**Supplement to**

# Reduction of AMP-activated Protein Kinase Alpha 2 Increases Endoplasmic Reticulum Stress and Atherosclerosis *In Vivo*

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## Materials and Methods

### Materials

A23187 (calcimycin), tempol, Ionomycin, and Indo-1 were from Sigma-Aldrich Inc (St. Lois, MO). BAPTA was purchased from Invitrogen Inc (Carlsbad, CA). Fluo-4 NW for free cytosolic $Ca^{2+}$ measurement kit was from Invitrogen. TUDCA, Compound C (cat# 171261), and peroxynitrite ($ONOO^-$) were from Calbiochem (San Diego, CA). Antibodies against p-AMPK, p-ACC, p-eIF2α, p-JNK, anti-3-nitrotryosine and anti-pan-AMPK were purchased from Cell Signaling Biotechnology (Danvers, MA). Antibodies against AMPKα1 and AMPKα2 were obtained from Bethyl Laboratories, Inc (Montgomery, TX) and Cell Signaling. The specificity of antibodies against AMPKα1, AMPKα2 and pan-AMPKα were double confirmed from different resources. Antibodies against p-PERK, anti-XBP1, PP2B, SERCA2, SERCA3, and AMPK siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against SERCA (monoclonal), GRP78, and Calmodulin (2D1) were bought from Abcam (Cambridge, MA). The antibody against ATF6 was from Imgenex (San Diego, CA). CaMKII activity assay kit was

bought from Upstate Biotechnologies (Long Island, NY). DHE for superoxide measurement was bought from Invitrogen. For immunohistochemistry staining, antibody against KDEL, which recognize GRP78 and GRP94, was from Stressgen; antibody against MDA was from Abcam, antibody against 4-HNE was from Cosmo Bio Co.; antibody against ATF6 was from Lifespan Biosciences, and 3-nitrotryosine was from Millipore.

## Methods

### Evaluation of aortic lesions

Evaluations of aortic lesions were performed, as described previously.[1] The animal protocol was reviewed and approved by the University of Oklahoma Institute Animal Care and Use Committee.

**Isolation and culture of mouse aortic endothelial cells**. Mouse aortic endothelial cells (MAEC) were isolated as described by Hewett PW et al [2] with minor modifications.[3] Briefly, after removing fat tissue and connective tissue in cold PBS buffer, mouse aorta were cut into pieces of approximately 3-cm and incubated with 0.2% collagenase solution at 37°C for 30 min with gentle agitation. After centrifugation at 3000 rpm for 3 min at 4°C, the pellets (enriched endothelial cells) were washed with cold PBS and seeded in culture dishes in endothelial basal media (EBM) containing 5% FBS. After the first 2-3 passages, the FBS concentration was gradually decreased to normal levels in subsequent passages (with 2% FBS after passage 3). MAEC were positively stained with vWF, eNOS, angiotensin-converting enzyme (ACE), but negatively stained with α-actin and bFGF. The purity of endothelial cells isolated by this method is usually over 90%. MAECs were used within 10 passages.

**Measurement of intracellular $Ca^{2+}$**. Intracellular $Ca^{2+}$ concentration was measured using a Fluo-4 NW kit from Invitrogen, according to instructions from the supplier. Relative intracellular $Ca^{2+}$ was expressed as % of control.[4]

**Assays of AMPK and $Ca^{2+}$-dependent CaM Kinase II**. AMPK activity was measured, as previously reported by our laboratory.[5] CaM Kinase II activity assay was carried out according to the manufacturer's instructions.

**Microsome preparation**. Microsomes were prepared according to the method of Hojmann et al [6] with minor modifications. In brief, cultured endothelial cells were homogenized in Tris-maleate buffer containing 30 mM Tris, 0.3 M sucrose, 0.1 mM PMSF, and 1x protease inhibitor cocktail (Roche, USA) (solution I pH 7.0.), spin at 1000 g for 10 min, then spin the supernatant at 10,000 g for 10 min, and the pellets were resuspended in solution II containing 30 mM Tris, 0.3 M sucrose, 0.6 M KCl, and protease inhibitors and spun at 100,000 g for 1 hr. The final pellets, which were considered the microsomal rich fraction with Sarco-endoplasmic reticulum $Ca^{2+}$ -ATPase, were resuspended in solution I for the SERCA activity assay.

**Assay of Sarco-endoplasmic reticulum $Ca^{2+}$-ATPase (SERCA) activity**. SERCA activity assay was carried out using [$\gamma$-$^{32}$P], as described by Authi KS et al [7] with minor modifications. Briefly, 50 µg microsomes were incubated with 120 mM KCl, 5 mM $MgSO_4$, 20 mM Hepes, pH 7.2, 1 mM MgATP containing [$\gamma$-$^{32}$P]-ATP, and 50 µM $CaCl_2$. Incubations were performed for 15 min at 37°C and stopped by addition of 0.2 ml Norit A (activated charcoal, 25 mg/ml in 0.1 M

$H_3PO_4$ solution). After centrifugation (1500 g for 20 min), 0.2 ml of the supernatant was taken and the radioactivity representing released [$^{32}$P]-Pi was measured by liquid scintillation spectrometry. The background was calculated from the control samples containing 5μM thapsigargan (TPG), a highly selective inhibitor of SERCA. The relative SERCA activity was expressed as % controls.

**Ratiometric $Ca^{2+}$ measurement and dynamic SERCA function**. Ratiometric $Ca^{2+}$ measurement was conducted as described by Ma R et al.[8] In brief, compound C treated HUVEC cells, or primary cultured cells from AMPK-KO mice, or siRNA treated HUVE cells were trypsinized and harvested by centrifuge. Cell number was counted using standard protocol. Each assay contains 1X10 $^6$ cells. Cells were washed with ECS buffer containing 140mM NaCl, 5mM KCl, 1.8mM $CaCl_2$, 10 mM glucose, 0.1% BSA, 15mM Hepes, pH7.4 and resuspended in ECS buffer, loaded with 2 μM Indo-1/AM in the presence of 0.05% Pluronic F-127(Molecular Probes), and the mixture was shaken for 45min at room temperature. The Indo-1/AM loaded cells were washed with ECS buffer without $CaCl_2$ three times. Cells were then resuspended in ECS/EGTA buffer with 10 μM calcium free EGTA. Two mls of Indo-1/AM loaded cells were subjected to ratiometric measurement. After 30 sec balance, 10μM Ionomycin was added to cells to spike calcium release from the ER store. Ratiometric measurements representing free intracellular $Ca^{2+}$ concentrations were obtained by a PTI QuantaMaster spectrofluorometer equipped with an excitation monochromator set at 350nm and two emission monochromators set at 405 and 485 nm. The relative calcium store in ER, the degree of sharpness of curve, and the timing for cytosolic calcium to return to basal level (homeostasis) was comparatively analyzed.

**Biotinlated-iodoacetamide (b-IAM) labeling of SERCA cysteine-674**. Biotinlated-iodoacetamide (b-IAM) labeling of SERCA cysteine-674 was performed according to the methods described by Tong et al (2008).[9] Proteins were separated by SDS-PAGE and b-IAM labeled Cys674-SERCA was detected using SERCA specific antibody.

**Overexpression of SERCA2b in endothelial cells**. Myc-tagged SERCA2b mammalian expression vector was constructed under a CMV promoter for mammalian cell expression. Plasmid was prepared using a kit for large-scale plasmid preparation from Qiagen,Inc. Plasmid DNA was delivered into primary cultured endothelial cells *via* electroporation (Amaxa, Germany). GFP served as a control, and the optimized program was selected after visualizing GFP transient expression efficiency under microscopy. In general, the GFP transient expression in primary cultured endothelial cells reached up to 70% after 24 hr transfection.

**Immunohistochemistry.** The aortic arch was dissected, fixed in 4% paraformaldehyde for 16 h, and embedded in paraffin. Four-micrometer thick sections were deparaffinized, rehydrated, and microwaved in citrate buffer for antigen retrieval. Sections were successively incubated in endogenous peroxidase and alkaline phosphatase block buffer (DAKO), protein block buffer, and primary antibodies, which were incubated with blots overnight at 4°C. After rinsing in wash buffer, sections were incubated with labeled polymer-horseradish peroxidase anti-mouse or anti-rabbit antibodies and DAB chromogen. Alternatively, they were incubated with polymer-alkaline phosphatase anti-mouse or anti-rabbit antibody and Permanent Red chromogen (EnVision™

G|2 Doublestain System, DAKO). After the final wash, the sections were counterstained with hematoxylin.

**Western blot**. Total proteins were analyzed by SDS-PAGE and blotted using standard protocols.[10] Densitometric quantification was performed using Quantity One software (Bio-Rad). Protein levels (arbitrary units) were normalized to β-actin and expressed as fold induction over control values.

## References


**1.** Fernandez-Hernando C, Ackah E, Yu J, Suarez Y, Murata T, Iwakiri Y, Prendergast J, Miao RQ, Birnbaum MJ, Sessa WC. Loss of Akt1 leads to severe atherosclerosis and occlusive coronary artery disease. *Cell Metab.* 2007;6(6):446-457.

**2.** Hewett PW, Murray JC, Price EA, Watts ME, Woodcock M. Isolation and characterization of microvessel endothelial cells from human mammary adipose tissue. *In Vitro Cell Dev Biol Anim.* 1993;29A(4):325-331.

**3.** Wang S, Xu J, Song P, Wu Y, Zhang J, Chul Choi H, Zou MH. Acute inhibition of guanosine triphosphate cyclohydrolase 1 uncouples endothelial nitric oxide synthase and elevates blood pressure. *Hypertension.* 2008;52(3):484-490.

**4.** Dong Y, Wu Y, Wu M, Wang S, Zhang J, Xie Z, Xu J, Song P, Wilson K, Zhao Z, Lyons T, Zou MH. Activation of Protease Calpain by Oxidized and Glycated LDL Increases the Degradation of Endothelial Nitric Oxide Synthase. *J Cell Mol Med.* 2008.

**5.** Xie Z, Dong Y, Scholz R, Neumann D, Zou MH. Phosphorylation of LKB1 at serine 428 by protein kinase C-zeta is required for metformin-enhanced activation of the AMP-activated protein kinase in endothelial cells. *Circulation.* 2008;117(7):952-962.

**6.** Hojmann Larsen A, Frandsen A, Treiman M. Upregulation of the SERCA-type Ca2+ pump activity in response to endoplasmic reticulum stress in PC12 cells. *BMC Biochem.* 2001;2:4.

**7.** Authi KS, Bokkala S, Patel Y, Kakkar VV, Munkonge F. Ca2+ release from platelet intracellular stores by thapsigargin and 2,5-di-(t-butyl)-1,4-benzohydroquinone: relationship to Ca2+ pools and relevance in platelet activation. *Biochem J.* 1993;294 ( Pt 1):119-126.

**8.** Ma R, Li WP, Rundle D, Kong J, Akbarali HI, Tsiokas L. PKD2 functions as an epidermal growth factor-activated plasma membrane channel. *Mol Cell Biol.* 2005;25(18):8285-8298.

**9.** Tong X, Ying J, Pimentel DR, Trucillo M, Adachi T, Cohen RA. High glucose oxidizes SERCA cysteine-674 and prevents inhibition by nitric oxide of smooth muscle cell migration. *J Mol Cell Cardiol.* 2008;44(2):361-369.

**10.** Xie Z, Dong Y, Zhang M, Cui MZ, Cohen RA, Riek U, Neumann D, Schlattner U, Zou MH. Activation of protein kinase C zeta by peroxynitrite regulates LKB1-dependent AMP-activated protein kinase in cultured endothelial cells. *J Biol Chem.* 2006;281(10):6366-6375.