Inflammatory Monocytes Determine Endothelial Nitric-oxide Synthase Uncoupling and Nitro-oxidative Stress Induced by Angiotensin II

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Background: Inflammatory monocytes are drivers of vascular injury and disease.

Results: Depletion of lysozyme M-positive monocytes prevents eNOS uncoupling and iNOS-derived nitro-oxidative stress.

Conclusion: Monocytes determine eNOS and iNOS function by directly modulating tetrahydrobiopterin bioavailability.

Significance: Understanding the impact of inflammation on endothelial function in detail is essential to identify tailored therapeutic strategies.

Endothelial nitric-oxide synthase (eNOS) uncoupling and increased inducible NOS (iNOS) activity amplify vascular oxidative stress. The role of inflammatory myelomonocytic cells as mediators of these processes and their impact on tetrahydrobiopterin availability and function have not yet been defined. Angiotensin II (ATII, 1 mg/kg/day for 7 days) increased Ly6C-high leukocytes and up-regulated levels of eNOS glutathionylation in aortas of C57BL/6 mice. Vascular iNOS-dependent NO formation was increased, whereas eNOS-dependent NO formation was decreased in aortas of ATII-infused mice as assessed by electron paramagnetic resonance (EPR) spectroscopy. Diphtheria toxin-mediated ablation of lysozyme M-positive (LysM+) monocytes in ATII-infused transgenic mice prevented eNOS glutathionylation and eNOS-derived Nω-nitro-L-arginine methyl ester-sensitive superoxide formation in the endothelial layer. ATII increased vascular guanosine triphosphate cyclohydrolase I expression and biopterin synthesis in parallel, which was reduced in monocyte-depleted LysM+DTR mice. Vascular tetrahydrobiopterin was increased by ATII infusion but was even higher in monocyte-depleted ATII-infused mice, which was paralleled by a strong up-regulation of dihydrofolate reductase expression. EPR spectroscopy revealed that both vascular iNOS- and eNOS-dependent NO formation were normalized in ATII-infused mice following monocyte depletion. Additionally, deletion as well as pharmacologic inhibition of iNOS prevented ATII-induced endothelial dysfunction. In summary, ATII induces an inflammatory cell-dependent increase of iNOS, guanosine triphosphate cyclohydrolase I, tetrahydrobiopterin, NO formation, and nitro-oxidative stress as well as eNOS uncoupling in the vessel wall, which can be prevented by ablation of LysM+ monocytes.

Angiotensin II (ATII2)–induced vascular dysfunction is characterized by increased vascular oxidative stress and loss of NO bioavailability. As predominant sources of superoxide (O2-), the vascular NADPH oxidase, an uncoupled endothelial nitric-oxide synthase (eNOS), xanthine oxidase, and mitochondria have been identified (1–5). The uncoupled eNOS has gained growing attention, because a dysfunctional eNOS leads to a decreased nitric oxide (NO) bioactivity in the vasculature and turns into a source of O2·− by transferring electrons to molecular oxygen in the uncoupled state, thereby shifting the O2\textsuperscript{−}/NO\textsuperscript{−} equilibrium toward O2\textsuperscript{−} (6). The uncoupling reaction of eNOS is triggered by oxidation (e.g. by peroxynitrite, ONOO−) of the eNOS cofactor tetrahydrobiopterin (BH3) leading to the formation of the BH3 radical and subsequently to dihydrobiopterin (BH2) (7). Intracellular depletion of BH2 is counteracted mainly by the activity of the biopterin-synthesizing enzyme guanosine triphosphate cyclohydrolase I (GTPCH) and the BH2-reducing enzyme dihydrofolate reductase (DHFR). Importantly, the cascade of eNOS uncoupling depends on excess production of O2·− which avidly reacts with NO to form ONOO•. Therefore, superoxide anion acts as a so-called “kindling radical” to fuel eNOS uncoupling.
making identification and control of the source of O$_2^-$ a promising therapeutic target.

In parallel, GTPCH as the rate-limiting enzyme of BH$_4$ synthesis has classically been defined as constitutively active in macrophages and can be induced by proinflammatory cytokines like tumor necrosis factor $\alpha$ and interferon $\gamma$ (IFN-$\gamma$) (8). In inflammatory cells, it is necessary to supply BH$_4$ to the inducible NO synthase (iNOS), a signature enzyme of inflammatory cells like proinflammatory monocytes or macrophages. Equipped with this machinery and combined with their nicotinate amide dinucleotide phosphate (NADPH) oxidase activity, inflammatory cells are capable of performing their innate immune functions like cytotoxic microbial killing but also of exerting harmful nitro-oxidative stress in inflammatory diseases like atherosclerosis. Monocytes that express high levels of lymphocyte antigen 6 complex locus C1 (Ly6C) have been shown to dominate monocytosis in atherosclerosis, to adhere to endothelium, and to give rise to inflammatory macrophages in atheromata (9).

Recently, we identified infiltrating lysozyme M-positive (LysM$^+$) CD11b$^+$/Gr-1$^-$ monocytes and vascular CD11b$^+$/F4/80$^+$ macrophages as critical mediators of ATII-induced vascular dysfunction and arterial hypertension. In that study, we found that both eNOS-dependent vascular relaxation and overall aortic expression of iNOS can be normalized by depletion of inflammatory monocytes. In addition, we found that iNOS-derived oxidative stress is largely determined by the mutual activation of proinflammatory natural killer cells and monocytes and intact IFN-$\gamma$/interleukin-12 signaling in the vasculature of ATII-infused mice (10, 11).

We therefore wanted to investigate the end point that inflammatory monocytes determine eNOS uncoupling via controlling BH$_4$ availability and function. We set out to test the hypothesis that depletion of inflammatory LysM$^+$ myelomonocytic cells would prevent both iNOS activity and eNOS uncoupling and thereby preserve NO bioavailability in ATII-induced vascular dysfunction. We found that ATII induces a Ly6C$^{high}$ inflammatory cell-dependent increase of iNOS, GTPCH, BH$_4$, NO$^+$ formation, and nitro-oxidative stress as well as eNOS uncoupling in the vessel wall, which can be prevented by ablation of LysM$^+$ monocytes.

**EXPERIMENTAL PROCEDURES**

**Animals**—All animal experimental work was in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and the study was approved by the University Hospital Mainz Ethics Committee and the authorities (Landesuntersuchungsamt Rheinland-Pfalz, Germany). LysM$^{Cre/Cre}$ and ROSA26$^{DTR/DTR}$ (all backcrossed more than 10 generations to the C57BL/6 background) were crossed to generate male LysM$^{Cre/wt}$ and LysM$^{Cre/wt}$/ROSA26$^{DTR/wt}$ mice (abbreviated LysM and LysM$^{DTR}$, respectively) as published before (10). In addition, male C57BL/6, eNOS$^{-/-}$, iNOS$^{-/-}$, Agrp1$^{-/-}$, and hemizygous gp91$^{phox-/-}$/Nox2 knock-out; all C57BL/6 background) mice were used (purchased from The Jackson Laboratory, Bar Harbor, ME). For diphertheria toxin receptor (DTR)-mediated cell ablation, LysM$^{DTR}$ mice along with their LysM controls received intraperitoneal injections with diphertheria toxin (Sigma) once daily (solved in PBS; 25 ng/g from days 1–3 then 5 ng/g thereafter) (12, 13). ATII (1 mg/kg/day for 7 days) versus sham (NaCl 0.9%) was delivered s.c. using miniosmotic pumps (model 1007D, ALZET, Cupertino, CA) from days 4 to 10. After day 10, mice were killed by exsanguination in isoflurane anesthesia, and blood and aortas were collected.

**Flow Cytometric Analysis of Aortic Lysates**—Prior to analysis, mouse aortas were lysed by 6.5 units/ml liberase TM (Roche Applied Science) for 20 min at 37 °C. To block nonspecific Fc receptor-mediated binding, single cell suspensions were preincubated with unlabeled mAb against CD16/CD32 against CD16/CD32. Cells were stained for 20 min with CD45 APC-eFluor-780, CD11b PE, Gr-1 Horizon, and Ly6c PerCP-Cy5.5 and for outgating of dead cells additionally with Fixable Viability Dye eFluor506 (CD11b from Pharmingen; all other antibodies from eBioscience, San Diego). iNOS FITC was stained after fixation with Fixation/Permeabilization solution from Pharmingen. A minimum of 100,000 events was acquired using the FACS Canto II (BD Biosciences), and viable cells were analyzed with FACSDiva software. For analysis of iNOS expression, isolated cells were stimulated with 10 ng/ml mIFN-$\gamma$ and 1 μg/ml LPS in the presence of 10 μg/ml brefeldin A overnight. After stimulation and surface staining, cells were fixed with the Cytofix/Cytoperm kit as indicated in the manufacturer’s instructions (Pharmingen) and stained for intracellular iNOS FITC (Pharmingen) or matched isotype control. Additionally, iNOS-positive cells A549 cells were used as a positive control (14).

**Reconstitution of Depleted Mice with Monocytes and Neutrophils**—CD11b$^+$Gr-1$^-$ monocytes were prepared from venous blood of C57BL/6, Agrp1$^{-/-}$, and gp91$^{phox-/-}$/y (Nox2 knock-out; all C57BL/6 background) mice by negative selection using magnetic activated cell sorting after discarding granulocytes following Histopaque 1083 gradient. LysM$^{DTR}$ mice were monocyte-depleted by diphertheria toxin injections according to our protocol (diphertheria toxin solved in PBS once daily, 25 ng/g from days 1 to 3 then 5 ng/g thereafter) and ATII-infused (1 mg/kg/day for 7 days, starting at day 4 of depletion protocol) and were reconstituted in vivo by single i.v. injection with 1.5 × 10$^6$ monocytes at day of the depletion protocol.

**Oxidative Fluorescent Microtopography**—Isolated aorta was cut into 3-mm rings, incubated in Krebs/Hepes solution for 15 min at 37 °C in the presence or absence of the eNOS inhibitor N$^e$-nitro-L-arginine methyl ester (l-NAME, 10 μM), embedded in aluminum cups of about 1 ml of a polymeric resin (Tissue Tek®, Sakura Finetek, Alphen aan den Rijn, Netherlands), and frozen in liquid nitrogen. Cryosections (6 μm) were stained with the superoxide-sensitive dye dihydroethidium (DHE, 1 μM) and 4′,6-diamidino-2-phenylindole (DAPI) for nuclear staining. Sections of all four study arms were analyzed in parallel with identical imaging parameters.

**Oxidative Burst of Whole Blood**—Venous blood was drawn into 0.1 volume of 3.8% sodium citrate. The blood was kept at room temperature and diluted 1:50 in Dulbecco’s PBS (without Mg$^{2+}$, Ca$^{2+}$, and bicarbonate). The L-012 (100 μM)-enhanced chemiluminescence (ECL) signal was counted in 0.5-ml samples in the absence or presence of phorbol 12,13-dibutyrate (10...
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µM) at intervals of 30 s for 10 min. ECL was expressed as counts/min/ml after incubation for 10 min.

Protein and mRNA Expression—Protein expression was assessed using SDS-PAGE and Western blotting as described previously. mRNA expression was analyzed by quantitative real-time RT-PCR as described previously (11). Aortas were cleaned of adipose tissue, rinsed, and snap-frozen. For protein expression analyses, protein suspensions from homogenized aortic tissue were submitted to SDS-PAGE and immunoblotting (Bio-Rad), using antibodies against α-actinin (mouse, monoclonal, Sigma), eNOS (mouse, monoclonal, 1:1000, BD Biosciences), heme oxygenase-1 (HO-1, mouse, monoclonal, StressGen, San Diego, GTPCH (mouse, monoclonal, Abnova, Heidelberg, Germany), dihydrofolate reductase (DHFR, mouse, monoclonal, Abnova, Heidelberg, Germany), and Nox4 (rabbit, polyclonal, Novus Biologicals, Littleton, CO) followed by peroxidase-labeled secondary antibody against mouse or rabbit IgG (Vector Laboratories, Burlingame, CA). Immunodetection was accomplished with either SuperSignal Substrate (Pierce) or ECL Reagent (Amersham Biosciences). Bands were evaluated by densitometry. mRNA expression was analyzed by quantitative real-time RT-PCR using a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Briefly, total RNA from mouse aorta was isolated according to the manufacturer’s protocol of the RNeasy fibrous tissue mini kit (Qiagen, Hilden, Germany). 0.5 µg of total RNA was used for real-time RT-PCR analysis with the QuantiTect™ Probe RT-PCR kit (Qiagen, Hilden, Germany). TaqMan® Gene Expression assays (Applied Biosystems, Foster City, CA) for TATA box-binding protein (TBP; Mm00446973_m1) and inducible nitric-oxide synthase (iNOS, Nos2; Mm00440485_m1) were purchased as probe and primer sets. The comparative ∆Ct method was used for relative mRNA quantification (15). Gene expression was normalized to the endogenous control, TBP mRNA, and the amount of target gene mRNA expression in each sample was expressed relative to that of control.

eNOS S-Glutathionylation—Briefly, M-280 sheep anti-mouse IgG-coated beads from Invitrogen were used along with a monoclonal mouse eNOS antibody (BD Transduction Laboratory). The beads were loaded with the eNOS antibody and cross-linked according to the manufacturer’s instructions. Next, the aortic homogenates were incubated with the eNOS antibody beads, precipitated with a magnet, washed, transferred to gel, and subjected to SDS-PAGE followed by a standard Western blot procedure using a monoclonal mouse antibody against S-glutathionylated proteins from Virogen (Watertown, MA) at a dilution of 1:1000 under nonreducing conditions. Disappearance of the signal on incubation with 2-mercaptoethanol served as a control. After the membrane was stripped, the bands were stained for eNOS to allow normalization of the signals. All signals were normalized on the eNOS staining of the same sample.

NO Measurement by Electron Paramagnetic Resonance Spectroscopy—Aortic NO’ formation was measured using EPR-based spin trapping with iron-dithyldithiocarbamate (Fe(DETC))2 colloid as described in general previously (5, 16). Whole aortas were cleaned of fat and connective tissue and cut into 3-mm rings. For iNOS stimulation, aortic rings were incubated in RPMI 1640 medium, 10% FCS + 1% penicillin/streptomycin + 10 µg/ml LPS (from Escherichia coli, Sigma) for 19–24 h at 37 °C, 5% CO2. LPS-pretreated or freshly prepared rings were transferred in 1 ml of Krebs/Hepes buffer on a 24-well plate. In selected experiments, 10 µM iNOS inhibitor N-[3(aminomethyl)benzyl]acetamidine, dihydrochloride (1400W) was added. For eNOS stimulation, samples were incubated with 10 µM calcium ionophore (A23187) for 2 min on ice before the colloidal solution was added. NaDETC (5.4 mg) and FeSO4·7H2O (3.4 mg) were separately dissolved under argon gas bubbling in 15 ml volumes of ice-cold PBS with Ca2+3/3Mg2+. These solutions were rapidly mixed to obtain a colloid Fe(DETC)2 solution (0.4 mM), which was added immediately to the rings (1 ml). After 60 min of incubation at 37 °C, aortic rings were placed at a fixed position in a 1-ml syringe with the top removed in Krebs/Hepes buffer and frozen in liquid nitrogen (so that the entire aortic sample was placed within a 100-µl volume of the syringe). For measurement, the frozen cylinder with the aortic sample was pressed out of the syringe and placed in a special Dewar vessel (Magnettech, Berlin, Germany) filled with liquid nitrogen. The localization of the aortic sample was adjusted to the middle of the resonator. EPR conditions were as follows: B0 = 3274 G, sweep = 110 G, sweep time = 60 s, modulation = 7000 mG, power = 10 milliwatts, using a Miniscope MS400 (Magnettech, Berlin, Germany).

Measurement of Aortic Levels of BH4, BH2, and Bioterin—Whole mouse aorta was homogenized in ice-cold HCl (0.1 N). BH4, BH2, and bioterin content were assessed by high-performance liquid chromatography using sequential electrochemical and fluorescence detection. Isocratic elution was performed (flow rate 0.85 ml/min) using a Nucleosil 100–5C18 column (25 cm, 4.6 mm, 5 µm, Supelco) and mobile phase (octyl sulfate sodium salt 0.6 mM, EDTA 0.5 mM, diethylenetriaminepentaacetic acid 0.25 mM, DL-dithiothreitol 1.25 mM, NaH2PO4 50 mM, pH 2.8), containing 2% acetonitrile (v/v). The electrochemical detection system for measuring BH4 consisted of an ESA Coulochrom III detector equipped with a boron-doped diamond electrochemical cell (model 5040) at a potential of +450 mV and a guard cell set at a potential of +800 mV to oxidize contaminants in the mobile phase. BH2 was converted to a fluorescent form by post-column electrochemical oxidation. An ESA Coulochrom II with model 5011 analytical cell set at a potential of +800 mV was used. The electrochemical electrodes were followed in series by a Jasco FP-2020 Plus Intelligent fluorescence detector. The excitation and emission wavelengths were set at 348 and 444 nm, respectively. Data acquisition and analysis were performed using the Chromelon® software (Dionex). BH4, BH2, and bioterin concentrations were expressed as picomoles/µg protein.

Vascular Relaxation Studies—Vascular relaxation of isolated aortic rings of C57BL/6 and iNOS−/− mice subjected to ATII infusion or sham was assessed. Isolated aortas were cut into 4-mm segments and mounted on force transducers (Kent Scientific Corp., Torrington, CT, and PowerLab, AD Instruments, Spechbach, Germany) in organ chambers filled with Krebs-Henseleit. In selected experiments, 10 µM iNOS inhibitor 1400W was added to the organ bath. To test for vasorelaxation...
in response to acetylcholine (ACh), aortic segments were stretched gradually over 1 h to reach a resting tension of 3.0 g. Following preconstriction with prostaglandin F₂ (3 nM), cumulative concentration-relaxation curves were recorded in response to increasing concentrations of ACh (10⁻⁷ to 10⁻⁵.5 M).

Blood Pressure Recordings—Systolic blood pressure was measured using the tail-cuff method in conscious trained mice (tail-cuff plethysmography, Kent Scientific CODA STD). Three measurements were taken for each mouse and averaged to yield one data point.

Statistical Analysis—Data are expressed as mean ± S.E. Statistical calculations were performed with GraphPad Prism 5 (GraphPad Software Inc, San Diego). Mann-Whitney test, paired or unpaired t test, one-way ANOVA, or Kruskal-Wallis test with post hoc Dunn test or Bonferroni test was used as appropriate. One asterisk indicates p values <0.05; two asterisks indicate p < 0.01, and three asterisks indicate p < 0.001, considered to be statistically significant.

RESULTS

ATII-induced Aortic Infiltration of Inflammatory Myelomonocytic Cells Impairs eNOS Function and Amplifies iNOS-derived NO’ Formation—In C57BL/6 mice, ATII infusion induced a drastic expansion of CD45⁺ leukocytes in the aorta (Fig. 1A) with a strong and selective increase of CD11b⁺Ly6C<sub>high</sub> monocytes, although the number and percentage of CD11b⁺Ly6C<sub>−</sub> monocytes was not significantly altered (Fig. 1B). Importantly, ATII sharply increased iNOS (Nos2) mRNA expression in whole aortic lysates in parallel with the number of iNOS-positive CD11b⁺ inflammatory cells in the aortic wall (Fig. 1, C and D). Levels of eNOS protein were increased in the aorta of ATII-infused mice. However, eNOS protein isolated from the aorta of ATII-infused mice showed a significantly higher rate of S-glutathionylation compatible with eNOS uncoupling (Fig. 1, E and F). Indeed, when we assessed endothelial reactive oxygen species formation with the O₂⁻-sensitive dye DHE, we observed an increase in vessel sections obtain from ATII-infused mice that was inhibitable by L-NAME, indicating eNOS uncoupling and activity in aortas of ATII-infused mice (Fig. 3, and Table 1). In contrast to ATII-infused control mice (Fig. 3, G and H), leaving only a background of LPS-inducible NO formation, which can be related to iNOS expressed by vascular smooth muscle cells or endothelial cells. These findings indicate the following: (i) ATII causes an increased demand for BH₄ in the vasculature, presumably to feed iNOS; and (ii) depletion of LysM<sup>−</sup> inflammatory cells has a differential and beneficial impact on eNOS function via handling BH₄ bioavailability.

iNOS Inhibition Abolishes ATII-induced Nitro-oxidative Stress and Vascular Dysfunction—To test the functional implication of iNOS for vascular dysfunction with a pharmacologic approach, we used 1400W, a specific irreversible inhibitor of iNOS (18). Acute ex vivo blockade of iNOS completely abolished the vascular NO’ signal provoked by LPS incubation (Fig. 4A) and significantly attenuated ATII-induced vascular endothelial dysfunction (assessed by the acetylcholine concentration-relaxation curve, Fig. 4B and Table 1). In contrast to C57BL/6 mice (see Fig. 1G), ATII-infused iNOS<sup>−/−</sup> mice had increased endothelial O₂⁻ formation that was not significantly different from iNOS<sup>−/−</sup> controls (Fig. 4C). Oxidative burst in whole blood and endothelial dysfunction was prevented in ATII-infused iNOS<sup>−/−</sup> mice (Fig. 4, E and F, and Table 2) that

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had an increase in systolic blood pressure comparable with ATII-infused C57BL/6 mice (Fig. 4D).

These results suggest that nitro-oxidative stress mediated by inflammatory cells in the vasculature is iNOS-derived that the majority of ATII induces iNOS expression and activity can be attributed to inflammatory cells (compare Figs. 3H and 4A), and that iNOS-derived nitro-oxidative stress causes vascular endothelial dysfunction evoked by ATII in vivo.

DISCUSSION

We present here novel data showing that CD11b\(^+\) Ly6C\(^{\text{high}}\) iNOS\(^+\) monocytes containing a functional Nox2 and the angiotensin II receptor type 1 (AT1R) are mediators of eNOS uncoupling and dysfunction in response to ATII. ATII-induced iNOS activity and expression in the vasculature critically depends on inflammatory cells and can be normalized by LysM\(^+\) cell depletion.
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The rate-limiting enzyme of BH₄ synthesis, the GTPCH, is induced in the vessel wall of ATII-infused mice as are vascular BH₄ levels; these processes are determined by the presence of LysM⁺ inflammatory cells that contain iNOS. We also provide evidence that ablation of LysM⁺ cells is beneficial for eNOS uncoupling. This results from a net effect of three synergistic processes as follows: 1st, prevention of oxidative decay of BH₄ by eliminating the leading source of nitro-oxidative stress from the system; 2nd, prevention of inflammation-induced up-regulation of the pacemaker enzyme of BH₄, the GTPCH; and 3rd, activation of the salvage pathway of BH₄ by up-regulation of vascular DHFR.

The NADPH oxidase has been implicated in eNOS uncoupling because of the fact that it is one of the predominant sources of O₂⁻ in the vasculature. For instance, deoxycorticosterone acetate salt-induced experimental hypertension is characterized by eNOS uncoupling depending on intact NADPH oxidase activity (19), and eNOS uncoupling was shown to be driven by NADPH oxidase-derived O₂⁻ in diabetes and ATII-induced vascular disease (20, 21). With our study, we can experimentally support the concept that ROS from a phagocytic NADPH oxidase acts as kindling radicals to fuel eNOS-derived O₂⁻. eNOS uncoupling is thought to be mechanistically linked to BH₄ depletion (6) presumably caused by BH₄ oxidation mediated by radicals, which may originate from phagocyte type, gp91phox-agtr1 and lev6g-high myelomonocytic cells expressing high levels of iNOS in the vessel wall. This finding supports the concept that ATII/AT,R signaling works as a fundamental proinflammatory stimulus in myelomonocytic cells in general (26, 27). It is also in line with previous data showing that ATII-induced vascular dysfunction and inflammation are IFN-γ-dependent mechanisms and that mice defective in IFN-γ formation are marked by a decreased iNOS expression and peroxynitrite burden in the vessel wall (11).

In this regard, it is conclusive that GTPCH is concordantly expressed with iNOS to supplement it with BH₄. Oxidation of BH₄ to BH₃ with a consecutive drop of vascular BH₄ levels as anticipated in a disease state of increased vascular oxidative stress (6) obviously does not outweigh the augmentation of vascular BH₃ in ATII-infused mice. Here, vascular BH₃ levels are rather coupled with an increased need for this essential cofactor of NO⁺ synthesis in a setting of an up-regulated iNOS-dependent NO⁺ formation in response to ATII. Regarding iNOS expression, our findings also indicate that ATII is a particularly potent proinflammatory impulse (10, 11), comparable with the power of classical iNOS inducers like LPS (24).

Targeted GTPCH overexpression in the endothelium has been shown to improve vascular endothelial dysfunction in diabetes and atherosclerosis (28, 29). Supplementation of exogenous BH₄ or its precursors or stimulation of endogenous BH₄ synthesis has been shown to attenuate endothelial dysfunction in experimental and/or human arterial hyper-
tension (30), hyperglycemia (31), diabetes (32), atherosclerosis (33), or cigarette smoking (34). In our hands, depletion of inflammatory cells not only prevents GTPCH overexpression (which can be harmful because it feeds iNOS with BH4) but also increases DHFR expression. This results in a net effect of elevated BH4 levels in the vessel wall sufficient to recouple eNOS, thereby providing an endogenous source of BH4 replenishment triggered by depletion of inflammatory cells. In a reversed approach, Gao et al. (35) had shown in a mouse model of eNOS uncoupling that ATII-induced abdominal aortic aneurysm formation hallmarked by vascular macrophage infiltration can be prevented by overexpression of DHFR. Together with our findings, this indicates a mutual repressive effect between DHFR expression and vascular inflammation.

Our findings strengthen the point that inflammatory cells are drivers of both eNOS uncoupling and iNOS-derived nitro-oxidative stress. A, O2•− formation in aortic tissue (endothelial scan) of sham-treated and ATII-treated LysM and monocyte-depleted LysMΔTR transgenic mice, incubated with L-NAME, or buffer. Left panel, representative DHE-photomicrotopographs. O2•− formation appears in red, in part highlighted by white arrows. Right panel, quantification, three independent experiments; one-way ANOVA, Bonferroni’s multiple comparison test are shown. B, O2•− formation in aortic tissue (endothelial scan) of sham-treated and ATII-treated monocyte-depleted LysMΔTR transgenic mice, reconstituted either with gp91phox−/− or Agtr1−/− monocytes or buffer. Upper panel, representative DHE photomicrotopographs. Lower panel, quantification, n = 4 independent experiments; paired t test is shown. C, Western blot analysis of eNOS S-glutathionylation in mouse aorta after eNOS immunoprecipitation, normalized to eNOS expression. Top, densitometry; bottom, representative original blot. Data are mean ± S.E. of protein of aortic lysates sham-treated and ATII-treated monocyte-depleted LysMΔTR and LysM controls from two to three animals/group. One-way ANOVA, Bonferroni’s multiple comparison test are shown. 2-Me, 2-mercaptoethanol; IP, immunoprecipitation. D, Western blot (WB) analysis of aortic HO-1 protein expression of sham-treated and ATII-treated monocyte-depleted LysMΔTR and LysM controls. Top, densitometry; bottom, representative original blot; one-way ANOVA, Newman-Keuls post-test n = 13–15 animals per group. *, p < 0.05; **, p < 0.01; and ***, p < 0.001.

It is important to note that depletion of LysM+ cells is an effective measure to improve vascular endothelial dysfunction (10). To further address this topic, we inhibited iNOS acutely by 1400W and could show that ATII-induced endothelial dysfunction was strongly attenuated. In addition, iNOS−/− mice...
were partially protected from eNOS uncoupling, leukocyte-derived oxidative burst, and endothelial dysfunction in ATII-induced arterial hypertension. In earlier reports, iNOS inhibition could block ATII-induced aortic aneurysm formation in SMAD3−/− mice (37) and restore endothelial function in models of vascular inflammation elicited by LPS, TNFα, or IFN-γ (38). Our data therefore indicate that iNOS induction sequelled by eNOS uncoupling substantially contributes to the pathophysiology of ATII-induced vascular injury.

In this study, we identify both inflammatory cell-determined iNOS activity and eNOS uncoupling as crucial factors for endothelial dysfunction and vascular inflammation in ATII-induced arterial hypertension. Depletion of inflammatory cells was able to restore iNOS-derived nitro-oxidative function and prevent iNOS-dependent NO formation in the aorta of ATII-infused mice.

FIGURE 3. Depletion of LysM+ cells determines BH4 bioavailability and function and prevents iNOS-dependent NO formation in the aorta of ATII-infused mice. A and C, Western blot analysis of GTPCH (n = 10–11) and DHFR (n = 13–16) protein expression in aortic homogenates of monocyte-depleted LysMIDTR and LysM controls. Top, densitometry; bottom, representative original blots. One-way ANOVA, Bonferroni’s post-test (DHFR), or Newman-Keuls post-test (GTPCH), n = 10–16 animals per group are shown. B and D, quantification of total biopterin (B) and BH4 levels (D) by HPLC with electrochemical detection in aortas of monocyte-depleted LysMIDTR and LysM controls. D, upper panel, representative chromatograms. Lower panel, quantification, n = 3–5 animals per group. E, aortic BH4/biopterin. F, representative chromatograms. G and H, aortas of LysM and LysMIDTR mice sham-treated or ATII-infused were stimulated with calcium ionophore A23187 (10 μM, G) or LPS (10 μg/ml for 19 h at 37 °C, H). eNOS activity (G) and iNOS-derived NO formation (H) was determined by measurement of NO-Fe(DETC), EPR signals. Left, mean spectra; right, quantification of signal intensity differences. Data are mean ± S.E. of n = 4–5 animals per group; one-way ANOVA, Bonferroni’s post-test are shown. *, p < 0.05; **, p < 0.01; and ***, p < 0.001.
stress and to recouple eNOS by readjusting BH₄ synthesis and bioavailability. Targeting iNOS specifically on inflammatory Ly6C<sup>high</sup> monocytes inside the vascular wall might represent a therapeutic option to attenuate vascular inflammation in hypertension and cardiovascular disease without interfering with overall nitric oxide bioavailability or innate host defense. Identification of integrating pathways that involve adaptive immune cells like T cells might offer a possibility to regulate monocyte/macrophage responses in vascular inflammation (39). This approach is appealing, because

FIGURE 4. iNOS inhibition abolishes ATII-induced vascular dysfunction. A, EPR spectra for NO-Fe²⁺-DETC complex formed from isolated mouse aorta of sham-infused and ATII-treated C57BL/6 mice after 24 h of LPS stimulation (10 μg/ml). Trap incubation was performed in the presence of 10 μM iNOS inhibitor 1400W. Treatment of aorta with 1400W totally abolished NO-Fe²⁺-DETC signal for NO formation, indicating that the signal of LPS-stimulated ATII-treated aortas is specific for iNOS. Left, mean spectra; right, quantification of signal intensity differences. Data are mean ± S.E. of n = 4 animals per group; one-way ANOVA, Bonferroni’s post-test are shown. B, relaxation of aortic segments of sham-infused and ATII-treated C57BL/6 mice in response to the endothelium-dependent vasodilator ACh was measured by isometric tension recordings. Two dose-response curves to ACh were performed consecutively. Before the second curve 10 μM iNOS inhibitor 1400W was added to aortic segments. Data are mean ± S.E. of n = 7–10 animals per group; one-way ANOVA and Bonferroni’s post hoc test for EC₅₀ and maximal relaxation (see also Table 1) are shown. C, O₂ formation in aortic tissue (endothelial scan) of sham-treated and ATII-treated iNOS<sup>+/−</sup> mice, incubated with L-NAME, or buffer. Left panel, representative DHE photomicrotopographs. O₂ formation appears in red; right panel, quantification, three independent experiments are shown; one-way ANOVA, Bonferroni’s multiple comparison test are shown. D, systolic blood pressure of sham-treated and ATII-treated C57BL/6 and iNOS<sup>+/−</sup> mice. Data are mean ± S.E. of n = 4–6 animals per group; one-way ANOVA, Bonferroni’s multiple comparison test are shown. *, p < 0.05 versus C57BL/6; #, p < 0.05 versus C57BL/6+ATII.

**TABLE 1**

| Parameter | C57BL/6 | C57BL/6 + ATII | C57BL/6 + ATII + 1400W |
|-----------|---------|----------------|------------------------|
| ACh efficacy (maximum relaxation, %) | 58.5 ± 2.5 (n = 7) | 39.7 ± 4.8 (n = 10)<sup>a</sup> | 69.7 ± 3.7 (n = 6)<sup>b</sup> |
| ACh potency (EC₅₀, log M%) | 7.1 ± 0.1 (n = 7) | 6.6 ± 0.1 (n = 10)<sup>a</sup> | 6.5 ± 0.2 (n = 6) |

<sup>a</sup> p < 0.05 versus C57BL/6.

<sup>b</sup> p < 0.05 versus C57BL/6 + ATII.
an arsenal of therapeutics is already available to modulate T cell function.

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TABLE 2

Efficacy and potency of the concentration-relaxation curves in isolated aortic rings

| Parameter | C57BL/6 | C57BL/6 + ATII | iNOS | iNOS + ATII |
|-----------|---------|---------------|------|------------|
| ACh efficacy (maximum relaxation, %) | 7.5 ± 1.0 (n = 4) | 4.3 ± 0.5 (n = 4) | 5.8 ± 0.6 (n = 6) | 7.0 ± 0.5 (n = 4) |
| ACh potency (EC50, nM) | 7.1 ± 0.1 (n = 4) | 6.5 ± 0.1 (n = 4) | 6.9 ± 0.1 (n = 6) | 6.8 ± 0.2 (n = 4) |

* p < 0.05 versus C57BL/6.
* p < 0.02 versus C57BL/6 + ATII.
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