SUPPLEMENTAL DATA

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

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Short title: Magnesium effects in aldosterone-infused mice

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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$^{2+}$, n=8), magnesium-supplemented group (Mg$^{2+}$ group) (0.75% dietary Mg$^{2+}$, n=9), aldosterone-infused group (Aldo group) (300µg/kg/day for 2 weeks then 400µg/kg/day by Alzet osmotic mini-pumps (Cupertino, CA) and 0.9% NaCl drinking water, n=9) and Aldo+Mg$^{2+}$ group (aldosterone infusion, 0.9% NaCl drinking water and 0.75 % dietary Mg$^{2+}$ n=9). To ensure consistency and accuracy of the Mg$^{2+}$ content in the Mg$^{2+}$-enriched diet, we did not simply supplement normal mouse chow with Mg$^{2+}$, but had the special diet custom prepared in pellet form by Harlan Teklad, to make certain that the diet contained 0.75% Mg$^{2+}$ 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 TRPM7 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 Na$_3$VO$_4$, 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


3. Touyz RM, Yao GJ. Inhibitors of Na+/Mg2+ exchange activity attenuate the development of hypertension in angiotensin II-induced hypertensive rats. *Hypertension* 2003; 21(2):337-344

**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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<tr>
<td>Forward</td>
<td>5'-AAACCGGAAGACAAACAATCAA</td>
<td>5'-CAATCCAATGTGTCCATCTCA</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-GCGATGTACATTTGCAATGAAGCT</td>
<td>5'-TGTGTATGCTATCTGTAGGTTTCCT</td>
</tr>
<tr>
<td>Probe</td>
<td>5'-AGGATGGTATTTGACCCGCC</td>
<td>5'-AGCAGGGTAATCTCCCCCGGG</td>
</tr>
</tbody>
</table>
Figure S1

The figure shows the change in systolic blood pressure (mmHg) over time (days) for different groups: Control, Mg²⁺, Aldo, and Aldo + Mg²⁺. The graph illustrates the comparative effects of these treatments on blood pressure levels.
Figure S3

A

B

Control      Mg²⁺   Aldo   Aldo + Mg²⁺

VCAM1

GAPDH

Control      Mg²⁺   Aldo   Aldo + Mg²⁺

COX2

GAPDH

VCAM1/GAPDH (%)

VCAM1/GAPDH (%)

COX2/GAPDH (%)

Control      Mg²⁺   Aldo   Aldo + Mg²⁺

*
Figure S4

A

B

VCAM1

α-actin

COX2

α-actin

Cont        Mg²⁺        Aldo        Aldo+Mg²⁺

Cont        Mg²⁺        Aldo        Aldo+Mg²⁺

0

2

4

6

8

10

V/CAM1/α-actin

0

250

500

750

1000

Cox2/α-actin (%)

0

250

500

750

1000

Control     Mg²⁺     Aldo     Aldo+Mg²⁺

Control     Mg²⁺     Aldo     Aldo+Mg²⁺