Supplemental Material

MAP Kinase-Activated Protein Kinase 2 in Angiotensin II-induced Inflammation and Hypertension: Regulation of Oxidative Stress

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Short Title: MK2 and Vascular Inflammation

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Methods

Nuclear extract preparation

VSMC nuclear extracts were prepared as previously described[1] with a minor modification. Nuclei were resuspended in 50 µl of ice cold lysis buffer and incubated on ice for 15 min. The suspension was centrifuged at 13000 rpm for 10 min. Protein concentration in the supernatant was determined using the Bradford method.

Western-Blotting

Total or fractioned proteins (15-20 µg) were extracted from VSMCs, separated by SDS-PAGE, transferred to nitrocellulose membranes and incubated overnight at 4°C with antibodies (1:1000) against the following: MK2, p38 MAPK and NF-κB p65 subunit from Cell Signaling Technology (Danvers, MA), and ICAM-1, VCAM-1, Ets-1 and p47phox from Santa Cruz Biotechnology (Santa Cruz, CA). After incubation with secondary antibodies, signals were revealed by chemiluminescence (SuperSignal West Pico chemiluminescent signal, Thermo scientific, Rockford, IL), with the Molecular Imager Chemidoc XRS system (Bio-Rad, Mississauga, ON, Canada), and quantified by densitometry using Quantity one software (Bio-Rad). Membranes were subsequently stripped and reprobed with anti-ß-actin antibody (Sigma Chemicals) to verify equal loading. VCAM-1 was also determined in protein extracts of mesenteric arteries stripped of fat from *Mk2* KO and WT mice.

Quantitative RT-PCR

The following primers were used with an annealing temperature of 58°C. MCP-1, 5′-AGCTCTCTCTTCCTCCACCAC-3′ (sense) and 5′-TCTGGACCCATTCCTTCTTG-3′ (antisense) and S16, 5′-TCTGGGCAAGGAGAGATTTG-3′ (sense) and 5′-CCGCCAAACTTCTTGGATTC-3′ (antisense).

Reactive oxygen species levels

Intracellular ROS levels were evaluated using 5-(and 6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester mixed isomers (CM-H_2DCFDA, Invitrogen). Briefly, VSMCs plated on black 96-well plate were transfected with siMK2 or siLuc, serum starved, treated or not with Nox2ds-tat and then stimulated with or without Ang II for 5 min as above. Then, cells were washed with phosphate-buffered saline, and incubated with CM-H_2DCFDA (10 µmol/L) for 30 minutes at room temperature and fluorescence red every 2 minutes for 60 minutes using a Fluoskan Ascent FL (Thermo Fischer Scientific Inc., Waltham, MA).

Dihydroethidium (DHE) was used to evaluate *in situ* production of superoxide ($\bullet O_2^-$)[2] in VSMCs and 10 µm-thick cryosections of aorta of WT and *Mk2* KO. VSMCs were transfected for with siMK2 or siLuc, serum starved, stimulated with or without Ang II for 10 min as above and then incubated at 37°C with DHE (2 µmol/L) or vehicle for 30 min. Fluorescence was visualized with a DM2000 microscope with a CY3 filter (555 nm) (Leica Microsystems, Richmond Hill, ON, Canada). Frozen sections of aorta were incubated with DHE (100 µmol/L) for 5 min at room temperature and fluorescence determined as above.

NADPH oxidase activity

The lucigenin-enhanced chemiluminescence assay was used to determine NADPH oxidase activity in VSMC homogenates as previously described.[3] VSMCs were transfected with

siMK2 or siLuc, serum starved and pre-exposed or not to Nox2ds-tat or its scrambled control and stimulated with or without Ang II for 5 min as above. Briefly, samples were homogenized in ROS lysis buffer. One hundred µL of homogenate was used for the lucigenin-based chemiluminescence assay in an Orion II microplate luminometer (Berthold detection systems Gmbh, Pforzheim, Germany). Background was determined over 10 s, 100 µl of lucigenin (12.5 µmol/L) were injected and light measured for 1 s every ~6 s for 3 min. Then 50 µL of NADPH (0.5 mmol/L) were injected and light was measured for an additional 3 min as for lucigenin. NADPH oxidase activity was calculated by subtracting the results of luminescence before from after NADPH addition. Activity was corrected for protein concentration and expressed as % of negative control.

Proliferation Assay

Ang II-stimulated DNA synthesis was determined by measuring incorporation of [^{3}H]thymidine into DNA.[(4)] VSMCs were cultured in a 96 well-microplates. At 70-80 % confluency, cells were transfected with siMK2 or siLuc, serum starved and stimulated with or without Ang II or 10% fetal bovine serum for 24 h as above. During the last 6 hours of incubation, 1 µCi/ml of [^{3}H]thymidine was added to the media. The medium was removed and the cells were washed with cold PBS. [^{3}H]thymidine incorporation into DNA was assessed using a TopCount NXT scintillation counter (Perkin Elmer, Waltham, MA).

Reference List

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