Death-Associated Protein Kinase 3 Mediates Vascular Inflammation and Development of Hypertension in Spontaneously Hypertensive Rats

Tatsuya Usui, Muneyoshi Okada, Yukio Hara, Hideyuki Yamawaki

Abstract—Death-associated protein kinase (DAPK) is a Ca\(^{2+}\)/calmodulin-regulated serine/threonine kinase that mediates cell death. Our recent study demonstrated that DAPK3 protein increases in the mesenteric artery from spontaneously hypertensive rats compared with Wistar Kyoto rats. Pathogenesis of hypertension is modulated at least in part by vascular inflammation. We examined whether DAPK3 mediates vascular inflammatory responses and development of hypertension. In rat mesenteric arterial smooth muscle cells, small interfering RNA against DAPK3 inhibited vascular cell adhesion molecule 1 expression and monocyte adhesion induced by tumor necrosis factor-α. DAPK3 small interfering RNA inhibited phosphorylation of c-Jun amino-terminal kinase, p38, and Akt, as well as reactive oxygen species (ROS) production induced by tumor necrosis factor-α. In human umbilical vein endothelial cells, expressions of vascular cell adhesion molecule 1, endothelial selectin, and cyclooxygenase 2, as well as ROS production induced by tumor necrosis factor-α, were inhibited by DAPK3 inhibitor. In vivo, blood pressure, ROS production, inflammatory molecule expression (vascular cell adhesion molecule 1 and endothelial selectin), and hypertrophy in isolated mesenteric artery were elevated in spontaneously hypertensive rats (10 weeks old), which were prevented by long-term treatment with a DAPK inhibitor (500 μg/kg per day for 6 weeks). In isolated mesenteric artery, the increased angiotensin II–induced contraction and the impaired acetylcholine-induced endothelium-dependent relaxation in spontaneously hypertensive rats were reversed by a DAPK inhibitor. The present results for the first time demonstrated in cultured smooth muscle cells and endothelial cells that DAPK3 mediates tumor necrosis factor–induced inflammatory responses via ROS-dependent mechanisms. It is also suggested that DAPK3 mediates the development of hypertension in spontaneously hypertensive rats likely via ROS-dependent inflammation, hypertrophy, and hypercontractility. (Hypertension. 2012;60:1031-1039.) • Online Data Supplement

Key Words: hypertension ■ inflammation ■ reactive oxygen species ■ vasoconstriction ■ hypertrophy

Calmodulin (CaM) is recognized as a key regulatory molecule for diverse biological functions, such as muscular contraction. In addition, a recent study demonstrated that CaM-dependent protein kinases, such as CaM-dependent protein kinase II, may regulate hypertension through the mechanisms including promotion of vascular smooth muscle hypertrophy. In the previous study, we compared expression levels of several CaM-related proteins with almost unknown functions in vasculature. As a result, we demonstrated that protein expression of eukaryotic elongation factor 2 kinase (also known as CaM-dependent protein kinase III), histone deacetylase 4, and death associated protein kinase (DAPK) 3 increased in the mesenteric artery from spontaneously hypertensive rats (SHRs) compared with Wistar Kyoto rats (WKYs). In the recent study, we also showed that histone deacetylase 4 promotes reactive oxygen species (ROS)–dependent vascular inflammation and mediates the development of hypertension in SHRs. However, it remains to be clarified how DAPK3, which affects vascular pathophysiology and control the development of hypertension.

The DAPK protein has been considered as a Ca\(^{2+}\)/CaM-regulated serine/threonine kinase that mainly mediates cell death. The DAPK protein consists of multiple domains, including an N-terminal kinase domain (known as a Ca\(^{2+}\)/CaM regulatory region), ankyrin repeats, a cytoskeleton binding region, and a C-terminal death domain. Various stimulation-induced intracellular calcium spikes promote the CaM binding to the CaM regulatory region of DAPK, which in turn activates DAPK. Five protein kinases, including DAPK1, DAPK2, DAPK3 (also known as Zipper-interacting protein kinase), DAPK-related apoptosis-inducing protein kinase 1, and DAPK-related apoptosis-inducing protein kinase 2, are known as DAPK family proteins. DAPK1, DAPK2, and DAPK3 are all ubiquitously expressed in various tissues from mice and rats. Recently, the functions of DAPK3 were gradually clarified. The mutation of DAPK3...
promoted increased cell survival, proliferation, and resistance to chemotherapy in cancer cells. In addition, it was shown that DAPK3 phosphorylated myosin light chain kinase, which may lead to apoptotic membrane blebbing. Enhanced phosphorylation of myosin light chain by DAPK3 was shown to increase smooth muscle contractility and cell motility. It was further reported that DAPK3 regulates inflammatory signalings, including ribosomal protein L13a, extracellular signal–regulated kinase, and interferon-γ–activated inhibitor of translation in macrophages.

Vascular inflammation is known to be an important process for the development of hypertension at an early stage. In addition, a critical role for inflammation induced by ROS has been suggested in hypertension. However, it remains to be clarified how DAPK3 controls hypertensive vascular diseases via inflammation. We, therefore, examined whether DAPK3 affects vascular smooth muscle and endothelial inflammatory responses and the development of hypertension. Here, we for the first time demonstrate that DAPK3 mediates ROS-dependent vascular inflammation and perhaps the development of hypertension via propagating vascular hypercontractility and hypertrophy in SHRs.

Materials and Methods

The detailed methods are available in the online-only Data Supplement.

Results

Effect of DAPK3 Knockdown on Tumor Necrosis Factor-α–Induced Inflammatory Responses in Vascular Smooth Muscle Cells

We first examined whether DAPK3 mediates inflammatory responses in smooth muscle cells (SMCs). For this purpose, we used 2 kinds of small interfering RNA (siRNA) against the DAPK3 gene and explored their effects on tumor necrosis factor (TNF)-α–induced inflammatory responses. We confirmed that...
DAPK3 protein was significantly silenced by each of the siRNAs (Figure 1A). It was further confirmed that TNF-induced DAPK3 expression was also significantly inhibited by DAPK3 siRNA (Figure S1, available in the online-only Data Supplement). TNF (10 ng/mL, 24 hours)-induced vascular cell adhesion molecule (VCAM)-1 expression was significantly inhibited by each DAPK3 siRNA (Figure 1B). We next examined whether DAPK3 knockdown inhibits monocyte adhesion to SMCs. The DAPK3 siRNA significantly decreased the number of monocyte adhesions to SMCs (Figure 1C). To explore upstream mechanisms of inhibition of VCAM-1 expression, effects of DAPK3 knockdown on inflammatory signals were examined. TNF (10 ng/mL, 20 minutes)–induced phosphorylation of c-Jun amino-terminal kinase (JNK; Figure 2A), p38 (Figure 2B), and Akt (Figure 2C) was significantly inhibited by DAPK3 siRNA. To further investigate the upstream mechanisms of proinflammatory effects of DAPK3, we examined whether DAPK3 knockdown prevents TNF-induced ROS production in SMCs. TNF (10 ng/mL, 20 minutes) increased an $2',7'$-dichlorodihydrofluorescein diacetate–sensitive fluorescent intensity compared with nonstimulated control (Figure 2D). DAPK3 siRNA significantly inhibited the TNF-induced ROS production (Figure 2D). DAPK inhibitor (DI; 3 μmol/L) also inhibited TNF-induced phosphorylation of JNK, p38, and Akt in the presence of control siRNA, whereas DI had no effects in the presence of DAPK3 siRNA (Figure S2), suggesting the specificity of this compound to DAPK3.

**Effect of DI on TNF-α–Induced Inflammatory Responses in Human Umbilical Vein Endothelial Cells**

We next examined whether DAPK3 also mediates inflammatory responses in human umbilical vein endothelial cells.
TNF (10 ng/mL, 24 hours)–induced expression of VCAM-1 (Figure 3A), endothelial-selectin (e-selectin; Figure 3B), and cyclooxygenase (COX)-2 (Figure 3C) in cultured human umbilical vein endothelial cells (HUVECs). After HUVECs were treated with 10 ng/mL of TNF for 6 hours in the absence or presence of DI (3 μmol/L, pretreatment for 30 minutes), expression of VCAM-1 (n=3–7), e-selectin (n=3–7), and COX-2 (n=3–7) was determined by Western blotting and shown as fold increase relative to control. Equal protein loading was confirmed using total actin antibody. The same actin equal loading was used in A and C. **P<0.01 vs control; ##P<0.01 vs TNF. Effect of DI on TNF–α–induced reactive oxygen species (ROS) production (D). ROS production was determined by a fluorescence staining using 2′,7′-dichlorodihydrofluorescein diacetate (H$_2$DCFDA). After HUVECs were treated with 10 ng/mL of TNF for 20 minutes in the absence or presence of DI (3 μmol/L, pretreatment for 30 minutes), they were loaded with H$_2$DCFDA (10 μmol/L) for 30 minutes. Scale bar, 50 μm. Fluorescent intensity was shown as fold increase relative to control (n=3–5). **P<0.01 vs control; ##P<0.01 vs TNF.

**Figure 3.** Effect of death-associated protein kinase (DAPK) inhibitor (DI) on tumor necrosis factor (TNF)-α–induced expression of vascular cell adhesion molecule (VCAM)-1 (A), endothelial-selectin (e-selectin; B), and cyclooxygenase (COX)-2 (C) in cultured human umbilical vein endothelial cells (HUVECs). After HUVECs were treated with 10 ng/mL of TNF for 6 hours in the absence or presence of DI (3 μmol/L, pretreatment for 30 minutes), expression of VCAM-1 (n=3–7), e-selectin (n=3–7), and COX-2 (n=3–7) was determined by Western blotting and shown as fold increase relative to control.

Effect of Long-Term DI Treatment on Blood Pressure of SHRs

We next examined the effects of long-term DI treatment (for 6 weeks) on blood pressure (BP) of SHRs (from 4 to 10 weeks old). The systolic BP, mean BP, and diastolic BP were significantly higher in SHRs than in WKYs (Figure 4). DI (500 μg/kg per day) significantly decreased the BP in SHRs. Treatment of WKY with DI (500 μg/kg per day) had no influence on the BP. The heart rate was significantly higher in SHRs than in WKYs (Figure S4). DI had no effect on heart rate in SHRs and WKYs.

Effect of Long-Term DI Treatment on Inflammatory Responses in Mesenteric Arteries From SHRs

To check whether DAPK3 mediates vascular inflammation in vivo, we examined the effects of long-term DI treatment on the expression of VCAM-1 and e-selectin in isolated mesenteric arteries from SHRs (10 weeks old). We confirmed that DI treatment significantly inhibited the increased DAPK3 expression in SHRs (Figure S5). The expression of VCAM-1 (Figure 5A) and e-selectin (Figure 5B) significantly increased in SHRs compared with WKYs. DI (500 μg/kg per day, 6 weeks) treatment significantly normalized it. Long-term DI treatment had no influence on the expression of VCAM-1 and e-selectin in WKYs (Figure 5A and 5B). We next examined whether long-term DI treatment attenuates ROS production. In
mesenteric arteries from SHRs (10 weeks old), fluorescence intensity of ROS-sensitive 2′,7′-dichlorodihydrofluorescein diacetate significantly increased compared with WKYs (Figure 5C). DI (500 μg/kg per day, 6 weeks) treatment significantly inhibited the increased ROS production in SHRs. Long-term DI treatment had no influence on the ROS production in WKYs (Figure 5C).

**Effect of Long-Term DI Treatment on Angiotensin II–Induced Contraction in Isolated Mesenteric Arteries From SHRs**

To further explore mechanisms of the normalization of the increased BP in SHRs, we examined whether long-term DI treatment affects hypercontractility of the mesenteric artery from SHR. Angiotensin II (Ang II; 0.1–30.0 nmol/L) induced a slight contraction in mesenteric arteries from WKYs (Figure 6A). In mesenteric arteries from SHRs, the Ang II–induced contraction was significantly augmented. Long-term DI (500 μg/kg per day, 6 weeks) treatment reversed the augmented Ang II–induced contraction in SHRs. DI treatment had no influence on the Ang II–induced contraction in WKYs.

**Effect of Long-Term DI Treatment on Acetylcholine-Induced Endothelium-Dependent Relaxation in Isolated Mesenteric Arteries From SHRs**

We next examined the effects of long-term DI treatment on acetylcholine (ACh)–induced endothelium-dependent relaxation. In mesenteric arteries from WKYs (10 weeks old), ACh (0.1 nmol/L to 10 μmol/L) induced relaxation in a concentration-dependent manner (Figure 6B). In mesenteric arteries from SHRs, the ACh-induced relaxation was significantly impaired. Long-term DI (500 μg/kg per day, 6 weeks) treatment prevented the impaired ACh-induced relaxation in SHRs. DI treatment had no influence on the ACh-induced relaxation in WKYs.

**Effect of Long-Term DI Treatment on Hypertrophy in Mesenteric Arteries From SHRs**

To further explore mechanisms of the normalization of the increased BP in SHRs, we examined whether long-term DI treatment affects hypertrophy. In mesenteric arteries from SHRs, wall thickness of arterial media significantly increased compared with WKYs (Figure 7). Long-term DI (500 μg/kg per day, 6 weeks) treatment inhibited the increased wall thickness in SHRs. DI treatment had no influence on the wall thickness in WKYs.

**Discussion**

In the present study, we examined whether DAPK3 mediates vascular inflammatory responses and the development of hypertension. The major findings of the present study are that inhibition of DAPK3 prevents TNF-induced VCAM-1 expression and activation of JNK, p38, and Akt, as well as ROS production, in cultured vascular SMCs. It is also found that inhibition of DAPK3 prevents TNF-induced expression of...
VCAM-1, e-selectin, and COX-2, as well as ROS production, in ECs. In the previous study, we showed that an antioxidant drug, N-acetyl l-cysteine inhibited TNF-induced expression of VCAM-1 and e-selectin, as well as activation of Akt, JNK, and p38, in cultured SMCs or endothelial cells. In addition, we confirmed that the inhibitor of Akt, JNK, or p38 prevented TNF-induced expression of VCAM-1 or e-selectin. Collectively, our results indicate that DAPK3 mediates TNF-induced inflammation via induction of proinflammatory molecules (VCAM-1, e-selectin, and COX-2) through activation of ROS-dependent signals in cultured SMCs and endothelial cells. It is also suggested that DAPK3 may mediate the development of hypertension in SHRs likely via ROS-dependent inflammation, hypertrophy, and hypercontractility.

DAPK3 has been implicated mainly in the regulation of cell death, including apoptosis and autophagy, in various types of cells. In the recent study, we demonstrated that DAPK3 expression increases in aortas and mesenteric arteries from SHRs. Nevertheless, it is unknown how DAPK3 mediates hypertensive vascular diseases via inflammation. In the present study, knockdown or inhibition of DAPK3 prevented TNF-induced inflammatory responses in vascular SMCs (Figures 1 and 2) and endothelial cells (Figure 3). Although little is known about the roles of DAPK3 on inflammation, recent reports showed that DAPK family proteins modulate inflammatory responses in addition to cell death. It was reported that knockdown of DAPK1 attenuated interleukin 1β production in macrophages. On the other hand, it was shown that DAPK1 and DAPK3 had negative regulatory roles in the expression of inflammatory genes, including ribosomal protein L13a, extracellular signal–regulated kinase, and interferon-γ–activated inhibitor of translation in macrophages. DAPK3 has also been recognized as a novel transcriptional regulator. It was reported that leukemia
inhibitory factor-induced phosphorylation of DAPK3 at Thr265 activated signal transducer and activator of transcription 3 signaling in HeLa cells.\textsuperscript{17,18} Because it was reported that signal transducer and activator of transcription 3 activity was regulated by JNK in cancer cells\textsuperscript{19} and our data showed that DAPK knockdown inhibited the TNF-induced JNK activation, it is likely that DAPK3 mediates TNF-induced vascular inflammatory responses via the activation of transcriptional factors, including signal transducer and activator of transcription 3 through activation of JNK.

It was reported that DI inhibited activation of both DAPK1 and DAPK3 with enzyme selectivity (IC\textsubscript{50}=69 nmol/L for DAPK1 and 225 nmol/L for DAPK3).\textsuperscript{20} In addition, it was shown that IP DI treatment at a concentration of 5 mg/kg improved rat cerebral ischemia.\textsuperscript{21} Considering these reports, we used the concentration of DI (500 μg/kg per day) for in vivo study and showed that DI inhibited the increased DAPK3 expression in SHRs (Figure S5). Because we confirmed that DAPK1 expression did not change in mesenteric arteries from SHRs compared with WKYs (n=4; data not shown), it is suggested that DI treatment may normalize the increased BP in SHRs mainly via the inhibition of DAPK3.

In the present study, long-term DI treatment reversed the augmented Ang II–induced contraction in mesenteric arteries from SHRs (10 weeks old). It was reported previously that Ang II type 2 receptor expression increased in mesenteric arteries from young SHRs (6 weeks old), which mediated the augmented Ang II–induced contraction.\textsuperscript{22} Therefore, it might be possible that DI reversed the augmented Ang II–induced contraction via inhibition of Ang II type 2 receptor expression. On the other hand, it was reported that DAPK3 mediated calyculin-induced smooth muscle contraction via activation of myosin light chain kinase in aortas from SHRs.\textsuperscript{23} In addition, it was shown that DAPK3 induced activation of regulatory myosin light chain 20 and contraction of smooth muscle.\textsuperscript{24} These reports suggest that DAPK3 may augment Ang II–induced contraction via myosin light chain

\textbf{Figure 6.} Effect of long-term death-associated protein kinase inhibitor (DI) treatment to spontaneously hypertensive rats (SHRs) on angiotensin (Ang) II–induced contraction (A) and acetylcholine (ACh)–induced endothelium-dependent relaxation (B) in an isolated mesenteric artery. After DI was administered to SHRs (4 weeks old) or Wistar Kyoto rats (WKYs) SC at a dose of 500 μg/kg per day for 6 weeks, superior mesenteric artery was harvested. Concentration-contraction relationships to Ang II (WKY, n=9; SHR, n=8; WKY+DI, n=6; and SHR+DI, n=6) were shown (A). Ang II (0.1–30.0 nmol/L) was cumulatively applied. Results were expressed as mean±SEM, and 100% represents the 72 mmol/L of KCl–induced maximal contraction. Concentration-contraction relationships to ACh (WKY, n=5; SHR, n=6; WKY+DI, n=3; and SHR+DI, n=8) were shown (B). ACh (0.1 nmol/L to 10 μmol/L) was cumulatively added after the precontraction induced by submaximal doses of noradrenaline (100 nmol/L to 1 μmol/L) had reached a steady state, and 100% represents the noradrenaline–induced precontraction. Results were expressed as mean±SEM. *P<0.05, **P<0.01 vs WKY; #P<0.05, ##P<0.01 vs SHR.

\textbf{Figure 7.} Effect of long-term death-associated protein kinase inhibitor (DI) treatment to spontaneously hypertensive rats (SHRs) on hypertrophy in an isolated mesenteric artery. After DI was administered to SHRs (4 weeks old) or Wistar Kyoto rats (WKYs) SC at a dose of 500 μg/kg per day for 6 weeks, superior mesenteric artery was harvested. The frozen sections (5 μm) were stained with hematoxylin and eosin, and the images were obtained. Thickness of arterial media (M) was shown as fold increase relative to WKY (n=5). Scale bar, 50 μm. **P<0.01 vs WKY; ##P<0.01 vs SHR.
kinase and/or regulatory myosin light chain 20 activation. We also showed that long-term DI treatment reversed the impaired ACh-induced relaxation in mesenteric arteries from SHRs. It was previously reported that the ACh-induced relaxation in mesenteric arteries from SHRs (14 weeks old) was impaired compared with WKYs. It was also reported in aortas from SHRs that endothelial NO synthase uncoupling mediated increased ROS production. Therefore, we suppose that DI inhibited ROS production and, in turn, increased bioactive NO production, which led to the enhancement of ACh relaxation.

Structural change of the resistance artery is one of the important factors in the development of hypertension. It was shown that medial volume in the small muscular artery from SHRs increased compared with WKYs. Another report showed that long-term treatment with sodium hydrosulfide, which possesses anti-inflammatory and anti-oxidant roles, decreased medial thickening and fibrosis of intramyocardial coronary arteries via inhibition of ROS production in SHRs. Activation of mitogen-activated protein kinase families (JNK, p38, and extracellular signal–regulated kinase) and Akt mediates cell proliferation of SMCs. In this study, we showed that knockdown of DAPK3 inhibited TNF-induced activation of JNK, p38, and Akt in SMCs. Considering these results, we suppose that DI may inhibit hypertrophy via inhibition of cell proliferation caused by ROS-dependent activation of JNK, p38, and Akt.

**Perspectives**

In the present study, we for the first time demonstrated that DAPK3 promotes ROS-dependent vascular inflammation in vascular smooth muscle and endothelial cells, which may lead to the development of hypertension via propagating vascular hypercontractility and hypertrophy in SHRs. Further studies on DAPK3 might contribute to develop new pharmaceutical therapy for the prevention of hypertensive cardiovascular diseases.

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**Disclosures**

None.

**References**


**What Is New?**

- DAPK3 acts mainly in the regulation of cell death.
- We for the first time revealed in vascular smooth muscle and endothelial cells that DAPK3 mediates TNF-α–induced inflammatory responses via ROS-dependent mechanisms.
- It was also demonstrated that DAPK3 mediates the development of hypertension in SHRs likely via ROS-dependent inflammation, hypertrophy, and hypercontractility.

**What Is Relevant?**

- DAPK3 may be a novel candidate gene that is related to the pathogenesis of hypertension in SHRs.

- Therefore, further studies on DAPK3 might contribute to develop new pharmaceutical therapy for the prevention of hypertensive cardiovascular diseases.

**Summary**

DAPK3 mediates ROS-dependent vascular inflammation and perhaps the development of hypertension via propagating vascular hypercontractility and hypertrophy in SHRs.
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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, UCAUAGUUCACAGCGAGAtt, DAPK3 siRNA-2, AUCAGCACCACAGCGGUCAtt) (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 3. 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 3. 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 Finetechanical 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 4. 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% O₂–5% CO₂ 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 (x 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.