Renal Denervation

Resting Afferent Renal Nerve Discharge and Renal Inflammation

Elucidating the Role of Afferent and Efferent Renal Nerves in Deoxycorticosterone Acetate Salt Hypertension

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Abstract—Renal sympathetic denervation (RDNx) has emerged as a novel therapy for hypertension; however, the therapeutic mechanisms remain unclear. Efferent renal sympathetic nerve activity has recently been implicated in trafficking renal inflammatory immune cells and inflammatory chemokine and cytokine release. Several of these inflammatory mediators are known to activate or sensitize afferent nerves. This study aimed to elucidate the roles of efferent and afferent renal nerves in renal inflammation and hypertension in the deoxycorticosterone acetate (DOCA) salt rat model. Uninephrectomized male Sprague–Dawley rats (275–300 g) underwent afferent-selective RDNx (n=10), total RDNx (n=10), or Sham (n=10) and were instrumented for the measurement of mean arterial pressure and heart rate by radiotelemetry. Rats received 100-mg DOCA (SC) and 0.9% saline for 21 days. Resting afferent renal nerve activity in DOCA and vehicle animals was measured after the treatment protocol. Renal tissue inflammation was assessed by renal cytokine content and T-cell infiltration and activation. Resting afferent renal nerve activity, expressed as a percent of peak afferent nerve activity, was substantially increased in DOCA than in vehicle (35.8±4.4 versus 15.3±2.8 %Amax). The DOCA–Sham hypertension (132±12 mm Hg) was attenuated by ≈50% in both total RDNx (111±8 mm Hg) and afferent-selective RDNx (117±5 mm Hg) groups. Renal inflammation induced by DOCA salt was attenuated by total RDNx and unaffected by afferent-selective RDNx. These data suggest that afferent renal nerve activity may mediate the hypertensive response to DOCA salt, but inflammation may be mediated primarily by efferent renal sympathetic nerve activity. Also, resting afferent renal nerve activity is elevated in DOCA salt rats, which may highlight a crucial neural mechanism in the development and maintenance of hypertension. (Hypertension. 2016;68:1415-1423. DOI: 10.1161/HYPERTENSIONAHA.116.07850.)

Key Words: afferent neurons ■ arterial pressure ■ denervation ■ deoxycorticosterone acetate ■ hypertension ■ inflammation

Arterial hypertension continues to be a primary predictor of future cardiovascular disease morbidity and mortality in the United States and worldwide. Unfortunately, the development of effective treatment and preventative modalities has not met the increasing demand. Although the pathogenesis of hypertension is complex, the contribution of renal dysfunction and increased activity of the sympathetic nervous system has been extensively documented and studied.

The concept that increased renal sympathetic nerve activity contributes to hypertension development, and maintenance is supported by studies showing renal denervation (RDNx) attenuating and reversing experimental hypertension. These findings led to the first clinical trials to treat patients with drug-resistant hypertension with RDNx, which reported an effective and sustained decrease in arterial pressure after RDNx. However, recent clinical trials have failed to support this antihypertensive effect, which has led to an intense debate over the effectiveness of RDNx and the need for further preclinical study.

The antihypertensive effect of RDNx was initially hypothesized to result from the ablation of renal efferent nerves, which contribute to tubular sodium reabsorption, renin release, and renal vascular resistance. However, several studies conducted in the 1980s suggested an important role for renal afferent nerves in the pathogenesis of hypertension because the interruption of visceral afferent input to the spinal cord by dorsal rhizotomy attenuated several preclinical models of hypertension. These studies suggested that increased afferent renal nerve activity (ARNA) contributes to an increase in SNA to renal and nonrenal vascular beds, resulting in an...
increase in arterial pressure. Interestingly, RDNx in patients with drug-resistant hypertension reportedly reduces muscle SNA,14 plasma glucose,15 arrhythmias,16,17 and even episodes of sleep apnea.18,19 Altogether, these findings are consistent with the hypothesis that a portion of the beneficial responses to RDNx may be due, in part, to the ablation of afferent rather than efferent renal nerves.

Our group has recently developed a novel method for selective renal afferent nerve ablation (A-RDNx).20 Also, we observed both total (T-RDNx) and A-RDNx attenuated deoxycorticosterone acetate (DOCA) salt hypertension by ≈50%, suggesting that the antihypertensive response to RDNx is primarily because of the ablation of afferent nerves.20 However, the question of how afferent-targeted RDNx attenuates hypertension remains unanswered.

Renal nerves, specifically efferent nerves,21 reportedly mediate renal inflammation through the trafficking and activation of T-cells and increased inflammatory cytokine content.22 Furthermore, several inflammatory proteins (eg, IL [interleukin]-6, IL-1β, MCP [monocyte chemoattractant protein]-1, and GRO/KC [chemokine ligand 1])23,24 that are increased in the models of hypertension may directly modify the sensitivity of peripheral sensory nerves.25 Together, these data suggest that the renal inflammation drives renal afferent activity and, in turn, contributes to the increase in arterial pressure.

This study was designed to investigate the antihypertensive effect of RDNx on DOCA salt hypertension and the roles of efferent and afferent renal nerves in this response. We directly tested the hypothesis that afferent renal nerve discharge and sensitivity to stimulation would be increased after 21 days of DOCA salt. Next, we tested the hypothesis that total RDNx (T-RDNx) and selective afferent RDNx (A-RDNx) would attenuate the development of DOCA salt hypertension. Moreover, we hypothesized that A-RDNx would lower arterial pressure independent of a decrease in renal inflammation.

Methods
All procedures were approved by the University of Minnesota Institutional Animal Care and Use Committee and were in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals. A detailed description of the Methods and Materials can be found in the online-only Data Supplement.

Experiment 1: Direct Recording of Resting Afferent Renal Nerve Activity in Vehicle and DOCA Salt Rats
These experiments were conducted using the DOCA salt rat, a well-established model that recapitulates moderate-to-severe human salt-sensitive hypertension and renal inflammation.26 Twenty male Sprague–Dawley rats (weight, 275–300 g; age, 10–12 weeks) underwent a unilateral nephrectomy 14 days before DOCA salt treatment (Figure 1). All rats were nephrectomized and allowed 3 weeks to recover. Half of the rats were switched to 0.9% saline and, 5 days later, were administered 100-mg DOCA in a silicone implant subcutaneously (DOCA, n=10). Controls remained on distilled water and received a silicone vehicle implant (vehicle, n=10). After the 21-day treatment, tissues were collected for further analysis. Expanded Methods are available in the online-only Data Supplement.

The mean value of the 10-minute baseline integrated voltages (∫ ARNA) was used to quantify the resting ARNA. Furthermore, to control for intraexperimental variability in the use of multiunit nerve recording,27 resting afferent nerve activity was also normalized to the peak ∫ ARNA response to intraperitoneal 50 μmol/L capsaicin (in 150 mmol/L NaCl), defined as the peak afferent activity (∫ ARNApeak), and expressed as a percent of ∫ ARNAmax using the following calculation:

\[
\%A_{\text{max}} = 100 \times \frac{\text{∫ ARNA}_{\text{peak}}}{\text{∫ ARNA}_{\text{baseline}}}
\]

Experiment 2: Effect of Total and Selective Afferent Renal Denervation on DOCA Salt Hypertension and Renal Inflammation
A separate experiment was designed to determine the role of efferent and afferent renal nerves in the development of DOCA salt hypertension and the associated renal inflammation. Thirty-eight male Sprague–Dawley rats (weight, 275–300 g; age, 10–12 weeks) underwent a unilateral nephrectomy 14 days before DOCA salt treatment (Figure 1). Rats were then randomly assigned to 1 of 4 experimental groups: (1) 100-mg DOCA salt+total renal RDNx (T-RDNx, n=12); (2) 100-mg DOCA salt+selective afferent ablation (A-RDNx, n=11); (3) 100-mg DOCA salt+sham denervation (DOCA–Sham, n=10), or (4) 0 mg DOCA+sham denervation (vehicle–Sham, n=5). T-RDNx was achieved by a perivascular application of 33 mmol/L renal RDNx was achieved by a perivascular application of 33 mmol/L capsaicin (in 5% Tween 80; 5% ethanol; 90% 150 mmol/L NaCl). Animals were instrumented with radiotelemeters for the continuous measurement of arterial pressure and heart rate. After the 21-day treatment, tissues were collected for further analysis. Expanded Methods are available in the online-only Data Supplement.

Statistical Analysis
Temporal data were analyzed by a repeated measurement, 2-way ANOVA with a Bonferroni post hoc test (α=0.05). All other data (>2 groups) were analyzed by 1-way ANOVA with a Bonferroni post hoc test (α=0.05) or a Student t test (2 groups). Statistical analyses were performed with GraphPad Prism 6.0 software. *P<0.05 versus vehicle–Sham; #P<0.05 versus DOCA–Sham. Data are presented as means±SEM.

Results

Experiment 1: Resting ARNA in the DOCA Salt Model
Resting Afferent Nerve Activity and Sensitivity in DOCA Salt Rats
Resting ARNA was measured under 2% isoflurane anesthesia. Mean arterial pressure (MAP) was elevated in anesthetized DOCA (151±5 mm Hg) rats than in vehicle (106±4 mm Hg). Sample nerve tracings of resting renal sympathetic nerve activity, resting ARNA, and background noise signals from both vehicle and DOCA rats are depicted in Figure 2.

Figure 1. The deoxycorticosterone acetate (DOCA) salt protocol timeline for experiments 1 and 2. A-RDNx indicates afferent-selective renal denervation; and T-RDNx, total renal denervation.
Before cutting the central end of the nerve, the integrated voltage of total multunit nerve activity (afferent+efferent) was no different between vehicle and DOCA (Figure 3B). After sectioning the central end of the nerve, activity fell to <10% of this initial level, but note that resting ∫ARNA was higher in DOCA (Figure 3C). No difference was observed in peak response to intrapelvic capsaicin between groups (Figure 3D). Finally, when resting ∫ARNA is expressed as percentage of peak ∫ARNA to intrapelvic capsaicin (%Amax), resting ARNA was significantly higher in DOCA (Figure 3E). Notably, resting ARNA was ≈2.5-fold higher in DOCA regardless of whether it was expressed as ∫ARNA or %Amax.

In a subset of 6 rats in each group, the responses to iso-baric intrapelvic perfusion of isotonic saline (150 mmol/L NaCl), hypertonic saline (600 mmol/L NaCl), and bradykinin (20 µg/mL) were assessed. Response to increased pelvic pressure from 0 to 20 mm Hg was also measured. No difference between groups was observed in ARNA response to any stimuli (see online-only Data Supplement).

Figure 3. After deoxycorticosterone acetate (DOCA) salt treatment, resting afferent renal nerve activity (ARNA) was measured in anesthetized rats. A, A sample tracing of raw (top), rectified (middle), and integrated (bottom) nerve activity from a vehicle–Sham animal is depicted, in which ARNA was recorded before and during the response to intrapelvic administration of 50 µmol/L capsaicin to establish the peak level of afferent renal nerve discharge. ARNA was quantified from the integrated (∫ ARNA) signal. B, No difference between vehicle and DOCA rats in raw integrated signal of resting total renal nerve activity. C, After proximal sectioning of the renal nerve, signal recorded represents only ∫ARNA. The resting ∫ARNA was elevated in DOCA than in vehicle (0.24±0.04 vs 0.10±0.01 µV·s). D, Peak afferent nerve response to intrapelvic perfusion of 50 µmol/L capsaicin was not different between vehicle and DOCA. E, Basal ARNA, expressed as a percentage of peak ARNA response to intrapelvic capsaicin (%Amax). After normalization, resting activity remained increased in DOCA salt rats compared with vehicle (32.0±5.7 vs 13.8±2.7 %Amax). All data are presented as mean±SEM (n=10/group). *P<0.05 vs vehicle–Sham.
Experiment 2: Response to Total and Selective Afferent Renal Denervation in DOCA Salt Hypertension and Renal Inflammation

Cardiovascular Responses
Before DOCA implantation, baseline MAP was lower in T-RDNx than in DOCA–Sham (96±1 versus 105±2 mm Hg) rats (Figure 4A). In contrast, basal MAP was unaffected by A-RDNx (103±4 mm Hg). The response to treatment was calculated as percent change from baseline (Figure 4B). The percent increase in MAP in DOCA–Sham was greater than vehicle–Sham, which was attenuated equally by T-RDNx and A-RDNx (Figure 4B). The steady-state absolute change in MAP (Figure 4C), averaged over the final 3 days (days 19–21), was greater in DOCA–Sham than in vehicle–Sham (Δ27±2 versus Δ4±1 mm Hg). Furthermore, this response was halved by T-RDNx and A-RDNx (Δ15±1 and Δ14±2 mm Hg). Baseline heart rate before DOCA salt treatment was similar across experimental groups (Figure 4D). The percent decrease in heart rate was greater in DOCA–Sham than in vehicle–Sham (Figure 4E), and T-RDNx and A-RDNx also mitigated this response. Steady-state heart rate response (Figure 4F) to DOCA–sham (−56±5 bpm) was also attenuated by T-RDNx and A-RDNx (−45±4 versus −39±4 bpm, respectively). No effect of T-RDNx or A-RDNx versus DOCA–Sham was observed in daily or cumulative sodium intake (Figure S2 in the online-only Data Supplement).

Renal Inflammatory Chemokine and Cytokine Content
As shown in Figure 5, several proinflammatory markers (GRO/KC, MCP-1, IL-2, and IL-6) were increased in DOCA–Sham than in vehicle–Sham. Moreover, compared with DOCA–Sham, T-RDNx decreased nearly all inflammatory markers (GRO/KC, MCP-1, IL-1β, IL-2, IL-6, IL-17a, and TNF-α). Similarly, A-RDNx attenuated several proinflammatory cytokines (GRO/KC, MCP-1, and IL-2), but fewer markers and to a lesser extent than T-RDNx. Within DOCA-treated rats (DOCA–Sham, T-RDNx, and A-RDNx), no significant correlation between arterial pressure and cytokine content was observed (see online-only Data Supplement)

Renal T-Cell Infiltration and Activation
To elucidate the role of renal efferent and afferent nerves in renal inflammation, renal single-cell lymphocyte suspensions were analyzed by flow cytometry. Renal T-cells and subpopulations, including CD4+ and CD8+ subsets (Figure 6), are nearly increased in DOCA–Sham rats. T-RDNx tended to prevent this effect, where CD4+ regulatory T-cells (CD25high), CD8+ T-cells, and CD8+ central memory T-cells (CD44high/CD62Lhigh) were lowered (P<0.05) by T-RDNx than by DOCA–Sham. No differences were observed between A-RDNx and DOCA–Sham.
RDNx Efficacy
T-RDNx reduced renal norepinephrine content to nearly 0 (<5% DOCA–Sham). In contrast, norepinephrine content in A-RDNx remained similar to DOCA–Sham. T-RDNx and A-RDNx markedly and similarly reduced CGRP (calcitonin gene related peptide) content, indicating that a similar efficacy was achieved. Together, these data indicate that T-RDNx and A-RDNx techniques ablated the majority of the targeted nerves. Data are available in the online-only Data Supplement.

Discussion
The objective of the present study was to investigate the role of afferent and efferent renal nerves in the pathogenesis of a model of salt-sensitive hypertension that is associated with renal inflammation—the DOCA salt rat. There were 4 important findings: first, using direct multiunit in vivo recording, we observed resting ARNA was elevated in the DOCA salt rat. Second, T-RDNx and selective A-RDNx mitigated both the rate and magnitude of DOCA salt hypertension development equally. Third, nearly all activated renal T-cells were lowered by T-RDNx compared with DOCA–Sham rats, but not A-RDNx. Finally, T-RDNx also lowered the renal proinflammatory marker content than DOCA–Sham, whereas A-RDNx attenuated or had no effect.

Collectively, these findings support the hypothesis that afferent and efferent renal nerves both play a central role in the pathogenesis of DOCA salt hypertension and renal inflammation. Our interpretation of these findings in the context of previous studies is discussed in greater detail below.

Renal Nerves and Hypertension: Disparate Roles for Afferent and Efferent Sympathetic Nerves
Increased renal sympathetic nerve activity and renal inflammation are both recently hypothesized to contribute to the development and maintenance of many forms of hypertension. In this study, and previous reports from our laboratory,6,20 T-RDNx abated the rate of development of DOCA salt hypertension by ≈50%. In addition, as we have previously observed,23 T-RDNx decreased the basal level of arterial pressure (before DOCA salt) by ≈10 mm Hg, suggesting that renal efferent nerves contribute to blood pressure homeostasis under normotensive conditions in the rat. In contrast, whereas A-RDNx had no effect on the basal level of arterial pressure, it attenuated the rate of development and magnitude of hypertension in a temporal pattern identical to T-RDNx. This finding, combined with the observation that the resting level of ARNA was substantially increased in DOCA salt, leads us to conclude that the renal nerve-dependent component of DOCA

| Figure 5. Renal cortical and medullary (mixed) tissue inflammatory marker content was measured by Luminex multiplex immunoassay. Several proinflammatory markers (GRO/KC [chemokine ligand 1], MCP [monocyte chemoattractant protein]-1, IL [interleukin]-2, and IL-6) were increased (*P<0.05) in DOCA–Sham rats than in vehicle–Sham. Total renal denervation (T-RDNx) greatly decreased (#P<0.05) the levels of GRO/KC, MCP-1, IL-1β, IL-2, IL-6, IL-17a, and TNF-α vs DOCA–Sham. Afferent-selective renal denervation (A-RDNx) attenuated fewer proinflammatory cytokines (GRO/KC, MCP-1, and IL-2) than DOCA–Sham. All data are presented as mean±SEM. *P<0.05 vs vehicle–Sham; #P<0.05 vs DOCA–Sham. |
salt hypertension is primarily mediated by increased afferent, rather than by efferent, renal nerve activity.

To our knowledge, there is only one other report where resting ARNA was measured in a model of hypertension. In agreement with the present study, Moss and Stoltock\textsuperscript{29} observed an increased resting firing rate of single-unit afferent renal nerves in the spontaneously hypertensive rat compared with a normotensive control. Whereas single-unit recordings are favorable because of simple quantification and low-experimental variability, this technique is dependent on large sample sizes to appropriately represent whole-organ activity. Alternatively, multiunit recording offers general sampling of multiple units within a bundle that may be missed using single-unit recording. Also, we feel that the use of normalization to a maximal response avoids the traditional complication of intraexperimental variability and group comparison. Importantly, the observation from this study using multiunit recordings and the previous use of single-unit recordings both confirm an elevation in ARNA in hypertensive models.

A more direct approach to elucidate this mechanism is the monitoring of arterial pressure response to ARNA stimulation. Notably, Kotke et al\textsuperscript{11} reported in 1945 that long-term direct stimulation of renal nerves produced chronic hypertension in dogs. Although efferent renal nerve stimulation was the presumed cause, the role of afferent renal nerve stimulation cannot be ruled out. More recently, studies from Patel and Kneuper\textsuperscript{32} and Simon et al\textsuperscript{33} found that acute stimulation of renal afferent nerves in conscious rats elicited an increase in vasopressin and arterial pressure. However, no follow-up

\begin{figure}
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\includegraphics[width=\textwidth]{figure6}
\caption{Renal T-cell infiltration and activation were assessed by flow cytometry. Nearly all T-cells subtypes were elevated with deoxycorticosterone acetate (DOCA) salt salt treatment. Total renal nerve ablation (T-RDNx) tended to lower renal T-cell infiltration and activation than DOCA–Sham. Compared with afferent-targeted RDNx (afferent-selective renal denervation [A-RDNx]), CD4+ central memory (Tcm), effecter memory (Tem), and regulatory (Treg) were lower in T-RDNx animals. All data are presented as mean±SEM. *P<0.05 vs vehicle–Sham; #P<0.05 vs DOCA–Sham.}
\end{figure}
studies examined whether this response is maintained longer than 1 hour, so the arterial pressure response to chronic ARNA stimulation remains speculative.

Afferent renal nerves modulate autonomic neural pathways in the brain integral in neuroendocrine control of cardiovascular function. For example, afferent renal nerve stimulation was recently reported to directly modulate the activity of premotor autonomic neurons in the rostral ventrolateral medulla and paraventricular nucleus in rats. Moreover, hypothalamic lesions attenuate DOCA salt hypertension and the two-kidney-one-clip model of hypertension, a model which is also proposed to be dependent on afferent renal nerves. Although peripheral sympathetic activity or neural signaling in the hypothalamus was not measured in the current study, we hypothesize that the attenuation of DOCA salt hypertension by A-RDNx is likely because of a disruption of afferent signal to the paraventricular nucleus and rostral ventrolateral medulla resulting in an attenuation of sympathetic activity and vasopressin release—both of which are reported to be elevated in DOCA salt hypertension. The neural and hormonal mechanisms by which A-RDNx attenuates DOCA salt hypertension remained to be established.

Notably, this study contrasts with our recent report using a similar approach in the Dahl salt-sensitive (Dahl S) rat on high-salt diet. Although T-RDNx decreased arterial pressure similarly in both the early and late phases of Dahl S hypertension, A-RDNx had no effect on arterial pressure. Based on these findings, we concluded that afferent renal nerves do not contribute to the hypertension in the Dahl S rat. Importantly, it is clear that A-RDNx does not uniformly prevent all experimental models of hypertension. The explanation for these differences is unfortunately unknown. One possibility is that renal inflammation, which hypothetically drives ARNA, is less pronounced in the Dahl S rat compared with the DOCA salt model. In other words, despite reports of renal inflammation in the Dahl S rats, it may not be of the type or magnitude that assymmetrically alters ARNA. Additional examination of the mechanistic relationship between renal inflammatory mediators and renal afferent nerves is currently underway.

Renal Nerves and Renal Inflammation: A Bidirectional Communication?

Renal nerve ablation is reported to have an anti-inflammatory effect, a concept that is also supported by this study. We found that the combination of efferent and afferent denervation (T-RDNx) abated the renal inflammation induced by DOCA salt. This is consistent with the previous report that T-RDNx attenuated the development of hypertension and renal inflammation in the angiotensin II mouse model. Moreover, these studies suggest that renal efferent nerves regulate the trafficking of inflammatory mediators. Interestingly, in contrast to the previous study where Xiao et al observed A-RDNx had no effect on the renal inflammation or hypertension in the angiotensin II mouse model, we observed an equal attenuation of the hypertension with A-RDNx and T-RDNx. It is important to note that this crucial difference cannot be directly attributed to the renal inflammation, as A-RDNx had a lesser effect on renal inflammatory cytokine content or T-cell activation than T-RDNx. Moreover, based on the findings of the current study, we conclude that the afferent renal nerves directly contribute to the DOCA salt hypertension, and the prohypertensive effects of renal inflammation may be relayed by the afferent renal nerves. Although the role of afferent nerves in the blood pressure effect is clear, the mechanism linking afferent nerves and renal inflammation remains speculative.

Peripheral and central sensitization to afferent sensory input has been reported in models of systemic inflammation and inflammation isolated in the dorsal root ganglion. This raises the question of whether nerves can become activated or sensitized through the interaction with proinflammatory mediators. Although we did not observe an increased sensitivity, we cannot rule out tonic activation because we observed ARNA was elevated under resting conditions in the DOCA salt rat. Moreover, in a study by Veelken et al using a rat model of glomerulonephritis, the investigators suggested that inflammatory immune cells (eg, macrophage and dendritic cells) may be indirectly communicating with the central nervous system through the direct interaction with afferent nerves. When considering our inflammatory protein content data, which suggest that a myriad of proinflammatory cell types and signals are contributing to this cytokine storm, several candidates for the chronic sensory nerve stimuli remain targets for future study.

About the anti-inflammatory effect of T-RDNx and partial effect A-RDNx, we think that this is in part because of a reduced renal perfusion pressure. Klaneke et al elegantly demonstrated a pressure-mediated renal inflammatory response in the DOCA salt rat. These investigators reported that both mineralocorticoid and pressure effects individually contribute to the renal inflammation induced by DOCA salt in rats. Although we observed a greater reduction in renal inflammation after T-RDNx compared with A-RDNx, it is important to note that blood pressure reduction remained similar. Interestingly, the prohypertensive effect of renal inflammation may be mediated through renal afferent nerves, as indicated by the antihypertensive effect of A-RDNx and the increased resting ARNA in DOCA salt rats. Further studies are required to elucidate the direct activation of renal afferent nerves by renal inflammation and its role in chronic blood pressure control.

Summary and Clinical Perspectives

With the recent conflicting clinical reports of the efficacy of RDNx, it is crucial to elucidate the mechanisms of the effect of RDNx and if it may be improved or tailored for the treatment of hypertension complicated by renal disease. Collectively, the data from the present study suggest that DOCA salt hypertension is mediated by both efferent and afferent renal nerves. First, efferent renal nerves seem to contribute to renal inflammation development, and the prohypertensive effect of renal inflammation is likely mediated through the presence and activation of afferent renal nerves. In concert with our other recent findings where no preventative or reversal effect of afferent-specific denervation was observed in Dahl S rats, perhaps these data collectively suggest that RDNx is not a universal treatment for all hypertension types. Furthermore, perhaps RDNx would be most effective in patients with confirmed renal inflammation and elevated peripheral or renal SNA. Indeed, development of diagnostic tests for renal inflammation and elevated renal sympathetic nerve activity could isolate this patient population. Furthermore, our current study...
highlights an important role for renal afferent nerves to consider in the future study of RDNx.

Limitations

Anesthesia
Measurement and analysis of renal sympathetic nerve activity is potentially complicated by anesthesia. It is not clear whether renal afferent nerve activity is affected because recordings were made directly from axonal projections of sensory nerves, where anesthetic modulations of synaptic transmission is not an issue. Although we did use a normalization procedure to minimize this confounder, we cannot rule out the possibility that isoflurane modulates afferent nerve discharge. We are currently developing a method to record ARNA in conscious rats to explore this possibility.

Denervation Efficacy

Based on our previous study,28 we have validated the efficacy of periaxonal capsaicin for targeted ablation of renal sensory neurons. We observed that pelvic CGRP content is undetectable 10 days post capsaicin treatment, but becomes detectable after 4 weeks. This time-dependent recovery of neurotransmitter content suggests that reinnervation may be occurring; however, it is not clear whether the detectable levels of neurotransmitter content in this study is because of partial denervation attainment or reinnervation. Moreover, although the loss of pelvic CGRP content is consistent with the ablation of periaxonal capsaicin for targeted ablation of renal sensory nerves, it is unknown whether a single capsaicin treatment results in the loss of renal dorsal root ganglion cell bodies and axons. Further studies are needed to more clearly establish the extent and duration of targeted renal afferent denervation using this method.

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Disclosures

None.

References

The present study suggests that afferent renal nerves may contribute directly to the development and maintenance of salt-sensitive hypertension complicated by renal inflammation.
SUPPLEMENTAL MATERIAL

Resting afferent renal nerve discharge and renal inflammation: Elucidating the role of afferent and efferent renal nerves in DOCA-salt hypertension

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SUPPLEMENTAL METHODS AND MATERIALS

Animals

Male Sprague Dawley rats (Weight: 275-300g; Age: 10-12 weeks) were purchased from Charles River Laboratories (Wilmington, MA) and housed in pairs in a temperature and light controlled room. Rats were allowed access to standard rat chow and distilled water ad libitum during this pre-experimental period.

Experiment 1: Direct recording of resting afferent renal nerve discharge in DOCA-salt rats

General Procedures

Rats were anesthetized with 5% isoflurane, and maintained at 2-3% during surgical preparation. The femoral vein and artery were cannulated with PE50 tubing for 0.9% saline perfusion (50µl/min) to establish steady state urine production and for arterial pressure measurement respectively. The renal pelvis was cannulated with a 32G triple-lumen catheter (Part Number: 0040EO; ReCathCo, Allison Park, PA) for intrapelvic perfusion and withdrawal of infusates and monitoring of pelvic pressure. A renal nerve bundle near the renal hilus was isolated and gently placed on an electrode consisting of two single stainless steel wires (0.003”; Sigmund Cohn Corp., Mount Vernon, NY). A ground wire was placed nearby in the abdominal muscle. Excess fluid was removed from the site by vacuum, and the nerve and electrode were encased in silicone (Kwik-Sil, WPI, Sarasota, FL) once a recording was obtained. Finally, the central end of the nerve was cut to eliminate efferent nerve traffic in the signal leaving only afferent nerve discharge and background noise. Following the surgical preparation, animals were maintained at 1.5% isoflurane for 30-minutes prior to initiating the protocol.

To determine the background noise contribution to the signal, the distal end of the nerve was cut upon completion of the experiment. This integrated voltage was subtracted from the final integrated ARNA signal (∫ARNA) during subsequent analysis. The nerve signal was amplified (Gain: 20,000X) and filtered by band-pass (0.05-3kHz) using a single-channel amplifier (Nihon Kohden, Model: Meg2100). All data was recorded at 2kHz and analyzed using LabChart 8.0 software (ADInstruments, USA).

Establishment of Maximal Afferent Renal Nerve Activity for Normalization Analysis

A separate pilot study was conducted in young male Sprague Dawley rats (n=6) to establish a stimulus that would induce maximal activation of afferent renal nerve activity (ARNa) in each animal to be used for normalizing multiunit recording of ARNA between animals (see Figure S1). We compared the ARNA response to non-selective depolarization by intrapelvic administration of increasing concentrations of potassium chloride (KCl; 40, 80, 60, and 320 mM) to increasing concentrations of the TRPV1 agonist capsaicin (10, 50, and 100 µM). Each stimulation was followed by flushing the renal pelvis with isotonic saline. Resting afferent activity was allowed to return to baseline between each stimulation.

Measurement of Resting ARNA and Responsiveness to Stimuli in DOCA-Salt Rats

This experiment was designed to test the hypothesis that DOCA-salt treatment alters resting ARNA. Twenty male Sprague Dawley rats (Weight: 275-300g; Age: 10-12 weeks) underwent the 21-day DOCA-salt treatment as previously described \(^1\), \(^2\). All rats were nephrectomized, and allowed three weeks to recover. Half of the rats were switched to 0.9% saline, and five days later, administered 100mg DOCA in a subcutaneous silicone implant (DOCA; n=10). Controls remained on distilled water and received a silicone vehicle implant (Vehicle; n=10). After 21 days of DOCA-salt (DOCA; n=10) or Vehicle (n=10) treatment, animals were anesthetized with 5% isoflurane, and maintained at 2-3% during surgical preparation.
Resting ARNA was recorded over a 10-minute period. Next, we measured the ARNA responses to randomized mechano- and chemo-sensitive stimuli. These included a 20mmHg increase in pelvic pressure with isotonic saline, intrapelvic perfusion of hypertonic saline (600mM NaCl), and intrapelvic administration of 20µg/mL bradykinin. A minimum of a five-minute recovery period was allowed between each stimulus to allow activity to return to resting level. Finally, a 50µM capsaicin solution was perfused into the renal pelvis to elicit a maximum level of afferent renal nerve discharge.

Nerve Activity Quantification and Analysis

The mean value of the 10-minute baseline \( \int \text{ARNA} \) was used to quantify resting ARNA. Further, to control for intra-experimental variability of the electrode placement and nerve units, resting afferent nerve activity was also normalized to the peak \( \int \text{ARNA} \) response to intrapelvic 50µM capsaicin (\( \int \text{ARNA}_{\text{peak}} \)). Resting \( \int \text{ARNA} \) was expressed as a percent of \( \int \text{ARNA}_{\text{peak}} \) using the following calculation:

\[
\%A_{\text{max}} = 100 \times \frac{\int \text{ARNA}_{\text{baseline}}}{\int \text{ARNA}_{\text{peak}}}
\]

Experiment 2: Effect of total and selective afferent renal denervation on DOCA-salt hypertension and renal inflammation

Experimental Groups and Surgical Procedures

Thirty-eight rats underwent a unilateral nephrectomy 14 days prior to treatment as previously described. Rats were then randomly assigned to one of four experimental groups: (1) 100 mg DOCA-salt + total renal denervation (DOCA T-RDNx; n=12), (2) 100 mg DOCA-salt + selective afferent ablation (DOCA A-RDNx; n=11), (3) 100 mg DOCA-salt + sham denervation (DOCA Sham; n=10), or (4) 0 mg DOCA + sham denervation (Control; n=5).

For all surgical procedures, rats were anesthetized with 2-3% isoflurane (Phoenix Pharmaceutical, St. Joseph, MO). Atropine sulfate (0.2 mg/kg, i.p.; West-Ward Pharmaceuticals, Eatontown, NJ), ketoprofen (5 mg/kg, s.c.; Fort Dodge Animal Health, Overland Park, KS) and gentamicin sulfate (2.5 mg/kg, i.m.; Hospira, Lake Forest, IL) were administered prior to surgery. Total RDNx was achieved via a midline abdominal incision by surgical sectioning of the renal nerves followed by a perivascular application of 10% phenol solution (in 100% ethanol) as previously reported. Selective afferent renal nerve ablation (A-RDNx) was performed by periaxonal application of a concentrated capsaicin solution (33mM capsaicin in 5% ethanol; 5% Tween 80; 90% 150mM NaCl) to the renal artery and vein as recently described. Sham procedures were performed with 0.9% saline. Following renal denervation or sham, rats were instrumented with a radiotelemeter for continuous measurement of arterial pressure and heart rate. Rats were allowed a seven-day post-operation recovery period and then housed individually in metabolic cages for the duration of the experiment (Techniplast, USA). For the three-day post-operation recovery, rats were given ketoprofen (2.5 mg/kg, s.c; s.i.d.), and the drinking water was supplemented with amoxicillin (1 mg/ml; Sandoz International, Holzkrichen, Germany).

Experimental Protocol

Following the seven-day recovery period, drinking water was replaced with a 0.9% saline solution for the three DOCA-salt groups (the Control group remained on normal drinking water) and baseline measurements of arterial pressure, heart rate and sodium intake were begun. At the end of the 5-day control period, rats were briefly anesthetized with isoflurane and received either a subcutaneous implant of 100mg DOCA in silicone or silicone vehicle (Sylgard 184 silicone elastomer base; Dow Corning, Midland, MI). Arterial pressure (AP) and heart rate (HR) were monitored continuously by radiotelemetry (Model PA-C10, Data Sciences International, USA).
AP was sampled at 500 Hz for 10 seconds every four minutes using commercially available software (Dataquest A.R.T., Data Sciences International, USA). HR was determined from the AP profile. Mean 24-hour (24h) averages of AP (MAP) and HR were calculated and plotted for each day of the study. Daily food and water intake were measured gravimetrically. Sodium intake was calculated by summing the intake of the 0.1% (g/g) NaCl chow diet and 0.9% (g/ml) saline intake. Cumulative sodium intake was calculated by area-under-the-curve analysis of 21 days of daily sodium intake data following DOCA or silicone vehicle implantation. Individual curves for each rat were analyzed and then group averages were analyzed for each treatment group. At the end of the three weeks of treatment, rats were anesthetized and euthanized by exsanguination. Kidneys were collected and dissected to isolate the renal pelvis from the renal cortical-medullary tissue (combined) sample. Tissues were flash-frozen in liquid nitrogen and stored for subsequent assessment of efficacy of T-RDNx and A-RDNx and measurement of renal inflammatory protein content.

_Condition of T-RDNx and A-RDNx_

Renal norepinephrine (NE) content was measured to assess whether efferent renal nerves were ablated effectively. Protein homogenates were assayed by high-performance liquid chromatography (HPLC) analysis with electrochemical detection 3. Effectiveness of afferent nerve ablation was assessed through the enzyme linked immunosorbent assay (ELISA) detection of calcitonin gene-related peptide (CGRP) renal tissue content as recently described 2. In isolated renal pelvic samples, CGRP tissue content was assayed according to the assay’s manufacturer’s instructions (Cayman Chemicals, Ann Arbor, MI). Data is expressed in picograms of analyte per milligrams of total protein. Homogenate total protein concentration was measured by Bradford assay.

_Renal Cytokine Analysis_

The renal inflammatory profiles of DOCA-salt and control rats were determined by measuring several pro-inflammatory chem- and cytokines. Pro-inflammatory analytes measured were as follows: GRO/KC, MCP-1, IL-1β, IL-2, IL-6, IL-17a, TNFα, and IFNγ. Data is expressed in picograms of analyte per milligrams of total protein. Homogenate total protein concentration was measured by Bradford assay.

_Staining and Flow Cytometry_

At day of necropsy, the kidney was halved and further dissected into approximately 2-3mm cubes with a sterile scalpel. The tissue contents were incubated in Collagenase Type I buffer (100 U/mL) (Worthington Biochemical Corporation) containing RPMI 1640/5%FCS/2mM MgCl2/2mM CaCl2 for 45 min at 37°C. Samples were homogenized gently with MACS C-tubes and poured through a 70µm filter. To isolate renal leukocytes, cell solutions further isolated by 44%/67% percoll gradient (800 x g at 20°C) (GE Heathcare, USA), and the leukocyte interface was transferred to a new tube and washed with FACS buffer to prepare for cell staining.

Cells were stained with either a T-effector or T-regulatory panel. T-Effector Panel: Anti-CD44 (Clone: IM7); Anti-CD3 (Clone: IF4); Anti-CD4 (Clone: OX-35); Anti-CD8 (Clone: OX-8); Anti-CD62L (Clone: OX-85); Anti-CD80 (Clone: 3H5); Anti-CD86 (Clone: 24F). T-regulatory panel: Anti-CD25 (Clone: OX-39); Anti-CD3; Anti-CD4; Anti-CD8; Anti-FoxP3 (Clone: FJK-16S); Anti-CD80; Anti-CD86. All antibodies were diluted 1:200 (5ug/ml). Intracellular staining with APC anti-FOXP3 was performed using the FoxP3 kit in accordance with the manufacturer’s directions (eBioscience). Samples were measured with the flow cytometer (BD LSR II, BD Biosciences, USA) and data was analyzed by FlowJo Software 8.0 (Ashland, OR, USA).
REFERENCES


SUPPLEMENTAL DATA

Necropsy Data

Body weight (BW) was similar in all four groups at the end of the protocol (Vehicle-Sham 478±21; DOCA-Sham 485±12; T-RDNx 496±24; A-RDNx 516±15g). Heart size was increased in DOCA-Sham rats compared to Vehicle-Sham, and T-RDNx attenuated (#p<.05) this hypertrophic effect (Vehicle-Sham 2.71±0.09; *DOCA-Sham 3.25±0.14; #T-RDNx 3.02±0.05; *A-RDNx 3.22±0.10g/kg BW). Similarly, renal mass was increased (*p<.05) in DOCA-Sham rats compared to Vehicle-Sham, and remained increased in the T-RDNx or A-RDNx rats (Vehicle 3.79±0.27; *DOCA-Sham 4.59±0.21; *T-RDNx 5.10 ±0.21; A-RDNx 5.59±0.19g/kg BW).
Capsaicin and Potassium Chloride Afferent Activation Response. Pilot experiments in six male Sprague Dawley rats were designed to determine the appropriate chemical and dose for the peak afferent activation normalization. We tested both capsaicin (TRPV-1 agonist) and KCl (non-specific nerve depolarization). The peak afferent nerve response was achieved with 50µM capsaicin. Response to intrapelvic KCl was lesser, even at supraphysiological (320mM) concentrations. All data presented as mean±SEM.
**Responsiveness to Chemical and Mechanical Afferent Stimulation.** Sensitivity of intrapelvic afferent renal nerves was assessed in DOCA and Vehicle rats using mechano- and chemo-sensitive stimuli. Represented in raw integrated voltage and as a percent of peak response to capsaicin, no difference in sensitivity was observed between DOCA and Vehicle rats across all stimuli. Abbreviations: IS=isotonic saline; HS=600mM hypertonic saline; BK=20ug/mL bradykinin; PP: 20mmHg increased pelvic pressure. All data presented as mean±SEM (n=6/group).
**Daily and Cumulative Sodium Intake.** Panel A: Daily sodium intake (mmol Na/day) was increased in all DOCA-salt groups (Sham, T-RDNx, and A-RDNx) compared to Vehicle-Sham rats. Panel B: Cumulative sodium intake (mmol) over the 21-day DOCA-salt treatment was no different between Sham, T-RDNx, and A-RDNx animals. All data presented as mean±SEM. *p<.05 vs. Vehicle-Sham; #p<.05 vs. DOCA-Sham.
Confirmation of Denervation Efficacy. To confirm efferent and afferent nerve ablation efficacy in each subject, renal norepinephrine (NE) and calcitonin gene-related peptide (CGRP) were measured in each subject. T-RDNx substantially (>90%) decreased (#p<.05) renal NE content and A-RDNx had no observed effect (DOCA 59±5; #T-RDNx 3±1; A-RDNx 56±5 ng/mg tissue). Further, T-RDNx and A-RDNx both decreased (#p<.05) CGRP content vs. sham DOCA+salt rats (DOCA 6287±1238; #T-RDNx 1720±423; #A-RDNx 1230±336 pg/mg tissue). The average CGRP and NE values are statistically significantly different from the theoretical value of zero. All data presented as mean±SEM. *p<.05 vs. Vehicle-Sham; #p<.05 vs. DOCA-Sham. Vehicle n=5; DOCA n=10; T-RDNx n=12; A-RDNx n=11.
Correlation Between Mean Arterial Pressure and Renal Cytokine Content. The relationship between final mean arterial pressures (MAP) and renal cytokine content in DOCA-treated animals (DOCA, T-RDNx, and A-RDNx) was assessed to elucidate a potential effect of blood pressure on cytokine content. No significant (p>.05) correlation was observed between MAP and any individual cytokine. DOCA n=6; T-RDNx n=5; A-RDNx n=5.
Sample Gating for Flow Cytometry. The gating samples for the T cell effector (left) and regulatory (right) panels are depicted above.