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Pivotal Role of AT_{1A} Receptors in the Development of Two-Kidney, One-Clip Hypertension: Study in AT_{1A} Receptor Knockout Mice

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Abstract

Objective—The present study was performed to examine in two-kidney, one clip (2K1C) Goldblatt hypertensive mice, first, the relative contribution of angiotensin II (ANG II) receptor subtypes 1A (AT_{1A}) and 1B (AT_{1B}); second, the role of ANG II type 2 (AT₂) receptors in the development of hypertension in wild-type (AT_{1A}^{+/+}) and AT_{1A} receptor knockout (AT_{1A}^{−/−}) mice and third, the role of increased nitric oxide synthase (NOS) activity in counteracting the hypertensinogenic action of ANG II in this model.

Methods—AT_{1A}^{+/+} and AT_{1A}^{−/−} mice underwent clipping of one renal artery and were infused with either saline vehicle or with the selective AT₂ receptor agonist CGP-42112A (CGP). Blood pressure (BP) was monitored by radiotelemetry. BP responses to the NOS inhibitor nitro-L-arginine-methyl-ester (L-NAME) were evaluated.

Results—AT_{1A}^{+/+} mice responded to clipping by a rise in BP which was not modified by CGP infusion. Clip placement caused a slight increase in BP in AT_{1A}^{−/−} mice which remained significantly lower than in AT_{1A}^{+/+} mice. Acute NOS inhibition caused greater increases in BP in 2K1C/AT_{1A}^{+/+} than in AT_{1A}^{+/+} mice.

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Conclusions—The present data support the critical role AT_{1A} receptors in the development of 2K1C hypertension, whereas AT_{1B} receptors play only a minor role in BP regulation in this model of ANG II-dependent hypertension. Activation of AT₂ receptors does not play an antagonistic role in the AT₁ receptor-mediated hypertensinogenic actions of ANG II in this model. Finally, enhanced NOS activity plays a protective role by counteracting the vasoconstrictor influences of ANG II in 2K1C hypertensive mice.

Keywords

renin-angiotensin system; two-kidney, one-clip Goldblatt hypertension; AT_{1A} receptor knockout mice; AT_{1B} receptor, nitric oxide synthase, AT₂ receptor, plasma and kidney ANG II levels

Introduction

It is well established that the renin-angiotensin system (RAS) contributes critically to the pathophysiology of two-kidney, one-clip (2K1C) Goldblatt hypertension [1]. Normal or enhanced intrarenal angiotensin II (ANG II) levels in the non-clipped kidney increase tubular sodium reabsorption via stimulation of the ANG II type 1 (AT₁) receptors. The resulting impairment of pressure-natriuresis is a pivotal mechanism leading to hypertension in this model [1–3]. In addition, although renal renin content is substantially suppressed in ANG II-dependent models of hypertension, AT₁ receptor-mediated internalization of ANG II from the circulation and AT₁ receptor-dependent augmentation of intrarenal angiotensinogen are two major mechanisms responsible for the increased intrarenal ANG II levels in the non-clipped kidney [4,5]. Two AT₁ receptor subtypes have been identified in rodents (AT_{1A} and AT_{1B}) and despite the fact that they are products of separate genes, they share a homology of over 90% nucleotide sequence [6]. Unfortunately, no AT₁ receptor antagonists are available to discriminate between the relative contributions of AT_{1A} or AT_{1B} receptors to the physiological regulation of blood pressure (BP) and between their pathophysiologic roles in this 2K1C Goldblatt model of ANG II-dependent hypertension. However, the development of genetically engineered mice with targeted disruption of the AT_{1A} receptor gene, i.e. AT_{1A} receptor knockout mice (AT_{1A}^{−/−}), offered a novel means to explore ANG II receptor function [7]. Thus, studies in AT_{1A}^{−/−} mice have documented the essential role of AT_{1A} receptors in BP regulation and in the development of 2K1C Goldblatt hypertension [8]. However, recent observations have demonstrated that under certain conditions, especially in the absence of AT_{1A} receptors, AT_{1B} receptors may play a limited role in the regulation of BP, ANG II-mediated renal vasoconstriction and intrarenal ANG II accumulation [9–13]. In view of this new information, we tested the hypothesis that AT_{1B} receptors contribute to intrarenal ANG II augmentation and to the development and maintenance of 2K1C Goldblatt hypertension. Accordingly, the first aim of the present study was to evaluate the relationship between changes in plasma and tissue ANG II levels and the course of BP changes after unilateral renal artery constriction (clipping) in wild-type (AT_{1A}^{+/+}) and in knockout (AT_{1A}^{−/−}) mice.

Several studies suggest that activation of ANG II type 2 (AT₂) receptors counteracts the AT₁ receptor-mediated hypertensinogenic actions of ANG II and that AT₂ receptor-mediated vasodilation is more pronounced during concomitant AT₁ receptor blockade [14–17]. Taken together, these data suggest that selective AT₂ receptor stimulation provides a novel therapeutic approach to the treatment of hypertension. However, the cardiovascular effects of chronic selective AT₂ receptor stimulation per se have not yet been evaluated in 2K1C Goldblatt hypertension. Accordingly, the second aim of the present study was to evaluate the effects of acute and chronic AT₂ receptor stimulation on the course of BP after unilateral renal artery clipping in AT_{1A}^{+/+} and AT_{1A}^{−/−} mice.

Finally, it has been suggested that enhanced nitric oxide (NO) formation counteracts the vasoconstrictor actions of ANG II during the early phases of hypertension in ANG II-dependent models of hypertension [18,19]. Thus, on one hand, NO may serve as an important vasodilatory system modulating the renal vascular and systemic BP responses to ANG II [20,21]. On the other hand, experimental data indicate that, during established hypertension in ANG II-dependent models, ANG II increases the generation of superoxide anion (O_2^-) via activation of AT_1 receptors and O_2^- may then decrease the biological availability of NO resulting in endothelial dysfunction [22]. In addition, impaired release of NO synthase (NOS)-dependent NO is considered an important mechanism in established ANG II-induced hypertension [22–24] but it is not clear whether impaired NO formation occurs in 2K1C Goldblatt hypertension. The third aim of the present study was, therefore, to evaluate the effects of acute NOS inhibition on BP in 2K1C $AT_{1A}^{+/+}$ and 2K1C $AT_{1A}^{-/-}$ mice during the initial and the maintenance phases of high BP in this model of ANG II-dependent hypertension.

Methods

The studies were performed in accordance with guidelines and practices established by the Institute for Clinical and Experimental Medicine Animal Care and Use Committee. All animals used in the present study were housed in facilities accredited by the Czech Association for Accreditation of Laboratory Animal Care and were bred from stock animals supplied from the Durham Veterans Affairs Medical Center (we acknowledge the generous gift of Professor Coffman). The genotype of animals was confirmed by Southern blot analyses of DNA isolated from the tail as described previously [25].

Series 1: Direct Measurement of BP by Radiotelemetry Methods

In accordance with the recently published recommendation for BP measurement in experimental animals we employed a radiotelemetry system for direct BP measurements [26]. Mice were anaesthetized with thiopental sodium (60 mg/kg i.p.) and TA11PA-C20 radiotelemetric probes (Data Sciences International, St. Paul, MN, USA) were implanted in the left carotid artery for direct BP measurements as described in detail previously [27]. Mice were allowed 7 days to recover, after which time basal BP was recorded and subsequently 2K1C Goldblatt hypertensive and sham-operated mice were prepared.

Preparation of 2K1C Goldblatt Hypertensive Mice

Male $AT_{1A}^{+/+}$ and $AT_{1A}^{-/-}$ mice weighing 29 to 33 g (at age of 5 months) were anesthetized with thiopental sodium (60 mg/kg i.p.). The right renal artery was isolated through a flank incision and, as described previously [8,28], a silver clip (0.12-mm internal diameter) was placed on the renal artery. Sham-operated mice that underwent the same surgical procedure except for placement of the renal artery clip served as controls. Osmotic minipumps (M 1002, Alzet Co.) containing saline vehicle or CGP-42112A (CGP), a selective AT_2 receptor agonist (Sigma Chemical Co.), at concentrations that allowed the delivery of $70 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ were implanted into the abdominal cavity of the 2K1C $AT_{1A}^{+/+}$ and $AT_{1A}^{-/-}$ mice (vehicle or CGP-treated 2K1C/ $AT_{1A}^{+/+}$ and vehicle or CGP-treated 2K1C/ $AT_{1A}^{-/-}$, respectively). This dose of CGP has been demonstrated previously to chronically and selectively stimulate AT_2 receptors [15]. Osmotic minipumps containing saline vehicle or CGP were also implanted into sham-operated $AT_{1A}^{+/+}$ and $AT_{1A}^{-/-}$ mice. Because the osmotic minipumps have an operating time of 14 days, they were replaced by new minipumps containing saline vehicle or CGP on day 13.

Direct BP measurements by radiotelemetry was conducted in the following experimental groups of mice:

1. sham-operated vehicle-treated $AT_{1A}^{+/+}$ (n = 6)

2. sham-operated CGP-treated AT_{1A}^{+/+} (n = 6)
3. vehicle-treated 2K1C/AT_{1A}^{+/+} (n = 8)
4. CGP-treated 2K1C/AT_{1A}^{+/+} (n = 9)
5. sham-operated vehicle-treated AT_{1A}^{-/-} (n = 7)
6. sham-operated CGP-treated AT_{1A}^{-/-} (n = 7)
7. vehicle-treated 2K1C/AT_{1A}^{-/-} (n = 9)
8. CGP-treated 2K1C/AT_{1A}^{-/-} (n = 9)

Determinations of Plasma and Tissue ANG II Concentrations in Conscious Mice

It is now generally recognized that plasma and tissue ANG II concentrations in anaesthetized animals are higher than those obtained from decapitated conscious rats and that normotensive animals exhibit a greater increase in renin secretion in response to anaesthesia and surgery than ANG II-induced hypertensive intrarenal renin-depleted animals [29]. Therefore, for the present we determined ANG II levels in separate groups of conscious mice that were decapitated. Heart (HW) and kidney (KW) weights (mg) were normalized per gram of body weight (BW).

ANG II levels were determined in plasma and in clipped and non-clipped kidneys and hearts of the following groups: 2K1C/AT_{1A}^{+/+}, CGP-treated/2K1C/AT_{1A}^{+/+}, 2K1C/AT_{1A}^{-/-} and CGP-treated/2K1C/AT_{1A}^{-/-} (n = 10 in each group) before clip placement (day -3) and on days 3,6,9,12,15,18,21,24 and 28 after clip placement. Sham-operated AT_{1A}^{+/+}, CGP-treated/AT_{1A}^{+/+}, AT_{1A}^{-/-} and CGP-treated/AT_{1A}^{-/-} mice (n = 8 in each group) served as controls. Mice were sacrificed by decapitation, and plasma, whole kidneys and left heart ventricular tissue ANG II were assessed by radioimmunoassay (RIA) according to the procedure developed and confirmed by Fox et al. and Navar et al., respectively, as described in detail previously [30,31]. In accordance with the manufacturer's instruction (Euro-Diagnostica Co., Malmö, Sweden), the ANG II RIA kit used in the present study does not cross-react with CGP. The procedure of blood sampling and the ANG II assay are routinely employed in our laboratory and this standardized approach allows us to compare the results with those of our previous studies evaluating the role of the RAS in the pathophysiology of hypertension [29, 32–34].

Assessment of the Effects of Acute AT₂ Receptor Stimulation and of NOS inhibition on BP

Separate groups of mice were anesthetized with thiopental sodium and placed on a servo-controlled surgical table that maintained body temperature at 37°C. A tracheostomy was performed using a PE-90 tubing. The mice were allowed to breathe air enriched with O₂. The right carotid artery was cannulated with a PE-10 catheter connected to a PE-50 catheter for continuous measurement of mean arterial pressure (MAP) with a pressure transducer (model MLT 1050). For recording a computerized data-acquisition system (PowerLab/4SP, ADInstruments, UK) was used. In addition, the right jugular vein was catheterized with PE-10 tubing for infusion of saline containing 1% bovine serum albumin (BSA; Sigma Chemical Co.) at a rate of 5 µl/min throughout the experiment. After surgery mice were allowed a 10-minute recovery period which was followed by a 25-minute control period for measurements of basal MAP. After an additional 25-minute period of saline vehicle infusion acute NOS inhibition was achieved by intravenous infusion of nitro-L-arginine-methyl-ester (L-NAME; Sigma Chemical Co.) (0.25 µg. g body wt⁻¹. min⁻¹) in saline vehicle-treated mice and in mice exposed to acute AT₂ receptor stimulation. The dose of L-NAME used in the present study was used and validated in our previous study [28]. The acute AT₂ receptor stimulation was achieved by intravenous infusion of CGP at the dose of 1ng/g of body wt⁻¹. min⁻¹. This dose of CGP was employed in previous studies evaluating the effects of acute AT₂ receptor stimulation on BP

[15,16] and has been shown to provide circulating plasma concentrations that are highly selective for AT₂ receptors on the basis of binding data [35]. Time-control mice received a constant saline vehicle infusion throughout the experiment. At the end of the experiment mice were sacrificed with an excess dose of intravenous thiopental sodium. The following experimental groups of mice (n = 9 in each group) were examined on days 7,14,21,25 and 28 after clip placement:

1. sham-operated vehicle-treated AT_{1A}^{+/+}
2. sham-operated CGP-treated AT_{1A}^{+/+}
3. vehicle-treated 2K1C/AT_{1A}^{+/+}
4. CGP-treated 2K1C/AT_{1A}^{+/+}
5. sham-operated vehicle-treated AT_{1A}^{-/-}
6. sham-operated CGP-treated AT_{1A}^{-/-}
7. vehicle-treated 2K1C/AT_{1A}^{-/-}
8. CGP-treated 2K1C/AT_{1A}^{-/-}

Statistical Analysis

Results are expressed as mean $\pm$ SEM. Two-way repeated-measures ANOVA were used to detect differences within each experimental group. For comparison between AT_{1A}^{+/+} and AT_{1A}^{-/-} mice, repeated-measures ANOVA was used with a test of interaction to determine whether the average changes after experimental manipulations (clip placement and pharmacological treatment) were significantly different between AT_{1A}^{+/+} and AT_{1A}^{-/-} mice. With Graph-Pad Prism software (Graph Pad Software, San Diego, CA, USA) statistical analysis was performed using Student's t-test, Wilcoxon's signed-rank test for unpaired data, or one-way analysis of variance (ANOVA) when appropriate. Values exceeding the 95% probability limits (p<0.05) were considered statistically significant.

Results

MAP in 2K1C/AT_{1A}^{+/+} and 2K1C/AT_{1A}^{-/-} Mice and Effects of Chronic AT₂ Receptor Stimulation

As shown in figures 1A and 1B basal MAP was significantly higher in AT_{1A}^{+/+} than in AT_{1A}^{-/-} mice (96 ± 2 vs. 72 ± 2 mmHg, p<0.05). As shown in figure 1A, beginning with day 5 after clipping of the right renal artery in wild-type (2K1C/AT_{1A}^{+/+}) mice MAP increased progressively to 141 ± 2 mmHg by day 28 and was unaffected by chronic CGP infusion (144 ± 3 mmHg).

MAP remained unchanged in sham-operated AT_{1A}^{+/+} and AT_{1A}^{-/-} mice without or with CGP-treatment (figures 1A and 1B). As shown in figure 1B, clipping of the renal artery in AT_{1A}^{-/-} mice increased MAP moderately but significantly to 81 ± 2 mmHg on day 17 when compared with basal levels. Chronic CGP infusion again did not influence the course of MAP elevation in 2K1C AT_{1A}^{-/-} mice.

Plasma and Tissue ANG II Levels and Heart (HW) and Kidney (KW) Weights in 2K1C AT_{1A}^{+/+} and AT_{1A}^{-/-} Mice and Effects of Chronic AT₂ Receptor Stimulation

Figures 2A and 2B show that basal plasma ANG II levels were significantly lower in sham-operated AT_{1A}^{+/+} mice than in AT_{1A}^{-/-} mice (66 ± 6 vs. 144 ± 27 fmol/ml, p<0.05) and were not altered by chronic CGP infusion (68 ± 8 vs. 163 ± 14 fmol/ml, p<0.05). On day 3 after clipping, plasma ANG II in vehicle- and CGP-treated AT_{1A}^{+/+} mice started to increase

significantly reaching its peak on day 9 after clipping (324 ± 49 and 250 ± 33 fmol/ml, respectively). They then returned to normal levels by day 28 after clip placement (79 ± 1 and 62 ± 8 fmol/ml, respectively). In contrast, clip placement did not significantly change plasma ANG II levels in vehicle- and CGP-treated $AT_{1A}^{-/-}$ mice throughout the experiment (figures 2A and 2B).

Figures 3A and 3B show that basal ANG II content in the left non-clipped kidney was significantly higher in vehicle- and CGP-treated $AT_{1A}^{+/+}$ mice than in vehicle- and CGP-treated $AT_{1A}^{-/-}$ mice (109 ± 8 and 110 ± 17 vs. 61 ± 7 and 63 ± 9 fmol/g, $p < 0.05$, respectively). There were no time-dependent changes during the experimental period in kidney ANG II concentrations in sham-operated vehicle- or CGP-treated $AT_{1A}^{+/+}$ and $AT_{1A}^{-/-}$ mice. In $AT_{1A}^{+/+}$ mice, whole kidney ANG II levels in the non-clipped kidney increased markedly and progressively after clip placement reaching 199 ± 16 fmol/g on day 28 after clipping. Chronic CGP infusion did not influence the course of intrarenal ANG II augmentation in the nonclipped kidney. In contrast, clip placement did not increase intrarenal ANG II levels in the non-clipped kidney of vehicle- and CGP-treated $AT_{1A}^{-/-}$ mice. Whole kidney ANG II levels in the non-clipped kidney on day 28 were not significantly different from basal values (72 ± 9 vs. 75 ± 10 fmol/g).

As shown in Figures 3C and 3D, clip placement resulted in immediate and striking increases in intrarenal ANG II content in the clipped kidney of vehicle- and CGP-treated $AT_{1A}^{+/+}$ mice when compared with basal levels, i.e. levels before clipping. On day 28 after clipping they were 3-fold higher than those of sham-operated vehicle- and CGP-treated $AT_{1A}^{+/+}$ mice (346 ± 24 and 342 ± 29 vs. 119 ± 10 and 104 ± 9 fmol/g, $p < 0.05$, respectively). In contrast, clip placement did not alter intrarenal ANG II levels in the clipped kidney of $AT_{1A}^{-/-}$ mice, whose basal levels were significantly lower than in $AT_{1A}^{+/+}$ mice (72 ± 8 vs. 122 ± 14 fmol/g, $p < 0.05$). There were no time-dependent changes during the experimental period in sham-operated and CGP-treated $AT_{1A}^{+/+}$ and $AT_{1A}^{-/-}$ mice.

Basal ANG II concentrations in left heart ventricle were not significantly different between $AT_{1A}^{+/+}$ and $AT_{1A}^{-/-}$ mice (46 ± 7 vs. 51 ± 8 fmol/g). They were not affected by either clip placement or chronic CGP infusion.

Heart (HW) and Kidney (KW) Weights in 2K1C $AT_{1A}^{+/+}$ and $AT_{1A}^{-/-}$ Mice

Since there were no significant differences in BW between $AT_{1A}^{+/+}$ and $AT_{1A}^{-/-}$ mice throughout the experiment, we employed HW/BW ratio as marker for cardiac hypertrophy. Basal HW/BW ratio was significantly higher in $AT_{1A}^{+/+}$ mice than in $AT_{1A}^{-/-}$ mice (5.50 ± 0.20 and 5.58 ± 0.27 vs. 4.49 ± 0.17 and 4.42 ± 0.19 , mg/g, $p < 0.05$, respectively). Beginning with day 12 after clip placement progressive increases in HW/BW ratio were observed reaching a maximum on day 28 (6.52 ± 0.21 mg/g). They were unaffected by chronic CGP infusion (6.41 ± 0.17 mg/g). HW/BW ratio remained unchanged in vehicle- and CGP-treated sham-operated $AT_{1A}^{+/+}$ and $AT_{1A}^{-/-}$ mice and 2K1C/ $AT_{1A}^{-/-}$ mice, respectively.

There were no significant differences between basal KW in $AT_{1A}^{+/+}$ and $AT_{1A}^{-/-}$ mice (339 ± 24 vs. 309 ± 21 mg); therefore, we employed the ratio of non-clipped/clipped KW as marker to appropriately evaluate the development of 2K1C Goldblatt hypertension. In $AT_{1A}^{+/+}$ and $AT_{1A}^{-/-}$ mice clip placement resulted in a progressive increase in the non-clipped/clipped KW ratio from 1.04 ± 0.09 to 2.68 ± 0.27 and from 1.06 ± 0.06 to 2.78 ± 0.14 , respectively ($p < 0.05$), which was not altered by chronic CGP infusion. In sham-operated $AT_{1A}^{+/+}$ and $AT_{1A}^{-/-}$ mice this ratio remained unchanged throughout the whole experiment.

MAP in Anaesthetized Mice and Effects of Acute AT₂ Receptor Stimulation and NOS Inhibition

Figures 4A and 4B show that similar to radiotelemetric data in conscious mice basal values of MAP in sham-operated AT_{1A}^{+/+} mice are significantly higher than in AT_{1A}^{-/-} mice. At 7 days after clipping MAP started to be significantly higher in 2K1C/AT_{1A}^{+/+} than in sham-operated AT_{1A}^{+/+} mice. As shown in figure 4B, clip placement in vehicle- and CGP-treated AT_{1A}^{-/-} mice caused significant elevations of MAP as compared with that of sham-operated vehicle- and CGP-treated AT_{1A}^{-/-} mice on days 25 (80 ± 2 and 81 ± 2 vs. 73 ± 2 and 74 ± 2 mmHg, respectively ($p < 0.05$)), and 28 after clipping (81 ± 2 and 81 ± 2 vs. 74 ± 2 and 73 ± 2 mmHg, respectively ($p < 0.05$)). Acute CGP infusion did not affect basal MAP in any of the experimental groups.

Figure 4C shows, that 2K1C/AT_{1A}^{+/+} mice responded to acute NOS inhibition with L-NAME with significantly greater increases in MAP than sham-operated AT_{1A}^{+/+} mice. The responses of MAP to L-NAME were unaffected by CGP treatment and were significantly greater on days 7, 14 and 21 than on days 25 and 28 after clipping ($+43 \pm 4$, $+52 \pm 5$ and $+41 \pm 4$ vs. $+28 \pm 3$ and $+22 \pm 4$ mmHg, respectively ($p < 0.05$)).

As shown in figure 4D, acute NOS inhibition induced similar increases of MAP in vehicle- and CGP-treated AT_{1A}^{-/-} and 2K1C/AT_{1A}^{-/-} mice on day 7, 14, 21, 25 and 28 days after clipping (or sham operation). Acute CGP infusion did not modify MAP responses to acute NOS inhibition in any of the experimental groups. However, when expressed as percent changes, MAP responses to acute NOS inhibition were similar in sham-operated AT_{1A}^{+/+} ($+15 \pm 2\%$) and sham-operated AT_{1A}^{-/-} mice ($+14 \pm 2\%$) in the absence and also in the presence of CGP infusion ($+14 \pm 2$ and $13 \pm 2\%$).

After acute AT₂ receptor stimulation followed by acute NOS inhibition with L-NAME, the final MAP in vehicle- and CGP-treated 2K1C/AT_{1A}^{+/+} mice was significantly lower on days 25 and 28 than on days 14 and 21 after clipping (170 ± 2 , 169 ± 2 and 167 ± 2 , 167 ± 2 vs. 178 ± 2 , 180 ± 2 and 180 ± 2 , 181 ± 3 mmHg, respectively ($p < 0.05$)), despite the fact that there were no significant differences in basal MAP.

In addition, after acute AT₂ receptor stimulation followed by acute NOS inhibition with L-NAME, the final MAP in vehicle- and CGP-treated 2K1C/AT_{1A}^{-/-} mice was significantly higher on days 25 and 28 than on days 7, 14 and 21 after clipping (90 ± 2 , 88 ± 2 and 90 ± 2 , 89 ± 1 vs. 81 ± 2 , 79 ± 1 , 81 ± 2 , 82 ± 2 and 79 ± 2 , 81 ± 2 mmHg, respectively, $p < 0.05$), which is in accordance with the finding that, in the absence of CGP, MAP in 2K1C/AT_{1A}^{-/-} mice was also higher on days 25 and 28 than on days 7, 14 and 21 after clip placement.

Discussion

To our knowledge, this is the first study evaluating the course of plasma and kidney ANG II levels in AT_{1A}^{+/+} and AT_{1A}^{-/-} mice after clipping of the renal artery in 2K1C Goldblatt hypertension. As first important finding our study provides strong evidence that AT_{1A} receptor activation plays the predominant role in the pathogenesis of 2K1C Goldblatt hypertension. Thus, targeted disruption of the AT_{1A} receptor gene in mice prevented the development of hypertension after clipping of one renal artery. This was associated with significantly lower ANG II concentrations in the clipped and non-clipped kidneys of AT_{1A} knockout mice when compared with those of AT_{1A} of wild-type mice. In contrast, plasma ANG II levels during the course of the experiment were significantly higher in sham-operated AT_{1A} knockout mice than in sham-operated AT_{1A} of wild-type mice and, moreover, clip placement did not alter plasma ANG II levels in AT_{1A}^{-/-} mice. In addition, by the end of the experiment (starting with day 24 after clipping) plasma ANG II levels were even significantly higher in 2K1C/AT_{1A}^{-/-} than

in 2K1C/AT_{1A}^{+/+} mice. We found, that plasma ANG II levels in AT_{1A}^{+/+} mice responded to clip placement with rapid increases and remained elevated until 18 days after clipping. They then returned to normal levels observed in sham-operated AT_{1A}^{+/+} mice. In addition, we found that ANG II levels in the clipped and non-clipped kidneys of AT_{1A}^{+/+} mice significantly increased after unilateral clip placement, but these increases were more profound in clipped kidneys. The findings in AT_{1A}^{+/+} mice are compatible with those previously obtained in rats, namely that plasma ANG II levels are elevated during the initial phase and return in the maintenance phase of 2K1C hypertension to levels observed in normotensive animals. Nevertheless, intrarenal ANG II concentrations in the non-clipped kidney are increased even when renin content is reduced to barely detectable levels [1,2]. Our observation that intrarenal concentrations of ANG II in the clipped kidney of 2K1C/AT_{1A}^{+/+} mice are markedly elevated is consistent with previous findings in 2K1C hypertensive rats and demonstrates the powerful well-known effect of increased intrarenal renin synthesis after clipping on the intrarenal ANG II generation in the clipped kidney [1]. Of special interest is our finding that intrarenal ANG II levels in the non-clipped kidney of 2K1C/AT_{1A}^{+/+} mice were already elevated on day 3 after clip placement suggesting that the increased ANG II concentrations in the non-clipped kidney precede the elevation of BP in these animals. Altogether, our data further emphasize the critical role of AT_{1A} receptors in determining the intrarenal ANG II content [3,5] and strongly indicate that the nonclipped kidney of 2K1C/AT_{1A}^{+/+} mice has an impaired ability to appropriately suppress intrarenal ANG II content in relation to their BP level. They support the concept that this inappropriately high intrarenal ANG II activity leads to insufficient sodium excretion by the non-clipped kidney and to a compromised pressure natriuresis which then contribute to the development and maintenance of hypertension in this model [1–3].

The second new finding of our present study documents that clipping of the renal artery in AT_{1A}^{-/-} mice does not cause hypertension. Nevertheless, it elicits a slight but significant increase in BP suggesting a minor, but still discernible, role for AT_{1B} receptors in BP regulation. The role of AT_{1B} receptors was noticeable from day 17 on after clipping, when considering MAP. However, further studies using AT₁ receptor antagonists, e.g. losartan, are needed to definitely confirm that increase in BP after clip placement in AT_{1A}^{-/-} mice is due to activation of AT_{1B} receptors.

Our notion regarding a minor, though still distinguishable role of AT_{1B} receptors in BP regulation is supported by the results of previous studies suggesting that in the absence of AT_{1A} receptors, AT_{1B} receptors mediate the effects of ANG II and contribute to the regulation of extracellular fluid volume homeostasis, renal function and BP and to the hypertensinogenic actions of ANG II [for review see 7]. Thus, it has been suggested previously that, after lowering endogenous plasma ANG II concentrations in AT_{1A}^{-/-} mice, the acute BP response to exogenous ANG II is mediated by AT_{1B} receptors [9]. It has also been demonstrated that ANG II can activate AT_{1B} receptors to stimulate calcium entry and calcium mobilization in vascular smooth cells isolated from AT_{1A}^{-/-} mice [10]. In addition, it has been suggested that the persistent ANG II-induced renal vasoconstriction in AT_{1A}^{-/-} mice is mediated via AT_{1B} receptors [11]. Moreover, using the mouse in vitro blood-perfused juxtamedullary nephron technique, in which the kidney is perfused with blood obtained from a donor rat with normal plasma ANG II concentration, Harrison-Bernard et al. [12] reported that afferent arteriolar vasoconstriction responses to low-dose ANG II were similar in AT_{1A}^{+/+} and AT_{1A}^{-/-} mice. Furthermore, Harrison-Bernard et al. [36] demonstrated in their study performed in kidneys from AT_{1A}^{-/-} and AT_{1B} receptor knockout (AT_{1B}^{-/-}) mice that vasoconstrictor responses to ANG II are mediated solely by AT_{1A} and AT_{1B} receptors present in afferent arterioles. These findings have been confirmed by studies performed in mice lacking both AT₁ receptor genes (AT₁ double nullizygotes) which showed that both AT₁ receptor subtypes mediate the afferent arteriolar vasoconstriction [37]. In addition, it has been recently reported that AT_{1B} receptors also mediate vasoconstriction in the mouse aorta [38]. Finally, Li et al. [13] found in their

recent study that ANG II infusion for 2 weeks increased SBP significantly in $AT_{1A}^{-/-}$ mice, although to a significantly lower extent than in $AT_{1A}^{+/+}$ mice (+11 vs. +55 mmHg, $p < 0.05$). Since treatment with the AT_1 receptor antagonist losartan completely blocked the rise in BP, AT_{1B} receptors are implicated in this BP response to chronic ANG II infusion.

In view of the critical importance of the intrarenal ANG II augmentation in the development of ANG II-dependent hypertension [3], low intrarenal ANG II levels combined with the absence of AT_{1A} receptors in $AT_{1A}^{-/-}$ mice may be responsible for the lack of development of hypertension after clipping the renal artery in $AT_{1A}^{-/-}$ mice. This notion is supported by the recent study performed by Crowley et al. [39]. Using a kidney cross-transplantation strategy in $AT_{1A}^{+/+}$ and $AT_{1A}^{-/-}$ mice they developed knockout mice either with specific deficiency of AT_{1A} receptors in the kidney or with normal expression of AT_{1A} receptors in the kidney but missing expression in non-renal tissues. These authors demonstrated that ANG II produces hypertension primarily through AT_{1A} receptors expressed in the kidney. Taken together, our present findings indicate that, at least in the model of 2K1C Goldblatt hypertension model, AT_{1B} receptors play a relative minor role in BP regulation and that their activation is not sufficient to mediate the hypertensinogenic actions of ANG II to cause hypertension in this model.

Of special interest are our findings regarding the development of cardiac hypertrophy and ANG II levels in heart tissue. Our data show that neither AT_{1A} receptor gene targeting nor clip placement altered ANG II concentrations in left heart ventricle. In addition, there was a tight linear correlation between heart weight and BP level indicating that the development of cardiac hypertrophy was entirely dependent on the development of hypertension. This view is also supported by results obtained by Crowley et al. [39] in systemic or kidney $AT_{1A}^{-/-}$ mice, showing that the severity of cardiac hypertrophy in ANG II-infused mice was exclusively dependent on the BP level. An important limitation of our study is the fact that we did not evaluate the concentration of ANG II in the peripheral vasculature since it is now well-known that local vascular production of ANG II plays an important role in hypertension-induced end-organ damage [40,41]

Our third major finding concerns the role of AT_2 receptors in 2K1C Goldblatt hypertension. Since present evidence suggests that activation of AT_2 receptors counteracts AT_1 receptor-mediated vasoconstrictor actions of ANG II and causes vasodilatation with a decrease in BP [14–17], it was expected that selective stimulation of AT_2 receptors should attenuate the development of 2K1C Goldblatt hypertension. However, our data show that chronic AT_2 receptor stimulation does not significantly change the course of hypertension in 2K1C $AT_{1A}^{+/+}$ mice. In addition, since it has been demonstrated that AT_2 receptor-mediated decreases in BP are more pronounced during concomitant AT_1 receptor blockade when elevated plasma ANG II levels may stimulate unopposed AT_2 receptors [14–17], one would anticipate that the BP lowering effect of AT_2 receptor stimulation should be more pronounced in $AT_{1A}^{-/-}$ mice. However, we did not observe significant changes in BP in response to chronic AT_2 receptor stimulation in either sham-operated or 2K1C $AT_{1A}^{-/-}$ mice. Moreover, acute AT_2 stimulation did not significantly influence MAP in anesthetized sham-operated or 2K1C $AT_{1A}^{+/+}$ and $AT_{1A}^{-/-}$ mice after clipping of the renal artery at any time during the course of the experiments. These data are in agreement with our previous findings that neither acute nor chronic AT_2 receptor blockade altered BP in sham-operated $AT_{1A}^{+/+}$ and $AT_{1A}^{-/-}$ mice and did not worsen the course of hypertension in 2K1C $AT_{1A}^{+/+}$ mice [8]. In addition, it has been reported that activation of AT_2 receptors inhibits renal renin synthesis and ANG II formation [42]. Therefore, we reasoned that chronic AT_2 receptor stimulation could alter plasma and tissue ANG II concentrations. However, we found that chronic CGP infusion did not change plasma or tissue ANG II levels in any of the experimental groups of $AT_{1A}^{+/+}$ and $AT_{1A}^{-/-}$ mice throughout the experiment. This finding suggests that activation of AT_2 receptors does

not play any important role in the regulation of ANG II production in sham-operated and 2K1C AT_{1A}^{+/+} and AT_{1A}^{-/-} mice.

The fourth major finding of the present study concerns the role of NO in the development of 2K1C-hypertension. Acute NOS inhibition with L-NAME led to greater increases in MAP in 2K1C/AT_{1A}^{+/+} than in sham-operated AT_{1A}^{+/+} mice. This response of MAP to NOS inhibition was significantly greater on days 7, 14 and 21 than on days 25 and 28 after clipping of the renal artery. In contrast, in all groups of AT_{1A}^{-/-} mice acute NOS inhibition elicited similar BP responses. These findings further support the notion that enhanced NOS activity plays a protective role during the early phases of hypertension in order to compensate, at least in part, the vasoconstrictor actions of elevated ANG II levels in models of ANG II-dependent hypertension [18–21]. This notion is further supported by our findings that final levels of MAP after acute NOS inhibition were lower in 2K1C/AT_{1A}^{+/+} mice on days 25 and 28 than on days 14 and 21 days after clipping despite the fact that basal MAP were significantly higher on days 25 and 28 than on days 14 and 21 after clipping. This increased NOS activity during the initial phase of 2K1C hypertension may have been caused either by the rise in BP with its greater endothelial shear stress, a potent stimulus for NO synthesis and release, or by a direct action mediated by the sustained stimulation of AT₁ receptors by ANG II [20,43–45]. Both mechanisms are compatible with our findings in AT_{1A}^{-/-} mice and point to the prominent role of intact AT_{1A} receptors in the development of 2K1C hypertension [8].

Of special interest is our observation in 2K1C/AT_{1A}^{+/+} mice that NOS activity is enhanced during the developmental phase but not in established hypertension. ANG II is a powerful stimulator of O₂⁻ generation [22] and previous studies have implicated that in chronic phases of 2K1C hypertension, when plasma ANG II levels are within the normal range, increased oxidative stress and subsequently reduced NO bioavailability contribute to the enhanced vascular responsiveness to the slow pressor effects of ANG II [23,24]. In addition, it has been proposed that ANG II-dependent hypertensive models show an increased O₂⁻ production and that the impaired release or bioavailability of NOS-dependent endothelium-relaxing factor contributes to the persistence of chronic hypertension [for review see 23]. Moreover, it has been shown that 2K1C hypertensive mice exhibit an enhanced protein expression of phosphorylated endothelial NOS (eNOS) in the thoracic aorta during the initial phase but not during the maintenance phase of hypertension [45]. In agreement with the aforementioned studies, our findings suggest that diminished NOS-dependent vasodilatation, most likely due to enhanced oxidative stress, might be an important factor contributing to the maintenance of hypertension in 2K1C/AT_{1A}^{+/+} mice. Further studies, e.g. with the O₂⁻ scavenger tempol, are needed to clarify the role of oxidative stress in the maintenance of hypertension in 2K1C/AT_{1A}^{+/+} mice.

Taken together, our present results clearly show that targeted disruption of AT_{1A} receptor gene prevents the intrarenal rise of ANG II content and the development of 2K1C Goldblatt hypertension in mice. We therefore suggest a critical role of AT_{1A} receptors in the development of hypertension in this model. In addition, we conclude from our data that AT_{1B} receptors play a relatively minor role in BP regulation and do not play a key role in the hypertensinogenic actions of ANG II after unilateral renal artery stenosis. Furthermore, since acute or chronic AT₂ receptor stimulation did not modify the development of hypertension or the BP responses to acute NOS inhibition in 2K1C hypertensive mice, activation of AT₂ receptors does not appear to play a major antagonistic role in the AT₁ receptor-mediated hypertensinogenic actions of ANG II in this mouse model of hypertension. Finally, our data suggest that an enhanced NOS activity plays a protective role by counteracting the vasoconstrictor effects of ANG II during the initial but not during the maintenance phase of high BP in 2K1C hypertensive mice.

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Figure 1.

Mean arterial blood pressure (measured by radiotelemetry) after clip placement or sham operation in conscious AT_{1A} receptor wild-type ($AT_{1A}^{+/+}$) (A) and knockout ($AT_{1A}^{-/-}$) mice (B). CGP indicates infusion of the AT_2 receptor agonist CGP-42112A via osmotic minipumps; 2K1C indicates two-kidney, one-clip Goldblatt hypertensive mice. * $P < 0.05$ versus basal values.

Figure 2.

Plasma angiotensin II (ANG II) after clip placement or sham operation in AT_{1A} receptor wild-type ($AT_{1A}^{+/+}$) and knockout ($AT_{1A}^{-/-}$) mice. CGP indicates infusion of the AT_2 receptor agonist CGP-42112A via osmotic minipumps; 2K1C indicates two-kidney, one-clip Goldblatt hypertensive mice. * $P < 0.05$ versus basal values.

Figure 3. Whole kidney angiotensin II (ANG II) content in nonclipped (A and B) and clipped (C and D) kidneys after clip placement or sham operation in conscious AT_{1A} receptor wild-type ($AT_{1A}^{+/+}$) and knockout ($AT_{1A}^{-/-}$) mice. CGP indicates infusion of the AT_2 receptor agonist CGP-42112A via osmotic minipumps; 2K1C indicates two-kidney, one-clip Goldblatt hypertensive mice.
* $P < 0.05$ versus basal values.

Figure 4.

Basal mean arterial pressure in AT_{1A} receptor wild-type ($AT_{1A}^{+/+}$) (A) and knockout ($AT_{1A}^{-/-}$) mice (B) after clip placement or sham operation. Changes in mean arterial pressure (C and D) after acute nitric oxide synthase inhibition achieved by intravenous (i.v.) infusion of nitro-L-arginine-methyl-ester (L-NAME) in AT_{1A} receptor wild-type ($AT_{1A}^{+/+}$) and knockout ($AT_{1A}^{-/-}$) mice. CGP indicates infusion of the AT_2 receptor agonist CGP-42112A via osmotic minipumps; 2K1C indicates two-kidney, one-clip Goldblatt hypertensive mice. * $P < 0.05$ versus unmarked values. # $P < 0.05$ versus * marked values.