Prostacyclin protects against elevated blood pressure and cardiac fibrosis

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Summary

Specific inhibitors of COX-2 have been associated with increased risk for cardiovascular complications. These agents reduce prostacyclin (PGI2) without affecting production of thromboxane (Tx) A2. While this abnormal pattern of eicosanoid generation has been implicated in the development of vascular disease associated with COX-2 inhibition, its role in the development of hypertension, the most common cardiovascular complication associated with COX-2 inhibition, is not known. We report here that mice lacking the receptor for PGI2 (IPKOs) develop salt-sensitive hypertension, cardiac hypertrophy, and severe cardiac fibrosis. Coincidental deletion of the TxA2 (TP) receptor does not prevent the development of hypertension, but cardiac hypertrophy is ameliorated and fibrosis is prevented in IPTP double knockouts (DKOs). Thus, deletion of the IP receptor removes a constraint revealing adverse cardiovascular consequences of TxA2. Our data suggest that adjuvant therapy that blocks unrestrained Tx actions might protect against end-organ damage without affecting blood pressure in patients taking COX-2 inhibitors.

Introduction

In the APPROVe study, an increased rate of cardiovascular events in patients treated with the specific COX-2 inhibitor rofecoxib led to its withdrawal from the market (FitzGerald, 2004; http://www.vioxx.com). Although the results leading to withdrawal of rofecoxib were unequivocal, they involved a relatively small number of individuals—an excess of just 20 events in a trial of 2,600 subjects. Like traditional nonsteroidal anti-inflammatory drugs (NSAIDs), which inhibit coincidentally both COX-1 and COX-2, selective inhibitors of COX-2 elevate blood pressure (Aw et al., 2005). However, experiments in mice suggest that the propensity to elevate blood pressure may relate not just to inhibition of COX-2 but also to the selectivity with which it is attained (Athirakul et al., 2001; Qi et al., 2002). Hypertension was detected early on in the trial of rofecoxib, and the development of excess cardiovascular events was not observed until 18 months into the trial. While the cardiovascular consequences of even small increases in blood pressure have been clearly documented (Chobanian et al., 2003), a direct relationship with hypertension and cardiovascular outcomes associated with COX-2 inhibitors has yet to be established. Moreover, the precise prostanoid pathways responsible for blood-pressure elevations caused by COX-2 inhibitors are not clear.

In humans, COX-2 inhibitors reduce prostacyclin levels without affecting generation of thromboxane (Tx) A2 (McAdam et al., 1999), which in the basal state appears to depend primarily on COX-1 pathways in platelets. This altered profile of eicosanoid synthesis may be a cardiovascular hazard, and experiments in mouse models suggest that reduced prostacyclin (PGI2) signaling when Tx actions are preserved promotes atherogenesis (Egan et al., 2004) and vascular injury (Cheng et al., 2002). However, the contribution of this altered profile of eicosanoids to the development of hypertension is not clear. Based on its actions as a vasodilator and natriuretic agent (Hilchey and Bell-Quilley, 1995; Hill and Moncada, 1979), inhibition of PGI2 synthesis could play a causal role in the elevation in blood pressure commonly seen in patients treated with these agents. However, previous studies in mouse models have suggested that prostacyclin may have little effect on resting blood pressure (Cheng et al., 2002; Murata et al., 1997; Watanabe et al., 2005; Xiao et al., 2001) or may actually promote hypertension caused by renal artery stenosis (Fujino et al., 2004).

To address the consequences of suppression of PGI2 on blood pressure and end-organ damage, we generated mice lacking the IP receptor for PGI2 on an inbred, 129/SvEv background. In this setting, we find that the absence of IP receptors is sufficient to cause hypertension with cardiac hypertrophy and fibrosis. Furthermore, when IP receptors are absent, we find that unrestrained actions of Tx acting through its TP receptor accentuate the intensity of hypertension-induced cardiac damage.

Results

Salt-sensitive hypertension in IPKOs (Figure 1A) In mice fed a conventional 0.4% NaCl diet, we found that blood pressures were significantly elevated in the
IPKOs (126 ± 2 mm Hg; n = 12) compared to wild-type (wt) controls (118 ± 2 mm Hg; p = 0.02, n = 10). We next evaluated the effects of altering dietary sodium intake on blood pressures in the two groups. There was a modest, nonsignificant fall in blood pressure (Figure 1A) in wt mice from 119 ± 5 to 114 ± 4 mm Hg (p = 0.18) after 3 weeks on a low-salt diet (<0.02 NaCl). By contrast, there was a more marked and significant fall in blood pressures in the IPKOs from 129 ± 5 to 119 ± 5 mm Hg (p < 0.05) during low-salt feeding. Moreover, at the end of the low-salt feeding period, blood pressures in the wt and IPKOs were not significantly different (p = 0.42). Three weeks of feeding the high-salt diet (6% NaCl) caused blood pressure to increase significantly in the wts to 137 ± 5 mm Hg (p < 0.05), consistent with previous reports of sodium sensitivity in this strain (Ryan et al., 2002; Wang et al., 2002). Therefore, it is unlikely that enhanced activity of the renin-angiotensin-aldosterone system can explain the exaggerated cardiac hypertrophy and fibrosis observed in the IPKOs. Moreover, the extent of interstitial cardiac fibrosis did not correlate with the degree of cardiac hypertrophy.

**Cardiac hypertrophy in IP-deficient mice**

To seek evidence of end-organ damage from chronic hypertension, we compared heart weights between *Ip*+/− and *Ip*+/+ mice at 3, 6, and 17 months of age. While heart-to-body weight ratios were similar in 3-month-old *Ip*+/+ and *Ip*−/− mice (5.0 ± 0.1 versus 5.0 ± 0.2), they were significantly increased (Figure 1B and Table 1) in the IPKOs by 6 months of age (6.3 ± 0.1 versus 5.3 ± 0.3; p < 0.05). Persistent cardiac hypertrophy was apparent in IPKOs by 17 months of age (7.2 ± 0.2 versus 6.3 ± 0.3; p < 0.05). Echocardiography was performed in conscious, 6-month-old mice to determine whether IP deficiency was associated with defects in cardiac function. Fractional shortening was virtually identical in wt (0.65 ± 0.02) and IPKOs (0.60 ± 0.05; p = 0.42) as were LV dimensions (3.28 ± 0.13 versus 3.41 ± 0.06 mm for LVEDD and 1.16 ± 0.06 versus 1.36 ± 0.18 mm for LVEDD). Thus, IP-deficient mice have cardiac hypertrophy with normal chamber dimensions and preserved systolic function. This phenotype of concentric LV hypertrophy with preserved systolic function is similar to that seen in patients with chronic hypertension where LVH is a potent risk factor for cardiovascular events such as MI and stroke (Verdecchia et al., 2001).

**IP deficiency causes cardiac fibrosis**

To develop a more complete characterization of cardiac damage associated with IP deficiency, we also examined cardiac histopathology, comparing wt and IP-deficient mice. Broad areas of interstitial fibrosis were evident throughout the left ventricle in IPKOs (Figure 2A), but there was no evidence of cardiac fibrosis in wts (Figure 2B). Indeed, myocardial fibrosis was detectably increased in the IPKOs as early as 6 months of age (4.9% ± 1.4% versus 1.6% ± 0.1%; p < 0.001) in the IPKOs (Figure 2C). These abnormalities were even more pronounced in the 17-month-old cohort (5.8% ± 1.1% versus 1.9% ± 0.2%; p < 0.001). Fibrosis was confined to the left ventricle as we did not observe lesions in the right ventricle of the IPKOs. Moreover, the extent of interstitial cardiac fibrosis did not correlate with the degree of cardiac hypertrophy.

**Renin and aldosterone levels are not affected by IP deficiency**

Because the IP receptor is a physiological regulator of renin (Fujino et al., 2004) and activation of the renin-angiotensin-aldosterone system can promote the development of cardiac hypertrophy and fibrosis, we compared renin and aldosterone levels in *Ip*+/+ and *Ip*−/− mice. Renin mRNA levels, measured by RT-PCR from total RNA isolated from kidneys after feeding a control (0.4% NaCl) diet, tended to be slightly lower but not significantly different in IPKOs (110 ± 19 pg/µg total RNA; n = 10) compared to wt controls (126 ± 29 pg/µg total RNA; n = 13). Urinary aldosterone excretion was virtually identical between the two groups (15 ± 2.3 versus 14.4 ± 1.4 ng/mg creatinine; n = 7 wt versus n = 6 IPKO). Therefore, it is unlikely that enhanced activity of the renin-angiotensin-aldosterone system can explain the exaggerated cardiac hypertrophy and fibrosis observed in the IP-deficient mice.

**Antihypertensive therapy prevents cardiac hypertrophy and fibrosis in IP-deficient mice**

To determine whether cardiac hypertrophy and fibrosis resulted from the increase in blood pressure, we treated the IPKOs with hydralazine, a vasodilator antihypertensive agent, from 3 to 6 months of age. Compared to pretreatment values, administration of hydralazine significantly lowered blood pressures in...
Table 1. Heart and body weights

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>3</th>
<th>6</th>
<th>17</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype</td>
<td>IP−/−, n = 6</td>
<td>IP−/−, n = 7</td>
<td>IP+/+, N = 6</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>27.1 ± 1</td>
<td>25.7 ± 0.4</td>
<td>30.5 ± 0.4</td>
</tr>
<tr>
<td>Heart weight (mg)</td>
<td>135 ± 7</td>
<td>129 ± 4</td>
<td>191 ± 5</td>
</tr>
<tr>
<td>H/B ratio (mg/g)</td>
<td>5.0 ± 0.1</td>
<td>5.0 ± 0.1</td>
<td>6.3 ± 0.1</td>
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p < 0.05 versus IP−/−.

IPKOs (130 ± 3 mm Hg versus 113 ± 2 mm Hg; p < 0.0001, n = 7) as expected. The blood pressures achieved in hydralazine-treated animals were equivalent to that of wt 129/SvEv mice (113 ± 2 versus 120 ± 2 mm Hg; n = 6). Reducing blood pressure to normal levels prevented both the increase in heart weight and the cardiac fibrosis seen in untreated IP-deficient mice (Figure 3), indicating their dependence on elevated blood pressure.

TP-receptor activation contributes to end-organ injury in IP deficiency

The predominant suppression of PGI2 synthesis and sparing of TxA2 synthesis caused by COX-2 inhibitors has been associated with increased cardiovascular risk (FitzGerald, 2004; FitzGerald and Patrono, 2001). To determine whether hypertension and the cardiovascular phenotype of IP deficiency resulted from unrestrained activation of the TP receptor, we generated inbred IPTP DKO 129/SvEv mice. Coincidental deletion of the TP did not rescue the hypertensive phenotype of IP deficiency, as there was no difference in blood pressures in the IPTP double knockouts (DKOs) (134 ± 5 mm Hg; n = 7) and the IPKOs (130 ± 3 mm Hg; n = 7). However, despite their similar blood pressures, both heart weights (5.8 ± 0.1 versus 6.3 ± 0.1 mg/gm; p < 0.01) and the extent of cardiac fibrosis (1.63% ± 0.2% versus 4.9% ± 1.4%; p < 0.01) were limited significantly by combined IPTP receptor deficiency compared to IP deletion alone (Figure 3). Indeed, the quantitative scores for cardiac fibrosis were virtually identical in the IPTP DKO mice and the wt controls (1.6% ± 1.0% versus 1.6% ± 1%; p = NS). Heart-to-body weight ratios in mice with isolated TP deficiency were similar to wt controls (5.0 ± 0.2 versus 5.2 ± 0.1 mg/gm), and the hearts were pathologically indistinguishable.

To determine whether the generation of TxA2 might be enhanced in hypertensive, IP-deficient mice, we measured urinary excretion of thromboxane (Tx) B2 (a stable metabolite of TxA2). There was no significant difference in the levels of uri-

Figure 2. Myocardial fibrosis in IP-deficient mice

A) Representative Masson trichrome-stained section of the left ventricle of a 17-month-old IP-deficient mouse showing prominent myocardial fibrosis. The black line represents 100 µm.

B) Similarly stained section of a 17-month-old heart from a wt control showing normal myocardium without fibrosis.

C) Morphometric analysis of the extent of cardiac fibrosis in wt (white bars) and IPKO (black bars) mice at 3 (n = 13), 6 (n = 18), and 17 months of age (n = 12). (*p < 0.01 versus wt.) Error bars represent SEM.
Discussion

Prostanoids have potent actions to regulate vascular tone and renal sodium handling, thereby impacting control of blood pressure (Smith, 1992). The most compelling evidence supporting a role for prostanoids in blood pressure homeostasis comes from clinical experiences with NSAIDs. In susceptible patients, inhibition of prostanoid generation by NSAIDs causes sodium retention with edema and hypertension (Gurwitz et al., 1994; Pope et al., 1993). In addition, NSAIDs may interfere with the actions of antihypertensive therapies, particularly loop diuretics and ACE inhibitors (Conlin et al., 1994; Johnson et al., 1994; Pope et al., 1993). However, sodium retention and hypertension associated with IP deficiency do not cause enhanced thromboxane generation.

Figure 3. Morphometric analysis of cardiac fibrosis in 6-month-old mice

The extent of cardiac fibrosis was determined as described in groups of 6-month-old wt mice (white bar; n = 12), IPKO mice (black bar; n = 6), IPKO mice that had been treated with hydralazine for 3 months (striped bar; n = 7), and mice with combined IP and TP deficiency (IPTP DKOs) (gray bar; n = 10). Thus, the absence of IP receptors and/or hypertension associated with IP deficiency do not cause enhanced thromboxane generation.

Prostanoids have potent actions to regulate vascular tone and renal sodium handling, thereby impacting control of blood pressure. They also play a critical role in the development of hypertension. The capacity of COX inhibitors to cause hypertension is well documented. However, NSAIDs only cause significant elevations in blood pressure in a subset of susceptible patients, including those with pre-existing hypertension, on active antihypertensive therapy or with specific risk factors for hypertension. In this regard, recent studies by Fujino et al. suggest that the IP receptors contribute to the pathogenesis of hypertension caused by renal artery stenosis (Fujino et al., 2004).

The capacity of COX inhibitors to cause hypertension is well documented. However, NSAIDs only cause significant elevations in blood pressure in a subset of susceptible patients, including those with pre-existing hypertension, on active antihypertensive therapy or with specific risk factors for hypertension (Gurwitz et al., 1994; Johnson et al., 1994; Pope et al., 1993). In this regard, exaggerated blood pressure response to sodium loading and depletion, also known as salt sensitivity, is an important risk factor for hypertension in humans (Weinberger, 1996). As mice of the 129/SvEv strain have salt sensitivity (Ryan et al., 2002; Wang et al., 2002), we reasoned that this strain of mice would be a useful platform for modeling NSAID-induced hypertension. To this end, we generated inbred IP-deficient mice on the 129/SvEv background and found that these animals have significant elevations in blood pressure. Moreover, the tendency for sodium sensitivity in wt 129/SvEv mice is markedly accentuated by IP deficiency, indicating a critical role for PGI2 in regulating pressure-natriuresis relationships.

Our findings differ from previous studies of blood pressure in IP-deficient mice (Cheng et al., 2002; Fujino et al., 2004; Murata et al., 1997; Watanabe et al., 2005; Xiao et al., 2001). For example, IP-deficient mice generated on an inbred C57BL/6 background had either normal or reduced blood pressures (Fujino et al., 2004; Watanabe et al., 2005). In contrast to the 129 strain, C57BL/6 mice are resistant to sodium-dependent blood-pressure changes (Ryan et al., 2002; Wang et al., 2002). Therefore, this difference in genetic backgrounds may explain the different blood-pressure responses in these studies. Nonetheless, in the study by Watanabe and associates, female C57BL/6 mice with IP deficiency acquire a sodium-sensitive phenotype (Watanabe et al., 2005), consistent with our assertion that PGI2 is an important regulator of renal sodium excretion. These differing susceptibilities to prostanoid-dependent hypertension among inbred mouse strains are consistent with the variable risk for NSAID-induced hypertension across human populations. Furthermore, these findings suggest that genetic factors might contribute to determining susceptibilities to this disorder in patients. Accordingly, understanding the nature of these strain differences may provide insights into the mechanisms of NSAID-associated hypertension in humans.

Hypertension in IP-deficient mice is associated with the development of robust cardiac hypertrophy and fibrosis. The characteristics of left ventricular hypertrophy with preserved synthesis of the vasoconstrictor prostanoid TxA2 (McAdam et al., 1999). Along with its vasodilator properties, PGI2 also promotes sodium and water excretion (Hilchey and Bell-Quilley, 1995; Hill and Moncada, 1979). Accordingly, reduced generation of PGI2 could contribute to sodium retention and blood-pressure elevation associated with COX-2 inhibition. However, the potential impact of PGI2 on blood pressure is complex. PGI2 acting through the IP receptor at the juxtaglomerular apparatus (JGA) is an important stimulatory pathway for release of renin, the rate-limiting enzyme in generation of angiotensin II (Gerber et al., 1979). Accordingly, activation of IP receptors in some circumstances could promote the development of hypertension. In this regard, recent studies by Fujino et al. suggest that the IP receptors contribute to the pathogenesis of hypertension caused by renal artery stenosis (Fujino et al., 2004).
systolic function in IP-deficient mice are very similar to LVH in humans with chronic hypertension where the presence of LVH is a potent risk factor for cardiovascular morbidity and mortality (Verdecchia et al., 2001). Myocardial fibrosis is also associated with poor cardiovascular outcomes (Klug et al., 1993; Weber and Brilla, 1991). Thus, in this mouse model, isolated absence of IP receptors is associated with hypertension and cardiac abnormalities that would be expected to confer cardiovascular risk in humans. While it is not clear whether hypertension and its accompaniments play a role in the cardiovascular risk associated with COX-2 inhibitors, a number of the patients in the APPROVe trial developed hypertension, and the excess in CV events in the patients receiving COX-2 inhibitor did not become apparent until more than 18 months into the study. Such a delayed appearance of risk might be expected if hypertension is a contributor.

Relative to other mouse models, the degree of cardiac fibrosis in the IPKOs was out of proportion to their level of blood-pressure elevation. Nonetheless, the pathogenesis of these abnormalities clearly depends on concomitant hypertension since lowering blood pressure with hydralazine completely prevented both hypertrophy and fibrosis. Thus, elevated blood pressure is essential for the development of cardiac injury in IP deficiency. Insofar as our findings might relate to patients with predominant inhibition of PGI2 from COX-2 inhibitors, this would suggest that control of hypertension could ameliorate this tendency for exaggerated end-organ injury. Nonetheless, the development of florid end-organ damage in this circumstance is more complex than a simple response to elevated blood pressure. Our data indicate a direct role for TP signaling to promote cardiovascular pathology associated with IP deficiency. That is, when hypertension associated with IP deficiency is not controlled, unrestrained activation of the TP receptor promotes cardiac fibrosis. This finding is consistent with observations in other models showing that activation of the TP receptor can promote end-organ injury (Cheng et al., 2002; Francois et al., 2004).

TP receptors contribute to blood-pressure elevation in experimental models of hypertension (Francois et al., 2004). Yet, concomitant deletion of TP receptors does not rescue the hypertensive phenotype of IP deficiency. Despite their similar blood pressures, cardiac hypertrophy and fibrosis are ameliorated in mice with concomitant deletion of both IP and TP receptors compared to those with IP deficiency alone. Thus, deletion of the IP receptor removes a constraint revealing potent effects of TxA2 to promote cardiac pathology. However, this is not due to exaggerated generation of TxA2 associated with IP deficiency and its associated hypertension. These findings provide additional evidence to support the thesis that the actions of COX-2 inhibitors to suppress PGI2 synthesis without affecting TxA2 levels have maladaptive consequences in the cardiovascular system. Such an effect would not be anticipated with conventional NSAIDs that cause more global inhibition of both PGI2 and TxA2. As TxA2 agonists promote inflammation (Thomas et al., 2003) and fibrosis (Bruggeman et al., 1993) in cell culture systems independent of any hemodynamic effects, these pathways may contribute to exaggerated cardiovascular injury associated with unrestrained activation of TP receptors in IP deficiency.

We have relied exclusively on tail-cuff measurements to determine blood pressures in this study. In our view, the tail-cuff technique has the major advantage of providing blood-pressure measurements in conscious mice using a completely non-invasive approach. However, there is evidence that this method may induce some stress in mice (Gross and Luft, 2003), and it is not as sensitive for blood-pressure measurement as radiotelemetry approaches. Nonetheless, previous studies have clearly documented its validity and tight correlations with the other available invasive approaches (Ito et al., 1995; Krege et al., 1995), including radiotelemetry (Crowley et al., 2005; Wong et al., 2002).

In summary, our study has several important implications. Firstly, the propensity for sodium retention and hypertension seen in patients taking COX-2 inhibitors is mechanistically consistent with suppression of PGI2. Although there is evidence from other studies that COX-1-derived eicosanoids, such as TxA2, augment the response to hypertensive stimuli (Francois et al., 2004), this was not true of the hypertension resultant from PGI2 deficiency. Despite a lack of an effect on blood pressure, our results suggest that blocking TP receptors, or perhaps reducing TxA2 generation by low-dose aspirin, might protect against cardiac end-organ damage associated with the predominant reduction of PGI2 associated with COX-2 inhibition. However, the practical utility of such an approach would require demonstration that GI protection is retained.

Experimental procedures

Mouse lines
Mice with targeted disruptions of the Ip and Tp genes were generated as described previously (Cheng et al., 2002; Thomas et al., 1998). As both mutations were originally generated in embryonic stem cells derived from 129 mice, chimeras were initially crossed with 129/SvEva females to immediately generate progeny bearing either the Ip or Tp mutations on a homogenous 129 background. Double-knockout mice (IP/TP DKO) were generated by intercrossing the IP- and TP-deficient lines. In our experiments, 129 wt littersmates were used as controls. Animals were maintained in the animal facility of the Durham VA Medical Center, and experimental procedures described below were approved by the Institutional Animal Care and Use Committees of the Durham VA and Duke University Medical Centers. The studies were limited to male mice.

Blood-pressure measurements in conscious mice
Systolic blood pressures were measured in conscious mice using a computerized tail-cuff system after 2 weeks of daily training, as described previously (Ito et al., 1995; Krege et al., 1995). Data were recorded at baseline for 3 weeks (one set of 10 measurements per day). Measurements with a standard deviation of more than 20 mm Hg for the systolic blood pressure were discarded. This method has been extensively validated and correlates well with direct measurements of intra-arterial pressure (Ito et al., 1995; Krege et al., 1995). Baseline measurements were carried out on conventional mouse chow (0.4% NaCl). To determine the effects of altered dietary salt intake on blood pressure, mice were first fed a low-salt diet (<0.02% NaCl, Harlan Teklad, Wisconsin) for 3 weeks while their systolic blood pressure was recorded daily as described above. After a washout period of feeding the conventional (0.4% NaCl) diet for one week, mice were then fed a high-salt diet (6% NaCl, Harlan Teklad) for 3 weeks while their systolic blood pressure was measured daily.

Echocardiography
Thoracic 2D guided M-mode echocardiography was performed in conscious mice using an HDI 5000 echocardiograph (ATL, Bothell, Washington) as previously described (Esposito et al., 2000). The following parameters were measured: LVEDD, LV end-diastolic dimension; LVESD, LV end-
systolic dimension; FS, fractional shortening calculated as (LVEDD-LVESD)/LVEDD. The individual performing the echocardiograms was blinded to the experimental groups.

**Analysis of cardiac histopathology**

At 3, 6, and 17 months of age, hearts were excised from mice and immediately weighed. The tissue was fixed in 10% formalin containing PBS, embedded in paraffin, and 4 µm sections were stained with Masson’s trichrome to highlight extracellular matrix deposition. Digital images were captured using an Olympus (Melville, New York) BX40 microscope and an Optronics (Goleta, California) DEI 750 3 CCD digital camera. The digital images were analyzed using Optimas v6.5 image analysis software (Media Cybernetics, Del Mar, California) by keying on color-specific features. Two color parameters were measured representing fibrosis (green color spectrum) and cardiac muscle (red color spectrum). The collagen fraction was calculated as the ratio of the sum of the total area of interstitial fibrosis to the sum of the interstitial fibrosis tissue area plus the myocyte area in the entire visual field of the section. All procedures and measurements were performed twice for each histologic cross-section and then averaged. Two surgical pathologists analyzed the slides without knowledge of the experimental groups.

**Hydralazine treatment**

Beginning at 3 months of age, a separate group of male Ip−/− mice were treated with hydralazine (150 mg/kg/day, Sigma Chemical) in drinking water for 3 months. Blood pressures were measured before and during hydralazine treatment. At the end of the study period, heart weights and cardiac fibrosis were measured as described above.

**Measurement of renin mRNA**

Relative levels of mRNA for renin were determined by real-time (RT) PCR with the ABI Prism 7700 Sequence Detection System as described previously (Crowley et al., 2005). Kidneys were harvested from IPKO and wt mice fed a control (0.4% NaCl) diet, and total RNA was isolated in using an RNeasy Mini Kit per the manufacturer’s instructions (Qiagen Inc., Valencia, California). RT-PCR amplifications were performed in a 96-well plate in the ABI Prism 7700 sequence detector (PE-Biosystem Inc., Foster City, California). During the amplification, the fluorescence of FAM (or TET), TAMRA, and ROX (a passive reference dye) was measured by the 7700 sequence detector in each well of the 96-well plate. The numbers of copies of the PCR template in the starting sample were calculated using the Sequence Detector Software (SDS) incorporated in the ABI Prism 7700 Sequence Detector System. A standard plasmid containing a DNA fragment for the renin gene was used as an external control, and amplification of the β-actin gene was used as an endogenous control. For each experimental sample, the amounts of the target and of the endogenous control were determined from the appropriate standard curves.

**Urinary excretion of aldosterone and TxB2**

Mice were individually housed in specially designed metabolic cages that accommodate individual mice with free access to tap water. Twenty-four hour urine was collected in wt and IPKO mice fed a control (0.4% NaCl) diet. Urine was clarified by 15 min of centrifugation (14000 g). Urinary aldosterone concentrations were determined by radioimmunoassay according to the manufacturer’s instructions (Diagnostic Labs). TxB2 was measured using an ELISA assay on fresh urine samples (Cayman Chemical, Ann Arbor, Michigan). Urine creatinine concentration was quantified using the alkaline picrate method (The Creatinine Companion, Excocell Inc.).

**Statistical analysis**

Data are expressed as mean ± SEM. Data were analyzed using paired or unpaired Student’s t test for parametric variables and Mann-Whitney or Fisher χ2 test for nonparametric variables.

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**References**


Hypertension and heart fibrosis in IP deficiency


