Thromboxane Receptors in Smooth Muscle Promote Hypertension, Vascular Remodeling, and Sudden Death

Matthew A. Sparks, Natalia A. Makhanova, Robert C. Griffiths, John N. Snouwaert, Beverly H. Koller, Thomas M. Coffman

Abstract—The prostanoid thromboxane A₂ has been implicated to contribute to the pathogenesis of many cardiovascular diseases, including hypertension. To study the role of vascular thromboxane-prostanoid (TP) receptors in blood pressure regulation, we generated mice with cell-specific deletion of TP receptors in smooth muscle using Cre/LoxP technology. We crossed the KI SM22α-Cre transgenic mouse line expressing Cre recombinase in smooth muscle cells with a mouse line bearing a conditional allele of the Tbxα2r gene (TbpαLox). In KI SM22α-Cre TbpαLox (TP-SMKO) mice, TP receptors were efficiently deleted from vascular smooth muscle cells. In TP-SMKOs, acute vasoconstrictor responses to the TP agonist U46619 were attenuated to a similar extent in both the peripheral and renal circulations. Yet, acute vascular responses to angiotensin II were unaffected at baseline and after chronic angiotensin II administration. Infusion of high-dose U46619 caused circulatory collapse and death in a majority of control mice but had negligible hemodynamic effects in TP-SMKOs, which were completely protected from U46619-induced sudden death. Baseline blood pressures were normal in TP-SMKOs. However, the absence of TP receptors in vascular smooth muscle cells was associated with significant attenuation of angiotensin II–induced hypertension and diminished vascular remodeling. This was also associated with reduced urinary thromboxane production after chronic angiotensin II. Thus, TP receptors in vascular smooth muscle cells play a major role in mediating the actions of thromboxane A₂ in TP agonist-induced shock, hypertension, and vascular remodeling of the aorta. (Hypertension. 2013;61:00-00.) • Online Data Supplement

Key Words: Ang II • thromboxanes • death • muscle, smooth • hypertension

Thromboxane A₂ (TXA₂) is a prostanoid mediator produced by the metabolism of arachidonic acid through the cyclooxygenase pathway. Although many prostanoid metabolites are vasodilatory, TXA₂ stimulation of smooth muscle cells leads to potent vasoconstriction. TXA₂ has been implicated to be detrimental in the pathogenesis of a number of cardiovascular diseases, including ischemic heart disease,1 atherosclerosis,2 hypertension,3 preeclampsia,4 and stroke.5 Its actions are mediated by the thromboxane-prostanoid (TP) receptor, a member of the 7-transmembrane, G protein–coupled receptor superfamily.6 These receptors are expressed on a number of cell types and tissues relevant to cardiovascular pathogenesis, including the heart, platelets, various inflammatory cell populations including macrophages, and in the vasculature on vascular smooth muscle cells (VSMCs) and endothelium.7,9 Mass concentrations of TP agonists in the circulation can lead to hemodynamic collapse and shock.10

TXA₂ appears to have little influence on baseline regulation of blood pressure, because administration of blockers of thromboxone synthase or TP receptors have negligible effects on baseline blood pressure.11,12 Likewise, basal blood pressures are normal in mice completely lacking TP receptors.13

On the other hand, several studies suggest that TP receptors make significant contributions to the pathogenesis of hypertension, especially hypertension associated with activation of the renin-angiotensin system.3,14,15 During angiotensin II (Ang II)–induced hypertension, urinary levels of the renal TXA₂ metabolite thromboxane B₂ (TxB₂) increase significantly.14,16 Thromboxane antagonists attenuate the development of hypertension associated with chronic infusion of Ang II and obesity.17 Similarly, TP receptor–deficient mice are resistant to the development of Ang II–dependent hypertension and have an attenuated increase in blood pressure with N⁵-nitro-1-arginine methyl ester administration.3,14,18

TP receptors are expressed in a number of cell lineages with the potential to influence blood pressure, including VSMCs,19 renal epithelium,20 inflammatory cells, and the central nervous system.20 However, it has been difficult to determine the precise cell lineages contributing to the ability of TP receptors to promote hypertension. Because of the well-recognized...
vasoconstrictor actions of TxA₂, we considered the possibility that TP receptors in the vasculature play a key role in promoting hypertension. Accordingly, to determine the contribution of TP receptors in VSMCs to blood pressure regulation and hypertension, we generated mouse lines lacking TP receptors only in smooth muscle using Cre/loxP gene targeting. Using these mice, we find that TP receptors in smooth muscle contribute to the pathogenesis of hypertension and are essential for the development of TP agonist-induced shock and death.

**Methods**

**Generation of Mice With Deletion of TP Receptors From Smooth Muscle**

To confirm the expression pattern of Cre recombinase in the KISM22α-Cre and, in particular, to document expression in small resistance vessels, we crossed the KISM22α-Cre mouse line with the mTmG dual-reporter mouse line. At baseline, all of the tissues in these mice express red fluorescence, whereas, in the presence of Cre recombinase, expression of an enhanced green fluorescent protein cassette is triggered, providing a histological footprint of Cre expression that was identified by green fluorescence. As shown in Figure S1 of the online-only Data Supplement, robust green fluorescent protein fluorescence was seen in the media of the aorta (Figure S1C), throughout glomerular afferent arterioles, and in presumably mesangial cells within the glomerulus (Figure S1D) in KISM22α-Cre-mTmG mice, thereby confirming expression of Cre in VSMCs of both conduit and resistance vessels.

To produce mice lacking TP receptors in VSMC (TP-SMKOs), we carried out successive intercrosses between the KISM22α-Cre line and mice homozygous for the conditional floxed Tbx2α allele (Tpfllox/lox) to generate KISM22α-Cre-Tpfllox/lox (TP-SMKO) and KISM22α-Cre-Tpfllox/lox (Control) mice for experiments. To document elimination of TP receptors from various vascular beds, levels of expression for TP receptor mRNA were measured by real-time reverse-transcription polymerase chain reaction. Segments of aorta were isolated from TP-SMKO and control mice, and the adventitia and endothelium were removed by dissection and stripping. As shown in Figure S2, mRNA for the TP receptor was easily detected in aortas from control mice but not from TP-SMKOs (P<0.0005). Similarly, TP receptor mRNA expression in mesenteric arteries, with intact endothelium and adventitia, was decreased by 80% in TP-SMKO mice compared with controls (P=0.05; Figure S2), but TP mRNA levels were similar in RNA extracted from whole kidney. Thus, in TP-SMKOs, TP receptor mRNA expression was efficiently and specifically extinguished from vascular smooth muscle.

**Vascular Responses to Thromboxane Agonist Are Attenuated With Deletion of TP Receptors From VSMCs**

TxA₂ is a potent vasoconstrictor eicosanoid. To determine whether loss of TP receptors in VSMCs affected this response, we compared systemic vasoconstrictor responses in anesthetized TP-SMKOs and controls. As shown in Figure 1, we observed robust vasoconstriction in response to acute administration of the TP receptor agonist (100 μg/kg of U46619) in the controls. The acute vasoconstrictor response to 100 μg/kg of U46619 was reduced by ≈60% in TP-SMKOs (Figure 1A and 1B).

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**Figure 1.** Diminished response to acute thromboxane agonist U46619 but intact response to acute angiotensin II (Ang II) administration in vivo. Acute vasoconstrictor responses to U46619 and Ang II were measured in anesthetized mice. Blood pressure was measured continuously, whereas U46619 (100 μg/kg) and Ang II (0.1, 1, and 10 μg/kg) were administered at 5- to 10-minute intervals in separate experiments. A, A significant decrease in peak pressor response was seen with 100 μg/kg of U46619; n=7−10; **P<0.0005 in the KISM22α-Cre-Tpfllox/lox (TP-SMKO) vs controls. B, Representative tracings from control (black circles) and TP-SMKO (white circles) mice after receiving 100 μg/kg of U46619. Data are expressed as mean change in systolic blood pressure from baseline value starting 15 seconds before injection (data points represent average change in systolic blood pressure every 5 seconds). C, No difference was seen in peak pressor response to Ang II at any dose between TP-SMKO and control mice (P value not significant at 0.1, 1, and 10 μg/kg doses; n=7−10). Student unpaired t test was used to make comparisons.
Renal blood flow was measured in anesthetized mice. Reduction in mean renal blood flow was compared with baseline values. A. Renal blood flow was significantly diminished after U46619 administration in control mice; however, no change was observed in TP-SMKO mice. *P<0.05, 1, 100 µg/kg; **P<0.005, 10 µg/kg; ***P<0.0005, 1000 µg/kg; n=3 in each group. B. No difference was seen in renal blood flow response to Ang II at any dose between TP-SMKO and control mice both at baseline and during Ang II–induced hypertension (P value not significant at 0.03, 0.10, and 1.00 µg/kg doses; n=4–8). Student unpaired t test was used to make comparisons.

Absence of TP Receptors From VSMCs Protects Mice From Thromboxane-Induced Shock and Death

High levels of TP agonist in the bloodstream cause a characteristic syndrome of hemodynamic collapse and death associated with systemic platelet aggregation, pulmonary thrombosis, and coronary spasm. This syndrome can also be induced after administration of arachidonic acid. In both cases, complete absence of TP receptors is protective, indicating a critical role for TP receptors. Accordingly, we examined whether TP receptors in smooth muscle contribute to this response. After acute intravenous administration of 1000 µg/kg of U46619, 60% (6 of 10) of control mice experienced hemodynamic collapse and death within 5 minutes, whereas none (0 of 7) of the TP-SMKOs died (Figure 3B; P=0.0345 Fisher exact test). The hemodynamic responses to this higher dose of U46619 were also strikingly different. As shown in Figure 3A, there was a marked but transient drop in blood pressure (=50–60 mm Hg) immediately after U46619 administration in all of the controls. Within ∼50 seconds, blood pressures recovered to baseline but then fell inexorably in the nonsurviving control mice. In contrast, in the small group of control animals, the initial drop in blood pressure was followed by a sustained pressor response for >200 seconds. On the other hand, the marked reduction in blood pressure after U46619 was not observed in the TP-SMKOs. Instead, there was a transient, very modest (=10 mm Hg) increase in pressure that resolved within 50 seconds (Figure 3A).

Effects of Eliminating TP Receptors From VSMCs on Blood Pressure and the Development of Hypertension

To define the impact of TP receptors in the vasculature on control of blood pressure, radiotelemetry units were implanted into 8- to 12-week-old male TP-SMKO (n=8) and control (n=8) mice to measure intra-arterial pressure continuously in the conscious, unrestrained state. As shown in Figure 4, baseline blood pressures were similar in control and TP-SMKO mice (control mean arterial pressure 114±1 mm Hg versus TP-SMKO mean arterial pressure 111±1 mm Hg; P value not significant [NS]). With 1 week of feeding a low-sodium (<0.02% NaCl) diet, mean arterial pressure fell significantly (P<0.05) and to a similar extent in both groups (TP-SMKOs, 107±1 mm Hg; controls, 109±2 mm Hg; P=NS). Similarly, with a high-sodium (6% NaCl) diet, blood pressure increased (P<0.01) but was not different between groups (TP-SMKOs, 118±3 mm Hg; controls, 121±3 mm Hg; P=NS). This is
consistent with previous studies showing that elimination of TP receptors from all tissues does not affect baseline blood pressure.13

On the other hand, previous studies using gene deletion and pharmacological antagonists indicate a significant contribution of TP receptors to the pathogenesis of hypertension.3,14 To induce hypertension, osmotic minipumps were implanted subcutaneously to infuse Ang II at 1000 ng/kg per minute while blood pressures were continuously monitored. With Ang II infusion, blood pressures increased significantly in control and TP-SMKO mice compared with baseline (Figure 4A; \( P < 0.005 \) for both control and TP-SMKO versus baseline). However, at >3 weeks of Ang II infusion, mean blood pressures in the TP-SMKO mice were \( \approx 25\% \) lower than controls (Figure 4B; controls, 159±2 mm Hg; TP-SMKO, 145±8 mm Hg; \( P < 0.05 \)). Thus, TP receptors in VSMCs play a significant role in driving blood pressure elevation associated with Ang II–induced hypertension.

Reduced Urinary TxB2 Excretion With Hypertension in Mice Lacking TP Receptors in VSMCs

To determine whether vascular TP receptors affect thromboxane generation, we measured urinary TxB2 excretion at baseline and after 2 weeks of Ang II infusion in controls and TP-SMKOs. At baseline, urinary TxB2 excretion was similar between controls and TP-SMKOs (Figure 5; controls, 2509±346

![Figure 3. Protection from death after acute thromboxane agonist U46619 administration in vivo.](http://hyper.ahajournals.org/)

**Figure 3.** Protection from death after acute thromboxane agonist U46619 administration in vivo. Acute vasoconstrictor responses to higher dose U46619 was measured in anesthetized mice. Blood pressure was measured continuously, while 1000 μg/kg of U46619 was administered via jugular vein. **A,** Representative tracings from control mouse that died (black circles), control mouse that lived (half black and white circles), and KI\( \text{SM22α-Cre} \cdot Tp^{\text{floxed}} \) (TP-SMKO; white circles) mice after receiving 1000 μg/kg of U46619. Data are expressed as mean change in systolic blood pressure from baseline value starting 15 seconds before injection (data points represent average change in systolic BP every 5 seconds). **B,** After administration of the highest dose of U46619 (1000 μg/kg), TP-SMKO mice had complete protection from acute cardiovascular collapse and death as compared with control mice. \( P = 0.0345 \), Fisher exact test.

![Figure 4.](http://hyper.ahajournals.org/)

**Figure 4.** Thromboxane-prostanoid (TP) receptors in vascular smooth muscle cells promote angiotensin II (Ang II)–dependent hypertension. **A,** With infusion of Ang II (1000 ng/kg per minute), blood pressures (BPs) increased significantly in both control and KI\( \text{SM22α-Cre} \cdot Tp^{\text{floxed}} \) (TP-SMKO) mice (\( \ast \)\( P < 0.005 \) both TP-SMKOs and controls, baseline as compared to Ang II), but the hypertensive response to Ang II was significantly attenuated in the TP-SMKOs (\( \ast \)\( P < 0.05 \); \( n = 8 \) in both groups). **B,** The increase in mean arterial pressure (MAP) during the Ang II infusion was significantly less in the TP-SMKOs (white bars; 145.8±5.0 mm Hg) vs controls (black bars; 159.1±4.0 mm Hg; \( \ast \)\( P < 0.05 \)). Student unpaired t test was used to compare MAP at baseline to Ang II in both TP-SMKOs and controls, whereas Mann-Whitney U test was used to compare MAP after Ang II infusion in TP-SMKO to control mice.
Hypertensive End-Organ Damage Is Diminished in Mice Lacking TP Receptors in Smooth Muscle

Chronic infusion of Ang II causes hypertension and significant end-organ injury. To examine the contribution of TP receptors in VSMCs to this injury, we assessed cardiac hypertrophy and the extent of vascular remodeling in TP-SMKOs. Among the prostanoids, TxA2 has been implicated in a variety of cardiovascular diseases, including atherosclerosis, myocardial infarction, stroke, and hypertension. Activation of TP receptors by TxA2 induces broad actions that could contribute to cardiovascular pathogenesis including vasoconstriction, platelet activation, oxidative stress, and thromboxane production after angiotensin II (Ang II)–induced hypertension. Urinary thromboxane (TxB2) was similar at baseline between controls and KISM22α-Cre-Tpflx/flox (TP-SMKO; controls, 2509±346 pg/24 h; TP-SMKO, 2114±437 pg/24 h; P value not significant; n=10). Urinary TxB2 excretion significantly increased in controls after 2 weeks of Ang II infusion (5406±721 pg/24 h; P<0.05; n=8). However, TP-SMKO mice did not have an increase in urinary TxB2 excretion vs baseline TP-SMKO (3635±707 pg/24 h; n=8) and was significantly less than controls at 2 weeks of Ang II infusion (P<0.05). Student unpaired t test was used to make comparisons.

Figure 5. Thromboxane-prostanoid (TP) receptors in vascular smooth muscle cells promote vascular remodeling after angiotensin II (Ang II)–induced hypertension. Representative hematoxylin/eosin-stained sections taken at [times]40 of thoracic aortas from control (n=6; A and C) and KISM22α-Cre-Tpflx/flox (TP-SMKO; n=5, B and D) mice before (A and B) and after (C and D) 4 weeks of Ang II infusion (n=5 control mice; n=5 TP-SMKO mice). E. Aortic medial thickness was quantified using morphometry. There were no differences in medial thickness at baseline between the groups; medial thickness increased significantly and to a similar extent in control and TP-SMKO mice during Ang II infusion. (**P<0.0005 control baseline vs control Ang II; #P<0.01 TP-SMKO baseline vs TP-SMKO Ang II; *P<0.05 TP-SMKO Ang II vs control Ang II). Black bars, 50 μm.
In this study, we were interested in precisely defining the contribution of vascular actions of TP receptors to the regulation of blood pressure. To this end, we generated mice with cell-specific deletion of TP receptors in smooth muscle, including VSMCs of conduit and resistance vessels, using Cre/loxP technology. Acute vasoconstrictor responses to TP agonists were markedly diminished in these TP-SMKOs.

In the basal state, blood pressures in the TP-SMKOs were not different from controls. Likewise, there were no differences in blood pressure responses between TP-SMKOs and controls during high- or low-sodium feeding. This finding is consistent with previous studies showing that pharmacological antagonists of the TxA2–TP pathway11,12 or global deletion of the Tbxa2r gene11 do not affect baseline blood pressures. Moreover, this is a characteristic of the prostaglandin system in general, where the impact on blood pressure and hemodynamic functions is minimal in healthy individuals and only becomes apparent in the face of physiological stresses or disease.25

On the other hand, we and others have shown that production of thromboxane is enhanced in models of hypertension caused by Ang II infusion or administration of Nω-nitro-L-arginine methyl ester.14,18,29 Moreover, blockade of TP signaling either through pharmacological inhibition or complete deletion of the TP receptor gene reduces the severity of hypertension in these models.13,14 This suggests that activation of the TxA2–TP axis is a final common pathway contributing to hypertension of diverse etiologies. In these previous studies, the specific populations of TP receptors that are critical to the development of hypertension cannot be determined. Accordingly, we used our TP-SM KO mouse line to define the contribution of TP receptors in VSMCs to the development of hypertension. In Ang II–dependent hypertension, we find that the extent of blood pressure elevation is reduced by ≈25% in TP-SMKOs compared with controls, indicating a significant contribution of vascular TP receptors to the pathogenesis of hypertension.

There are ≥2 ways that TP receptors in VSMCs could affect vascular resistances in Ang II–dependent hypertension. The first is through direct effects of TP receptors to trigger signaling pathways linked to vasoconstriction. Alternatively, as suggested previously,30 TP receptors may affect vascular tone indirectly by modulating contractile responses mediated by Ang II type 1 receptors. Because the acute vasoconstrictor responses to Ang II were not affected in the TP-SMKOs either at baseline or after chronic infusion of Ang II, our studies indicate that the vascular actions of TP receptors in Ang II–dependent hypertension are direct, not involving modulation of activity or sensitivity of Ang II type 1 receptors.

Through its actions to control vascular tone, TP receptors may impact blood pressure either through effects on peripheral vascular resistance or by reducing renal blood flow and thereby amplifying sodium and fluid reabsorption by the kidney. Because we found equivalent attenuation of vasoconstriction to TP agonist in the systemic and renal vasculatures of TP-SMKOs, our studies cannot distinguish the relative importance of TP effects on peripheral versus renal hemodynamics in the pathogenesis of hypertension. Although measurement of renal blood flow with flow probe in anesthetized mice is more precise at measuring changes in blood flow than in establishing a baseline measurement, we nonetheless compared the baseline renal blood flow between the groups after 2 weeks of Ang II infusion. Although these values were numerically higher in the TP-SMKOs (6.4±0.8 versus 5.5±0.3 mL/min per 100 g of body weight), this difference did not achieve statistical significance perhaps related to the imprecision of the flow probe measurement. Alternatively, there may be differences in regional blood flow to the cortex or medulla that would not be detected by this technique.

Previous studies have suggested that activation of TP receptors in the vasculature may stimulate further production of thromboxane production in a feed-forward mechanism.31 To determine whether TP receptors might participate in this pathway, we measured urinary excretion of TxB2 in controls and TP-SMKOs. Although there was no difference at baseline, urinary thromboxane excretion failed to increase in the TP-SMKOs, indicating that TP receptors in VSMCs promote thromboxane generation in Ang II–dependent hypertension. Moreover, although platelets and various inflammatory cells are capable of generating substantial quantities of thromboxane, our data suggest that a TP receptor–driven pathway in VSMCs is the predominant source of thromboxane in this setting. These findings are consistent with the previous study by Rocca et al31 showing increased urinary TxB2 excretion in mice with more than expression of TP receptors in VSMCs. Furthermore, the attenuation of thromboxane generation in TP-SMKOs during Ang II infusion likely contributes to the reduced severity of hypertension.

Along with sustained increases in blood pressure, chronic Ang II infusion also causes end-organ damage resembling that seen in humans with hypertension, including left ventricular hypertrophy and pathological vascular remodeling. Indeed, both the TP-SMKOs and controls developed robust cardiac hypertrophy, but the degree of hypertrophy was similar between the groups. Vascular remodeling, assessed by the development of medial expansion in the descending thoracic aorta, was also observed in both groups. However, the extent of remodeling was much more marked in the controls where medial thickness increased by ≈84% compared with only ≈30% in the TP-SMKOs. This difference far exceeds the relatively modest differences in blood pressure, suggesting that direct actions of TP receptors in VSMCs contribute to vascular remodeling above and beyond effects on blood pressure. This finding is consistent with other studies indicating significant contributions of direct actions of TP receptors in VSMCs to vascular remodeling, above and beyond the effects of blood pressure.32 Yet, in data not shown from isolated aortic segments, we found no differences in mRNA expression for several potential mediators of VSMC hypertrophy, including plasminogen activator inhibitor 1, interleukin 6, platelet-derived growth factor A, chemokine ligand 2 (monocyte chemotactic protein-1), chemokine ligand 5, and transforming growth factor β. Accordingly, lower blood pressure may have a predominant effect.

Rapid cardiovascular collapse after the acute administration of either arachidonic acid or TP receptor agonists has
been a consistent finding in various animal models.\textsuperscript{13,19,33} Whatever the precise mechanism, TP receptors are absolutely required, because mice with global deficiency of TP receptors are completely protected.\textsuperscript{32} The concentration of U46619 used in these studies exceeds those of TxA\textsubscript{2} metabolites typically found in the circulation. However, these concentrations are required to achieve measurable, TP-dependent vascular responses in vivo. Our current studies indicate that it is the population of TP receptors on smooth muscle that are critically important mediators of this response, because TP-SMKOs are also protected from hypotension and death. Although coronary thrombosis has been suggested to be a key feature of this syndrome, our data indicate that TP activation in platelets is not sufficient to trigger the response, because platelet TP receptors are preserved in the TP-SMKOs. These findings are in agreement with previous work by Pfister et al.,\textsuperscript{19} who found that a subset of New Zealand white rabbits with reduced numbers of TP receptors in aortic VSMCs but normal levels of TP receptors in platelets were protected from TP agonist-induced death.

In control mice given high doses of TP agonist, there was an immediate drop in blood pressure in the range of 50 to 60 mm Hg (Figure 3). We speculate that this may reflect transient cardiac ischemia and dysfunction resulting from coronary artery vasoconstriction and that more severe injury may occur in the subgroup of control mice with progressive deterioration of hemodynamic function and death. Alternatively, this could conceivably be a consequence of acute respiratory impairment attributed to profound bronchoconstriction caused by the TP agonist. Because TP-SMKOs also lack TP receptors on bronchial smooth muscle,\textsuperscript{34} such a response would also be abrogated in the TP-SMKOs.

**Perspectives**

Enhanced activation of the TP receptor is involved in the pathogenesis of hypertension from diverse causes. Here we show that TP receptors in VSMCs are primarily responsible for this effect by altering systemic and renal vascular responses to TP receptor agonists and by augmenting urinary thromboxane production. Along with their contribution to elevated blood pressure, TP receptors in VSMCs may have direct actions to promote vascular remodeling of the aorta. These findings suggest that blockade of TP receptor signaling in VSMCs should be useful for reducing blood pressure and preventing pathological remodeling of the vasculature in hypertension.

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**Disclosures**

None.

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**Novelty and Significance**

**What Is New?**

- We show that smooth muscle TP receptors play an important role in Ang II–induced hypertension and subsequent vascular remodeling. This augmentation of blood pressure is associated with decreased urinary thromboxane production and is independent of acute peripheral and renal vasoconstrictor responses to Ang II. We also show that vascular smooth muscle TP receptors are responsible for cardiovascular collapse secondary to TP agonists.

**What Is Relevant?**

- This is relevant because the pathogenesis of hypertension is not completely elucidated. Finding novel therapies to treat hypertension are greatly needed. Targeting smooth muscle TP receptors could provide novel therapies for hypertension.

**Summary**

- TP receptors in VSMCs play a role in hypertensive response to chronic administration of Ang II in mice. Vascular smooth muscle–specific TP receptor deletion alters both the systemic and renal vascular responses to TP receptor agonists and augments urinary thromboxane production after Ang II–induced hypertension. Along with their contribution to elevated blood pressure, TP receptors in VSMCs may have direct actions to promote vascular remodeling of the aorta. Lastly, VSMC TP receptors appear to mediate the acute hemodynamic response and sudden death associated with TP receptor agonism. These findings suggest that blockade of TP receptor signaling in VSMCs should be useful for reducing blood pressure and preventing pathological remodeling of the vasculature in hypertension and might be useful in treating shock.
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THROMBOXANE RECEPTORS IN SMOOTH MUSCLE PROMOTE

HYPERTENSION, VASCULAR REMODELING AND SUDDEN DEATH

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SUPPLEMENT MATERIALS AND METHODS

**Generation of experimental animals.** KI Sm22α-Cre mice were purchased from the Jackson Laboratory (stock number 006878, Bar Harbor, ME). These mice were intercrossed with a conditional Tbxα2 allele mouse line that were generated using homologous recombination in embryonic stem cells as previously described. Mice were maintained on a mixed C57Bl/6 and 129/SvEv background. The membrane-tomato/membrane-green (mT/mG) reporter mice were purchased from the Jackson Laboratory (stock number 006767, Bar Harbor, ME). Mice were bred and maintained in the AAALAC-accredited animal facilities at the Durham VA Medical Center according to NIH guidelines. All of the animal studies were approved by the Duke University and Durham Veterans' Affairs Medical Center Institutional Animal Care and Use Committee and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animals had free access to standard rodent chow and water unless specified. 8-12 week-old male mice KI Sm22α-Cre^+^ TP^{flox/flox} (TP-SMKO) and littermate KI Sm22α-Cre^-^ TP^{flox/flox} (Controls) were used for experiments.

**Measurement of TP-R mRNA levels and RT-PCR.** Relative levels of mRNA for the TP-receptor in various tissues were determined by real time RT-PCR with the ABI Prism 7700 sequence detection system as described using Taq-Man reagents. For each experimental sample, the amounts of target and endogenous control were determined by the ΔΔCT method. PCR probes were obtained from Applied Biosystems (TP receptor- Mm00436917_m1 and GAPDH- 4308313, Carlsbad, CA).

**Urinary Excretion TxB2 Metabolites.** Twenty-four-hour urine samples were collected by metabolic cage at baseline and after 2 weeks of Ang II infusion. Urine samples were centrifuged briefly to remove particulate matter and then immediately aliquoted and frozen at −80°C until assay. Stable thromboxane (Tx)B2 (TxB2) in urine was measured using a specific competitive enzyme immunoassay (Cayman Chemical, Ann Arbor, MI).

**Measurement of medial thickness of aortae.** The extent of vascular remodeling was assessed by measuring medial thickness of descending thoracic aorta. 2 cm of descending aorta was dissected and placed in 10% formalin overnight. 10µm sections were obtained after paraffin embedding. Sections were stained with hematoxylin and eosin then photographs were taken at 40X (Zeiss Axio Imager, QImaging MicroPublisher 5.0 MP colour camera). Medial thickness of the descending aorta using 4 random sections throughout the specimen was quantified using MetaMorph (Molecular Devises, Sunnyvale, CA) in a blinded fashion.

**Assessment of acute vasoconstrictor responses.** We examined acute pressor responses to Ang II (Sigma Aldrich, Saint Louis, MO) and the TxA2 receptor agonist U46619 (Caymen Chemicals, Ann Arbor, MI) in mice anesthetized with isoflurane. A catheter (PE-50) was inserted into the left jugular vein for the administration of basal fluids and vasoconstrictors. A second catheter (Millar Mikro-Tip 1.4F, Houston TX) was placed in the carotid artery. Intra-arterial blood pressure was recorded continuously through the right carotid catheter using the PowerLab data acquisition system and LabChart software (ADInstruments, Colorado Springs, CO). At 5-min intervals, increasing doses (0.1, 1 and 10 μg/kg) of Ang II or (100 μg/kg and 1000 μg/kg) of U46619 were injected intravenously whilst intra-arterial pressures were continuously monitored.

**Blood pressure measurements in conscious mice.** Blood pressures were measured continuously in 8-12 week-old male conscious TP-SMKO (n=8) and control (n=8) mice using radiotelemetry as described previously. Ang II was infused chronically (1000ng/kg/min) by an osmotic mini-pump (Alzet, Cupertino, CA).

**Assessment of renal blood flow.** We examined acute renal blood flow responses to Ang II and U46619 in mice anesthetized with isoflurane. A catheter (PE-50) was inserted into the left jugular vein for the administration of basal fluids and vasoconstrictors. A small incision was made on the right
flank to expose the kidney and a non-cannulating ultrasonic flowmeter (Transonic Systems Inc., Ithaca, NY) interfaced with a 5 mm V-shaped probe was placed around the right renal artery. Mice were allowed to stabilize for 30-min. before measurements were started. Vascular reactivity to increasing doses of Ang II (0.3, 0.1, 0.3 and 1 µg/kg) or U46619 (1, 10, 100 and 1000 μg/kg) were injected intravenously while renal blood flow is continuously monitored. Data were expressed as percent change in renal blood flow from baseline value determined just before injection.

**Statistical analysis**- The values for each parameter within a group are expressed as the mean ± the standard error of the mean (SEM). For comparisons between groups with normally distributed data, statistical significance was assessed using ANOVA followed by unpaired t-test adjusted for multiple comparisons. For comparisons between groups with non-normally distributed data, the Mann Whitney U test was employed. For comparisons within groups, normally distributed variables were analyzed by a paired t-test, whereas non-normally distributed variables were analyzed by the Wilcoxon signed rank test. Normality was determined using the Shapiro-Wilk W test.

**REFERENCES**


Supplemental Figure S1. Verification of smooth muscle specific Cre recombinase expression. Specific expression of the KI-SM22α-Cre transgene was verified by inter-crossing with mTmG reporter mice. No GFP expression was seen in the smooth muscle layer of aorta (A) and small vessels of the kidney (B) from Cre- mTmG+ mice. However, specific GFP expression was seen in medial layer of the aorta (C) and along the afferent arteriole of the kidney (D) of Cre+ mTmG+ mice.

Supplemental Figure S2. The TP receptor is deleted from conduit vessels. Real-time RT-PCR performed on aorta stripped of endothelium and adventitia arteries, showed that mRNA for the TP receptor was easily detected from control mice (black bars) but not from aorta in TP-SMKO mice (n=4; *P<0.0005). Similarly, TP receptor mRNA expression was decreased in the mesenteric artery of TP-SMKO mice (white bars; n=4; #P=0.05 vs control). No difference was seen in TP receptor expression in whole kidney between TP-SMKO and control mice (n=4; P=NS). Students unpaired T test was used to make comparisons. Each experimental (TP-SMKO) tissue sample (aorta, mesenteric artery and kidney) was compared to its own control tissue sample respectively.