ORIGINAL RESEARCH ARTICLE

NLRP3 Inflammasome Mediates Aldosterone-Induced Vascular Damage

BACKGROUND: Inflammation is a key feature of aldosterone-induced vascular damage and dysfunction, but molecular mechanisms by which aldosterone triggers inflammation remain unclear. The NLRP3 inflammasome is a pivotal immune sensor that recognizes endogenous danger signals triggering sterile inflammation.

METHODS: We analyzed vascular function and inflammatory profile of wild-type (WT), NLRP3 knockout (*NLRP3*$^{-/-}$), caspase-1 knockout (*Casp-1*$^{-/-}$), and interleukin-1 receptor knockout (*IL-1R*$^{-/-}$) mice treated with vehicle or aldosterone (600 µg·kg^{-1}·d^{-1} for 14 days through osmotic mini-pump) while receiving 1% saline to drink.

RESULTS: Here, we show that NLRP3 inflammasome plays a central role in aldosterone-induced vascular dysfunction. Long-term infusion of aldosterone in mice resulted in elevation of plasma interleukin-1β levels and vascular abnormalities. Mice lacking the IL-1R or the inflammasome components NLRP3 and caspase-1 were protected from aldosterone-induced vascular damage. In vitro, aldosterone stimulated NLRP3-dependent interleukin-1β secretion by bone marrow–derived macrophages by activating nuclear factor-κB signaling and reactive oxygen species generation. Moreover, chimeric mice reconstituted with NLRP3-deficient hematopoietic cells showed that NLRP3 in immune cells mediates aldosterone-induced vascular damage. In addition, aldosterone increased the expression of NLRP3, active caspase-1, and mature interleukin-1β in human peripheral blood mononuclear cells. Hypertensive patients with hyperaldosteronism or normal levels of aldosterone exhibited increased activity of NLRP3 inflammasome, suggesting that the effect of hyperaldosteronism on the inflammasome may be mediated through high blood pressure.

CONCLUSIONS: Together, these data demonstrate that NLRP3 inflammasome, through activation of IL-1R, is critically involved in the deleterious vascular effects of aldosterone, placing NLRP3 as a potential target for therapeutic interventions in conditions with high aldosterone levels.

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Key Words: aldosterone ■ immunology ■ inflammasomes ■ inflammation

Clinical Perspective

What Is New?

- We identify a new mechanism involving the NLRP3 inflammasome whereby aldosterone, through interleukin-1 receptor, influences vascular function.
- Cells from the bone marrow are involved in the NLRP3 inflammasome axis underlying aldosterone-induced vascular dysfunction.
- Aldosterone mediates the first and second signals necessary for inflammasome activation through nuclear factor-κB stimulation and reactive oxygen species generation, respectively.
- NLRP3 inflammasome activation is increased in immune cells from hypertensive patients, including those with hyperaldosteronsim.
- Our data indicate that the immune system, through the NLRP3 inflammasome, has a central role in aldosterone- and hypertension-associated vascular abnormalities.

What Are the Clinical Implications?

- Aldosterone contributes to hypertension, atherosclerosis, and cardiometabolic abnormalities by inducing endothelial dysfunction, vascular inflammation, fibrosis, and remodeling.
- NLRP3 inflammasome activation in leukocytes is key to aldosterone-induced vascular dysfunction and is potentially involved in the cardiovascular complications in patients with hyperaldosteronism and hypertension.
- Our data identify the NLRP3 inflammasome as a putative novel therapeutic target in pathologies associated with vascular dysfunction, especially in those conditions in which aldosterone plays a role.

Aldosterone is a steroid hormone synthesized mainly by the adrenal glomerulosa in response to various stimuli, including angiotensin II and adrenocorticotropic hormone. Classically, aldosterone, through activation of mineralocorticoid receptors (MRs), upregulates apical epithelial sodium channels and increases sodium reabsorption at the level of the distal nephron.[1,2] However, excessive aldosterone production results in vascular damage, dysfunction, and remodeling.[1–6]

Inflammation is a key feature in the pathogenesis of many cardiovascular disorders, including atherosclerosis, acute coronary syndromes, stroke, and arterial hypertension.[4–8] Aldosterone participates in the physiopathological processes, including vascular injury, and in cardiovascular and metabolic diseases, at least in part by causing endothelial dysfunction and vasoconstriction, remodeling, oxidative stress, and inflammation.[1,4,5,7,9,10] However, molecular mechanisms by which aldosterone triggers inflammation remain unclear.

Members of the Nod-like receptor (NLR) family have emerged as important damage innate immune sensors.[11,12] NLRP3, one of the most well-characterized members of the NLR family, regulates the assembly of the inflammasome, a multimeric complex that contains active caspase-1, which processes and increases interleukin (IL)-1β and IL-18 secretion. IL-1β is transcriptionally regulated when damage components are sensed by pattern recognition receptors.[11–14] NLRP3 inflammasome responds to a variety of signals that are indicative of damage to the host, including environmental irritants, endogenous danger signals, pathogens, and mitochondria-derived reactive oxygen species (ROS).[11,15,16] In addition to infection,[11,12] NLRP3 inflammasome contributes to target organ damage in type 2 diabetes mellitus[17] and cardiovascular and renal diseases.[18–22]

Here, we addressed the molecular mechanisms of aldosterone-induced vascular dysfunction. We found that the NLRP3 inflammasome is engaged in the vascular complications provoked by aldosterone. It is worth noting that NLRP3 inflammasome in immune cells triggers vascular dysfunction, inflammation, and remodeling through activation of the IL-1 receptor. Of importance, increased NLRP3 inflammasome activation occurs in immune cells of patients with hyperaldosteronism and hypertension. NLRP3 inflammasome activation may represent a major trigger of vascular disease in conditions associated with aldosterone excess or high levels of blood pressure (BP).

METHODS

Animals

Eight- to 10-week-old male C57BL6/J mice (wild-type [WT]) and knockout mice for IL-1 receptor (IL-$1R^{-/-}$), caspase-1 ($Casp$-$1^{-/-}$), and NLRP3 ($NLRP3^{-/-}$) were infused with vehicle or aldosterone (600 μg/kg per day) for 14 days with ALZET osmotic minipumps (Durect, Cupertino, CA) while receiving 1% saline in the drinking water.[4] Mice were kept in the animal facility of the Department of Pharmacology, Ribeirao Preto Medical School, University of Sao Paulo, under controlled temperature (22°C–24°C) and humidity and 12-hour light/dark cycles, were fed a standard diet, and were given water ad libitum. All experimental procedures were approved by the Ethics Committee on Animal Research of the Ribeirao Preto Medical School, University of Sao Paulo (protocol 012/2013-1) and are in accordance with the Guidelines of the Brazilian College of Animal Experimentation.

Bone Marrow Transplantation

Bone marrow transplantation was performed according to previous studies.[23,24] Recipient WT mice at 10 weeks of age were exposed to 9-Gy total body irradiation. After 1 day, whole bone marrow cells collected from the femur of WT or $NLRP3^{-/-}$ donor mice were injected intravenously (through the tail vein) into the recipient mice. Three groups of bone marrow chimera were produced as follows: bone marrow from WT to WT recipient

mice treated with vehicle (WT→WT), WT to WT mice infused with aldosterone (WT→WT+aldosterone), and bone marrow from *NLRP3*−/− to WT recipient mice infused with aldosterone (*NLRP3*−/−→WT+aldosterone). Aldosterone infusion was performed 6 to 8 weeks after bone marrow transplantation. The effectiveness of the irradiation procedure was analyzed by survival in mice that were not submitted to the bone marrow transplantation or treated with antibiotics (Figure I in the online-only Data Supplement).

BP Measurement

After 11 days of aldosterone infusion, mice were anesthetized with a mixture of isoflurane 2% and O_2, and the surgical procedures were carried out in aseptic conditions. The animals were placed in the supine position, and a cervical incision was made. Polyethylene catheters (Clay-Adams, Northridge, CA) were implanted in the left carotid artery for BP measurement. Catheters were filled with heparinized saline (100 IU/mL), capped, and tunneled subcutaneously to the back of the neck. All animals received penicillin and streptomycin (200 IU/g and 80 μg/g, respectively) and were allowed to recover for 2 days. BP measurements were carried out in conscious, unrestrained mice in their own cages 3 days after surgery. On the day of the experiment, the arterial catheter was connected to a pressure transducer (model DPT-100 Deltran, Utah Medical Products, Midvale, UT), and the venous catheter was connected to a polyethylene (Clay-Adams) extension. Mice were allowed to rest for 10 minutes (acclimatization period), and BP was continuously recorded for 20 minutes at 4 kHz (data record) with a computer equipped with an analog-to-digital interface (PowerLab/4SP, ADInstruments, Colorado Springs, CO).

IL-1β Measurement

IL-1β concentration in cell culture supernatant, plasma, and serum samples was assessed with an IL-1β ELISA kit (BD Biosciences).

Vascular Structure Analysis

Mesenteric resistance arteries were cannulated in a pressure myograph (Living System Instrumentation) and incubated in calcium-free Krebs solution containing 2 mmol/L EGTA for analysis of passive structure, as previously described.[25] Cross-sectional area (CSA) and wall:lumen ratio were analyzed in pressurized mesenteric resistance arteries (80 mmHg).

Vascular Reactivity Studies

Rings from second-order mesenteric resistance arteries were mounted (Danysh MyoTechnology) for isometric tension recordings with PowerLab software (AD Instruments). Rings were placed under a resting tension of 2 mN in tissue baths containing warmed (37°C), aerated (95% O_2, 5% CO_2) standard physiological saline solution (in mmol/L: 130 NaCl, 4.7 KCl, 1.17 $MgSO_4$, 0.03 EDTA, 1.6 $CaCl_2$, 14.9 $NaHCO_3$, 1.18 KH_2PO_4, and 5.5 glucose). Administration of 10 μmol/L phenylephrine was used to test arterial viability, and the presence of intact endothelium was verified by acetylcholine (1 μmol/L)–induced relaxation in vessels contracted with phenylephrine 1 μmol/L. Concentration-response curves for phenylephrine, acetylcholine, and sodium nitroprusside were performed. In a set of experiments, mesenteric resistance arteries from WT mice were preincubated for 2 hours with 2.5 ng/mL IL-1β.

Real-Time Polymerase Chain Reaction

Total mRNA was extracted from mice mesentery beds (Trizol Plus, Invitrogen, Carlsbad, CA), purified with RNeasy spin columns, and eluted from the column in 30 μL diethylpyrocarbonate-treated water, and the concentration was established with a NanoDrop 1000 (NanoDrop Technologies, Wilmington, DE). cDNA was generated by real-time polymerase chain reaction with SuperScript III (Invitrogen) from 400 ng total RNA. The gene targets used were vascular cell adhesion protein-1 (VCAM-1; Mm01320970_m1), intercellular adhesion molecule-1 (ICAM-1; Mm00516023_m1), and GAPDH (4352339E) as a housekeeping control. The relative mRNA expressions (target gene/GAPDH, housekeeping gene) were calculated by ΔΔCt method, and the results were reported as arbitrary units expressed relative to control mice.

Vascular Inflammatory Response: Macrophage Adhesion

Adapted from our previous work,[26] the adhesion assay was performed in a 24-well plate coated with 4% agarose. Aortic rings (5 mm) were cleaned and opened longitudinally. The vascular segments were positioned with the endothelium side up in the solid agarose surface (1 segment per well), fixed with sharp-pointed pins, and kept in F12 medium at 37°C. Bone marrow–derived macrophage (BMDM) adherent cells were cultured in RPMI supplemented with 30% L929 cell-conditioned medium and 20% FBS. For cell fluorescent labeling, macrophages (10^5 cells/mL) were suspended in 1% BSA-supplemented PBS containing 1 μmol/l F4/80 (Molecular Probes) and incubated for 20 minutes at 37°C. Labeled macrophages were washed twice with PBS and suspended in Krebs solution. Fluorescence-labeled cells (10^5 cells per well) were then added to the wells containing the vascular segments and were allowed to adhere for 30 minutes at 37°C in 5% CO_2. Nonadherent cells were removed by gentle washing with prewarmed Krebs solution. The number of adherent cells was counted with fluorescence microscopy. Four fields were evaluated per segment.

Isolation of Mouse Peritoneal Cavity Cells

Male WT mice were anesthetized with a mixture of isoflurane 2% and O_2, and the peritoneal cavity was washed with 5 mL PBS containing EDTA (0.01g/mL). The 5-mL lavage was centrifuged at 12 000*g* for 10 minutes to obtain the cell pellets, which contained only leukocytes.[27] These pellets were washed 3 times and frozen at −80°C for future analyses.

BMDM Isolation and Stimulation

BMDMs from WT and *NLRP3*−/− mice were cultured in RPMI 1640 supplemented with 10% FBS, 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.05 mol/L 2-mercaptoethanol, and macrophage colony-stimulating factor (20 ng/mL) for 6 days.[28]

After 7 days, BMDMs were incubated with aldosterone (0.1 μmol/L) for 4 to 8 hours and with 500 ng/mL ultrapure

lipopolysaccharides (InvivoGen tlrl-peklps) for 6 hours and then stimulated with 20 µmol/L nigericin for 40 minutes (Sigma-Aldrich). The combination of lipopolysaccharides plus nigericin is considered a classic stimulus for NLRP3 inflammasome activation. To analyze whether aldosterone activates the NLRP3 inflammasome by itself, cells were stimulated with lipopoly-saccharides (6 hours)+aldosterone (2 hours) and aldosterone (6 hours)+nigericin (40 minutes). Gene expression of NLRP3 (Mm00840904_m1) and IL-1β (Mm00434228_m1) was determined by real-time polymerase chain reaction. To determine the contribution of nuclear factor-κB (NF-κB) to aldosterone-induced NLRP3 and IL-1β gene expression, aldosterone incubation (4 and 6 hours) was also performed in the presence of an NF-κB inhibitor (pyrrolidine dithiocarbamate 100 µmol/L).

Western Blot Analysis

Total protein was extracted from peritoneal cavity cells and from 5×10^6 BMDM lysate or 5×10^6 cultured human leukocytes. The supernatants from cells were also collected. Cells were homogenized in 50 mmol/L Tris/HCl (pH 7.4) lysis buffer (containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 150 mmol/l NaCl, 1 mmol/l EDTA, 0.1% SDS, 2 mmol/l sodium orthovanadate [Na_3VO_4], 1 mmol/L phenylmethylsulfonyl fluoride, 1 µg/mL pepstatin A, 1 µg/mL leupeptin, and 1 µg/mL aprotinin). Total protein was cleared by centrifugation at 12000g for 10 minutes, and the pellet was discarded. Proteins (25 µg) were separated by electrophoresis on a polyacrylamide gel (12%) and transferred to Immobilon-P polyvinylidene fluoride membranes. Nonspecific binding sites were blocked with 5% skim milk or 1% BSA in Tris-buffered saline solution with Tween for 1 hour at 24°C. Membranes were then incubated with specific antibodies overnight at 4°C. Antibodies were as follows: anti-NLRP3 (R&D Systems), anti–caspase-1 (Imgenex), and anti–IL-1β (Santa Cruz Biotechnology). Antibody to β-actin (Sigma) was used as an internal housekeeping control for animal samples. After incubation with secondary antibodies, the enhanced chemiluminescence luminol reagent (GE Healthcare) was used for antibody detection.

NF-κB Activation

To analyze whether aldosterone contributes to the priming process of NLRP3 inflammasome, we used the NF-κB luciferase–stable RAW264.7 cell line. Cells were stimulated with 0.1 µmol/L aldosterone for 2 to 6 hours or with 500 ng/mL ultrapure lipopolysaccharides used as a positive control. Cells were then lysed, and luciferase activity was measured with the Luciferase Assay System (Promega), according to the manufacturer's instructions.

MR Cytosolic and Nuclear Fractions

To evaluate nuclear accumulation of MRs, BMDMs were stimulated with aldosterone (0.1 µmol/L) for 4 and 6 hours. Nuclear protein extracts were obtained with a nuclear extract kit (Active Motif), according to the manufacturer's protocol. Western blot was used to measure MR expression (Abcam) in cytosolic and nuclear fractions. β-Actin expression (antibody from Sigma) was used as an internal housekeeping control. Values were obtained by densitometry with ImageJ software (National Institutes of Health).

Human Leukocytes Isolation and Response to Aldosterone Stimulation

Human leukocytes were obtained from heparinized venous blood (10 mL) of healthy subjects and isolated by Percoll gradient, as previously described.[8] A total of 5×10^6 leukocytes were seeded per well. After 12 hours, leukocytes were primed with 500 ng/mL ultrapure lipopolysaccharides (InvivoGen tlrl-peklps) for 6 hours and then stimulated with 20 µmol/L nigericin (Sigma-Aldrich) for 40 minutes, which was used as a positive control. In the absence of FBS, a total of 5×10^6 BMDM cells were stimulated with aldosterone (0.1 µmol/L) for 6 and 8 hours.

Caspase-1 Activity–FAM-FLICA Assay

BMDMs cells were stained for 1 hour with a FAM-FLICA caspase-1 assay (FAM-YVAD-FMK) kit (Immunochemistry Technologies, Bloomington, MN) according to the manufacturer's instructions. Active caspase-1 was then measured by flow cytometry. Data were acquired on a FACSCanto II flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star).

Detection of Mitochondrial Superoxide

BMDM cells were labeled with MitoSOX Red (5 µmol/L; Life Technologies) to detect mitochondrial superoxide. Mitochondrial ROS were measured by flow cytometry. The data were acquired on a FACSCanto II flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star).

Primary Hyperaldosteronism in Humans

To analyze whether high aldosterone levels also activate NLRP3 inflammasome in human leukocytes, blood samples were taken from patients diagnosed with hyperaldosteronism with Percoll gradient methodology. The procedure was approved by the Ethics Committee on Human Research of the Ribeirao Preto Medical School, University of Sao Paulo.

Hyperaldosteronism was diagnosed by the ratio of aldosterone to renin and confirmed by the saline infusion test. Reference values are shown in Table I in the online-only Data Supplement. NLRP3 activation components were measured in polymorphonuclear cells by Western blot, and IL-1β was measured in serum by ELISA. Age-matched healthy volunteers were used as controls, and samples from hypertensive patients were also used.

Statistical Analysis

For comparisons of multiple groups, 2-way ANOVA followed by the Bonferroni posttest was used. The differences in values obtained for 2 different groups were determined with the Student t test. For comparison of IL-1β levels in serum from humans, the Welch modified t test for unequal variances was used. Contraction to phenylephrine is expressed as percentage of KCl-induced response. The relaxation in response to acetylcholine and sodium nitroprusside is expressed as a percentage of contraction induced by phenylephrine. The individual concentration-response curves were fitted by nonlinear regression analysis. pD_2 (defined as the negative logarithm of the EC_{50} values) and maximal response (Emax) were determined.
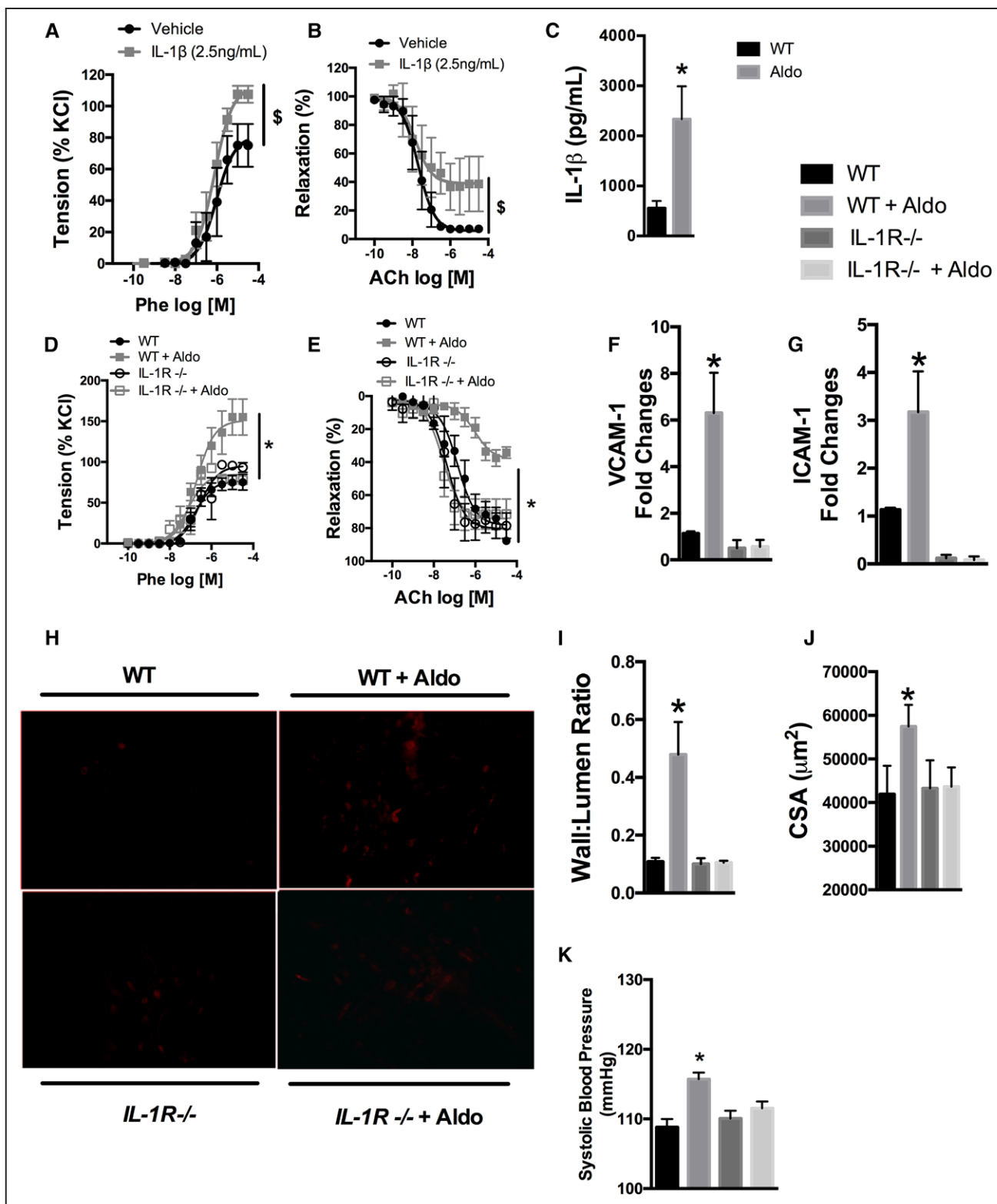
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Figure 1. Interleukin (IL)-1 receptor (IL-1R) is involved in aldosterone (Aldo)-induced vascular damage.

A, Constrictor responses to phenylephrine (Phe) and (**B**) relaxation response to acetylcholine (ACh) in mesenteric arteries from C57BL6/J mice incubated with IL-1β (2.5 ng/mL) for 2 hours (n=5 mice per group). **C**, IL-1β plasma levels in C57BL6/J mice infused with Aldo (WT+Aldo) or vehicle (WT). (**D**) Phe-induced constriction and (**E**) relaxation responses to ACh in mesenteric arteries from C57BL6/J and *IL-1R−/−* mice infused with Aldo (WT+Aldo and *IL-1R−/−*+Aldo, respectively) or vehicle (WT and *IL-1R−/−*, respectively; n=4–6 mice per group). **F**, Vascular cell adhesion protein-1 (VCAM-1) and (**G**) intercellular adhesion molecule-1 (ICAM-1) mRNA expression in mesentery isolated from WT+Aldo and *IL-1R−/−*+Aldo or WT and *IL-1R−/−* mice (*Continued*)

Analyses were performed with Prism 5.0 software (GraphPad). A difference was considered statistically significant at $P \leq 0.05$.

RESULTS

IL-1β Is Critical in Aldosterone-Induced Vascular Damage

Continuous infusion of aldosterone for 14 days in mice promoted an important vascular dysfunction, characterized by increased vascular contractility to phenylephrine and decreased endothelium-dependent vasodilation to acetylcholine (Figure IIa and IIb in the online-only Data Supplement). Moreover, aldosterone infusion markedly increased the expression of VCAM-1 and ICAM-1 in the mesenteric bed (Figure IIc and IId in the online-only Data Supplement) and the number of adherent macrophages in the aorta (Figure IIe in the online-only Data Supplement). Aldosterone also induced vascular remodeling characterized by increased CSA and wall:lumen ratio (Figure IIf and IIg in the online-only Data Supplement). Aldosterone infusion produced a small increase in the systolic BP (SBP) in C57BL/6 mice (Figure IIh in the online-only Data Supplement). Aldosterone did not change sodium nitroprusside-induced vascular relaxation (Figure III in the online-only Data Supplement).

Considering that proinflammatory cytokines, particularly IL-1β, induce vascular dysfunction,[29–31] we investigated whether IL-1β is critical to aldosterone-induced vascular damage. Mesenteric resistance arteries incubated with IL-1β exhibited increased phenylephrine-induced contractions and decreased acetylcholine-induced relaxation (Figure 1A and 1B). Aldosterone infusion in WT mice increased IL-1β levels, as determined by ELISA (Figure 1C). $IL\text{-}1R^{-/-}$ mice infused with aldosterone were completely protected against aldosterone-induced vascular dysfunction; that is, they did not exhibit increased vascular contractions to phenylephrine or decreased vascular relaxation to acetylcholine (Figure 1D and 1E). IL-1R deletion also prevented aldosterone-induced increased vascular VCAM-1 and ICAM-1 gene expression (Figure 1F and 1G). In addition, aldosterone-treated $IL\text{-}1R^{-/-}$ mice exhibited a lower number of adherent macrophages in aortic segments compared with WT mice treated with aldosterone (Figure 1H). $IL\text{-}1R^{-/-}$ mice did not develop vascular remodeling in response to aldosterone infusion, which was clearly observed in WT mice (Figure 1I and 1J). Last, $IL\text{-}1R^{-/-}$ mice were protected against an aldosterone-induced increase in SBP (Figure 1K).

Caspase-1 Is Involved in Aldosterone-Induced Vascular Damage

Because activation of caspase-1 induces processing and secretion of IL-1β,[12] aldosterone-induced vascular abnormalities were also determined in $Casp\text{-}1^{-/-}$ mice. Caspase-1 deletion partially blunted aldosterone-induced hypercontractility to phenylephrine and prevented decreased vascular relaxation to acetylcholine (Figure 2A and 2B). The lack of caspase-1 prevented the effects of aldosterone in the expression of the inflammatory markers VCAM-1 and ICAM-1 and in the adherence of macrophages to aortic segments (Figure 2C–2E). Aldosterone-induced vascular remodeling, represented by increased CSA and wall:lumen ratio, and increased SBP were attenuated in $Casp\text{-}1^{-/-}$ mice (Figure 2F–H).

NLRP3 Plays an Essential Role in Aldosterone-Induced Vascular Damage

Caspase-1 contributes to innate immunity through the assembly of NLRP3 inflammasome, which has been associated with several inflammatory and cardiovascular diseases.[11,20–22,32] To evaluate whether NLRP3 is involved in aldosterone-induced vascular damage, $NLRP3^{-/-}$ mice were infused with aldosterone. NLRP3 deletion also partially prevented the hypercontractility to phenylephrine and abrogated impaired vascular relaxation to acetylcholine in aldosterone-treated mice (Figure 3A and 3B). $NLRP3^{-/-}$ mice presented lower VCAM-1 and ICAM-1 gene expression and number of adherent macrophages in aortic segments in response to aldosterone (Figure 3C–3E). $NLRP3^{-/-}$ mice were also protected from aldosterone-induced vascular remodeling (CSA and wall:lumen ratio) and high SBP (Figure 3F–3H). Taken together, these results indicate that NLRP3 inflammasome plays a key role in aldosterone-induced vascular abnormalities.

Inflammasome Is Activated in Response to Aldosterone

To confirm that aldosterone activates the inflammasome platform and that NLRP3 is involved in this activation,

Figure 1 Continued. (n=5 mice per group). **H**, Adherent macrophages (red) in aortic segments from WT+Aldo and $IL\text{-}1R^{-/-}$+Aldo or WT and $IL\text{-}1R^{-/-}$ mice (n=5 mice per group). **I**, Wall:lumen ratio and (**J**) cross-sectional area (CSA) parameters in isolated mesenteric segments from WT+Aldo and $IL\text{-}1R^{-/-}$+Aldo or WT and $IL\text{-}1R^{-/-}$ mice measured with a pressure myograph (n=4–6 mice per group). **K**, Systolic blood pressure in WT and $IL\text{-}1R^{-/-}$ mice infused with Aldo (WT+Aldo; $IL\text{-}1R^{-/-}$+Aldo) or vehicle (WT; $IL\text{-}1R^{-/-}$). Emax and pD_2 values were determined in the concentration-response curves to Phe and ACh. Two-way ANOVA followed by the Bonferroni posttest was used for comparisons of multiple groups, whereas for 2 different groups, the Student t test was used. Values are reported as mean±SEM. $P<0.05 vs without IL-1β. *$P<0.05 vs WT mice.

BMDMs from WT and *NLRP3*^{-/-} mice were stimulated with aldosterone. Aldosterone increased mitochondrial ROS production (Figure 4A and 4B). Caspase-1 activation was measured by Western blot in the lysate of BMDMs and by FLICA. Aldosterone (for 6 and 8 hours) increased

cleaved caspase-1, although not to levels observed in the positive control (lipopolysaccharides+nigericin). BM-DMs from *NLRP3*^{-/-} mice averted caspase-1 activation (Figure 4C and 4D). Lipopolysaccharides+nigericin, the positive control, increased IL-1β levels in the superna-
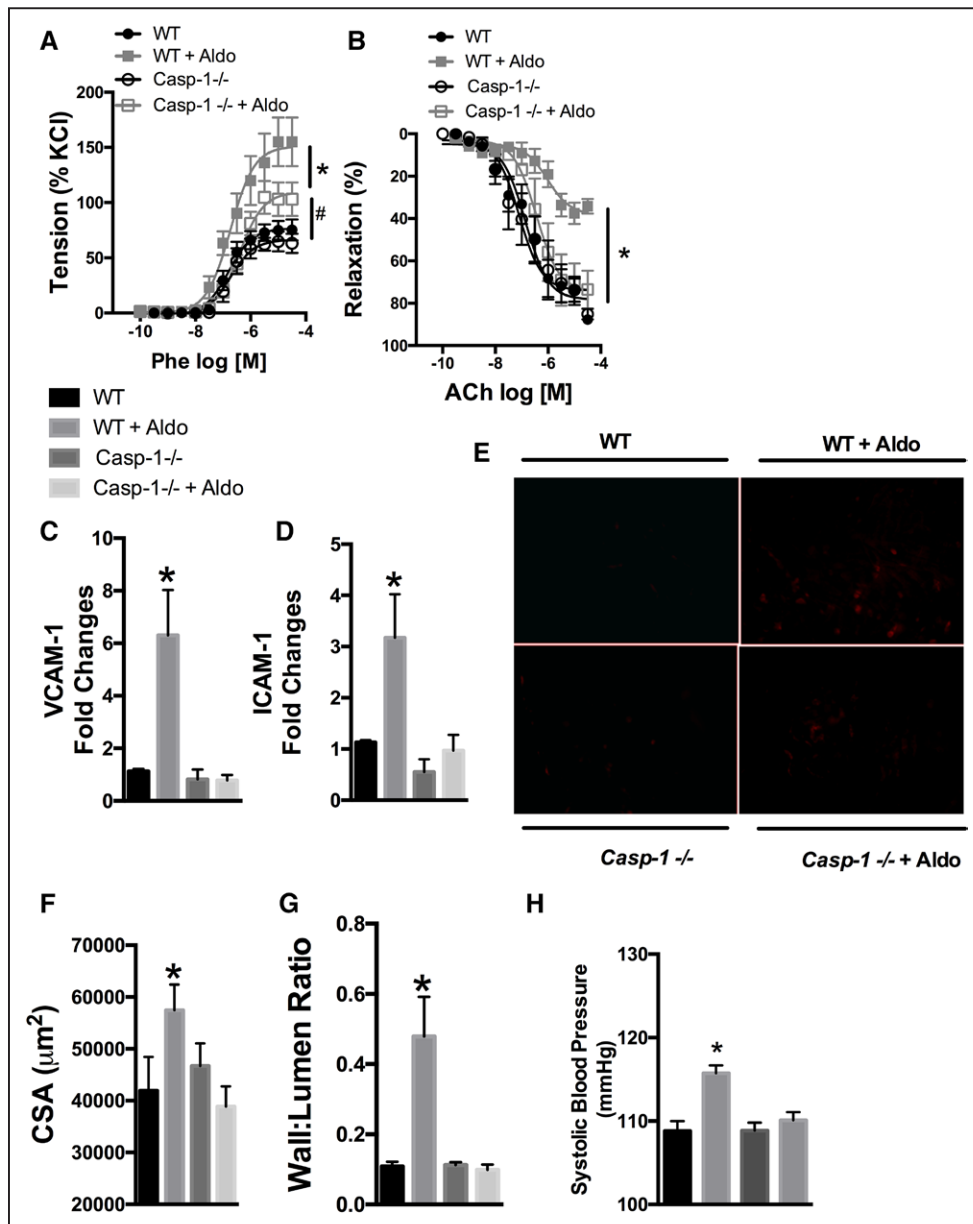
Figure 2. Caspase-1 is involved in aldosterone (Aldo)-induced vascular damage.
A, Constrictor responses to phenylephrine (Phe) and (**B**) relaxation responses to acetylcholine (ACh) in mesenteric arteries from in C57BL6/J and *Casp-1*^{-/-} mice infused with Aldo (WT+Aldo and *Casp-1*^{-/-}+Aldo, respectively) or vehicle (WT and *Casp-1*^{-/-}, respectively; n=6–7 mice per group). **C**, Vascular cell adhesion protein-1 (VCAM-1) and (**D**) intercellular adhesion molecule-1 (ICAM-1) mRNA expression, determined by quantitative real-time polymerase chain reaction, in mesentery isolated from WT+Aldo and *Casp-1*^{-/-}+Aldo or WT and *Casp-1*^{-/-} mice (n=5 mice per group). **E**, Adherent macrophages (red) in aortic segments from WT+Aldo and *Casp-1*^{-/-}+Aldo or WT and *Casp-1*^{-/-} mice analyzed by fluorescence microscopy (n=5 mice per group). **F**, Cross-sectional area (CSA) and (**G**) wall:lumen ratio parameters in isolated mesenteric segments from WT+Aldo and *Casp-1*^{-/-}+Aldo or WT and *Casp-1*^{-/-} mice measured with a pressure myograph (n=5 mice per group). **H**, Systolic blood pressure in WT and *Casp-1*^{-/-} mice infused with Aldo (WT+Aldo; *Casp-1*^{-/-}+Aldo) or vehicle (WT; *Casp-1*^{-/-}). Emax and pD$_2$ values were determined in the concentration-response curves to Phe and ACh. Values are reported as mean±SEM. Two-way ANOVA followed by the Bonferroni posttest was used for comparisons of multiple groups. *$P<0.05$ vs WT mice. #$P<0.05$ vs *Casp-1*^{-/-} mice treated with aldosterone.
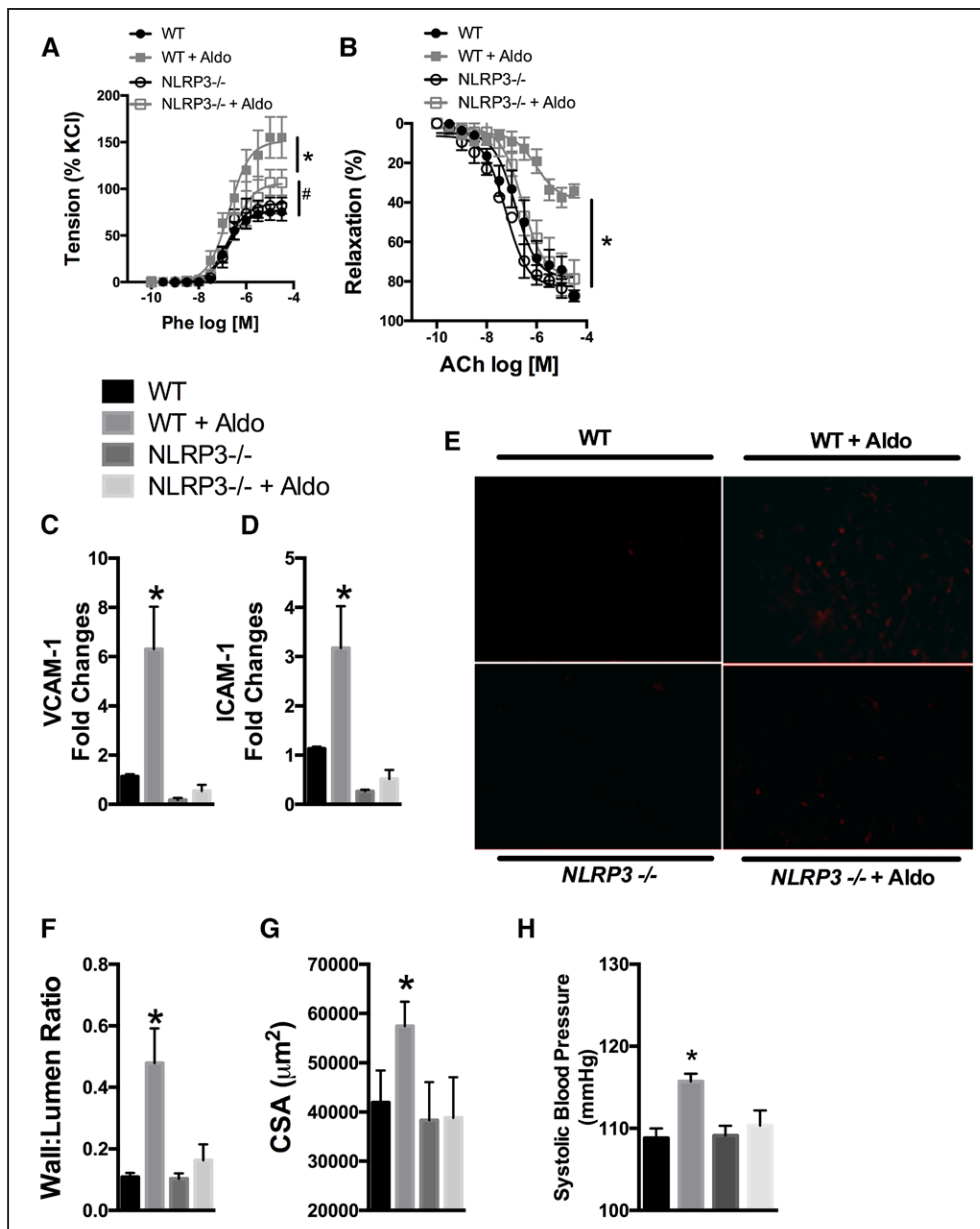
Figure 3. NLRP3 is involved in aldosterone (Aldo)-induced vascular damage.
A, Constrictor responses to phenylephrine (Phe) and (**B**) relaxation responses to acetylcholine (ACh) in mesenteric arteries from C57BL6/J and *NLRP3*−/− mice infused with Aldo (WT+Aldo and *NLRP3*−/−+Aldo, respectively) or vehicle (WT and *NLRP3*−/−, respectively; n=6–7 mice per group). **C**, Vascular cell adhesion protein-1 (VCAM-1) and (**D**) intercellular adhesion molecule-1 (ICAM-1) mRNA expression, determined by quantitative real-time polymerase chain reaction, in mesentery isolated from WT+Aldo and *NLRP3*−/−+Aldo or WT and *NLRP3*−/− mice (n=5 mice per group). **E**, Adherent macrophage (red) in aortic segments from WT+Aldo and *NLRP3*−/−+Aldo or WT and *NLRP3*−/− mice analyzed by fluorescence microscopy (n=5 mice per group). **F**, Wall:lumen ratio and (**G**) cross-sectional area (CSA) parameters in isolated mesenteric segments from WT+Aldo and *NLRP3*−/−+Aldo or WT and *NLRP3*−/− mice measured with a pressure myograph (n=5 mice per group). **H**, Systolic blood pressure in WT and *NLRP3*−/− mice infused with Aldo (WT+Aldo; *NLRP3*−/−+Aldo) or vehicle (WT; *NLRP3*−/−). Emax and pD$_2$ values were determined in the concentration-response curves to Phe and ACh. Two-way ANOVA followed by the Bonferroni posttest was used for comparisons of multiple groups. Values are reported as mean±SEM. *$P<0.05$ vs WT mice. #$P<0.05$ vs *NLRP3*−/− mice treated with Aldo.

tant, measured by ELISA and Western blot. Aldosterone for 6 and 8 hours also enhanced, although less than lipopolysaccharides+nigericin, IL-1β levels in the supernatant (Figure 4E and 4F). *NLRP3* deletion prevented lipopolysaccharides+nigericin– and aldosterone-induced IL-1β production by BMDMs (Figure 4E and 4F). Our data suggest that aldosterone induces NLRP3 inflammasome activation, which may be mediated by oxidative stress.
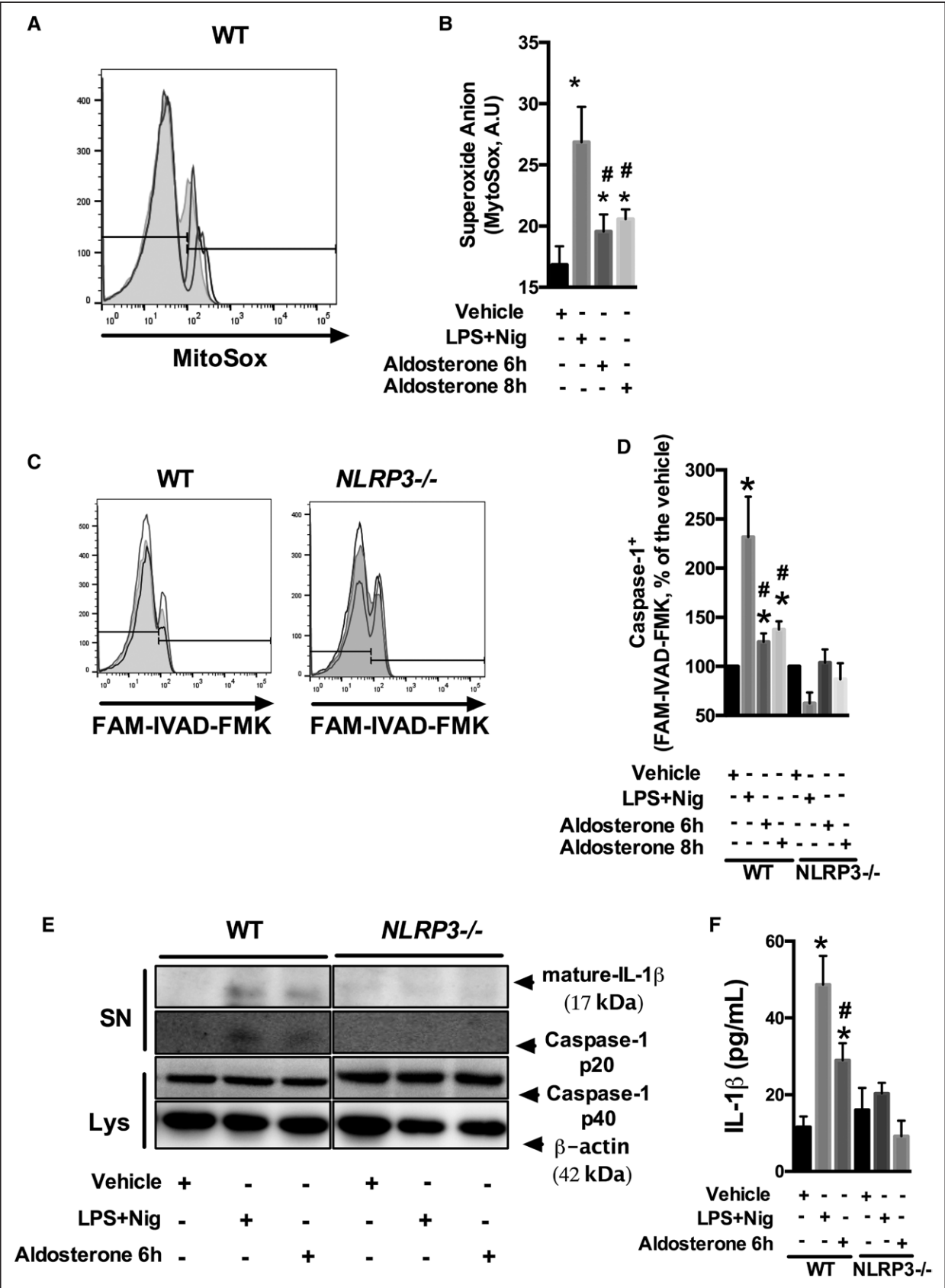
Figure 4. Aldosterone increases reactive oxygen species (ROS) generation and activates NLRP3 inflammasome in bone marrow–derived macrophages (BMDMs).

A, ROS production by mitochondria in C57BL6/J and *NLRP3⁻/⁻* BMDMs treated with aldosterone (0.1 μmol/L) for (*Continued*)

Aldosterone seems to activate NLRP3 inflammasome by genomic mechanisms because the aldosterone-induced increase in caspase-1 activity (Figure IVa–IVd in the online-only Data Supplement) was correlated with increased MR translocation to the nucleus (Figure IVe in the online-only Data Supplement).

Aldosterone Increases NLRP3 and IL-1β Expression Through NF-κB Activation

Inflammasome activation is a 2-step process. The first step, the priming process, leads to the expression of NLRP3 and pro–IL-1β through activation of NF-κB. To analyze whether aldosterone induces the first signal for NLRP3 inflammasome activation, macrophages were submitted to 2 different stimuli (aldosterone+nigericin and lipopolysaccharides+aldosterone; lipopolysaccharides+nigericin was used as a positive control). Both stimuli, aldosterone+nigericin and lipopolysaccharides+aldosterone, increased NLRP3 inflammasome activation, measured by FLICA and flow cytometry (Figure Va and Vb in the online-only Data Supplement). Aldosterone also triggered NF-κB activation after 4 hours of incubation (Figure Vc in the online-only Data Supplement). In addition, aldosterone increased NLRP3 and IL-1β gene expression, and pyrrolidine dithiocarbamate, an NF-κB inhibitor, prevented aldosterone-induced NLRP3 and IL-1β gene expression (Figure Vd and Ve in the online-only Data Supplement).

NLRP3 in the Immune System Is Key to Aldosterone-Induced Vascular Damage

Considering the link between the immune system and cardiovascular diseases, we analyzed whether aldosterone treatment activates NLRP3 inflammasome in cells from the peritoneal cavity (peritoneal macrophages) in WT mice. Aldosterone infusion increased NLRP3 protein expression, caspase-1 activity, and mature IL-1β in peritoneal cavity cells (Figure 5A–5C). Although our data clearly show that vascular damage induced by aldosterone depends on NLRP3 inflammasome activation and that aldosterone activates inflammasome in immune cells through NLRP3, it was not clear whether NLRP3 inflammasome activation in the immune system or in the vasculature contributes to vascular damage induced by aldosterone. To address this question, we transplanted the bone marrow from *NLRP3⁻/⁻* mice into WT mice. Aldosterone increased contractions to phenylephrine and decreased vascular relaxation to acetylcholine in WT→WT mice. However, aldosterone-induced changes in vascular reactivity were partially abrogated in *NLRP3⁻/⁻*→WT mice (Figure 5D and 5E). Furthermore, *NLRP3⁻/⁻*→WT mice were moderately protected from aldosterone-induced vascular inflammation, determined by VCAM-1 and ICAM-1 mRNA expression and adherence of macrophages to aortic segments (Figure 5F–5H). *NLRP3⁻/⁻*→WT mice treated with aldosterone exhibited attenuated increases in CSA and wall:lumen ratio compared with aldosterone-infused WT→WT mice (Figure 5I–5J). Overall, these findings suggest that NLRP3 inflammasome in the immune system has a very important role in vascular damage induced by aldosterone.

Hyperaldosteronism and Hypertension Trigger NLRP3 Inflammasome Activation in Isolated Human Polymorphonuclear Cells

Hyperaldosteronism presents a straight relationship with vascular damage. To determine whether patients with hyperaldosteronism exhibit NLRP3 inflammasome activation, polymorphonuclear cells from a patient diagnosed with primary hyperaldosteronism were isolated (Table I in the online-only Data Supplement), and NLRP3 activation was assessed. In addition, serum IL-1β levels were determined in these patients. Hyperaldosteronism increased NLRP3 protein expression, caspase-1 activation, and mature IL-1β in human leukocytes. Serum levels of IL-1β levels were also increased in patients with hyperaldosteronism compared with healthy human volunteers (Figure 6A and 6B). Polymorphonuclear cells from hypertensive patients that do not exhibit increased aldosterone levels also exhibited increased NLRP3 inflammasome activity (Figure 6C).

To confirm the potential ability of aldosterone to activate NLRP3 inflammasome, leukocytes were isolated from a healthy human volunteer, seeded in 24-well plates, and stimulated with aldosterone, with lipopolysaccharides+nigericin used as a positive control. In human leukocytes, aldosterone increased NLRP3 protein expression, triggered caspase-1 activation, and increased mature IL-1β in the lysate and in the supernatant (Figure 6D).

Figure 4 Continued. 6 and 8 hours by flow cytometry analysis. **B**, Quantification of the average of arbitrary units (A.U) of MytoSox in C57BL6/J and *NLRP3⁻/⁻* BMDMs. C, Caspase-1 activity in C57BL6/J and *NLRP3⁻/⁻* BMDMs treated with aldosterone (0.1 μmol/L) for 6 and 8 hours by flow cytometry analysis. **D**, Quantification of the average percentages of FAM-YVAD⁺ in C57BL6/J and *NLRP3⁻/⁻* BMDMs. **E**, Immunoblotting for caspase-1 p20 and interleukin (IL)-1β p17 in cell-culture supernatants (SN) and cell lysates (Lys) in BMDMs stimulated for 6 hours with lipopolysaccharides (LPS; 500 ng/mL) plus 20 μmol/L nigericin (Nig) for 1 hour or with aldosterone (0.1 μmol/L) for 6 hours. **F**, IL-1β production measured by ELISA in C57BL6/J and *NLRP3⁻/⁻* BMDMs stimulated for 6 hours with LPS (500 ng/mL) plus 20 μmol/L Nig for 1 hour or with aldosterone (0.1 μmol/L) for 6 hours (n=5 per group). One- or 2-way ANOVA followed by the Bonferroni posttest was used for comparisons of multiple groups. Values are reported as mean±SEM. *$P<0.05$ vs vehicle. #$P<0.05$ vs LPS+Nig.
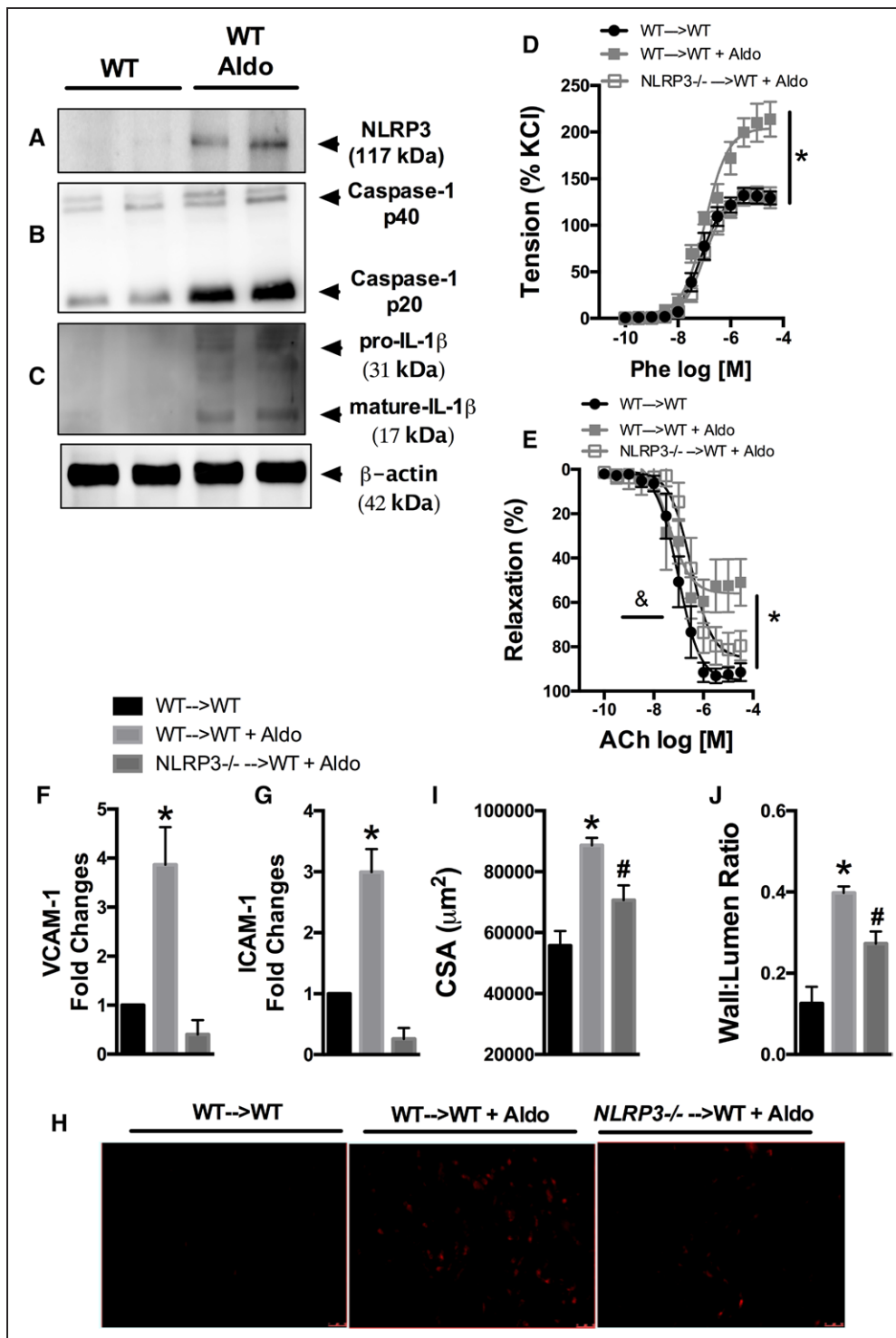
Figure 5. NLRP3 inflammasome in bone marrow–derived cells is involved on aldosterone (Aldo)-induced vascular damage.
WT→WT and *NLRP3*^{-/-} →WT chimeric mice were produced, and Aldo infusion was performed. Immunoblotting for (**A**) NLRP3, (**B**) caspase-1 p20, and (**C**) interleukin (IL)-1β p17 in peritoneal cavity cells from C57BL6/J mice treated with Aldo (WT+Aldo) or vehicle (WT). **D**, Constrictor responses to phenylephrine (Phe) and (**E**) relaxation responses to acetylcholine (ACh) in mesenteric arteries from WT→WT and *NLRP3*^{-/-} →WT mice infused with aldosterone (WT→WT+Aldo and *NLRP3*^{-/-} →WT+Aldo) or vehicle (WT→WT; n=5–7 mice per group). **F**, Vascular cell adhesion protein-1 (VCAM-1) and (**G**) intercellular adhesion molecule-1 (ICAM-1) mRNA expression, determined by quantitative real-time polymerase chain reaction, in mesentery isolated from WT→WT, WT→WT+Aldo, and *NLRP3*^{-/-} →WT+Aldo mice (n=5 mice per group). **H**, Adherent macrophage (red) in aortic segments from WT→WT, WT→WT+Aldo, and *NLRP3*^{-/-} →WT+Aldo mice analyzed by fluorescence microscopy (n=5 mice per group). (*Continued*)
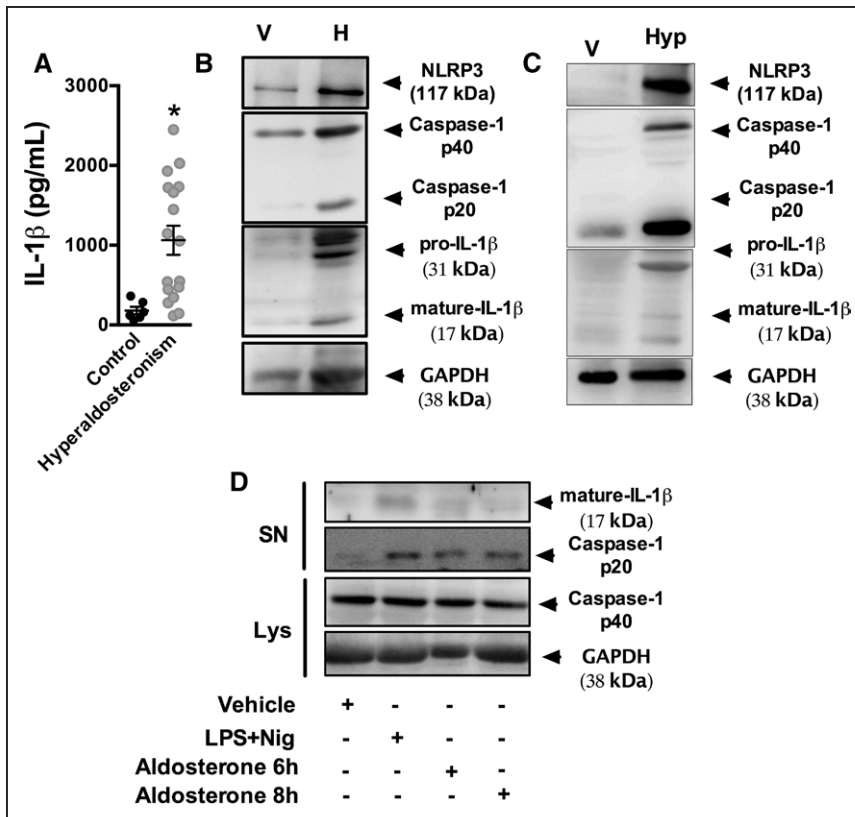
Figure 6. Hyperaldosteronism and hypertension activate NLRP3 inflammasome.
A, Interleukin (IL)-1β production measured by ELISA in healthy human volunteers (controls) and in patients with hyperaldosteronism (H). **B**, Immunoblotting for NLRP3, caspase-1 p20, and IL-1β p17 in polymorphonuclear cells (PMNCs) from a healthy human volunteer (V; control), a patient with hyperaldosteronism, and (**C**) a hypertensive patient. **D**, Immunoblotting for caspase-1 p20 and IL-1β p17 in cell-culture supernatants (SN) and cell lysates (Lys) of PMNCs from a healthy human volunteer stimulated for 6 hours with lipopolysaccharides (LPS; 500 ng/mL) plus 20 μmol/L nigericin (Nig) for 40 minutes or with aldosterone (0.1 μmol/L) for 6 and 8 hours (n=6–16 per group). The Welch modified *t* test for unequal variances was used to analyze the IL-1β levels. Values are reported as mean±SEM. *P<0.05 versus control.

DISCUSSION

Our data demonstrate for the first time that NLRP3 inflammasome in bone marrow–derived cells plays a crucial role in aldosterone-induced vascular damage by processing and secreting IL-1β. In addition, our clinical studies indicate that vascular damage associated with hyperaldosteronism might be dependent on NLRP3 inflammasome activation in immune cells. Because NLRP3 inflammasome activation in immune cells was also found in hypertensive patients with normal aldosterone levels, it is possible that aldosterone-induced NLRP3 inflammasome activation is mediated through high BP.

It is well established that aldosterone leads to vascular damage[1,3,4,33] and to small to mild increases in BP levels[4,34,35] in mice. Clinical and experimental studies have shown that salt and aldosterone independently induce vascular abnormalities and that aldosterone plus salt intake accelerates organ damage or, more specifically, vascular damage,[36–39] possibly through inflammatory-related processes.[1,40] In the present study, we have not addressed whether salt directly induces or contributes to aldosterone-induced NLRP3 inflammasome activation,

but our study is in line with previous reports because aldosterone triggered a minor increase in SBP.

The immune system, through innate and adaptive immune responses, modulates vascular function in different conditions.[4,7,9,10,41–43] Long-term angiotensin II infusion in control mice impairs endothelium-dependent vasodilatation. In contrast, angiotensin II causes minimal perturbation of endothelium-dependent vasodilatation in *Rag1*^{−/−} mice (knockout mice for mature B and T lymphocytes).[9] Furthermore, adoptive transfer of T-regulatory lymphocytes, but not T-effector cells, blunts angiotensin II-induced vascular damage.[7] Last, similar to T-regulatory lymphocyte effects on angiotensin II–induced vascular injury, T-regulatory lymphocyte adoptive transfer also prevents aldosterone-induced vascular damage.[4] B and T lymphocytes are the major cellular components of the adaptive immune response, whereas T-regulatory lymphocytes are considered suppressors of the innate and adaptive immune responses.[4,41,42] Evidence indicates that innate immune responses control vascular function through Toll-like receptors, a subfamily of pattern recognition receptors.[44,45] Circulating mtDNA and impaired DNase activity lead to activation of the innate immune system through Toll-like receptor 9 and

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Figure 5 Continued. I, Cross-sectional area (CSA) and (**J**) wall:lumen ratio parameters in isolated mesenteric segments from WT→WT, WT→WT+Aldo, and *NLRP3*^{−/−}→WT+Aldo mice measured with a pressure myograph (n=5 mice per group). Emax and pD₂ values were determined in the concentration-response curves to Phe and ACh. Two-way ANOVA) followed by the Bonferroni posttest was used for comparisons of multiple groups. Values are reported as mean±SEM. *P<0.05 vs *NLRP3*^{−/−}→WT+Aldo. #P<0.05 vs WT→WT. &P<0.05, WT→WT+Aldo vs *NLRP3*^{−/−}→WT+Aldo on pD₂ value.

contribute to vascular dysfunction in spontaneously hypertensive rats.[45] In addition, treatment with a neutralizing anti–Toll-like receptor4 reduces vascular injury in C57BL6/J mice infused with angiotensin II, likely by inhibition of oxidative stress–related mechanisms.[46] Although various studies have shown a link between cardiovascular disease and the immune system, the involvement of NLRP3 inflammasome on vascular dysfunction has not been clarified.

NLRP3 forms a multiprotein inflammasome complex that activates caspase-1 and leads to the maturation of several key proinflammatory cytokines, including IL-1β and IL-18. Mitochondrial ROS are one of the main activators of NLRP3 inflammasome.[11,47] There is indirect evidence that NLRP3 inflammasome activity plays a role in the development of vascular damage. For example, mice with type 1 diabetes mellitus treated with anakinra (IL-1R antagonist) are protected from vascular dysfunction.[29] Anakinra treatment improves vascular and left ventricular function in patients with rheumatoid arthritis,[48] and *Casp-1*^{−/−} mice with pristine-induced murine lupus do not exhibit vascular damage.[49] In addition, endothelial dysfunction in obese Otsuka Long-Evans Tokushima Fatty rats seems to occur through NLRP3 activation and mitochondrial dysfunction.[16] Last, patients with coronary artery disease present high NLRP3 RNA expression, which is positively correlated with IL-1β and IL-18 levels.[50]

Few studies have shown a direct effect of inflammasome activation on specific organ injury. The inflammasome assembly in mouse heart under acute myocardial infarction causes additional loss of functional myocardium, leading to heart failure.[22] Mice deficient in *ASC* and *Casp-1* present diminished inflammatory responses and subsequent injuries, including infarct development, myocardial fibrosis, and dysfunction induced by myocardial ischemia/reperfusion injury, which depend on bone marrow and myocardial resident cells.[21] In addition, *NLRP3*^{−/−} mice present improved cardiac function and reduced hypoxic damage when subjected to global ischemia and reperfusion compared with WT mice.[19]

Uninephrectomized mice treated with deoxycorticosterone acetate and saline, a model of mineralocorticoid hypertension, present inflammasome activation in the kidney, which is associated with increased collagen deposition and inflammatory markers. *ASC* knockout mice are protected against these abnormalities. In addition, NLRP3 antagonist (MCC950) treatment blunts deoxycorticosterone acetate and saline–induced hypertension and kidney inflammation.[18] Uninephrectomized WT mice infused with aldosterone present increased inflammasome activation in the kidney, which is abrogated by eplerenone treatment (MR antagonist), suggesting that aldosterone activates the inflammasome through MR. Moreover, the lack of *ASC* gene prevents increased renal inflammation and remodeling, which are dependent of bone marrow–derived cells. Completing this interesting finding, macrophages primed with lipopolysaccharides

and stimulated with aldosterone present augmented inflammasome activation, mediated by mitochondrial ROS.[15] Our study, at least in isolated macrophages, is in line with these findings. Although Kadoya et al[15] addressed the inflammasome activation in the development of renal fibrosis in mice, the involvement of NLRP3 on aldosterone effects was indirectly assessed.

Using knockout mice for NLRP3, Casp-1, and IL-1R, the present study shows that aldosterone activates NLRP3 inflammasome, leading to vascular dysfunction, inflammation, and remodeling that are dependent on IL-1R activation, placing the NLRP3 inflammasome as the major platform in aldosterone-induced vascular damage. Protective vascular effects associated with the deletion of inflammasome components do not seem to depend on BP differences, although the small increase in BP produced by aldosterone in WT mice was not observed in mice deficient in NLRP3 and inflammasome components. In addition, aldosterone-induced vascular dysfunction occurs through NLRP3 inflammasome activation in cells from the bone marrow. In BMDMs, aldosterone seems to induce both the first and second signals necessary for inflammasome activation. The priming signal leads to NF-κB activation, whereas ROS may be the second signal. Our data suggest that the immune system has a major impact on aldosterone-induced abnormalities of vascular tone and structure. Of importance, NLRP3 inflammasome activation is observed in patients with hyperaldosteronism and in hypertensive patients who do not exhibit high levels of aldosterone, which places NLRP3 inflammasome as a possible clinical target for these patients.

Our data add one more piece to the puzzle linking cardiovascular diseases and the immune system by showing and reinforcing the role of innate immune responses on vascular injury or, more precisely, on aldosterone-induced vascular disease. We identified NLRP3 inflammasome–dependent IL-1β secretion as a pivotal pathway that can be targeted for the development of future therapeutic strategies to prevent aldosterone- and hypertension-associated vascular damage.

DISCLOSURES

None.

AFFILIATIONS

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FOOTNOTES

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