Galectin-3 Mediates Aldosterone-Induced Vascular Fibrosis

Laurent Calvier, Maria Miana, Pascal Reboul, Victoria Cachofeiro, Ernesto Martinez-Martinez, Rudolf A. de Boer, Françoise Poirier, Patrick Lacolley, Faiez Zannad, Patrick Rossignol, Natalia López-Andrés

Objective—Aldosterone (Aldo) is involved in arterial stiffness and heart failure, but the mechanisms have remained unclear. Gal-3 (Galectin-3), a β-galactoside-binding lectin, plays an important role in inflammation, fibrosis, and heart failure. We investigated here whether Gal-3 is involved in Aldo-induced vascular fibrosis.

Methods and Results—In rat vascular smooth muscle cells Gal-3 overexpression enhanced specifically collagen type I synthesis. Moreover Gal-3 inhibition by modified citrus pectin or small interfering RNA blocked Aldo-induced collagen type I synthesis. Rats were treated with Aldo-salt combined with spironolactone or modified citrus pectin for 3 weeks. Hypertensive Aldo-treated rats presented vascular hypertrophy, inflammation, fibrosis, and increased aortic Gal-3 expression. Spiro lactone or modified citrus pectin treatment reversed all the above effects. Wild-type and Gal-3 knockout mice were treated with Aldo for 6 hours or 3 weeks. Aldo increased aortic Gal-3 expression, inflammation, and collagen type I in wild-type mice at both the short- and the long-term, whereas no changes occurred in Gal-3 knock-out mice.

Conclusion—Our data indicate that Gal-3 is required for inflammatory and fibrotic responses to Aldo in vascular smooth muscle cells in vitro and in vivo, suggesting a key role for Gal-3 in vascular fibrosis. (Arterioscler Thromb Vasc Biol. 2013;33:67-75.)

Key Words: aldosterone • collagen type I • fibrosis • galectin-3 • vascular smooth muscle cells

Aldosterone (Aldo), a mineralocorticoid hormone that acts classically via an intracellular mineralocorticoid receptor (MR), is a well-known key regulator of blood pressure (BP) and electrolytic balance. A growing body of clinical and preclinical evidence suggests that Aldo plays an important pathophysiological role in cardiovascular remodeling and diseases by promoting changes involving cardiac hypertrophy, fibrosis, arterial stiffness, as well as in inflammation and oxidative stress. In rats, Aldo increases inflammation through an MR-dependent pathway both in vitro and in vivo. Concerning fibrosis, evidence from animal experiments as well as Randomized Aldactone Evaluation (RALES) and Eplerenone Post-acute Myocardial Infarction Heart Failure Efficacy and Survival (EPHESUS) studies in patients with heart failure (HF) (with or without previous myocardial infarction) suggest that chronic MR blockade reduces markedly blood collagen peptides. These peptides are the biological markers of cardiovascular fibrosis, suggesting that Aldo is an important determinant of cardiovascular collagen turnover. In rats, Aldo infusion concomitantly with a high-sodium intake increases arterial stiffness with development of extracellular matrix (ECM) protein synthesis and inflammation. These findings are reinforced at the cellular level in vascular smooth muscle cells (VSMCs) where Aldo increases collagen synthesis via MR. Taken together, the published data indicate the potential contribution of Aldo in vascular remodeling, acting as a collagen regulator. However, the precise mechanisms responsible for Aldo-induced collagen synthesis in VSMCs remain to be determined.

Gal-3 (Galactosialactoside binding lectin family. It is present in the cytoplasm, nucleus, extracellular space, and bound to the cell surface. It is composed of a highly conserved N-terminal domain, which is required for its multimerization or for binding unglycosylated molecules, and a C-terminal carbohydrate recognition domain (CRD), which interacts preferentially with glycoproteins (extracellular and at the cell surface). It has been proposed that Gal-3 interacts with numerous ligands such as cell surface receptors (integrins) and ECM proteins (collagen, elastin, fibronectin). The expression of Gal-3 has been reported in many tissues (including myocardium). Gal-3 is expressed in fibroblasts, endothelial cells, and inflammatory cells such as macrophages which appear to be involved in most models of injury.

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This lectin is involved in numerous physiological and pathological processes some of which, inflammation and fibrosis, are pivotal contributing to pathophysiological mechanisms in the development and progression of arterial stiffness. Indeed, it has been demonstrated that Gal-3 is a central contributor to the pathology of atherosclerotic plaque progression by amplification of key pro-inflammatory molecules in the aorta. Moreover, Gal-3 is associated with cardiac dysfunction through induction of cardiac fibroblast proliferation, collagen deposition, and ventricular dysfunction. However, its vascular effects have never been investigated. Previous studies have demonstrated that several cytokines (interleukin-2, -4, and -7) regulate Gal-3 expression in T lymphocytes. Of particular interest was the finding that myocardial Gal-3 expression was enhanced in Ren-2 rats and in angiotensin II-treated mice, suggesting a role for the renin–angiotensin system in Gal-3 regulation. Indeed, it has been recently demonstrated that angiotensin II stimulates Gal-3 expression in cardiac myofibroblasts. Several inhibitors of Gal-3 have been well described, such as lactose, N-Acetyl-D-lactosamine, and the modified citrus pectin (MCP) (a complex water soluble indigestible polysaccharide riche in β-galactose). MCP contains fragments of the original pectin molecule, including rhamnogalacturonan 1 regions which contain galactan side-chains which are recognized CRD of Gal-3, with a high affinity and block the lectin’s activity. These inhibitors have been used to determine the role of Gal-3 in cell migration and adhesion as well as in a metastasis model. In humans, plasma Gal-3 emerges as a biomarker associated with HF onset, morbidity, and mortality.

Therefore, our hypothesis was that Gal-3 may be a new important factor inducing not only cardiac, but also vascular remodeling via facilitating collagen synthesis in VSMCs. Moreover, we propose that this lectin could be involved in Aldo-induced vascular fibrosis. The aim of this study was to investigate the expression and the effects of Gal-3 in VSMCs and to determine whether Gal-3 could be a mediator of Aldo effects in VSMCs in vitro and in vivo.

Materials and Methods

Detailed Materials and Methods are available in the online-only Data Supplement.

Animals

The investigation was performed in accordance with the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (publication no. 82-23, revised in 1996). For the Aldo+salt model, adult male Wistar rats were treated for 3 weeks with vehicle (n=10), Aldo-salt (1 mg/kg per day, n=10 and 1% NaCl as drinking water), Aldo-salt plus Spironolactone (Spirono) (200 mg/kg per day, n=10), Aldo-salt plus MCP (100 mg/kg per day, n=9), Spirono (n=7) or MCP (n=5) alone. The model and the dose of Aldo were chosen from previous studies in which the treatment increased systolic BP and diastolic BP.

As described previously, to analyze short-term effects of Aldo, adult male C57Bl6 wild-type (WT) mice and Gal-3 knock-out (KO) mice received 1 intraperitoneal injection of Aldo (0.1 mL injected, 1 mg/kg) or vehicle (0.1 mL injected, 150 mmol/L NaCl, 5% ethanol) (n=6, each group). Tail cuff BP was measured 12 hours before and 6 hours after injection. Animals were sacrificed 6 hours after the injection. To analyze long-term effects of Aldo, adult male C57Bl6 WT mice and Gal-3 KO mice were infused for 3 weeks with Aldo (1 mg/kg per day) or vehicle (150 mmol/L NaCl, 5% ethanol) using an osmotic minipump (Alzet) (n=7, each group). Tail cuff BP was monitored throughout the treatment.

VSMCs Isolation and Culture

Primary rat aortic VSMCs were isolated from the thoracic aorta of male Wistar rats as described previously. VSMCs were stimulated with Aldo at 10−9 mol/L and inhibitors at 10−6 mol/L. Transient transfections with plasmid were performed in VSMCs by XtremGENE9 (Roche) according to the manufacturer’s recommendations, with a vector construct containing either the recombinant human full length sequence of Gal-3 and cloned in pcDNA3.1 plasmid (Invitrogen) or a sequence coding for a truncated form of recombinant human Gal-3 (coding amino acids 104 to 250; recombinant human CRD) and an empty vector was used as control. Transfection of VSMCs with small interfering RNA (siRNA) was realized with Gal-3 mRNA target specific and using MATra-si (IBA) according to the manufacturer’s recommendations, and scramble siRNAs were used as control.

Statistics

Results are presented as mean±SEM, computed from the average measurements obtained from each group of cells or animals. Normal distribution of data was checked by means of the Shapiro Wilks test and a Levene statistic test was performed to check the homogeneity of variances. Differences among >2 experimental conditions were tested by the ANOVA 1-way test, followed by the Scheffé test to analyze differences between groups. The unpaired Student t test or the Mann Whitney U test was used to assess statistical differences between 2 experimental conditions. P<0.05 was considered significant. A sensitivity analysis adjusting for BP variations in the rat model was also performed, using a 2-way ANOVA.

Results

Aldo Induced Gal-3 Upregulation

Gal-3 expression was detected at the mRNA and the protein levels in primary cultured quiescent VSMCs (Figure 1) and was localized in the perinuclear area (data not shown). Treatment with Aldo increased Gal-3 protein levels in a concentration-dependent manner in primary VSMC cultures. This increase was significant compared with the control for the 2 concentrations of Aldo, 10−9 mol/L, (2.3-fold, P<0.05) and 10−8 mol/L (3.6-fold, P<0.05) (Figure 1A). Time-course examination showed that induction of Gal-3 expression by Aldo started at 12 hours (1.6-fold) and peaked significantly at 24 hours (2.3-fold, P<0.05) (Figure 1B).

We investigated next the involvement of MR in Aldo-induced Gal-3 upregulation. Both eplerenone and RU28318 abolished (P<0.05) Aldo-induced Gal-3 upregulation at the mRNA and protein levels (Figure 1C and 1D). Neither eplerenone nor RU28318 affected Gal-3 levels in the absence of Aldo (data not shown). To evaluate whether Aldo-induced Gal-3 increase involved de novo synthesis of intermediary proteins, we analyzed the effects of transcription (actinomycin D) inhibitor. It completely inhibited (P<0.05) the increase in Gal-3 mRNA (Figure 1E) and protein (Figure 1F) induced by Aldo. Actinomycin D did not affect Gal-3 levels in the absence of Aldo (data not shown).

Gal-3 Overexpression Induced Collagen Type I Protein Synthesis

VSMCs were transfected with either the control vector (empty) or the vector containing recombinant human Gal-3 or recombinant human CRD sequence (Figure 2). Cells overexpressing recombinant human Gal-3 presented both native Gal-3 (molecular weight=30 kDa) and recombinant Gal-3 (molecular weight=35 kDa) with a higher molecular weight compared...
with native because of the polyhistidine tail. As our antibody was unable to recognize the recombinant human CRD, we have performed complementary experiments of transfection and analyzed the messengers of each rh protein by reverse transcription-polymerase chain reaction with primers able to discriminate human from rat to validate the expression of the recombinant forms (data not shown). Cells overexpressing recombinant human Gal-3 exhibited increased (1.6-fold, \( P < 0.05 \)) collagen type I deposition, without changes in collagen type III, fibronectin, or elastin levels as compared with cells transfected with the empty vector. Cells overexpressing recombinant human CRD did not show significant variation in any protein of interest.

**Gal-3 Inhibition Blocked Aldo-Induced Collagen Type I Deposition**

To investigate the role of Gal-3 in Aldo-induced ECM changes, Gal-3 silencing was used. Gal-3 siRNA reduced efficiently its target decreasing Gal-3 level (0.5-fold versus scramble, \( P < 0.05 \); Figure 3A). However, Gal-3 silencing had no significant effect on ECM protein deposition at the basal level compared with the scramble. Primary VSMC cultures treated with Aldo showed a significant increase in collagen type I (1.6-fold versus scramble, \( P < 0.05 \)) and elastin (1.6-fold versus scramble, \( P < 0.05 \)). Gal-3 knockdown blocked Aldo increased collagen type I deposition (\( P < 0.05 \)). However, Gal-3 silencing did not modify the elastin increase induced by Aldo. Collagen type III level was not affected by Gal-3 siRNA when VSMCs were treated with Aldo. Moreover, using MR antagonists eplerenone and RU28318, Aldo-induced collagen type I expression was blocked and therefore was mediated by MR whereas elastin expression was MR independent (data not shown).

To confirm whether Gal-3 is necessary for Aldo to induce collagen synthesis, 2 Gal-3 inhibitors binding with high affinity to the sugar-recognizing site were used in primary VSMC
Densitometry values were normalized to represent the mean±SEM of the 3 independent experiments. Conditions were performed at least in triplicate. Histogram bars of VSMCs from at least 3 different rats were used and all conditions were performed at least in triplicate. *P<0.05 vs empty vector; †P<0.05 vs Gal-3.

First, we used immunochemistry techniques to localize which marker of VSMC. Spiro or MCP Treatment in Rats and Vascular Fibrosis Were Blocked by Aldo-Salt-Induced Hypertension, Inflammation, and Vascular Fibrosis Were Blocked by Spiro or MCP Treatment in Rats

Aldo-Salt-Induced Hypertension, Inflammation, and Vascular Fibrosis Were Blocked by Spiro or MCP Treatment in Rats

First, we used immunochemistry techniques to localize which cells express Gal-3 in the aortic tunica media (Figure 4A). Gal-3 was co-localized with α-smooth muscle actin, a specific marker of VSMC.

Average body weight as well as BP and heart rate for the 6 groups of rats are presented in the Table. Body weight did not differ between all the groups. Aldo-salt treatment induced an increase in systolic BP, diastolic BP, and mean BP as well as pulse pressure, and a decrease in heart rate. Hypertension as well as the decrease in heart rate was prevented by Spiro and MCP treatments. All the above parameters were unafacted by Spiro or MCP without Aldo. These changes were accompanied by morphometric modifications of the aorta. Aldo-treated rats presented increased aortic media thickness (1.2-fold, P<0.05) as well as aortic media cross-sectional area (1.2-fold, P<0.05) as compared with the control rats. Morphological changes were normalized by concomitant treatment with Spiro or MCP (P<0.05). Both parameters were unaffected by treatment with Spiro or MCP without Aldo.

The thoracic aortic media wall composition is described in the Table. Dry weight per centimeter and the cell protein content were significantly increased by the Aldo-salt treatment compared with control rats, indicating that hypertrophy of the media had occurred. These significant increases were not observed under co-treatment with Spiro or MCP. The increased dry weight reflects not only the increase in VSMC content but also an increase in some extracellular components. A significant increase in collagen (2.1-fold, P<0.05) and elastin (1.2-fold, P<0.05) content was observed, and consequently, there was a significant increase in the collagen to elastin ratio after Aldo-salt treatment that was corrected by both Spiro and MCP.

These results on ECM remodeling were confirmed by complementary approaches and completed by inflammatory response analysis. Aortic mRNA analysis (Figure 4B) revealed that Aldo-induced Gal-3, col1a1, monocyte chemoattractant protein-1, and osteopontin (OPN) synthesis were inhibited by both Spiro and MCP treatment (P<0.05). Aldo-induced interleukin-6 synthesis was partially blocked by Spiro and completely inhibited by MCP treatment (P<0.05). Aortic protein productions of Gal-3, collagens (type I and type III), elastin, monocyte chemoattractant protein-1, and OPN (P<0.05, Figure 4C and 4D) were enhanced by Aldo treatment. These increases were normalized by treatment with Spiro or MCP (P<0.05). Neither Spiro nor MCP treatment in the absence of Aldo affected measured protein levels (data not shown).

Sensitivity analyses were performed to investigate the influence of BP variations on Gal-3 and collagen expressions. Systolic BP was found as the parameter the most strongly associated with these factors. The BP component has been removed by adjustment for systolic BP variations and the association of Spiro with Gal-3 and collagen variations was no longer found significant. However, the effect of MCP on these factors remained significant, thereby suggesting that MCP exerted anti-Gal-3 and antifibrotic activities independently of BP variations.

Aldo Induces the Short-Term Expression of col1a1 mRNA in WT But Not in KO Gal-3 Mice

To complete the understanding of Gal-3 actions in Aldo-induced vascular collagen accumulation, WT and Gal-3 KO mice were challenged in a short-term model with acute exposure to Aldo. No differences in baseline aortic morphology (media thickness and media cross-sectional area) were observed between the 2 strains WT and KO (data not shown). No differences in BP levels were noticed between WT and KO mice at baseline or after treatment (data not shown). Gal-3 mRNA was present in the media of aorta even under basal conditions (Figure 5A). Moreover, Aldo-treated WT mice presented increased aortic Gal-3 (4.7-fold, P<0.05), col1a1 (2.2-fold, P<0.05), and OPN (3.9-fold, P<0.05) mRNA.
expressions but no significant changes in col3a1 mRNA expression. In Gal-3 KO mice, Aldo treatment did not modify col1a1, col3a1, or OPN mRNA expressions.

**Gal-3 KO Mice Are Protected From Inflammation and Collagen Type I Deposition Induced by the Long-Term Aldo Treatment**

The treatment by Aldo of WT and Gal-3 KO mice was extended to a 3-week infusion of Aldo. This treatment did not alter BP levels in WT nor in Gal-3 KO mice (data not shown). Basal levels of col1a1, col3a1, OPN and brain natriuretic peptide were similar in untreated WT and Gal-3 KO mice, whereas Gal-3 KO mice presented increased bone morphogenetic protein-4 level as compared with WT. Aldo-treated WT mice presented increased aortic mRNA expression of Gal-3 (1.6-fold, P < 0.05), col1a1 (2.5-fold, P < 0.05), col3a1 (2.1-fold, P < 0.05), OPN (2.7-fold, P < 0.05), and decreased brain natriuretic peptide mRNA expression (0.3-fold, P < 0.05) (Figure 5B) as compared with control WT mice. Meanwhile, no significant changes in bone morphogenetic protein-4 mRNA expression were observed. In Gal-3 KO mice, Aldo treatment did not modify col1a1, col3a1, OPN, bone morphogenetic protein-4 nor brain natriuretic peptide mRNA expressions. Aortic Gal-3 protein concentration was increased in Aldo-treated WT mice (2.1-fold, P < 0.05) compared with control WT mice (Figure 5C). Aldo-treated WT mice presented increased protein expression of collagen type I (1.9-fold, P < 0.05) and type III (1.5-fold, P < 0.05), whereas Gal-3 KO mice were specifically resistant to Aldo-dependent increase in collagen type I (Figure 5D). Aldo-induced collagen type III expression was not affected in Gal-3 lacking mice. KO mice were also resistant to Aldo-induced monocyte chemotactant protein-1 and OPN expressions in the aortic tunica media (Figure 5E).

**Discussion**

The purpose of this study was to investigate Gal-3 effects on VSMCs and its role as a mediator of Aldo-induced ECM remodeling in vitro and in vivo. We demonstrate that Gal-3 is spontaneously expressed in primary VSMCs, and its overexpression increases specifically collagen type I production. This effect depends on the multimerization of Gal-3 because only the full length and not the truncated protein (CRD) is effective. Furthermore, Aldo increases Gal-3 expression in cultured VSMCs and in aortic tunica media from rats and mice. In primary VSMC cultures and in aorta, this effect is dependent on MR activation. Additionally, the inhibition of Gal-3 in vitro (siRNA and blockade of its lectin-binding activity) or in vivo (MCP-treated rats, Gal-3 KO mice) blocks specifically Aldo-induced inflammation, collagen type I deposition, and vascular remodeling. Thus, Gal-3 emerges as a key pathway involved in collagen regulation in VSMCs and as a potential mediator of Aldo-induced vascular fibrosis.
It is generally believed that the composition of the ECM is a critical determinant of arterial stiffness, but the signaling pathways involved in this process are not fully understood. In our study, we describe for the first time that Gal-3 overexpression in primary VSMCs results in increased collagen type I synthesis without changes in collagen type III or elastin levels, therefore increasing collagen type I/III and collagen type I/elastin ratios favoring matrix stiffness. Moreover, overexpression of truncated Gal-3 does not affect ECM protein synthesis, suggesting that multimerization is necessary for its fibrotic role. Interestingly, in accordance with our observations, it has been demonstrated in rat acute kidney injury that Gal-3 plays a major role in renal collagen deposition, because its specific blockade with MCP prevents specially collagen type I accumulation but not collagen type III. To explain this specificity of Gal-3 on collagen type I, the authors propose that the collagen type I promoter contains a high number of SP-1 binding sites through which Gal-3 might act but they are not present for collagen type III. This increase in collagen deposition is responsible for a large part of vascular fibrosis, an important factor leading to arterial stiffness. In addition to vascular fibrosis, accumulating evidence shows that Gal-3 is a profibrotic factor mediating aldosterone-induced arterial inflammation and fibrosis. Representative fluorescent photomicrographs of confocal microscopy sections of the aortic tunica media from rats are shown (A). Aortas are staining for nuclei (blue), α-smooth muscle actin (α-SMA) (green), and Gal-3 (red). Arrows show Gal-3 and α-SMA staining merged (yellow/orange). Aortas from rats treated with Aldo-salt (1 mg/kg per day) in combination with Spiro (200 mg/kg per day) or modified citrus pectin (MCP) (100 mg/kg per day) were analyzed. mRNA expression of Gal-3, col1a1, interleukin-6 (IL-6), monocyte chemotactant protein-1 (MCP-1), and osteopontin (OPN) are shown (B). Spiro and MCP co-treatment blocked Aldo-induced mRNA synthesis. Protein expression of Gal-3, collagen type I, and type III as well as elastin are shown (C). Spiro and MCP co-treatment reversed Aldo-induced extracellular matrix protein deposition. Representative pictures of slides stained for elastin as well as immunostained for collagen type I, collagen type III, MCP-1, and OPN are presented (D). Spiro and MCP co-treatment reversed Aldo-induced inflammation and fibrosis. All conditions were performed at least in triplicate. Histogram bars represent the means±SEM of the 3 independent experiments, in arbitrary units normalized to β-actin and HPRT, respectively, for protein and mRNA. *P<0.05 vs control; †P<0.05 vs Aldo.
lung, cardiac, and renal failure,\textsuperscript{18,28,41} suggesting a major role for this lectin in organ fibrosis.

In our experimental models, in response to stimulation with Aldo, VSMCs show MR-dependent increased expression in Gal-3, in vitro and in vivo. Furthermore, as reported previously\textsuperscript{28} in kidney, our results show that Gal-3 inhibitors block the lectin's activity and decrease Gal-3 levels in VSMCs. However, the mechanism remains unclear and warrants further dedicated studies.

In spite of studies unveiling partial mechanisms to explain Aldo-induced ECM protein production,\textsuperscript{1} the effect of Aldo per se in matrix remodeling is still under debate.\textsuperscript{12,42} As regards the inflammatory response, our results show that Gal-3 inhibitors block the lectin's activity and decrease Gal-3 levels in VSMCs. However, the mechanism remains unclear and warrants further dedicated studies.

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<th>Table.</th>
<th>Hemodynamic Parameters and Aortic Wall Composition in Rats</th>
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Spiro indicates spironolactone; MCP, modified citrus pectin; BW, body weight; SBP, systolic blood pressure; DBP, diastolic blood pressure; MBP, mean blood pressure; PP, pulse pressure; HR, heart rate; MCSA, media cross-sectional area of the aorta; and Aldo, aldosterone.

Dry weight and contents in proteins are expressed in mg/cm, media thickness in µm and MCSA in 10¹ µm². Values are mean±SEM.

\*P<0.05 vs control group; †P<0.05 vs Aldo-treated group.

Results demonstrate that Gal-3, via its lectin activity, is a necessary mediator allowing Aldo-induced collagen type I synthesis, because the blockade of Gal-3 with MCP (in cell cultures and in rats) or N-Acetyl-D-lactosamine (in cell cultures) abolishes Aldo-induced collagen type I deposition. In confirmation of the pharmacological data, Gal-3-depleted VSMCs and Gal-3 KO mice are resistant to Aldo profibrotic effects, especially collagen type I deposition. Indeed, in mice deficient for Gal-3, Aldo increases collagen type III expression but fails to increase collagen type I, thus, normalizing the collagen type I/III ratio and the matrix stiffness. In comparison, WT mice treated with Aldo present an increase in the collagen type I/III ratio. This observation reinforces the specificity of Gal-3 action on collagen type I. In addition, KO mice are also resistant to Aldo-induced decrease in the antifibrotic factor brain natriuretic peptide. Hence, Aldo fails specifically to induce collagen type I expression in VSMCs and mice lacking Gal-3, suggesting that the absence of this lectin blocks Aldo effects on vascular fibrosis. Regarding elastin production, in primary rat VSMC cultures, Aldo induces elastin deposition in an MR and Gal-3 independent manner. This result is in accordance with previous studies that describe Aldo-induced elastogenesis as not dependent on MR.\textsuperscript{25} Thus, our current findings provide a new mechanism MR and Gal-3 dependent underlying vascular fibrosis and remodeling caused by Aldo.

That MCP alone does not alter BP levels but blunts Aldo-induced hypertension was unexpected because such an effect on BP levels has not been reported previously, and is an exciting finding. The fact that rats co-treated with Aldo+MCP present normal levels of all vascular ECM proteins (not only the collagen type I but also collagen type...
III and elastin) could be partly a consequence of the reduction in BP induced by MCP because it has been widely described that changes in vascular remodeling and BP have close links, acting on each other. Interestingly, sensitivity analyses adjusted for BP variation show the persistence effect of MCP (but not Spiro) on fibrosis inhibition in rats, a finding corroborated by our results in the Aldo-treated mice model (short- and long-term), without BP variation. However, additional studies focusing on heart, kidney, and small vessel interactions may highlight the mechanisms involved in BP normalization.

In conclusion, we propose that Gal-3 is a key player in Aldo-induced vascular inflammation as well as vascular remodeling, and a potential therapeutic target in vascular stiffness occurring during hypertension in which Aldo is involved. Moreover, it has been suggested that Gal-3 is involved in cardiovascular diseases as a mediator of HF development and progression. It has been further described that Gal-3 plasma levels increase during HF development, associated with cardiac dysfunction. Further clinical studies are required to establish a relation between this lectin and Aldo in cardiovascular pathologies (including hypertension and HF) and the potential therapeutic benefit of Gal-3 inhibition with MCP.

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References


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SUPPLEMENTAL MATERIAL

MATERIALS AND METHODS

Animals

The investigation was performed in accordance with the Guide for Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (publication no. 82-23, revised in 1996). Male Wistar rats (250 g; Harlan Iberica) were treated for 3 weeks with vehicle (sunflower oil, subcutaneous injection, n=10), Aldo-salt (1 mg/kg/day, subcutaneous injection, n=10 and 1% NaCl as drinking water), Aldo-salt plus Spironolactone (Spiro) (200 mg/kg/day, subcutaneous injection, n=10), Aldo-salt plus MCP (100 mg/kg/day, in food, n=9), Spiro (n=7) or MCP (n=5) alone. The model and the dose of Aldo were chosen from previous studies in which the treatment increased systolic (SBP) and diastolic blood pressure (DBP) ¹.

Adult male C57BJ6 wild-type mice and Gal-3 KO mice ² were infused for 3 weeks with Aldo (1 mg/kg/day) or vehicle (150 mmol/liter NaCl, 5% ethanol) using an osmotic minipump (Alzet) (n = 7, each group). Tail cuff blood pressure was monitored throughout the treatment. As described previously ³, to analysed short effects of Aldo mice received one intraperitoneal injection of Aldo (0.1 ml injected, 1 mg/kg) or vehicle (0.1 ml injected, 150 mmol/liter NaCl, 5% ethanol) (n = 6, each group). Tail cuff blood pressure was measured 12 h before and 6 h after injection. Animals were sacrificed 6 h after the injection under 3% isoflurane/O2 anesthesia.

Haemodynamic parameters

Rats were anaesthetized with ketamine (Imalgene 1000, Merial; 70 mg/kg, I.P.) plus xylazine (Rompun 2%, Kup Pharma; 6 mg/kg, I.P.), and a catheter (Sciense FT211B, 1.5mm diameter) was advanced into the left ventricle through the right carotid artery. The catheter
was connected to a data acquisition system (PowerLab/800, ADInstruments), and signals were monitored and digitally stored for analysis with the software Chart for Windows (version 4.2).

**VSMCs isolation and culture**

Primary rat aortic VSMCs were isolated from the thoracic aorta of male Wistar rats as described previously. VSMCs were used between passages 3 and 8. For experiments, cells were seeded into 6-well plates at 90% confluence and serum starved (1 %) for 12h. Then, cells were cultured in the same medium and stimulated with Aldo (Sigma) at $10^{-10}$ to $10^{-8}$ mol/liter for 6 or 24 h respectively for mRNA and protein determination, except for the Aldo ($10^{-8}$ mol/liter) time-response experiments (from 0 to 48 h). To investigate the intracellular pathways, the following reagents were added 30 min before Aldo ($10^{-8}$ mol/liter) addition: RNA synthesis inhibitor actinomycin D ($10^{-6}$ mol/liter; Calbiochem), two MR antagonists eplerenone ($10^{-6}$ mol/liter; Sigma) and RU28318 ($10^{-6}$ mol/liter; Tocris Bioscience), two Gal-3 inhibitors MCP ($10^{-6}$ mol/liter; Econugenics) and LacNac ($10^{-6}$ mol/liter; Sigma).

**Western Blot analysis**

The aortas were dissected, cut longitudinally (to remove the endothelium), and the adventitia layer was removed. Then aortic tunica medias were immediately frozen in liquid nitrogen for molecular studies. Aliquots of 20 µg of proteins were electrophoresed on SDS polyacrylamide gels and transferred to Hybond-c Extra nitrocellulose membranes (Amersham Biosciences). The following specific antibodies were used: Gal-3 at 1:500 (Thermo Scientific), collagen type I at 1:500 (Biogenesis), collagen type III at 1:500 (Santa Cruz), fibronectin at 1:500 (Chemicon), elastin at 1:250 (Abcam), and β-actin at 1:1000 (Sigma). Bound antibodies were detected by peroxidase-conjugated secondary antibodies (Amersham Biosciences) and visualized using the Immun-Star western kit (Bio-rad) or ECL-Advance chemiluminescence detection system (Amersham). After densitometric analyses, optical density values were expressed as arbitrary units and β-actin was used to normalise protein.
loading. All Western Blots were performed at least in triplicate for each experimental condition.

**ELISA**

Gal-3 concentrations were measured by ELISA according to the manufacturer’s instructions (R&D Systems).

**Reverse-transcription PCR**

Total RNA was extracted with Trizol Reagent (Euromedex) and purified using the RNeasy kit, according to the manufacturer’s instructions (Qiagen). First strand cDNA was synthesized according to the manufacturer’s instructions (Roche). Quantitative PCR analysis was then performed with SYBR green PCR technology (ABGene). List of the primers (Invitrogen) used in rat:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>Sequence (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gal-3</td>
<td>Forward</td>
<td>AGC CCA ACG CAA ACA GTA TC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GGC TTC AAC CAG GAC CTG TA</td>
</tr>
<tr>
<td>HPRT</td>
<td>Forward</td>
<td>AGG ACC TCT CGA AGT GT</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ATT CAA ATC CCT GAA GTA CTC AT</td>
</tr>
<tr>
<td>IL-6</td>
<td>Forward</td>
<td>GCC CTT CAG GAA CAG CTA TG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GTC TCT TCT CCG GAC TTG TG</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Forward</td>
<td>TTC CTT ATT GGG GTC AGC AC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CAG TTA ATG CCC CAC TCA CC</td>
</tr>
<tr>
<td>OPN</td>
<td>Forward</td>
<td>ATG AGA CTG GCA GTG GTT</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GCT TTC ATT GGA GTT GCT</td>
</tr>
<tr>
<td>Col1a1</td>
<td>Forward</td>
<td>GCC TCC CAG AAC ATC ACC TA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ATG TCT GTG TTG CCC CAA GT</td>
</tr>
</tbody>
</table>

And in mouse:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>Sequence (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col1a1</td>
<td>Forward</td>
<td>TAC TCG AAC GGG AAT CCA TC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ACT GGT ACA TCA GCC CGA AC</td>
</tr>
<tr>
<td>Col3a1</td>
<td>Forward</td>
<td>ACC AAA AGG TGA TGC TGG AC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GAC CTC GTG CTC CAG TTA GC</td>
</tr>
<tr>
<td>Gal-3</td>
<td>Forward</td>
<td>GCT TAT CCT GGC TCA ACT GC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TTC ACT GTG CCC ATG ATT GT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward</td>
<td>AAC TTT GGC ATT GTG GAA GG</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>GGA TGC AGG GAT GAT GTT CT</td>
</tr>
<tr>
<td>BMP-4</td>
<td>Forward</td>
<td>TGA GCC TTT CCA GCA AGT TT</td>
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</table>
Reverse  CTT CCC GGT CTC AGG TAT CA
BNP  Forward  AGA CCC AGG CAG AGT CAG AA
Reverse  CAG CTC TTG AAG GAC CAA GG
OPN  Forward  GAC TGG TGC GTG ACC CAT CT
Reverse  TTC ATT GGA ATT GCT TGG AAG A

Relative quantification was achieved with MyiQ (Bio-rad) software according to the manufacturer's instructions. HPRT or GAPDH, in rat or mouse models respectively, was used as the endogenous control. All PCRs were performed at least in triplicate for each experimental condition.

**Transient transfection with plasmid**

VSMCs were seeded into 6-well plates at 70% confluence, and serum starved for 12h, then transfected with 1 µg each of a vector construct containing either the recombinant human full-length sequence of Gal-3 (rhGal-3) cloned in pcDNA3.1 plasmid (Invitrogen) or a sequence coding for a truncated form of recombinant human Gal-3 (coding amino acids 104 to 250; rhCRD). An empty vector was used as a control. Transfections were performed with XtremGENE9 (Roche) according to the manufacturer's recommendations for 24h. Expressed proteins are tagged with a polyhistidine tail. Cells were then incubated in medium with 1% FBS for 48h before cell harvest. Validation of the transfections was made by Western Blot and RT-PCR.

**Transfection of VSMCs with siRNA**

VSMCs were seeded into 6-well plates at 70% confluence and transfected with a pool of three siRNAs (GeneCust) Gal-3 target-specific and using MATra-si (IBA) according to the manufacturer's recommendations. Cells were allowed to recover for 24h before serum privation and stimulation. Scramble siRNAs were used as a control.

**Aortic composition**

Aortic segments were opened longitudinally, the media separated from the adventitia and the medial length measured under a microscope. Media were then defatted, dried and
Medial cell proteins were extracted by 0.3% sodium dodecyl sulfate (SDS) and subsequently assayed, insoluble elastin was purified by the hot alkali method and quantified by weighing. Proteins in the NaOH extract were then hydrolysed, and total medial collagen was quantified by assaying hydroxyproline in the hydrolysate, using a colorimetric assay.

**Morphological and histological evaluation**

Sections (5µm-thick) were stained with orcein for elastin content. Media thickness of the aorta was measured in five different regions and media cross sectional area of the aorta (MCSA) was measured in triplicate with NIS software (Nikon).

**Immunohistochemistry**

Paraffin-embedded aorta sections of 5 µm were used. Slides were treated with H₂O₂ for 10 min to block peroxidase activity. All sections were blocked with 5% normal goat serum in PBS for 1 h and incubated overnight with collagen type I, collagen type III, monocyte chemoattractant protein-1 (MCP-1) or osteopontin (OPN) (1:50 dilution; Abcam), washed three times, and then incubated for 30 min with the horseradish peroxidase-labeled polymer conjugated to secondary antibodies (Dako Cytomation, Carpentaria, CA). The signal was revealed by using DAB Substrate Kit (BD Pharmingen).

**Double staining immunohistochemistry**

Arterial segments were placed in Krebs buffer containing 30% sucrose, transferred to a cryomold containing Tissue-Tek OCT embedding medium (Jung Tissue Freezing Medium, Leica), and frozen in liquid nitrogen. Tissues were kept at -80°C until the day of the experiments. Frozen transverse sections (6 µm) were cut onto APS-coated slides and air-dried for at least 60 min. Preincubation was carried out for 60 minutes in a PBS solution containing 30% normal goat serum. Slides were then incubated overnight at 4 °C in a solution containing a mouse monoclonal antibody against alpha smooth muscle actin (α-SMA) (1:100, Biocare Medical, USA) and a rabbit monoclonal antibody against Gal-3 (1:50, Epitomics,
USA). After three washings (5 min each) with the secondary antibody, a donkey anti-mouse or anti-rabbit IgG conjugated to Alexa 488 or Alexa 546 (Molecular Probes, Life Technologies, Madrid, Spain) for 1 h at 37ºC. Cells' nuclei were staining using 4'-6-Diamidino-2-phenylindole (1mg/ml; DAPI; Sigma; USA). After washing, immunofluorescent signals were viewed using an inverted Leica TCS SP2 confocal laser scanning microscope with oil immersion lens (x40). The specificity of the immunostaining was evaluated by omission of the primary antibody and processed as above. Under these conditions, no staining was observed in the vessel wall.

Statistics

Results are presented as mean±SEM, computed from the average measurements obtained from each group of cells or animals. Normal distribution of data was checked by means of the Shapiro Wilk's test and a Levene statistic test was performed to check the homogeneity of variances. Differences among more than 2 experimental conditions were tested by the ANOVA one way test, followed by the Scheffé test to analyze differences between groups. The unpaired Student’s t test or the Mann Whitney U tests were used to assess statistical differences between two experimental conditions. P values lower than 0.05 were considered significant. A sensitivity analysis adjusting for BP variations in the rat model was also performed, using a two way ANOVA.

REFERENCES