Aldosterone

Neutrophil Gelatinase–Associated Lipocalin, a Novel Mineralocorticoid Biotarget, Mediates Vascular Profibrotic Effects of Mineralocorticoids

Antoine Tarjus, Ernesto Martínez-Martínez, Cristian Amador, Céline Latouche, Soumaya El Moghrabi, Thorsten Berger, Tak W. Mak, Renaud Fay, Nicolette Farman, Patrick Rossignol, Faiez Zannad, Natalia López-Andrés, Frédéric Jaisser

See Editorial Commentary, pp 20–22

Abstract—Activation of the mineralocorticoid receptor has been shown to be deleterious in cardiovascular diseases (CVDs). We have recently shown that the lipocalin 2 (Lcn2), or neutrophil gelatinase–associated lipocalin (NGAL), is a primary target of aldosterone/mineralocorticoid receptor in the cardiovascular system. Lcn2 is a circulating protein, which binds matrix metalloproteinase 9 and modulates its stability. We hypothesized that Lcn2 could be a mediator of aldosterone/mineralocorticoid receptor profibrotic effects in the cardiovascular system. Correlations between aldosterone and profibrotic markers, such as procollagen type I N-terminal peptide, were investigated in healthy subjects and subjects with abdominal obesity. The implication of Lcn2 in the mineralocorticoid pathway was studied using Lcn2 knockout mice subjected to a nephrectomy/aldosterone/salt (NAS) challenge for 4 weeks. In human subjects, NGAL/matrix metalloproteinase 9 was positively correlated with plasma aldosterone and fibrosis biomarkers. In mice, loss of Lcn2 prevented the NAS-induced increase of plasma procollagen type I N-terminal peptide, as well as the increase of collagen fibers deposition and collagen I expression in the coronary vessels and the aorta. The lack of Lcn2 also blunted the NAS-induced increase in systolic blood pressure. Ex vivo, treatment of human fibroblasts with recombinant Lcn2 induced the expression of collagen I and the profibrotic galectin-3 and cardiotrophin-1 molecules. Our results showed that Lcn2 plays a key role in aldosterone/mineralocorticoid receptor–mediated vascular fibrosis. The clinical data indicate that this may translate in human patients. Lcn2 is, therefore, a new biotarget in cardiovascular fibrosis induced by mineralocorticoid activation. (Hypertension. 2015;66:158-166. DOI: 10.1161/HYPERTENSIONAHA.115.05431.) • Online Data Supplement

Key Words: aldosterone □ collagen type I □ fibroblast □ lipocalin 2 □ mineralocorticoid receptor

Aldosterone via the activation of the mineralocorticoid receptor (MR) is a main actor of renal sodium reabsorption and water homeostasis. Extra-renal effects of the mineralocorticoid pathway have now been characterized, especially in the cardiovascular system, opening on new dimensions of the aldosterone/MR pathway in physiology, pathophysiology, and diseases. In the cardiovascular system, mineralocorticoid signaling has been shown to play an important role in the progression of cardiovascular diseases (CVDs). Previous reports using transgenic mouse models or pharmacological approaches demonstrated adverse effects of aldosterone on cardiac remodeling and fibrosis, inflammation, and hypertension.1 These features are prevented by the pharmacological blockade of the MR. In humans, several clinical studies showed the beneficial effects of MR antagonism in addition to standard care, to reduce mortality and morbidity in patients with severe2 or mild heart failure (HF)3 or after acute myocardial infarction.4

Understanding the molecular mechanisms of mineralocorticoid activation pathway in cardiovascular pathophysiology remains incomplete. Indeed, a better knowledge of the underlying mechanisms may highlight novel mediators of the MR signaling cascade. These newly identified intermediates could be good candidates as biotargets for novel pharmacological approaches, especially in diseases where the aldosterone/MR pathway is involved. Moreover, these biotargets may as well be considered as potent biomarkers of MR activation,
allowing a better selection and survey of patients that could benefit from MR antagonists as a therapeutic opportunity.

We identified lipocalin 2 (Lcn2), also known as neutrophil gelatinase–associated lipocalin (NGAL) or 24p3, as a novel MR target in the cardiovascular system in mice, using a pan-nectomic analysis with dedicated transgenic animal models with targeted MR alteration in cardiomyocytes. We showed that MR, as transcription factor, can bind directly the promoter of Lcn2 and controls its transcription. Lcn2 is a 25-kDa secreted glycoprotein belonging to the lipocalin superfamily, which binds small lipophilic substances.

Increased systemic levels and myocardial expression of Lcn2/NGAL have been reported in several experimental models and CVDs, such as HF and atherosclerosis; it is also considered as a biomarker of renal dysfunction because its plasma and urinary concentrations increase in kidney diseases. At difference to rodents, human Lcn2/NGAL has a cysteine residue at position 87 allowing a disulfide bond with matrix metalloproteinase 9 (MMP-9) that stabilizes MMP-9, increasing its activity. In clinical studies, increased Lcn2/NGAL concentrations have been reported in obese patients and overweight subjects as such prone to develop both hyperaldosteronism and HF.

We took advantage of a previously characterized human cohort of asymptomatic obese subjects but as such prone to develop CVDs, including HF, to question whether Lcn2/NGAL is correlated with plasma aldosterone levels and markers of extracellular matrix (ECM) remodeling. To get further insights into the role of Lcn2 as an actor of the MR signaling cascade in the cardiovascular system and its involvement in the occurrence of fibrosis, we used a genetic approach using an Lcn2 knockout mouse model that was submitted to mineralocorticoid challenge. We also tested the ability of Lcn2 to induce expression of ECM proteins in human fibroblasts.

Materials and Methods

Human Studies

A subset of 133 nonhypertensive subjects with available plasma samples, from a cohort of 140 subjects that has been previously characterized (R2C2, role of the renin–angiotensin–aldosterone system in the mechanisms of transition to HF in abdominal obesity [AO]), was studied. Subjects were classified according to smoking status, age, sex, and body mass index (BMI). Body mass index was calculated as weight in kilograms divided by height in meters squared. Subjects were then categorized into two groups: nonobese (BMI <25 kg/m²) and obese (BMI ≥25 kg/m²) according to the Inserm animal care and use committee guidelines. Mice were housed in a climate-controlled facility on a 12-hour light/dark cycle and provided free access to food and water. Experiments were approved by the Darwin ethics committee. Mice were uninephrectomized and osmotic minipumps (Charles River Laboratories, L'Arbresle, France) of aldosterone (200 μg/kg per day; Sigma-Aldrich, St-Quentin-Fallavier, France) were implanted subcutaneously. The day after surgery, the drinking water was replaced with 1% saline. Mice were on normal chow. In sham-treated animals, the kidney was exposed but not removed. Four weeks later, mice were euthanized by decapitation. Systolic blood pressure was measured by tail cuff plethysmography in trained conscious mice. Ten measurements per mouse were taken every day between 10 AM and 12 PM during 5 days using a BP2000 Visitech model (Bioseb, Vitrolles, France) as described previously.

Culture of Human Cardiac Fibroblasts

Human cardiac fibroblasts (Promocell, Heidelberg, Germany) were cultured in the medium fibroblasts media 3 (Promocell) according to the manufacturer's instructions. Human cardiac fibroblasts were used between passages 5 to 7. Cells were incubated for 24 hours with different concentrations of recombinant NGAL (5, 50, and 500 ng/mL; R&D Systems).

Statistics

All analyses were performed using GraphPad Prism V6.01 (GraphPad Software, San Diego, CA) or SAS 9.3 (SAS Institute, Cary, NC) software. The two-tailed significance level was set to P<0.05. Results were expressed as mean±SEM, median (interquartile range) or correlation coefficient.
coefficient and its 95% confidence interval ($r_s$ [95% confidence interval]) as appropriate. Univariate analyses were carried out using non-parametric Mann–Whitney (2 groups) or Kruskal–Wallis (>2 groups) tests for between-group means comparisons, Spearman method for correlation analyses and $\chi^2$ test for percent. Multivariate analyses were performed using ANOVA with Bonferroni adjustment for post hoc tests.

Results

Lcn2/NGAL is Related to Cardiovascular Remodeling in Humans

In a cohort of normotensive asymptomatic obese subjects and age–sex matched healthy volunteers, plasma-free NGAL and Gal-3 concentrations did not significantly differ between the 2 populations, whereas subjects with AO displayed a 3.8-fold higher NGAL/MMP-9 concentration ($P<0.0001$), 1.4-fold in plasma aldosterone levels ($P<0.0001$), 1.9-fold higher in PINP ($P<0.0001$), 4.1-fold higher in cardiotrophin-1 (CT-1) ($P<0.0001$), and 20% lower in type I collagen telopeptide concentration ($P<0.0001$) versus controls (Table 1). No significant correlation was observed between free NGAL and plasma aldosterone, or with ECM biomarkers (data not shown). In contrast, in the whole study population, plasma NGAL/MMP-9 complexes were found positively correlated with plasma aldosterone (Figure 1A; Table 2). NGAL/MMP-9 complexes were also positively correlated with PINP ($r_s=0.49; P<0.0001$), PIIINP ($r_s=0.23; P=0.01$; Figure 1B and 1C; Table 2; Fig. S1) and negatively correlated with type I collagen telopeptide ($r_s=-0.38; P<0.0001$; Figure 1D; Table 2). In healthy subjects, none of the parameters are correlated together (Table 2). In the subjects with AO, NGAL/MMP-9 was significantly correlated with plasma PINP, PIIINP, and type I collagen telopeptide (Table 2).

Lcn2 Gene Inactivation Limits the Vascular Fibrosis Induced by NAS Challenge in Mice

To progress in the understanding of the role of Lcn2, we characterized its involvement in the fibrotic phenotype induced by

Table 2. Spearman Correlation Between Neutrophil Gelatinase–Associated Lipocalin/Matrix Metalloproteinase 9 Complex and Several Factors of the Human Cohort

<table>
<thead>
<tr>
<th>Factor</th>
<th>Controls</th>
<th></th>
<th></th>
<th>Abdominal Obesity</th>
<th></th>
<th></th>
<th>All Subjects</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$r_s$ (95% CI)</td>
<td>$P$ Value</td>
<td></td>
<td>$r_s$ (95% CI)</td>
<td>$P$ Value</td>
<td></td>
<td>$r_s$ (95% CI)</td>
<td>$P$ Value</td>
</tr>
<tr>
<td>Aldosterone</td>
<td>0.01 (−0.26 to 0.28)</td>
<td>0.94</td>
<td></td>
<td>0.19 (−0.02 to 0.40)</td>
<td>0.081</td>
<td></td>
<td>0.29 (0.13 to 0.44)</td>
<td>0.0006</td>
</tr>
<tr>
<td>PINP</td>
<td>0.61 (0.40 to 0.76)</td>
<td>&lt;0.0001</td>
<td></td>
<td>0.28 (0.06 to 0.47)</td>
<td>0.012</td>
<td></td>
<td>0.49 (0.35 to 0.61)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PIIINP</td>
<td>0.11 (−0.17 to 0.37)</td>
<td>0.45</td>
<td></td>
<td>0.24 (0.02 to 0.45)</td>
<td>0.034</td>
<td></td>
<td>0.23 (0.05 to 0.39)</td>
<td>0.010</td>
</tr>
<tr>
<td>ICTP</td>
<td>−0.07 (−0.34 to 0.20)</td>
<td>0.61</td>
<td></td>
<td>−0.28 (−0.47 to −0.07)</td>
<td>0.011</td>
<td></td>
<td>−0.38 (−0.52 to −0.23)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Gal-3</td>
<td>−0.19 (−0.44 to 0.09)</td>
<td>0.19</td>
<td></td>
<td>0.21 (−0.01 to 0.41)</td>
<td>0.066</td>
<td></td>
<td>0.11 (−0.07 to 0.27)</td>
<td>0.23</td>
</tr>
</tbody>
</table>

$P$ value from the Spearman nonparametric correlation. CI indicates confidence interval; Gal-3, galectin-3; ICTP, type I collagen telopeptide; PINP, procollagen type I N-terminal peptide; PIIINP, procollagen type III N-terminal peptide; and $r_s$, Spearman’s rank correlation coefficient.
mineralocorticoid challenge in mice with genetic deletion of Lcn2. Lcn2 knockout mice displayed normal baseline characteristics (Table 3). NAS challenge induced cardiac and renal hypertrophy in both genotypes as shown by the augmentation of cardiac weight/tibia length and renal weight/tibia length ratios (Table 3). WT mice submitted to the NAS challenge developed an increase in systolic blood pressure (Table 3). Of note, the increase in blood pressure elicited by NAS was reduced in mice with Lcn2 inactivation, compared with that of WT mice (Table 3).

NAS challenge in WT mice decreased the aortic vasodilatory response to acetylcholine (endothelium-dependent vasorelaxation; Figure 2A; Table S2) and sodium nitroprusside

Table 3. Physiological Parameters of Mice

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Wild-Type Sham</th>
<th>Wild-Type NAS</th>
<th>Lcn2 KO Sham</th>
<th>Lcn2 KO NAS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body mass index, g</td>
<td>26.3±0.5</td>
<td>27.3±0.9</td>
<td>28.1±0.6</td>
<td>26.0±0.4</td>
</tr>
<tr>
<td>Tibia length, mm</td>
<td>17.6±0.3</td>
<td>17.8±0.3</td>
<td>18.2±0.2</td>
<td>17.5±0.3</td>
</tr>
<tr>
<td>HW/TL ratio, mg/mm</td>
<td>6.01±0.22</td>
<td>7.74±0.36***</td>
<td>6.46±0.09</td>
<td>8.00±0.39**</td>
</tr>
<tr>
<td>KW/TL ratio, mg/mm</td>
<td>11.3±0.4</td>
<td>18.9±0.7****</td>
<td>11.1±0.3</td>
<td>17.3±0.8****</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>107±3.0</td>
<td>133±5****</td>
<td>109±3</td>
<td>115±3†</td>
</tr>
<tr>
<td>Na+ excretion, U Na+/U Creat</td>
<td>36.1±3.8</td>
<td>658.8±84.6**</td>
<td>30.8±4</td>
<td>933.9±130.8**</td>
</tr>
</tbody>
</table>

HW indicates heart weight; KO, knockout; KW, kidney weight; Lcn2, lipocalin 2; NAS, nephrectomy/aldosterone/salt challenge; and TL, tibia length.

**P<0.01, ***P<0.001, and ****P<0.0001, NAS challenge vs sham mice. †P<0.001, Lcn2 KO mice vs wild-type mice. No. of animals per condition is 12 except for blood pressure (n=6–7).

Figure 2. Lipocalin 2 (Lcn2) does not modify the vascular dysfunction induced by nephrectomy/aldosterone/salt (NAS) treatment. Graphs represent the vascular responses to increased acetylcholine (Ach; A), sodium nitroprusside (SNP; B), and phenylephrine (Phe; C). Histograms correspond to the area under curve of the respective graphs. Data are mean±SEM (n=5–7 mice per group). **P<0.01, ***P<0.001, and ****P<0.0001. KO indicates knockout; and WT, wild-type.
Lcn2 prevented the induction of a profibrotic perivascular phenotype in mice (Figure 4A and 4B). Consistent with this observation, collagen I/collagen III protein ratio was increased in the heart from WT mice under NAS treatment, but not altered in NAS-treated Lcn2 knockout mice (Figure 4C). Cardiac expression of Gal-3 and CT-1 was not modified by NAS (Figure S2). Fibrosis (Figure 4D and 4E) also occurred in the aorta of WT, not Lcn2 knockout mice, after NAS challenge, with parallel changes in collagen I expression (Figure 4F). Thus, loss of Lcn2 prevented the induction of a profibrotic perivascular phenotype.

**Lcn2 Gene Inactivation Blunted the Increased in a Plasma Biomarker of ECM Remodeling Induced by NAS Challenge in Mice**

Because loss of Lcn2 prevented ECM remodeling in cardiovascular tissues after NAS challenge in mice, we next analyzed a circulating biomarker to evaluate the profibrotic status of the mice treated or not with NAS. In the plasma of WT mice submitted to NAS treatment, PINP (a marker of collagen type I synthesis) was increased by 50%, as compared with sham-operated mice (Figure 5A). The absence of Lcn2 blunted the PINP production induced by NAS (Figure 5A). The plasma levels of CT-1 and Gal-3, 2 profibrotic molecules previously reported to be involved in the aldosterone-induced ECM remodeling in the cardiovascular system were analyzed. CT-1 plasma level was not detectable. Gal-3 plasma level was increased by the NAS challenge, an effect blunted in Lcn2 knockout mice (Figure 5B).

**Lcn2 Induces a Fibrotic Phenotype in Cultured Fibroblasts**

In mice, Lcn2 is an aldosterone target in cardiomyocytes, endothelium, and vascular smooth muscle cells. Lcn2 is a secreted protein and can act as autocrine and paracrine agent. We, therefore, analyzed whether recombinant NGAL could be a direct modulator of profibrotic genes in vascular smooth muscle cells and fibroblasts that play a key role in cardiovascular remodeling. NGAL has no effect on collagen type I expression in vascular smooth muscle cells (Figure S3). In human fibroblasts, recombinant human NGAL application for 24 hours induced a dose-dependent increase in collagen type I protein expression (Figure 6A) but not in fibronectin (Figure 6B). Moreover, recombinant NGAL increased the expression of Gal-3 (Figure 6C) and CT-1 (Figure 6D), as well as secretion of Gal-3 and CT-1 in the medium (Figure 6E and 6F).

**Discussion**

Upregulation of Lcn2 expression has been described in several pathological conditions, such as obesity, inflammation, and cancer and more recently in the cardiovascular system. Polymorphisms in the human NGAL promoter correlated with blood pressure levels in a population-based prospective cohort study of cardiovascular risk factors.
in a general population of Hong Kong.33 However, correlation between NGAL plasma levels, which may be affected by these polymorphisms and blood pressure was not studied.33 Another study reported that patients with essential hypertension had higher NGAL plasma levels than normotensive patients, and that plasma NGAL was significantly correlated with systolic blood pressure levels.34

We cannot explain why loss of Lcn2 prevents blood pressure increase induced by a mineralocorticoid challenge. Renal sodium handling and vascular reactivity are similar in

Figure 4. Lipocalin 2 (Lcn2) inactivation prevents extracellular matrix remodeling induced by nephrectomy/aldosterone/salt (NAS) treatment in the cardiovascular system. A and D, Histology of heart (A) or aorta (D) stained for collagen deposition (Sirius Red). B and E, Quantification of vascular fibrosis in the heart (B) or the aorta (E). C and F, Western blot of collagen I/Col I/collagen III (Col III) ratio in the heart (C) and collagen I/elastin in the aorta (F). Data are mean±SEM (n=6–7 mice per group), *P<0.05, **P<0.01. KO indicates knockout; and WT, wild-type.

Figure 5. Lipocalin 2 (Lcn2) inactivation prevents the nephrectomy/aldosterone/salt (NAS)-dependent increase of a plasma biomarker of extracellular matrix remodeling. Bar graph represents the plasma concentration of procollagen type I N-terminal peptide (PINP), a marker of collagen type I maturation (A) and plasma concentration of galectin-3 (B), in the 4 groups of mice. Data are mean±SEM (n=6–7 mice per group), *P<0.05, **P<0.01. KO indicates knockout; and WT, wild-type.
WT and Lcn2 knockout mice. We noticed, however, that vascular oxidative stress is blunted. Further studies are required to explore whether oxidative stress is part of the benefits of Lcn2 inactivation on blood pressure. Because the increase in blood pressure by the aldosterone-salt challenge is blunted in Lcn2 knockout mice, we cannot exclude a consequence of lower blood pressure on reduced vascular remodeling. The role of Lcn2 in the interplay between blood pressure and ECM remodeling, therefore, affecting arterial stiffness is, however, difficult to assess because both the parameters influence each other. We provide evidences that Lcn2 and perivascular fibrosis are linked: loss of Lcn2 prevents the increase in cardiac and aortic collagen I, as well as ECM remodeling assessed by collagen fibers deposition into aorta. Interestingly, loss of Lcn2 also blunts the increase in plasma PINP, a marker of procollagen type I processing in mice challenged by mineralocorticoids. This preclinical observation extends to humans: within a cross-sectional study of asymptomatic normotensive obese participants at risk to develop cardiovascular disease but without left ventricular hypertrophy (ie, HF stage A), we observed that plasma NGAL/MMP-9 was significantly associated with fibrosis biomarkers previously associated with mineralocorticoid effects in HF. This suggests that NGAL/MMP-9 is already altered at the

Figure 6. Fibrotic effect of Lipocalin 2/neutrophil gelatinase–associated lipocalin (NGAL) in human fibroblasts. Western-blot analysis of protein expression of collagen type I (Col I; A), fibronectin (FN; B), galectin-3 (Gal-3; C), and cardiophin-1 (CT-1; D) in human fibroblasts incubated with different NGAL concentrations. E and F, ELISA quantification of secreted galectin-3 (E) and cardiophin-1 (F) in the supernatant. Data are mean±SEM of 4 assays, in arbitrary units normalized to β-actin. Values are expressed in fold from the control group. *P<0.05, **P<0.01 vs control.
early stage of cardiac remodeling that precedes clinical HF, and may, therefore, be a key factor in the aldosterone signaling pathway leading to tissue remodeling. This is further strengthened by cellular data using human cardiac fibroblast where we unveiled a direct profibrotic effect of recombinant human NGAL via increased collagen I production and the synthesis and secretion of the profibrotic molecules, such as Gal-3 and CT-1.

This study has several limitations. Because of the lack of a specific cysteine residue, Lcn2/MMP-9 complex is not present in rodents opposed to humans. Therefore, we cannot assess the potential role of the interaction between Lcn2 and MMP-9 in the beneficial effects we observed in the mouse model. A second limitation is the use of a global Lcn2 knockout. This model does not allow us to identify the cell type(s) involved in the observed effects in Lcn2 knockout mice. We cannot rule out that Lcn2 has other targets than fibroblasts in vivo. Moreover, because Lcn2 is a secreted protein, it may act at distance from its origin of synthesis.

In conclusion, Lcn2 seems as a novel actor in the pathological activation of the cardiovascular mineralocorticoid cascade, mandatory for the elevation of blood pressure and vascular fibrosis. This study demonstrates that Lcn2/NGAL is a key element in the signaling events modulated by mineralocorticoid challenge, in relation with blood pressure increase and ECM remodeling. Whether Lcn2/NGAL may represent a novel therapeutic target in ECM remodeling is an exciting perspective.

Perspectives

We demonstrate in this study that Lcn2 plays a key role in the increased blood pressure and remodeling process induced by mineralocorticoid excess, therefore, representing a potential biotarget for pharmacological modulation of the mineralocorticoid system. The rise of NGAL/MMP-9 and its association with plasma aldosterone levels and fibrosis biomarkers in subjects with stage A HF suggest that it may also serve as a biomarker of excessive MR activation, and help identifying populations more prone to respond to mineralocorticoid receptor antagonists prescribed to prevent adverse cardiovascular remodeling.

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We thank Brigitte Beauvois and Sandrine Bouchet for their suggestions.

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Disclosures

None.

References

17. Eschalier R, Rossignol P, Kearney-Schwartz A, Adamopoulos C, Karatzidou K, Fay R, Mandler D, Marie PY, Zannad F. Features of cardiac remodeling, associated with blood pressure and fibrosis biomarkers, are
**What Is New?**

- Lcn2, a target gene of the mineralocorticoid receptor in the cardiovascular system, is mandatory for the development of perivascular fibrosis induced by nephrectomy/aldosterone/salt challenge through direct modulation of profibrotic molecules expression.

**What Is Relevant?**

- Human clinical data findings indicate that the plasma level of neutrophil gelatinase-associated lipocalin (NGAL), the human Lcn2 isoform, correlated with aldosteronemia and collagen processing markers.

**Summary**

This study demonstrates that Lcn2/NGAL is a key element in the signaling events modulated by mineralocorticoid challenge, in relation with blood pressure increase and extracellular matrix remodeling.
Neutrophil Gelatinase–Associated Lipocalin, a Novel Mineralocorticoid Biotarget, Mediates Vascular Profibrotic Effects of Mineralocorticoids

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Neutrophil Gelatinase Associated Lipocalin, a novel mineralocorticoid biotarget, mediates vascular pro-fibrotic effects of mineralocorticoids

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This file includes:
1 supplemental method
1 supplemental references
2 supplemental tables
3 supplemental figures
SUPPLEMENTAL METHODS

Tissue sampling
Hearts were rinsed in cold PBS (Phosphate Buffer Solution), weighted and cut into 3 parts (transversal cut). The base and the apex were used for biochemical studies, the mid-part for morphological studies. Aortas were rinsed in cold PBS. Thoracic aorta was used to assess vascular reactivity; a small piece was used for morphological studies. Abdominal aorta was used for biochemical studies. Left tibia length was used for organ weight normalization.

Vascular Reactivity Analysis
Vascular contractile and relaxing responses were assessed in isolated thoracic aortas as described. Aortic rings were maximally contracted with isotonic potassium chloride solution (KCl, 80mM) and then contractile capacity of each ring was measured with a submaximal concentration of phenylephrine (Phe, 10^{-5}M). The contraction evoked by Phe (10^{-9} to 10^{-5}M) tested the α-adrenergic pathway. The dose response to acetylcholine (Ach, 10^{-9} to 10^{-6}) was determined in rings precontracted by 10^{-5}M Phe to evaluate endothelium dependent relaxation. Dose-response to the NO donor sodium nitroprusside (SNP, 10^{-9} to 10^{-4}M) was used to measure vascular smooth muscle cell (VSMC) sensitivity to nitric oxide (NO) pathway. All the chemicals were purchase from Sigma-Aldrich if not specified otherwise. pEC50 were calculated using a non-linear regression with three parameters (GraphPad Prism V.6.01, San Diego, USA).

Circulating marker aminoterminal propeptide of type I collagen (PINP) in mice
Blood was collected during sacrifice using Microvette® capillary tube coated with lithium heparin (Sarstedt, Marnay, France). Plasma was retrieved after 5 minutes centrifugation (2000g, room temperature). PINP concentrations were measured by ELISA according to the manufacturer's instructions (R&D Systems).

Histological procedures
Sirius red staining to show collagen content was performed on 5 μm sections of thoracic aortas and mid-part the heart fixed with 4% paraformaldehyde for 24 hours and embedded in paraffin. Image acquisition was made using a Leica DM4000B microscope and Leica Application Suite software (Leica Microsystems, Nanterre, France). Collagen content was quantified by surface of Sirius red staining over vessel surface using ImageJ software, and expressed as percentage of the total cross-sectional area.

Culture of vascular smooth muscle cells
Rat A7R5 cell line (ATCC, Molsheim, France) and vascular smooth muscle cell primary culture were cultured in Dulbecco’s Modified Eagle Medium (Life Technologies, Saint Aubin, France) according to the manufacturer’s instructions. Cells were used between passages 5-7. Cells were incubated for 24 hours with different concentrations of recombinant NGAL (5, 10, 50, 100, 500 ng/ml, R&D Systems).

cDNA isolation and Real-Time PCR
Total RNA from cells were extracted using the TRIZOL® reagent (Life Technologies , Saint Aubin, France), according to manufacturer protocol. Reverse transcription of mRNA (500ng)
was performed using Superscript II Reverse Transcriptase KIT (Life Technologies). Transcript levels of genes were analyzed by real-time PCR (fluorescence detection of SYBR Green) in an iCycler iQ (Bio-Rad, Cergy-St-Christophe, France). For each sample, mRNA levels were normalized to the housekeeping gene 18S.

**Western Blotting**
Western blots analyses were performed in aorta and heart from WT and Lcn2 KO mice and in human cardiac fibroblasts. Cell and tissue samples were homogenized using a Roche cOmplete lysis-M buffer with protease inhibitors and phosphatases inhibitors cocktail (Roche, Neuilly, France). Extracts were centrifuged at 14000 rpm for 10 minutes at 4°C and protein concentration in the supernatant was determined by the Pierce protein assay (Bio-Rad). Thirty micrograms of total proteins were loaded on 4-15% polyacrylamide gel and transferred to nitrocellulose membranes (Bio-Rad). Membranes were incubated with primary antibodies for: Collagen type I (Abcam, Cambridge, UK, dilution 1:1000 for tissues or Santa Cruz Biotechnology, Heidleberg, Germany, dilution 1:250 for cell samples), Collagen type 3 (Santa Cruz Biotechnology, dilution 1:250), Elastin (Abcam, dilution 1:250), Fibronectin (Millipore, St-Quentin-en-Yvelines, France, dilution 1:250), Galectin-3 (Santa Cruz Biotechnology, dilution 1:1000), Cardiotrophin-1 (Abcam, dilution 1:500) and β-actin (Sigma-Aldrich, dilution 1:10000) as a loading control. After washing, detection was made through incubation with peroxidase-conjugated secondary antibody, and developed using an ECL chemiluminescence kit (Bio-Rad). After densitometric analyses, optical density values were expressed as arbitrary units. Results are expressed as fold increase over the values of the control group. Western Blots were performed at least in triplicate for each experimental condition.

**ELISA**
Galectin-3 and Cardiotrophin-1 levels in the supernatant of human cardiac fibroblasts and mice cardiac tissue were measured using ELISA (R&D Systems). ELISA were performed according the manufacturer’s instructions.
SUPPLEMENTAL REFERENCES

SUPPLEMENTAL TABLES

Table S1: Study population feature

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Controls (n = 52)</th>
<th>Abdominal obesity (n = 81)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender male: n (%)</td>
<td>24 (46%)</td>
<td>36 (44%)</td>
<td>0.85</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>61 (57 - 71)</td>
<td>88 (77 - 96)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>1.69 (1.62 - 1.74)</td>
<td>1.66 (1.58 - 1.72)</td>
<td>0.17</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>22.6 (21.1 - 24.0)</td>
<td>31.4 (29.6 - 33.4)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>79 (71 - 85)</td>
<td>102 (96 - 107)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>71 (66 - 76)</td>
<td>73 (68 - 78)</td>
<td>0.33</td>
</tr>
<tr>
<td>Mean arterial pressure (mmHg)</td>
<td>86 (81 - 91)</td>
<td>89 (83 - 95)</td>
<td>0.047</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>60 (57 - 66)</td>
<td>70 (63 - 77)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>5.53 (4.88 - 5.80)</td>
<td>5.91 (5.26 - 6.47)</td>
<td>0.018</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/l)</td>
<td>3.38 (2.99 - 3.99)</td>
<td>3.65 (3.21 - 4.14)</td>
<td>0.12</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/l)</td>
<td>1.58 (1.34 - 1.88)</td>
<td>1.29 (1.14 - 1.66)</td>
<td>0.009</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>0.76 (0.65 - 1.01)</td>
<td>1.33 (0.92 - 1.92)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Fasting blood glucose (mmol/l)</td>
<td>4.86 (4.44 - 5.19)</td>
<td>5.00 (4.72 - 5.33)</td>
<td>0.071</td>
</tr>
<tr>
<td>C-reactive protein (mg/l)</td>
<td>0.85 (0.51 - 1.44)</td>
<td>2.51 (1.20 - 4.75)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Estimated GFR (ml/min/1.73m²)</td>
<td>76 (66 - 84)</td>
<td>78 (68 - 83)</td>
<td>0.99</td>
</tr>
<tr>
<td>Left ventricle mass</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- g</td>
<td>80 (71 - 98)</td>
<td>90 (75 - 106)</td>
<td>0.081</td>
</tr>
<tr>
<td>- g/m² BSA</td>
<td>48 (43 - 52)</td>
<td>43 (38 - 52)</td>
<td>0.032</td>
</tr>
</tbody>
</table>

Data are median and interquartile range (IQR: interquartile range = first and third quartile).
Table S2: Contraction and relaxation responses in aortas of Lcn2 KO mice and their littermates under NAS treatment.

<table>
<thead>
<tr>
<th>Agonists</th>
<th>Wild type Sham (n=5)</th>
<th>Wild type NAS (n=7)</th>
<th>Lcn2 KO Sham (n=5)</th>
<th>Lcn2 KO NAS (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylcholine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Emax (%)</td>
<td>62.1 ± 6.3</td>
<td>39.0 ± 6.5</td>
<td>81.0 ± 2.7</td>
<td>49.1 ± 7.5*</td>
</tr>
<tr>
<td>pEC50</td>
<td>-7.83 ± 0.41</td>
<td>-6.10 ± 0.17**</td>
<td>-7.25 ± 0.06</td>
<td>-6.75 ± 0.17</td>
</tr>
<tr>
<td>Sodium Nitroprusside</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Emax (%)</td>
<td>104.9 ± 2.0</td>
<td>93.3 ± 1.3***</td>
<td>100.4 ± 1.4</td>
<td>95.6 ± 1.2</td>
</tr>
<tr>
<td>pEC50</td>
<td>-7.80 ± 0.05</td>
<td>-7.33 ± 0.05**</td>
<td>-7.61 ± 0.05</td>
<td>-7.20 ± 0.05**</td>
</tr>
<tr>
<td>Phenylephrine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Emax (%)</td>
<td>62.0 ± 8.9</td>
<td>166.9 ± 15.3**</td>
<td>59.7 ± 11.0</td>
<td>163.9 ± 17.0***</td>
</tr>
<tr>
<td>pEC50</td>
<td>-6.47 ± 0.32</td>
<td>-6.93 ± 0.09</td>
<td>-6.44 ± 0.17</td>
<td>-6.96 ± 0.11</td>
</tr>
<tr>
<td>Potassium Chloride</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Emax (mN)</td>
<td>2.35 ± 0.34</td>
<td>6.08 ± 0.48****</td>
<td>2.42 ± 0.16</td>
<td>5.38 ± 0.28***</td>
</tr>
</tbody>
</table>

Emax: maximal efficiency, pEC50: negative logarithm to base 10 of 50% efficiency. Data are mean ± SEM (n=5-7 mice per group), * p<0.05, ** p<0.01, *** p<0.001 and **** p<0.0001 nephrectomy aldosterone treatment NAS vs sham mice.
Figure S1: Non-hypertensive subjects of the R2C2 (Role of the Renin Angiotensin Aldosterone System in the Mechanisms of Transition to Heart Failure in Abdominal Obesity) study: scatterplots of NGAL/MMP-9 vs. Galectin-. Correlation coefficients and 95% confidence intervals from non-parametric Spearman's analysis are indicated. Solid and dashed lines: unadjusted regression lines in the whole sample and Abdominal Obesity (AO) subgroups.)
Figure S2: Protein expression of Galectin-3 and Cardiotrophin-1 in mice heart. Bar graphs represents the cardiac concentration of Galectin-3 (Gal-3)(A) and Cardiotrophin-1 (CT-1)(B) in the 4 groups of mice. Data are mean ± SEM (n=10 mice per group), ** p<0.01.
Figure S3: Effects of Lcn2 on Collagen Type I expression in several vascular smooth muscle cell lines. mRNA levels of Collagen Type I represented on 18S expression (A) in A7R5 cell line and (B) in vascular smooth muscle cell primary culture, after 24 hours of incubation with recombinant Lcn2.