Macrophage depletion lowers blood pressure and restores sympathetic nerve \(\alpha_2\)-adrenergic receptor function in mesenteric arteries of DOCA-salt hypertensive rats

Loc V. Thang,1 Stacie L. Demel,2 Robert Crawford,1,3 Norbert E. Kaminski,1,3 Greg M. Swain,2,4 Nico Van Rooijen,5 and James J. Galligan1,2

1Department of Pharmacology and Toxicology, Michigan State University, East Lansing, Michigan; 2Neuroscience Program, Michigan State University, East Lansing, Michigan; 3Center for Integrative Toxicology, Michigan State University, East Lansing, Michigan; 4Department of Chemistry, Michigan State University, East Lansing, Michigan; and 5Department of Molecular Cell Biology, Vrije Universiteit Medical Center, Amsterdam, The Netherlands

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Thang LV, Demel SL, Crawford R, Kaminski NE, Swain GM, Van Rooijen N, Galligan JJ. Macrophage depletion lowers blood pressure and restores sympathetic nerve \(\alpha_2\)-adrenergic receptor function in mesenteric arteries of DOCA-salt hypertensive rats. Am J Physiol Heart Circ Physiol 309: H1186–H1197, 2015.—We tested the hypothesis that vascular macrophage infiltration and \(O_2\) release impairs sympathetic nerve \(\alpha_2\)-adrenergic autoreceptor (\(\alpha_2\)AR) function in mesenteric arteries (MAs) of DOCA-salt hypertensive rats. Male rats were uninephrectomized or sham operated (sham). DOCA pellets were implanted subcutaneously in uninephrectomized rats who were provided high-salt drinking water or high-salt water with apocynin. Sham rats received tap water. Blood pressure was measured using radiotelemetry. Treatment of sham and DOCA-salt rats with liposome-encapsulated clodronate was used to deplete macrophages. After 3–5, 10–13, and 18–21 days of DOCA-salt treatment, MAs and peritoneal fluid were harvested from euthanized rats. Norepinephrine (NE) release from periartrial sympathetic nerves was measured in vitro using amperometry with microelectrodes. Macrophage infiltration into MAs as well as TNF-\(\alpha\) and p22phox were measured using immunohistochemistry. Peritoneal macrophage activation was measured by flow cytometry. \(O_2\) was measured using dihydroethidium staining. Hypertension developed over 28 days, and apocynin reduced blood pressure on day 10. \(O_2\) and macrophage infiltration were greater in DOCA-salt MAs compared with sham MAs after day 10. Peritoneal macrophage activation occurred after day 10 in DOCA-salt rats. Macrophages expressing TNF-\(\alpha\) and p22phox were localized near sympathetic nerves. Impaired \(\alpha_2\)AR function and increased NE release from sympathetic nerves occurred in MAs from DOCA-salt rats after day 18. Macrophage depletion reduced blood pressure and vascular \(O_2\) while restoring \(\alpha_2\)AR function in DOCA-salt rats. Macrophage infiltration into the vascular adventitia contributes to increased blood pressure in DOCA-salt rats by releasing \(O_2\), which disrupts \(\alpha_2\)AR function, causing enhanced NE release from sympathetic nerves.

salt-sensitive hypertension; immune activation; sympathetic nervous system; \(\alpha_2\)-adrenergic autoreceptors; amperometry

NEW & NOTEWORTHY

We have identified a novel mechanism by which salt-sensitive hypertension disrupts normal function of the sympathetic nervous system. Antioxidants may be helpful in treating some forms of hypertension.

INCREASED SYMPATHETIC nerve activity contributes to high blood pressure in some animal models of hypertension, including the DOCA-salt model (10, 57). Hypertension in the DOCA-salt model is driven by reduced renal mass (removal of one kidney), high circulating mineralocorticoid levels, and high salt intake (57). Elevated salt increases central sympathetic drive to the cardiovascular system (17, 48), but there are also alterations in the local mechanisms that modulate sympathetic neurotransmission in salt-sensitive hypertension, including impaired function of \(\alpha_2\)-adrenergic autoreceptors (\(\alpha_2\)ARs) on sympathetic nerves associated with mesenteric arteries (MAs) (41, 49). \(\alpha_2\)ARs provide negative feedback control over norepinephrine (NE) release, and MAs are part of the splanchnic circulation, a major resistance arterial bed critical for blood pressure regulation (2, 28, 30). MA diameter (and therefore vascular resistance) is controlled partly by NE released from periartrial sympathetic nerves (51) as removal of the celiac ganglion, which contains the cell bodies of sympathetic nerves supplying MAs, reduces blood pressure in DOCA-salt-treated rats (28). These data indicate that NE released from sympathetic nerves supplying MAs contributes to blood pressure regulation. Impaired function of \(\alpha_2\)ARs in the mesenteric circulation will increase NE release, MA constriction, and blood pressure (61). Identification of the mechanism by which \(\alpha_2\)AR function is disrupted will help elucidate the pathophysiology of salt-sensitive hypertension.

The specific factors contributing to DOCA-salt hypertension are time-dependent. Yemane et al. (69) described three phases of DOCA-salt hypertension: 1) an early phase (1–5 days) driven by increased plasma Na\(^+\), volume expansion, and circulating vasopressin levels; 2) a developed phase (2–6 wk) driven by increased sympathetic nervous system activity and elevated vasopressin and endothelin-1 levels; and 3) the malignant phase (>6 wk) driven by increased vasopressin, endothelin-1, and vascular and cardiac remodeling. Therefore, studies of the pathophysiology of DOCA-salt hypertension need to consider phase specific variations.

ROS, especially \(O_2\), contribute to the pathophysiology of hypertension (35, 40, 60). This is particularly true for DOCA-salt hypertension as the superoxide dismutase mimetic tempol lowers blood pressure and sympathetic nerve activity in DOCA-salt hypertensive rats (68). A major source of \(O_2\) is...
NADPH oxidase, and increased NADPH oxidase activity contributes to hypertension (60, 70). Macrophage NADPH oxidase has an intracellular p47phox subunit and membrane-bound Nox-1/gp91phox and p22phox subunits (34). Hypertensive stimuli upregulate the p22phox subunit and enzyme activity (70). DOCA-salt mice deficient in NADPH oxidase have reduced blood pressure compared with wild-type DOCA-salt mice (44). Vascular inflammation also contributes to the initiation and development of hypertension (4, 18, 21, 31). Vascular inflammation is due partly to the infiltration of lymphocytes (19, 20, 21, 25, 43) and macrophages (3, 6, 11, 23, 39), which release O2-. A previous study (40) has shown that NADPH oxidase activation by ANG II contributes to hypertension development by activating the sympathetic nervous system via an action in the brain. However, the effects of macrophage infiltration and oxidative stress on the function of periartrial sympathetic nerves have not been studied. This is important because the activation and proliferation of macrophages in the vascular adventitia contributes to inflammation, vascular remodeling, fibrosis, and endothelial dysfunction in hypertension (8, 12, 15, 17, 47). For example, mice deficient in macrophage colony-stimulating factor have reduced vascular inflammation and damage in ANG II-mediated hypertension (11). Furthermore, an antagonist of chemokine (C-C motif) receptor type 2 prevents vascular macrophage infiltration and reduces blood pressure in DOCA-salt mice (5). It is unclear whether macrophage-derived O2- disrupts α2AR function, causing further increases in blood pressure in the DOCA-salt model. In the present study, we tested the hypothesis that there is a time-dependent infiltration of activated macrophages into the adventitia of MAs of DOCA-salt hypertensive rats causing increased vascular O2-, leading to impaired α2AR function. To test this hypothesis, we capitalized on the innate ability of macrophages to engulf cellular debris and pathogens. We introduced liposome-embedded clodronate (Lipo-Clod) to our animal model, which was engulfed by macrophages. Macrophages were subsequently killed by clodronate via apoptosis (63, 64).

METHODS

DOCA-salt hypertension. All animal use procedures were approved by the Institutional Animal Care and Use Committee of Michigan State University. Male Sprague-Dawley rats (~200 g, Charles River Laboratories, Portage, MI) were acclimated for 5 days before entry into experimental protocols. Rats were anesthetized via isoflurane inhalation; sham-operated (sham) control rats were uninephrectomized and placed on normal tap water. DOCA-salt rats were uninephrectomized, and a DOCA (150 mg)-containing silastic pellet was implanted subcutaneously in the midscapular region. After surgery, all rats received enrofloxacin antibiotic (5 mg/kg im) and carprofen analgesic (5 mg/kg sc). DOCA pellet-implanted rats were placed on high-salt drinking water (1% NaCl + 0.2% KCl) available ad libitum.

Blood pressure measurements and in vivo drug treatments. In some experiments, blood pressure was measured in conscious rats using the

Table 1. Sources of primary and secondary antibodies and working dilutions

<table>
<thead>
<tr>
<th>Primary Antibodies</th>
<th>Source</th>
<th>Host Species</th>
<th>Dilution</th>
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<td>AbD Serotec</td>
<td>Mouse</td>
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<td>NADPH oxidase</td>
<td>Santa Cruz Biotechnology</td>
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<tr>
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<td>Inflammatory cytokine</td>
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<table>
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<tr>
<td>Goat</td>
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![Fig. 1. Time course of DOCA-salt hypertension development and effects of apocynin (A) and liposome-embedded clodronate (Lipo-Clod; B) treatment. A: DOCA-salt hypertension developed with a biphasic time course. Mean arterial pressure (MAP) rose steadily through day 15 (~20-mmHg increase) and reached a plateau near day 16. MAP rose again after day 21. DOCA-salt treatment began on day 2 of the protocol. The NADPH oxidase inhibitor apocynin (2 mM in drinking water) reduced blood pressure between days 15 and 21. B: MAP of sham-operated (sham) normotensive and DOCA-salt hypertensive rats treated with liposome-embedded PBS (Lipo-PBS) or Lipo-Clod. Lipo-Clod reduced blood pressure during days 23–28 compared with DOCA-salt rats treated with Lipo-PBS. Lipo-Clod treatment did not affect MAP in sham groups. Data are means ± SEM and were analyzed by two-way ANOVA and a Bonferroni’s post hoc test; n = 6–8 for all groups. *P < 0.05 vs. DOCA alone (A) or vs. DOCA Lipo-PBS (B).](http://ajpheart.physiology.org/Downloadedfrom)
automated CODA tail-cuff system (Kent Scientific, Torrington, CT). Rats were restrained and allowed to sit quietly to prewarm for 10 min. The tail cuff was inflated five times to 250 mmHg and slowly deflated over a period of 15 s. Blood pressure was obtained during each inflation cycle by a volume recording sensor. Blood pressure values were an average of five readings.

In some experiments, blood pressure was recorded continuously in conscious unrestrained rats used radiotelemetry. In these experiments, rats were uninephrectomized, and the catheter of a radiotelemetry-based pressure transmitter (TA11PA-D70, DSI) was implanted into the femoral artery and the body of the transmitter was placed subcutaneously at the inner thigh. Rats were allowed 4 days to recover with free access to food and water. Rats were housed in an individual cage on top of a radiotelemetry receiver (RPC-1, DSI) that was connected to a data exchange matrix and computerized data-acquisition program (Dataquest ART 3.0, DSI) to monitor arterial pressure remotely. Mean arterial pressure (MAP) was sampled for 10 s every minute for 24 h. Rats were placed on either high-salt drinking water (1% NaCl + 0.2% KCl, n = 13) or high-salt drinking water containing the NADPH oxidase inhibitor apocynin (2 mM, n = 8) available ad libitum. All rats were fed standard rat chow. On day 3, rats were anesthetized with isoflurane, a DOCA (150 mg)-containing pellet was implanted as described above, and blood pressure monitoring resumed. As the DOCA-salt protocol is used routinely in our laboratory and the model reliably produces hypertension, sham treated rats were not included in the apocynin-treatment protocol.

In macrophage depletion experiments, rats were treated with Lipo-Clod. Clodronate was incorporated into liposomes as previously described (60, 61). Control groups received liposome-encapsulated PBS (Lipo-PBS). After sham or DOCA-salt treatment and radiotelemetry implantation (as described above), Lipo-Clod or Lipo-PBS was injected intravenously at a dose of 50 mg/kg clodronate. Thereafter, 25 mg/kg clodronate was administered intraperitoneally every 7 days. Control rats received 1 ml/kg Lipo-PBS.

**Immunohistochemistry.** MAs (diameter: 200–300 μm) from DOCA-salt and sham rats were excised and cleaned of perivascular fat. MAs were fixed in 4% paraformaldehyde overnight at 4°C and then permeabilized with 0.1% Triton X-100 in PBS (0.1 M, pH 7.2) containing 4% goat serum for 1 h at room temperature. Tissues were then incubated in the same buffer solution but containing the appropriately diluted primary antibodies for 2 h at room temperature followed by a 1-h incubation with the appropriate secondary antibodies (Table 1). MAs were washed three times with 0.1 M PBS between primary and secondary antibody incubations and after secondary antibody incubation. MAs were then mounted on glass microscope slides using Prolong antifade mounting medium (Life Technologies, Grand Isle, NY) and a glass coverslip. Images were acquired using a TCS SL laser scanning confocal microscope (Leica Microsystems, Heidelberg, Germany).

When assessing the levels of expression of CD163, TNF-α, and p22phox using fluorescence microscopy, we measured fluorescence intensity associated with each antigen in five random areas (each 0.1 mm²) from a single MA from each of five sham and five DOCA-salt hypertensive rats. Image acquisition parameters were kept constant across all tissues.

**Dihydroethidium staining.** When dihydroethidium (DHE) reacts with O₂, ethidium bromide is formed, and it intercalates into DNA, yielding a red fluorescent signal when excited at 488 nm. MAs were removed from euthanized rats in chilled Krebs-Ringers-HEPES (KRH) solution of the following composition (in mM): 130 NaCl, 1.3 KCl, 2.2 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, and 1.0 HEPES, and 0.09 glucose (pH 7.4). MAs were incubated in DHE (2 μM) solution for 1 h at 37°C. After DHE incubation, MAs were washed with Krebs-Ringer-HEPES solution and mounted for microscopy. Confocal fluorescence images were obtained with 488-nm excitation and 560-nm emission wavelengths.

Amperometric measurement of NE release from periarterial sympathetic nerves in vitro. Tertiary MAs were isolated, cleaned of fat and connective tissue, carefully pinned to the base of a silastic elastomer-lined chamber recording chamber (4-ml volume), and then perfused with warmed (36°C) and oxygenated (95% O₂-5% CO₂) Krebs buffer [composed of (in mM) 117 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgCl₂, 25 NaHCO₃, 1.2 NaH₂PO₄, and 11 dextrose] at a flow rate of 4 ml/min. Tissues were allowed to equilibrate for 1 h before experiments begun.

Continuous amperometry with a carbon fiber microelectrode was used to measure dynamic changes in NE concentration at the blood vessel surface. The carbon fiber microelectrodes were prepared as

![Fig. 2. Time-dependent macrophage infiltration into the adventitia of mesenteric arteries (MAs) but not skeletal muscle arteries of DOCA-salt hypertensive rats. A and B: whole mount immunohistochemical labeling of CD163-positive macrophages in the adventitia of MAs from DOCA-salt (A) and sham control rats (day 21; B). C: normalized number of macrophages per region. Macrophage numbers were four to five times higher in arteries from DOCA-salt rats compared with those from sham control rats beginning on days 10–13. Data are means ± SE and were analyzed by one-way ANOVA and a Bonferroni’s post hoc test; n = 5. *P < 0.05 vs. control and days 3–5. D and E: whole mount immunohistochemical labeling of MA (D) and skeletal muscle (E) peri-vascular sympathetic nerves labeled by neuropeptide Y (NPY) immunoreactivity along with macrophage labeling with anti-CD163. Macrophages were found in close proximity to periarterial sympathetic nerves in MAs but not skeletal muscle arteries from DOCA-salt rats.](http://ajpheart.physiology.org/)

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cells were collected via lavage using 1/1000 perfused for 40 min before the recording of NE oxidation currents. This applied potential was used to detect NE release elicited by short trains of electrical stimulation (10 Hz, 5 s, 80 V) using a bipolar focal stimulation electrode positioned on the artery surface (49, 50). A control oxidation current in response to nerve stimulation was obtained in each tissue, and either UK-14304 (α2AR receptor agonist) or idazoxan (α2AR receptor antagonist) were then applied by addition to the Krebs solution. The drug solution was perfused for 40 min before the recording of NE oxidation currents.

Isolation of peritoneal macrophages. Rats were anesthetized with pentobarbital (50 mg/kg ip), and the abdomen was exposed. Peritoneal cells were collected via lavage using 1× Ca2+-free HBSS (Invitrogen-Life Technologies, Grand Island, NY). The cell suspension was centrifuged at 300 g (4°C). Erythrocytes were removed using ACK lysing buffer (Invitrogen-Life Technologies). The total number of viable leukocytes was determined using trypan blue exclusion and an automatic cell counter (Bio-Rad, Hercules, CA).

Flow cytometry. Harvested peritoneal cell suspensions (1 × 10⁶ cells/sample) were treated with Fc Block (BD Biosciences, San Jose, CA), and macrophages were identified by incubation with anti-CD11b Alexa Fluor- and anti-CD163 RPE-conjugated antibodies (AbD Se-rotec, Raleigh, NC). Cells were then fixed with Cytofix (BD Biosciences). Data were acquired using a FACSCanto II cell analyzer (BD Biosciences) and analyzed using FlowJo software (version 8.8.6, TreeStar, Ashland, OR).

Drugs. All drugs were obtained from Sigma-Aldrich Chemical (St. Louis, MO), and concentrated stock solutions were prepared using deionized water. Working dilutions were made in Krebs buffer at the time of the experiment.

Statistics. Data are presented as means ± SE; n is the number of animals from which the data were obtained. Data were analyzed with Graphpad Prism (version 5.0) using a Student’s t-test or one-way or two-way ANOVAs with Bonferroni’s post hoc test as appropriate. Nonparametric data were analyzed with a Kruskal-Wallis test. For multivariate analysis of flow cytometry data, FlowJo 8.8.6 was used for probability binning comparisons. Differences were considered significant at P < 0.05.

RESULTS

Time course of DOCA-salt hypertension: effects of apocynin and Lipo-Clod. After 5 days of DOCA-salt treatment, MAP increased slightly by days 10–13; it had increased by >20 mmHg and reached a plateau by day 13 in untreated DOCA-salt rats. By day 18, MAP began to increase again and continued to increase until day 25, when the experiment was completed (Fig. 1, A and B). MAP rose with a similar time course in apocynin-treated DOCA-salt rats, but MAP increases were smaller than in untreated DOCA-salt rats. This difference was most prominent between days 17 and 21, where MAP plateaued in untreated DOCA-salt rats (Fig. 1A). Similar increases in MAP occurred in DOCA-salt rats treated with Lipo-PBS, whereas MAP was significantly lower during days 23–28 in DOCA-salt rats treated with Lipo-Clod (Fig. 1B). Lipo-Clod treatment did not affect MAP of sham rats (Fig. 1B).

Time-dependent macrophage infiltration into the adventitia of MAs. As MAP increased in two phases (an early phase insensitive to apocynin and Lipo-Clod and a later phase sensitive to apocynin and Lipo-Clod), we tested the possibility that macrophage infiltration into MAs might follow a similar biphasic time course. CD163 is a hemoglobin-haptoglobin scavenger receptor expressed by activated macrophages (59). The number of CD163-positive macrophages in the adventitia of DOCA-salt MAs was higher than in arteries from sham control rats on day 28 (Fig. 2, A and B). Macrophage infiltration was first detected between days 10 and 13 and remained high through day 28 (Fig. 2C). Some macrophages were in close apposition to perivascular sympathetic nerves identified by neuromereptide Y immunoreactivity (Fig. 2D). This response was specific for MAs as we did not detect adventitial macrophages in abdominal skeletal muscle arteries (Fig. 2E).

Time-dependent increase in O2− levels in the adventitia of MAs. The relative level of O2− (measured using DHE fluorescence) in the adventitia was significantly higher in MAs from DOCA-salt rats compared with sham rats on day 10 (Fig. 3, A and B). Semiquantification of O2− revealed that DHE-derived fluorescence intensity increased in DOCA-salt rats relative to sham control rats starting on day 10 and remained high through day 21 (Fig. 3C).

![Comparison of DHE fluorescence intensity between DOCA-salt and sham rats](image-url)

**Fig. 3.** Detection of O2− in the adventitia of MAs. A and B: photomicrographs showing dihydroethidium (DHE; arrows) fluorescence in MAs from DOCA-salt (A) and sham control (B) rats. C: normalized mean DHE fluorescence intensity showing twofold higher DHE labeling in DOCA-salt compared with sham control MAs beginning after day 10 of DOCA-salt hypertension. Data are means ± SE and were analyzed by one-way ANOVA and a Bonferroni’s post hoc test; n = 5. *P < 0.05 vs. control and days 3–5. AJP-Heart Circ Physiol • doi:10.1152/ajpheart.00283.2015 • www.ajpheart.org
Adventitial macrophages in DOCA-salt MAs express high levels of TNF-α. On day 21, adventitial macrophages in DOCA-salt MAs expressed higher levels of TNF-α compared with those found in MAs from sham rats (Fig. 4, A–D). TNF-α expression in macrophages was evaluated semiquantitatively, and we found that adventitial macrophages from DOCA-salt MAs expressed higher levels of TNF-α than those in MAs from sham rats (Fig. 4E).

**Adventitial macrophages in DOCA-salt MAs express high levels of TNF-α.**

**Increased expression of p22^phox in MAs of DOCA-salt rats.** CD163-positive macrophages in the adventitia of MAs from DOCA-salt rats expressed higher levels of the NADPH oxidase subunit p22^phox (Fig. 5, A and B). There was relatively little p22^phox expression in adventitial macrophages from sham rats (Fig. 5, C and D). Semiquantitative analysis of fluorescence intensity showed higher expres-
sion of p22^phox in macrophages in MAs from DOCA-salt rats compared with those from sham rats (Fig. 5E).

**Time-dependent activation of peritoneal macrophages.** CD11b is an integrin protein expressed by activated macrophages (25). Using flow cytometry, we detected three populations of peritoneal macrophages: CD11b low, CD11b intermediate, and CD11b high (Fig. 6A). On days 3–5, there were no differences in the proportions of CD11b low, intermediate, or high macrophages between samples taken from sham versus DOCA-salt rats (Fig. 6B). By day 10 and continuing through day 21, sham rats had a significantly lower percentage of CD11b high macrophages and a significantly higher percentage of CD11b intermediate macrophages (Fig. 6B). CD11b high macrophages from DOCA-salt rats also expressed a higher level of CD163 compared with those from sham control rats (Fig. 6C).

**Lipo-Clod depleted macrophages.** Using DHE fluorescence, we found that relative levels of O$_2^-$ in MAs of DOCA-salt rats treated with Lipo-Clod were lower than in DOCA-salt rats treated with Lipo-PBS (Fig. 7, A and B). Semiquantification of O$_2^-$ levels revealed that the levels of O$_2^-$ in MAs of DOCA-salt rats treated with Lipo-Clod were significantly lower than in DOCA-salt rats treated with Lipo-PBS (Fig. 7C). Lipo-Clod also reduced the baseline levels of O$_2^-$ in control rats (Fig. 7C). The number of CD163-positive macrophages in the adventitia of MAs from DOCA-salt rats treated with Lipo-Clod was significantly lower than DOCA-salt rats treated with Lipo-PBS (Fig. 7, D–F).

Using flow cytometry, we found that the percentage of peritoneal macrophages detected in DOCA-salt rats treated with Lipo-Clod was significantly lower than in DOCA-salt rats treated with Lipo-PBS (Fig. 8, A and B). With further gating on the peritoneal macrophage population, we found that Lipo-Clod reduced the number of CD11b-positive/CD163-positive peritoneal macrophages in DOCA-salt rats (Fig. 8, C and D).

**Lipo-Clod restores the function of prejunctional α$_2$ARs in MAs from DOCA-salt hypertensive rats.** We used idazoxan (α$_2$AR antagonist)-induced facilitation of NE release as a measure of α$_2$AR function. Blockade of the prejunctional α$_2$AR increases the amplitude of NE oxidation current (11). In the early stages of hypertension development (days 3–5 and 10–13), idazoxan increased NE oxidation current equally well in MAs from sham and DOCA-salt rats (Fig. 9, A and B). However, by days 18–21, when DOCA-salt hypertension had established, idazoxan-induced enhancement of NE oxidation current was reduced compared with that in sham control MAs (Fig. 9C). We tested if macrophage depletion in DOCA-salt rats would restore the function of prejunctional α$_2$ARs in established DOCA-salt hypertension (>day 21) by testing the effects of UK-14304 and idazoxan in MAs from Lipo-PBS- and Lipo-Clod-treated rats. At low concentrations of both drugs (0.01 and 0.1 μM), there were no differences in oxidation currents between tissues obtained from Lipo-PBS- and Lipo-Clod-treated DOCA-salt rats. However, UK-14304 (1 μM) produced a larger inhibition in DOCA-salt rats treated with Lipo-Clod (Fig. 9D). Similarly, idazoxan (1

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**Fig. 6. Flow cytometry analysis of the time course of peritoneal macrophage activation in DOCA-salt hypertensive rats.** A: dot plot of DOCA-salt hypertensive peritoneal macrophages showing three populations of macrophages: CD11b low, intermediate, and high on day 21 of DOCA-salt hypertension. B: the percentage of CD11b high macrophages was significantly higher in DOCA-salt MAs beginning on days 10–13. The percentage of CD11b intermediate macrophages was lower in DOCA-salt compared with sham MAs during the same time period. There were no changes in the CD11b low macrophage population. Data are means ± SE and were analyzed using a Kruskal-Wallis test; n = 5. #P < 0.05 vs. sham rats. C: CD163 fluorescence intensity histogram of the CD11b high macrophage population showing the higher expression of CD163 in DOCA-salt compared with sham control MAs.
μM) increased NE oxidation currents in MAs from DOCA-salt rats treated with Lipo-PBS, but idazoxan produced a much larger increase in MAs from Lipo-Clod-treated DOCA-salt rats (Fig. 9E). These pharmacological data indicate that macrophage depletion minimizes α2AR dysfunction in DOCA-salt rats.

DISCUSSION

The results from this study indicate that 1) DOCA-salt hypertension develops with a biphasic time course; 2) the initial elevation of blood pressure recruits macrophages, which are a source of O2− in the adventitia of MAs; 3) sustained increases in vascular O2− impair α2AR function, leading to increased NE release from periarterial sympathetic nerves; and 4) macrophage depletion attenuates the development of the later phase of DOCA-salt hypertension and restores α2AR function by reducing vascular O2−. We propose that the modest increase in blood pressure during the early stage of DOCA-salt hypertension causes vascular damage and neoantigen formation. These changes lead to macrophage infiltration into the vascular adventitia and initiation of an inflammatory response that results in a dysregulation of NE release from periarterial sympathetic nerves and further increases in blood pressure. There is substantial evidence pointing to increased sympathetic nerve activity in DOCA-salt hypertension. For example, there is increased lumbar sympathetic nerve activity (48), increased circulating plasma catecholamines (5), and greater decreases in blood pressure caused by ganglion blockade (15). In addition, removal of the celiac ganglion (which provides sympathetic innervation of the splanchnic circulation) lowers blood pressure in DOCA-salt rats (28). In addition to the increase in sympathetic nerve activity, local changes at the level of the vascular neuroeffector junction may also contribute to increased sympathetically mediated vasoconstriction in DOCA-salt hypertension. These changes include impaired function of the presynaptic α2AR, which regulates neurotransmitter release from sympathetic nerve fibers (12, 41, 49).

Immune activation in hypertension. Hypertension is a multiorgan disease that involves activation of the immune system and vascular inflammation (13, 21, 37, 53, 58, 65). Mice lacking T and B cells have blunted hypertension and do not develop vascular remodeling during Ang II infusion or DOCA-salt treatment, whereas adoptive transfer of T cells, but not B cells, restores these changes (19). In addition to lymphocytes, macrophage infiltration into blood vessels as well as the brain, heart, and kidneys also contribute to the pathophysiology of hypertension and its consequences (23). Macrophage accumulation in the vascular wall during hypertension contributes to oxidative stress, endothelial dysfunction, and inflammation (14, 16, 44). Although hypertension involves inflammation and periarterial sympathetic nerve dysfunction, the interaction among inflammatory mechanisms and sympathetic nerve function (particularly at the vascular neuroeffector junction) and hypertension is poorly understood.

Fig. 7. Lipo-Clod treatment reduces adventitial O2− and macrophage infiltration in MAs from DOCA-salt rats. A: photomicrograph showing DHE fluorescence in a MA from a DOCA-salt rat treated with Lipo-PBS. B: photomicrograph showing DHE fluorescence in a MA from a DOCA-salt rat treated with Lipo-Clod. C: semiquantitative measurement of DHE fluorescence intensity in MAs of DOCA-salt rats treated with Lipo-PBS or Lipo-Clod. Data are means ± SE; n = 5 rats/group. *P < 0.05. D: whole mount immunohistochemical labeling of CD163-positive macrophages in the adventitia of DOCA-salt rats treated with Lipo-PBS. E: there were fewer CD163-positive macrophages in the adventitial of MAs from DOCA-salt rats treated with Lipo-Clod. F: the mean number of adventitial macrophages in MAs from Lipo-Clod-treated DOCA-salt rats was lower than that in MAs from Lipo-PBS-treated DOCA-salt rats. Data are means ± SEM and were analyzed by a Mann-Whitney test; n = 5. *P < 0.05.
One component of vascular inflammation in hypertension is the accumulation of high levels of ROS, including O$_2^-$ (8, 68). Adventitial fibroblasts are one source of O$_2^-$ (8), but activated macrophages express high levels of NADPH oxidase, an enzyme that produces O$_2^-$, suggesting that adventitial macrophages are a source of vascular O$_2^-$ (5). Macrophages are phagocytic cells that release O$_2^-$ in response to a variety of stimuli (3, 11). We show here that perivascular macrophages contribute to the increased levels of O$_2^-$ in the adventitia of MAs, as macrophage depletion using Lipo-Clod reduced vascular O$_2^-$ in DOCA-salt rats.

In the present study, we examined two macrophage markers: CD163 and CD11b. Macrophages in the adventitial layer of MAs from DOCA-salt rats with established hypertension expressed high levels of CD163, a hemoglobin/haptoglobin scavenger receptor (52). CD163 expression is typically associated with alternative activation of macrophages and an anti-inflammatory response (47). However, CD163 activation is also linked to an upregulation of inflammatory cytokines, including TNF-α (52). CD11b is an integrin $\alpha_M$-subunit that forms the heterodimeric integrin $\alpha_M\beta_2$ molecule (59). $\alpha_M\beta_2$ is a cell surface receptor expressed by many leukocytes, including macrophages. $\alpha_M\beta_2$ is linked to the activation of NF-κB, which upregulates proinflammatory cytokines (38, 42). While we did not measure the coexpression of CD11b and TNF-α, we did find that CD163-positive macrophages in the adventitia of MAs of DOCA-salt hypertensive rats expressed elevated levels of TNF-α. High levels of TNF-α would enhance the inflammatory response in the blood vessel wall, leading to vascular injury. Vascular macrophage infiltration may be somewhat specific for the splanchnic circulation as we found macrophages in MAs but not in skeletal muscle arteries. Macrophage infiltration into the wall of mesenteric resistance arteries is a common finding in DOCA-salt and ANG II-induced systemic hypertension (11, 31, 36). Macrophage infiltration into the wall of the pulmonary artery also occurs in animal models of pulmonary hypertension (45). Others (5, 31, 54) have found that macrophages infiltrate into the wall of the thoracic aorta in DOCA-salt, ANG II-infused, and stroke-prone spontaneously hypertensive rats, so the macrophage response may not be specific for MAs; however, macrophage infiltration into resistance arteries, other than MAs, has not been reported to our knowledge. In addition, other investigators used different macrophage/monocyte marker (F4/80, CD63, and MOMA-2) antibodies to identify aortic macrophages. We used CD163 as a macrophage marker, which might identify a different macrophage subpopulation (7). In addition, macrophages infiltrating into the adventitia of MAs may come from the large population of peritoneal macrophages. Our data showed that, like macrophages in the MA adventitia, peritoneal macrophages expressed CD163 and hypertension upregulated the expression of CD163. Furthermore, peritoneal macrophages express the $\alpha_M\beta_2$-integrin cell adhesion molecule, and integrin expression is upregulated in hypertensive rats. Most interestingly, the time
at which the peritoneal macrophage intergrins are upregulated in the hypertensive animals was similar to that of macrophage infiltration into the mesenteric adventitia. It is tempting to speculate that these integrin adhesion molecules contribute to the recruitment and attachment of macrophages to sites of inflammation (32) and that increased \( \alpha_\delta \beta_2 \)-integrin expression enhanced the attachment of peritoneal macrophages to the adventitia of MAs. The somewhat selective macrophage infiltration into the adventitia of MAs with subsequent disruption of \( \alpha_\delta \)AR function may be sufficient to alter systemic blood pressure. The sympathetic nerve supply of the mesenteric circulation is critical for the regulation of systemic blood pressure, as discussed in more detail below.

**\( \alpha_\delta \)AR impairment is caused by \( O_2^- \).** Many animal and clinical studies have shown an increase in \( O_2^- \) production during hypertension (9, 34, 35, 44, 60, 68). Vascular \( O_2^- \) is produced primarily by NADPH oxidase (39). Under physiological conditions, \( O_2^- \) is maintained at a low level by the enzymes superoxide dismutase, catalase, and glutathione peroxidase. However, in hypertension, there is increased \( O_2^- \), which activates signaling pathways involved in smooth muscle cell growth and proliferation and inflammation (39). Uncontrolled \( O_2^- \) causes cellular damage and eventually apoptosis, because \( O_2^- \) damages proteins, lipids, and DNA (20). \( O_2^- \) is a reactive and short-lived molecule. However, we showed that some macrophages are in close apposition with sympathetic nerves supplying MAs, which could allow \( O_2^- \) to disrupt \( \alpha_\delta \)AR function. \( \alpha_\delta \)Rs couple to inhibition of NE release by activation of the \( \mathrm{G}_6 \) subtype of \( G \) protein with subsequent inhibition of activation of the Ca\(^{2+} \) channels needed for neurotransmitter release (27). Inhibition of Ca\(^{2+} \) channel function is caused by binding of the \( \beta_\gamma \) subunit of the \( G \) protein directly to the channel (55). ROS can directly disrupt \( G \) protein-coupled receptor signaling. For example, the oxidant molecule \( \mathrm{H}_2\mathrm{O}_2 \) causes \( G \) protein uncoupling from the D1-dopamine receptor in renal proximal tubule cells maintained in cell culture (1). Thus, a possible mechanism of \( O_2^- \) disruption of \( \alpha_\delta \)R function is through receptor-\( G \) protein-effector uncoupling. This proposed general mechanism is supported by our recent data showing that the function of prejunctional \( A_1 \) adenosine receptors on perirterial sympathetic nerves is also impaired in DOCA-salt hypertensive rats (56).

We have previously shown that apocynin [a drug that can block NADPH oxidase (51)]-treated DOCA-salt rats with established hypertension (>day 21) reduced MA and sympathetic ganglion \( O_2^- \) levels and improved \( \alpha_\delta \)AR function (12). In the present study, we show that chronic apocynin treatment reduced blood pressure in the later phase (>day 21) of DOCA-salt hypertension. This suggests that the later phases of DOCA-salt hypertension may be exacerbated by oxidative damage of \( \alpha_\delta \)AR and/or its signaling pathway in perivascular sympathetic nerves, resulting in increased NE release.
Our study supports the hypothesis that macrophage-derived O$_2$ disrupts $\alpha_2$AR function because depletion of macrophages restores its function. Inflammation through recruitment, activation, and proliferation of macrophages in the vascular adventitia is part of the pathophysiology of hypertension (39), as mice deficient in macrophage colony-stimulating factor exhibit reduced vascular inflammation and are protected against the damage caused by DOCA-salt hypertension (11). Our data suggest that one putative protective mechanism is the prevention of impaired $\alpha_2$AR function. Impaired function of $\alpha_2$-ARs is not specific for DOCA-salt hypertension or MAs. There is an increase in circulating catecholamines in DOCA-salt rats due to reduced $\alpha_2$AR function, and this would come from sympathetic nerves supplying multiple organ systems as well as the adrenal gland (47). $\alpha_2$AR function is also reduced in sympathetic nerves supplying the caudal artery and portal vein of spontaneously hypertensive rats (24). Although impaired $\alpha_2$AR function is not specific for the mesenteric circulation, removal of the celiac ganglion attenuates hypertension in DOCA-salt rats (24). The celiac ganglion provides much of the sympathetic nerve supply of the mesenteric circulation, which plays a key role in the regulation of systemic blood pressure. Impaired $\alpha_2$AR function is also only one component of the overall pathophysiology of DOCA-salt hypertension and probably contributes to the developed phase (>2 wk) of DOCA-salt hypertension (69), when increased sympathetic nervous system activity is a major contributor to blood pressure elevation.

Conclusions. The present study suggests that inflammatory mechanisms play an important role in blood pressure regulation through their effect on sympathetic nerve function. In the future, it is possible that vascular macrophage markers could be used in patients with hypertension to identify those at risk for long-term changes in sympathetic nerve function or as a target for antihypertensive treatment.

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No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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