Hemodynamic and Neural Responses to Renal Denervation of the Nerve to the Clipped Kidney by Cryoablation in Two-kidney One-clip Hypertensive Rats

by

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Abstract

Renal artery stenosis is increasing in prevalence. Angioplasty plus stenting has not proven better than medical management. There has been a reluctance to use available denervation methodologies in this condition. We studied conscious, chronically-instrumented two-kidney one-clip (2K-1C) Goldblatt rats, a model of renovascular hypertension, to test the hypothesis that renal denervation by cryoablation (cryoDNX) of the renal nerve to the clipped kidney decreases mean arterial pressure (MAP), plasma and tissue Ang II, and contralateral renal sympathetic nerve activity (RSNA). Five week-old male Sprague Dawley rats underwent sham (ShC) or right renal artery clipping (2K-1C), placement of telemetry transmitters, and pair-feeding with a 0.4% NaCl diet. After six weeks, rats were randomly assigned to cryoDNX or sham cryo-treatment (shamDNX) of the renal nerve to the clipped kidney. MAP was elevated in 2K-1C and decreased significantly in both ShC cryoDNX and 2K-1C cryoDNX. Tissue norepinephrine was ~85% lower in cryoDNX kidneys. Plasma Ang II was higher in 2K-1C shamDNX but not in 2K-1C cryoDNX vs ShC. Renal tissue Ang II in the clipped kidney decreased after cryoDNX. Baseline integrated RSNA of the unclipped kidney was 3-fold higher in 2K-1C vs ShC and decreased in 2K-1C cryoDNX to values similar to ShC. Maximum reflex response of RSNA to baroreceptor unloading in 2K-1C was lower after cryoDNX. Thus, denervation by cryoablation of the renal nerve to the clipped kidney decreases not only MAP but also plasma and renal tissue Ang II levels and RSNA to the contralateral kidney in conscious, freely moving 2K-1C rats.
Introduction

Activation of the sympathetic nervous system plays an important role in the development and maintenance of several forms of human hypertension (16, 23, 53) and animal hypertension (31, 42, 45, 46). Specifically, neuroexcitation contributes to the high arterial pressure and high plasma renin and angiotensin II (Ang II) levels in the two-kidney, one-clip (2K-1C) Goldblatt rat, a model of renovascular hypertension (17, 26, 39, 51). Early in the course of 2K-1C hypertension, renin secretion by the clipped kidney increases and that of the contralateral kidney is suppressed by the elevated blood pressure. Approximately six weeks after clipping of the renal artery the elevated arterial pressure transitions from a primarily renin dependent to a more neurogenically mediated mechanism (40).

Stimulation of the renal sympathetic nerves results in renin secretion as well as renal tubular sodium reabsorption (4, 34). Inhibitory renorenal reflexes are impaired in 2K-1C rats, thereby leading to enhanced efferent renal sympathetic nerve activity (RSNA) and urinary sodium retention (30). Denervation of the clipped kidney by surgical transection of the nerve with application of phenol to the nerve and artery of the clipped kidney interrupts nerve activity to the ischemic kidney. In addition, physiologic indices consistent with diminished RSNA to the contralateral kidney such as renin secretion and renal sodium reabsorption are reduced (7, 19, 26, 28, 30, 50). Potential decreases in other sympathetic outputs resulting from interruption of afferent signals from the kidney may result in vasodilation of other vascular beds as well (32). Except for one study showing decreased RSNA in the contralateral kidney only 1.5 hours after recovery from anesthesia (30), direct measurements of contralateral RSNA, certainly in fully awake and freely moving rats several days after recovery, has not been reported. Together, inhibition of the renin angiotensin system, higher urinary sodium excretion and vasodilation of selective vascular beds contribute to the reduction in arterial pressure observed with renal denervation of the stenotic kidney.
Over the last few years, renal sympathetic denervation for uncontrolled essential hypertension in humans has become an area of intense study (6, 33, 53), but significant renal artery stenosis has typically been an exclusion criterion (24). Importantly, renovascular hypertension due to atherosclerotic disease is increasing with up to 20-54% prevalence in high risk groups with diabetes mellitus, heart failure or peripheral vascular disease (1, 15). Many such patients suffer from hypertension resistant to multidrug therapy (8). Nearly one-third of these individuals die within five years, even with current interventional modalities (2). Moreover, angioplasty with or without stenting of the renal arteries has proven of little benefit in controlling arterial pressure, decreasing cardiac or renal events, or reducing mortality (2, 13, 61). A recent study in a limited number of patients who had already undergone renal artery stenting reported some success in further decreasing systolic pressure after radiofrequency denervation (3). Given technical considerations such as avoiding the area near the stenosis and delivery of lower power radiofrequency energies, there is still concern as to whether renovascular patients are suitable for current approaches for renal sympathetic denervation (60).

Cryotherapies are used to ablate aberrant cardiac conduction pathways (52), treat malignancies (14) and ameliorate peripheral vascular disease (35). It is, therefore, possible that emerging endovascular cryotechnologies using very low temperatures may successfully ablate renal sympathetic nerves (36). The present studies were designed as proof-of-principle experiments to test the hypothesis that cryo-treatment of the renal nerve to the clipped kidney will decrease systemic arterial pressure, reduce plasma Ang II levels, and decrease contralateral RSNA in conscious, chronically-instrumented 2K-1C hypertensive rats. Since changes in baseline arterial pressure as well as alteration in afferent inputs from the kidney could very well modulate the arterial baroreflex, whenever possible, the baroreflex response of RSNA before and after cryo-treatment was assessed in the 2K-1C rats.


Methods

Male Sprague Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) were used in all protocols. Rats were permitted to acclimate after arriving in the vivarium for a minimum of 3 days. They were housed under controlled conditions (ambient temperature 21-23°C; lights on from 0700-1900). They were permitted free access to water and standard rat chow containing 0.4% NaCl, except where noted by protocol. All rats were cared for in compliance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals (8th edition, 2011). All procedures and protocols were reviewed and approved by the Wayne State University Institutional Animal Care and Use Committee.

Renal artery clipping. Five-week old rats were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (8 mg/kg). Additional doses (25-50% of the initial dose) were administered if needed to maintain a plane of anesthesia. The right renal artery was exposed via a right flank incision and visualized under a stereomicroscope so that the renal nerves as well as blood vessels could be carefully identified. Then, a 0.2 mm silver clip was placed around the artery (2K-1C) carefully avoiding the renal nerves. This resulted in the clip typically being placed more proximal to the aorta and avoiding the hilus of the kidney. Sham clipped rats (ShC) underwent identical surgery but no clip was placed. The flank incision was closed with surgical staples.

Hemodynamic radiotelemetry transmitter placement. Immediately after renal artery clipping, the femoral artery was exposed via a groin incision and the proximal end occluded briefly so that the gel-filled catheter attached to the radiotelemetry transducer (TA11PA-C40; Data Sciences International, St. Paul, MN) could be inserted into the artery and then advanced into the distal aorta. The catheter was secured with medical adhesive and the transmitter device placed subcutaneously and secured to the underlying muscle. The skin was closed with surgical staples. The rat then received a dose of buprenorphine SR (0.3 mg/kg i.p.) for analgesia. Each rat was returned to its home cage with its individual receiver and permitted to recover for 3 days prior to initiating hemodynamic recordings.
Dual hemodynamic and renal nerve radiotelemetry transmitter placement. Rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.). If required, supplemental doses were given as needed. The telemetry unit (Telemetry Research TR46S, Auckland, New Zealand) which has both blood pressure and nerve electrode components was placed using a modification of the technique described by Stocker and Muntzel (57). Briefly, two incisions were made: one over the left flank and the other in the left groin. A separate venous catheter was first inserted into the left femoral vein, secured, and then tunneled subcutaneously and exteriorized posteriorly at the base of the neck. To maintain patency, the catheter was filled with heparinized saline (100 U/ml). Then, a tunnel was made subcutaneously from the femoral area to the flank. The electrode wires from the transmitter were passed through this tunnel for subsequent placement. First, the catheter with the arterial pressure transducer was inserted into the left femoral artery and secured with sutures. Then, the left renal nerve was identified under a stereomicroscope. The nerve was carefully placed onto the exposed ends of the electrode wires from the transmitter. The silicone casing of the proximal ends of the wires was then stabilized by anchoring it with 6-0 sutures to the adventitia of the aortic wall. Placement of the electrodes and quality of the nerve signal was established by evaluating the nerve sound using an audio monitor and verified using an oscilloscope (Hameg, New Meadow, NY). Then, the nerve and electrodes were encased with silicone gel (Kwik-Sil, World Precision Instruments, Sarasota, FL). The muscles were then sutured closed in layers and the body of the transmitter inserted subcutaneously over the lower abdomen. The skin was closed with surgical staples. The rat was then returned to its home cage and permitted to recover.

Cryo-treatment of the renal nerve. Cryo-treatment was performed using the argon-based CryoCare system with a PERC-15 Percryo cryoprobe (Endocare, Austin, TX). Rats were anesthetized with ketamine and xylazine as before. A right flank incision was made along the scar from the previous surgery. The right renal nerve was identified and isolated under a stereomicroscope. The tip of the cryoprobe was carefully held under the nerve such that the tip was isolated from other tissues as the nerve lay on the
tip. Freezing was initiated at 100% power. Once the probe temperature reached -155 ± 5°C it was held for 30 seconds, followed by a thawing cycle (maximum temperature not exceeding 7°C) of 1 minute. The freeze-thaw cycle was repeated a total of three times (cryoDNX). Then, the muscle and skin layers were closed. Sham treated rats underwent the same procedures but without freezing or thawing (sham DNX). Rats were returned to their home cages and received Tylenol, 6mg/ml, in their drinking water for 3 days for analgesia in order to avoid potential effects of long acting opioids on blood pressure.

Protocol 1. The experimental design in Protocol 1 is depicted in Figure. 1. All protocol measurements were performed on conscious rats. After renal artery clipping or sham clipping and placement of hemodynamic telemetry transmitters, arterial pressure and heart rate was monitored for 6 weeks by telemetry. At the end of the 6th week, each rat was randomly assigned to undergo cryo-treatment of the right renal nerve or sham cryo-treatment. Hemodynamic parameters were then monitored for an additional 2 weeks in the resulting four groups of rats (ShC cryoDNX, ShC shamDNX, 2K-1C cryoDNX, 2K-1C shamDNX). Each rat was then placed into a metabolic cage and permitted to acclimate for 3 days. During the entire time, they were provided with free access to water. Food intake was measured daily. The 2K-1C rats typically ingest ~ 2 to 5 gm less food than ShC rats, so that on the third day, pair-feeding was begun so that each ShC rat was pair-fed to receive the same amount of 0.4% NaCl diet as its 2K-1C counterpart. Urine was collected daily from days 4 to 7 for measurement of volume, Na, K, creatinine and protein. Upon completion of the balance studies, the rats were returned to their home cages and hemodynamic recording was resumed for 1 week. At the end of the observation period, each rat was anesthetized with ketamine and xylazine as above, the aorta was exposed via a midline abdominal incision and aortic blood obtained for measurement of Na, K, creatinine and Ang II. Both kidneys were harvested, weighed and cortical tissue snap frozen for determination of tissue Ang II and norepinephrine.
For comparison with previously published data regarding renal tissue Ang II in 2K-1C rats (44), separate groups of sham-clipped and 2K-1C rats were anesthetized as above and renal cortical tissue taken for assessment of tissue Ang II 1 week after sham or renal artery clipping.

Protocol 2. After renal artery clipping or sham clipping, rats were returned to their home cages. Five and one-half weeks later, each rat was equipped with a dual hemodynamic and renal nerve telemetry transmitter and a venous catheter for infusions. Arterial pressure, heart rate and renal sympathetic nerve activity (RSNA) were recorded daily from 0800-1200 via a TR161 receiver. Data was digitized, recorded and analyzed using an analogue to digital converter and software platform (Power Lab 8/30 and LabChart Pro 7, ADInstruments, Colorado Springs, CO). Four days after placement of the transmitter, rats underwent either cryo-treatment or sham cryo-treatment of the right renal nerve and resulted in four groups of rats: ShC cryoDNX, ShC shamDNX, 2K-1C cryoDNX, and 2K-1C shamDNX. Recording of hemodynamic and RSNA parameters continued for up to 10 days or until the nerve recording lost fidelity. Values for RSNA were only used for analysis from day 3 after surgery to permit full recovery from anesthesia. In addition, RSNA was monitored daily and if the recording failed on a given day (e.g., day 6), values only from a ≥ 24 hr prior to the failure (day 4) were used to insure validity. On average, our nerve activity lasted 5 days.

Baroreflex testing of the heart rate and RSNA responses was performed on unrestrained conscious 2K-1C rats in their home cages two days prior and three days after cryo- or sham-treatment of the renal nerve. Baroreflex curves were generated by inducing ramp decreases and increases in arterial pressure Nitroprusside (200 µg/ml, Ohmeda) was infused intravenously at an increasing rate of 7.5 – 100 µg/kg/min and phenylephrine at 5 – 50 µg/kg/min (200 µg/ml, Sigma Aldrich, St. Louis, MO) so as to result in a ramp decrease or increase over a 2 minute period, respectively. Fifteen to 30 minutes were permitted between infusions for all parameters to return to baseline values. At the end of each
experiment, trimethaphan camsylate (20 mg/kg i.v., Hoffman-La Roche) was administered as a bolus dose to assess background noise.

**Plasma and urinary measurements.** Urine volume was measured gravimetrically. Plasma and urinary sodium concentrations were measured by flame photometry (Model 2655-10, Cole-Parmer, Vernon Hills, IL). Creatinine in plasma and urine was measured using a modified colorimetric Jaffe reaction (Pointe Scientific, Canton, MI)(47). Urinary protein was assessed by the method of Lowry(38).

**Plasma and tissue Ang II radioimmunoassay.** Plasma was assayed for Ang II by the method reported by Navar *et al* (44) and adapted by our laboratory(39). In brief, 1 ml of plasma was extracted with 90% methanol in water. The extracts were taken to dryness under nitrogen, stored at -70°C overnight. Renal cortical tissue was processed by homogenizing 50-100 mg tissue in ice cold methanol followed by centrifugation at 4000 *g* for 30 minutes at 0°C. The supernatant was aspirated into the assay tubes and the pellet resuspended in ice cold methanol, homogenized and centrifuged as before. The supernatants were then combined, dried down under nitrogen and stored at -70°C until assay.

Plasma and tissue extracts were reconstituted in assay buffer containing 50mM sodium phosphate, 1 mM EDTA, 0.25 mM thimerosal, and 0.25% peptidase-free human serum albumin. Each sample was assayed in duplicate. ¹²⁵I-labeled Ang II (Perkin-Elmer, Billerica, MA) was used as the tracer. The anti-Ang II antibody (Peninsula Laboratories, San Carlos, CA) was at a final dilution of 1:660,000. Nonspecific binding was 2.3%; the lower limit of detection was 0.6 fmol/tube; 50% binding was 16.0 fmol/tube.

**Tissue norepinephrine assay.** Renal tissue was homogenized in 0.1 N perchloric acid with 1 µM EGTA, centrifuged at 4000 *g* for 10 minutes. An aliquot of the supernatant was diluted 1:10 in perchloric-EGTA for assay, and the remainder was frozen at -70°C. The diluted sample was shipped on dry ice for analysis at the Vanderbilt Hormone Assay Core ([http://hormone.mc.vanderbilt.edu](http://hormone.mc.vanderbilt.edu)). Analysis was accomplished by high pressure liquid chromatography via electrochemical detection.
Dehydroxylbenzylamine was used as the internal standard with each extraction to monitor recovery and determine quantitation.

**Analyses and statistics.** Heart rate and arterial pressure were averaged over seven consecutive days unless otherwise specified. Resting integrated RSNA consisted of 1-second sequential averages over the 4-hr recording period. Values are expressed as the mean ± SE. Protocol 1 was designed to assess a difference of 10 mmHg with a standard deviation of 5 mmHg with 95% power at an \( \alpha \) level of 0.05, thus requiring 9 rats per group. The study in Protocol 2 was powered to assess a 50% change in resting integrated RSNA of the 2K-1C group (~4.5 \( \mu \)V.s with a standard deviation of 2 \( \mu \)V.s) three days after cryo-treatment with 95% power and an \( \alpha \) level of 0.05, thus requiring 4 rats per group. Comparison with baseline values in the same animal was accomplished by using the paired t-test. Comparisons among groups were made by one-way ANOVA followed by Tukey Kramer analysis. Comparison with baseline over time among groups was done by two-way ANOVA followed by Dunnett’s test.

Baroreflex curves were constructed by using maximum activity of the RSNA as the 100% value as well as the raw integrated nerve activity. RSNA-MAP curves were constructed for each rat as previously described (39). Resting values recorded before nitroprusside and phenylephrine were averaged for each curve. All data are reported as the mean ± SE. One-way ANOVA followed by Tukey-Kramer analysis was used for comparisons among groups.

Urinary sodium excretion was calculated as the product of urine sodium concentration and urine flow rate; sodium intake was the product of the weight of food ingested and its sodium content. Sodium intake and urine sodium excretion were averaged for each day (54). Creatinine clearance was calculated using the standard clearance formula (58). Body weights, kidney weights, plasma Ang II, tissue Ang II and tissue NE concentrations were averaged for each group. All data are expressed as the mean ± SE. Two-way ANOVA for independent measures was used for comparisons across time in the four groups of rats.
For comparisons of individual means among groups, one-way ANOVA followed by Tukey Kramer *post hoc* analysis was used. In all cases, a *P*-value less than 0.05 was accepted as significant.
Results

Baseline values for the rats in Protocol 1 are shown in Table 1. Body weights did not differ among the groups. The right (clipped) kidney weighed significantly less than the left kidney in both groups of 2K-1C rats; kidneys from ShC rats were of similar weight.

Figure 2 shows daily MAP and heart rate one day prior to cryo- or sham-treatment and daily averages over the next 7 days. Although MAP declined in the 2K-1C cryoDNX group as soon as one day after treatment, the decrease in MAP did not achieve significance until day 5. Thereafter, MAP tended to stabilize at this new level until euthanasia on day 21; MAP on day 7 was 129.8 ± 4.1 vs 130.5 ± 5.6 mmHg on days 18-21 (see Fig. 3). MAP after cryoDNX in the ShC group did not decrease over the first 7 days. Heart rate did not differ among the groups or over time (P > 0.05). The average MAP during the 3 days prior to sham cryo-treatment or cryo-treatment was significantly higher in both groups of 2K-1C rats compared with ShC rats. MAP averaged over days 18 to 21 after cryo-treatment of the nerve to the clipped kidney significantly decreased MAP in 2K-1C rats. Notably, ShC cryoDNX rats also displayed a significant decrease in MAP by the end of the three weeks. Sham cryo-treatment of the renal nerve resulted in no change in MAP in either ShC or 2K-1C rats (Fig. 3). Heart rate was similar to that observed in the first seven days of the protocol and did not differ among the four groups at the end of the 3 weeks (data not shown).

Three weeks after sham or cryo-treatment, tissue norepinephrine values from the right kidney were significantly lower in both ShC and 2K-1C cryo-treated groups (Table 1). Thus, renal tissue norepinephrine content was ~85% lower in the cryo-treated kidney compared with either the corresponding sham cryo-treated kidney or the contralateral kidney in the same rat. In a separate set of rats, tissue norepinephrine content was 86 ± 3% lower at 1 week (n = 5) and 81 ± 3% (n = 7) at 3 weeks after cryo-treatment of the renal nerve.
Creatinine clearance and protein excretion were similar among the groups (Fig. 4A and B). Sodium intake and urinary sodium excretion did not change over the 4 days of measurement and were similar among the groups (Fig. 4C and D). After completion of the metabolic studies, MAP remained significantly lower in the 2K-1C cryoDNX rats compared to pre-treatment MAP (-16 ± 5 mmHg, \( P < 0.05 \)). No differences were evident in ShC shamDNX (1.9 ± 3.9 mmHg) or 2K-1C sham cryoDNX rats (3.0 ± 3.0 mmHg). The ShC cryoDNX group did display a decrease in MAP compared with pre-cryotreatment MAP (-7.0 ± 3.5 mmHg; \( P < 0.05 \)).

Figure 5 shows that plasma Ang II values were ~3-fold greater in the 2K-1C sham cryoDNX rats compared with either ShC group. Cryo-treatment of the renal nerve to the clipped kidney significantly decreased plasma Ang II values in the 2K-1C rats to values no different from plasma Ang II concentrations in the ShC rats. Notably, renal tissue Ang II was similar in ShC rats at both 1 week and 9 weeks. Renal tissue Ang II content is significantly increased 2K-1C rats 1 week after renal artery clipping; however, this elevation was not evident in the 2K-1C rats after 9 weeks. Renal cortical tissue Ang II values were similar from both right and left kidneys of both groups of ShC rats. The clipped kidney of the 2K-1C sham cryo-treated rats displayed significantly higher tissue Ang II content compared with the non-clipped kidney; this increase was prevented by cryo-treatment (Fig. 6).

Table 2 shows the values for MAP at baseline and 4 days after sham cryo-treatment or cryo-treatment in Protocol 2. Cryo-treatment significantly lowered MAP in the 2K-1C group; sham cryo-treatment had no effect. As in Protocol 1, sham cryo-treatment did not change tissue norepinephrine content of the right kidney, but cryo-treatment decreased norepinephrine content of the right kidney in both ShC and 2K-1C rats.

Raw telemetry recordings of MAP, raw RSNA, integrated RSNA and heart rate before and after cryo-treatment from a freely moving 2K-1C are shown in Figure 7A. MAP and RSNA both decreased one day after cryo-treatment and continued to decline up to day 4. The decrease in RSNA was not due to
electrode malfunction or decreased nerve viability since a decrease in MAP induced by injection of nitroprusside four days after cryo-treatment evoked increases in both heart rate and RSNA. Sham cryo-treatment of a 2K-1C rat in Figure 7B shows that MAP continued to rise over time and RSNA was unchanged. Overall, integrated RSNA prior to cryo-treatment was 3- to 4-fold higher in both groups of 2K-1C rats compared with the ShC groups. Integrated RSNA decreased significantly in the 2K-1C cryo-treated group to a value no different from that in the ShC groups (Table 2).

Examples of the integrity of the telemetric nerve recordings at baseline and after responses to nitroprusside or phenylephrine are depicted in Figure 8. These recordings are from the same rat shown in Figure 9, nine days after cryo-treatment (Fig. 9C and D). Background RSNA after ganglionic blockade with trimethaphan camsylate is also shown.

Typical baroreflex curves of RSNA expressed as % maximum RSNA as well as in absolute units in a single 2K-1C rat before and after cryo-treatment are presented in Figure 9. In this rat, both upper and lower plateaus decreased. Baroreflex testing was not part of the original hypothesis and design, but was performed as an addendum to the study. Thus, full baroreflex curves were completed on day 3 in three of four 2K-1C rats; these results are shown in Table 3. In one rat were able to complete baroreflex curves after cryo-treatment on day 3 (Fig. 9A and B) and as late as day 9 (Fig. 9C and D). The major difference was a further decrease in resting MAP and range and a shift in the curve to the left. The upper plateau decreased significantly after cryo-treatment resulting in a concurrent decrease in the range. In contrast, the upper plateau, if anything, displayed a tendency to rise in the sham cryo-treated 2K-1C rats (Table 3), but this did not achieve significance since the study was not powered to evaluate baroreflex parameters.
Discussion

The present findings support the hypothesis that cryo-treatment of the renal nerve to the clipped kidney in 2K-1C rats decreases systemic arterial pressure comparable to decreases in pressure observed with surgical denervation. Arterial pressure in sham clipped rats was also significantly decreased by unilateral cryoablation of the renal nerve. In addition, these studies demonstrate for the first time that denervation of the renal nerves by cryoabation reduces plasma Ang II levels, lowers renal tissue Ang II content of the clipped kidney, and decreases contralateral RSNA in conscious, chronically-instrumented 2K-1C hypertensive rats. The decline in renal tissue norepinephrine content of the clipped kidney by as much as 85% provides proof-of-principle that cryotherapy may be used to effect sympathetic renal denervation comparable to that reported two weeks after severing the renal nerve and application of phenol in spontaneously hypertensive rats and suggests that reinnervation may be limited or delayed with this approach (43). Heart rate did not differ among the groups either before or after sham or cryoablative therapy. Moreover, a limited number of baroreflex observations suggest that denervation of the renal nerves by cryoablation results in a decreased reflex renal sympathoexcitatory response to a decline in arterial pressure.

Cryoablation of the renal nerve to the clipped kidney reproducibly decreased mean arterial pressure by ~15 mmHg in six-week 2K-1C rats in both protocols. This decline in arterial pressure after cryo-treatment was comparable to that observed six days after standard surgical denervation of the ischemic kidney in this model (26). The decrease in pressure was sustained through the third week after cryo-treatment and is similar in magnitude to the attenuation seen with bilateral renal denervation in the development of hypertension in spontaneously hypertensive rats (27) and DOCA salt rats (22) or after severing the nerve to the unclipped kidney in 2K-1C rats (50). Arterial pressure also declined significantly in the sham-clipped rats in Protocol 1 which was powered to assess mean arterial pressure but not in Protocol 2 which was powered to evaluate RSNA. This is consistent with the findings of Jacob
et al (21) who found ~10 mmHg difference in mean arterial pressure after standard denervation in normal rats. They were able to observe this difference as early as 7 days after bilateral renal denervation. In the present studies, mean arterial pressure in the sham-clipped group was not different at 7 days (Fig. 2 and Table 2) but did achieve significance three weeks after cryoablation of the renal nerve (Fig. 3). It may well be that unilateral denervation requires a longer period for its effect on normotensive rats. Moreover, the study in Protocol 2 was powered to evaluate RSNA and not mean arterial pressure. Taken together, these observations in both 2K-1C and sham-clipped rats show that cryoablation of the renal nerve is as effective as surgical denervation to decrease arterial pressure.

Just as with surgical denervation, several mechanisms contribute to the decrease in systemic pressure after cryoablation in the 2K-1C rats. Low renal perfusion pressure to the clipped kidney stimulates renin secretion via renal baroreceptor and macula densa mechanisms. Efferent renal sympathetic stimulation potentiates the renin secretory response to low perfusion pressure (56). Thus, while high plasma Ang II would otherwise depress renin secretion by a feedback mechanism (9), the concurrent sympathetic activation and low perfusion pressure in the clipped kidney together result in high circulating Ang II. The substantial decrease in plasma Ang II after cryo-treatment of the 2K-1C rats to levels no different than in sham-clipped rats is consistent with the absence of a renin secretory response to renal hypoperfusion in humans after preganglionic sympathetic blockade due to epidural anesthesia(20).

The potential mechanisms involving Ang II and renal nerves in 2K-1C hypertension are depicted in Figure 10. Besides raising systemic pressure by producing direct vasoconstriction via activation of AT₁ receptors on vascular smooth muscle cells (5), circulating Ang II also results in differential activation of regional sympathetic nerve activities (46). In contrast to chronic exogenous Ang II infusion which typically results in baroreflex inhibition of efferent RSNA (62), endogenous activation of the renin-angiotensin system in the 2K-1C model enhances cervical sympathetic nerve activity (59) and RSNA (7).
Afferent inputs from the stenosed kidney in 2K-1C hypertension (30) as in other ischemic renal conditions (29) paradoxically exert an excitatory influence on contralateral efferent RSNA despite elevated endogenous Ang II. Studies have shown that inhibitory renorenal reflexes that occur in kidneys from normal rats (12) do not occur from the clipped kidney in 2K-1C rats, but actually enhance efferent RSNA to the contralateral kidney (30). Moreover, afferent renal nerves also project to central structures that regulate sympathetic outputs to key vascular beds (55). Some of these nuclei, such as the subfornical organ and area postrema, lie outside the blood brain barrier and possess abundant AT$_1$ angiotensin receptors (49) so that plasma Ang II can then potentiate excitatory afferent renal nerve inputs further enhancing efferent sympathetic activity (10, 11, 39). Cryo-treatment performed in the present studies involved freezing the whole renal nerve, so that afferent as well as efferent nerves from the stenosed kidney were interrupted. Thus, the combined decline in plasma Ang II and excitatory afferent inputs in awake, freely moving rats after cryo-treatment contributed to the decrease in both systemic pressure and contralateral RSNA and confirm the earlier studies regarding surgical denervation by Kopp and Buckley-Bleiler (30) observed only 1.5 hours after recovery from pentobarbital anesthesia.

The high levels of intrarenal Ang II content in both clipped and non-clipped kidneys one week after clipping confirm the findings by Guan et al (18) who also showed that by 25 days tissue Ang II content, though significantly elevated, was not as pronounced. This present report is the first to show that there is no difference in renal tissue Ang II content between 2K-1C and sham clipped rats nine weeks after clipping. Nonetheless, similar to rats studied at 25 days (18), tissue Ang II content of the clipped kidney of 2K-1C rats was higher than that of the contralateral kidney and was significantly attenuated by cryo-treatment of the nerve. Elevated intrarenal Ang II has been shown to decrease sodium excretion and may thereby further contribute to hypertension (48). The present data show that denervation decreases intrarenal Ang II in the stenosed kidney and confirm the earlier findings in anesthetized 2K-1C rats (30) that sympathetic inputs to the contralateral kidney are suppressed leading to increased urinary sodium.
excretion. Thus, effects on sodium excretion by the reduced intrarenal Ang II and sympathetic drive may work in concert to lower systemic pressure and restore sodium balance chronically. We did not observe the increase in urinary sodium excretion which likely occurs early after renal denervation as the metabolic studies were performed 10 days after denervation at which time the rats were back into sodium balance. Since arterial pressure also declined in sham-clipped rats, our findings are consistent with earlier studies that renal nerves influence basal arterial pressure even in the normotensive state (21). However, it is also apparent that in 2K-1C rats there is an additional decrease in pressure that is likely due to changes in systemic and intrarenal Ang II. Further studies are certainly warranted to ascertain the role of renal nerves in normotensive rats as well.

The limited number of baroreflex observations suggests that cryoablation of the renal nerve decreases the reflex renal sympathoexcitatory response to baroreceptor unloading. The present data cannot distinguish whether this modulation of the baroreflex is due to interruption of direct neural inputs from renal afferent nerves or to lowering of circulating Ang II, or both. Since both the neural and Ang II inputs converge on the subfornical organ, it is of interest that the attenuation of the maximal response to baroreceptor unloading here is similar to that seen with electrolytic lesioning of the subfornical organ (39) or chronic administration of a centrally acting sympatholytic agent (7). In addition, although this study was not a priori powered to assess baroreflex function, there was no detectable difference in the lower plateau. The latter observation would be consistent with previous studies suggesting that the baroreflex is already maximally active to suppress arterial pressure in secondary hypertension (7, 37, 59), and suggests that further studies are warranted.

**Limitations**

Cryo-treatment resulted in a significant decrease in tissue norepinephrine content, however, not to the extent reported with severing of the renal nerve and application of phenol where tissue norepinephrine decreased by >95% (30). Without detailed longitudinal histologic studies, it is not
possible to distinguish definitively between lack of full denervation vs reinnervation. Previous studies with surgical denervation reported attenuation of the decrease in renal norepinephrine to 89% after two weeks and 76% after four weeks (27) while others have shown tissue norepinephrine to be only 50% of control values by 24 days (25). That the decrease in renal tissue norepinephrine was similar at 7 and 21 days post cryo-treatment is more consistent with cryoablation being initially less effective than surgical denervation. Nonetheless, if tissue norepinephrine is used as the index of denervation, reinnervation appears to be limited during the 3 week course of the present experiments. This contrasts with studies after standard denervation showing ~40% reinnervation after 3 to 4 weeks (43). Thus, cryoablation may be less effective initially but could conceivably be more long lasting. Corroboration of this finding as well as identifying the mechanism of the sustained decrease in norepinephrine would provide valuable information. Importantly, sodium intake was carefully controlled across groups in the present study. No differences were seen in creatinine clearance or sodium excretion at the time of the balance studies, but this does not exclude the possibility that sodium excretion differed acutely after sham or cryo-treatment such that cumulative sodium excretion may have differed prior to balance being restored. Split renal function would have provided additional insights but would have entailed anesthesia. Baroreflex testing was a post hoc addition to the studies and, hence, not adequately powered for definitive interpretation. Nonetheless, the findings indicate that the availability of nerve telemetry will prove pivotal in ascertaining the relationship between the arterial baroreflex in freely moving rats after chronic manipulations in disease models.

**Perspectives**

In summary, these studies support the proof-of-principle that cryoablation alone to the renal nerve is able to effect substantial sympathetic renal denervation to the clipped kidney in conscious, chronically-instrumented 2K-1C rats leading to reductions in systemic arterial pressure, plasma Ang II levels, renal tissue Ang II and norepinephrine content of the clipped kidney, and contralateral RSNA.
Given that renovascular hypertension due to atherosclerotic disease is increasing in prevalence (15), it is noteworthy that standard approaches using angioplasty and stenting of the renal artery have proven ineffective in lowering systemic pressure (13). Since the time of onset of stenosis in humans is impossible to judge, it is likely that the sympathetic mechanisms are engaged by the time the stenosis is addressed. Despite the early reports of successful treatment with radiofrequency denervation in resistant essential hypertension (33, 53), renal artery stenosis has generally been considered an exclusionary factor (60). Moreover, issues have more recently have come to light regarding the limited effectiveness of radiofrequency denervation such as proximity to the vessel wall, medication compliance, and operator experience among others. Other radiofrequency-based technology for renal denervation may improve on these technical aspects, but balloon cryoplasty has already been applied to peripheral vascular disease (41). Emerging cryotechnology has shown sufficient power to effect endovascular approaches to the highest heat-sink scenario of aberrant cardiac conduction pathways. Thus, cryoablation of the renal nerves with or without concurrent angioplasty appears increasingly feasible for future endovascular approaches and may be of benefit in individuals with renovascular hypertension to relieve both the stenosis as well as sympathoexcitation.
Acknowledgements

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Conflicts of Interest

None of the authors have conflicts of interest to declare.
References


Figure Legends

**Figure 1.** Timeline and procedures in the experimental design for rats in Protocol 1 (see Methods). 2K-1C, two-kidney one-clip; ShC, sham clipped; Cryo DNX, cryo-treated nerve on clipped side; Sham DNX, sham-treated nerve on clipped side; Ang II, angiotensin II; NE, norepinephrine.

**Figure 2.** Changes in mean arterial pressure (MAP; panel A) and heart rate (HR, panel B) as measured by telemetry 1 day prior to and daily for 7 days after cryo- or sham-treatment of the renal nerve to the clipped kidney in 2K-1C and sham clipped rats. ShC sham DNX (□- -, n = 11); ShC cryo DNX (■—, n = 9); 2K-1C sham DNX (○- -, n = 9); 2K-1C cryo DNX (● —, n = 11). * P < 0.05 vs baseline Day -1.

**Figure 3.** Mean arterial pressure (MAP) by telemetry averaged over the 3 days prior to cryo-treatment (clear bars) and 3 weeks later averaged over the 3 days prior to euthanasia (black bars) sham-treatment (sham DNX) or cryo-treatment (cryo DNX) of the renal nerve to the clipped kidney in 2K-1C rats or to the sham clipped kidney in sham clipped rats (ShC) rats, respectively. Values are mean ± SE; n = 11, 9, 9 and 11. * P < 0.01 vs corresponding ShC group; † P < 0.05 vs before cryoDNX same group; § P < 0.05 vs 2K-1C sham DNX post treatment.

**Figure 4.** Renal parameters in each of the four groups of rats in metabolic cages: (A) creatinine clearance; (B) 24-hr protein excretion on Day 4; and (C) Na intake and (D) Na excretion on Days 1 to 4. Values are mean ± SE; n as in Figure 2.

**Figure 5.** Plasma Ang II values in each of the four groups of rats at the end of the protocol (week 9). Values are mean ± SE; n as in Figure 2; * P < 0.001 vs both ShC groups; † P < 0.001 vs 2K-1C sham DNX.
Figure 6. Renal cortical tissue Ang II values in kidneys from ShC and 2K-1C rats 1 week after clipping (2K-1C) or sham clipping (ShC), and 9 weeks after clipping or sham clipping the right renal artery. Tissues in week 9 were obtained from rats with data depicted in Figures 2 – 4. Values are mean ± SE; for week 1, n = 6 and 6; for week 9, n as in Figure 2. * P < 0.05 vs ShC, same side; † P < 0.05 vs left kidney same treatment; § P < 0.05 vs right kidney 2K-1C sham DNX.

Figure 7. Representative recordings of MAP, RSNA, integrated RSNA (ʃRSNA) and heart rate (HR) two days (D -2) and one day (D -1) prior to treatment and then one (D +1), three (D +3) and four (D +4) days in individual 2K-1C rats after either (A) cryo-treatment (Cryo DNX) or after (B) sham cryo-treatment (Sham DNX). Dotted line indicates injection of nitroprusside (NP) to verify nerve response.

Figure 8. Representative MAP and RSNA recordings in a single 2K-1C rat immediately before and after injection with either nitroprusside, phenylephrine or trimethaphan camsylate during baroreflex testing. The expanded recordings are taken at the points of the dashed lines with the corresponding letters. Arrows indicate the time at the beginning of the injections.

Figure 9. Baroreflex curves of RSNA in a single 2K-1C rat before (baseline, solid circles) and on Day 3 (A and B) and on Day 9 (C and D) after cryo-treatment (Cryo DNX; open circles) of the nerve to the clipped kidney. Values are plotted as percent of maximum RSNA (A and C) and as raw nerve activity (µV.s; B and D). Resting values are shown in red for pre-treatment and green for post treatment.

Figure 10. Diagram of potential mechanisms involved in increased Ang II and efferent RSNA to the contralateral kidney in 2K-1C hypertension. Hypoperfusion of the stenotic kidney results in increased secretion of renin thereby initiating a cascade leading to elevated plasma Ang II. Plasma Ang II can act
to increase vascular resistance directly (not shown), but also act on brain nuclei that lie outside the blood brain barrier and possess AT₁ receptors such as the subfornical organ (SFO) or area postrema (AP). Projections from the AP or the SFO via paraventricular nucleus (PVN) can then send signals either via the brainstem cardiovascular regulatory centers or directly to spinal cord to enhance efferent RSNA. Notably, efferent RSNA may increase renin secretion from the contralateral kidney as well. Afferent renal nerves from the clipped kidney also project to SFO and to brainstem centers. These inputs, together with spinal cord renorenal reflexes initiating from afferent nerves of the clipped kidney, lead to increased efferent RSNA to the contralateral kidney.
### Table 1

**Body Weights, Kidney Weights and Cortical Norepinephrine Content**

in the Four Groups of Rats in Protocol 1

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Body Wt (g)</th>
<th>Left Kidney Wt (g)</th>
<th>Right Kidney Wt (g)</th>
<th>Left Kidney NE (ng/g)</th>
<th>Right Kidney NE (ng/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ShC sham cryo</td>
<td>11</td>
<td>442 ± 10</td>
<td>1.291 ± 0.098</td>
<td>1.278 ± 0.087</td>
<td>100 ± 6</td>
<td>92 ± 6</td>
</tr>
<tr>
<td>ShC cryo DNX</td>
<td>9</td>
<td>446 ± 6</td>
<td>1.267 ± 0.024</td>
<td>1.273 ± 0.083</td>
<td>106 ± 6</td>
<td>16 ± 4*†</td>
</tr>
<tr>
<td>2K-1C sham cryo</td>
<td>9</td>
<td>415 ± 8</td>
<td>1.264 ± 0.030</td>
<td>1.146 ± 0.026*</td>
<td>88 ± 8</td>
<td>82 ± 8</td>
</tr>
<tr>
<td>2K-1C cryo DNX</td>
<td>11</td>
<td>432 ± 15</td>
<td>1.369 ± 0.201</td>
<td>1.181 ± 0.038*</td>
<td>104 ± 11</td>
<td>24 ± 6*†</td>
</tr>
</tbody>
</table>

ShC, sham clipped; 2K-1C, two kidney one clip; cryo DNX, cryo treatment; sham cryo, sham cryo-treatment

Values are mean ± SE.

* $P < 0.05$ vs left kidney; † $P < 0.001$ vs corresponding sham cryo DNX group
**Table 2**  
Mean Arterial Pressure, Renal Tissue Norepinephrine Content and Renal Sympathetic Nerve Activity  
Before and 3 Days After Cryo-treatment in Protocol 2

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>MAP Baseline (mmHg)</th>
<th>MAP Post DNX (mmHg)</th>
<th>Left Kidney NE (ng/g)</th>
<th>Right Kidney NE (ng/g)</th>
<th>Integrated RSNA Baseline (µV.sec)</th>
<th>Integrated RSNA Post DNX (µV.sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ShC sham DNX</td>
<td>4</td>
<td>105.0 ± 4.9</td>
<td>108.7 ± 6.7</td>
<td>100 ± 3</td>
<td>87 ± 2</td>
<td>2.8 ± 2.2</td>
<td>3.3 ± 2.0</td>
</tr>
<tr>
<td>ShC cryo-DNX</td>
<td>4</td>
<td>107.3 ± 2.6</td>
<td>105.0 ± 4.9</td>
<td>134 ± 19</td>
<td>19 ± 4&lt;sup&gt;†&lt;/sup&gt;</td>
<td>3.1 ± 1.2</td>
<td>4.7 ± 1.1</td>
</tr>
<tr>
<td>2K-1C sham DNX</td>
<td>4</td>
<td>161.0 ± 12.6*</td>
<td>168.0 ± 8.7*</td>
<td>96 ± 2</td>
<td>75 ± 5</td>
<td>9.1 ± 1.3*</td>
<td>11.2 ± 3.5*</td>
</tr>
<tr>
<td>2K-1C cryo-DNX</td>
<td>4</td>
<td>162.0 ± 5.4*</td>
<td>152.0 ± 5.4&lt;sup&gt;†§&lt;/sup&gt;</td>
<td>101 ± 3</td>
<td>17 ± 2&lt;sup&gt;†&lt;/sup&gt;</td>
<td>12.2 ± 1.5*</td>
<td>3.1 ± 1.5&lt;sup&gt;†§&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are mean ± SE. ShC, sham clipped; 2K-1C, two-kidney one-clip; DNX, denervated

* P < 0.05 vs corresponding ShC group;  # P < 0.001 vs left kidney; † P < 0.001 vs corresponding sham DNX group; § P < 0.05 vs baseline same group
Table 3

Arterial Baroreflex Parameters of Renal Sympathetic Nerve Activity

Before and 3 Days after Sham or Cryo-treatment of the Renal Nerve in 2K-1C Rats

<table>
<thead>
<tr>
<th></th>
<th>2K-1C</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>Cryo</td>
<td>Sham</td>
<td>CryoDNX</td>
</tr>
<tr>
<td></td>
<td>pre</td>
<td>post</td>
<td>pre</td>
<td>post</td>
</tr>
<tr>
<td>upper plateau, %</td>
<td>100 ± 0</td>
<td>142.8 ± 55.8*</td>
<td>100 ± 0</td>
<td>55.6 ± 9.1*†</td>
</tr>
<tr>
<td>lower plateau, %</td>
<td>50.3 ± 13.0</td>
<td>41.0 ± 3.0</td>
<td>34.6 ± 1.0</td>
<td>28.4 ± 5.6</td>
</tr>
<tr>
<td>range, %</td>
<td>49.5 ± 12.8</td>
<td>101.8 ± 53.7</td>
<td>65.4 ± 1.0</td>
<td>27.2 ± 6.1*†</td>
</tr>
<tr>
<td>BP50, mm Hg</td>
<td>140.9 ± 21.7</td>
<td>139.7 ± 14.9</td>
<td>130.6 ± 12.5</td>
<td>137.7 ± 18.9</td>
</tr>
<tr>
<td>slope coefficient, -1/mmHg</td>
<td>0.17 ± 0.04</td>
<td>0.12 ± 0.02</td>
<td>0.14 ± 0.05</td>
<td>0.16 ± 0.02</td>
</tr>
<tr>
<td>Gmax, -% /mmHg</td>
<td>2.45 ± 0.51</td>
<td>3.02 ± 1.17</td>
<td>2.25 ± 0.86</td>
<td>1.16 ± 0.35</td>
</tr>
</tbody>
</table>

Values are mean ± SE; n = 3 for each group

* P < 0.05 vs pre treatment; † P < 0.05 vs sham-cryo post treatment
Figure 1

SD rat, 5 wk-old

2K-1C

- Telemetry 6 wks

- Cryo DNX
  - Telemetry 1 wk

- Sham DNX

ShC

- Telemetry 6 wks

- Cryo DNX
  - Telemetry 1 wk

- Sham DNX

Metabolic Cages

- 3 day, acclimation; 4 days: Urinary Na, K, Creatinine, Protein

- Telemetry 1 wk

Aortic blood for Na, K, Creatinine, Ang II

Telemetry 1 wk
Figure 2

A

MAP, mmHg

Days after ShamDNX or CryoDNX

B

HR, bpm

Days after ShamDNX or CryoDNX
Figure 3
Figure 4

A. Creatinine Clearance, ml/min

B. Urine Protein, mg/day

C. Na intake, mmol/day

D. $U_{Na}$, mmol/day
Figure 5
Figure 6

Renal Cortical Ang II, fmol/mg

1 week

9 weeks

- Left kidney
- Right kidney

ShC 2K-1C

Sham DNX ShC Cryo DNX 2K-1C Sham DNX 2K-1C Cryo DNX
Figure 7A
Figure 7B
Figure 8

Nitroprusside

Phenylephrine

Trimethaphan
Figure 10

Afferent Renal Nerves

Ang I

Ang II

Angiotensinogen (liver)

Brainstem CV Centers

Spinal Cord

Efferent Renal Sympathetic Nerves

Plateau (AP)

PVN

SFO

AT1

AT1

Renin

Sympathetic Nerves

Figure 10