Dimethylarginine dimethylaminohydrolase 1 modulates endothelial cell growth through NO and Akt

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Extended Materials and Methods

Cell culture and transfection. Primary human umbilical artery endothelial cells (HUVEC) (Cambrex) were maintained in EGM medium (Cambrex) under 5% CO2 in a humidified incubator at 37 °C. Cells at passage 4 to 7 were used for experiments. Cells with 30% confluence were transfected with Lipofectamin 2000 (LF-2000, Invitrogen). After 5 hours incubation, growth medium was replaced.

Measurements of Nitrite, cGMP and ADMA. ADMA was measured using an ADMA-ELISA kit (DLD). Nitrite, the stable end product of NO degradation, was measured using the colorimetric Griess assay kit (Oxford Biomedical Research), cGMP was measured using a direct immunoassay kit (BioVision).

Assays for tube formation, cell proliferation and cell migration. HUVEC were overlaid onto 50μl Matrigel (BD Biosciences) in 48-well plates for 18 hours. The formed tubes were labeled with 8μg/ml Calcein AM (Molecular Probes) for 30 minutes and digital photographs were then obtained. Each experiment was performed in triplicate, and 5 random pictures were taken of each well at a magnification of 10X. Linear “tube” formation was quantified using the method described by Wild et al (1).
Cell proliferation was determined with a CyQUANT® cell proliferation assay kit (Invitrogen).

**Cell migration assay:** Cell migration was determined as previously described (2). HUVEC grown to 75–80% confluence were trypsinized and suspended in EBM medium at a final concentration of \(5 \times 10^5\) cells per ml. In a 24-well plate, 300\(\mu\)l of this cell suspension was added to the upper filter chamber of the transwell insert (Costar) with a polycarbonate membrane (8 \(\mu\)m pore size). In the lower chamber 500 \(\mu\)l of EBM containing 2.5% FBS was added. The plate was incubated at 37°C with 5% CO2 for 18 h. After incubation, non-migratory cells on the upper surface of the filter were removed by wiping with a cotton swab. The migratory cells were fixed with 4% paraformaldehyde for 15 min, stained with 0.03% crystal violet (g/100ml) for 1 hour, and photographed under an inverted light microscope (20X). The average number of cells in 5 fields of each insert (3 inserts for each condition) was determined.

**Selective gene silencing with RNA interference.** DDAH1 siRNA was generated with BLOCK-iT Dicer RNAi kits (Invitrogen) following the manufacturer’s instructions. The DDAH1 cDNA (clone MGC: 45161) was from ATCC. Two pairs of specific primers were used to generate double strand siRNA (20-23 nucleotides), and both successfully silenced DDAH1 in HUVEC. Two controls were used: transfection with LacZ siRNA (Z-siRNA) and transfection without siRNA. The first primer pair produced a PCR product of 325bp of the coding sequence; sense: gcaactttagatgccgaga, antisense: gcctttgtggtatatat. The second primer pair produced a PCR product of 218bp of the non-coding sequence; sense aaggcccctctcctcat, antisense catgattggttttgac. Selective silencing of DDAH1 was achieved with no effect on DDAH2.

**Generation of recombinant adenovirus expressing flag-DDAH1 (Ad-DDAH1).** Ad-flag-DDAH1 was generated using AdEasy™ Adenoviral Vector System (Stratagene) with pShuttle-
CMV as shuttle vector following the manufacturer’s instructions. A redundant point mutation was made to remove the Pac1 site at 935 nt in the DDAH1 cDNA sequence. Viral stock was purified using Adeno-X™ Maxi Purification kit, and titrated using the Adeno-X™ rapid titer kit (Clontech). Adenovirus was introduced into cells with MOI 100; after 1 hour incubation, the media was removed, the cells washed twice with HBSS, and fresh culture media was added for another 48 hour culture. Treatments were added in basal EBM for 18 hours before collecting cell lysate.

**Generation of site mutation at Cys273 to Ser in Ad-flag-DDAH1.** Site mutation was generated using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene) following the manufacturer’s instructions. The mutation was confirmed by DNA sequencing.

**Endothelial sprouting assay:** EC regenerative capacity was determined by an ex vivo aortic ring assay (3). Descending thoracic aortas were isolated from wild type and endo-DDAH1 knockout mice and cut into 1-mm-thick rings under a dissecting microscope. These aortic rings were briefly washed in DPBS (Invitrogen) and placed between 2 layers of growth factor-reduced Matrigel (BD Biosciences) covered with EGM Medium. The media was changed every two to three days. After five days of culture in Matrigel, the number of sprouts was counted from each ring. For adenoviral infection, 10 aortic rings were incubated with $10^8$ pfu of adenovirus for 2 hours before being placed on the Matrigel (4).

**DDAH activity assay:** HL-1 cells (from Dr. Claycomb, Louisiana State University) were infected with Ad-GFP, Ad-DDAH1 or Ad-DDAH1 mutant at MOI:100; two 100mm dishes of the HL-1 cells were used for each of the three adenoviruses. 48 hours after infection, the cells were collected and DDAH activity was determined as previously described (5).

**Ras and Akt activity assays:** Ras activity assay kit (Product NO.17-218) was from Millipore (Upstate Biotechnology). Briefly, RBD (Ras binding domain) of Raf1 conjugated
agarose beads is used to pull down Ras-GTP (activated Ras) from cell lysates, and the amount of immunologically precipitated Ras-GTP indicates Ras activity. Akt activity assay kit (Product NO. 9840) was from Cell Signaling Technology, Inc. (Danvers, MA 01923).

**Western blot:** Tissue homogenate and cell lysate in lysis buffer were resolved on 8-12% SDS-polyacrylamide gels. After transfer protein from PAGE gel to PVDF membrane, the membrane was cut into strips based on molecular weight, membranes were incubated with primary antibodies at 4°C overnight. The primary antibody against DDAH1 was made in our laboratory as previously described (5). DDAH2 antibody (Product NO. AB1383; 1:500 dilution) was purchased from Abcam Inc. Antibodies against total-Akt (Product NO. G0804; 1:1000 dilution), GAPDH (Product NO.32233, 1:5000 dilution), GFP (Product NO. 9996, 1:1000 dilution) were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif); α-tubulin (product No.T6074, 1:5000 dilution) was from Sigma (St Louis, Mo). The following antibodies were purchased from Cell Signaling Technology, Inc (Danvers, MA 01923): p-Akt\textsuperscript{Ser473} (Product NO. 9271), p-eNOS\textsuperscript{Ser1177} (Product NO. 9571, 1:1000), eNOS (Product NO. 9572), PTEN (Product NO. 9552), p-PTEN\textsuperscript{Ser380} (Product NO. 9551), and PP2A (Product NO. 2259). The antibodies from Cell Signaling Technology were used at 1:1000 dilution in 5% BSA TBST.
Supplemental Data

**Supplemental Figure I.** DDAH1 siRNA had no effect on DDAH2 or eNOS expression in HUVEC.

Supplemental Figure II. Selective DDAH1 gene silencing decreased HUVEC cell proliferation that was not fully rescued by addition of L-arginine up to 5 mM. *p<0.05 as compared with corresponding groups treated with Ctr (control) siRNA. #<0.05 as compared with vehicle treated cells.
**Supplemental Figure III.** Selective DDAH1 gene silencing decreased NOx production by HUVEC. caAkt increased NOx production both in cells treated with control siRNA and DDAH1 siRNA. L-Arginine (1mM) caused a significant further increase in NOx production in DDAH1 siRNA cells. *p<0.05 as compared with corresponding groups treated with control siRNA. #<0.05 as compared with vehicle treated cells.

![Graph showing relative nitrite content](image)

**Supplemental Figure IV.** Western blot of PTEN and P-PTEN of lysate from control and Ad-DDAH1 infected HUVEC (A). Immunoprecipitation (IP) using Akt antibody conjugated agarose beads followed by PP2A western blot demonstrated that there was no interaction between PP2A and AKt (B); PP2A expression was not altered in the Ad-DDAH1 infected HUVEC.
Supplemental Figure V. Global DDAH1 KO attenuated pulmonary GTP-Ras level. (n=4, P<0.05). *p<0.05 as compared with the wild type (WT) group.

Supplemental Figure VI. Mutated DDAH1 associates with Ras in HUVEC.

Supplemental Figure VII: DDAH1 associates with Ras in lung tissue from wild type mice.
**Supplemental Figure VIII:** In HUVEC, Akt activity negatively regulated ERK activity. (A) Inhibition of Akt activity by ly294002 (10μM) increased p-ERK1/2 level. (B) Constitutively active Akt (Ad-caAkt) decreased p-ERK1/2 level.
References


