Hypertension

Novel Role for Inhibitor of Differentiation 2 in the Genesis of Angiotensin II–Induced Hypertension

Petra Gratze, MD; Ralf Dechend, MD; Carolin Stocker, PhD; Joon-Keun Park, PhD; Sandra Feldt, PhD; Erdenechimeg Shagdarsuren, MD; Maren Wellner, PhD; Faikah Gueler, MD; Song Rong, MD; Volkmar Gross, MD; Michael Obst, PhD; Ralph Plehm, MS; Natalia Alenina, PhD; Ana Zencussen, PhD; Jens Titze, MD; Kersten Small, PhD; Yoshifumi Yokota, MD, PhD; Martin Zenke, PhD; Friedrich C. Luft, MD; Dominik N. Muller, PhD

Background—Angiotensin (Ang) II–induced target-organ damage involves innate and acquired immunity. Mice deficient for the helix-loop-helix transcription factor inhibitor of differentiation (Id2−/−) lack Langerhans and splenic CD8a+ dendritic cells, have reduced natural killer cells, and have altered CD8 T-cell memory. We tested the hypothesis that an alteration in the number and quality of circulating blood cells caused by Id2 deletion would ameliorate Ang II–induced target-organ damage.

Methods and Results—We used gene-deleted and transgenic mice. We conducted kidney and bone marrow transplants. In contrast to Ang II–infused Id2−/−, Id2+/− mice infused with Ang II remained normotensive and failed to develop albuminuria or renal damage. Bone marrow transplant of Id2−/− bone marrow to Id2−/− mice did not restore the blunted blood pressure response to Ang II. Transplantation of Id2−/− kidneys to Id2−/− mice also could not prevent Ang II–induced hypertension and renal damage. We verified the Ang II resistance in Id2−/− mouse in a model of local tissue Ang II production by crossing hypertensive mice transgenic for rat angiotensinogen with Id2−/− or Id2+/− mice. Angiotensinogen-transgenic Id2−/− mice developed hypertension, albuminuria, and renal injury, whereas angiotensinogen-transgenic Id2−/− mice did not. We also found that vascular smooth muscle cells from Id2−/− mice showed an antisenescence phenotype.

Conclusions—Our bone marrow and kidney transplant experiments suggest that alterations in circulating immune cells or Id2 in the kidney are not responsible for Ang II resistance. The present studies identify a previously undefined role for Id2 in the pathogenesis of Ang II–induced hypertension. (Circulation. 2008;117:2645-2656.)

Key Words: hypertension ■ angiotensin ■ immune system ■ kidney ■ vasoconstriction

Hypertension increases the risk of stroke, renal failure, and ischemic heart disease.1 Angiotensin (Ang) II, aside from increasing peripheral vascular resistance and regulating extracellular volume through aldosterone, contributes to target-organ damage.2 The injurious effects of Ang II are mediated by the angiotensin II type 1 (AT1) receptor, which is widely expressed.3,4 Furthermore, Ang II activates the transcription factor nuclear factor-κB and promotes inflammation.5,6 We and others have found that suppression of the immune system ameliorates Ang II–induced target-organ damage in a blood pressure–independent manner.5–8 However, the relative contributions of the immune system and hypertension per se in the development of target-organ damage are debated. The helix-loop-helix transcription factor, inhibitor of differentiation (Id) 2, acts as a dominant-negative antagonist of other helix-loop-helix transcription factors, through heterodimerization and thereby inhibition of DNA binding. Id2 plays an important role in the differentiation, proliferation, invasion, and apoptosis of various cell types.9,10 Very recently, Id2 was found to be involved in a transcriptional network that differentiates ventricular myocytes into specialized conduction system cells. Id2-deficient...
**Blood Pressure**

A graph showing the effect of Ang II infusion on mean arterial pressure over days. The graph compares mean arterial pressure in two groups: $ld2^{+/+}$ and $ld2^{-/-}$ mice. The $ld2^{+/+}$ mice show a significant increase in blood pressure compared to the baseline, while the $ld2^{-/-}$ mice show a slight increase.

**Albuminuria**

A bar graph showing the effect of Ang II infusion on albuminuria in two groups: $ld2^{+/+}$ and $ld2^{-/-}$ mice. The $ld2^{+/+}$ mice show a significant increase in albuminuria, while the $ld2^{-/-}$ mice show a slight increase.

**AT1A Receptor in Renal Vessels**

Images showing the expression of AT1A receptor in renal vessels of $ld2^{+/+}$ and $ld2^{-/-}$ mice. The $ld2^{-/-}$ mice show a significantly lower expression of the receptor.

**AT1A Receptor mRNA in VSMC**

A bar graph showing the expression of AT1A receptor mRNA in VSMC in $ld2^{+/+}$, $ld2^{-/-}$, and $AT1AR^{-/-}$ mice. The $ld2^{-/-}$ mice show a significantly lower expression of the receptor mRNA.

**AT1B Receptor mRNA in VSMC**

A bar graph showing the expression of AT1B receptor mRNA in VSMC in $ld2^{+/+}$, $ld2^{-/-}$, and $AT1AR^{-/-}$ mice. The $ld2^{-/-}$ mice show a significantly lower expression of the receptor mRNA.

**i.v. Bolus Injections**

A bar graph showing the change in mean arterial blood pressure in $ld2^{+/+}$ and $ld2^{-/-}$ mice after i.v. bolus injections of PE (20 ng, 60 ng, 600 ng) and Ang II (6 μg, 375 ng). The $ld2^{-/-}$ mice show a significantly higher mean arterial blood pressure compared to the $ld2^{+/+}$ mice.
Id2−/− mice show structural and functional conduction system abnormalities, including left bundle-branch block.11

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Changes in vascular smooth muscle cell (VSMC) differentiation play a key role in vascular diseases, yet the mechanisms that control VSMC differentiation are largely undefined. A potential role for Id2 in the control of VSMC differentiation and phenotypic modulation has been suggested. Both enhanced VSMC growth and inhibition of VSMC differentiation marker genes have been observed in response to Id2 overexpression.12,13 Id2−/− mice lack Langerhans and splenic CD8α+ dendritic cells, have reduced natural killer cells, and have altered CD8 T-cell memory.14–17 We used Id2−/− mice to analyze whether or not alterations in circulating blood cells and function would ameliorate Ang II–induced target-organ damage.

Methods

Experimental Animals

Id2 mutants (Id2−/−) and Id2+/+ controls were backcrossed more than 7 generations on an NMRI (Naval Medical Research Institute) background. Mice were generated as described previously18 and were bred and maintained in the animal facility of the Max Delbrück Center. We followed the American Physiological Society guidelines for animal care, and the Animal Use Committee of Berlin approved the studies.

In protocol I, male age-matched Id2−/− and Id2+/+ controls were continuously infused with Ang II (1.44 mg·kg−1·d−1 SC by minipump infusion for 2 weeks; n=8 and 12, respectively). To dissect the role of circulating from tissueId2, we performed protocol II. After lethal irradiation, bone marrow from Id2−/− mice was transplanted to Id2−/− controls (n=11) or Id2+/+ mice (n=7), or bone marrow from Id2−/− mice was transplanted to Id2+/+ controls (n=12). In protocol III, we transplanted Id2−/− kidneys to Id2+/+ mice (n=5) as described in detail elsewhere.19 Mice in protocols II and III were infused with Ang II as described in protocol I. In protocol IV, we crossed WT×Id2−/− mice with rat angiotensinogen (Aogen)-transgenic (TGm) Id2−/− mice to investigate the role of tissue Ang II in this model (n=30 for TGm×Id2−/− and n=17 for TGm×Id2+/+). Mice transgenic for rat Aogen have been described elsewhere.19 Nontransgenic age-matched WT×Id2−/− (n=30) and WT×Id2+/+ (n=15) mice were used as controls. During our initial analysis, we compared Aogen-transgenic Id2−/− mice with Aogen-transgenic Id2+/+ mice to evaluate the impact of Id2 heterozygosity on target-organ damage. We found no difference with regard to mean arterial pressure (MAP), cardiac hypertrophy, albuminuria, and renal fibrosis between the groups (Data Supplement, Figure I). Therefore, we continued our further analysis with Id2−/− mice as controls.

MAP was measured by radiotelemetry via an intracarotid catheter.20 Baseline MAP was averaged over a 3-day period 10 days after implantation of the telemetry transmitter. In protocols I, II, and III, MAP was averaged from day 8 to day 10 after Ang II infusion (n=5 per group). Urine samples were collected during a 24-hour period 2 days before and 11 days after Ang II infusion. We measured urinary albumin by ELISA (CellTrend, Luckenwalde, Germany). Animals were euthanized at week 13 for protocols I and IV and at week 17 for protocols II and III, respectively.

Immunohistochemistry, Histology, Cell Culture, and Reverse-Transcription Polymerase Chain Reaction

Immunohistochemistry and histology were performed as described previously.21 Details about the procedure, including the quantification of infiltrated cells, are given in the Data Supplement. Aortic VSMCs were isolated from Id2−/−, Id2+/+ mice and AT1A−/− mice as described previously.21 and passages 5 to 9 were used for the experiments. For details about the cellular senescence analyses and the TaqMan real-time polymerase chain reaction experiments, please refer to the Data Supplement.

Statistical Analysis

Data are presented as mean±SEM. Statistically significant differences in mean values were tested by ANOVA, and when significance was indicated, the Scheffé post hoc test was used to discern differences between specific groups. In Figure 1A, the area under the curve was calculated for each individual experiment (n=5 for Id2−/− versus n=6 for Id2+/+). Thereafter, statistical differences were tested by ANOVA and post hoc Scheffé test. In addition, we also tested blood pressure differences by repeated-measures ANOVA followed by Scheffé test. In the renal transplant experiments in the present study, we used paired t test analysis to evaluate statistical differences for pre- and post-Ang II infusion. A value of P<0.05 was considered statistically significant. The data were analyzed with Statview statistical software.

The authors had full access to the data and take full responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results

Id2−/− Mice Are Resistant to Ang II–Induced Hypertension

To investigate the effect of Id2 on the development of hypertension and target-organ damage, we infused Ang II in Id2−/− and Id2+/+ mice. During the first day of Ang II infusion, both groups showed a transient increase in MAP that might have been due to postoperative stress and/or Ang II (Figure 1A). Thereafter, blood pressure in Id2−/− mice returned to normal and remained normal until the end of Ang II infusion. In contrast, Ang II induced significant hypertension in Id2+/+ mice, which persisted during the entire protocol (Figure 1A). The areas under the blood pressure curves, which indicate the pressure load over the entire experiment, were significantly different between the 2 groups. Furthermore, we found that from day 2 after Ang II infusion, blood pressure in Id2−/− mice was significantly increased at each time point compared with Id2+/+ mice. After Ang II treatment, albuminuria was significantly higher in Id2−/− mice than in Id2+/+ mice (240±37 versus 33±9 µg/d, respectively, P<0.05; Figure 1B). Masson trichrome stains of kidney

Figure 1. Role of Id2 in Ang II–induced hypertension and renal damage, A, Telemetric. MAP recording during 14 days of Ang II infusion. Id2−/− mice were not able to maintain high MAP over the infusion period. B, Ang II–infused Id2−/− mice showed significantly increased 24-hour albuminuria compared with Id2+/+ mice. C, Kidney damage after Ang II infusion. Masson trichrome stain shows that Id2−/− mice developed media hypertrophy, extracellular matrix depositions, and tubular changes, whereas no histopathological changes were observed in Id2+/+ mice. D, AT1 receptor immunoreactivity in renal vessels was higher in Id2−/− than in Id2+/+ mice. E, AT1A receptor mRNA expression was higher in Id2−/− than in Id2+/+. F, AT1A receptor mRNA expression was higher in Id2−/− than in Id2+/+ VSMCs. Nevertheless, Id2−/− VSMCs showed abundant expression compared with AT1A−/− VSMCs. G, Id2−/− mice had little expression. H, Dose-dependent blood pressure increase with intravenous bolus injections of phenylephrine (PE) and Ang II in Id2−/− and Id2+/+ mice. Results are mean±SEM. *P<0.05 vs Id2+/+ mice.
sections showed increased vascular and tubular damage and greater matrix production in Ang II–treated Id2+/− mice than in Id2−/− mice (Figure 1C). Ang II–treated Id2+/− mice showed normal histology without perivascular matrix formation.

We next investigated whether altered renal gene expression of renin-angiotensin system components was responsible for the effect on blood pressure and renal damage in Id2−/− mice. Total renal AT1A and AT1B receptor, AT2 receptor, AT1 receptor–associated protein (ATRAP), and ACE-2 mRNA expression was not altered either by Id2 deficiency or by Ang II infusion (Data Supplement Figure II). Interestingly, AT1A immunoreactivity from renal Id2−/− vessels was significantly higher than from Id2+/− vessels (Figure 1D). AT1A but not AT1B mRNA was decreased in primary VSMCs from Id2−/− compared with Id2+/− (Figure 1E and 1F). To better understand the qualitative change, we compared AT1 receptor expression with VSMCs isolated from AT1A receptor gene–deleted mice. AT1A and AT1B receptor expression was much higher in Id2−/− VSMCs than in AT1A receptor–deficient VSMCs (Figure 1E and 1F).

We next determined whether vessels from Id2−/− mice had a major defect in vascular contraction. Acute intravenous bolus injections of either phenylephrine or Ang II increased blood pressure to the same magnitude in both Id2 genotypes (Figure 1G). Furthermore, the lower AT1A receptor expression in renal vessels had no effect on the Ang II pressure response in acute experiments (Figure 1G). In addition, chronic administration of the nitric oxide synthase inhibitor N^γ^-nitro-L-arginine methyl ester (L-NNAME) increased MAP in Id2−/− mice (from 115±1 to 120±0.4 mm Hg after L-NNAME) and Id2+/− mice (from 105±3 to 121±8 mm Hg after L-NNAME). These data indicate that Id2−/− mice are able to respond to vasoactive stimuli.

**Tissue-Specific Expression of Id2 and Blood Pressure Regulation**

To dissect the role of Id2 in immune cells compared with other cell types in the development of hypertension, we performed bone marrow transfer after lethal irradiation (Figure 2A). After full engraftment, all 3 groups were normotensive (Figure 2B). Infusion of Ang II for 2 weeks in Id2+/− mice with bone marrow from Id2+/− or Id2−/− mice resulted in a significantly increased MAP (146±5 and 146±7 mm Hg, respectively, P<0.05; Figure 2B). In contrast, Ang II–induced hypertension in Id2−/− mice with bone marrow from Id2+/− mice was not sustained (Figure 2B). Furthermore, both hypertensive groups developed albuminuria (267±53 and 210±70 μg/d; Figure 2C) and histological alterations (Figure 2D), whereas Id2+/− mice with normal circulating blood cells were protected against albuminuria (26±6 μg/d, P<0.05) and did not develop renal pathology. The data indicate that Id2 in tissues, rather than the alterations in immune cells, is responsible for the reduced hypertensive response and tissue damage in Id2−/− mice.

To test the hypothesis that Id2 deficiency in the kidney plays a major role in the regulation of blood pressure, we performed kidney transplant experiments (Figure 2E). Uninephrectomized Id2+/− mice that received Id2−/− kidneys were normotensive (114±3 mm Hg). After Ang II infusion, MAP increased 51 mm Hg to 165±10 mm Hg (P<0.01 versus baseline; Figure 2F). Ang II infusion in uninephrectomized Id2+/− mice with Id2+/− kidneys resulted in a 60-fold increase in albuminuria compared with baseline (P<0.05; Figure 2G). Histology of the Ang II–infused transplanted Id2−/− kidneys showed severe renal damage (Figure 2H). These results suggest that renal Id2 does not mediate hypertension and renal damage.

**Effect of Local Ang II on Blood Pressure in Id2 Mutants**

Local Ang II formation depends on local synthesis and could possibly give results different from long-term Ang II infusion. We therefore next validated our results using a transgenic mouse (TGM) overexpressing rat angiotensinogen. In this model, mouse renal cleaves rat angiotensinogen to generate Ang I and II, which leads to hypertension and target-organ damage.20 To determine whether Id2 deficiency would lead to a similar phenotype in a model with endogenously elevated tissue Ang II, we mated TGM×Id2+/− mice with WT×Id2−/− mice (Figure 3A). Here, we found that TGM×Id2+/− mice were hypertensive compared with TGM×Id2−/− mice and the respective nontransgenic controls (141±3 versus 115±3 versus 107±2 and 107±2 mm Hg at week 12, respectively, P<0.05; Figure 3B). Furthermore, TGM×Id2+/− mice developed significantly higher albuminuria (192±31 μg/d at week 12; Figure 3C). In contrast, albuminuria was normal in TGM×Id2+/− mice and nontransgenic controls (26±4 versus 19±2 and 26±4 μg/d at week 12, respectively; Figure 3C). TGM×Id2−/− mice showed media hypertrophy, extracellular matrix deposition, and tubular damage, whereas TGM×Id2+/− mice showed no obvious pathological changes (Figure 3D). Histology in nontransgenic control groups was normal. Consistent with these histological data, we found significantly increased levels of renal collagen IV (2.4±0.3 versus 0.4±0.3 versus 0.5±0.2 versus 0.2±0.0 arbitrary units [AU], P<0.05 TGM×Id2+/− versus other groups), fibronectin (2.0±0.3 versus 0.3±0.2 versus 0.5±0.3 versus 0±0 AU, P<0.05 TGM×Id2+/− versus other groups), and CD4 T-cell and macrophage infiltration in TGM×Id2+/− compared with TGM×Id2−/−, WT×Id2−/−, and WT×Id2−/− mice (Figure 3E and 3F). To exclude the possibility that Id2 deficiency influenced the expression of renin-angiotensin system components in TGM mice in the present study, we screened for rat angiotensinogen (TGM×Id2+/− 48±8 versus TGM×Id2+/− 35±10 AU; P=NS) and AT1 receptor expression (TGM×Id2+/− 7.6±1.6 versus TGM×Id2−/− 8.5±1.2 AU; P=NS) in the liver (TGM×Id2+/− 6.3±1.2 versus TGM×Id2−/− 7.8±1.2 AU; P=NS) and in the heart (TGM×Id2+/− 2.0±0.5 versus TGM×Id2−/− 2.3±0.3 AU; P=NS), as well as in the kidney.

**Id2 Deficiency Shows Increased PPAR-γ and Decreased PPAR-α Expression in VSMCs**

We next wondered whether altered gene expression of Id2−/− VSMC might be involved in our phenotype. Therefore, we isolated primary aortic VSMCs from Id2+/− and Id2−/− mice, performed Affymetrix gene-array analysis, and identified genes...
Figure 2. Tissue-specific expression of Id2 and blood pressure regulation. Telemetric MAP recording and albuminuria in bone marrow (BM)-transplanted mice. A, Schematic study design. After lethal irradiation, BM from Id2+/- mice was transplanted into Id2+/- or Id2-/- mice, and BM from Id2+/- mice was transplanted into Id2-/- mice. B, MAP in all groups before and after Ang II infusion (MAP average from day 8 to 11; *P<0.05 vs baseline and #P<0.05 vs other Ang II-infused groups). C, Albuminuria concentrations after 2 weeks of Ang II infusion (*P<0.05 vs other groups). D, Renal damage was diminished in Id2-/- mice with Id2+/- BM. Masson trichrome stain of kidney paraffin sections from Ang II–treated mice. Histopathological changes were observed in kidneys of Id2-/- mice with BM from either Id2+/- or Id2-/- mice. Normal morphology was observed in Id2-/- mice with Id2+/- BM. E, Schematic study design of kidney transplant experiments. F, Uninephrectomized Id2+/- mice, which received an Id2-/- kidney, were normotensive before Ang II infusion but severely hypertensive thereafter. G, In parallel, albuminuria was 60-fold increased after Ang II. H, After Ang II, transplanted Id2-/- kidneys showed severe renal damage. Results are mean±SEM.
Figure 3. Role of Id2 in angiotensinogen-transgenic mice. A, Schematic study design. Id2−/− mice were crossed with Id2+/− mice overexpressing rat angiotensinogen (TGM x Id2+/−). B, TGM x Id2+/− mice developed significantly higher MAP. TGM x Id2−/− and nontransgenic control groups (WT x Id2+/− and WT x Id2−/−) were normotensive. C, Albuminuria was significantly elevated in TGM x Id2+/− mice compared with TGM x Id2−/− and respective nontransgenic control groups. D, TGM x Id2+/− showed media hypertrophy, extracellular matrix deposition, and tubular damage. No histopathological changes were observed in TGM x Id2−/− mice or the nontransgenic groups. E, Semiquantitative scoring of renal CD4 T cells and (F) F4/80-positive macrophages. Results are mean±SEM. *P<0.05 vs TGM x Id2+/−.
from the peroxisome proliferator activator protein (PPAR) family. First, we confirmed the absence of Id2 mRNA in Id2−/− VSMCs by TaqMan reverse transcription–polymerase chain reaction (Figure 4A). PPAR-γ expression was 8-fold higher in Id2-deficient VSMCs than in Id2+/- control cells. In contrast, PPAR-α expression (C) was reduced by 3-fold, and PPAR-β expression (D) was unchanged. Results are mean±SEM. *P<0.05 vs Id2+/− VSMCs.

Figure 4. Role of Id2 in PPAR-γ expression in VSMCs. Aortic VSMCs from Id2-deficient (Id2−/−) and Id2+/- control mice were analyzed. A, Id2 mRNA expression was absent in Id2−/− VSMCs compared with Id2+/- VSMCs. Loss of Id2−/− expression resulted in 8-fold increased PPAR-γ expression (B) compared with heterozygous control cells. In contrast, PPAR-α expression (C) was reduced by 3-fold, and PPAR-β expression (D) was unchanged. Results are mean±SEM. *P<0.05 vs Id2+/− VSMCs.

Id2 Deficiency Protects VSMCs From Cellular Senescence

We also identified a cluster of genes involved in the regulation of cellular senescence, which we investigated in more detail. Cellular senescence can be defined as cell-cycle arrest that accompanies the exhaustion of replication potential. Senescent cells have a characteristic morphology. Furthermore, a low proliferation rate, marker expression of senescence-associated (SA)-β-gal activity, and a typical pattern of cell-cycle regulators are also features of senescence. During the culturing process in higher passages (>6), Id2−/− and Id2+/− VSMCs became enlarged in morphology, a characteristic of the senescence phenotype, whereas Id2+/− VSMCs were smaller and flatter, with less cell-to-cell contact (Figure 5A). To ascertain whether Id2 is involved in senescence, we examined SA-β-gal activity, a biomarker for cellular senescence. At pH 6, SA-β-gal activity was increased in Id2+/− VSMCs compared with Id2+/− VSMCs (Figure 5B). The progressive decrease in the proliferation potential followed by irreversible growth arrest is an important feature of senescence. We determined the level of bromodeoxyuridine incorporation after 24-hour starvation. Bromodeoxyuridine incorporation into the DNA of Id2+/− VSMCs was decreased compared with Id2+/+ VSMCs (Figure 5C). These results were confirmed by cell proliferation analysis (Figure 5D). To characterize the nature of cell-cycle arrest, we examined
expression of cell-cycle regulatory proteins. We found that the presence of Id2 deficiency resulted in decreased mRNA expression of p16INK4a, p21Cip1, collagen 1a1, and bone morphogenetic protein-4 (Figure 5E).

Discussion

The novel finding in the present study is that the helix-loop-helix transcription factor Id2 plays a major role in the genesis of hypertension in response to Ang II. We showed in 2 Ang...
II–dependent models that Id2−/− mice were resistant to hypertension and did not develop target-organ damage, even though their response to acute vasoconstrictors appeared to be preserved. Angiotensinogen-transgenic mice with additional Id2 deficiency and Id2−/− mice infused with Ang II showed normal blood pressure and were protected from renal damage. In contrast, angiotensinogen-transgenic Id2+/− and Id2−/− mice infused with Ang II developed hypertension with elevated albuminuria, renal fibrosis, and inflammatory cell infiltration. Reconstitution of immune cells by bone marrow transplant did not restore the hypertension caused by Ang II in Id2−/− mice, which indicates that Id2 in circulating blood cells, regardless of their Id2 expression, does not modulate blood pressure. Kidney transplant experiments demonstrated that extrarenal Id2 is most likely responsible for the lack of blood pressure sensitivity. Alternatively, the vessel wall could be a candidate tissue that might have been responsible for the insensitivity to Ang II–induced hypertension. Id2 deficiency resulted in an altered gene expression of PPAR-α and -γ in primary VSMCs. Furthermore, the Id2 deficient-VSMCs showed an antisenescence phenotype.

Ang II plays a major role in the pathogenesis of cardiovascular disease. Most of the actions of Ang II (for instance, regulation of blood pressure and water/salt balance) are mediated via the AT1 receptor. We first wondered whether the lack of Ang II sensitivity to maintain hypertension was solely affected by a simple downregulation of the AT1 receptor. This speculation seemed quite plausible because Aoki et al. reported that mice lacking Id2 developed hydronephrosis and had reduced AT1 receptor expression in
the pelvis and ureter. These mice were bred on a mixed 129Sv background. In contrast, Id2-/- mice in the present study, which were bred on a pure NMRI background, did not develop hydropnephrosis and had unchanged AT1,- receptor mRNA expression in heart, liver, and total kidney. Therefore, genetic background might have been responsible for the development of hydropnephrosis. When we performed a detailed analysis in the kidney and isolated VSMCs, we found the AT1,- receptor was significantly reduced in renal vessels and VSMCs. Nevertheless, AT1A receptor mRNA was abundantly expressed in Id2-/- VSMCs compared with AT1A,- receptor VSMCs. In contrast, AT1B receptor expression in the kidney and in VSMCs was not affected by Id2 deficiency. Apparently, the remaining vascular AT1,- and increased AT1B receptor expression was sufficient for Ang II–induced vasoconstriction, because acute Ang II injections increased blood pressure to a similar extent in Id2-deficient and control mice. These results demonstrate that short-term Ang II–AT1 receptor signaling leads to vasoconstriction, whereas long-term blood pressure is impaired in Ang II-treated Id2-/- mice.

A recent study by Crowley and colleagues22 showed a very similar blood pressure profile as observed in the present study. They transplanted AT1A,-/ receptor kidneys into wild-type mice and observed a short transient rise in blood pressure after Ang II administration that could not be maintained over time. Increased natriuresis appeared to be the reason for the phenotype.22 We performed kidney transplant to address the hypothesis that Id2 deficiency in the kidney might have promoted the blood pressure insensitivity. Uninephrectomized Id2-/- mice that received Id2-/- kidneys were normotensive. After Ang II infusion, these mice showed a 51-mm Hg increase in MAP, severe albuminuria, and renal damage. In contrast to AT1A,-/ receptor transplant experiments, kidney-transplanted mice in the present study had a high total body sodium content (Jens Titze, unpublished data, 2007). One obvious difference between the 2 studies is the AT1 receptor expression level. Although AT1,- receptor knockout mice are deficient in all kidney cell types (vessels, tubules, and glomeruli), the Id2-/- mice in the present study had only decreased AT1 receptor expression in vessels, which was not sufficient to promote sodium excretion. These data clearly indicate that extrarenal Id2 deficiency is responsible for the phenotype.

Id2 contributes to tumorigenesis by inhibiting cell differentiation and stimulating proliferation;24 however, few studies have addressed the role of Id2 in cardiovascular disease. Matsumura et al13 used a rat carotid balloon injury model and found that Id2 was expressed in a time-dependent pattern similar to the kinetics of cell proliferation. They overexpressed Id2 in smooth muscle cells in vitro and demonstrated that the Id2 protein is integrated into the cell-cycle regulatory cascade that leads to smooth muscle cell proliferation.13 Interestingly, PPAR-γ agonist treatment reduced expression of Id2 in smooth muscle cells.25 PPAR-γ agonists have been reported to inhibit neointima formation after balloon injury in association with decreased DNA synthesis.26 PPAR-γ might inhibit vascular injury by reducing Id2. With regard to blood pressure, several studies in animal and humans with metabolic syndrome have shown that PPAR-γ agonists can reduce blood pressure or protect against the development of hypertension.27–29 To our surprise, we found that VSMCs from Id2-deficient mice showed 8-fold greater PPAR-γ and 3-fold decreased PPAR-α expression compared with control VSMCs. Very recently, Tordjman et al30 demonstrated that transgenic mice with high Ang II and PPAR-α deficiency could not develop hypertension. Therefore, we speculate that PPAR-α and -γ might have contributed to the lack of blood pressure sensitivity and vessel damage after Ang II.

The primary goal of the present study was to analyze the role of dendritic, natural killer, and memory CD8 T cells in Ang II–induced target-organ damage, because these cell types are either altered or absent in Id2-/- mice.14,15,17 There is increasing evidence that the immune system contributes actively to the pathogenesis of hypertension/Ang II–induced target-organ damage. We and others have demonstrated that immunosuppressive treatment could ameliorate target-organ damage in various nonimmune models of renal damage.7,31,32 Ang II activates innate and acquired immunity via the AT1,- receptor.33–35 We have shown previously that Ang II promotes dendritic cell migration but not maturation.7 Very recently, others reported that T cells and natural killer cells possess a functional renin-angiotensin system.36 To examine the role of Langerhans dendritic, natural killer, and memory T cells, we reconstituted the lacking immune cells by bone marrow transplant. Our results in Id2-/- mice reconstituted with wild-type bone marrow or Id2-/- mice with bone marrow from Id2-/- mice demonstrated that none of the 3 cell types played a major role in the genesis of hypertension and target-organ damage in this model. We were surprised, because depletion of natural killer cells reduces atherosclerotic damage.37

Despite intensive research, the relative contributions of the kidney, cardiovascular regulation, and the brain to the development of hypertension are still not fully understood. Vascular resistance is increased in hypertensive patients, and vasodilators such as calcium channel blockers and other vasodilatory drugs are generally effective in lowering blood pressure.38,39 Our speculation that Id2 would in particular modulate target-organ damage by influencing bone marrow–derived cells was not supported by our experiments. To our surprise, Id2 by its absence precluded not only target-organ damage but also an increase in blood pressure. The bone marrow transplant experiments did not provide an explanation. The kidney transplant experiments were negative, but they were positive in the sense that after transplantation, the same phenotype persisted. Alternatively, vascular cells might play a role in the genesis of hypertension. We isolated primary VSMCs and performed Affymetrix gene expression profiling. We found a cluster of genes, which indicated that Id2 deficiency might regulate vascular senescence. Id2-/- and Id2-/+ VSMCs, in contrast to Id2-/- VSMCs, showed increased SA-β-Gal activity, typical cell morphology, reduced cell proliferation, increased expression of p16INK4a and p21Cip1, and a lower level of cyclin A, all of which are features of cellular senescence. Interestingly, PPAR-α activation induces p16INK4a and PPAR-α gene-deleted VSMCs have decreased p16INK4a expression.40 Possibly, decreased PPAR-α expression in Id2-/- VSMCs led to decreased p16INK4a expression.
and thus regulated vascular senescence. The bone morphogenetic protein-4/transforming growth factor-β signaling pathway is also known to be involved in the promotion of senescence.41 We found decreased bone morphogenetic protein-4 expression in Id2-deficient VSMCs. Thus, alteration in the bone morphogenetic protein-4/transforming growth factor-β/Id2 axis might also have contributed to the antisenescence phenotype. We are aware that the present data do not provide a causal link between vascular senescence and the genesis of hypertension. Nevertheless, there is increasing evidence that vascular senescence contributes to the pathogenesis of atherosclerosis.42,43

We identified a new role for the helix-loop-helix transcription factor Id2 in regulation of blood pressure and renal damage. The role of vascular senescence in the genesis of hypertension is an attractive speculation but needs to be addressed in the future. In addition, the identification of Id2 target genes might enable a better understanding of the long-term regulation of Ang II–induced vasoconstriction and target-organ damage. Finally, Id2 plays an important regulatory role in immunity, such as dendritic and T-helper cell function. Immune mechanisms are unfolding in terms of Ang II–induced hypertension and vascular dysfunction. A recent report on the role of the T-cell in Ang II–mediated effects supports this view.44 The present study points in a similar direction.

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Disclosures

None.

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Novel Role For Inhibitor Of Differentiation Id2 In The Genesis Of Angiotensin II-Induced Hypertension

Running head: Id2 and hypertension

Petra Gratze, MD¹, Ralf Dechend, MD¹, Carolin Stocker¹, Joon-Keun Park, PhD³, Sandra Feldt¹, Erdenechimeg Shagdarsuren, MD⁶, Maren Wellner, PhD¹, Faikah Gueler, MD³, Song Rong, MD³, Volkmar Gross, MD², Michael Obst, PhD², Ralph Plehm, MS², Natalia Alenina, PhD², Ana Zenclussen, PhD⁴, Jens Titze, MD⁸, Kersten Small, PhD⁹, Yoshifumi Yokota, MD, PhD⁷, Martin Zenke, PhD⁵, Friedrich C. Luft, MD¹,², and Dominik N. Muller, PhD¹,²

¹Medical Faculty of the Charité, Experimental and Clinical Research Center, Franz Volhard Clinic, and HELIOS Clinic, Berlin Germany
²Max Delbrück Center for Molecular Medicine, Berlin-Buch, Germany
³Medical School of Hannover, Hannover, Germany
⁴Institute of Medical Immunology, Medical Faculty of the Charité, Berlin, Germany
⁵Institute for Biomedical Engineering, Aachen University Medical School, Germany
⁶Institute for Molecular Cardiovascular Research, RWTH Aachen University, Germany
⁷Department of Molecular Genetics, School of Medicine, University of Fukui, Fukui, Japan
⁸Friedrich-Alexander-University, Erlangen, Nürnberg, Germany
⁹Merck&Co. Inc., Research Laboratories, Rahway, NJ, USA

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Dominik N. Muller, PhD
Experimental and Clinical Research Center
Lindenberger Weg 80
13125 Berlin, Germany Tel: +49 30 9406 4581, Fax: +49 30 9406 4220
Email: dominik.mueller@mdc-berlin.de
Methods:

Immunohistochemistry and histology

Ice-cold acetone-fixed cryosections (6 µm) were stained using immunofluorescence techniques as described earlier.\textsuperscript{1,2} The sections were incubated with the monoclonal antibodies anti-CD4, anti-F4/80 (both serotec, UK) and the polyclonal antibodies anti-AT1\textsubscript{A} receptor (N10; Santa Cruz) anti-fibronectin (Paesel, Frankfurt, Germany) and anti-collagen IV (Southern Biotechnology, Birmingham, USA). The sections were analyzed with a Zeiss Axioplan-2 microscope (Carl Zeiss) and Axio Vision 2 multichannel image processing system (Carl Zeiss). Semi-quantitative scoring of infiltrated cells was performed in a blinded fashion without knowledge of the origin of the specimens. Since cell infiltration in mice is not distributed uniformly, but rather in cell cluster, we developed the following scoring system with a grading from 0-3. Semi-quantification analyses were performed in exclusively in cortical areas. The whole kidney was divided in 25 areas. Each area was evaluated whether or not a cluster of cells infiltrated. If all 25 areas showed infiltrated cells, the specimen was set as 100 %. If no area was positive it was set as 0%.

Scores:

0: No cells and/or cluster of cells were detected in the kidney.
1: Up to 25% showed infiltrated cells.
2: 25-50% showed infiltrated cells.
3: > 50% showed infiltrated cells.

Paraffin embedded kidney sections were stained with Masson’s trichrome stain by clinical routine protocol.
**Vascular smooth muscle cell (VSMC) culture**

VSMCs were cultured in smooth muscle basic medium (SmBM, Clonetics) supplemented with insulin, FGF, EGF, and 10% FBS at 37°C in a humidified 5% CO₂ – 95% air atmosphere. VSMCs from 5 to 9 passages were subjected to each of the following experiments.

**Immuncytochemical analyses**

VSMC were grown on a glass cover slip and cultured as described above. To examine the expression of α-smooth muscle actin antigen, the cells were fixed with 4% paraformaldehyde in PBS for 10 minutes and permeabilized with methanol. VSMCs were then incubated with a 1:40 dilution of mouse polyclonal anti-α-smoothmuscle-Actin (DAKO, Germany), followed by incubation with a 1:100 dilution of AlexaFluor 488-conjugated mouse anti-mouse IgG (Molecular Probes). The specimens were observed with a Nikon Diaphot 300 fluorescence microscope.

**Quantitative TaqMan RT-PCR**

Total kidney and cardiac RNA was isolated using the TRIZOL followed Qiagen protocol. Total RNA was isolated from VSMCs using an RNase kit (Qiagen) according to the manufacturer's protocol and treated with DNase. RNA yields were determined photometrically using a Nanodrop ND-1000 Spectrophotometer (NanoDrop Technologies, Germany). TaqMan RT-PCR was performed as described previously. Each sample was analyzed in triplicate. For quantification, the AT1$_{\alpha}$ and AT1$_{\beta}$ receptor, AT2 receptor, ATRAP, ACE-2, p16INK4a, p21$^{\text{cip1}}$, BMP-4, collagen 1a1, Id2 and peroxisome proliferator-activated receptor (PPAR)-γ, α and β sequences were normalized in relation to the GAPDH
or 18S product. The primers were synthesized by Biotez (Berlin, Germany). The sequences of the primers used for the amplifications were as follows:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>p21\text{Cip1} F</td>
<td>5’-GGT GGG CCC GGA ACA T-3’</td>
</tr>
<tr>
<td>p21\text{Cip1} R</td>
<td>5’-CGC CTT GGA GTG ATA GAA ATC TG-3’</td>
</tr>
<tr>
<td>p16\text{Ink4a} F</td>
<td>5’-GCC GCA CCG GAA TCC T-3’</td>
</tr>
<tr>
<td>p16\text{Ink4a} R</td>
<td>5’-TTG AGC AGA AGA GCT GCT ACG T-3’</td>
</tr>
<tr>
<td>Colla1 F</td>
<td>5’-AGG GTG CTC GTG GAT TGC-3’</td>
</tr>
<tr>
<td>Colla1 R</td>
<td>5’-AAG CCT CGG TGT CCC TTC A-3’</td>
</tr>
<tr>
<td>BMP4 F</td>
<td>5’-TGG GCT GGA ATG ATT GGA TT-3’</td>
</tr>
<tr>
<td>BMP4 R</td>
<td>5’-CCC CAT GGC AGT AGA AGG C-3’</td>
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<tr>
<td>AT1\text{A} receptor F</td>
<td>5’-TTGTCCACCCGATGAAGTCTC-3’</td>
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<tr>
<td>AT1\text{A} receptor R</td>
<td>5’-ATGCAGGTGACTTTTGCCA-3’</td>
</tr>
<tr>
<td>AT1\text{B} receptor F</td>
<td>5’-GGGCAGTTTATACCGCTATGGA 3’</td>
</tr>
<tr>
<td>AT1\text{B} receptor R</td>
<td>5’-TGGCCGAAGCGATCTTACAT-3’</td>
</tr>
<tr>
<td>ACE2 F</td>
<td>5’-TGGGATACCTACCCCTTCCACATCAG-3’</td>
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<tr>
<td>ACE2 R</td>
<td>5’-CCCCACATATACCAAGCAAA-3’</td>
</tr>
<tr>
<td>ATRAP F</td>
<td>5’-CCTCCGCCCCCGATTCTC-3’</td>
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<tr>
<td>ATRAP R</td>
<td>5’-TCTGATGATGAGTCAATTGTCTTGTA-3’</td>
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<tr>
<td>PPARR-\text{α} F</td>
<td>5’-AGGCTGTAAGGGCTTCTC-3’</td>
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<tr>
<td>PPARR-\text{α} R</td>
<td>5’-TGCAGCTCCGATCACAATTG-3’</td>
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<tr>
<td>PPAR-\text{β} F</td>
<td>5’-GCCACAACGCACTTCTT-3’</td>
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<tr>
<td>PPAR-\text{β} R</td>
<td>5’-CCACACCAGGCCCTTCTT-3’</td>
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<tr>
<td>PPAR-\text{γ} F</td>
<td>5’-GGATGTCTCAATGCCATCAG-3’</td>
</tr>
<tr>
<td>PPAR-\text{γ} R</td>
<td>5’-AGAAGGAGAAGCTGGCG-3’</td>
</tr>
</tbody>
</table>
**Senescence-associated (SA) β-Galactosidase Activity**

Briefly, the VSMCs were fixed in 4% paraformaldehyde in PBS for 30 minutes at room temperature and incubated for 72h at 37°C in freshly prepared SA-β-Gal staining solution containing 1mg/ml 5-bromo-4-chloro-3-indoly-D-galctopyranoside (X-gal), 5mM potassium ferrocyanide, 5mM potassioum ferricyanide, 150mM NaCl, 2mM MgCl₂, 40 mM citric acid, sodium phosphate, pH 6.0. Slides were washed in PBS and mounted in DAPI fluorescence mounting medium (Vector, Germany).

**Flow cytometric analysis**

Subconfluent cultures were growth-arrested for 24 hours and then stimulated with 10% FBS and labeled with 100µM BrdU for 24 hours. Cells were trypsinized, washed in 5% FBS/PBS, fixed in 4% paraformaldehyde for 10 minutes and permeabilized on ice for 30 minutes in Aceton/Methanol (50/50 %v/v). After 2 washes in 5% FBS/PBS, 25µg/ml DNase and anti-BrdU-FITC-antibody (Becton Dickinson, Germany) were added and incubated for 30min at room temperature. Cells were washed in 5% FBS/PBS, resuspended in FACS buffer and analyzed using a FACScan (Becton Dickinson, Germany) cytometer.

**Proliferation of VSMC**

VSMC (1 × 10³) were cultured in a 96-well plate, each well containing 100 µl of smooth muscle basic medium (SmBM, Clonetics, Germany) supplemented with insulin, FGF, EGF, and 10% FBS. The ATP content of cells was determined at the time points 0, 24, 48, and 72 h. Therefore fifty microliters of lysis buffer was added to each well and shaken at 700 rpm for 5 min before 50µl of substrate solution was added and shaken again. The luminescence was measured by a Scectrafluor Plus microplate scintillation counter (Tecan, Germany) according to the manufacturer’s instructions (ATP lite assay, Perkin Elmer, Germany).
Protein extraction and western blot

Cells were washed twice in PBS and lysated in RIPA-buffer containing a cocktail of protease inhibitors (Roche Molecular Biochemicals, Germany) and phosphatase inhibitors (Sigma-Aldrich). Protein extracts were boiled for 10 min and centrifuged at 13,000 g. Protein concentration was determined by the Bradford protein assay. Proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membrane (Hybond™-C, Amersham Biosciences, Germany). Membranes were blocked with 1% peptone and incubated overnight with specific antibodies against p21Cip1 and Cyclin A (both from Santa Cruz, Germany). Immunoreactive bands were detected by incubation with a secondary antibody conjugated to horseradish peroxidase and chemiluminescence substrate (Jackson Immunoresearch, Germany).

Results

Effect of Id2 heterozygosity on blood pressure and target organ damage

In our initial study we compared rat angiotensinogen-transgenic (TGM) Id2+/+ mice with TGM Id2+/- mice to evaluate the impact of Id2 heterozygosity on target organ damage. We found, that both groups TGMxId2+/+ and TGMxId2+/− mice were equally hypertensive (Figure I) showed no difference in cardiac hypertrophy and albuminuria with similar renal fibronectin expression (Figure I).

Renal mRNA Expression of Renin-Angiotensin System (RAS) Components and PPAR-α

We wondered if differences in the expression of components of the renal RAS could be responsible for the blood pressure phenotype in Id2+−. Therefore, we analyzed the mRNA levels from whole kidney extracts from Id2+/+ and Id2−/− (untreated and after 2 weeks of Ang II
infusion). We found no differences in renal $\text{AT1}_A$, $\text{AT1}_B$ and $\text{AT2}$ receptor expression between all groups (Figure II). $\text{AT1}$ receptor associated protein (ATRAP), a modulator of $\text{AT1}$ receptor signaling was also not regulated (Figure II). In addition, we analyzed renal ACE-2 and found similar expression levels in all groups (Figure II).

We also performed PPAR-α mRNA analyses in renal tissues (Figure III). Surprisingly, we found that PPAR-α expression was equally high expressed in all our 4 groups. We can only speculate about the reason for this finding. The kidney is derived out of various cell types (mesangial cells, proximal and distal tubules, medullary collecting ducts and vessels), which all express PPAR-α. Therefore, it might well be that other cell types besides VSMC have accounted for the total PPAR-α mRNA signal what we detected. The total contribution of VSMC to the entire renal contents is likely to be relatively small.

**Figure Legends**

**Figure I. Role of Id2 heterozygousity in Ang II-induced target organ damage. (A)** Telemetric mean arterial blood pressure (MAP) recording during week 12 of age. (B) Cardiac hypertrophy index (week 12). No difference in 24-hour albuminuria (C) and renal fibronectin expression (D) at the age of 12 weeks in Id2$^{+/+}$ and Id2$^{-/-}$ mice.

**Figure II. Renal expression of RAS components.** Untreated and Ang II-infused Id2$^{+/+}$ and Id2$^{-/-}$ mice show similar renal $\text{AT1}_A$, $\text{AT1}_B$ receptor and ATRAP expression. No differences could be found in AT2 receptor and ACE-2 expression.

**Figure III. Renal PPARα expression.** No differences could be detected in the four groups for PPAR-α expression.
References:

