Neutrophil Gelatinase-Associated Lipocalin Is a Novel Mineralocorticoid Target in the Cardiovascular System


Abstract—Mineralocorticoid receptor (MR) activation may be deleterious to the cardiovascular system, and MR antagonists improve morbidity and mortality of patients with heart failure. However, mineralocorticoid signaling in the heart remains largely unknown. Using a pan-genomic transcriptomic analysis, we identified neutrophil gelatinase-associated lipocalin (NGAL or lipocalin 2) as a strongly induced gene in the heart of mice with conditional and targeted MR overexpression in cardiomyocytes (whereas induction was low in glucocorticoid receptor–overexpressing mice). NGAL mRNA levels were enhanced after hormonal stimulation by the MR ligand aldosterone in cultured cardiac cells and in the heart of wild-type mice. Mineralocorticoid pathological challenge induced by nephrectomy/aldosterone/salt treatment upregulated NGAL expression in the heart and aorta and its plasma levels. We show evidence for MR binding to an NGAL promoter, providing a mechanism for NGAL regulation. We propose that NGAL may be a marker of mineralocorticoid-dependent injury in the cardiovascular system in mice. (Hypertension. 2012;59:966-972.) 

Key Words: transgenic mice ■ cardiomyocyte ■ cardiac cell lines ■ chromatin immunoprecipitation ■ mineralocorticoid receptor

Inappropriate mineralocorticoid signaling has been shown to play an important role in the progression of cardiovascular (CV) disease. Aldosterone (Aldo) is a main regulator of renal sodium reabsorption, with an overall effect on volemia and blood pressure. Aldo binds to the mineralocorticoid receptor (MR), a transcription factor of the nuclear receptor family present in the kidney. Extrarenal pathophysiological effects of this hormone have been characterized, extending its actions to the CV system, the brain, the adipose tissue, the skin, and the eye. Inappropriate MR activation has been shown to promote cardiac fibrosis in experimental models. The Randomized Aldactone Evaluation Study, Eplerenone Post-Acute Myocardial Infarction Heart Failure Efficacy and Survival Study, and Eplerenone in Mild Patients Hospitalization and Survival Study in Heart Failure clinical trials have demonstrated that the addition of the MR antagonists spironolactone or eplerenone to standard care markedly reduced the overall and CV mortality in patients with left ventricular systolic dysfunction and mild or severe symptoms of chronic heart failure (HF) or with HF signs after acute myocardial infarction.

The molecular mechanisms of Aldo and MR activation in the CV system are not yet fully established. A better understanding of these mechanisms may unveil novel biotargets for pharmacological modulation of the signaling cascades induced by mineralocorticoids in CV diseases.

In this study we identified neutrophil gelatinase-associated lipocalin (NGAL) in a pan-genomic transcriptomic analysis performed on hearts of transgenic mice that overexpress the MR in cardiomyocytes. NGAL (lipocalin 2 or 24p3) is a 25-kDa glycoprotein belonging to the lipocalin superfamily. The cell-specific roles of NGAL remain elusive, but enhanced NGAL in tissues, plasma, or urine has been reported in several pathological states, such as kidney failure and obesity, and many other situations. Increased systemic and myocardial expressions of NGAL have been observed in clinical and experimental HF. Hormonal regulation of NGAL expression has not been investigated. Here we document NGAL induction after mineralocorticoid challenge, in vivo in the mouse heart and ex vivo in cultured cells.
Cardiac NGAL mRNA was strongly enhanced in MRcardio mice; the induction is essentially MR dependent and not GR dependent, because only (MRcardio mice) identified NGAL as a gene highly induced in the heart of MRcardio mice; the induction is essentially MR dependent and not GR dependent, because cardiac NGAL mRNA was strongly enhanced in MRcardio mice (≈150 fold compared with the control littermates), whereas GR overexpression (in GRcardio mice) led to a much smaller (≈5-fold) increase in NGAL mRNA (Figure 1A). Treatment of MRcardio mice with the MR antagonist canrenoate (MR antagonist RU38486, or GR (GRcardio) in cardiomyocytes and their littermate controls. MRcardio mice were treated for 8 weeks with the MR antagonist canrenoate (++MRA) or with doxycycline (+Dox) to switch off the human MR expression. NGAL mRNA levels (quantitative PCR) were normalized for ubiquitin C expression as an internal control. Values in control mice were set as 1, and fold changes are shown on the Figure. Number of mice per condition is indicated below the bars. **P<0.01, *P<0.05 was considered significant.

**Methods**

**Animals**
The use of mice was in accordance with the guidelines of the European Community and approved by our institutional animal care and use committees. For conditional MR or glucocorticoid receptor (GR) overexpression in cardiomyocytes, the previously described double-transgenic MRcardio (α-miosin heavy chain-tetracyclin transactivator/tetracyclin response element [TetA/tetO]-human MR) and GRcardio (α-miosin heavy chain-TetA/tetO-human GR) were used and compared with their control littermates.

Wild-type mice were treated with Aldo alone (low or high doses) or submitted to nephrectomy/Aldo/salt treatment (NAS) as described in the supplementary Methods section (please see the online-only Data Supplement). MR antagonists used in vivo were either potassium canrenoate (30 mg/kg of body weight [BW] per day in the drinking water) for mice with chronic Aldo infusion.

**Cell Culture**
Reagents are from Sigma-Aldrich (Saint-Quentin Fallavier, France) unless specified. H9C2/αR cells, a clonal cell line of cardiomyocytes derived from the embryonic rat ventricle and stably expressing the MR (kindly provided by A. Naray-Fejes-Toth, Dartmouth School of Medicine, Lebanon, NH) were grown as described. Cells were treated with Aldo, corticosterone, the MR antagonist RU38486, or the MR antagonist RU28318 (Tocris Biosciences, Bristol, United Kingdom), as indicated in the legend of Figure 3.

HL-1 cells (kindly provided by W.C. Claycomb, Louisiana State University Health Sciences Center, New Orleans, LA), an immortalized cell line derived from neonatal mouse cardiac myocytes, were cultured as described and transiently transfected with a mouse MR-expressing plasmid. Cell transfection and chromatin immunoprecipitation are described in the supplementary Methods section.

Primary mouse aortic endothelial cells and primary mouse vascular smooth muscle cells derived from 8- to 10-week-old mice were isolated under sterile conditions as described. Endothelial cells were used at passages 2 to 3 and smooth muscle cells at passages 4 to 7. After 24 hours in serum-free medium, cells were incubated as indicated in legends of Figure 2.

**Cell Biology**
Real-time PCR, Western blot, immunolocalization, and measurements of plasma levels of NGAL are indicated in the supplementary Methods section.

**Statistical Analyses**
Results are provided as mean ± SEM. The nonparametric Mann-Whitney U test was used to assess statistical differences between 2 groups. Differences among >2 experimental conditions were tested by the ANOVA 1-way analysis followed by the Newman-Keuls test; P<0.05 was considered significant.

**Results**
Cardiac NGAL Expression Is Enhanced After MR Overexpression in Cardiomyocytes
A pan-genomic transcriptomic analysis on hearts of double-transgenic mice that overexpress the MR in cardiomyocytes only (MRcardio mice) identified NGAL as a gene highly induced in the heart of MRcardio mice; the induction is essentially MR dependent and not GR dependent, because cardiac NGAL mRNA was strongly enhanced in MRcardio mice (≈150 fold compared with the control littermates), whereas GR overexpression (in GRcardio mice) led to a much smaller (≈5-fold) increase in NGAL mRNA (Figure 1A). Treatment of MRcardio mice with the MR antagonist...
The NAS model is a classic model of mineralocorticoid stress that associates mineralocorticoid administration on top of salt excess and reduced renal mass. We found that NGAL mRNA expression was increased in the heart of mice with NAS challenge, whereas nephrectomy alone, Aldo alone, or excess salt intake alone had no effect (Figure 2C). Because NGAL may be secreted, its plasma concentration was estimated in mice challenged with NAS treatment followed by MR antagonism. Four weeks of NAS challenge enhanced plasma NGAL levels, which returned to low values when mice were treated by an MR antagonist (Figure 2D).

NAS challenge also enhanced by 2.5-fold NGAL mRNA expression in the aorta (data not shown). Immunolocalization experiments showed that NAS challenge led to clear NGAL signal over large cardiac vessels, whereas cardiomyocytes were barely positive; small arterioles and capillaries were negative (Figure 2E). Mineralocorticoid sensitivity of vascular NGAL expression was evidenced on primary cultures of aortic endothelial and smooth muscle cells: Aldo upregulated NGAL expression was evidenced on primary cultures of aortic endothelial and smooth muscle cells: Aldo upregulated

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NGAL transcripts in both cell types, and MR antagonist fully prevented the induction (Figure 2F).

**NGAL Is an Aldo/MR Target Gene in Cultured Cardiac Cells**

To distinguish consequences of enhanced MR signaling from long-term cardiac adaptation to chronic MR activation, we measured NGAL expression in a rat cardiomyocyte cell line stably transfected with the MR (H9C2/MR⁺ cells). Aldo induced NGAL mRNA expression after 24-hour incubation, in a dose-dependent manner (Figure 3A and 3B). Cells treated with corticosterone (Figure 3C) or Aldo (Figure 3D) exhibited MR-specific enhanced NGAL mRNA (Figure 3D) and protein (Figure 3E) expression that was prevented by coincubation with the MR antagonist RU28318, whereas the GR antagonist RU28486 was ineffective (Figure 3C through 3E). Thus, NGAL is upregulated by both MR-Aldo and MR-corticosterone complexes in this cell context.

To evaluate the mechanism of NGAL regulation by MR activation, we used the mouse cardiomyocyte cell line HL-1 cells that do not express detectable levels of endogenous MR activity²² and that were transiently transfected with mouse MR. Aldo produced a 6-fold increase in NGAL mRNA levels in HL-1 cells transfected with mouse MR but not in HL-1 cells transfected with empty vector (Figure 4A). Cotransfection of mouse MR and NGAL-luciferase reporter construct (using the −794 to +36 NGAL promoter region) that contains a hormone response element (Figure 4B) followed by Aldo treatment, resulted in a 4-fold increase in luciferase activity (Figure 4C) that was blocked by the MR antagonist spironolactone (Figure 4C). When the hormone response element was deleted, no significant increase in luciferase activity on Aldo treatment was detected (Figure 4C). This suggests that MR binds to the hormone response element present in the −794 to +36 NGAL promoter and that NGAL is an Aldo/MR responsive gene. Chromatin immunoprecipitation showed that the hormone response element–mediated Aldo/MR effect is a direct consequence of MR binding to this promoter element (Figure 4D). When MR was transfected, the NGAL promoter was enriched in the chromatin immunoprecipitation product by 4-fold. Aldo treatment produced further enrichment of the NGAL promoter (≥11-fold), indicating increased binding of MR to the promoter. These data show that the MR directly controls NGAL expression in cardiomyocytes by binding to its promoter.

**Discussion**

Inappropriate or excessive MR activation is a hallmark of a number of CV diseases that may benefit from treatments with MR antagonists. In this study we have provided evidence that NGAL is a mineralocorticoid-target gene in the CV system. We have shown that NGAL is a cardiac gene with high
Neutrophil gelatinase-associated lipocalin (NGAL) is a direct target of mineralocorticoid receptor (MR). NGAL mRNA abundance (quantitative PCR) in cardiac HL-1 cells transfected with mouse MR (HL-1 + MR) or with empty vector (HL-1) and treated or not with 10^{-8} M aldosterone for 16 hours. Schematic representation of mouse NGAL promoter. HRE indicates hormone response element. Arrowheads indicate the approximate location of primers used to analyze chromatin immunoprecipitation products. The line below represents the fragment of NGAL promoter fused to luciferase (NGAL-luc construct). Numbers refer to the transcription start sites. Luciferase expression mediated by the wild-type (NGAL[-794/+36]-luc) or mutated (NGAL[-794/+36]ΔHRE-luc) fragment of mouse NGAL promoter in transfected HL-1 cells, treated or not with 10^{-8} M aldosterone alone or with 10^{-6} M spironolactone (MRA) for 16 hours. Renilla luciferase was used as internal control. Luciferase values are normalized to control conditions. Quantitative PCR analysis of MR binding to the HRE in mouse NGAL promoter in HL-1 cells transfected or not with mouse MR and treated or not with 10^{-8} M aldosterone. Bars represent the fold enrichment of the 184-bp fragment surrounding the HRE over the value obtained with the irrelevant IgG. *P<0.05, treatment vs control (ANOVA analysis, Newman-Keuls test). Data are means of 3 to 4 independent experiments, each performed in triplicate.

It is highly plausible that several factors contribute to determine NGAL expression levels, in physiological situations and in pathology. Of interest, it has been shown that nuclear factor κB (NF-κB) can directly enhance NGAL expression, as demonstrated in vascular smooth muscle cells. Interleukin 1β triggers binding of NF-κB to the NGAL promoter. Further analysis of induction of NGAL through the NF-κB pathway was provided recently. Cross-talk between nuclear receptors and NF-κB has been reported. In the literature, there are data that show transcriptional antagonism between the MR and NF-κB. Aldo also inhibited NF-κB activation (leading to production of tumor necrosis factor-α) in neutrophils, and the authors concluded that MR mediates anti-inflammatory effects in these cells when they interact with the endothelium. Conversely, activation of NF-κB by Aldo has been reported in renal collecting duct cells; however, this may not be a direct effect but rather an adaptive phenomenon to limit excessive Aldo signaling. In cardiac cells, balance between MR and NF-κB may regulate NGAL expression; this balance may be different after short-term and long-term situations and even more in a pathological context. Further experiments are needed to test such hypotheses, to understand how and why NGAL levels vary. Altogether, these data illustrate the complexity of NGAL regulation that is far from being elucidated.

NGAL/lipocalin 2 is expressed at low levels in normal tissues and highly in a number of situations with tissue injury, where its roles are multiple and not fully understood.
NGAL/lipocalin 2 was initially identified as a critical component of innate immunity, limiting bacterial infection, through sequestration of iron. Later on, this protein was shown to be a modulator of cell proliferation, differentiation, apoptosis, and more generally in the reaction to various stress factors. Its expression is strongly enhanced in response to renal tubular injury. It has been shown that NGAL may protect the kidney from failure after ischemia/reperfusion; conversely, it was reported that it may belong to a critical pathway leading to progression toward renal failure.

Extensive literature data have been provided to designate plasma and/or urinary NGAL levels as sensitive markers of early prediction of kidney injury and its outcomes in humans. NGAL, as a biomarker of renal tubular damage, may also reflect renal consequences of cardiac impairment after cardiac surgery, myocardial infarction, or cardiorenal syndrome associated with HF. However, recent data suggest that cardiac NGAL and circulating NGAL levels may be altered by cardiac dysfunction, independent of renal dysfunction.

Plasma NGAL is high in patients with acute HF after myocardial infarction (Optimal Trial in Myocardial Infarction With the Angiotensin II Antagonist Losartan), as well as in patients with chronic HF or coronary heart disease and in the atherosclerotic plaque. We found that plasma NGAL concentration is enhanced on mineralocorticoid stress in mice (although it is difficult to determine the origin of plasma NGAL in the NAS model). Human trials should determine whether plasma NGAL correlates with circulating Aldo levels and MR antagonism in patients with HF.

**Perspectives**

We demonstrate in the present study that NGAL is upregulated by mineralocorticoids, through MR activation, in the CV system, where its cellular functions remain to be elucidated. Future studies should also determine whether the rise of plasma NGAL may serve as a biomarker of excessive MR activation in patients with HF, to select those who could benefit from treatment with MR antagonists.

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**Disclosures**

None.

**References**


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NEUTROPHIL GELATINASE-ASSOCIATED LIPOCALIN IS A NOVEL MINERALOCORTICOID TARGET IN THE CARDIOVASCULAR SYSTEM

SUPPLEMENTARY METHODS


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Animal treatments
To switch off the transgene expression in 3 months old MRcardio mice, doxycycline (dox, 2mg/ml in drinking water) was administered for 4 weeks before sacrifice. Adult mice were treated with aldosterone alone either acutely (bolus of 0.1 or 1 mg/kg BW six hours before sacrifice) or for 4 weeks (1 mg/kg BW/day delivered subcutaneously via ALZET® osmotic mini pumps (Charles River Laboratories, L’Arbresle, France). In other series, mice with nephrectomy/aldosterone/salt treatment (NAS) were uninephrectomized (xylazine/ketamine anesthesia) and received 60 µg/kg BW/day aldosterone, delivered subcutaneously via ALZET® osmotic mini pumps, and 1% sodium chloride to drink, for 4 weeks. The control mice were sham-operated and maintained on standard chow and tap water. In some series, mice were subjected to only one of the 3 components of the NAS challenge (uninephrectomy alone or aldosterone alone or 1% sodium chloride to drink).

Cell transfection and chromatin immunoprecipitation
HL-1 cells were transfected using Lipofectamine 2000. A NGAL promoter fragment including -794 to +36 relative to the transcription start site was cloned in pGL4.1-luc (Promega, Madison, WI). Deletion of a hormone responsive element (HRE) in the NGAL promoter (position -551 to -501) was performed by site-directed mutagenesis using a commercial kit (Quickchange, Agilent, Santa Clara, CA). The effect of MR activation on NGAL promoter activity was tested by co-transfecting these plasmids with mouse MR. Steroid treatments were performed after 24 h in charcoal-stripped medium. Luciferase activity was determined with a kit (DualGlo, Promega).

For chromatin immunoprecipitation (ChIP) cells were cross-linked with formaldehyde. Chromatin was purified, sonicated and used for ChIP as recommended by the manufacturer (ChIP-IT Express, Active Motif, Carlsbad, CA). Three reactions were performed: positive control with an anti-RNApolII antibody; a negative control with an irrelevant IgG; a test reaction using an anti-MR antibody (H-300, Santa Cruz Biotechnology, Santa Cruz, CA). ChIP products were then purified and the enrichment of NGAL promoter area containing the HRE was tested by qPCR.

Cell biology
RNA extraction and qPCR analyses were performed as described. Primers for NGAL were 5'-GGACCAGGGCTGCTCGTACT-3' and 5'-GTTGGGCACCTTGCACATTGT-3' in mouse samples, and 5'-TCACCCCTGTACGGAAGAACC-3' and 5'-GGTGGGAACAGAGAAAACGA-3' for H9C2 rat cells (forward and reverse primers); UBC mouse primers were 5'-AGCCCATGTTACCCACCAAG-3' and 5'-ACCCAGAAACGACAAGCAG-3'; rat β actin primers were 5'-TTCTACAATGAGCTGCGTGTG-3' and 5'-CAGGTCCAGACGAGGAT-3'; GAPDH primers were 5'-AAGGCTTCTGGATGAAGG-3' and 5'-GAATGAGGGATGTTCAGTCT-3'. Western blots were performed using goat polyclonal anti-NGAL antibody (1:1000, R&D Systems Europe, Lille, France). GAPDH was used as internal control for protein loading. For immunohistochemical detection of NGAL on paraformaldehyde-fixed tissues, the NGAL antibody was diluted 1:100. Mouse plasma samples were collected using lithium-heparin Microvette® CB300 (Sarstedt, Marnay, France) and plasma NGAL levels were measured by ELISA (Bioponto Diagnostics, Gentofte, Denmark).