraction was decreased after inactivation of p38 MAPK, pointing to a role for the p38 MAPK/HSP27 pathway in modulating the intensity and maintenance of vascular smooth muscle contraction. The ability of p38 MAPK to mediate the ET-1-induced contraction in peripheral

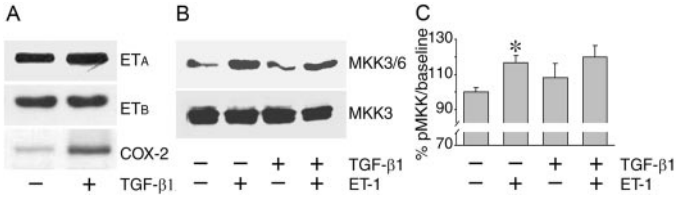

Fig. 5. A, representative Western blots for ETA and ETB receptors, and COX-2 in cerebrovascular SMC treated TGF-β1. Although the cells reacted to treatment, as shown by COX-2 induction, ETA and ETB receptors were unchanged. B and C, likewise, TGF-β1 did not alter ET-1-induced phosphorylation of MKK3/6, as shown in representative Western blots (B) and quantified by densitometry readings (C). *, $p < 0.05$ compared with baseline ($n = 3$ experiments).

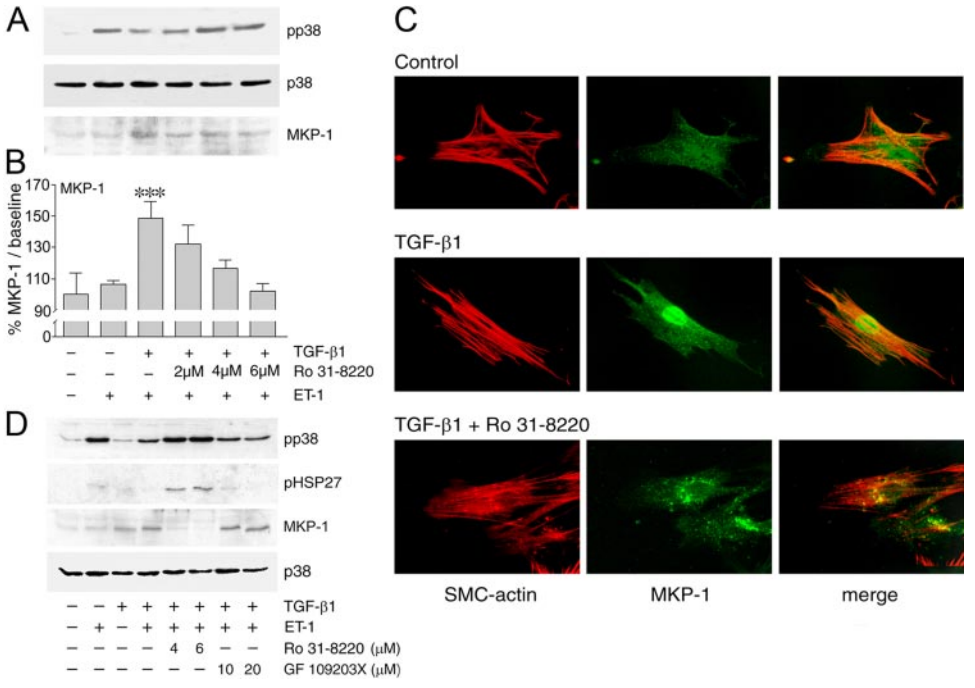

Fig. 6. A and B, exposure of cerebrovascular SMC to TGF-β1 inhibited the ET-1-mediated p38 MAPK activation concomitantly with increased MKP-1 expression, the latter was blocked by increasing concentrations of Ro-31-8220, as illustrated by representative Western blots (A) and quantified by densitometry (B, $n = 6$ different experiments) ***, $p < 0.001$. C, double-immunostaining for SMC-actin filaments (Cy3, red) and MKP-1 (Cy2, green) in SMCs at rest, after treatment with TGF-β1, or with TGF-β1 and Ro-31-8220 showed increased expression of MKP-1 primarily in the nucleus but also in the cytoplasm of cells treated with TGF-β1. In cells treated with Ro-31-8220, the TGF-β1-induced expression of MKP-1 was abrogated ($n = 5$ independent experiments). D, in contrast, the inhibitory effect of TGF-β1 on the ET-1-induced activation of the p38 MAPK/HSP27 pathway (pp38 and pHSP27, respectively) was not affected by selective inhibition of PKC with GF 109203X (representative Western blots from three different experiments). Loading is comparable for all samples as shown by p38 MAPK.

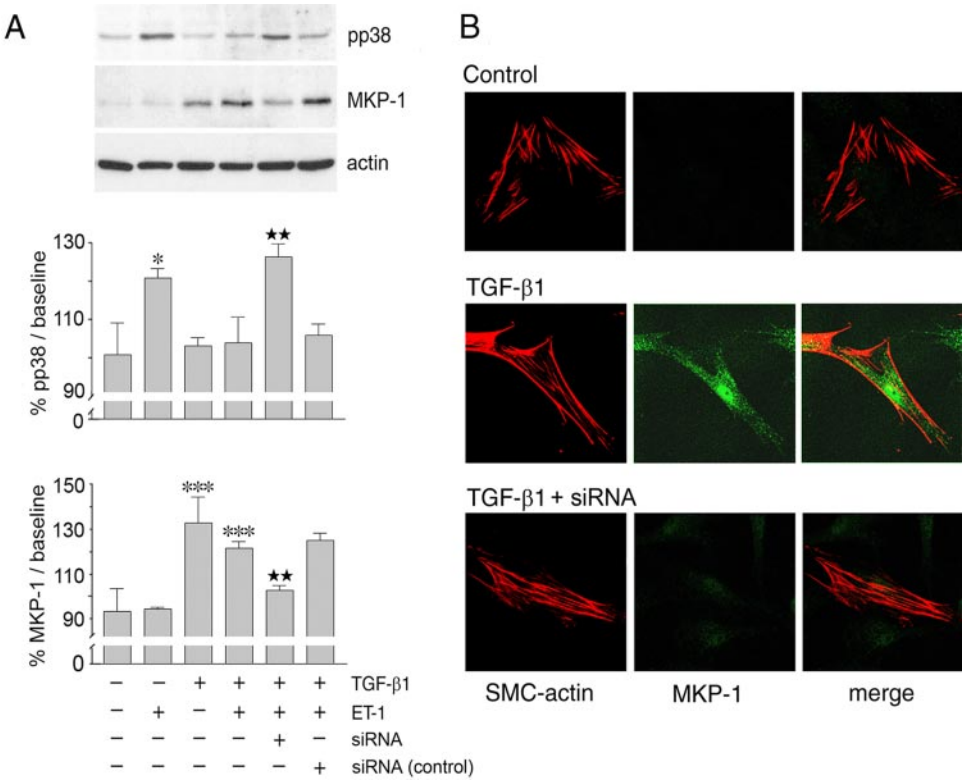

Fig. 7. A, in SMC transfected with MKP-1 siRNA, but not with control siRNA, the inhibitory effect of TGF-β1 on the ET-1-induced p38 MAPK activation was prevented concurrently with down-regulation of MKP-1 expression. *, $p < 0.05$ and ***, $p < 0.001$ compared with control (−) conditions, and ★★, $p < 0.01$, compared with the ET-1 effect in the presence of TGF-β1 without siRNA. B, this was further confirmed in MPK-1 siRNA transfected SMC immunodetected for SMC actin (Cy3, red) that displayed no detectable MKP-1 protein expression (Cy2, green) similar to unstimulated cells (control).

arteries has been attributed to its capacity to phosphorylate MAP-kinase-activated protein-2 kinase, which then translocates to the cytoplasm and phosphorylates HSP27 (Yamboliev et al., 2000). HSP27 is an F-actin binding protein that, upon phosphorylation, facilitates actin-myosin coupling, the major mechanism in smooth muscle contraction (Yamboliev et al., 2000; Bitar, 2002).

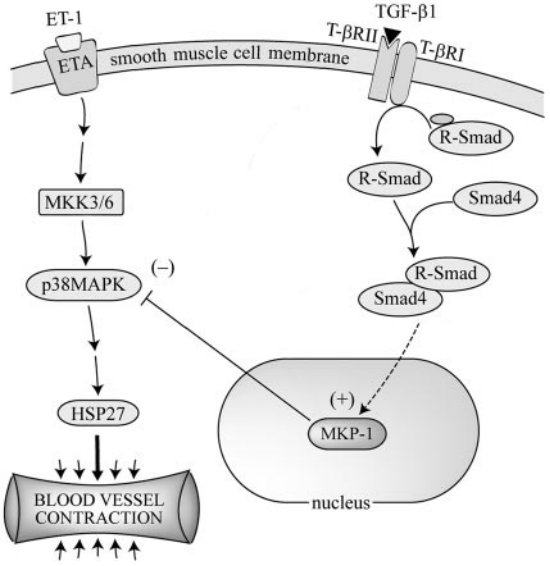

Fig. 8. Schematic representation of the signaling pathways involved in the ETA receptor-mediated ET-1-induced contraction in mouse cerebral arteries and how TGF-β1 can affect this response. ET-1 binds to ETA receptors on SMC membrane and stimulates the p38 MAPK/HSP27 pathway to induce contraction. TGF-β1, through binding to its receptor complex and, probably, activation of the Smad pathway, would induce MKP-1 expression, a negative regulator of p38 MAPK activity, which results in impaired signaling of ET-1A contractile receptors.

Regulation of p38 MAPK Activation. In cerebrovascular SMC cultures exposed persistently to TGF-β1, the time course for p38 MAPK and ERK activation differed greatly, consistent with previous reports in other cell types (Pratt et al., 2003). Moreover, although TGF-β1-mediated activation of ERK and p38 MAPK had both returned to basal levels by 72 h, the ET-1-induced activation of p38 MAPK was selectively impaired at that time, and this without any disruption in MKK3/6 phosphorylation, indicating an effect at the p38 MAPK step. What is more, the ET-1-mediated p38 MAPK/HSP27 activation was restored upon inhibition of MKP-1 expression with Ro-31-8220 but not after PKC inhibition with GF 109203X, indicating that Ro-31-8220 rescuing effect of p38 MAPK is due to its ability to inhibit MKP-1 expression and not PKC activity (Beltman et al., 1996; Harkin et al., 1998; Davies et al., 2000). This was further confirmed after silencing endogenous MKP-1 expression with siRNA, which resulted in the concurrent loss of MKP-1 protein expression (~70% decrease) and normalization of p38 MAPK activity. Together, these findings indicate that MKP-1 expression is involved in the impaired ET-1-induced activation of p38 MAPK after long-term TGF-β1 treatment (Fig. 8).

This conclusion is consistent with the marked reduction in p38 phosphorylation in hearts of MKP-1 transgenic mice (Bueno et al., 2001), and the sustained activation of p38 MAPK, but not ERK, in macrophages from MKP-1 knockout mice after lipopolysaccharide stimulation (Chi et al., 2006). It is also in line with the fact that p38 MAPK is the preferred substrate for MKP-1 over ERK (Yue et al., 2000; Pratt et al., 2003). Together with the fact that MKP-1 requires hours to increase after stimulation (McMullen et al., 2005), we conclude that long-term exposure to TGF-β1 impairs ET-1-mediated contraction in cerebral arteries by selectively inhibiting p38 MAPK activation through induction of MKP-1 expression. Because TGF-β1 re-

ceptors signal primarily through the Smad pathway, which is known to affect MKP-1 expression (Jono et al., 2003), we suggest that this mechanism is involved in the cellular cascade leading to p38 MAPK inhibition in cerebrovascular smooth muscle cells (Fig. 8). Furthermore, our findings of no deleterious effect of long-term $A\beta_{1-40}$ peptide on the ET-1-mediated p38 MAPK activation in brain SMC are consistent with a preserved ET-1 contractile response in transgenic mice overexpressing the Aβ precursor protein (Tong et al., 2005) and with the idea that $A\beta_{1-40}$ is stimulatory rather than inhibitory on p38 MAPK (Paris et al., 2003).

Our findings highlight a new role for MKP-1 in regulating vascular p38 MAPK activity and cerebrovascular tone. The selective impairment of the ET-1 response could be attributed, at least in part, to its high dependence on the p38 MAPK/HSP27 compared with 5-HT (Banes et al., 1999) or PE (Zhao et al., 2003), which use primarily other pathways to induce contraction. This mechanism may be of particular importance in AD, in which increased cerebrovascular levels of TGF-β1 (Grammas and Ovase, 2002) may reduce p38 MAPK/HSP27 signaling and, consequently, the ET-1-induced contractions. Such an effect could attempt maintain cerebral homeostasis and perfusion by compensating for the impaired dilatory function elicited by increased brain levels of both Aβ and TGF-β1 in AD (Iadecola et al., 1999; Tong et al., 2005).

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