

Effects of Restoration of Blood Flow on the Development of Aortic Atherosclerosis in ApoE^{-/-} Mice With Unilateral Renal Artery Stenosis

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Background—Chronic unilateral renal artery stenosis (RAS) causes accelerated atherosclerosis in apolipoprotein E-deficient (ApoE^{-/-}) mice, but effects of restoration of renal blood flow on aortic atherosclerosis are unknown.

Methods and Results—Male ApoE^{-/-} mice underwent sham surgery (n=16) or had partial ligation of the right renal artery (n=41) with the ligature being removed 4 days later (D4LR; n=6), 8 days later (D8LR; n=11), or left in place for 90 days (chronic RAS; n=24). Ligature removal at 4 or 8 days resulted in improved renal blood flow, decreased plasma angiotensin II levels, a return of systolic blood pressure to baseline, and increased plasma levels of neutrophil gelatinase associated lipocalin. Chronic RAS resulted in increased lipid staining in the aortic arch (33.2% [24.4, 47.5] vs 11.6% [6.1, 14.2]; *P*<0.05) and descending thoracic aorta (10.2% [6.4, 25.9] vs 4.9% [2.8, 7.8]; *P*<0.05), compared to sham surgery. There was an increased amount of aortic arch lipid staining in the D8LR group (22.7% [22.1, 32.7]), compared to sham-surgery, but less than observed with chronic RAS. Lipid staining in the aortic arch was not increased in the D4LR group, and lipid staining in the descending aorta was not increased in either the D8LR or D4LR groups. There was less macrophage expression in infrarenal aortic atheroma in the D4LR and D8LR groups compared to the chronic RAS group.

Conclusions—Restoration of renal blood flow at either 4 or 8 days after unilateral RAS had a beneficial effect on systolic blood pressure, aortic lipid deposition, and atheroma inflammation. (*J Am Heart Assoc.* 2016;5:e002953 doi: 10.1161/JAHA.115.002953)

Key Words: atherosclerosis • hypertension • inflammation • renal • renal artery stenosis

The Cardiovascular Outcomes in Renal Atherosclerotic Lesions (CORAL) trial, the largest randomized study of patients with renal artery stenosis (RAS) ever performed, found that renal artery stent implantation did not provide any benefit beyond optimal medical therapy in the occurrence of death, cardiovascular events, or renal events in patients with moderately severe atherosclerotic RAS.¹ There was a modest improvement in systolic blood pressure favoring the stent group, but no difference in adverse clinical outcomes. The

CORAL results were similar to findings from the Angioplasty and Stenting for Renal Artery Lesions (ASTRAL) trial² and the Stent Placement and Blood Pressure and Lipid-Lowering for the Prevention of Progression of Renal Dysfunction Caused by Atherosclerotic Ostial Stenosis of the Renal Artery (STAR) trial.³

The reasons that successful restoration of renal blood flow did not improve cardiovascular outcomes in patients with RAS in ASTRAL, CORAL, and STAR are not well understood. The majority of adverse clinical events in these trials were attributed to coronary artery disease, strongly suggesting that a better understanding is needed of the interaction between renal ischemia and atherogenesis. One potential explanation for the lack of benefit in these trials is that renal ischemia causes irreversible atherogenic changes and that restoration of renal blood flow has no effect on mitigating the damage to the vasculature.⁴ Biological plausibility of this hypothesis is provided by numerous studies showing that decreased renal perfusion is associated with RAS activation,⁵ and data from animal models showing that even short-term elevation in angiotensin II (Ang-II) levels can accelerate the development of atherosclerosis and lead to changes in the arterial wall that persist even after Ang-II levels return to

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baseline.^{6–11} In addition, RAS has been shown to result in elevated oxidative stress, sympathoadrenergic activation, and impaired vasoactive responses, both within the kidney and the systemic microcirculation.^{12,13}

Although there are extensive data from animal models showing that unilateral RAS leads to accelerated atherosclerosis, there is surprisingly little information on what extent, if any, these adverse vascular effects respond to revascularization. Past studies in the rat^{14,15} have shown that blood pressure normalizes and plasma renin activity decreases after unclipping either 6 weeks or 4 months after unilateral RAS, but effects on vascular pathology have not been studied. Thus, the purpose of the present study was to examine the effects of transient periods of partial unilateral renal artery constriction on aortic atherogenesis in apolipoprotein E-deficient (ApoE^{-/-}) mice.

Methods

Surgery and Blood Pressure Measurements

Male ApoE^{-/-} mice (C57BL/6 background) purchased from The Jackson Laboratory (Bar Harbor, ME) were fed a standard chow diet (Certified Rodent Chow 5001; Purina Mills, Kansas City, MO) and had free access to water. Animals were housed and cared for according to the guidelines proposed by the National Institutes of Health (NIH) for the care and use of experimental animals (NIH publication No. 85-23).

Surgery and laser Doppler imaging were performed as previously described on 18-week-old male ApoE^{-/-} mice.¹⁶ Briefly, mice were anesthetized and a vertical incision made in the midline of the back. The right renal artery was exposed and a needle with an outside diameter of 0.2096 to 0.235 mm positioned on top of the artery. The vessel was ligated by tying the sutures down firmly around the needle and the vessel and then removing the needle. In the groups that underwent ligature removal, mice were anesthetized on the defined day and a vertical incision made in the midline of the back. The right renal artery was exposed and the ligature removed. Blood flow through the renal artery was assessed using a 10-MHz pulsed Doppler probe before ligation, immediately postligature placement, before ligature removal and before being killed.

Systolic blood pressure was measured before surgery and then at 10 and 15 days after surgery and then at 15-day intervals in a core laboratory at the University of North Carolina (Chapel Hill, NC) using a computerized, noninvasive, tail-cuff system (Hatteras Instruments, Cary, NC), which has been validated for reproducibility in acclimatized mice.¹⁷ At each time point, the reported number is the mean of 10 measurements made at the same time each day for 5

consecutive days. Animals were habituated to the device for 1 week before blood pressures were obtained.

Tissue Analysis

Mice were euthanized 90 days after surgery, and 4% paraformaldehyde in PBS was infused for 10 minutes at a pressure of 100 mm Hg. After tissue fixation, aortic tissue was resected from the aortic valve to the iliac bifurcation and adventitial tissue was carefully removed. Lipid deposition was assayed using 0.3% Oil Red O solution as described by Weiss et al.⁸ Briefly, the intimal surface was exposed by a longitudinal cut along the inner curvature of the aortic arch and descending thoracic aorta. To quantify the extent of intimal surface covered by Oil Red O staining, images from a digital camera were analyzed using NIH software (ImageJ; NIH, Bethesda, MD).

The infrarenal abdominal aorta was embedded in paraffin blocks, sectioned, and stained with Mason's trichrome stain or a rat antimouse monoclonal antibody that recognizes the murine F4/80 macrophage antigen (Serotec, Raleigh, NC). Antibody staining was followed by secondary antibodies and HRP-conjugated streptavidin. Staining was then visualized by reaction with DAB (Sigma-Aldrich, St. Louis, MO).

Measurement of Plasma Levels of Ang II, NGAL and Total Cholesterol

Plasma Ang-II concentrations were determined using a commercially available Radioimmunoassay kit (Phoenix Pharmaceuticals Inc., Burlingame, CA), according to the manufacturer's instructions. Plasma samples prepared from blood collected in EDTA tubes at various time points by tail bleed were assayed in duplicates. A gamma counter was utilized to measure counts per minute of the pellets. Results were interpreted by natural log transformation of the data and then compared to a standard curve.

Mouse plasma neutrophil gelatinase associated lipocalin (NGAL) concentrations were determined utilizing by a commercially available ELISA kit (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. Plasma samples prepared from blood collected at various time points by mice tail bleed in EDTA tubes were assayed in duplicates. After addition of stop solution, optical density of samples was immediately read in a plate reader at 450 nm along with specified wavelength correction. Results were interpreted in correlation with the standard curve.

Mouse plasma cholesterol levels were determined by utilizing the commercially available Amplex Red assay kit (catalog No.: A12216; Molecular Probes, Eugene, OR), according to the manufacturer's instructions, using plasma samples prepared from blood collected at various time points

by mice tail bleed in EDTA tubes.¹⁸ Results were interpreted utilizing a standard curve developed using cholesterol samples provided in the kit.

Data Analysis and Statistics

Normally distributed data are presented as mean±SD and non-normally distributed data as median [25%, 75%]. Comparison between groups was done by Student *t* test, ANOVA, 1-way repeated-measures ANOVA, or Kruskal–Wallis 1-way ANOVA (for non-normally distributed continuous variables) followed by the Holm–Sidak method or Dunn’s multiple range test (if groups were unequal in size). The Mantel–Haenszel chi-square was used to analyze differences between categorical variables. Differences were considered significant at a *P* value of ≤0.05.

Results

Quantification of Reduction in Blood Flow With Partial Constriction of Right Renal Artery

RAS was elicited in male ApoE^{-/-} mice by partial constriction of the right renal artery resulting in an average reduction of blood flow to the right kidney to 60±6% of baseline values as measured using scanning laser Doppler perfusion imaging (Figure 1A and 1B). Right renal blood flow was significantly reduced compared to baseline immediately after ligature placement and when measured 4, 8, or 90 days later (Figure 1B). Mice that underwent sham surgery in which the renal artery was exposed, but not ligated, had no change in renal blood flow during the 90 days of the study (n=16).

Mice with RAS were assigned to groups in which the ligature was removed either 4 days (D4LR group; n=6) or 8 days after surgery (D8LR group; n=11) or maintained for 90 days (chronic RAS group; n=24). At the time of killed, renal blood flow was the same as baseline in the sham surgery and D4LR groups, mildly impaired in the D8LR group (86±4%; *P*<0.05), and severely reduced in the chronic RAS group (44±17%; *P*<0.05; Figure 1B). No mouse developed complete occlusion of the right renal artery.

Chronic RAS was Associated With Loss of Renal Mass and a Sustained Increase in Systolic Blood Pressure

At the time of killed, 90 days after the initial surgery, there was no difference in weight between the left and right kidneys in the sham-operated group, the D4LR group, or the D8LR group, whereas the right kidney was significantly smaller than the left kidney in the chronic RAS group (Figure 1C). Body

weight was similar in all groups at the time of killed (data not shown).

Chronic RAS elicited a mild, sustained increase in systolic blood pressure with statistically significant blood pressure elevation at all time points (Figure 1D; *P*<0.05 compared to blood pressure before surgery). In the D8LR group, systolic blood pressure was elevated 7 days after partial renal artery constriction, but returned to baseline levels at 15 days and later time points. In the D4LR group, blood pressure could not be measured at 7 days because of the recent surgery, but there was no elevation in systolic blood pressure at 15 days or any later time point. Sham surgery had no effect on systolic blood pressure during the duration of the experiment (*P*=0.87).

NGAL Levels Were Increased and Ang-II Levels Were Decreased After Restoration of Renal Blood Flow in Mice With Partial Unilateral RAS

NGAL levels were elevated in both the D4LR and D8LR groups, compared to the sham surgery group, when measured at 15 days. In contrast, NGAL levels were lower in the chronic RAS group at this time point (Figure 1E). By 30 days, NGAL levels were similar in chronic RAS and sham surgery controls, but remained elevated in the D4LR and D8LR groups. The increases were similar in magnitude in both groups. At 90 days, NGAL levels in the D4LR and D8LR groups had returned to baseline values; results similar to past studies in humans, which showed no difference in NGAL levels measured before and 3 to 4 months after revascularization.¹⁹

Plasma Ang-II levels were increased in the chronic RAS group at 15 days, but were similar to the sham surgery group at 30 and 90 days (Figure 1F). Ang-II levels were decreased in the revascularization groups after ligature removal (when measured at 15 days) and remained suppressed for the duration of the experiment. These results are similar to studies in the rat two-kidney-one-clip (2K1C) model, in which plasma renin concentrations were only 64% of baseline levels when measured 60 days after restoration of renal blood flow.^{14,15}

Average total cholesterol values in mice was 277±114 µg/mL. Plasma total cholesterol levels did not change over time with chronic RAS or with restoration of renal blood flow (Figure 2).

RAS was Associated With Increased Lipid Deposition in the Aortic Arch and Descending Thoracic Aorta

ApoE^{-/-} mice develop atherosclerotic lesions in the aortic arch, primarily in the lesser curvature and at the origin of the

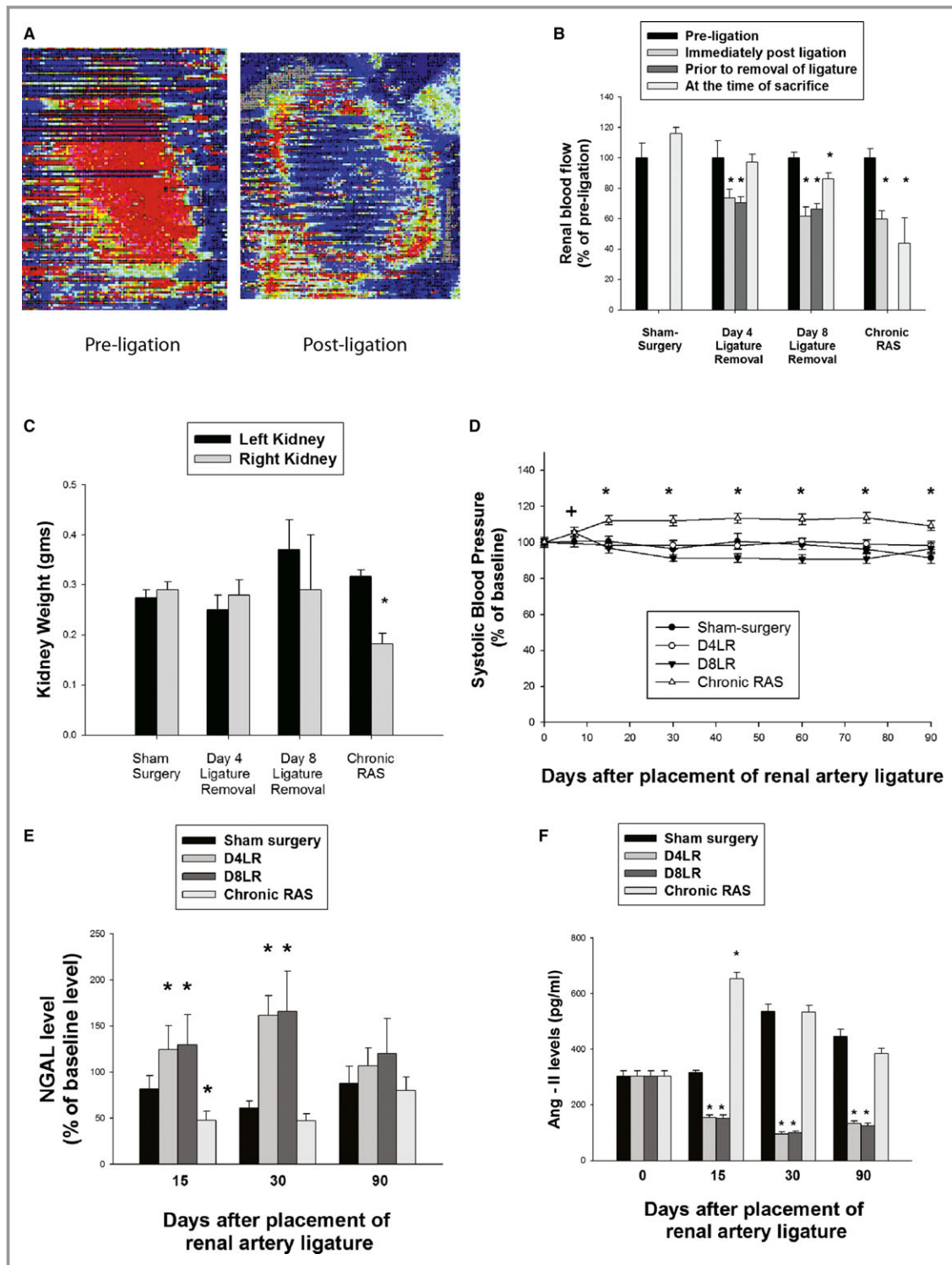


Figure 1. Renal perfusion and physiological effects after unilateral renal artery ligation. Representative examples of laser Doppler imaging of renal perfusion from the same mouse before constriction and immediately after constriction (A) and perfusion of the right kidney at various time points in each group (B). Effects of RAS are shown by kidney weight (mean±SEM, C), systolic blood pressures measured on 5 successive days both before surgery and then 10, 15, 30, 45, 60, 75, and 90 days after surgery (D), plasma NGAL levels measured at various time points (E), and plasma Ang-II levels measured at various time points (F). Ang-II levels were numerically higher at 30 and 90 days in the sham surgery group compared to baseline, but these differences were not statistically significant (* $P < 0.05$ for comparison to sham surgery group; $^+P < 0.05$ for D8LR group compared to sham surgery group). Ang-II indicates angiotensin II; NGAL, neutrophil gelatinase associated lipocalin; RAS, renal artery stenosis.

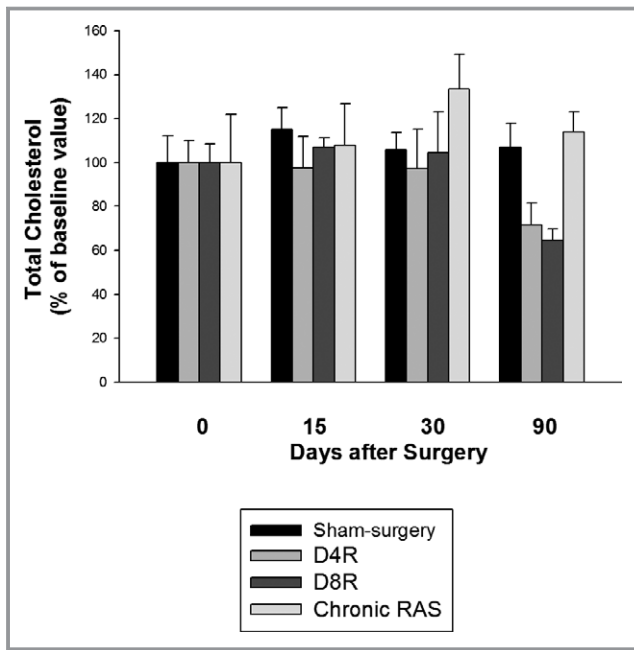


Figure 2. Total cholesterol levels after unilateral renal artery constriction with and without revascularization. Plasma total cholesterol levels were measured at baseline and then 15, 30, and 90 days after surgery. There were no statistically significant differences at any point in any group as determined by ANOVA.

great vessels. These lesions progress with age in chow-fed mice, such as used in the present study. Interventions such as a high-fat, high-cholesterol diet,²⁰ infusion of Ang II,^{6,8} and unilateral RAS^{10,11,16,21} have been shown to accelerate lipid deposition in this area.

Lipid deposition, as determined by Oil Red O staining, was detected in 11.6% [6.1, 14.2] of the area of the aortic arch in the sham surgery group, similar to results from past studies in chow-fed ApoE^{-/-} mice (Figure 3A and 3B).^{6,22} Chronic RAS resulted in an approximate 3-fold increase in lipid staining in the aortic arch compared to sham surgery (33.2% [24.4, 47.5] vs 11.6% [6.1, 14.2]; *P*<0.05). In the D8LR group, there was a 2-fold increase in the aortic arch area, which stained with Oil Red O compared to the sham surgery group (22.7% [22.1, 32.7]; *P*<0.05). There was no increase in lipid deposition in the aortic arch in mice in the D4LR group (10.8% [9.1, 11.9]; *P*=ns [not significant]).

To determine the effect of restoration of renal blood flow on atherogenesis within areas of low shear stress, the descending thoracic aorta was stained with Oil Red O. There was less lipid deposition in the descending thoracic aorta, compared to the aortic arch, in all groups (Figure 3C). The area of the descending thoracic aorta which stained for lipids was similar in the sham surgery (4.9% [2.8, 7.8]), D4LR (8.9% [4.3, 10.7]), and D8LR (5.3% [4.2, 16.0]) groups. The area of the descending thoracic aorta, which stained for lipid

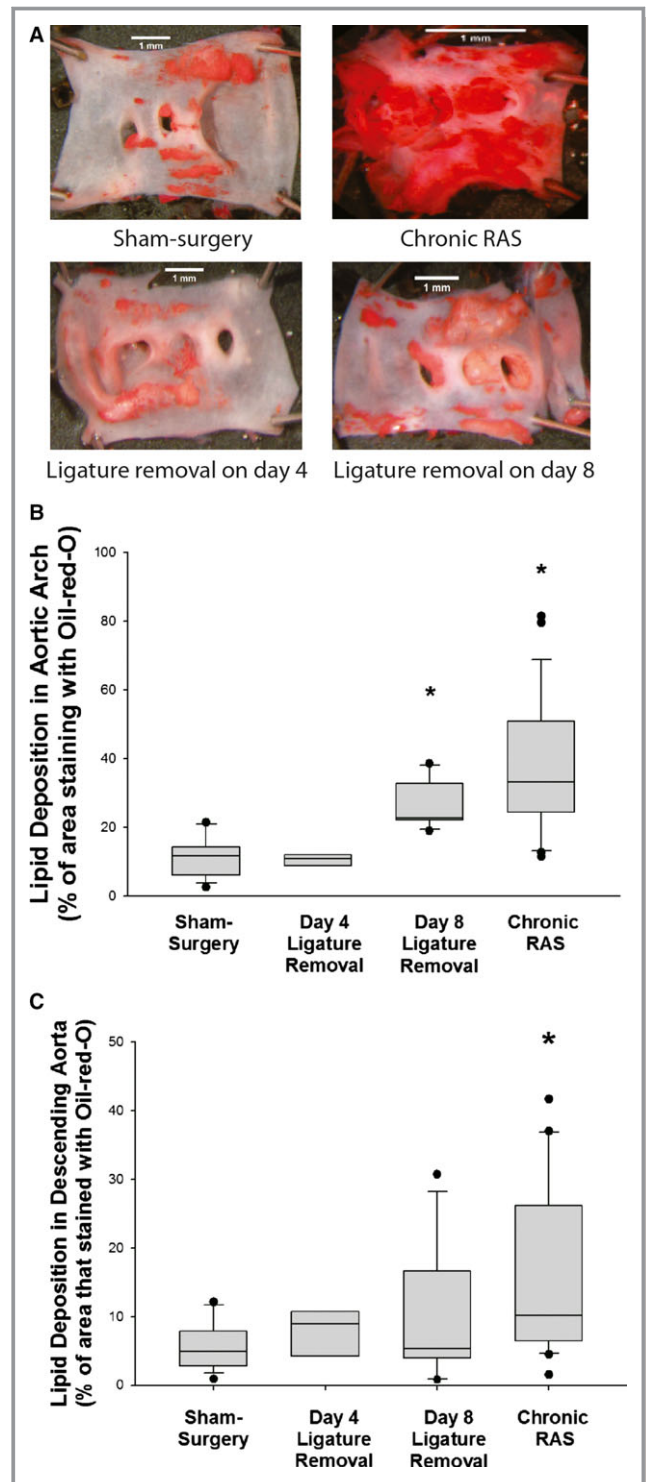


Figure 3. Aortic lipid deposition after unilateral renal artery constriction with and without revascularization. Representative examples of the aortic arch stained with Oil Red O from mice in the sham surgery, chronic RAS, D4LR, and D8LR groups are shown in (A). The area of the aortic arch (B) and descending thoracic aorta (C) that stained with Oil Red O as a percentage of total area for all mice in each of the groups (**P*<0.05 vs sham surgery). RAS indicates renal artery stenosis.

deposition, was ≈ 2 -fold higher in the chronic RAS group compared to the sham-surgery group (10.2% [6.4, 25.9]; $P < 0.05$).

Restoration of Renal Blood Flow Was Associated With Less Inflammation in Aortic Atheroma

Atheroma having cholesterol clefts, calcification, acellular necrotic core, a lipid core, and/or a fibrous cap were identified in the infrarenal aorta in 2 of 6 (33%), 4 of 11 (36%), and 13 of 24 (54%) mice in the D4LR, D8LR, and chronic RAS groups, respectively (Figure 4). In contrast, no atheroma were observed in the 16 mice in the sham surgery group. Comparing the 3 groups with renal artery ligation versus sham surgery showed that any renal ischemia was associated with a statistically significant increase in the presence of infrarenal aortic atheroma (19 of 41 [46%] vs 0 of 16; $P = 0.008$).

There was significant macrophage expression within atheroma from mice with chronic RAS (Figure 5A), consistent with previous studies showing that chronic RAS is associated with a vascular inflammatory reaction.^{10,11,16} Macrophages were detectable within atheroma from mice in the D4LR and

D8LR groups, but at levels significantly less than observed in atheroma from mice in the chronic RAS group (Figure 5B).

Discussion

These results demonstrate that restoration of renal blood flow after either 4 or 8 days of renal ischemia was associated with decreased aortic atherosclerotic changes compared to chronic RAS. Importantly, however, RAS for 8 days was associated with accelerated atherogenesis in the aorta of ApoE^{-/-} mice, compared to the sham surgery group, as determined by the presence of atheroma in the infrarenal aorta and lipid deposition in the aortic arch. The increased atherogenesis in the aorta was observed despite normalization of renal blood flow, a decrease in plasma Ang-II levels to subnormal levels, and a return of systolic blood pressure to baseline after ligation removal. These results demonstrate that even relatively brief periods of renal ischemia cause accelerated atherosclerosis, and strongly suggest that the benefits of restoration of renal blood flow in ApoE^{-/-} mice with unilateral RAS are dependent on the length of time of renal ischemia.

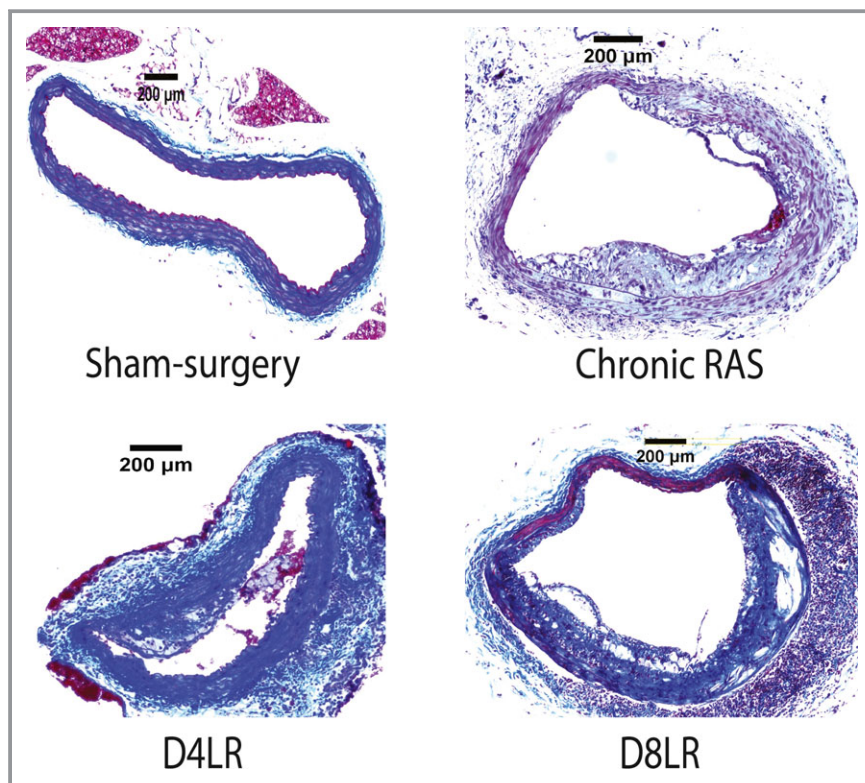


Figure 4. Atheroma in the infrarenal abdominal aorta. Representative sections from the distal one third of the descending aorta from various groups stained with Trichrome showing the formation of atheroma (original magnification, 100 \times). RAS indicates renal artery stenosis.

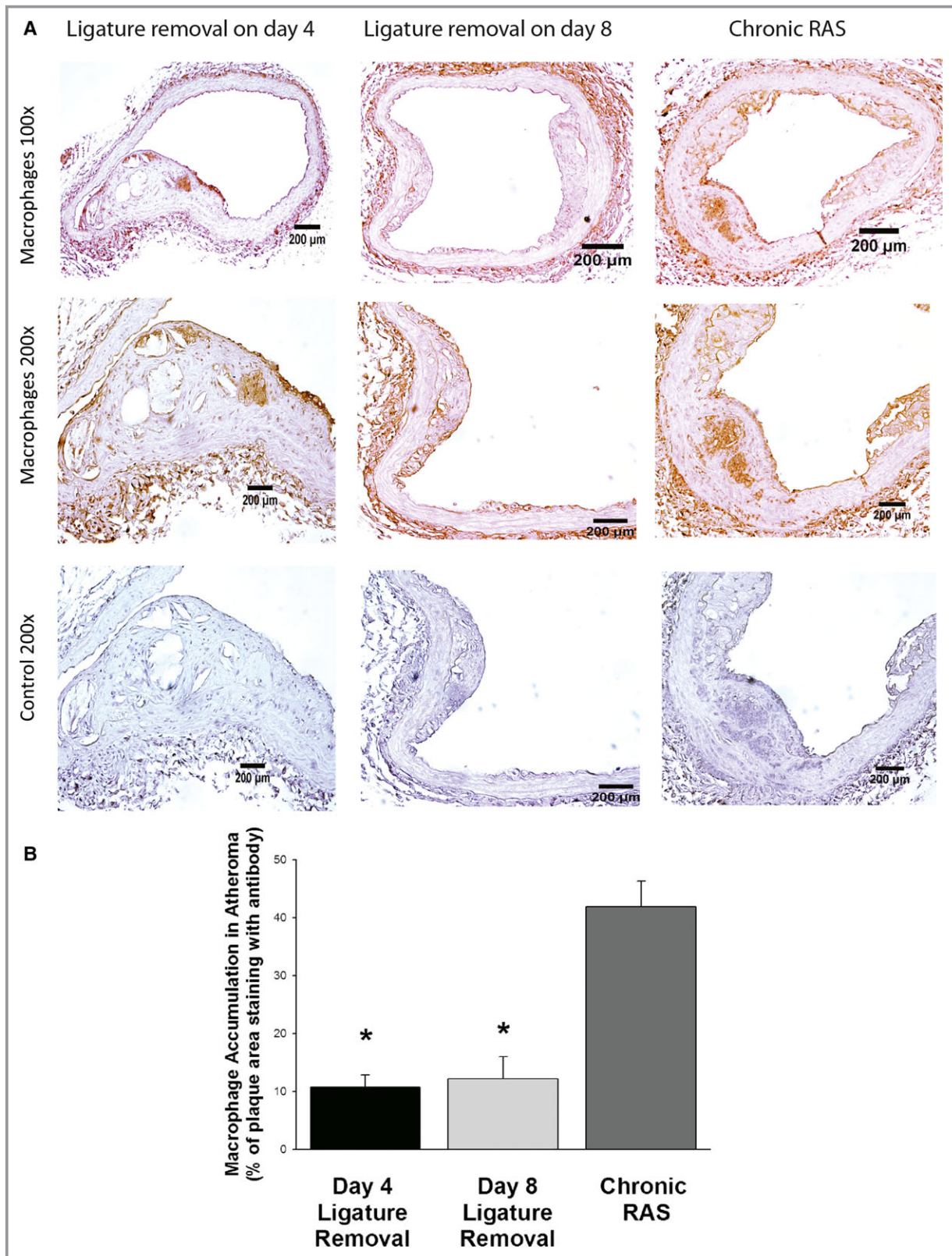


Figure 5. Atheroma inflammation after unilateral renal artery constriction with and without revascularization. Representative sections from atheroma in the distal one third of the descending aorta from various groups stained with a rat antimouse monoclonal antibody that recognizes the murine F4/80 macrophage antigen (A). The area of the atheroma that had visible staining for macrophages is quantified in (B). There were no atheroma in the mice that underwent sham surgery ($*P<0.05$ vs sham surgery group). RAS indicates renal artery stenosis.

A novel finding of this study is that early restoration of renal perfusion reduced the inflammatory response observed in aortic atheroma in mice with chronic RAS. Abdominal aortic atheroma were present in mice in the D4LR, D8LR, and chronic RAS groups, whereas there were no atheroma in the 16 mice that underwent sham surgery. There was significant accumulation of macrophages within atheroma in the chronic RAS group, consistent with past studies that showed pronounced inflammatory response in the aorta 4 weeks^{10,11} and 90 days¹⁶ after unilateral renal artery clipping in ApoE^{-/-} mice. Restoration of renal blood flow either 4 or 8 days after unilateral partial RAS was associated with lower levels of macrophage infiltration than chronic RAS, strongly suggesting that restoration of renal blood flow has an anti-inflammatory effect. A past study in pigs with unilateral RAS found that renal revascularization reduced, but did not normalize, either systemic or renal markers of inflammation.²³

In addition to an anti-inflammatory effect, there are several other mechanisms that may partially explain how restoration of renal blood flow reduces aortic atherosclerosis. Abrupt onset of renal ischemia in the 2K1C model is associated with RAS activation, and Ang-II has profound effects on the vasculature, including modulating vascular tone, stimulating smooth muscle cell responses, regulating production of growth factors and extracellular matrix, and enhancing macrophage infiltration into the vascular wall.^{6–11} Exposure to elevated levels of exogenous Ang-II^{6,8,24} and endogenous Ang-II^{10,11} have been shown to stimulate formation of atherosclerotic lesions in ApoE^{-/-} mice. The elevation of Ang-II levels observed 15 days after surgery in mice with chronic RAS in the current study was in contrast to the persistent reduction in Ang-II levels observed in mice that had restoration of renal blood flow. Other mechanisms that have been shown to contribute to atheroma formation in various models of RAS and which may be modulated by a reduction in the time of renal ischemia include regulation of nitric oxide levels, generation of reactive oxygen species, low-density lipoprotein oxidation, and upregulation of early growth response-1, an immediate early gene product and zinc finger transcription factor that has been implicated in atherogenesis.^{13,25,26}

Another novel finding of this study is the time course of NGAL levels in the 2K1C model of ApoE^{-/-} mice with and without revascularization. Plasma levels of NGAL were decreased after 15 days of renal ischemia in the chronic RAS model and then returned to baseline at 30 and 90 days. This study is the first to show a decrease in NGAL levels in a mouse model of renal injury. Past studies demonstrating that NGAL is rapidly and consistently upregulated in the ischemic mouse kidney^{27–29} examined early time points (usually within a few hours of injury) and/or ischemia-reperfusion injury. In contrast, the present study examined

NGAL levels at delayed time points in the presence of persistent renal injury.

In contrast to the decrease in NGAL levels observed at 15 days in the chronic RAS group, NGAL levels were increased at 15 and 30 days in mice that had restoration of renal blood flow. Because plasma NGAL is a powerful and independent predictor of acute kidney injury and renal repair,^{30,31} the increased levels of NGAL observed after revascularization would suggest that restoration of renal blood flow leads to a prolonged renal repair process.^{27,28} Additional studies are needed to define whether the changes in plasma NGAL levels were renal in origin given that NGAL exists in blood in 3 different molecular forms—as a monomer, homodimer, and heterodimer³²—with activated neutrophils predominantly releasing homodimeric NGAL, whereas injured distal tubule renal epithelial cells largely secrete monomeric NGAL.^{33,34}

This study provides further evidence that restoration of renal blood flow in rodents with unilateral RAS results in a rapid and persistent decrease in plasma markers of RAS activation. In both of the revascularization groups, Ang-II levels were decreased when measured after restoration of renal blood flow (at 15 days) and remained suppressed for the duration of the experiment (in contrast, plasma Ang-II levels were increased at 15 days in the chronic RAS group). Although numerous studies have documented activation of the RAS with abrupt onset of unilateral renal ischemia, much less is known about the time course of RAS activation after revascularization. Results of the current study are similar to those noted in past studies of the 2K1C model in female, white Wistar rats. In that model, unilateral renal artery clipping was associated with a marked increase in plasma renin concentration when measured 30.9±1.3 days later (638% of baseline). Removal of the clip resulted in a marked decrease in plasma renin concentration to 82% of baseline at 7 days and 64% of baseline at 60 days.^{14,15} Plasma renin levels in 2K1C hypertensive rats declined to levels below those of sham controls in as little as 3 hours after unclipping.³⁵

The results of this study should be interpreted with several important caveats. One is that the mice in the transient RAS groups underwent a second surgery for ligature removal. The comparison groups had a single surgery, and thus we cannot exclude the possibility that the second surgery contributed to vascular pathology. Second, we did not establish the time course over which renal artery flow returned to baseline levels. Doing this with laser Doppler perfusion imaging would have required additional surgeries, which would have confounded the results. Third, we were unable to quantify blood pressure early after renal artery constriction because BP measurements were unreliable in the perioperative period.

Clinical studies examining the effects of percutaneous revascularization in patients with severe RAS have failed to

show any benefit on all-cause mortality or cardiovascular outcomes.^{1–3} This lack of benefit is despite successful restoration of blood flow in >95% of cases. The current study suggests that the duration of renal ischemia is an important variable in determining effects on atherogenesis and that timely, and not just successful, reestablishment of normal renal blood flow is necessary to have beneficial effects on RAS-induced development of atherosclerosis.

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Disclosures

None.

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