Role for Thromboxane Receptors in Angiotensin-II–Induced Hypertension

Helene Francois, Krairerk Athirakul, Lan Mao, Howard Rockman, Thomas M. Coffman

Abstract—To evaluate the role of thromboxane in hypertension and its complications, we studied mice with targeted disruption of the TXA2 receptor gene in an angiotensin-II–dependent model of hypertension. To determine whether genetic background might alter the physiological actions of the TP receptor, we studied two lines of TP knockout (Tp−/−) mice with distinct genetic backgrounds (C57BL/6 and BALB/c). During chronic angiotensin II infusion (1000 ng/kg per minute × 28 days by subcutaneous osmotic pump), TP deficiency prevented mortality in the C57BL/6 background but not in the BALB/c strain. Chronic angiotensin II infusion also caused a rapid and significant increase in blood pressure in wild-type (WT) C57BL/6 and BALB/c animals, which was significantly attenuated in Tp−/− mice on either background. After 28 days of infusion, cardiac hypertrophy only occurred in the C57BL/6 strain: heart/body weight ratio increased by 57% ± 8% in WT mice compared with 17% ± 6.5% for the Tp−/− mice (P<0.01). Chronic angiotensin II infusion caused albuminuria only in the C57BL/6 strain, and TP deficiency did not alter its development. Cyclooxygenase-1 knockout mice also had attenuated blood pressure increase during chronic angiotensin II infusion, suggesting that cyclooxygenase-1 metabolites are involved in angiotensin-II–dependent hypertension. Thus, on the C57BL/6 background, TP receptors contribute to cardiac hypertrophy but not proteinuria. However, irrespective of genetic background, the TP receptor makes a robust contribution to the pathogenesis of angiotensin II-dependent hypertension. (Hypertension. 2004;43[part 2]:364-369.)

Key Words: thromboxane ■ hypertension ■ angiotensin II ■ hypertrophy ■ proteinuria ■ cyclooxygenase

Thromboxane A2 (TxA2) is produced by the metabolism of arachidonic acid through the cyclooxygenase-1 (COX1) and cyclooxygenase-2 pathway. TxA2 is a potent vasoconstrictor and platelet aggregate.1 In addition, TxA2 regulates renal hemodynamics and sodium handling.2–4 Based on its biological actions, TxA2 has been implicated in the pathogenesis of cardiovascular diseases including ischemic heart disease,5 atherosclerosis,6 and eclampsia,7 although the precise COX isoform mediating these actions is still unclear. Interactions between TxA2 and the renin-angiotensin system have also been established. For example, angiotensin II (Ang II) stimulates TxA2 synthesis in vascular and renal tissues.8,9 Moreover, there is evidence for common actions of Ang II and TxA2 to promote systemic and renal vasoconstriction, sodium handling,10 and vascular smooth muscle cell proliferation.11 These interactions suggest a potential contribution of TxA2 acting through the thromboxane A2 (TP) receptor to the pathogenesis of hypertension and its complications. Here, using a genetic approach, we examine the role of TP receptors and the COX1 pathway in a model of Ang-II–dependent hypertension and its cardiac and renal complications.

Methods

Establishment of the TP Receptor and COX1 Knockout Mice Lines

The thromboxane A2 (TP) knockout (Tp−/−) mice were generated as previously described.12 The TP mutation was backcrossed onto two different inbred genetic backgrounds for more than 12 generations using C57BL/6 and BALB/c mice that were purchased from the Jackson Laboratory (Bar Harbor, ME). The cyclooxygenase-1 (COX1) knockout mice (Cox1−/−) mice were generated as previously described.13 The genetic background of these mice consists of a random mix 129/SvEv and C57BL/6. Matched Cox1−/− littermates generated from heterozygous crosses were used as controls.

Maintenance of Mice

Three to 4-month-old male Tp−/−, wild-type (WT) mice, Cox1−/− and matched littermates were used for experiments. Animals were maintained in the Durham VA medical center facility and were fed normal diet (0.4% NaCl) and provided water ad libitum. The experimental procedures described were approved by the animal research committees of the Durham VA and Duke University Medical Centers.

Model of Ang II–Dependent Hypertension

Ang II (1000 ng/kg per minute; Sigma chemical; n=6 animals per group) dissolved in sterile saline or sterile saline alone for control
animals (n=6 in each group) was infused using an Osmotic Minipump (Alzet model 2004; Alza Corp) that was inserted subcutaneously during an isoflurane anesthesia (3%).

**Physiological Studies**

**Systolic Blood Pressure and Heart Rate**

Systolic blood pressure (SBP) and heart rate (HR) were determined in conscious mice by the noninvasive computerized tail-cuff method after 2 weeks of daily training. This method was validated previously and correlates well with direct measurements of intraarterial pressure.\(^1^4\) Data were recorded at baseline for 1 week. After initiation of the infusion, additional measurements were performed after 7, 14, 21, and 28 days for the TP\(^{-/-}\) mice and WT animals. Three sets of 10 measurements were recorded for each day. Measurements with SD>20 mm Hg for SBP were not accepted. For the 28 days of infusion.

**Transthoracic Echocardiography**

After 28 days of Ang II infusion, transthoracic echocardiography was performed in conscious mice. Two-dimensional-guided M-mode echocardiography was performed using an HDI 5000 echocardiograph (ATL, Bothell, Wash). The following parameters were measured: left ventricular end-diastolic dimension (LVEDD); left ventricular end-systolic dimension (LVESD); fractional shortening (FS), and the average of three beats was recorded for each parameter. Transthoracic echocardiograms was blinded to the genotype of the animals. All measurements were made manually, and the average of three beats was recorded for each parameter.

**Urinary Albumin Excretion**

Urinary albumin excretion was quantified by the albuminuria/creatinuria (A/C) ratio. Mice were individually housed in specially designed metabolic cages with free access to tap water, which accommodate individual mice. The 24-hour urine sample was collected before and 14 and 28 days after Ang II infusion. Urine was clarified by 15 minutes of centrifugation (14 000g) and then stored at −80°C. Albuminuria was determined by ELISA (Albuwell; Exocell Inc.) and creatinuria was measured using the alkaline picrate method. The same dilution of urine was used for both methods (1:20) on the same day.

**Statistical Analysis**

Data are expressed as mean±SEM. Paired and unpaired data were analyzed with Student t test. ANOVA was used to detect differences related to the strain and the genotype and was followed by unpaired Student t test. A two-way ANOVA followed by a post hoc least significant difference analysis was applied to test for interactions between genotype and Ang II responses. For the survival analysis, \(\chi^2\) test was used to compare the mortality rate within the strains.

**Results**

**Baseline HR and SBP**

Because our long-term interest is understanding the role of TP receptors in cardiovascular disease in humans who are genetically heterogeneous, we wished to determine whether and how TP physiology might be affected by genetic background. We used two common mouse strains for these studies: C57BL/6 and BALB/c. The C57BL/6 strain has been previously shown to have a robust hypertensive response to chronic Ang II infusion,\(^1\) whereas resting HRs are reduced in BALB/c mice.\(^1\) At baseline, there was no difference in SBP or HR between TP\(^{-/-}\) and WT mice on either background (data not shown). In addition, there was no difference in SBP between the 2 strains of mice (115±2 versus 114±2 mm Hg for C57BL/6 and BALB/c mice, respectively; \(P=\)nonsignificant).

However, the BALB/c mice had a significantly lower HR than the C57BL/6 mice, irrespective of the TP genotype (526±15 versus 646±13 bpm, BALB/c versus C57BL/6, respectively; \(P<0.001\)).

**Survival**

On both inbred backgrounds, chronic Ang II was associated with significant mortality in WT mice (40% versus 39% for the BALB/c and the C57BL/6, respectively) (Table). The cause of death could not be determined with certainty, but severe hypertensive responses and/or strokes were observed in the C57BL/6 mice. Decompensated heart failure developed in BALB/c mouse. Most of the animals died after the second week of Ang II infusion (data not shown). On the C57BL/6 background, TP deficiency completely prevented mortality (0% versus 39%, TP\(^{-/-}\) and WT, respectively; \(P<0.05\)). In contrast, the absence of TP receptors had no effect on mortality on the BALB/c background (50% versus 40%; nonsignificant).

**Ang-II–Dependent Hypertension**

Chronic Ang II infusion induced a rapid and sustained increase in SBP in the WT C57BL/6 animals (\(P<0.001\)) (Figure 1A and 1B). The elevation in SBP during Ang II infusion was associated with a decrease in HR that was also sustained for the entire experiment: from 537±21 (D0) to 502±23 bpm (D28) for the WT (\(P<0.001\)) and from 643±10 (D0) to 527±15 (D14) and 508±27 bpm (D28) for the TP\(^{-/-}\) mice (\(P<0.001\) and paired t test). On the C57BL/6 background, Ang-II–dependent hypertension was significantly blunted in TP\(^{-/-}\) mice. SBP was significantly lower by 30 mm Hg on day 7 (\(P<0.001\)) and by 20 mm Hg until day 28 (\(P<0.05\)). Consistent with a hypertension-induced baroreflex, the HR of the C57BL/6 TP\(^{-/-}\) mice was significantly higher than that of the WT animals on day 7, when the greatest difference between the SBP of the 2 genotypes was observed (617±13 versus 528±18 bpm for the TP\(^{-/-}\) and the WT C57BL/6, respectively; \(P<0.001\)). The significant difference in mortality between the groups may have attenuated the difference in SBP, because death was observed in mice manifesting the most severe hypertension.

Chronic Ang II infusion also caused a robust hypertension in WT BALB/c mice (\(P<0.001\)). However, the magnitude of

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\(^*P<0.05\) vs C57BL/6 WT Ang II.
We have relied on tail-cuff manometry as our primary method for BP measurement. Although the reliability of this method has been questioned by some, published studies have repeatedly validated its correlation with direct measurements of intra-arterial pressure. In our experience, the major problem with the tail-cuff method is sensitivity and ability to discriminate small differences in BP. This was not an issue in these studies.

**Cardiac Parameters**

In comparing WT and Tp<sup>-/-</sup> on the 2 different genetic backgrounds, we found no differences in cardiac function assessed by FS in the saline-infused animals (data not shown) and after 28 days of Ang II infusion (0.66±0.06 versus 0.66±0.05, C57BL/6 Tp<sup>-/-</sup> versus WT mice, respectively; P=nonsignificant; and 0.52±0.07 versus 0.56±0.05, BALB/c Tp<sup>-/-</sup> versus WT mice, respectively; P=nonsignificant) (Figure 2). We did not observe differences in cardiac chamber dimensions as measured by LVEDD and LVEDS, either (data not shown). All these echocardiographic parameters were not modified during Ang II infusion (data not shown). Thus, the lower SBP observed in the knockout animals was not caused by decreased cardiac function during chronic Ang II infusion. However, between the 2 strains, the FS was significantly lower in the BALB/c animals compared with the C57BL/6 (0.56±0.02 versus 0.66±0.01 for the BALB/c and the C57BL/6, respectively; P<0.01). LVEDD was also significantly larger in BALB/c versus C57BL/6 mice (3.7±0.1 versus 3.15±0.08 mm, BALB/c versus C57BL/6 mice, respectively; P<0.001).

Despite the lack of effect of Ang II on echocardiographic parameters, heart/body weight (H/B) ratio only increased significantly in the WT Ang II C57BL/6 animals compared with saline-infused WT C57BL/6 animals (H/B=4.75±0.21 versus 7.49±0.41 mg/g, n=6 saline WT mice versus n=11 Ang II WT mice, respectively; P<0.01). The development of cardiac hypertrophy was significantly ameliorated in the C57BL/6 Tp<sup>-/-</sup> mice compared with WT animals.
AT1 receptors in the kidney plays a key role in the generation (56% versus 130%/H11006 lower in the BALB/c mice than in the C57BL/6 animals after 28 days of Ang II infusion, the A/C ratio was significantly resistant to the albuminuric effects of Ang II infusion. After 28 days of Ang II infusion. In contrast, BALB/c mice were resistant to the albuminuric effects of Ang II infusion. After 28 days of Ang II infusion, the A/C ratio was significantly lower in the BALB/c mice than in the C57BL/6 animals (56±9 versus 130±23 µg/g; P<0.01, unpaired t test).

Role for COX1 in Ang-II–Dependent Hypertension

TXA2 is produced by the metabolism of arachidonic acid via the COX pathway. Of the two COX isoforms, COX1 has been linked to generation of TxA2 in platelets. To examine the role of COX1 in Ang-II–dependent hypertension, we compared BP responses in Cox1+/+ and Cox1−/− mice during chronic infusion of Ang II. In the Cox1+/+ mice, chronic Ang II infusion caused a significant increase in SBP similar to that seen in the other experiments with WT animals (from 104±1 to 162±9, P<0.01). Compared with the WT controls, this increase in SBP with Ang II was significantly blunted in the Cox1−/− mice (139±4 versus 162±9 for Cox1−/− and WT, respectively; P<0.05). The magnitude of BP reduction in the Cox1−/− mice (approximately 20 mm Hg) was similar to that seen in the Tp−/− mice.

Discussion

Activation of the renin-angiotensin system has a number of physiological effects that work in concert to increase BP. Most of these effects are mediated by activation of AT1, angiotensin receptors by Ang II. In particular, stimulation of AT1 receptors in the kidney plays a key role in the generation of hypertension by promoting retention of sodium and impairing pressure natriuresis. Along with its direct effects, Ang II also activates other hormonal mediators that contribute to the pathogenesis of hypertension including aldosterone and endothelin. Ang II also stimulates the generation of prostanoids, lipid mediators that are synthesized by the cyclooxygenase metabolism of arachidonic acid. Previous studies have suggested that prostanoids modulate the hemodynamic actions of angiotensin II.

Among the prostanoids, TxA2 is a potent vasoconstrictor that promotes platelet aggregation. We used mouse lines with targeted disruption of the TP receptor to examine the role of TxA2 in Ang-II–dependent hypertension on 2 different inbred backgrounds. In the basal state, the Tp−/− mice on 2 different inbred genetic backgrounds have a normal cardiovascular phenotype and normal BPs, consistent with previous studies suggesting that TxA2 does not play a role in BP regulation when the renin-angiotensin system is not
activated.\textsuperscript{22} Although their BPs were similar, we found significant differences in certain cardiovascular parameters between the WT BALB/c and the C57BL/6 mice. HRs were reduced in the BALB/c mice by almost 20%. This lower HR in the BALB/c strain was associated with larger left ventricular diameters and lower FS. These findings provide another illustration of the variable cardiovascular characteristics of common inbred strains of laboratory mice. These strain differences at baseline were not affected by the \textit{Tp} mutation.

We used chronic Ang II infusion as a model of Ang-II–dependent hypertension. This model has high levels of circulating Ang II that mimics two-kidney one-clip hypertension.\textsuperscript{23} Ang II infusion caused significant hypertension in WT mice on both backgrounds, but the magnitude of BP elevation was significantly higher in the C57BL/6 animals. Although there was significant mortality in WT mice on both backgrounds, it was our impression that the causes of death may have been somewhat different between the two strains. In the C57BL/6 animals, death was often preceded by marked elevation in BP and partial paralysis suggestive of stroke. In contrast, dilated cardiomyopathy and florid heart failure with Ang II infusion appeared to develop in at least 1 of the BALB/c mice. Perhaps consistent with these differing cardiovascular responses to Ang II, the absence of TP receptors completely prevented mortality on the C57BL/6 but had no effect on mortality rate on the BALB/c background. The genetic mechanism for differences in TP receptor physiology between these strains is not clear. One possible explanation that would be important to identify might be differences in the pattern or levels of TP receptor expression in key target organs.

On both genetic backgrounds, the absence of TP receptors significantly ameliorated the development of hypertension. On average, BPs were approximately 20 mm Hg lower in Ang-II–infused \textit{Tp}\textsuperscript{−/−} mice compared with \textit{Tp}\textsuperscript{+/+} controls, and the magnitude of BP lowering was similar on C57BL/6 and BALB/c backgrounds. These data are consistent with previous studies showing that TxA2 synthase inhibitors lower BP in Ang-II–induced hypertension\textsuperscript{22} or in two-kidney, one-clip hypertension.\textsuperscript{24} However, interpretation of studies using TxA2 synthase inhibitors is problematic because when TxA2 synthase is inhibited, prostaglandin endoperoxides such as PGH\textsubscript{2} may accumulate. These compounds can act as agonists at the TP receptor and thus attenuate efficacy.\textsuperscript{25,26} Moreover, accumulated PGH\textsubscript{2} may be used as substrate for synthesis of other prostanoids. This shunting of endoperoxide substrate can increase synthesis of other prostanoids, such as PGE\textsubscript{2} or PGI\textsubscript{2}, that are vasodilators.\textsuperscript{27} Our study emphasizes not only a role for TxA2 through the TP receptor in Ang-II–induced hypertension but also a role for other TP ligands such as PGH\textsubscript{2} and isoprostane.

It has been suggested that TxA2 can have positive inotropic effects.\textsuperscript{28} Therefore, echocardiograms were performed in conscious mice to determine whether the lower BP in the \textit{Tp}\textsuperscript{−/−} animals might be caused by altered cardiac responses during chronic Ang II infusion. However, echocardiographic findings were virtually identical in \textit{Tp}\textsuperscript{+/+} and \textit{Tp}\textsuperscript{−/−} animals on both backgrounds. Furthermore, within the groups, Ang II infusion did not alter these findings compared with baseline.

Taken together, our findings clearly show that activation of TP receptors makes a significant contribution to BP elevation in Ang-II–dependent hypertension. This action of TP receptors is robust and easily detected across 2 different genetic backgrounds. We also tested whether TP receptors also contribute to end-organ injury in this model. As discussed, Ang II infusion did not significantly alter echocardiographic findings. Significant cardiac hypertrophy developed with Ang II infusion in WT C57BL/6 mice. This effect was blunted in the C57BL/6 TP-deficient mice. TP receptors are expressed in cardiac myocytes and receptor density may increase in pathological conditions, suggesting the possibility that direct cellular actions of TP receptors might promote cardiac hypertrophy.\textsuperscript{29} Yet we found a strong positive correlation between the heart weight and BP pressure, suggesting that the effect of TP receptors to promote cardiac hypertrophy is likely caused by their actions to increase BP. In contrast, hypertrophy with Ang II infusion did not develop in BALB/c mice, illustrating another significant difference in cardiovascular responses between these strains.

There were also strain differences in the susceptibility to kidney injury in this model. The BALB/c mice were resistant to the development of proteinuria, whereas significant proteinuria developed after 28 days of Ang II infusion in the C57BL/6 strain. However, levels of proteinuria were not different between the C57BL/6 \textit{Tp}\textsuperscript{+/+} and \textit{Tp}\textsuperscript{−/−} groups indicating that, unlike the situation with cardiac hypertrophy, TP receptor activation does not play a significant role in proteinuria in this model. Moreover, BPs were significantly lower in the C57BL/6 \textit{Tp}\textsuperscript{−/−} compared with the \textit{Tp}\textsuperscript{+/+} mice, yet the magnitude of their proteinuria was similar. This suggests that the effects of Ang II to cause proteinuria in this model do not depend directly on BP and may instead be driven by direct cellular actions of Ang II in the kidney.

Previous studies have established the actions of Ang II infusion to promote the synthesis of various eicosanoids, especially TxA2 and PGH\textsubscript{2}, in vascular and renal tissues.\textsuperscript{8,9} Our findings suggest that chronic Ang II induces the synthesis of TxA2 or another TP receptor ligand, such as PGH\textsubscript{2} or isoprostanes, contributing\textsuperscript{30,31} to the pathogenesis of hypertension and end-organ injury. Because COX1 is the predominant COX isoform in platelets and is the major source of COX1-deficiency on Ang II hypertension. Similar to the \textit{Tp}\textsuperscript{+/−} animals, we observed significant abrogation of hypertension in \textit{Cox1}\textsuperscript{−/−} mice. However, because thromboxane production was not measured in these animals, we cannot be certain that the beneficial effect of COX1-deficiency was caused by reduced generation of TXA2. Nonetheless, we speculate that COX1-derived prostanoids may promote vasoconstriction and sodium retention in this model. Moreover, this finding is consistent with previous studies indicating a role for COX1 in acute vascular responses to Ang II.\textsuperscript{32}

Perspectives

Using a genetic approach, we demonstrated a major role for the TP receptor pathway in Ang-II–induced hypertension. This effect is robust and independent of genetic background. On susceptible backgrounds, TP receptor-deficiency pre-
vented mortality and Ang II-induced cardiac hypertrophy but did not alter the development of proteinuria. COX1-deficiency had a very similar effect to attenuate Ang-II-induced hypertension, suggesting the possibility that TxA2 is derived from COX1 in this model. Accordingly, reducing TxA2 synthesis with TxA2 synthase inhibitors or even low-dose aspirin or blocking TP receptor signaling may be useful in preventing Ang-II–dependent hypertension and associated left ventricular hypertrophy.

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