Macrophage Mineralocorticoid Receptor Signaling Plays a Key Role in Aldosterone-Independent Cardiac Fibrosis

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Mineralocorticoid receptor (MR) activation promotes the development of cardiac fibrosis and heart failure. Clinical evidence demonstrates that MR antagonism is protective even when plasma aldosterone levels are not increased. We hypothesize that MR activation in macrophages drives the profibrotic phenotype in the heart even when aldosterone levels are not elevated. The aim of the present study was to establish the role of macrophage MR signaling in mediating cardiac tissue remodeling caused by nitric oxide (NO) deficiency, a mineralocorticoid-independent insult. Male wild-type (MRflox/flox) and macrophage MR-knockout (MRflox/flox/LysMCre/+; mac-MRKO) mice were uninephrectomized, maintained on 0.9% NaCl drinking solution, with either vehicle (control) or the nitric oxide synthase (NOS) inhibitor NG-nitro-l-arginine methyl ester (L-NAME; 150 mg/kg/d) for 8 wk. NO deficiency increased systolic blood pressure at 4 wk in wild-type L-NAME/salt-treated mice compared with all other groups. At 8 wk, systolic blood pressure was increased above control in both L-NAME/salt treated wild-type and mac-MRKO mice by approximately 28 mm Hg by L-NAME/salt. Recruitment of macrophages was increased 2- to 3-fold in both L-NAME/salt treated wild-type and mac-MRKO. Inducible NOS positive macrophage infiltration and TNFα mRNA expression was greater in wild-type L-NAME/salt-treated mice compared with mac-MRKO, demonstrating that loss of MR reduces M1 phenotype. mRNA levels for markers of vascular inflammation and oxidative stress (NADPH oxidase 2, p22phox, intercellular adhesion molecule-1, G protein-coupled chemokine receptor 5) were similar in treated wild-type and mac-MRKO mice compared with control groups. In contrast, L-NAME/salt treatment increased interstitial collagen deposition in wild-type by about 33% but not in mac-MRKO mice. mRNA levels for connective tissue growth factor and collagen III were also increased above control treatment in wild-type (1.931 ± 0.215 *vs.* 1 ± 0.073) but not mac-MRKO mice (1.403 ± 0.150 *vs.* 1.286 ± 0.255). These data demonstrate that macrophage MR are necessary for the translation of inflammation and oxidative stress into interstitial and perivascular fibrosis after NO deficiency, even when plasma aldosterone is not elevated. **(*Endocrinology* 153: 3416–3425, 2012)**

The mineralocorticoid receptor (MR) is best known for its role in regulating electrolyte and fluid homeostasis in the body. A role for MR signaling in cardiovascular disease progression has now been demonstrated in the experimental and clinical setting, although the mechanisms underpinning the translation of MR signaling into heart failure remain poorly defined. Clinical use of MR antagonists, spironolactone or eplerenone, in addition to

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Abbreviations: ANP, Atrial natriuretic peptide; CCR5, G protein-coupled chemokine receptor 5; COL3, collagen III; CTGF, connective tissue growth factor; DOC, deoxycorticosterone; ICAM, intercellular adhesion molecule-1; iNOS, inducible NOS; LysM, lysozyme M; mac-MRKO, macrophage MR knockout; MR, mineralocorticoid receptor; MRKO, MR knockout; NADPH, reduced nicotinamide adenine dinucleotide phosphate; L-NAME, NG-nitro-l-arginine methyl ester; SBP, systolic blood pressure.

current best practice therapy, has been shown to significantly reduce morbidity and mortality in patients with heart failure (1, 2). In these trials the cardiac benefits were seen even though most patients had normal or low plasma levels of aldosterone and support the hypothesis that MR in nonepithelial tissues may be activated in a mineralocorticoid-independent manner in these patients (3).

Mineralocorticoid-independent activation of the MR has now been described in several studies (4, 5). Recent evidence supports the hypothesis that in tissues where MR selectivity is not protected by 11β hydroxysteroid dehydrogenase type 2 activity, changes in redox state or tissue damage enable glucocorticoid hormones to produce equivalent agonist responses at the MR to aldosterone (6, 7). In this study we use long-term treatment with the NG-nitro-l-arginine methyl ester (L-NAME) as an aldosterone-independent mediator of cardiovascular dysfunction. Loss of NOS leads to vascular dysfunction and hypertension, which in turn leads to vascular and cardiac tissue remodeling and cardiac failure in the longer term (8–12). Although plasma aldosterone levels do not increase with L-NAME, eplerenone is protective in this model in a blood pressure-independent manner, suggesting a role for MR activation in the disease process (13). The mechanisms by which MR antagonism protects against the development of fibrosis in NO deficiency are still unknown.

Immune cell infiltration into the myocardium is a key feature of cardiac inflammation, and fibrosis and infiltrating macrophages play an essential role in the ongoing inflammatory response in the myocardium via secretion of cytokines and profibrotic factors that stimulate fibroblast differentiation and collagen production; limiting macrophage recruitment is protective in both cardiac and renal ischemia-induced apoptosis and fibrosis (14, 15). More recently, selective macrophage MR knock-out mice (mac-MRKO) have been demonstrated to be protected from mineralocorticoid-dependent [deoxycorticosterone (DOC)/salt] cardiac fibrosis, inflammation, and hypertension, thus identifying a key role for macrophage MR in the development of cardiac fibrosis (16).

The aim of the present study is to thus to establish the role of macrophage MR signaling specifically in the generation of inflammation, macrophage phenotype, and tissue fibrosis in response to long-term (8 wk) NO deficiency in which plasma aldosterone is not elevated. We hypothesized that L-NAME/salt-induced cardiac oxidative stress, inflammation, and cardiac fibrosis would be attenuated by cell-specific deletion of the MR in macrophages, independent of macrophage recruitment or blood pressure changes. Our data show that mac-MRKO mice are protected from cardiac fibrosis despite the presence of oxidative stress, inflammation, inflammatory cell infiltration, and hypertension.

Materials and Methods

Macrophage-specific MRKO mice

All protocols were approved by the Monash University Animal Ethics Committee. Macrophage-specific MRKO mice were generated using the cre-lox approach where mice expressing cre recombinase under the control of the lysozyme M (LysM) promoter were crossed with mice containing the MRflox allele. The LysM promoter can regulate Cre recombinase in multiple cell types in the myeloid lineage. These mice thus have MR deleted specifically from macrophages and other cells of the myeloid lineage including neutrophils and granulocytes. MR deletion was validated by PCR analysis of genomic DNA from tail tips and Western blot analysis of bone marrow macrophages from KO and MR*flox/flox* control mice as previously described (16). Clausen *et al.* (17) demonstrated a deletion efficiency of 83–98% in mature macrophages using this technique. Based on the characteristics of the LysM promotor employed, it should be noted that there is potential for loss of MR signaling in other cell types in addition to monocytes/macrophages including granulocytes and, to a lesser extent, dendritic cells.

L-NAME/salt model of hypertension and cardiac fibrosis

Male mice at 8 wk of age were uninephrectomized and given 0.9% sodium chloride (NaCl) drinking solution containing L-NAME (Sigma Chemical Co., St. Louis, MO). A preliminary study was carried out in wild-type mice to determine the optimum dose of L-NAME to be administered (150 mg/kg/d). Wild-type and mac-MRKO mice were then randomized to receive either vehicle or L-NAME/salt treatment for 8 wk resulting in four treatment groups: wild-type control treated, wild-type L-NAME treated (WT L-NAME), macrophage MRKO control-treated, mac-MRKO L-NAME treated (KO L-NAME).

Systolic blood pressure (SBP) measurement

SBP measurements were taken from pretrained, warmed mice using tail-cuff plethysomography (ITTC Life Science, Woodland Hills, CA) as previously described (16). To ensure reliable and reproducible SBP readings, mice were trained biweekly for 3 wk before SBP was recorded (18, 19).

Cardiac tissue collection and analysis

All mice were killed after 8 wk of treatment by 70% CO_2 in air. Blood was collected by cardiac puncture. The heart was excised, weighed, and divided along the mid-coronal plane. The apex was snap frozen and processed for quantitative real-time RT-PCR analysis for mRNA expression as previously described (Supplemental Table 1, published on The Endocrine Society's Journals Online website at http://endo.endojournals.org) (16).

RNA extraction and real time RT-PCR

RNA was isolated from snap-frozen cardiac tissue samples using the RNeasy RNA extraction kit (Qiagen Pty Ltd., Hilden, Germany) and incubated with Ambion DNA-free Dnase treat-

ment (Applied Biosystems, Foster City, CA) according to the manufacturer's guidelines. Quantitative RT-PCR amplification was performed on the Lightcycler (Roche Diagnostics GmbH, Mannheim, Germany), using the Lightcycler FastStart DNA Master SYBR Green 1 Kit (Roche Diagnostics) and primer sets listed in Supplemental Table 1.

Immunohisctochemistry

Paraffin-embedded, 5 μm-thick heart sections were incubated overnight at 4 C with MAC2 (1:200; eBioscience, San Diego, CA), vascular endothelial growth factor (1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), connective tissue growth factor (CTGF) (1:400; Abcam, Cambridge, MA), TGF-β (1:600; Santa Cruz Biotechnology) or inducible NOS (iNOS) (Abcam) followed by the appropriate biotinylated secondary antibody for 2 h at room temperature and then incubated with ABC complex (Vectastain, Vector Laboratories, Burlingame, CA) for 45 min. Positive staining was visualized by incubation with 3,3′-diaminodenzidine (DAKO Corp., Carpinteria, CA), and the tissues were counterstained with hemotoxylin. Immunostaining with NADPH oxidase 2 (NOX2) (1:100, BD Biosciences, Palo Alto, CA) and osteopontin (1:100; Developmental Studies Hybridoma Bank, University of Iowa, IA) was performed using the Dako Animal Research Kit (DAKO) according to the manufacturer's instructions. The appropriate negative IgG was used as a negative control for each primary antibody. Histological analysis for collagen was performed on tissues stained with 0.1% Sirius Red (Sigma-Aldrich, Castlehill, Australia) as previously described (16, 19, 20). Tissue sections were sampled using a systematic, unbiased approach in which the identity of each section was masked. Approximately 20 fields were sampled per tissue for determination of percent collagen area. A semiquantitative scoring approach was used to asses vessel staining, and an optical dissector approach was used to quantify infiltration of macrophages using the Computer-Assisted Stereological Toolbox (C.A.S.T.-GRID) software package, version 1.10 (Olympus DK A/s, Albertslund, Denmark).

RIA

Plasma concentration of atrial natriuretic peptide (ANP), a hormone secreted by cardiomyocytes involved in vasodilation, corticosterone, and aldosterone were determined using ImmunChem Double Antibody RIA kits (MP Biomedicals, Irvine, CA) as per manufacturer's instructions.

Statistical analysis

All data sets, excluding vessel staining and plasma corticosterone, were analyzed using a two-way ANOVA. The Bonferroni *post hoc* test was used to identify significant differences between means of different groups. For nonparametric data (vessel staining), a median value was determined for each heart, and data are presented as the median for each treatment group ± interquartile range. For data that did not follow a Gaussian distribution the Kruskal-Wallis test with the Dunns *post hoc* test was applied. Differences between group means were considered significant at $P < 0.05$, and data are reported as mean ± SEM. All data were analyzed using Graphpad Prism statistical software package (Version 5.03; GraphPad Software Inc., San Diego, CA).

Results

mac-MRKO mice

Untreated mac-MRKO mice exhibit normal phenotype, body, and heart weight. Deletion of MR from macrophages was validated using Western blots and PCR as previously described (16). Wild-type control mice had higher plasma ANP compared with all other treatment groups and higher plasma corticosterone compared with mac-MRKO control mice. Mac-MRKO mice showed no increase in plasma levels of aldosterone, corticosterone, or ANP compared with wild-type mice (Table 1).

NO deficiency-induced cardiac fibrosis

Structural changes in the heart have been shown to be associated with both qualitative and quantitative changes in fibrillar collagen content in the heart (21, 22). L-NAME/salt treatment for 8 wk significantly increased interstitial collagen content in wild-type but not mac-MRKO mice (Fig. 1A and Supplemental Fig. 1, A–D). Perivascular fibrosis around large, multilayered blood vessels was more extensive in wild-type compared with mac-MRKO mice, indicating a significant genotype effect (Fig. 1B and Sup-

TABLE 1. Hemodynamic, cardiac, and plasma parameters at 8 wk of age

Parameter	WT CON	WT L-NAME	mac-MRKO CON	mac-MRKO L-NAME
Body weight (g)	30.0 ± 1.00	29.6 ± 0.47	29.6 ± 0.67	28.5 ± 0.57
Heart weight/body weight (mg/g)	5.41 ± 0.27	5.85 ± 0.13	5.64 ± 0.15	5.89 ± 0.15
SBP at 8 wk (mm Hg)	99.0 ± 1.00	128 ± 5.68[a]	100 ± 4.08	127 ± 8.01[a]
SBP at 4 wk (mm Hg)	104 ± 2.91[bc]	131.67 ± 3.82[ac]	97.5 ± 1.34	100.0 ± 0
Brain natriuretic peptide (relative mRNA expression)	1.00 ± 0.12	1.43 ± 0.13[b]	1.02 ± 0.11	1.33 ± 0.19[b]
Plasma ANP (pg/ml)	737 ± 25.1[c]	479 ± 39.5	339 ± 82.7	337 ± 17.0
Plasma aldosterone (pg/ml)	46.6 ± 12.7	17.7 ± 7.02	19.4 ± 11.5	10.1 ± 2.75
Plasma corticosterone (ng/ml)	281 ± 49.6[e]	183 ± 62.3	84.1 ± 15.5	208 ± 43.5

Treatment groups are as follows: WT CON, untreated wild-type mice; WT L-NAME, wild-type mice treated with L-NAME/salt; KO CON, untreated macrophage-specific MRKO mice; KO L-NAME, macrophage-specific MRKO mice treated with L-NAME/salt.

[a] $P < 0.001$ *vs.* WT CON and KO CON; [b] $P < 0.05$ *vs.* WT CON and KO CON. [c] $P < 0.05$ *vs.* KO L-NAME. [d] P]lt] 0.05 *vs.* WT L-NAME; KO CON, KO L-NAME; [e] $P < 0.05$ *vs.* KO CON.

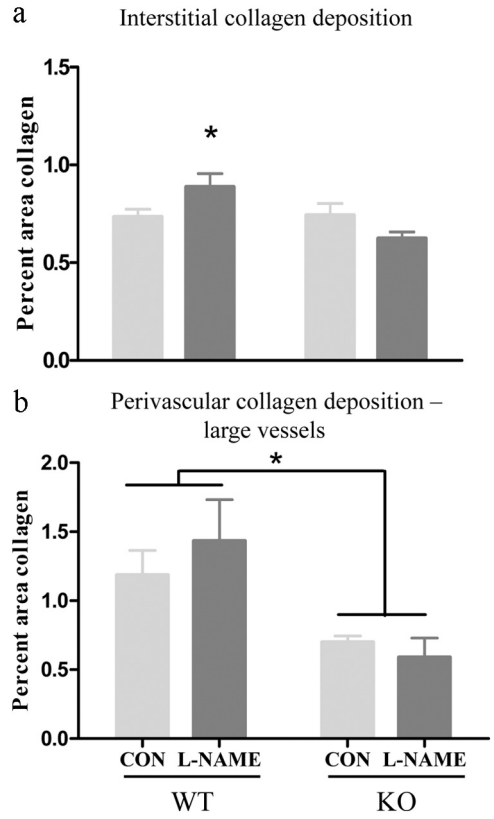

FIG. 1. mac-MRKO are protected from increased cardiac tissue fibrosis. Treatment groups as follows: WT CON, untreated wild-type mice; WT L-NAME, wild-type mice treated with L-NAME/salt; KO CON, untreated macrophage-specific MRKO mice; KO L-NAME, macrophage-specific MRKO mice treated with L-NAME/salt. A, L-NAME/salt for 8 wk significantly increased interstitial collagen in WT L-NAME compared with all other treatment groups (*, $P < 0.05$ *vs.* WT CON, KO CON, KO L-NAME). B, Perivascular collagen was increased around large blood vessels in wild-type mice compared with KO (genotype effect, *, $P < 0.05$ WT *vs.* KO). Values are expressed as mean ± SEM (n = 8–12).

plemental Fig. 1, A–D); perivascular fibrosis around small and medium vessels was not different between groups.

NO deficiency significantly increased mRNA expression for the profibrotic factor CTGF (23) and the structural collagen isoform collagen III (COL3) in wild-type but not mac-MRKO mice (Fig. 2, A and B). CTGF protein expression in small and medium vessels was also increased in wild-type L-NAME/salt-treated mice only (Fig. 3, A and B).

Qualitative assessment of COL3 immunostaining showed a tissue distribution similar to that for Sirius red staining. mRNA levels for type 1 plasminogen activator inhibitor, a member of the serine protease inhibitor gene family involved in the tissue fibrotic response (24), the profibrotic cytokine TGF-β (25), and collagen isoform COL1 were unchanged (Fig. 2, C and D, and Supplemental Fig. 2A). These data show that loss of macrophage MR function reduces expression of L-NAME/salt-induced profibrotic signals (CTGF) and interstitial collagen deposition.

NO deficiency-induced oxidative stress

To understand the mechanism of cardiac fibrosis suppression in mac-MRKO mice, effectors of fibrosis were examined. To determine the level of cardiac oxidative stress caused by L-NAME/salt treatment, gene expression of two subunits of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a key source of vascular and inflammatory cell reactive oxygen species, were assessed by quantitative RT-PCR. NOX2, the phagocytic oxidase component expressed in macrophages and vascular endothelial cells, and the more widely expressed p22phox both showed increased mRNA levels in wild-type and mac-MRKO mice compared with control-treated mice (Fig. 4, A and B). mRNA for glucose-6-phosphate dehydrogenase, a rate-limiting enzyme in the pentose phosphate pathway that is involved in NADPH production and preserving NO levels, showed no significant change in any treatment group (Supplemental Fig, 2B) (26). These data indicate that there is a similar increase in superoxide production in L-NAME/salt-treated mice in both genotypes.

NO deficiency-induced expression of proinflammatory genes and vascular inflammation

To further characterize the cardiac response to L-NAME/salt, markers of inflammation and macrophage infiltration were assessed. L-NAME/salt treatment for 8 wk significantly increased mRNA levels for intercellular adhesion molecule-1 (ICAM-1), but not macrophage chemoattractant protein 1, in both wild-type and mac-MRKO mice compared with control mice (see Fig. 5 and Supplemental Fig. 2C). Expression of G protein-coupled chemokine receptor 5 (CCR5) was also significantly increased in both wild-type and mac-MRKO mice compared with control mice (Fig. 6B). TNFα expression was significantly increased in L-NAME/salt-treated mice compared with control mice of the same genotype. Wild-type L-NAME/salt-treated mice had significantly higher expression of TNFα compared with mac-MRKO L-NAME/salt-treated mice (Fig. 6C). Immunostaining analysis demonstrated that protein levels of vascular endothelial growth factor, ICAM-1, and osteopontin were not significantly altered by genotype or treatment. These data show that L-NAME/salt equivalently increased the expression of a subset of inflammatory genes in wild-type and mac-MRKO mice.

Macrophage recruitment and phenotype in response to NO deficiency

The inflammatory response to L-NAME/salt administration evaluated by determining the number of infiltrating macrophages that stained positive for MAC2 and

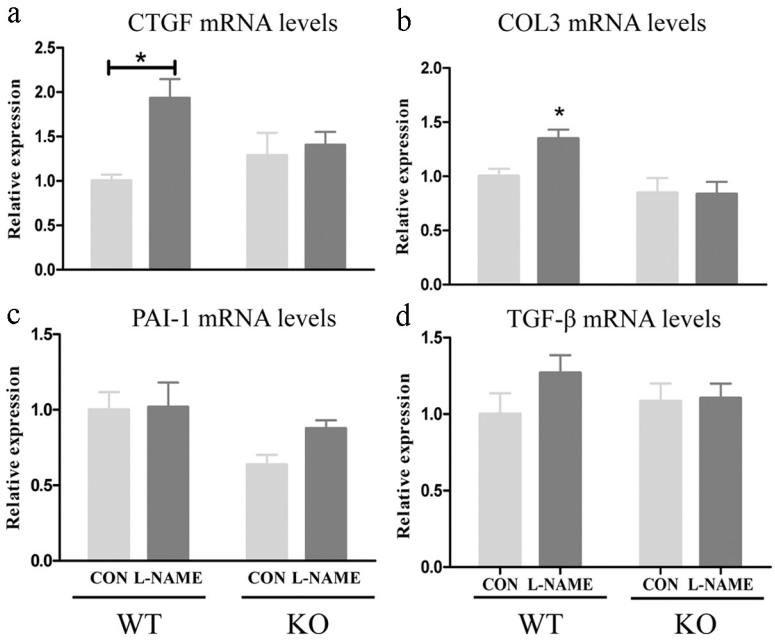

FIG. 2. Profibrotic gene expression is not increased in mac-MRKO in response to NO deficiency. Treatment groups as for Fig. 1. A, L-NAME/salt treatment increased cardiac mRNA levels for CTGF in WT L-NAME compared with WT CON (*, $P < 0.05$ *vs.* WT CON). B, L-NAME/salt treatment increased cardiac mRNA levels for COL3 in WT L-NAME compared with all other treatment groups (*, $P < 0.05$ *vs.* WT CON, KO CON, KO L-NAME). C, L-NAME/salt did not alter mRNA expression of type 1 plasminogen activator inhibitor (PAI-1) in either genotype. D, L-NAME/salt did not alter mRNA expression of TGF-β in either genotype. Values are expressed as mean ± SEM (n = 8–12).

NOX2. L-NAME/salt treatment resulted in an equivalent 2- to 3-fold increase in the number of infiltrating MAC2- and NOX2-positive macrophages in both wild-type (2.8 ± 0.2 cells per field) and mac-MRKO mice (2.8 ± 0.4 cells per field) compared with control-treated mice (~1.1 ± 0.1 cells per field; Fig. 5, A and B). The extent of macrophage infiltration was not different in mac-MRKO and wild type with either treatment (Supplemental Fig. 3, A–H), indicating that loss of macrophage MR signaling does not alter macrophage infiltration. These data suggest that MR activation in other cell types, such as endothelial cells, is responsible for chemoattractant signaling and macrophage infiltration in NO deficiency. Markers of macrophage phenotype were evaluated by immunostaining and RT-PCR for M1 and M2 cytokines. INOS-positive macrophage infiltration was increased in wild-type L-NAME/salt-treated mice compared with all other treatment groups (Fig. 5C), and the ratio of iNOS/Mac2 macrophages was greater in wild-type L-NAME/salt treated mice compared with mac-MRKO L-NAME treated mice (Fig. 5D). mRNA levels of the M2 markers Arg1 and CD163 were not different across genotype or treatment groups (Supplemental Table 2). These data suggest that loss of MR in macrophages decreases M1 phenotype, but does not change the M2 phenotype, in response to NO deficiency (Fig. 6).

NO deficiency-induced hypertension and cardiac hypertrophy

To examine the role of macrophage MR signaling on blood pressure regulation, SBP was measured at 4 and 8 wk of L-NAME/salt treatment. At 4 wk, SBP was significantly increased in wild-type L-NAME/salt-treated mice compared with all other groups. Wild-type control mice had higher SBP compared with mac-MRKO control mice. At 8 wk of L-NAME/salt treatment the mean SBP of control treated mice was 99 ± 1 mm Hg in wild-type and 100 ± 4.1 mm Hg in mac-MRKO mice. These SBP are considered normal mouse SBP. L-NAME/salt treatment for 8 wk significantly increased mean SBP in both wild-type (128.3 ± 5.7 mm Hg) and mac-MRKO (127.8 ± 8.0 mm Hg) mice (Table 1). This increase of approximately 20% was a statistically significant treatment effect, and no difference was observed between the means of L-NAME/salt-treated wild-type and mac-MRKO mice. The heart weight/body weight ratio was not significantly increased by L-NAME/salt treatment in wild-type or mac-MRKO mice (Table 1). mRNA expression levels of brain natriuretic peptide, a protein secreted by cardiac ventricles in response to cardiomyocyte stretching and hypertrophy, were significantly increased by NO deficiency in wild-type and mac-MRKO mice compared with

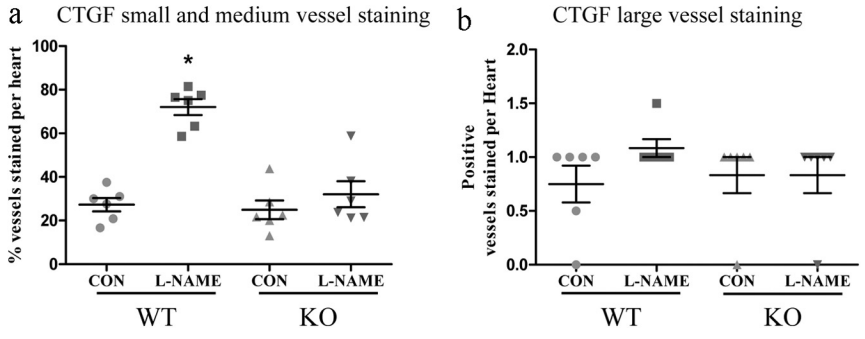

FIG. 3. L-NAME-induced vascular CTGF expression is attenuated in mac-MRKO. Treatment groups as for Fig. 1. A, Quantification of CTGF staining in small and medium vessels (*, $P < 0.05$ *vs.* WT CON, KO CON, KO L-NAME). B, Quantification of CTGF staining in large vessels. Values are expressed as mean ± SEM (n = 6).

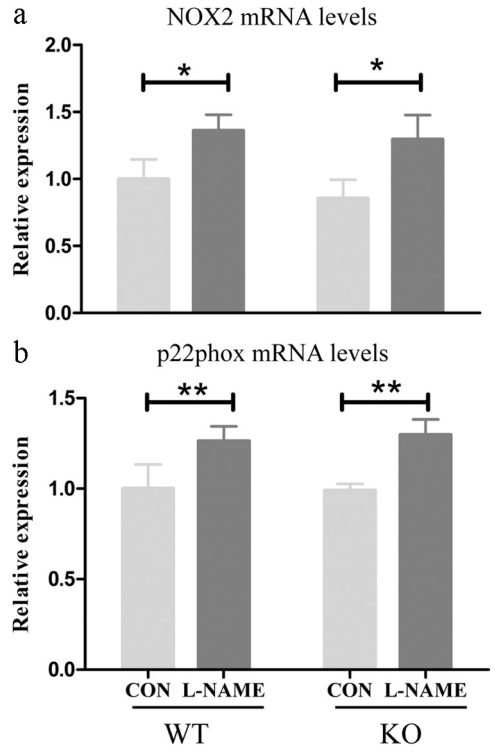

FIG. 4. Oxidative stress is equivalently increased in wild-type and mac-MRKO with L-NAME treatment. Treatment groups as in Fig. 1. A, The NADPH oxidase subunit NOX2 is increased by L-NAME/salt treatment in WT L-NAME and KO L-NAME mice (*, $P < 0.05$ *vs.* WT CON, KO CON). B, L-NAME treatment significantly increases expression of the NADPH oxidase subunit p22phox in WT L-NAME and KO L-NAME mice (**, $P < 0.001$ *vs.* WT CON, KO CON). Values are expressed as mean ± SEM (n = 8–12).

control treatment (Table 1). These data show that changes seen between wild-type and mac-MRKO are not due to hemodynamic effects.

Discussion

This study demonstrates that mac-MRKO mice are selectively protected from increased interstitial fibrosis in response to L-NAME/salt. These studies have identified a role for macrophage MR signaling in regulating CTGF and COL3 mRNA and protein levels that are major contributors to the fibrotic response. Moreover, the reduced fibrotic response in mac-MRKO mice is independent of vascular oxidative stress, macrophage infiltration, and increased SBP, which were equivalent to wild-type mice. Together, these data demonstrate a central role for macrophage MR signaling in cardiac tissue remodeling independent of mineralocorticoid status.

Macrophage MR signaling and cardiac fibrosis

We recently identified a key role for macrophage MR signaling in driving cardiac oxidative stress, inflammation, fibrosis, and hypertension in DOC/salt hypertensive mice (16). The present study demonstrates that mac-MRKO mice are similarly protected when subjected to a model of cardiovascular fibrosis independent of mineralocorticoid excess, which suggests both a central role for the macrophage MR in driving the tissue fibrotic response and a mechanism that may contribute to the protective effects of eplerenone/spironolactone in the absence of high-plasma aldosterone observed in a series of large clinical trials (1, 2).

Our data support the hypothesis that MR activation specifically in macrophages determines the development of tissue fibrosis. Previous studies in mice lacking expression of chemoattractant signals, and thus a global reduction in macrophage number, show limited tissue remodeling and damage (14, 15). The present study provides evidence that the MR determines the profibrotic signaling function of the macrophage and facilitates the onset of fibrosis. Our new data also show tissue fibrosis to be independent of cardiac hypertrophy, consistent with previous studies. A significant increase in fibrosis was detected in wild-type mice treated with DOC/salt. The possibility exists that some small degree of myocyte loss may occur (given lack of hypertrophy), but given that less than 1% of tissue volume is involved this level of myocyte loss could not be quantified in this context.

A recent study using the same mac-MRKO mouse model but using a 2-wk L-NAME/angiotensin II treatment similarly demonstrated a decrease in cardiac fibrosis in the knockout mice and also a decrease in macrophage infiltration into the myocardium (27). This raises the question whether the reduction in fibrosis is due to lack of macrophage MR signaling or lack of macrophage infiltration into the myocardium. Our data clearly demonstrate that macrophage infiltration into the myocardium is intact but the actions of the macrophages are altered. Further work to evaluate different structural portions of the myocardial vasculature with respect to macrophage infiltration will be informative.

The mRNA levels for profibrotic mediators such as CTGF and COL3 were up-regulated in WT compared with mac-MRKO mice, suggesting a potential signaling mechanism for the protective effects observed. A reduction in COL3 levels is consistent with our previous study in which tissue fibrosis was limited in macrophage MR-null mice (16). Here we show a reduction in CTGF by RT-PCR and immunostaining. Prominent CTGF expression was seen in the vessel wall and, to a lesser extent, in the inflammatory infiltrate and cardiomyocytes. Significantly less CTGF staining was seen in the vessel wall in

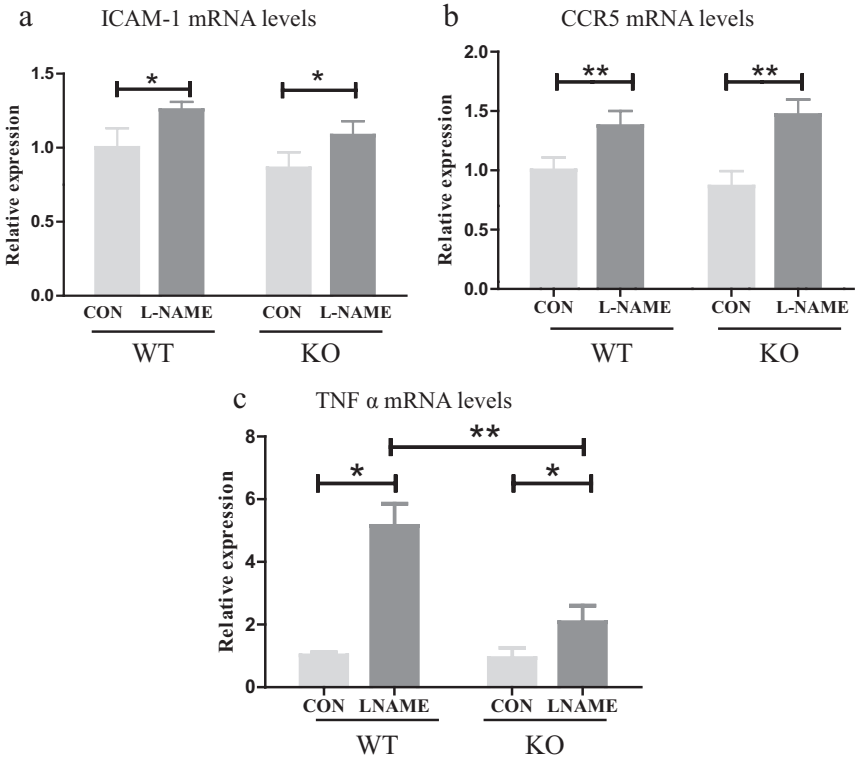

FIG. 5. NO deficiency increases inflammatory gene expression in wild-type and mac-MRKO. Treatment groups as for Fig. 1. A, L-NAME/salt for 8 wk significantly increased mRNA levels of ICAM-1 in mice of both genotypes (*, $P < 0.05$ *vs.* WT CON, KO CON). B, L-NAME/salt for 8 wk significantly increased mRNA levels of CCR5 in mice of both genotypes (**, $P < 0.001$ *vs.* WT CON, KO CON). C, L-NAME/salt for 8 wk significantly increased mRNA levels of TNFα in mice of both genotypes (*, $P < 0.05$ *vs.* WT CON, KO CON). TNFα expression was increased in wild-type treated mice compared with mac-MRKO treated mice (**, $P < 0.01$ *vs.* KO CON, KO L-NAME). Values are expressed as mean ± SEM (n = 8–12).

mac-MRKO mice. These differences suggest that macrophage MR signaling may regulate CTGF synthesis in other cell types, *e.g.* the cardiomyocyte that produces high levels of CTGF. Taken together, data for COL3 and CTGF suggest a mechanism whereby macrophage MR activation regulates collagen production in the myocardium. The present study thus builds on current knowledge and is the first to demonstrate that mac-MRKO mice are protected in an aldosterone-independent treatment model and that cardiac responses downstream of NO deficiency are macrophage-MR dependent (27).

The role of macrophage MR signaling in NO deficiency

After 8 wk of L-NAME/salt administration, vascular oxidative stress and inflammation (NOX2, p22PHOX) and expression of chemoattractant markers (CCR5, ICAM-1) are similar between the two genotypes, consistent with well-characterized responses to L-NAME/salt administration in cell types other than macrophages. However, NO signaling is critical for not only vascular tone but for macrophage function in both normal and disease conditions (28). Low levels of NO in macrophages are protective whereas high levels, due to iNOS activation, can induce apoptosis (29–31). Given that a recent study suggests that MR-null macrophages cannot increase iNOS activity in response to angiotensin II plus L-NAME (27), it may be that by limiting the level of NO production in macrophages and thus limit inflammation and fibrosis within the myocardium. A recent study suggests L-NAME/salt is sufficient to increase iNOS-positive macrophage infiltration in wild-type but not mac-MRKO mice and that the ratio of iNOS-positive macrophages to total macrophage number is also greater in L-NAME/salt-treated wild-type mice *vs.* mac-MRKO mice. These data demonstrate that although total macrophage infiltration is increased in both genotypes with L-NAME/salt treatment, loss of MR results in a reduction in iNOS expression. A second marker of M1 macrophage polarization, TNFα, was also decreased, consistent with a reduction in classical (M1) macrophage activation in macrophage MR-null mice.

Aldosterone-independent regulation of macrophage MR signaling

Given the absence of 11β-hydroxysteroid dehydrogenase type 2 (11-βHSD2) activity in macrophages, under normal circumstances MR signaling in these cells is not expected to be driven by mineralocorticoids but rather by the more abundant glucocorticoids (cortisol or corticosterone) (32–34). Glucocorticoids have equivalent high affinity for the MR and are therefore most likely to occupy the receptor in nonepithelial tissues, as is the case in cardiomyocytes and neurons (6, 35) Evidence shows that signaling by glucocorticoid-bound MR may oppose aldosterone responses but, in the presence of oxidative stress or tissue injury, are equivalent to aldosterone-bound MR, even without a change in receptor occupancy by corticosterone (5, 36, 37). In our study L-NAME promoted tissue inflammation and oxidative stress in an MR-dependent manner without change in either corticosterone or aldosterone level (WT L-NAME *vs.* KO L-NAME). Several recognized molecular markers of MR activation are elevated by the L-NAME treatment in both WT and KO (*i.e.*

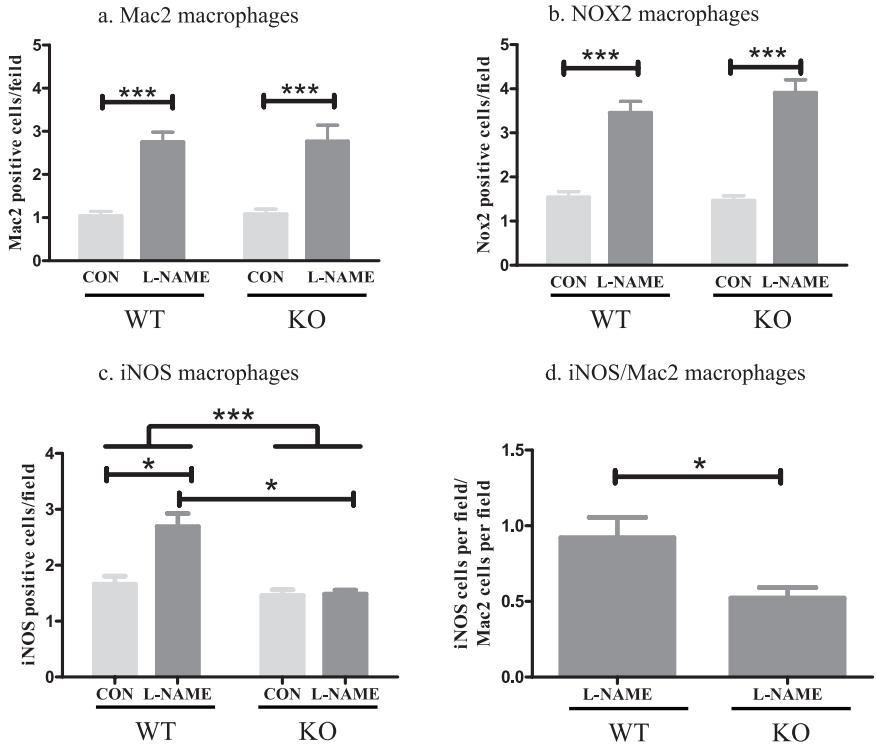

FIG. 6. NO deficiency increases macrophage infiltration in wild-type and mac-MRKO. Treatment groups as for Fig. 1. A, L-NAME/salt for 8 wk significantly increased the number of infiltrating Mac2-positive macrophage in mice of both genotypes (***, $P < 0.0001$ *vs.* WT CON, KO CON). B, L-NAME/salt for 8 weeks significantly increased the number of infiltrating Nox2-positive macrophage in mice of both genotypes (***, $P < 0.0001$ *vs.* WT CON, KO CON). C, L-NAME/salt treatment significantly increased iNOS-positive macrophage infiltration in wild-type treated mice compared with all other treatment groups (*, $P < 0.05$ *vs.* WT CON, KO CON, KO L-NAME). Mac-MRKO mice had decreased number of iNOS-positive macrophages compared with wild type regardless of treatment (***, $P < 0.0001$ WT CON and L-NAME *vs.* KO COM and L-NAME). D, L-NAME/salt treatment increased the ratio of iNOS-positive macrophage to total macrophage number in wild-type mice compared with mac-MRKO mice (*, $P < 0.05$). Values are expressed as mean ± SEM (n = 8–12).

ICAM1, NOX2, and p22 phox). This indicates that altered levels of these markers reflect up-regulated MR signaling in nonmacrophage cell types, probably cardiomyocytes and/or vascular cell types. Changed levels of some other molecular markers known to be MR activated can only be seen in WT-treated but not KO-treated animals (*i.e.* CTGF and COL3), indicating that MR signaling in macrophages may be deemed responsible. These data suggest a potential mechanism for the protection seen in clinical settings of cardiovascular disease without elevated plasma aldosterone. Further studies to directly demonstrate nuclear MR localization are now warranted to further probe the mechanisms at a cellular level.

Although MR activation can directly increase macrophage superoxide production, consistent with promoting a proinflammatory (M1, Th1) phenotype (38, 39), it is normally alternatively activated macrophages that are involved in type II inflammation, tissue remodeling, and angiogenesis (M2, Th2 responses) (40, 41). The role of MR in regulating one or both of the M1 or M2 phenotypes remains to be clearly defined. Previous studies have demonstrated that loss of macrophage MR drives an M2 phenotype during lipopolysaccaride stimulation. It is important to note that although lipopolysaccaride, an endotoxin that derives from gram-negative bacteria, is a strong stimulator of inflammation, these responses may not be relevant to cardiovascular disease; a more relevant pathophysiological stimulus is interferon-γ. Our data show, in contrast to Usher *et al.*, that *in vivo* loss of macrophage MR causes loss of M1 phenotype but does not increase M2 phenotype, which suggests that cardiac tissue macrophages have lost their M1 proinflammatory phenotype but have not progressed into a secondary stage of the inflammatory response leading to increased fibrosis. It may be that instead of macrophages being either M1 or M2, an intermediate phenotype exists in these mice. In any case, our data are consistent with a central role for macrophage MR in cardiac remodeling, regardless of the disease stimulus. Further studies using cultured macrophages are an important next step in identifying the signaling molecules involved in the development of cardiac fibrosis.

Macrophage MR signaling and SBP regulation

Mac-MRKO mice treated with L-NAME/salt showed an equivalent increase in SBP to wild-type mice at 8 wk. In wild-type mice this SBP could be detected earlier, at 4 wk, but interestingly in neither case could significant cardiac hypertrophy be detected in association with SBP elevation. These data differ from our previous study in DOC/salt-treated mac-MRKO mice, which were protected from increased blood pressure at both 4 and 8 wk of treatment. Although the magnitude of increase in SBP to L-NAME is similar to that observed in previous studies (16), the mechanisms inducing hypertension clearly differ for these treatments. That NO deficiency promotes hypertension independently of macrophage function may not be surprising given the importance of NO signaling in endothelial-mediated vascular reactivity (42). Importantly, the development of cardiac fibrosis in this model

is clearly independent of SBP changes, consistent with previous work (43, 44).

Conclusion

Mac-MRKO mice are protected from L-NAME/salt induced cardiac fibrosis. Our study has demonstrated increased macrophage infiltration in response to 8 wk of NO deficiency, supporting our previous data showing similar cardiac macrophage infiltration in a state of mineralocorticoid excess (16). In both studies mac-MRKO mice are protected from increased interstitial fibrosis. The current study clearly demonstrates that attenuation of macrophage MR signaling is beneficial even when tissue damage such as oxidative stress has occurred and a clear shift in macrophage phenotype away from a proinflammatory state. These data suggest that some of the clinical benefits of MR antagonists observed when plasma aldosterone levels are normal may be due, at least in part, to a macrophage MR-dependent mechanism and further suggest that targeting MR activation in macrophages may have potential clinical benefits for the treatment of heart failure.

Differences in macrophage infiltration and SBP between this and other studies indicate that MR activation in other cell types or other unknown signaling pathways are also playing a role in heart failure. Evidence emerging from other cell-selective MRKO mouse models, such as the cardiomyocyte MRKO, demonstrates a role for MR signaling in many cell types within the myocardium in the development of cardiac fibrosis (16, 45, 46). Together, these data indicate that the progression of heart failure and fibrosis is complex but that the macrophage MR is a central mechanism through which multiple pathological pathways converge to promote cardiac fibrosis.

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