ONLINE SUPPLEMENT

DAPK3 mediates vascular inflammation and development of hypertension in spontaneously hypertensive rats

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Short title: DAPK3 mediates vascular inflammation
Supplemental Materials and Methods

**Materials**

Reagent sources were as follows: TNF-α (Roche Applied Science, Mannheim, Germany); angiotensin (Ang) II and noradrenaline (NA) (Sigma, St. Louis, MO, USA); acetylcholine (ACh) (Daiichi Pharmaceutical, Tokyo); Death associated protein kinase (DAPK) inhibitor (DI) “Death-Associated Protein Kinase Inhibitor (4Z)-4-(3-Pyridylmethylene)-2-styryl-oxazol-5-one” (Merck KGaA, Darmstadt, Germany); N-Acetyl-L-Cysteine (NAC) (Sigma Aldrich, St. Louis, MO, USA). Antibody sources were as follows: phospho-JNK (Promega, Madison, WI, USA); phospho-p38, phospho-Akt (Ser473), total-JNK and total-Akt (Cell Signaling, Beverly, MA, USA); total-p38, vascular cell adhesion molecule-1 (VCAM-1) and cyclooxygenase (COX)-2 (Santa Cruz Biotech, Santa Cruz, CA, USA); endothelial-selectin (e-selectin) (R&D System, Minneapolis, MN, USA); total-actin (Sigma Aldrich, St. Louis, MO, USA); α-actin (DAKO, Glostrup, Denmark); DAPK3 (Gene Tex, Irvine, CA, USA).

**Culture of vascular smooth muscle cells (SMCs)**

Male Wistar rats (7-9-week-old) were anesthetized with urethane (1.5 g/kg, i.p.) and euthanized by exsanguination. The superior mesenteric artery was isolated. SMCs isolated from mesenteric artery were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA) 1. Passage 4 to 20 SMCs at 80 to 90 % confluence were growth arrested by incubating in DMEM containing 0.5 % FBS for 24 hours before stimulation. Animal care and treatment were conducted in conformity with institutional guidelines of The Kitasato University and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animal research was approved by ethical committee of School of Veterinary Medicine, The Kitasato University.

**Culture of human umbilical vein endothelial cells (HUVECs)**

HUVECs were obtained from Kurabo (Osaka, Japan), and cultured in Medium 200 supplemented with low serum growth supplement (Cascade Biologics, Portland, OR, USA) as described previously 2. Cells at passages from 3 to 8 were used.

**Western blotting**

Western blotting was performed as described previously 3. Protein lysates were obtained by homogenizing SMCs, HUVECs or tissue samples with triton-based lysis buffer (1 % Triton X-100, 20 mM Tris, PH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM Na3VO4, 1 μg/ml leupeptin, and 0.1 % protease inhibitor cocktail;
Nacalai Tesque, Kyoto, Japan). Protein concentration was determined using the bicinchoninic acid method (Pierce, Rockford, IL, USA). Equal amount of proteins (8-10 µg) were separated by SDS-PAGE (7.5 %), and transferred to a nitrocellulose membrane (Pall, Ann Arbor, MI, USA). After being blocked with 3 % bovine serum albumin (for phosphorylation-specific antibodies) or 0.5 % skim milk (for others), membranes were incubated with primary antibodies at 4 °C overnight, and then visualized using horseradish peroxidase-conjugated secondary antibodies (1:10,000 dilution, 1 h) and the EZ-ECL system (Biological industries, Kibbutz Beit Hesmek, Israel). Equal loading of protein was confirmed by measuring total protein or actin expression. The results were analyzed using CS Analyzer 3.0 software (ATTO, Tokyo, Japan).

**Small interfering RNA (siRNA) transfections**

One day after SMCs were subcultured, they (30-40% confluent) were transfected for 24 h with siRNA against DAPK3 (DAPK3 siRNA-1, UCAUAGUUCACAGCCGAGAtt, DAPK3 siRNA-2, AUCAGCACCACACUGUCUtt) (Nippon EGT, Toyama, Japan) or non-silencing control siRNA (control siRNA) (Qiagen, Valencia, CA, USA) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) dissolved in Optimem (Invitrogen) at a final concentration of 40 nM. And then, SMCs were serum-starved by incubating in DMEM containing 0.5% FBS for 24 h before stimulation.

**Cell adhesion assays**

U937 (monocytes) cells were obtained from RIKEN Cell Bank (Tsukuba, Japan) and cultured in RPMI-1640 Medium supplemented with 5% FBS. After SMCs transfected with DAPK3 siRNA or control siRNA (40 nM, 24 h) and HUVECs treated with or without DI (3 µM, 30 min) in a 6-well culture plate were stimulated with TNF-α (10 ng/ml) for 24 h (SMCs) or 6 h (HUVECs), they were washed with Tris-Buffered Saline (TBS). And then, U937 cells (~8.5 x 10^5 cells /well) were co-incubated for 1 h with SMCs or HUVECs at 10 rpm at 37 °C. Non-attached cells were removed by the several washings and then the cells were fixed with 4% paraformaldehyde at 37 °C for 10 min. The number of attached U937 cells was randomly counted in three areas per well at x200 filed, and averaged.

**Measurement of intracellular ROS production in vitro**

Intracellular ROS production in SMCs or HUVECs was examined by a fluorescence staining using 2’, 7’-dichlorodihydrofluorescein diacetate (H2DCFDA, Invitrogen) \(^1,2\). After treatment for 20 min with TNF-α in the presence of DAPK3 siRNA, control siRNA or DI (pretreatment for 30 min), SMCs or HUVECs were loaded with H2DCFDA (10 µM) for 30 min. Fluorescence images were obtained using a fluorescence microscope (BX-51, Olympus, Tokyo, Japan) equipped with cooled CCD camera (MicroPublisher 5.0 RTV, Roper Japan, Tokyo, Japan). The Image J software was used for the quantitative analysis of the images.
Animal studies

DI or vehicle (DMSO) was subcutaneously administered to male SHR (4-week-old; Hoshino Laboratory Animals, Inc., Ibaragi, Japan) and age-matched male WKY at a dose of 500 µg/kg/day for 6 weeks. Animal care and treatment were conducted in conformity with institutional guidelines of The Kitasato University and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animal study was approved by ethical committee of School of Veterinary Medicine, The Kitasato University.

Blood pressure (BP) and heart rate measurement

Systolic BP (SBP), diastolic BP (DBP), mean arterial BP (MBP) and heart rate of SHR and WKY were measured using a tail-cuff system (Softron, Tokyo, Japan) in the conscious conditions. SBP and MBP were measured based on photoelectric volume and vibrational pattern of the pulse wave, respectively. The DBP was provided based on the calculations. We measured BP six times for each rat, and three stable values were averaged.

Tissue preparation

SHR (0.25–0.29 kg: 10-week-old) and age-matched WKY (0.27–0.31 kg) were anesthetized with urethane (1.5 g/kg, i.p.) and euthanized by exsanguination. The superior mesenteric arteries were isolated as described previously. After the fat and connective tissues were removed, the arterial samples were used for the extraction of protein, the measurement of isometric tension and the histological analysis.

Histology

After isolated mesenteric artery was embedded in OTC compound (Sakura Finetechical Co., Ltd., Tokyo, Japan) and quickly frozen in liquid nitrogen, the thin frozen sections (5 µm) were made using a cryostat (Leica, Solms, Germany) and then stained with hematoxylin and eosin using standard protocols. The images were obtained using a light microscope (BX-51, Olympus, Tokyo, Japan). For measurement of ROS, the frozen sections were loaded with H2DCFDA (5 µM) for 30 min. The sections treated with anti-oxidant, NAC (10 mM, pretreatment for 30 min) or non-H2DCFDA-treated sections were also prepared as positive or negative control, respectively. Fluorescence images were obtained using a fluorescence microscope. The Image J software was used for the quantitative analysis of the images.

Measurement of smooth muscle contraction

The contractility of isolated, endothelium-intact mesenteric artery was measured as described previously. The arterial rings were placed in normal physiological salt solution (PSS), which contained (mM): NaCl 139.9, KCl 5.4, CaCl2 1.5, MgCl2 1.0, NaHCO3 23.8, and glucose 5.5.
Ethylendiaminetetraacetic acid (EDTA), 1 µM, was also added to remove the contaminating heavy metal ions which catalyze oxidation of organic chemicals. The high $K^+$ (72.4 mM) solution was prepared by replacing NaCl with equimolar KCl. These solutions were saturated with a 95% $O_2$–5% $CO_2$ mixture at 37 °C and pH 7.4. Smooth muscle contractility was recorded isometrically with a force-displacement transducer (Nihon Kohden, Tokyo, Japan). Each muscle ring was attached to a holder under a resting tension of 0.5 g. After equilibration for 30 min in a 3 ml organ bath, each ring was repeatedly exposed to high $K^+$ solution until the responses became stable (60–90 min). Concentration-response curves to AngII (0.1-30 nM) were obtained by the cumulative application. The 72.4 mM KCl-induced maximal contractions were used for normalization. ACh (1 nM – 300 µM) was cumulatively applied to the arteries pre-contracted to the similar level with submaximal concentrations of NA (100 nM -1 µM).

*Statistical Analysis*

Data are shown as means ± SEM. Statistical evaluations were performed using one-way ANOVA followed by Bonferroni test. Values of $P < 0.05$ were considered statistically significant.
References


**Figure S1.** Effect of death associated protein kinase (DAPK)3 knockdown on TNF-α-induced DAPK3 expression. After SMCs were transfected with control or DAPK3 small interfering (siRNA), they were treated with 10 ng/ml TNF-α for 1 h. DAPK3 expression (n=4) was determined by Western blotting and shown as fold increase relative to control siRNA without TNF stimulation. Equal protein loading was confirmed using α-actin antibody. **P<0.01 vs. control siRNA without TNF stimulation; ##P<0.01 vs. cont siRNA+TNF.**
**Figure S2.** Effect of co-treatment with DAPK inhibitor (DI) and DAPK3 siRNA on TNF-α-induced phosphorylation of JNK (A), p38 (B) and Akt (C). After transfection with DAPK3 or control siRNA (40 nM, 24 h) in the presence or absence of DI (3 µM, 30 min), SMCs were stimulated with 10 ng/ml TNF-α for 20 min. Phosphorylation of JNK (n=5), p38 (n=6) and Akt (n=5) was determined by Western blotting and shown as fold increase relative to control siRNA without TNF stimulation. Equal protein loading was confirmed using total antibody. **P< 0.01 vs. control siRNA without TNF; #P<0.05, ##P<0.01 vs. cont siRNA+TNF.
Figure S3. Effect of DI on TNF-α-induced monocyte adhesion to human umbilical vein endothelial cells (HUVECs). After HUVECs were pretreated with DI (3 µM, 30 min), TNF-α (10 ng/ml, 6 h) was treated. After adding U937 cells for 1 h to HUVECs, non-adherent cells were removed and the number of adhering U937 cells was randomly counted in three areas (×200 fields) and averaged (n=3-5). Scale bar: 50 µm. The number of U937 cells adhering to HUVECs was shown as fold increase relative to control. **P<0.01 vs. cont; #P<0.05 vs. TNF.
**Figure S4.** Effect of long-term DI treatment on heart rate of spontaneously hypertensive rats (SHR). DI was administered to SHR (4-week-old) or age-matched Wistar Kyoto rats (WKY) subcutaneously at a dose of 500 µg/kg/day for 6 weeks (n=6). Heart rate (beat per min) was measured using a tail-cuff system at weekly intervals. Results were expressed as mean ± S.E.M. *P<0.05, **P<0.01 vs. WKY.
**Figure S5.** Effect of long-term DI treatment to SHR on DAPK3 expression in an isolated mesenteric artery. After DI was administered to SHR (4-week-old) or WKY subcutaneously at a dose of 500 µg/ kg/day for 6 weeks, superior mesenteric artery was harvested. DAPK3 expression (n=3-4) was determined by Western blotting and shown as fold increase relative to WKY. Equal protein loading was confirmed using total actin antibody. **P<0.01 vs. WKY; #P<0.05.
Figure S6. Summary of the present results. DAPK3 promotes reactive oxygen species (ROS)-dependent inflammatory responses in vascular smooth muscle and endothelial cells, which may lead to the development of hypertension via propagating vascular hypercontractility and hypertrophy in SHR.