Renal Denervation Prevents Immune Cell Activation and Renal Inflammation in Angiotensin II–Induced Hypertension

Liang Xiao, Annet Kirabo, Jing Wu, Mohamed A. Saleh, Linjue Zhu, Feng Wang, Takamune Takahashi, Roxana Loperena, Jason D. Foss, Raymond L. Mernaugh, Wei Chen, Jackson Roberts II, John W. Osborn, Hana A. Itani, David G. Harrison

Rationale: Inflammation and adaptive immunity play a crucial role in the development of hypertension. Angiotensin II and probably other hypertensive stimuli activate the central nervous system and promote T-cell activation and end-organ damage in peripheral tissues.

Objective: To determine if renal sympathetic nerves mediate renal inflammation and T-cell activation in hypertension.

Methods and Results: Bilateral renal denervation using phenol application to the renal arteries reduced renal norepinephrine levels and blunted angiotensin II–induced hypertension. Bilateral renal denervation also reduced inflammation, as reflected by decreased accumulation of total leukocytes, T cells, and both CD4+ and CD8+ T cells in the kidney. This was associated with a marked reduction in renal fibrosis, albuminuria, and nephrinuria. Unilateral renal denervation, which partly attenuated blood pressure, only reduced inflammation in the denervated kidney, suggesting that this effect is pressure independent. Angiotensin II also increased immunogenic isoketal-protein adducts in renal dendritic cells (DCs) and increased surface expression of costimulation markers and production of interleukin (IL)-1α, IL-1β, and IL-6 from splenic DCs. Norepinephrine also dose dependently stimulated isoketal formation in cultured DCs. Adoptive transfer of splenic DCs from angiotensin II–treated mice primed T-cell activation and hypertension in recipient mice. Renal denervation prevented these effects of hypertension on DCs. In contrast to these beneficial effects of ablating all renal nerves, renal afferent disruption with capsaicin had no effect on blood pressure or renal inflammation.

Conclusions: Renal sympathetic nerves contribute to DC activation, subsequent T-cell infiltration and end-organ damage in the kidney in the development of hypertension. (Circ Res. 2015;117:547-557. DOI: 10.1161/CIRCRESAHA.115.306010.)

Key Words: dendritic cells ■ inflammation ■ lymphocyte ■ norepinephrine ■ sympathetic nerve block

Inflammation, and in particular adaptive immunity, contributes to the development of hypertension. Recombination activating gene 1–deficient (RAG-1−/−) mice lacking lymphocytes are relatively resistant to hypertension caused by angiotensin II, norepinephrine, or deoxycorticosterone acetate–salt challenge.1,2 Similar protective effects have also been observed in mice with severe combined immune deficiency and in Dahl salt–sensitive rats with deletion of the RAG-1 gene.3,4 Adoptive transfer of T cells to RAG-1−/− mice restores hypertension and end-organ dysfunction in response to hypertensive stimuli.1,2 T lymphocytes and other inflammatory cells accumulate in the kidneys and vasculature during hypertension and produce cytokines, such as interleukin (IL)-17A, interferon (IFN)-γ, and tumor necrosis factor α, which in turn lead to vascular remodeling and renal sodium retention, augmenting blood pressure elevation.5,6 The mechanisms underlying T-cell activation are not yet fully understood.

Editorial, see p 487
In This Issue, see p 483

T-cell activation requires antigen presenting cells, in particular dendritic cells (DCs) and the phenomenon of costimulation.5 We have previously shown that DCs from hypertensive mice have increased surface expression of the costimulatory B7 ligands (CD80 and CD86), suggestive of DC maturation and activation.6 Pharmacological blockade or genetic deletion of CD80/CD86 prevents T-cell activation in response to angiotensin II and reverse both angiotensin II and DOCA-salt...
implicated in both pro- and anti-inflammatory responses.10–12 Immune cells possess adrenergic receptors, which have been shown to slow the progression of chronic renal disease.14,15 Independent of its therapeutic efficacy in humans, RDN provides a useful platform on which to study the relationship between sympathetic nerves and renal inflammation and T-cell activation.2,8,9 The mechanisms underlying a potential anti-inflammatory role of RDN. We determined the efficacy of RDN in both preventing and reversing hypertension and examined the effect of both renal efferent and afferent nerves in modulating renal inflammation.

Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR7</td>
<td>C–C chemokine receptor type 7</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>interferon-γ</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>Klf4</td>
<td>Kruppel-like factor 4</td>
</tr>
<tr>
<td>RAG-1</td>
<td>recombination activating gene 1</td>
</tr>
<tr>
<td>RDN</td>
<td>renal denervation</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>vascular cell adhesion molecule 1</td>
</tr>
</tbody>
</table>

The central nervous system orchestrates much of the inflammation caused by angiotensin II and probably other hypertensive stimuli. Inhibition of sympathetic outflow by AV3V lesioning or by deletion of the NADPH oxidase subunit p22phox in the subfornical organ reduces hypertension and T-cell activation, whereas activation of sympathetic outflow by deletion of the extracellular superoxide dismutase in the subfornical organ enhances T-cell activation.2,8,9 The mechanisms underlying this link between the central nervous system and T-cell activation are, however, not well understood. In keeping with this, a recent study by Mathis et al16 showed that RDN reduces albuminuria and renal cortical monocyte chemoattractant protein expression in mice with experimental systemic lupus erythematosus. In this study, we tested the hypothesis that renal sympathetic nerves modulate renal inflammation and T-cell activation in hypertension and sought to understand mechanisms underlying a potential anti-inflammatory role of RDN.

Methods

The Institutional Animal Care and Use Committee of Vanderbilt University approved all animal protocols. A detailed description of the materials and methods can be found in the Online Data Supplement.

Results

Effects of RDN on Catecholamine Content and Hypertension

In initial studies, we examined the efficacy of renal artery phenol application in producing RDN. Norepinephrine content was markedly decreased in denervated kidneys compared with the sham-treated kidneys (Figure 1A). In contrast, renal epinephrine, which is largely derived from the adrenal glands, was not altered by phenol application. Western blots for tyrosine hydroxylase, the rate-limiting enzyme for catecholamine biosynthesis, confirmed successful denervation (Figure 1B). We also confirmed that this technique does not interrupt innervation of adjacent lymph nodes and the adrenal gland, as verified by the neuronal marker β3 tubulin expression and catecholamine content in lymph nodes and adrenal glands, respectively (Online figure I).

The hypertensive response to angiotensin II, assessed by radiotelemetry, was markedly blunted in mice that had previously undergone bilateral RDN. In sham-operated mice, systolic pressure increased to 163±4 mm Hg in response to 2 weeks angiotensin II infusion. A–C, Western blot showing tyrosine hydroxylase (TH) in innervated and denervated kidneys. C and D, Effect of renal denervation on the hypertensive response to 2 weeks angiotensin II infusion (490 ng/kg per minute). Data in (A) and (B) were analyzed by paired t tests and data in (C) and (D) with 2-way ANOVA with repeated measurements, n=10 and 8 in each group. BP indicates blood pressure. *P<0.05, **P<0.01, ***P<0.001.
diastolic pressure was reduced from 131±5 mm Hg to 111±5 mm Hg (P<0.001) by RDN (Figure 1D).

**Effects of RDN on Renal Inflammation and T-Cell Activation in Angiotensin II–Induced Hypertension**

To examine the effect of denervation on renal inflammation, we prepared single-cell suspensions of renal homogenates and performed flow cytometry. As apparent in Figure 2A and in the mean data, angiotensin infusion increased the presence of all leukocyte subsets, including total leukocytes, identified by CD45, total T cells, and both CD4+ and CD8+ T cells. RDN prevented the increased renal accumulation of leukocytes in response to angiotensin II infusion (Figure 2B–2G). Of note, the number of memory T cells, as defined as the CD44high, was also increased by angiotensin II, and this was normalized by RDN. RDN has recently been reported to have beneficial effects on vascular inflammation in apolipoprotein−/− mice.17 In keeping with this, we also found that RDN reduced aortic infiltration of total leukocytes, total T cells, CD4+ T cells, and CD8+ T cells (Online figure II).

To confirm our flow cytometry results and to localize inflammatory cells in the kidney, CD3+ and F4/80+ cells in the kidney were visualized using immunohistochemistry. T cells were increased in both the renal cortex and the medulla by angiotensin II infusion (Figure 3A and 3B) and this was prevented by RDN. Monocyte/macrophages, as identified by

---

**Figure 2. Effects of bilateral renal denervation on renal leukocyte and T-cell infiltration.** Mice underwent bilateral renal denervation and 1 week later had osmotic minipumps for angiotensin II infusion implanted. Two weeks later, kidneys were harvested for flow cytometry. Representative flow cytometry of kidneys from mice with sham surgery or bilateral renal denervation are shown in (A). Live singlet cells were gated for total leukocytes (CD45+), total T cells (CD3+), CD4+ and CD8+ T cells. CD44 expression was examined in CD4+ and CD8+ T cells. Fluorescence-minus-one controls for CD44high cells are shown as dotted lines. Mean data are shown for total leukocytes (B), T cells (C–E), and memory T-cell accumulation (F and G) in response to angiotensin II was examined in either sham-treated or denervated kidneys. Data were analyzed using 2-way ANOVA, n=5 to 10 in each group. RDN indicates renal denervation. *P<0.05, **P<0.01.
F4/80 staining, were also increased by angiotensin II infusion, and were reduced in the renal medulla by denervation. Further flow cytometry studies using a gating strategy recently described by Jakubzick et al. showed that the majority of these F4/80+ cells were monocytes and not macrophages (Online figure III). Taken together, these data indicate that sympathetic innervation plays a major role in mediating renal inflammation in the angiotensin II–induced hypertension.

We further examined the effect of RDN on renal injury in hypertension. Angiotensin II infusion caused collagen deposition in both the renal cortex and the medulla (A and B, n=6 per group). DHE fluorescence was visualized at 530 nm to 560 nm and relative fluorescence intensity was quantified (C, n=4 per group). Twenty-four urine collections were analyzed for albumin content and nephrin (D and E, n=6 per group). Data in (A) to (C) were analyzed using 1-way ANOVA and data in (D) and (E) were analyzed using 2-way ANOVA. Ang II indicates angiotensin II; and RDN, renal denervation. *P<0.05, **P<0.01, ***P<0.001.

Local Effects of Sympathetic Nerves on Renal Inflammation in a Unilateral RDN Model
To determine if renal nerves directly contribute to inflammation or if renal inflammation is predominantly because of high blood pressure, we performed unilateral RDN. Using this approach, both kidneys are exposed to a similar pressure, but only 1 is denervated. Unilateral RDN also reduced the hypertensive response to a 2-week infusion of angiotensin II, albeit not as effectively as bilateral denervation (146±5 versus 164±3 mm Hg; P<0.05; Figure 4A). Interestingly, significantly fewer total leukocytes (Figure 4B), lymphocytes (Figure 4C), and both CD4+ and CD8+ T cells (Figure 4E and 4F) accumulated in the denervated kidney as compared with the innervated kidney. Combining with the results from bilateral denervation, this suggests that there is a significant proinflammatory effect of sympathetic innervation, independent of pressure elevation (Online figure IV).

Effect of RDN on Chemoattractant and Adhesion Molecules
The homing of inflammatory cells to an affected tissue depends on local levels of adhesion and chemoattractant molecules. We, therefore, used polymerase chain reaction to detect mRNA levels of selected proteins known to attract immune cells. Hypertension caused a striking increase in the vascular cell adhesion molecule 1 (VCAM-1), intercellular cell adhesion molecule 1 monocye chemotactic protein 1, and regulated on activation, normal T cell expressed and secreted. RDN significantly attenuated, and in the case of VCAM-1 and regulated on activation, normal T cell expressed and secreted, completely prevented this response (Figure 5A–5D). Nitric oxide has anti-inflammatory properties, and we found that mRNA for the endothelial nitric oxide synthase (eNOS) was markedly reduced by angiotensin II–induced hypertension.
and this was partly corrected by RDN (Figure 5E). An important transcription factor that modulates eNOS transcription is Kruppel-like factor 4 (Klf4). Hypertension was associated with a striking reduction of Klf4, and this was completely prevented by RDN (Figure 5F).

We considered the hypothesis that RDN increases renal blood flow, as eNOS and Klf4 are known to be shear responsive. We, therefore, measured renal blood flow using magnetic resonance imaging in angiotensin II–infused mice subjected to unilateral RDN surgery and found no significant change in renal perfusion caused by denervation (Online figure V).

**Effect of RDN on DC Activation in Angiotensin II–Induced Hypertension**

T-cell activation is dependent on antigen presenting cells, and in particular DCs. These cells present antigen and provide costimulation to initiate T-cell proliferation and activation. On maturation, DCs increase expression of CD80 and CD86. We, therefore, performed additional studies to determine if RDN affects DC activation and their ability to promote hypertension. Subtypes of splenic DCs were characterized according to the surface markers I-Ab, CD11c, CD11b, and B220 (Online figure VI). Angiotensin selectively increased CD80 and CD86 expression by ≈26% and 15% in CD11b+/CD11c+ DCs, and this was prevented by RDN (Figure 6A and 6B).

We have recently found that isoketal-protein adducts are formed in hypertension. These accumulate in DCs and promote DC activation, causing DCs to express costimulatory molecules, produce cytokines, and stimulate T-cell proliferation.7 We performed additional studies using flow cytometry to detect intracellular isoketal-protein adducts in sham-operated and RDN mice. As we previously reported, splenic CD11c+/CD11b+ DCs from hypertensive mice robustly accumulated isoketal adducts, and RDN prevented this (Figure 6C). One explanation for these findings is that renal sympathetic nerves stimulate formation of isoketal adducts in the kidney, perhaps directly in DCs. To address this, we examined isoketal adducts in the DCs within the kidney shortly after the onset of angiotensin II infusion. In sham mice not receiving angiotensin II, only 25% of DCs stained positively for isoketal-protein adducts. In contrast, nearly 80% of the DCs isolated from kidneys 5 days after the onset of angiotensin II contained isoketal-adducted proteins. RDN markedly reduced these (Figure 6D).

Activated DCs produce cytokines that influence the inflammatory milieu, in large part by guiding T-cell polarization. To examine if RDN alters DC cytokine production, splenic DCs were cultured in RPMI for 24 hours and cytokines released into the media were measured using Luminex. Angiotensin II infusion increased splenic DC IL-1α, IL-1β, and IL-6 production 2- to 6-fold, and these increases were completely prevented by RDN (Figure 6E and 6F).
prevented by bilateral RDN (Figure 6E–6G). Other cytokines, including IL-12, IL-23, GM-CSF, and TGF-β were not altered by RDN (Online Table I).

Role of Activated DCs in Priming Hypertension and Effect of RDN

To further determine if renal innervation affects the ability of DCs to promote hypertension and renal inflammation, we performed adoptive transfer experiments. Mice underwent RDN or sham surgery as described above, and 10 days later underwent infusion of angiotensin II (490 ng/kg per minute) for 2 weeks. 1×10⁶ splenic DCs were isolated from these animals and injected by tail vein into recipients. Ten days later, the recipients were treated with low-dose angiotensin (140 ng/kg per minute) for 2 weeks. This experimental paradigm is illustrated in Figure 7A. DCs from mice with intact renal nerves markedly augmented the hypertensive response to this generally subpressor dose of angiotensin II (Figure 7B and 7C). In contrast, DCs from denervated animals did not alter the hypertensive response to low-dose angiotensin II. Flow cytometry revealed that adoptive transfer of DCs from angiotensin II–infused mice with intact renal nerves increased renal accumulation of total leukocytes, CD3+ cells, and both CD4+ and both CD8+ T cells in response to low-dose angiotensin II. These values were reduced by 30% to 50% in mice that had received DCs from mice with RDN (Figure 7D–7H). These data illustrate an important contribution of DCs to hypertension, and show that this is modulated by the sympathetic nervous system.

Role of Afferent Renal Nerves in Angiotensin II–Induced Hypertension

Activation of renal afferent nerves has been reported to produce hypertension in response to calcineurin,19 renal failure,20 and renal injury.21 To examine a role of afferent renal nerves in modulating inflammation, we applied capsaicin to the renal arteries. This markedly reduced the afferent nerve marker calcitonin gene-related peptide in the renal pelvis, but had no effect on renal norepinephrine levels (Online Figure VIIA and VIIB). Disruption of the afferent nerves using this approach did not alter either the hypertension (Online Figure VIIC) or renal inflammation (Online Figure VIID–VIIH) caused by angiotensin II.

Effect of RDN After the Onset of Hypertension

In clinical trials, RDN is usually performed after the onset of hypertension. To mimic this clinical scenario, we infused...
angiotensin II for 4 weeks in mice, and performed RDN after 2 weeks. Other mice underwent a sham denervation procedure at 2-week angiotensin II infusion. As shown in Figure 8A and 8B, RDN after the onset of hypertension lowered blood pressure by ~15 mm Hg, compared with that observed in mice undergoing sham denervation, during the ensuing 2 weeks. This value was significantly less that the blood pressure reduction observed when denervation was performed at the onset of angiotensin II infusion (149±4 mm Hg versus 129±3 mm Hg; P<0.001). Of note, this 4-week protocol resulted in substantially more leukocytes in the kidney than observed after 2 weeks of angiotensin II, but RDN substantially reduced total leukocytes and all T-cell subsets as demonstrated by flow cytometry (Figure 8C–8F).

Direct Effect of Sympathetic Stimuli in Isoketal Adduct Formation in DCs
To examine if sympathetic stimuli directly affect DC function in hypertension, adrenergic receptor expression in splenic DCs was quantified using real-time polymerase chain reaction (Online figure VIIIA), and further confirmed by Western blot (Online figure VIIIB). We found that DCs contain mRNA for α1D, α2A, α2B, α2C, β1, and β2 adrenergic receptors. In most cases, adrenergic receptors were downregulated by angiotensin II infusion, with the exception of β2 adrenergic receptors, which were increased. To further determine if adrenergic signaling contributes to DC activation, bone marrow–derived DCs were cultured with norepinephrine, and isoketals were measured by flow cytometry 1 week later. Norepinephrine dose...
dependently increased isoketal-protein adducts in those cells. A similar response was observed with DCs obtained from β2-receptor–deficient mice (Figure 8C and 8D). In contrast to the effects of norepinephrine, neuropeptide Y, another sympathetic nerve transmitter, had no effect on DC isoketal-protein content (Online figure VIIIE).

Potential Role of C–C Chemokine Receptor Type 7 in Homing of DCs From the Kidney to Secondary Lymphoid Organs

Our data about the effect of RDN on splenic DC function and isoketal content are compatible with the hypothesis that these cells are initially activated in the kidney and migrate to secondary lymphoid organs such as the spleen, where they interact with T cells to promote T-cell activation (Figure 8G). To gain further insight into this hypothesis, we performed additional studies on C–C chemokine receptor type 7 (CCR7)–deficient mice. CCR7 is used by DCs to allow homing to secondary lymphoid organs. The hypertensive response to angiotensin II was identical to that observed in wild-type mice (Online figure IIXA). Likewise, DCs in the kidney demonstrated an increase in CD80 and CD86 (Online figure IXB); however, there was no effect of angiotensin II infusion on these DC surface markers in the spleen (Online Figure IXC). Angiotensin infusion failed to induce inflammatory cell infiltration in the kidneys of CCR7−/− mice (Online Figure IXC–IXF). However, memory CD8+ T cells were increased (Online Figure IXH).

Discussion

In this study, we show that the renal sympathetic nerves play an important role in activation of adaptive immunity in hypertension. RDN attenuates the hypertension caused by chronic angiotensin infusion and prevents T-cell infiltration and subsequent renal injury. Renal sympathetic outflow also promotes DC maturation as defined by upregulation of costimulatory molecules and augmented proinflammatory cytokine production during angiotensin II infusion, and all these changes are reversed by RDN. In accordance with these, DC adoptive transfer experiments further indicate that renal nerves indeed contribute to DC activation, which in turn promote T-cell activation and hypertension. Our data also indicate that efferent but not afferent renal nerves mediate these effects.

Before our present study, the interplay between renal sympathetic nerves and inflammation was poorly defined. As in previous studies, we found that hypertension is associated accumulation of leukocytes, T cells, and both CD4+ and CD8+ T cells in the kidney. In recent studies, we have shown that T cells that infiltrate the kidney in hypertension have an effector memory phenotype. Importantly, RDN not only reduces the total number of immune cells in the kidney but also memory T cells. We have found that activated T cells in hypertension produce IL-17A, TNF-α, and IFN-γ, and we and others have shown that these affect vascular and renal function. For example, prolonged exposure of renal proximal tubular cells to IFN-γ enhances production of angiotensinogen by renal

Figure 8. Effect of renal denervation (RDN) on blood pressure and renal inflammation in established hypertension. Mice received angiotensin II (Ang II, 490 ng/kg per minute) infusion for 4 weeks, and bilateral RDN or sham surgery were performed after 2 weeks. Systolic blood pressure was measured by tail cuff (n=6 per group) and by telemetry for at baseline, and after 2, 3, and 4 weeks of Ang II infusion (n=5 per group). Dotted lines indicate the time when RDN or sham surgeries were performed. Renal infiltration of total leukocytes (CD45+, C), T cells (CD3+, D), and both CD4+ (E) and CD8+ T cells (F) were quantified with flow cytometry (n=6 per group). Blood pressures were compared using 2-way ANOVA with repeated measures. Data in (C)–(F) were analyzed with t test, *P<0.05, **P<0.01. G, Proposed model of the effect of renal sympathetic nerves on activation of adaptive immunity in hypertension. BP indicates blood pressure.
epithelial cells, which modulates tubular sodium transport. In keeping with this concept, we have recently shown that angiotensin II infusion increases phosphorylation of the Na–K–2Cl cotransporter the NaCl cotransporter and Ste20/SPS-1–related proline–alanine-rich kinase in wild-type mice but not in IFN-γ−/− mice. In this study, we also found evidence that IL-17A and IFN-γ modulate abundance of Na/H-exchanger isoform 3 and the motor myosin VI during angiotensin II infusion. It is therefore likely that the reduction of effector memory T cells in the kidney contributes to the antihypertensive effect of RDN.

The homing of immune cells to sites of inflammation is mediated by local increases in adhesion and chemoattractant molecules. In keeping with this, we found that angiotensin II infusion increases renal expression of VCAM-1, intercellular cell adhesion molecule 1, monocyte chemotactic protein 1, and regulated on activation, normal T cell expressed and secreted, and that these are all attenuated by RDN. In contrast, angiotensin II–induced hypertension is associated with a marked decrease in eNOS mRNA expression, and this is completely prevented by denervation. Nitric oxide has myriad anti-inflammatory effects on the endothelium, reducing expression of VCAM-1, monocyte chemotactic protein 1, regulated on activation, normal T cell expressed and secreted, intercellular cell adhesion molecule 1, and monocyte adhesion. In many cases, this has been attributed to modulation of transcription factors, such as nuclear factor-κB, activator protein 1, and cAMP response element-binding protein. In this regard, the KIf4 modulates endothelial function by promoting eNOS expression, reducing VCAM-1 expression and exerting antithrombotic effects. Thus, the enhancement of eNOS and KIf4 expression could explain at least a part of the anti-inflammatory effects of RDN.

A critical finding in this study is that RDN reduces DC activation and the ability of these cells to convey hypertension. Recently, we showed that isoketal-adducted proteins accumulate in DCs and seem to act as neoantigens, which lead to T-cell activation and ultimately hypertension. In this study, we find that isoketals accumulate in DCs within the kidney within 5 days of angiotensin II infusion, and that this is prevented by RDN. In accord with our previous study, we found that adoptive transfer of DCs from an angiotensin II–infused mouse to naive recipients markedly enhances the hypertensive response to a generally subpressor dose of angiotensin II. We now demonstrate that this prohypertensive effect of DCs is eliminated if the donor mouse has undergone RDN. When DCs process antigens, they undergo a maturation step involving increased surface expression of the B7 ligands CD80 and CD86, which we have shown are essential for development of hypertension. Importantly, denervation reduces DC expression of these costimulatory molecules and also markedly reduces DC production of the proinflammatory cytokines IL-1α, IL-1β, IL-2, and IL-6. These cytokines skew T cells to Th1 and Th17 phenotypes, and thus promote inflammation.

Our present studies also define mechanisms by which sympathetic nerves affect DC function. We found that DCs express numerous adrenergic receptors, and that β2 receptors are increased during angiotensin II infusion. Long-term exposure of DCs to norepinephrine in vitro dose dependently increased isoketal-adduct formation in DCs. Our data also indicate that β3 receptor signaling is not involved in this process as the increase in DC isoketal-adducts was unchanged in mice lacking this receptor. We also provide evidence that angiotensin II–induced hypertension causes a fairly striking increase in vascular superoxide production in the kidney, and that this is prevented by RDN. It is, therefore, possible that ROS such as hydrogen peroxide or peroxynitrite produced by vascular cells could diffuse into DCs and lead to lipid oxidation and isoketal formation. Our data exclude a role of neuropeptide Y in this process.

It is possible that many of the beneficial effects of RDN are simply related to a decrease in blood pressure and the resultant decrease in pressure-induced damage in the kidney. In an effort to separate the effects of pressure and sympathetic denervation, we used unilateral denervation. In this setting, T-cell accumulation was reduced in the denervated, but not in the innervated kidney. These findings suggest that innervation per se, rather than pressure elevation, participates in the renal inflammatory response in hypertension. We cannot completely exclude a role of pressure, as vascular stretch is known to increase production of reactive oxygen species and monocyte chemotactic protein 1, VCAM-1, and IL-6 expression. We have previously found that antihypertensive treatment with hydralazine and hydrochlorothiazide prevents vascular inflammation, however, these agents have off-target effects that directly affect immune responses. Data from previous studies by our group and others suggest that various hypertensive stimuli such as angiotensin II and salt promote modest increases in blood pressure that are augmented by inflammation in a feed-forward fashion. It is likely that sympathetic innervation plays an important intermediate role in such interplay.

A striking finding in this study is that RDN affected the function of DCs in the spleen. One explanation for this is that DCs arising from the kidney migrate to the spleen, where they can activate T cells that promote systemic inflammation (Figure 8G). Our data in CCR7−/− mice are compatible with this concept. CCR7 is used by DCs as a homing marker that allows their transmigration to secondary lymphoid organs. In CCR7−/− mice, we continued to observe DC activation in the kidney, but not in the spleen, suggesting that their movement from the kidney to the spleen might be defective. These studies must be interpreted with caution, as CCR7 is also critical for T-cell homing to secondary lymphoid organs. Nevertheless, findings in these animals suggest an interplay between the kidney and spleen, and perhaps other sites of immune activation.

There has been substantial debate as to the role of renal afferent nerves in hypertension. Intrarenal injection of phenol in rats leads to sustained hypertension and increased hypothalamic norepinephrine production, thought secondary to afferent nerve activity. Circulating calcitonin-related peptide level, reflecting afferent nerve activity, is elevated in this model. Likewise, activation of renal afferent nerves enhances hypertension in response renal wrapping. In contrast, activation of sensory nerves has been proposed to buffer the increase in blood pressure caused by angiotensin II. Sensory afferents could conceivably lead to reflex activation of cells within the spleen, as observed in this study. Our data using capsaicin renal sensory denervation...
suggest that afferent nerves have little role in blood pressure, regulation of blood pressure, or immune cell activation in response to chronic angiotensin II infusion, but do not discount a role of these in the hypertension caused by other stimuli.

An important issue related to this study is the clinical benefit of RDN in humans. The SYMPLICITY HTN-1 and 2 trials reported a striking lowering of blood pressure by catheter-based radiofrequency renal nerve ablation, with sustained reductions near 30 mmHg. The Global SYMPLICITY registry confirmed a similar effect on office blood pressure. These studies, however, were not sham controlled or blinded. Questions were raised about study design and the patients included in these trials. In contrast, the SYMPLICITY HTN-3 trial, which included sham controls and was blinded, showed essentially the same lowering of blood pressure in the sham-treated and denervation-treated patients. It has been suggested that the benefit observed in the earlier trials were because of the Hawthorne effect, regression of blood pressure values to a mean and placebo effects. In contrast, the SYMPLICITY HTN-3 trial suggested that a greater number of catheter ablations correlated with greater blood pressure lowering and that non-blacks responded more to this procedure. Another issue is that long-standing hypertension in humans, with attendant changes in renal and vascular function, might not be responsive to renal nerve ablation. Indeed, we found that the antihypertensive effect of denervation was less pronounced when applied after the onset of hypertension than when denervation was performed before the onset of angiotensin II infusion. Despite this, we observed a substantial anti-inflammatory effect of RDN performed after the onset of hypertension. It is, therefore, possible that the anti-inflammatory effects of RDN might not be reflected by simple measures of blood pressure, but might have long-term benefit on renal function or nonrenal cardiovascular events.

Acknowledgments

We are grateful to the Transitional Pathology Shared Resource core and the Epithelial Biology Center at Vanderbilt University for the preparation, imaging, and quantification of immunohistochemical staining, as well as the Vanderbilt Hormone Assay and Analytic Services Core for the measurements of catecholamines and cytokines. Confocal microscopy was performed, in part, through the use of the Vanderbilt University Medical Center Cell Imaging Shared Resource. We also thank Dr. Florent Elefteriou for supplying the β2AR−/− mice.

Sources of Funding

This work was supported by the National Institutes of Health Program Project Grants P01 HL58000 and GM015431 and R01 grants HL105294 and HL039006 (D.G. Harrison), R01 HL116476 (J.W. Osborn), an American Heart Association predoctoral fellowship to J. Wu, and postdoctoral fellowship awards to A. Kirabo, H.A. Itani, and M.A. Saleh.

Disclosures

None.

References


Renal Denervation Prevents Immune Cell Activation and Renal Inflammation in Angiotensin II–Induced Hypertension

Liang Xiao, Annet Kirabo, Jing Wu, Mohamed A. Saleh, Linjue Zhu, Feng Wang, Takamune Takahashi, Roxana Loperena, Jason D. Foss, Raymond L. Mernaugh, Wei Chen, Jackson Roberts II, John W. Osborn, Hana A. Itani and David G. Harrison

Circ Res. 2015;117:547-557; originally published online July 8, 2015; doi: 10.1161/CIRCRESAHA.115.306010

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2015 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/117/6/547

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2015/07/08/CIRCRESAHA.115.306010.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/
Supplemental Material

Renal Denervation Prevents Immune Cell Activation and Renal Inflammation in Angiotensin II–Induced Hypertension

Liang Xiao Ph.D.1
Annet Kirabo Ph.D.1
Jing Wu Ph.D.1
Mohamed A. Saleh Ph.D.1,2
Lijue Zhu1
Feng Wang Ph.D.3
Takamune Takahashi Ph.D.4
Roxana Loperena6
Jason D. Foss Ph.D.6
Raymond L. Mernaugh Ph.D.7
Wei Chen Ph.D.1
Jackson Roberts II M.D.1
John W. Osborn Ph.D.6
Hana A. Itani Ph.D.1
David G. Harrison, M.D.1

1Department of Medicine, Division of Clinical Pharmacology, Vanderbilt University, Nashville, TN
2Department of Pharmacology and Toxicology, Faculty of Pharmacy, Mansoura University, Egypt
3Department of Radiology and Radiological Sciences, Vanderbilt University, Nashville, TN
4Department of Medicine, Division of Nephrology and Hypertension, Vanderbilt University, Nashville, TN
5Department of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, TN
6Department of Integrative Biology and Physiology, University of Minnesota, Minneapolis, MN
7Department of Biochemistry, Vanderbilt University, Nashville, TN

Address for Correspondence:
David G. Harrison, M.D.
Betty and Jack Bailey Professor of Medicine, Pharmacology and Physiology
Director of Clinical Pharmacology
Room 536 Robinson Research Building
Vanderbilt University
Nashville, TN 37232-6602
Telephone 615-875-3049
Fax 615-875-3297
e-mail: david.g.harrison@vanderbilt.edu
Detailed Materials and Methods

Animals: All mice were obtained from Jackson Laboratories on a C57Bl/6 background. In the case of the β2AR-/- mice, these were outbred from β1/β2AR+ mice. At 3 months of age, mice were randomly selected for renal denervation or sham surgery. For renal denervation, mice were anesthetized by intraperitoneal ketamine (100 mg/kg) and xylazine (10 mg/kg) and the renal arteries were visualized via flank incisions. The renal nerves were ablated by encircling the renal artery with a 5-0 suture soaked with 10% phenol in ethanol. In sham-operated mice, normal saline was applied rather than phenol. In some experiments, unilateral denervation was performed by applying phenol to only the left renal artery. To selectively disrupt renal afferent nerves, a similar surgical approach was used except capsaicin (33 mmol/L) dissolved in 5% Tween 80, 5% ethanol and 90% normal saline was applied to the renal artery for 15 minutes as described recently. Blood pressure was measured either invasively using telemetry or non-invasively using an automated tail-cuff system as previously described. Hypertension was induced by the infusion of angiotensin II (490 ng/kg/min) via osmotic minipumps for two weeks to four weeks. At study termination, mice were euthanized by exposure to CO₂. One week later, osmotic minipumps were implanted subcutaneously for infusion of angiotensin II (490 ng/kg/min) or vehicle for 2 weeks unless otherwise indicated. In subsets of mice, telemetry units were implanted for measurement of blood pressure. After at least one week recovery from telemetry implant, blood pressure was recorded for 10 minutes every hour for the duration of the experiments (i.e. three days prior to osmotic minipump implantation and until the end of angiotensin II infusion at Day 14). At the end of each experiment, mice were sacrificed with CO₂ inhalation and the chest was rapidly opened and the superior vena cava sectioned. A catheter was placed in the left ventricular apex and the animals were perfused at a physiological pressure with Krebs-Hepes buffer until the effluent from the vena cava was cleared of blood. The Institutional Animal Care and Use Committee approved all experimental protocols.

Material: The antibodies and fluorophores were purchased from Biolegend (San Diego, CA) included: 7-AAD for live/dead staining; BV510-conjugated anti-CD45 (30-F11); APC-conjugated anti-CD4 (GK1.5); APC/Cy7-conjugated anti-CD8 (53-6.7); PE/Cy7-conjugated anti-CD3 (145-2C11); FITC-conjugated CD44 (IM7); PE-conjugated anti-CD80 (16-10A1); BV421-conjugated anti-CD86 (GL-1); PE/Cy7-conjugated anti-I-A^b (AF6-120.1); APC/Cy7-conjugated anti-CD11c (Bu15), APC-conjugated anti-CD11b (M1/70), FITC-conjugated anti-B220 (RA3-6B2). An Alexa 488 tagged single-chain antibody that has been previously described was used for detecting intracellular isoketal adducts D11. Primary antibodies for Western blot included rabbit polyclonal antibodies anti-tyrosine hydroxylase (AB152 from Millipore), rabbit monoclonal anti-d2 adrenergic receptor (ab151727), anti-β₁ adrenergic receptor (ab3442), anti-β₂ adrenergic receptor(ab182136, all from Abcam) and anti-GAPDH (sc-32233, from Santa Cruz Biotechnology). Enzyme immunoassay kits for urinary albumin and nephrin were purchased from Exocell (Philadelphia, PA). Enzyme immunoassay kit for calcitonin gene related peptide was from Cayman Chemical (Ann Arbor, MI). All primers and probes for real time PCR (NOS3: Mm00435217_m1, Klf4: Mm00516104_m1, MCP-1/Ccl2: Mm00441242_m1, VCAM-1: Mm01320970_m1, ICAM-1: Mm00516023_m1) gene expression assays as well as a GAPDH endogenous control) were from Applied Biosystems. Norepinephrine and neuropeptide Y were purchased from Sigma Aldrich (St Louis, MO).
Measurements of Albuminuria/Nephrinuria: Albumin and nephrin were measured by ELISA from 24 hour urine samples as described previously. All concentrations were multiplied by total urine volume to obtain the daily excretion rate.

Catecholamines: After euthanasia, the kidneys and adrenal glands were freeze-clamped and pulverized by a mortar and pestle chilled in liquid nitrogen. Norepinephrine and epinephrine contents were measured by high-performance liquid chromatography via electrochemical detection as previously described.

Calcitonin Gene Related Peptide: With kidneys harvested immediately after euthanasia, the renal pelvis using a dissecting microscope. Tissues were homogenized in 1M acetic acid and CGRP was measure according to the protocol provided in the ELISA kit.

Western Blot Analysis and Real-time PCR: Western blotting was performed using antibodies against tyrosine hydroxylase, α2b, β1 and β2 adrenergic receptors, and GAPDH. Goat anti-rabbit and goat anti-mouse secondary polyclonal antibodies were employed. Western blots were quantified by densitometry. Levels of endothelial nitric oxide synthase (eNOS), transcription factor Kruppel-like factor 4 (Klf4), intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), regulated on activation normal T cell expressed and secreted (RANTES), and monocyte chemotactic protein 1 (MCP-1) mRNA were measured using TaqMan real-time PCR.

Cytokine Detection: DCs were positively selected from the spleen using an autoMACS separator and CD11c magnetic beads (Miltenyi Biotech). The purity of these was confirmed to be >95% by flow cytometry. Splenic CD11c+ cells were placed in 96-well plates at a density of 5×10^5 per well and cultured in 200 μL RPMI1640 medium supplemented with 10% fetal bovine serum for 24 hours. Cytokines released into the medium were quantified via a Luminex assay. We have previously confirmed that this kit does not select mouse macrophages.

Confocal Microscopy: For superoxide detection, kidneys were rapidly removed after euthanasia, immersed in optimal cutting temperature (OCT) media and frozen in dry ice. Thirty μm sections were obtained and used for detecting superoxide by DHE as described previously. Sections were imaged using confocal microscopy with an excitation wavelength of 405 nm and an emission wavelength of 530-560 nm. For β3 tubulin staining, lymph nodes adjacent to the kidneys were carefully isolated, and 10-μm-thick frozen sections were prepared. Images were obtained using a primary anti-β3 tubulin antibody and the Alexa 488 goat anti-rabbit secondary antibody.

Dendritic Cell Adoptive Transfer: One million splenic DCs were obtained from mice that had undergone either sham surgery or bilateral renal denervation and subsequent angiotensin II or vehicle infusion for two weeks. These were suspended in 200 μL PBS, and adoptively transferred to naïve mice by tail vein injection. Telemetry transmitters were implanted in the recipient mice one week before adoptive transfer, and low dose (140 ng/kg/min) angiotensin II infusion were initiated 10 days later.

Preparation of Bone Marrow Derived Dendritic Cells: Bone marrow cells from C57BL/6 and β2AR−/− mice were cultured in 6-well plates at a concentration of 0.5×10^6 cells/ 2.5 ml in the presence of GMCSF and IL-4 as described previously. Norepinephrine (1 – 3 μmol/L) was added to the medium at the beginning of culture, and supplemented on day 3 and day 6. In other experiments neuropeptide Y was added to achieve a final concentration of 100 nmol/L.

Immunohistochemistry: Five micron sections were obtained from formalin fixed, paraffin embedded kidneys. Collagen was visualized by Masson Trichrome blue staining, and immunohistochemistry was used to detect CD3+ and F4/80+ cells in the kidney as previously described.
Magnetic Resonance Imaging: Relative renal blood flow (rRBF) and relative renal blood volume (rRBV) were measured by dynamic susceptibility contrast magnetic resonance imaging (DSC-MRI) with the administration of iron oxide nanoparticles as described. Six mice with unilateral renal denervation and two-week angiotensin II infusion were used in this experiment. One or two days before of scanning, a catheter was placed in the jugular vein, and externalized from the interscapular region for administration of contrast agent. Mice were anesthetized (isoflurane 1.5-2%) and scanned on a Varian 7T MRI system using a Doty 38 transmit/receive coil. Body temperature was maintained at 37° during the scan. An oblique axial plan through both kidneys was chosen. A dynamic susceptibility-weighted gradient-echo sequence was applied during an iv injection of monocrystalline iron oxide nanoparticles (MION, 6mg/kg), which was administered rapidly in less than one second. A dynamic series of 600 images (~1s/volume) was acquired up to 10 minutes. R₂ imaging was performed using a multiple spin echo sequence with refocusing pulses of 180 (repetition time=2500ms, 16 echoes, 4 averages) before and after MION injection. The averaged ΔR₂ across voxels of each kidney was used to represent rRBV, while peak amplitude/area from the relative DSC signal intensity time curve of each kidney was used to represent rRBF (sec⁻¹).

Statistics: Data in the manuscript are expressed as mean ± SEM. For telemetry and tail cuff blood pressure measurements over time, two-way ANOVA with repeated-measures was employed, followed with a Bonferroni post hoc test when significance was indicated. To compare the effect of renal denervation on renal inflammation, two-way ANOVA was used as indicated. Because of differences in variation between groups, we employed a non-parametric rank sum test with a Bonferroni correction for comparisons of cytokine release by DCs. P values are reported in the figures and a value less than 0.05 was considered significant.
References:


### Supplemental Table I: Effect of Renal Denervation on Cytokine Production in DCs

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Sham (n=5)</th>
<th>Ang II (n=8)</th>
<th>Ang II + RDN (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2</td>
<td>0.65±0.06</td>
<td>1.87±0.42 *</td>
<td>1.28±0.35</td>
</tr>
<tr>
<td>IL-12 p40</td>
<td>0.00±0.00</td>
<td>0.22±0.18</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>IL-12 p70</td>
<td>0.40±0.13</td>
<td>0.84±0.24</td>
<td>0.39±0.09</td>
</tr>
<tr>
<td>IL-23</td>
<td>820.4±328.16</td>
<td>672.2±245.6</td>
<td>377.6±60.62</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>13.9±3.46</td>
<td>76.04±35.65</td>
<td>13.65±3.60</td>
</tr>
<tr>
<td>TGFβ1</td>
<td>1.80±0.16</td>
<td>1.87±0.22</td>
<td>2.27±0.29</td>
</tr>
<tr>
<td>TGFβ2</td>
<td>1.75±0.14</td>
<td>1.70±0.05</td>
<td>1.88±0.10</td>
</tr>
<tr>
<td>TGFβ3</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
</tr>
</tbody>
</table>

In pg per 10⁶ cells. *p<0.05 vs. Sham.

IL: interleukin  
GM-CSF: granulocyte-macrophage colony-stimulating factor  
TGF: transforming growth factor
Online Figure I: Effects of renal denervation on adjacent lymph nodes and adrenal glands. The neuronal marker beta 3 tubulin was visualized in the lymph nodes adjacent to the kidneys of mice by confocal microscopy (A). Norepinephrine and epinephrine content in adrenal glands were measured by HPLC (B). Data were analyzed using unpaired t tests, n=5 in each group. No statistical significant difference was detected between groups.
Online Figure II: Effect of renal denervation on aortic inflammation in response to angiotensin II. Mice underwent bilateral renal denervation or sham surgery and then received angiotensin II (490 ng/kg/min) for 14 days. Aortas were harvested for flow cytometry and total leukocytes (A), T cells (B) and CD4$^+$ and CD8$^+$ T cells (C and D) quantified using flow cytometry. Data were analyzed using one-way ANOVA, n=5 in each group. *P < 0.05, **P < 0.01, ***P<0.001.
Online Figure III: Gating strategy for detection of macrophages, dendritic cells and monocytes from spleen and kidney.
Online Figure IV: Correlation between systolic pressure and renal T cell infiltration in mice after sham, unilateral (uniRDN) and bilateral renal denervation (biRDN). Mice underwent either sham surgery, unilateral or bilateral renal denervation and subsequently received either vehicle (Veh) or angiotensin II (Ang) infusion for two weeks. In unilateral denervated mice, the innervated (in) and denervated (de) kidneys are plotted separately. Flow cytometry was used to quantify total T cells (left panel), CD4+ and CD8+ T cells (middle and right panel).
Online Figure V: Effect of renal denervation on relative renal blood flow in mice with unilateral renal denervation after two-week angiotensin II infusion. Renal MRI was performed as described in the supplemental method. Data were analyzed with paired t test, n=5. *P < 0.05, **P < 0.01.
Online Figure VI: Effects of renal denervation on activation of other antigen presenting cells in the spleen in mice with angiotensin II infusion.
Online Figure VII: Ablation of afferent renal nerves does not prevent hypertension or renal inflammation. Selective ablation of afferent renal nerves by capsaicin treatment was validated by measurements of calcitonin gene related peptide (CGRP) (A). Renal norepinephrine and epinephrine contents were measured by HPLC as described in the methods (B). Systolic blood pressure was measured using the tail cuff method (C, n=8 for each group.) Flow cytometry of single cell homogenates was used to quantify renal infiltration of leukocytes and lymphocytes in the kidney (D-H). Data for panels A and B (n=5 per group) were analyzed using paired t test. Data in panel C was measured using two-way ANOVA. Data in panels E – H were analyzed by unpaired t tests.
Online Figure VIII: Effects of sympathetic signaling on isoketal-protein adduct formation in dendritic cells. DCs were magnetically isolated from spleens of mice that received vehicle or angiotensin II for two weeks. mRNA expression of adrenergic receptor (AR) subtypes were measured by realtime PCR (n=3). Protein levels of α2β, β1 and β2 adrenergic receptors in DCs were determined by Western blot and mouse brain was used as a positive control (B). DCs were derived from bone marrow of either wild type or β2AR−/− mice using GM-CSF and IL-4, and cultured with norepinephrine in vitro for 7 days (n=5 per group). In other experiments (E) wild type bone marrow-derived DCs were cultured in the presence of neuropeptide Y (n=4 per group). Isoketal-protein adducts were quantified by flow cytometry. Data in panels A and E were analyzed with t test. Data in panel D were analyzed using two-way ANOVA.
Online Figure IX: Effect CCR7 deficiency on hypertension, dendritic cell maturation and renal inflammation. CCR7−/− mice received angiotensin II (490 ng/kg.min) for two weeks. Blood pressure was measured by tail cuff (A). Flow cytometry was used to determine surface expression of CD80 and CD86 on DCs from the kidney (B) and spleen (C) and the renal presence of total leukocytes (D), T cells (E to G), and memory T cells (H and I). Data were analyzed using t tests, n=5 in each group. *P < 0.05.