**Downregulation of Renal TRPM7 and Increased Inflammation and Fibrosis in Aldosterone-Infused Mice**

Effects of Magnesium

Bruno Sontia, Augusto C.I. Montezano, Tamara Paravicini, Fatiha Tabet, Rhian M. Touyz

**Abstract**—Hyperaldosteronism is associated with hypertension, cardiovascular fibrosis, and electrolyte disturbances, including hypomagnesemia. Mechanisms underlying aldosterone-mediated Mg$^{2+}$ changes are unclear, but the novel Mg$^{2+}$ transporters TRPM6 and TRPM7 may be important. We examined whether aldosterone influences renal TRPM6/7 and the TRPM7 downstream target annexin-1 and tested the hypothesis that Mg$^{2+}$ administration ameliorates aldosterone-induced cardiovascular and renal injury and prevents aldosterone-associated hypertension. C57B6 mice were studied (12 weeks, n=8 to 9/group); (1) control group (0.2% dietary Mg$^{2+}$), (2) Mg$^{2+}$ group (0.75% dietary Mg$^{2+}$), (3) aldosterone group (Aldo, 400 µg/kg/min and 0.9% NaCl drinking water), and (4) Aldo+Mg$^{2+}$ group. Blood pressure was unaltered by aldosterone and was similar in all groups throughout the experiment. Serum Na$^+$ increased and serum Mg$^{2+}$ decreased in the Aldo group. Aldo mice had hypomagnesuria and proteinuria, and renal, cardiac, and aortic fibrosis, which were normalized by Mg$^{2+}$ supplementation. Renal and cardiovascular expression of interleukin-6, VCAM1 and COX2 was increased in the Aldo group. Magnesium attenuated renal and cardiac interleukin-6 content and decreased renal VCAM1 and cardiac COX2 expression (P<0.05). Aldosterone decreased expression of renal TRPM7 and the downstream target annexin-1 (P<0.05) without effect on TRPM6. Whereas Mg$^{2+}$ increased mRNA expression of TRPM6 and TRPM7, it had no effect on TRPM7 and annexin-1 protein content. Our data demonstrate that aldosterone mediates blood pressure–independent renal and cardiovascular fibrosis and inflammation through Mg$^{2+}$-sensitive pathways. We suggest that altered Mg$^{2+}$ metabolism in hyperaldosteronism may relate to TRPM7 downregulation and that Mg$^{2+}$ protects against cardiovascular and renal damaging actions of aldosterone. (Hypertension. 2008;51:915-921.)

**Key Words:** TRP channels ■ cations ■ cardiovascular remodeling ■ blood pressure ■ vascular cell adhesion molecule ■ COX2 ■ annexin-1

Aldosterone, classically thought to be produced by the zona glomerulosa of the adrenal cortex and implicated in the maintenance of sodium, potassium, and acid-base balance and blood pressure regulation, is now considered a hormone with pleiotropic actions, produced by multiple tissues, including the heart, vessels, kidney, and brain. In additional to regulating renal electrolyte excretion, aldosterone contributes to vascular inflammation, oxidative stress, collagen deposition, and endothelial dysfunction. As such, aldosterone has been implicated in the development of cardiovascular and renal remodeling, fibrosis, and injury. The importance of these processes in clinical medicine is being increasingly recognized by the cardiovascular and renal protective effects of the aldosterone antagonists, spironolactone and eplerenone.

The profibrotic and proinflammatory actions of aldosteronism are accompanied by disturbances in cation homeostasis including hypomagnesemia and decreased intracellular free magnesium concentration ([Mg$^{2+}$]). Recent evidence suggests that aldosterone-induced cardiovascular inflammation is induced, in part, by decreased [Mg$^{2+}$], with Ca$^{2+}$ loading leading to increased H$_2$O$_2$ formation and cellular oxidative stress. Hyperaldosteronism-associated renal magnesium wasting and cardiovascular injury and fibrosis are ameliorated by spironolactone and eplerenone and by Mg$^{2+}$ administration. Mechanisms whereby aldosterone causes Mg$^{2+}$ depletion are unclear, but the recently identified novel magnesium transporters, transient receptor potential melastatin 6 and 7 (TRPM6 and TRPM7), may be important. Both proteins share the unique feature of an atypical kinase domain at their C terminus and are negatively regulated by intracellular Mg$^{2+}$ levels. TRPM6 and TRPM7 have been described as the “gatekeepers” of human Mg$^{2+}$ metabolism, but may also play a role in intracellular signaling events, because TRPM7 kinase activates downstream targets annexin-1, calpain, and myosin II A heavy
Table 1. Body Weight, Heart and Kidney Size, and Biochemical Parameters in the Different Groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Mg2+</th>
<th>Aldo</th>
<th>Aldo + Mg2+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>27.9±0.40</td>
<td>31.5±0.74</td>
<td>31.3±0.71</td>
<td>29.2±0.56</td>
</tr>
<tr>
<td>Heart size, g/mm</td>
<td>6.8±0.17</td>
<td>7.4±0.14</td>
<td>8.4±0.46*</td>
<td>8.1±0.19</td>
</tr>
<tr>
<td>Kidney size, g/mm</td>
<td>1.9±0.04</td>
<td>2.1±0.05</td>
<td>2.6±0.12†</td>
<td>2.7±0.05†</td>
</tr>
</tbody>
</table>

Serum biochemistry

| Na⁺                        | 143±1.5  | 143±0.46§ | 148±0.4‡ | 146±0.7† |
| K⁺                        | 5.3±0.4  | 6.7±0.2  | 4.4±0.3* | 4.6±0.1 |
| Mg2⁺                      | 0.77±0.05 | 0.92±0.02‡ | 0.71±0.03 | 0.97±0.06‡ |
| Ca2⁺                      | 1.93±0.12 | 1.94±0.04 | 1.83±0.01 | 2.10±0.05 |

Urine biochemistry

| Na⁺                        | 149±9    | 180±24§  | 105±15†  | 200±59¶ |
| K⁺                        | 472±45   | 444±35   | 322±61*  | 430±47  |
| Mg2⁺                      | 33±3.9   | 95±15¶   | 12±2.0‡  | 50±13§  |
| Ca2⁺                      | 1.5±0.3  | 2.5±0.6  | 1.0±0.3  | 2.0±0.05 |
| Protein                   | 8.6±0.4  | 6.1±0.6§  | 11.8±1.8¶ | 6.7±2.2§ |

Heart and kidney size measured as heart mass/tibial length and kidney mass/mass tibial length.

Serum and urine Na⁺, K⁺, Mg2⁺, and Ca2⁺ values expressed as mmol/L.

Urine protein values expressed as mg/L.

*P<0.05 vs control, †P<0.01 vs control, ‡P<0.001 vs control, §P<0.05 vs Aldo group, ¶P<0.01 vs Aldo group.

An expanded Methods section is available in an online data supplement at http://hyper.ahajournals.org.

Materials and Methods

Blood Pressure Measurements and Serum and Urine Analysis

The systolic blood pressure (SBP) was measured in conscious mice every 3 days using the Visitech tail cuff system (BP 2000 Blood Pressure Analysis System, Visitech).

Blood and urine were collected at the beginning and end of the experiment. Levels of Mg2⁺, Ca2⁺, Na⁺, and K⁺ and urine protein were measured by automated methods at the hospital laboratory.

Analysis of TRPM6 and TPRM7 mRNA Levels With Real-Time Polymerase Chain Reaction

Frozen kidney samples were homogenized and RNA extracted using Trizol reagent (Invitrogen). 200 ng of each RNA sample was reverse transcribed using random hexamers and TaqMan reverse transcription (RT) reagents (Applied Biosystems). Specific primers and FAM-labeled probes for mouse TRPM6 and TRPM7 were designed using Primer Express software (Applied Biosystems) and sequences from the NCBI database (Table S1). All reactions were performed in triplicate, and a standard curve constructed on each plate using an independent control sample of kidney cDNA. The PCR conditions were: 50°C for 2 minutes, 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute (Applied Biosystems 7300 Real-time PCR system). Relative expressions of TRPM6 and TRPM7 in the unknown samples were determined from a standard curve and expressed relative to 18S.

Western Blotting

Proteins from kidney (renal cortex), heart, and aorta were extracted from frozen tissue as previously described.23 Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and incubated with specific antibodies to VCAM1 (Santa Cruz Biotechnology), COX2 (Cayman chemical), TRPM7 (Abcam), annexin-1 (Santa Cruz Biotechnology). Signals were revealed by chemiluminescence, visualized autoradiographically, and subsequently membranes were stripped (Pierce Biotechnology) and reprobed with GAPDH or a-actin antibodies (Chemicon International), which were used as internal controls. Optical density of bands was quantified densitometrically.
was significantly increased versus controls (heart, 6.88 ± 0.17 versus 2.63 ± 0.12, P < 0.001). In magnesium-supplemented aldosterone-treated animals (Table 1). Aldosterone infusion was associated with significantly reduced Na⁺ and Mg²⁺ levels, which were normalized by Mg²⁺ supplementation. Aldosterone-infused mice exhibited significant proteinuria, which was ameliorated by Mg²⁺.

**Renal Effects of Aldosterone and Magnesium Supplementation**

Renal fibrillar collagen deposition was increased in aldosterone-treated animals (Table 2). This was associated with an increased inflammatory response as evidenced by increased expression of IL-6, VCAM1, and COX2 (Figures 1 through 3). Magnesium supplementation did not influence renal fibrosis or inflammatory mediators in the control group, but significantly decreased effects in the aldosterone-treated group.

**Effects of Aldosterone and Magnesium on Renal Expression of TRPM6 and TRPM7**

As shown in (Figure 4), aldosterone had no effect on mRNA expression of TRPM6, but significantly reduced TRPM7 mRNA content (P < 0.05). Magnesium supplementation in-
creased expression of TRPM6 and TRPM7 in aldosterone-infused mice (P < 0.01). In control mice, TRPM7 mRNA expression was increased by Mg2+ (P < 0.05, t-test).

At the protein level, we evaluated expression of TRPM7, but not of TRPM6, because only TRPM7 gene seemed to be influenced by aldosterone, confirming our previous studies. Moreover it is very difficult to accurately assess TRPM6 protein because of the unavailability of sensitive anti-TRPM6 antibodies. As demonstrated in (Figure 5), protein content of renal TRPM7 and its downstream substrate annexin-1 was significantly reduced in the aldosterone group. This was unaffected by Mg2+ (Figure 5).

Cardiovascular Effects of Aldosterone and Magnesium Supplementation

As observed in the kidney, collagen deposition was significantly increased in the heart in response to aldosterone (Figure 6). This effect was attenuated in mice supplemented with Mg2+. Expression of cardiac IL-6, VCAM, and COX2 was significantly enhanced in the aldosterone group (Figures S2 and S3). Magnesium reduced IL-6 and COX2 expression, but did not influence cardiac VCAM1 expression.

Aortic collagen content and media thickness were significantly increased in the aldosterone-infused mice versus controls (53.91±1.8 versus 116±13.61, P < 0.01) (Table 2, Figure 7). Magnesium significantly reduced the media thickness of the aorta (116±13.61 versus 68.04±2.09, Aldo versus Aldo+Mg; P < 0.05). Vascular expression of VCAM1 and COX2 was significantly increased in the aldosterone-treated mice (P < 0.05) (Figure S4). Magnesium supplementation did not influence expression of proinflammatory mediators in the aorta.

Discussion

Major findings from the present study demonstrate that in C57B6 mice (1) aldosterone induces significant renal and cardiovascular hypertrophy, fibrosis, and inflammation independently of blood pressure elevation, (2) Mg2+ supplementation attenuates aldosterone-mediated cardiovascular and renal remodeling, (3) aldosterone decreases renal expression of the novel Mg2+ transporter TRPM7, but not TRPM6, and the TRPM7 downstream target annexin-1, and (4) Mg2+ administration prevents aldosterone-induced renal dysfunction (proteinuria) and electrolyte disturbances. Our findings highlight the potent profibrotic and proinflammatory actions of aldosterone in the heart, vasculature, and kidneys and suggest that Mg2+ plays a role in these processes. We also provide the first evidence that aldosterone modulates renal TRPM7 expression, which could be important in altered Mg2+ homeostasis associated with hyperaldosteronism.

An unexpected finding in our study was the failure of aldosterone to induce hypertension. This may relate to the fact that the mice investigated here were not uninephrectomized as in many other studies. Moreover, we studied C57B6 mice, which seem to be resistant to hypertensive-inducing stimuli. Other studies have also reported variable blood pressure actions of aldosterone, with some studies reporting a significant hypertensive effect and others showing no change in blood pressure. To ensure that the methodology used to measure blood pressure in our study was reliable, we performed a second set of experiments where mice were infused with Ang II (400 ng/mg/min). These mice developed hypertension within 3 days (SBP=160 mm Hg), similar to our previous studies indicating that the tail cuff technique used was functional.

![Figure 2](image-url) Immunohistochemical images demonstrate renal IL-6 content in the 4 groups. IL-6 is demonstrated as brown staining. IL-6 content was increased in the aldosterone group vs other groups. Magnesium reduced IL-6 content in aldosterone-treated mice. Images are representative of 8 to 9 mice in each group. Insert is a representative image from another section.

![Figure 3](image-url) Expression of proinflammatory mediators VCAM1 (A) and COX2 (B) in kidneys from control, magnesium-supplemented, aldosterone-treated, and aldosterone-magnesium groups. Upper panels are representative immunoblots. GAPDH was used as an internal control. Lower panels, Corresponding bar graphs representing data as means±SEM from 8 to 9 mice/group. Data expressed as VCAM1/GAPDH and as COX2/GAPDH and normalized to control, taken as 100%. *P<0.05, **P<0.01, ***P<0.001.
The fact that aldosterone did not cause hypertension yet induced marked cardiac, vascular, and renal remodeling as well as renal dysfunction (proteinuria) suggests that it has direct cardiovascular/renal effects independently of changes in blood pressure. This is supported by extensive experimental data indicating that aldosterone directly stimulates cell proliferation, hypertrophy, collagen deposition, and inflammation.27–29 Molecular mechanisms underlying these processes involve activation of MAP kinases, tyrosine kinases, NADPH oxidase-derived reactive oxygen species, and proinflammatory transcription factors, mediated through genomic and nongenomic pathways.1,30,31 Many of these signaling molecules are influenced by Mg2+/H⁺. We and others reported that MAP kinases, c-Src, cell cycle proteins, and NADPH oxidase are regulated by Mg2+ and that small changes in [Mg2+]i have significant effects on signal transduction and cellular functional responses.32–34 These processes may contribute, at least in part, to the protective effects of Mg2+ observed in the magnesium-supplemented group.

Considering that our experiment was a long-term study, we cannot exclude the possibility that some of the observed cardiovascular and renal changes may be secondary to aldosterone-induced electrolyte alterations. Nevertheless, the initiating stimulus was aldosterone, indicating that this hormone does play a role in the process. Hence, in chronic in vivo studies, aldosterone effects may be both direct and indirect.

Magnesium has important antiinflammatory and antioxidant properties. Mg2+-deficient mice exhibit severe cardiovascular inflammation and oxidative injury, which are normalized by Mg2+ supplementation.34,35 In our study, dietary Mg2+ ameliorated aldosterone-induced damaging actions and reduced fibrosis in the kidney, heart, and aorta, similar to effects previously reported.10 We examined expression of IL-6, a secondary cytokine, VCAM1, a proinflammatory adhesion molecule and COX2, a key enzyme involved in the inflammatory response. Although aldosterone significantly increased expression of all of these mediators, the antiinflammatory response of Mg2+ was variable, with major effects in kidney and heart and little effect in the aorta. Reasons for this regional heterogeneity are unclear but may relate to differences in [Mg2+]i in the different tissues. In support of our findings, Kramer et al also reported variable effects of dietary Mg2+ on circulating proinflammatory mediators in cardiac disease.36 It is also possible that Mg2+ actions are tissue-specific. For example in the aorta of Mg2+-supplemented mice, remodeling was markedly improved, whereas inflammatory responses were not, suggesting that in the vasculature effects on fibrosis and growth by Mg2+ may be more important than effects on inflammation.

Extensive clinical and experimental evidence indicates that hyperaldosteronism causes electrolyte changes, specifically hypokalemia, hypernatremia, and hypomagnesemia.8,9 In the aldosterone-infused mice, serum Na+ was increased and serum K+ was decreased confirming efficient absorption of the administered aldosterone. Reasons for these electrolyte changes are attributable to renal actions of aldosterone, which promote K+ excretion and Na+ reabsorption through activation of the Na+/H+ exchanger, ENaC, and Na+/K+ ATPase transporters.1 In our study, serum Mg2+ tended to be lower in
the aldosterone group. Urine Mg²⁺ was significantly reduced. These findings are in contrast to those reported by Runyan et al.⁸ and Chhokar et al.,³⁷ where aldosterone infusion induced hypermagnesuria. However, in those studies, ⁸,³⁷ rats, not mice, were investigated, uninephrectomy was performed, and animals were infused for 4 to 6 wk. Hence the experimental protocol was different from our study. Reasons for hypomagnesuria and relative hypomagnesemia in our aldosterone-infused mice may relate to downregulation of renal TRPM7 and to redistribution of Mg²⁺ in muscle, bone, and the gastrointestinal tract. Although we did not measure activity of TRPM7 directly, we assessed annexin-1 status, which paralleled TRPM7 changes. Annexin-1 is a TRPM7 kinase-sensitive substrate¹² that has been implicated in inflammation, cell proliferation, and apoptosis.³⁸ Another mechanism whereby aldosterone may influence urinary Mg²⁺ excretion is through the Na⁺/Mg²⁺ exchanger, which we demonstrated to be upregulated in hypertension.³⁹ Hence altered Mg²⁺ metabolism in hyperaldosteronism may be related to direct stimulation of the Na⁺/Mg²⁺ exchanger on the one hand and to downregulation of TRPM7 on the other.

Cellular models demonstrate that TRPM6 and TRPM7 are regulated by Mg²⁺.¹⁴,¹⁵ We speculated that Mg²⁺ supplementation would normalize TRPM7 in aldosterone-treated mice. This was confirmed at the gene level, where mRNA expression of TRPM6 and TRPM7 was significantly increased in aldosterone-infused mice receiving Mg²⁺. However, this was not evident at the protein level, because TRPM7 and annexin-1 content were not normalized in Mg²⁺ supplemented mice. Reasons for these differences between gene and protein status might relate to mRNA instability, transcriptional changes, or to changes at the posttranslational levels. It may also be possible that tissue [Mg²⁺], was not high enough to influence TRPM7 protein expression.

In conclusion, data from the present study demonstrate that aldosterone mediates blood pressure-independent renal and cardiovascular fibrosis and inflammation and electrolyte disturbances through Mg²⁺-sensitive pathways. We also report the novel findings that aldosterone induces downregulation of renal TRPM7 and annexin-1. These findings suggest that altered Mg²⁺ metabolism in hyperaldosteronism may relate to TRPM7 dysregulation and that Mg²⁺ protects against cardiovascular and renal damaging actions of aldosterone.

Perspectives

Hyperaldosteronism, the incidence of which is increasing in clinical medicine, is associated with hypomagnesemia, hypertension, and cardiovascular remodeling. Mechanisms contributing to these processes, and particularly to aldosterone-associated Mg²⁺ changes, are unclear. Experimental evidence suggests that impaired transmembrane Mg²⁺ transport through TRPM6/7 channels may be important. Our data demonstrate that aldosterone mediates blood pressure-independent renal and cardiovascular fibrosis and inflammation and renal dysfunction, which are ameliorated by dietary Mg²⁺-supplementation. We also show that aldosterone induces downregulation of renal TRPM7 and its target annexin-1. Hence altered Mg²⁺ metabolism in aldosteronism may relate to TRPM7 dysregulation. Magnesium has cardiovascular protective effects and prevents renal damage and...
dysfunction in aldosteronism. Such findings have important clinical implications in the understanding of mechanisms underlying hyperaldosteronism-associated hypomagnesemia and associated target-organ damage and suggest that Mg2+ supplementation may have potential therapeutic benefit in this condition.

Sources of Funding
This study was supported by grants from the Canadian Institutes of Health Research (CIHR) and the Heart and Stroke Foundation of Canada. R.M.T. is supported through a Canada Research Chair/ Canadian Foundation for Innovation award. A.C.I.M. received a fellowship from Amgen.

Disclosures
None.

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Hypertension. 2008;51:915-921; originally published online February 11, 2008;
doi: 10.1161/HYPERTENSIONAHA.107.100339

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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SUPPLEMENTAL DATA

Downregulation of Renal TRPM7 and Increased Inflammation and Fibrosis in Aldosterone-infused Mice: Effects of Magnesium.

Sontia B, Montezano ACI, Paravicini T, Tabet F, Touyz RM

Kidney Research Centre, Ottawa Health Research Institute, University of Ottawa, Ontario, Canada.

Short title: Magnesium effects in aldosterone-infused mice

Correspondence:
Rhian M Touyz MD, PhD
Kidney Research Centre
University of Ottawa/Ottawa Health Research Institute
451 Smyth Rd
Ottawa, ON, K1H 8M5
Tel: 613-562-5800 ext 8241, Fax: 613-562-5487
Email: rtouyz@uottawa.ca
SUPPLEMENTAL DATA

Materials and methods

Animals

This study was approved by the Animal Ethics Committee of the University of Ottawa and performed according to the recommendations of the Canadian Council for Animal Care. Four groups of 12 week-old male C57B6 mice (Jackson Laboratory, Maine, DE, USA) were studied: Control (normal mouse chow) (Harlan Teklad Global Diet:2018) (0.2% dietary Mg\textsuperscript{2+}, n=8), magnesium-supplemented group (Mg\textsuperscript{2+} group) (0.75% dietary Mg\textsuperscript{2+}, n=9), aldosterone-infused group (Aldo group) (300\textmu g/kg/day for 2 weeks then 400\textmu g/kg/day by Alzet osmotic mini-pumps (Cupertino, CA) and 0.9% NaCl drinking water, n=9) and Aldo+Mg\textsuperscript{2+} group (aldosterone infusion, 0.9% NaCl drinking water and 0.75 % dietary Mg\textsuperscript{2+} n=9). To ensure consistency and accuracy of the Mg\textsuperscript{2+} content in the Mg\textsuperscript{2+}-enriched diet, we did not simply supplement normal mouse chow with Mg\textsuperscript{2+}, but had the special diet custom prepared in pellet form by Harlan Teklad, to make certain that the diet contained 0.75% Mg\textsuperscript{2+} content. Mice tolerated the diet well as evidenced by the fact that they continued to thrive.

Blood pressure measurements

The systolic blood pressure (SBP) was measure in conscious mice every 3 days using the Visitech tail cuff system (BP 2000 Blood Pressure Analysis System, Visitech, Apex, NC). The mice were immobilized on a warmed platform at 37°C. One week before commencing the experiment, mice were trained daily using the Visitech system. For blood pressure measurements, the first 5 recordings were disregarded and the average of the 10 successive measurements taken as the final blood pressure reading.
Serum and urine analysis

Blood and urine were collected at the beginning and end of the experiment. Blood was collected by venesection from the saphenous vein (300-500 µL). Mice were placed in metabolic cages for 24 hours for urine collection. Serum and urine levels of Mg$^{2+}$, Ca$^{2+}$, Na$^{+}$ and K$^{+}$ and urine protein were measured by automated methods at the hospital laboratory.

Analysis of TRPM6 and TPRM7 mRNA levels with real-time PCR

Extraction of RNA and cDNA conversion. Frozen kidney samples were homogenized and RNA extracted using Trizol® reagent (Invitrogen) as per manufacturer's instructions. Extracted RNA was quantified by measuring absorbance at 260 nm. 200 ng of each RNA sample was then reverse transcribed using random hexamers and TaqMan® reverse transcription (RT) reagents (Applied Biosystems). The final RT reaction mixture contained: total RNA (200 ng), 1 x TaqMan® RT buffer, MgCl$_2$ (5.5 mol/L), dNTPs (500 µmol/L each of dCTP, dATP, dTTP, dGTP), random hexamers (2.5 µmol/L), RNase inhibitor (0.4 U/µL) and Multiscribe™ reverse transcriptase (1.25 U/µL) in a final volume of 10 µL. After 10 mins of incubation at room temperature, the RT reaction progressed for 30 min at 48°C before inactivating the reverse transcriptase by heating to 95°C for 5 min (Eppendorf Mastercycler).

Real-time PCR protocol. Specific primers and FAM-labeled probes for mouse TRPM6 and TRPM7 were designed using Primer Express software (Applied Biosystems) and sequences from the NCBI database (table S1). Primer and probe sets for both genes were designed to span an exon-exon junction to avoid amplification of genomic DNA. Primers and VIC-labeled probes for the housekeeping gene 18S were purchased from Applied
Biosystems. The final reaction mix for real-time PCR contained: 1 x TaqMan Universal RT-PCR master mix, 10 ng of cDNA, 900 nmol/L of forward and reverse primers for the target gene (TRPM6 or TRPM7) and 200 nmol/L of target gene probe in a final volume of 25 µL. For 18S amplification, 40 nmol/L of forward primer, 60 nmol/L of reverse primer and 200 nmol/L of probe were used. TRPM7 and 18S amplifications were multiplexed in the same tube, however competition between TRPM6 and 18S reactions meant that these were performed separately. All reactions were performed in triplicate, and a standard curve constructed on each plate using an independent control sample of kidney cDNA. The PCR conditions were: 50°C for 2 min, 95 °C for 10 min, then 40 cycles of 95°C for 15 s and 60°C for 1 min (Applied Biosystems 7300 Real-time PCR system). All experiments were analysed using the relative standard curve method. Relative expressions of TRPM6 and TRPM7 in the unknown samples were determined from the standard curve, and expressed relative to 18S.

**Western blotting**

Proteins from kidney (renal cortex), heart and aorta were extracted from frozen tissue as previously described (1-3). Tissues were homogenized in lysis buffer [50 mmol/l Tris/HCl (pH 7.4), 1% Nonidet P40, 0.5% Sodium deoxycholate, 1% SDS, 2 mmol/l Na₃VO₄, 1 mmol/l PMSF, 1 µmol/l pepstatin A, 1 µmol/l leupeptin, 1 µmol/l aprotinin] and the protein supernatant was separated by centrifugation. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes and incubated with specific antibodies to VCAM1 (Santa Cruz Biotechnology), COX2 (Cayman chemical), TRPM7 (Abcam), annexin-1 (Santa Cruz Biotechnology). Signals were revealed by chemiluminescence, visualized autoradiographically and subsequently membranes were
stripped (Pierce Biotechnology) and reprobed with GAPDH or α-actin antibodies (Chemicon International), which were used as internal controls. Optical density of bands was quantified densitometrically after scanning (Epson Perfection 4990 scanner, Epson America Inc. CA) Image-Quant software (Molecular Dynamics, Sunnyvale, CA, USA).

**Histopathological analysis**

Tissues were fixed in 4% formaldehyde solution for 24h at 4°C, dehydrated, embedded in paraffin and sectioned transversely (4 µm). Sections were stained with hematoxylin and Sirius red and scored for vascular damage and collagen deposition as we previously described (4). Samples were examined with a Nikon Eclipse E600 microscope (Nikon Corporation, Kanagawa, Japan). Images were captured using the image Pro Plus software computer program (Ipwin 32, Media Cybernetics, L.P., Maryland, USA).

**Immunohistochemistry**

Frozen tissue (kidney, heart apex and aorta) were cryosectioned (7 µm thickness) and fixed with cold acetone for 10 minutes. For direct immunohistochemistry, sections were incubated with 3 % H₂O₂ and a Pierce solution to block endogenous peroxidase and biotin, respectively, followed by overnight incubation (humidified box, 4°C) with a biotinated anti-goat interleukin-6 (IL-6) monoclonal antibody (Santa Cruz). Sections were incubated for 60 minutes with a secondary biotin-conjugated anti-rabbit antibody (1:1500 in 2 % horse serum: Rockland) and with streptavidin conjugated to horseradish peroxidases (Vector Labs). Color was developed by the addition of DAB (Sigma Chemicals). Sections were lightly stained in hematoxylin, dehydrated with alcohol and xylene, and scored by an independent observer unaware of the groups and treatments of
the mice. To normalize for background staining, procedures were also performed in sections incubated only with the secondary antibody.

Results

Blood pressure
Mice were treated for 3 months with aldosterone. They also received 0.9% NaCl in the drinking water. Aldosterone was infused initially at a dose of 300 µg/kg/min. Since blood pressure did not increase after 2 weeks infusion at this dose, we increased the aldosterone concentration to 400µg/kg/min until the end of the experiment. Aldosterone failed to increase SBP, even at the higher dose of infusion (Figure S1). Magnesium supplementation did not influence blood pressure in control or aldosterone-treated mice.

Cardiovascular effects of aldosterone and magnesium supplementation
As observed in the kidney, collagen deposition was significantly increased in the heart in response to aldosterone. This effect was attenuated in mice supplemented with Mg^{2+}. Expression of cardiac IL-6, VCAM and COX2 was significantly enhanced in the aldosterone group (figures S2, S3). Magnesium reduced IL-6 and COX2 expression, but did not influence cardiac VCAM1 expression.

Vascular expression of VCAM1 and COX2
Vascular expression of VCAM1 and COX2 was significantly increased in the aldosterone-treated mice (p<0.05) (figure S4). Magnesium supplementation did not influence expression of pro-inflammatory mediators in the aorta.
References


Figure Legends

Figure S1. Systolic blood pressure (SBP) in the control, magnesium-supplemented, aldosterone-infused and aldosterone+magnesium groups. SBP was measured every 3 days for 12 weeks. Data are means±SEM. n=8-9 mice/group

Figure S2. Immunohistochemical images demonstrate cardiac IL-6 content in the four groups. IL-6 is demonstrated as brown staining. IL-6 content was increased in the aldosterone group versus other groups. Magnesium reduced IL-6 content in aldosterone-treated mice. Images are representative of 8-9 mice in each group

Figure S3. Expression of pro-inflammatory mediators VCAM1 (A) and COX2 (B) in hearts from control, magnesium-supplemented, aldosterone-treated and aldosterone+magnesium groups. Upper panels are representative immunoblots. GAPDH was used as an internal control. Lower panels, corresponding bar graphs representing data as means±SEM from 8-9 mice/group. Data expressed as VCAM1:GAPDH and as COX2:GAPDH and normalized to control, taken as 100%. *p<0.05

Figure S4. Expression of pro-inflammatory mediators VCAM1 (A) and COX2 (B) in aortae from control, magnesium-supplemented, aldosterone-treated and aldosterone+magnesium groups. Upper panels are representative immunoblots. GAPDH was used as an internal control. Lower panels, corresponding bar graphs representing data as means±SEM from 8-9 mice/group. Data expressed as VCAM1:GAPDH and as COX2:GAPDH and normalized to control, taken as 100%. *p<0.05, **p<0.01, ***p<0.001.
Table S1. Specific primers for mouse TRPM6 and TRPM7. Primers were designed using Primer Express software (Applied Biosystems) and sequences from the NCBI database.

<table>
<thead>
<tr>
<th>Primer sequence</th>
<th>TRPM6 (NM_153417)</th>
<th>TRPM7 (NM_021450)</th>
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</thead>
<tbody>
<tr>
<td>Forward</td>
<td>5’-AAACCGGAAGACAAACAATCAA</td>
<td>5’-CAATCCAAATGGTTGTTCCATCTCA</td>
</tr>
<tr>
<td>Reverse</td>
<td>5’-GCGATGT'TTGTCAATGAAGC'T</td>
<td>5’-TGTGATATGTCATCTGTAGGTTCTC'T</td>
</tr>
<tr>
<td>Probe</td>
<td>5’-AGGGATGGTGT'TTGACCCGG'C</td>
<td>5’-AGCAGGGTAATCTCCCCCCGGG</td>
</tr>
</tbody>
</table>
Figure S1

The figure shows a graph representing systolic blood pressure over time for three different conditions: Control, Mg²⁺, and Aldo + Mg²⁺. The y-axis represents systolic blood pressure in mmHg, ranging from 40 to 200. The x-axis represents time in days, ranging from 0 to 120.

- The Control group shows a relatively stable blood pressure throughout time.
- The Mg²⁺ group shows a slight increase in blood pressure over time.
- The Aldo + Mg²⁺ group shows a substantial increase in blood pressure compared to the other groups.
Figure S3

A

B

Cont       Mg$$^{2+}$$   Aldo   Aldo+Mg$$^{2+}$$

VCAM1→

GAPDH→

Aldo+Mg$$^{2+}$$

Cont       Mg$$^{2+}$$   Aldo   Aldo+Mg$$^{2+}$$

COX2→

GAPDH→

0
50
100
150
200

VCAM1/GAPDH (%)

Control  Mg$$^{2+}$$  Aldo  Aldo+Mg$$^{2+}$$

200

COX2/GAPDH (%)
Figure S4

A

VCAM1

α-actin

Cont  Mg²⁺  Aldo  Aldo+Mg²⁺

***  ***

B

COX2

α-actin

Cont  Mg²⁺  Aldo  Aldo+Mg²⁺

**  *

VCM1/α-actin  COX2/α-actin (%)

Control  Mg²⁺  Aldo  Aldo + Mg²⁺  Control  Mg²⁺  Aldo  Aldo + Mg²⁺