Central and Peripheral Mechanisms of T-Lymphocyte Activation and Vascular Inflammation Produced by Angiotensin II–Induced Hypertension

Paul J. Marvar, Salim R. Thabet, Tomasz J. Guzik, Heinrich E. Lob, Louise A. McCann, Connie Weyand, Frank J. Gordon, David G. Harrison

Rationale: We have previously found that T lymphocytes are essential for development of angiotensin II–induced hypertension; however, the mechanisms responsible for T-cell activation in hypertension remain undefined.

Objective: We sought to study the roles of the CNS and pressure elevation in T-cell activation and vascular inflammation caused by angiotensin II.

Methods and Results: To prevent the central actions of angiotensin II, we created anteroventral third cerebral ventricle (AV3V) lesions in mice. The elevation in blood pressure in response to angiotensin II was virtually eliminated by AV3V lesions, as was activation of circulating T cells and the vascular infiltration of leukocytes. In contrast, AV3V lesioning did not prevent the hypertension and T-cell activation caused by the peripheral acting agonist norepinephrine. To determine whether T-cell activation and vascular inflammation are attributable to central influences or are mediated by blood pressure elevation, we administered hydralazine (250 mg/L) in the drinking water. Hydralazine prevented the hypertension and abrogated the increase in circulating activated T cells and vascular infiltration of leukocytes caused by angiotensin II.

Conclusions: We conclude that the central and pressor effects of angiotensin II are critical for T-cell activation and development of vascular inflammation. These findings also support a feed-forward mechanism in which modest degrees of blood pressure elevation lead to T-cell activation, which in turn promotes inflammation and further raises blood pressure, leading to severe hypertension. (Circ Res. 2010;107:263-270.)

Key Words: hypertension • vascular inflammation • T cells • central nervous system

The CNS plays an integral role in blood pressure regulation, primarily through sympathetic activation and mediation of various neurohumoral factors such as angiotensin II (Ang II) and vasopressin.1–4 Hormones such as these can access the circumventricular organs, which are adjacent to the cerebral ventricles and have a poorly formed blood brain barrier. The circumventricular organs implicated in blood pressure control include the organum vasculosum of the lamina terminalis (OVLT), the area postrema, the subfornical organ, and the anteroventral third ventricle (AV3V) region.5 The AV3V region includes the median preoptic nucleus, the OVLT, and the periventricular nucleus and has been shown to play an important role in several behavioral, neural and hormonal functions involved in body fluid and cardiovascular homeostasis.6,7 Electrolytic lesions that disrupt the AV3V region have been shown to abolish virtually all of the central actions of Ang II, including drinking behavior, sympathetic outflow, and vasopressin release.8 Electrolytic lesions of this brain region abolish the centrally mediated pressor response to central and peripheral infusions of Ang II, as well as preventing and/or reversing several other forms of experimental hypertension.5,6

In addition to central mechanisms, substantial evidence suggests that inflammation can contribute to the pathophysiology of hypertension. For example, inflammatory cells accumulate in the kidney and vasculature of hypertensive animals, and the prevention of this can lower blood pressure.8 Recently, our laboratory has found that mice lacking T-lymphocytes are resistant to the development of both Ang II and DOCA-salt induced hypertension.9 Adoptive transfer of T, but not B cells restored hypertension in these animals. Despite the growing evidence for the role of T cells in hypertension, the mechanisms underlying T-cell activation and vascular infiltration by T cells remain unclear. Given the importance of the CNS in regulating blood pressure, we hypothesized that the CNS could...
participate in activation of peripheral T cells and contribute to vascular inflammation in response to Ang II. We tested this hypothesis by producing electrolytic lesions of the AV3V region in mice and then examined properties of circulating T cells and the vascular accumulation of these cells in response to chronic Ang II infusion. We also examined the role of pressure elevation in T-cell activation and vascular inflammation. Our findings provide a new understanding for how the CNS, in particular the circumventricular organs, can contribute to the promotion of systemic inflammation in hypertension.

Methods

Animals

C57BL/6, RAG-1−/− and ovalbumin (OVA)-specific, MHC class II-restricted αβ T-cell receptor (TCR) transgenic mice (OT-II) were obtained from The Jackson Laboratory.

Production of Hypertension

Using an osmotic minipump (Alzet, Model 2002), either Ang II (490 ng·kg⁻¹·min⁻¹), norepinephrine (3.8 μg·kg⁻¹·min⁻¹), or vehicle was infused for 14 days. Blood pressure was measured by radiotelemetry or tail cuff method, as described previously. When radiotelemetry was used, the transmitters were implanted 1 week before the minipumps. In some experiments, hydralazine (250 mg/L) was administered in the drinking water to prevent the development of hypertension. Adaptive transfer of T cells into RAG-1−/− mice was performed using methods similar to those described previously and norepinephrine or vehicle infusion initiated 3 weeks later.

AV3V Lesions

Mice were anesthetized with intraperitoneal ketamine and acepromazine (90 mg/kg and 1.8 mg/kg, respectively) and mounted in a stereotactic frame with skull level between bregma and lambda. Using aseptic technique, a small hole was drilled in the skull, the midsagittal sinus was retracted, and a 200-μm diameter tungsten electrode (AM Systems), Teflon-insulated except at the tip, was inserted at the midline 0.4-mm anterior to bregma and 4.8 mm ventral to the dura. Lesions were produced by passing a 1.0-mA DC anodal current through the electrode for 3 seconds. Surgery for sham-operated mice was identical except that the electrode was lowered only 4.0 mm, and no current was applied. Because AV3V-lesioned animals do not drink water for several days after lesioning, mice were given a highly palatable 10% sucrose solution before and after lesioning to induce voluntary fluid intake and prevent dehydration. Following the lesion, AV3V-lesioned mice were weaned onto normal drinking water by gradually reducing the concentration of sucrose over a 2-week period.

AV3V Lesion Verification

Electrolytic lesioning in and around the third ventricle can produce neuronal destruction of variable extent as shown by the topography detailed in previous studies using AV3V-lesioned animals. Mice included in the AV3V-lesioned groups were functionally and histologically characterized as having sustained complete AV3V lesions using criteria (drinking behavior and histology) that were independent of the dependent variables of interest in these studies (blood pressure, superoxide production, T-cell function). Mice were considered to have complete AV3V lesions if they met the following 3 criteria: (1) failed to drink any water within 24 hours following surgery; (2) drank less than 1.0 mL of water within 2.0 hours following subcutaneous injection of 6.0% NaCl; and (3) histological confirmation following the experimental protocol of complete destruction of the AV3V region, including the OVLT, median preoptic and periventricular nuclei (Figure 1). Approximately 50% of mice that received AV3V lesions met the above criteria. In the AV3V mice that met these criteria, the 24-hour water intake was 0.08±0.03 mL compared to 2.65±0.27 mL in the sham-operated group, whereas the drinking volume in response to the hypertonic 6.0% NaCl thirst challenge was 0.56±0.17 and 2.16±0.13 mL in the AV3V-lesioned and sham-operated mice, respectively.

Superoxide Measurements, Fluorescent Cell Sorting, and Analysis of Cellular Inflammation

Vascular O₂⁻ production was measured by quantifying formation of 2-hydroxyethidium from dihydroethidium (25 μmol/L) by HPLC as described previously. Analysis of circulating T cells from the blood...
and inflammatory cells in vascular homogenates of the aorta was performed using fluorescent cell sorting as described previously. Antibodies used for staining were as follows: FITC anti-CD45 (30-F11); APC anti-CD4 (GK1.5); PerCP anti-CD8 (53-6.7); APC anti-CD3 (145-2C11); FITC CD44 (IM7); FITC CD69 (H1.2F3). After immunostaining, cells were resuspended in FACS buffer (0.5% BSA in PBS) and analyzed immediately on a LSR-II flow cytometer with DIVA software (Becton Dickinson). Data were analyzed with FlowJo software (Tree Star Inc). The institutional Animal Care and Use Committee at Emory University approved all of the above experimental protocols.

Data Presentation and Statistical Analysis
Summary data are expressed as the means±SEM, and values of P<0.05 were considered statistically significant. Comparisons between groups of animals or treatments were made by ANOVA. The Bonferroni post hoc test was used to make comparisons.

Results
Effects of AV3V Ablation on Blood Pressure and Vascular Superoxide
In sham-operated mice, Ang II infusion produced a progressive rise in blood pressure when measured either by tail cuff or radiotelemetry (Figure 2A through 2C). Infusion of vehicle alone had no effect on arterial pressure (Figure 2A). AV3V ablation did not affect baseline blood pressure but markedly reduced the hypertension produced by peripheral infusion of Ang II compared to the sham-operated group (Figure 2A through 2C). Ang II infusion in AV3V-lesioned mice produced a small rise in blood pressure after 7 and 14 days compared to sham vehicle (Figure 2A). In other groups of mice, direct measurement of arterial pressure by radiotelemetry largely confirmed the results obtained using the tail cuff method. AV3V lesions virtually eliminated increases in systolic and diastolic blood pressure normally produced by peripheral infusion of Ang II (Figure 2B and 2C).

Previous studies from our laboratory have shown that chronic Ang II infusion increased vascular O$_2^-$ production. We confirmed these results in C57BL/6 mice without CNS lesions, as shown in Figure 2D. Chronic infusion of Ang II in sham-operated mice elevated aortic superoxide levels to values similar to those without CNS lesions. In contrast, vascular superoxide levels of mice given Ang II were significantly reduced by AV3V lesioning and were not different from mice that did not receive Ang II infusion (Figure 2D).

Effects of AV3V Ablation on T-Cell Activation and Vascular Inflammation
Consistent with previous observations, Ang II infusion in sham-operated mice increased the percentage of circulating helper (CD4$^+$) T cells that expressed the early activation marker CD69 (Figure 2A through 2D). This increase in activated CD4$^+$ T cells was abolished by AV3V lesioning (Figure 2A through 2D). In sham-operated mice, Ang II infusion promoted vascular infiltration by inflammatory leukocytes, as evidenced by aortic accumulation of total CD45$^+$ leukocytes (Figure 2C) and CD3$^+$ T cells (Figure 2D). These data indicate that Ang II–induced activation of circulating T cells and infiltration of vascular tissue by inflammatory cells was completely prevented by the AV3V lesion. These data indicate that Ang II–induced activation of circulating T cells and infiltration of vascular tissue by inflammatory cells cannot be ascribed to a direct action of the circulating hormone on peripheral tissues but, instead, must be mediated by a CNS action of Ang II that is dependent on the AV3V region.

Effect of AV3V Ablation on Norepinephrine-Induced Hypertension, T-Cell Activation, and Vascular Inflammation
Systemic infusion of the peripherally acting vasoconstrictor norepinephrine was used as a positive control for any potential nonspecific or detrimental effects of the AV3V lesion on cardiovascular function. As shown in Figure 4A, AV3V lesions had no effect on hypertension produced by norepinephrine. When immune cell activation and vascular inflam-
mation were measured, similar increases in circulating T cells expressing the early activation marker CD69 and the tissue-homing marker CD44<sup>high</sup> were observed in both AV3V-lesioned and sham-operated animals (Figure 4B and 4C). Moreover, norepinephrine infusion also promoted vascular inflammation assessed by aortic accumulation of CD45<sup>+</sup> leukocytes and CD3<sup>+</sup> T cells, irrespective of whether mice had sustained AV3V lesions or not (Figure 4C through 4E). These results indicate that AV3V lesions do not impair the production of hypertension and T-cell activation produced by a peripherally acting stimulus. We further confirmed that, as in the case of Ang II, T cells largely mediate the hypertensive response to norepinephrine, because the blood pressure elevation to this catecholamine was markedly reduced in RAG-1<sup>−/−</sup> mice and restored by adoptive transfer of T cells (Figure 4F).

**Effect of Preventing Hypertension With Hydralazine on Vascular Superoxide, T-Cell Activation, and Vascular Inflammation**

Results from the previous experiments using Ang II and norepinephrine infusion to produce hypertension suggest that immune cell activation may be more dependent on elevated arterial pressure per se, rather than the specific stimulus that produces it. To determine whether the reduction in T-cell activation and vascular inflammation following AV3V ablation and Ang II infusion was a consequence of preventing hypertension, we administered hydralazine via the drinking
water during Ang II infusion. Following 2 weeks of Ang II infusion, hydralazine eliminated the elevation in blood pressure to Ang II (Figure 5A), as well as vascular superoxide production (Figure 5B). Hydralazine also prevented the activation of circulating T cells (Figure 5C and 5D), as well as vascular accumulation of inflammatory cells (Figure 5E and 5F). We also examined the effect of hydralazine administration during norepinephrine infusion. As in the case of Ang II, hydralazine prevented the increase in arterial pressure and T-cell activation and infiltration during norepinephrine-induced hypertension (Online Figure I, available at http://circres.ahajournals.org).

The effects of hydralazine on T-cell activation and vascular inflammation could be related to its antihypertensive actions or, alternatively, to a direct effect on T cells that might prevent their activation. To distinguish between these possibilities, we performed studies using OT-II mice. OT-II mice are transgenic for a T-cell receptor that induces production of CD4$^+$ activated T cells when the receptor is activated by the peptide OVA 323-339. OT-II mice were injected on day 1 intraperitoneally with OVA 323-339 peptide (0.5 mg/L) or vehicle [Al(OH)$_3$; 2.5 mg], and hydralazine was administered in the drinking water during the 7 days. Blood pressure was not significantly changed between groups [OVA, 122±5.7; Al(OH)$_3$, 122 ± 7.6 mm Hg; hydralazine+OVA: 113±1.3 mm Hg; hydralazine+Al(OH)$_3$, 113±10.1 mm Hg]. In control mice, OVA markedly enhanced T-cell activation, as evidenced by an increase in CD69 and in CD44$^+$ expressed on CD4$^+$ cells (Figure 6A and 6B) compared to OT-II mice treated with vehicle Al(OH)$_3$. Treatment of OT-II mice with hydralazine for 1 week did not diminish T-cell

Figure 5. Hydralazine prevents Ang II–induced hypertension, increased superoxide production, T-cell activation and vascular inflammation. A, Blood pressure measurements during 2 weeks of Ang II infusion and hydralazine (HYD) treatment (n=8 to 10). B, Aortic superoxide levels following Ang II infusion and hydralazine treatment (n=5 to 11 in each group). C and D, Percentage of circulating CD4$^+$ lymphocytes expressing the early activation marker CD69 and the T-cell tissue-homing marker CD44 following Ang II and hydralazine treatment (n=11 to 14). E and F, Absolute numbers of total leukocytes (CD45$^+$) and total T cells (CD3$^+$) in collagenase-digested whole aortas following Ang II infusion and hydralazine treatment (n=7 to 16). *P<0.05, Ang II vs vehicle; †P<0.05, Ang II+HYD vs Ang II; **P<0.05, vehicle+HYD vs Ang II+HYD.

Figure 6. Hydralazine does not prevent circulating T-cell activation in OT-II transgenic mice. A and B, The percentage of circulating CD4$^+$ lymphocytes expressing the early activation marker CD69 (A) and T-cell tissue-homing marker CD44 in OT-II transgenic mice immunized with OVA and Al(OH)$_3$ vehicle [n=3 OVA; n=2 Al(OH)$_3$] (B). Control represents mice that did not receive hydralazine. *P<0.05, OVA vs Al(OH)$_3$ in control and hydralazine groups.
activation evoked by OVA (Figure 6A and 6B). These results indicate that hydralazine does not impair T-cell activation in response to a nonhypertensive stimulus.

Discussion
The purpose of this study was to investigate the potential mechanisms by which Ang II increases blood pressure and T-cell activation. Anteroventral third cerebral ventricle (AV3V) ablation of the forebrain, which is known to prevent the central actions of Ang II, was found to prevent activation of circulating T cells and to inhibit the vascular inflammation caused by this octapeptide. In contrast, AV3V lesions did not alter the effect of norepinephrine on these parameters. AV3V lesioning also prevented the increase in vascular O$_{2}^{-}$ production caused by Ang II infusion. The antiinflammatory effects of AV3V lesioning could have been attributable to a reduction of neurohumoral stimuli or to prevention of pressure-induced tissue damage. Hydralazine, which prevented the hypertensive response to Ang II infusion, also prevented T-cell activation, vessel infiltration of leukocytes, and vascular superoxide production. In contrast, hydralazine did not block T-cell activation in response to a nonhypertensive stimulus in OT-II transgenic mice. This suggests that the reduction in T-cell activation and vascular infiltration caused by hydralazine during Ang II and norepinephrine infusion were likely a result of its blood pressure lowering effects rather than any direct actions it has on T cells. Our results suggest that Ang II–mediated hypertension is caused by central mechanisms and that T-cell activation is likely dependent on mechanical stimuli caused by elevations of arterial pressure induced by these central signals.

Before the present study, it was unknown whether T-cell activation and vascular infiltration in Ang II–induced hypertension was attributable to the direct effects of Ang II on T cells or whether T-cell activation was mediated by its CNS actions. To address this question, we created lesions of the AV3V region, which encompasses the OVLT, the ventral portion of the median preoptic nucleus (MnPO), and the preoptic periventricular nuclei.5 The AV3V region receives input from other circumventricular structures, such as the subfornical organ, which transduces peripheral angiotensin signals and sends them to the AV3V, thus contributing to the development of hypertension.20,21 These regions of the brain have a poorly formed blood brain barrier and are capable of responding to circulating hormones such as Ang II22,23 and modulating its central actions, which include drinking behavior, vasopressin secretion, sympathetic activity, and norepinephrine synthesis.5–24 Our studies confirm these effects in that the increase in blood pressure following systemic Ang II infusion was attenuated after AV3V ablation. Interestingly, AV3V lesioning also prevented activation of circulating T cells and vascular inflammation caused by Ang II. Thus, these results indicate that the central actions of Ang II contribute to its proinflammatory effects.

To demonstrate that the reduction of T-cell activation after AV3V lesioning was not a nonspecific effect of this intervention, we performed additional studies in which we infused norepinephrine. We reasoned that one of the major effects of AV3V lesioning was to abolish sympathetic outflow to the periphery and that this, therefore, could be bypassed by administration of norepinephrine, a peripherally acting vasoconstrictor stimulus. The degree of blood pressure elevation, T-cell activation, and vascular infiltration of CD45$^+$ and CD3$^+$ cells in response to norepinephrine was similar in the absence and presence of AV3V lesioning. These results demonstrate that AV3V lesioning does not nonspecifically block the inflammatory response to all hypertensive stimuli and verifies the specificity for the central actions of Ang II. Moreover, these experiments with norepinephrine demonstrate that other vasoconstrictor stimuli are capable of mediating vascular inflammation during hypertension.

Ang II is known to promote sympathetic outflow and therefore one interpretation of our results is that immune cell activation is dependent on sympathetic activation and catecholamine release. T cells possess adrenergic receptors, and sympathetic nerves richly innervate lymphatic tissues.25 Ang II could, therefore, stimulate T cells via activation of the sympathetic nervous system. These considerations are in keeping with the findings of Ganta et al, who showed that Ang II administration in the lateral cerebral ventricle increased splenic sympathetic nerve activity, which, in turn, causes increased mRNA expression of various proinflammatory cytokines in splenocytes. Splenic sympathetic denervation abrogated these responses, thus clearly linking the central effects of Ang II to peripheral immune activation and cytokine production.26

Given the above considerations, it is possible that the consequent release of central mediators in response to Ang II, such as catecholamines, could directly lead to T-cell activation in the absence of blood pressure elevation. To address this, we performed studies using hydralazine to prevent hypertension during Ang II and norepinephrine infusion. The results of these studies suggest that pressure elevation per se could lead to T-cell activation and vascular infiltration. In contrast to AV3V lesioning, hydralazine activates the sympathetic nervous system,27 in part via baroreflex-mediated mechanisms, but in the present study, it completely prevented T-cell activation. These results would seem to exclude a direct effect of sympathetic nerves on T-cell activation and would favor an effect of pressure per se in T-cell activation.

We have previously found that hydralazine inhibits the NADPH oxidase.28 The NADPH oxidase has been identified in T cells,29 and its blockade might have prevented T-cell activation. Hydralazine also opens calcium-sensitive potassium channels,30 which are present in T cells,31 and, therefore, may have direct effects on T-cell function. To determine whether hydralazine directly inhibits T-cell activation in response to nonhypertensive stimuli, we studied OT-II transgenic mice. These animals express a T-cell receptor that leads to CD4$^+$ cell activation in response to OVA peptide administration. We found that hydralazine did not block T-cell activation caused by OVA peptide administration in these animals, suggesting that it does not prevent T-cell activation.
by stimuli that do not cause hypertension. These findings support the notion that the effect of hydralazine during Ang II infusion was not attributable to a direct effect of the drug on T cells but more likely to its prevention of hypertension. We are not completely able, however, to delineate a direct effect of neurohumoral mediators, such as catecholamines, from mechanical stimuli caused by pressure elevation. Our findings are nevertheless compatible with a scenario in which the central effects of Ang II lead to pressure elevation, which, in turn, promotes T-cell activation and vascular inflammation.

These results should not be taken to indicate that inflammation, and in particular T-cell activation, is simply a result of hypertension and does not contribute to blood pressure elevation. As we previously demonstrated, in the absence of T cells, the hypertensive response to Ang II and DOCA-salt challenge is minimal. Our present findings suggest, however, that a modest increase in blood pressure may lead to an inflammatory response that further increases and sustains blood pressure in a feed-forward fashion (Online Figure III). This might explain the propensity of prehypertension to progress to overt hypertension. These considerations stress the benefit of lowering blood pressure by virtually any means to prevent and reverse this mechanism.

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Disclosures
None.

References


**Novelty and Significance**

**What Is Known?**

- We have previously shown that T cells are essential for development of hypertension.
- The circumventricular organs in the CNS are key targets for the peptide angiotensin II and are essential in the regulation of blood pressure.

**What New Information Does This Article Contribute?**

- Central effects of angiotensin II are required for T-cell activation and peripheral vascular inflammation in the setting of hypertension caused by this octapeptide.
- Modest elevations of blood pressure mediated by central stimuli promote inflammation, which leads to severe hypertension in a feed-forward fashion.

There is growing evidence for the role of T cells in hypertension; however, the mechanisms underlying T-cell activation and vascular infiltration by T cells remain unclear. The circumventricular organs are critical for the regulation of blood pressure, and, therefore, we hypothesized that the CNS could participate in activation of peripheral T cells and contribute to vascular inflammation in response to angiotensin II. Lesioning of the anteroventral third ventricle (AV3V) is known to prevent hypertension caused by angiotensin II. In the present study, we found that T-cell activation and vascular inflammation caused by angiotensin II infusion were completely blocked by AV3V lesioning. The increase in blood pressure and T-cell activation to peripherally acting norepinephrine was unaffected by AV3V ablation. Administration of an antihypertensive drug attenuated the elevation in blood pressure following angiotensin II infusion and also prevented the increase in T-cell activation and vascular inflammation. Our findings are compatible with a pathway in which central stimuli, such as angiotensin II, cause modest elevations of blood pressure, which leads to T-cell activation and, ultimately, more severe hypertension. This study provides a new understanding of the link among central signals, peripheral inflammation, and hypertension.
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On line supplement
Expanded Methods

Production of Hypertension (Angiotensin II and Norepinephrine):

Using an osmotic minipump (Alzet, Model 2002) either angiotensin II (490 ng · kg\(^{-1} \cdot \text{min}^{-1}\)), norepinephrine (3.8 ug · kg\(^{-1} \cdot \text{min}^{-1}\)), or vehicle was infused for 14 days. Blood pressure was measured by radiotelemetry or tail cuff method, as previously described.\(^1\) When radiotelemetry was employed, the transmitters were implanted 1 week prior to the minipumps. In some experiments, hydralazine (250mg/L) was administered in the drinking water to prevent the development of hypertension.\(^2\) Hydralazine treatment was initiated 3 days prior to the implantation of either norepinephrine or angiotensin II.

AV3V Lesions:

As described by Johnson, et al.\(^3\), mice were anesthetized (ketamine-90 mg/kg, acepromazine-1.8 mg/kg, i.p.) and mounted in a stereotaxic frame with skull level between bregma and lambda. Using aseptic technique, a small hole was drilled in the skull, the mid-sagittal sinus retracted, and a 200 µm diameter tungsten electrode (AM Systems), Teflon insulated except for 375 µm at the tip, was positioned on the midline 0.4 mm anterior to bregma and 4.8 mm ventral to dura. Lesions were produced by passing a 1.0 mA DC anodal current to a scalp cathode for 3 seconds. Sham lesions were produced identically except that the electrode was lowered only 4.0 mm, and no current was passed. Following AV3V ablation, animals completely lack thirst and can die of dehydration. To prevent this, mice were provided with a 10% sucrose solution to drink for the week prior to the lesion. Similar to rats, mice drink the sucrose solution because of the sweet taste and thus remain hydrated. Mice were weaned to tap water over the next 1-3 weeks by slowly reducing the concentration of sucrose in the drinking water. At least 2 wk’s were allowed for recovery before experimentation. At the conclusion of all experimentation, mice were sacrificed, and the brain harvested and placed in 4% paraformaldehyde. One to 3 wk’s later 50 µm frozen sections were cut and stained with cresyl violet for histological analysis. Only mice sustaining complete ablation of the organum vasculosum of the lamina terminalis, ventral median pre-optic nucleus, and pre-optic periventricular nuclei were included for analysis as sustaining complete AV3V lesions. Lesion scoring was done on coded slides so the scorer was unaware of the animal’s identity.

Vascular superoxide measurements:

For estimates of vascular \(O_2^-\) production using HPLC, five 2-mm segments of vessels were incubated at 37°C for 15 min with Krebs-HEPES buffer containing 50 µM dihydroethidium. The vessels were then washed of dihydroethidium and incubated in the Krebs-HEPES buffer for an additional 1 h. The vessels were then placed in 300 µl of cold methanol, homogenized, and filtered (0.22 µm). The filtrate was then analyzed by HPLC. Separation of ethidium, oxyethidium, and dihydroethidium was performed using a Beckman HPLC System Gold model with a C-18 reverse phase column (Nucleosil 250, 4.5 mm; Sigma-Aldrich, St. Louis, MO), equipped with both UV and fluorescence detectors. Fluorescence detection at 580 nm (emission) and 480 nm (excitation) was used to monitor oxyethidium production. UV absorbation at 355 nm was used for the detection of dihydroethidium. The mobile phase was composed of a gradient
containing 60% acetonitrile and 0.1% trifluoroacetic acid. Dihydroethidium, ethidium, and oxyethidium were separated by a linear increase in acetonitrile concentration from 37 to 47% over 23 min at a flow rate of 0.5 ml/min.

FACS staining for blood:

Total blood leukocytes were isolated from the whole heparinized blood after osmotic lysis of excess red blood cells. Cells were centrifuged (800 g), washed twice with PBS and 0.5% BSA (FACS buffer), counted, resuspended in 1% BSA/PBS, and stored on ice for <30 min. Within 30 min, 10⁶ cells were stained for 15 min at 4°C with antibodies and washed twice with FACS buffer. Antibodies used for staining, and in different multicolor combinations, are as follows: Antibodies used for staining were as follows: FITC anti-CD45 (30-F11); APC anti-CD4 (GK1.5); PerCP anti-CD8 (53–6.7); APC anti-CD3 (145-2C11); FITC CD44 (IM7); FITC CD69 (H1.2F3). After immunostaining, cells were resuspended in FACS buffer (0.5% bovine serum albumin in PBS) and analyzed immediately on a LSR-II flow cytometer with DIVA software (Becton Dickinson). Data were analyzed with FlowJo software (Tree Star, Inc.).

Adoptive transfer of T cells:

Adoptive transfer of T cells into RAG-1⁻ mice was performed using methods similar to those previously described ¹ and as follows: Total splenocytes were isolated from donor mice, and either T cells were isolated using cell-specific isolation kits (Miltenyi Biotech) and negative magnetic sorting (AutoMACS), yielding sterile and highly enriched T cells. The purity of these was confirmed to be ≥ 95% by flow cytometry before injection. Immediately after the cell isolation, 2 × 10⁷ cells were resuspended in 150 µl of sterile PBS, passed through a 70-µm filter, and injected into RAG-1⁻ mice via tail vein. Successful adoptive transfer was confirmed at the time of sacrifice using flow cytometry. 3 wk after adoptive transfer of T cells, the number of T cells in the spleen of RAG1⁻ mice averaged 2.6 ± 0.3 × 10⁶. Norepinephrine or vehicle infusion initiated 3 weeks later following adoptive transfer.

FACS staining of aorta:

Mouse aortas were digested using collagenase type IX (125 U/ml), collagenase type IS (450 U/ml), and hyaluronidase IS (60 U/ml) dissolved in 20 mM Hepes-PBS buffer containing calcium and magnesium for 60 min at 37°C, with constant agitation. Aortas were then passed through a 70-µm sterile cell strainer (Falcon; BD Biosciences) into a 50ml falcon tube using 0.5% BSA PBS buffer to facilitate the filtration process. Cells were then spun at 1200 rpm for 15min. Single-cell suspensions were acquired, cells counted, and stained, using multicolor flow cytometry as described in the manuscript. An initial gate was applied to exclude cell debris from further analysis and CD45 staining was used to identify leukocytes within the aortic cell suspension. Within the CD45⁺ gate, T cells were identified with anti-CD3, -CD4, and -CD8 antibodies, as well as antibodies to detect other supplementary surface molecules. Figure 3E illustrates the gating that was applied.
References to on-line material


Online Figure I: Hydralazine prevents norepinephrine induced hypertension, T cell activation and vascular inflammation. Panel A: Blood pressure measurements during 2 weeks of norepinephrine (NE) infusion and hydralazine (HYD) treatment (n = 9-13). Panel B: Aortic superoxide levels following NE infusion and HYD treatment (n = 5 per group). Panel C-D: Percentage of circulating CD4+ lymphocytes expressing the early activation marker CD69 and the T cell tissue homing marker CD44 following NE infusion and HYD treatment (n = 9-14). Panel E-F: Total number of CD45+ leukocytes and CD3+ T cells in aortas following NE infusion and HYD treatment (n= 9-12) after 14 day infusion with vehicle or NE. (*P<0.05 NE vs Vehicle) (†P<0.05 NE + HYD vs NE)(**P<0.05 Vehicle + HYD vs NE + HYD; No statistically significant difference = ns).
Online Figure II: Coronal sections of the rostral to caudal extent of a typical anteroventral third ventricle (AV3V) lesion. Panels (A-D): As indicated by the red arrows the appearance of the lesion starts at the level of 0.5mm anterior to bregma and becomes more apparent as it extends caudally to 0.14mm anterior to bregma (Panel D). The periventricular tissue destruction is greatest at approximately 0.4mm to 0.26mm (Panels B-C). Panels (E-F) the lesion is absent at the level 0.02mm anterior to bregma to -0.34mm posterior to bregma. (AC, anterior commissure; OC, optic chiasm; 3V, third ventricle). These coordinates were taken according to the atlas of Paxinos and Franklin.11
**Online Figure III: Proposed mechanism and hypothesis for T cell activation and vascular inflammation during hypertension.** Increased circulating levels of angiotensin II can have direct effects on the central nervous system via the circumventricular organs. Anteroventral third ventricle (AV3V) ablation prevents the central effects as well as downstream T lymphocyte activation and vascular inflammation. Independent of these central effects, peripherally acting stimuli such as norepinephrine lead to blood pressure elevation, T cell activation and vascular inflammation and these effects can be blocked with hydralazine. We speculate that these peripheral and central actions trigger an initial elevation in blood pressure that over time lead to neo-antigen formation, T cell activation and vascular inflammation thus ultimately contributing to the development of sustained hypertension. (Dashed arrow – indirect effects; solid arrow-direct effects; Reactive oxygen species = ROS).