Online Supplemental Methods and Data

RNAi silencing of brain klotho potentiates cold-induced elevation of blood pressure *via* the endothelin pathway

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Running title: Klotho and blood pressure

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Online Methods

shRNA design and selection-RNA interference (RNAi) technology is a powerful methodology recently developed for the specific knockdown of targeted genes. There are several criteria for ideal shRNA design (http://www.genelink.com/sirna/shRNAi.asp). Two to four shRNA sequences were designed within open reading frame of the target gene to select the most efficient functional sequence. In current study, we chose two shRNAs for klotho and ET1 respectively to construct two AAV.KL-shRNAs and two AAV.ET1-shRNAs. Before large scale packaging of the recombinant AAVs for in vivo study, the inhibition efficiency of ET1 was tested using qPCR, and the inhibition efficiency klotho was determined using western blot.

Determination of the inhibition efficiency of AAV.KL-shRNA by western blot-Two klotho-shRNAs were tested to confirm the inhibition efficiency in rat aorta smooth muscle (RASM) cells which were pre-transfected mouse klotho full length cDNA (pAAV.mKL). Briefly, the RASM cells were seeded in 6-well plate ($3x10^5$/well) 12 hours before transfection. The plasmid DNAs of AAV.mKL (4μg) and AAV.KL-shRNA (4μg) were mixed with 200μl of Opti-medium (Invitrogen, Carlsbad, CA, USA) respectively. Fifteen μl of the transfection reagent (Optifect, Invitrogen, Carlsbad, CA, USA) was mixed with 200μl of Opti-medium (Optifect-Opti-medium). The mix of AAV.mKL-Opti-medium was combined with Optifect-Opti-medium (mKL-Optifect), the mix of AAV.KL-shRNA-Opti-medium (KL-shRNA-Optifect) was combined with Optifect-Opti-medium. The mixtures were incubated at room temperature for 20min. The RASM culture medium was removed and the mixture of mKL-Optifect was added onto the cultured cells and incubated at 37ºC for 30min and then added the mixture of KL-shRNA-Optifect onto the cells again (without taking the previous medium), then incubated at 37ºC for additional 48 hours. The cells were harvested and lysed with RIPA buffer containing protease inhibitor (Sigma-Aldrich, Atlanta, GA, USA). The lysates were centrifuged at 10,000g for 10 min. The supernatant was ready for protein assay and western blot.

Quantitative RT-PCR-Total RNA was isolated from paraffin-embedded sections in cerebroventricle areas shown choroids plexus. The procedure of RNA isolation was performed according to the manual of RecoverALL Total RNA isolation kit (Ambion, Austin, USA). Two μg of total RNA was used for reverse transcription and 2μl of cDNA was used for PCR. Primers of klotho, ET1, ETA, and ETB used in qRT-PCR were concluded in table 1. The programs of qPCR for klotho, ET1, ETA, ETB and β-actin were concluded in table S1.

Determination of the titers of recombinant AAVs-After purification of recombinant AAVs, the titer of unconcentrated viral stock (PFU, plaque formation units) was determined by X-gal staining using HT1080 cells introduced with AAV.LacZ (Stratagene, La Jolla, CA, USA). Briefly, HT1080 cells were plated at a density of 3×10^5 per well in 12-well tissue culture plates containing 1 ml of DMEM growth medium. Ensure that cells are spread evenly in wells for accurate titer determination. After overnight incubation at 37°C, the cells should be ~80% confluent before proceeding. Add 0.2 ml of AAV permissive growth medium (DMEM Growth

Medium containing 10% (v/v) heat-inactivated FBS, 2mM L-glutamine, 0.8 μM camptothecin.) Mix well by swirling, then return the plates to 37°C incubator for 4 hours. Dilute the primary viral stock 100-fold. From this 100-fold dilution, perform 5-fold serial dilutions in 2.5-ml volumes over a dilution series from 2 × 10–3 to 8 × 10–5 in L-DMEM (DMEM Growth Medium containing 2% FBS). Add 0.5 ml of each dilution to separate wells of the 12-well plates. Perform the titer in triplicate, adding 0.5 ml of each dilution to each of three wells. In addition, include a no viral stock as a negative titer control. Incubate at 37°C for 1–2 hours. Swirl plates gently at 30-minute intervals during the incubation. Add 0.5 ml of pre-warmed H-DMEM (DMEM Growth Medium containing 18% FBS) per well, then incubate at 37°C for 40–48 hours. After incubation, the cells were fixed and stained using the X-gal staining protocol of choice. Count the blue-stained cells and calculate the viral particles (number of stained cells) per ml of stock solution.

Immunohistochemistry-Sections were stained following standard protocols by using goat-anti-klotho polyclonal, mouse anti-ET1 monoclonal, rabbit anti-ETA polyclonal, rabbit anti- ETB polyclonal antibodies (see table S2 for the sources and concentrations). After regular de-wax, rehydration, and antigen retrieving, the sections were rinsed in 0.1 M PBS (pH 7.6) for 3 times and incubated in 3% hydrogen peroxide (in PBS) for 15 minutes to block endogenous peroxidases, rinsed in PBS, and then pre-incubated in a blocking solution (background sniper, Cat#, BS966, Biocarta, San Diego, CA, USA) for 10min. Thereafter, the sections were incubated with primary antibody at 4ºC overnight and after rinsing, incubated for 1 hour in conjugated secondary antibody (see table S2). The reaction product was visualized with 3,3-diaminobenzidine (DAB) (Invitrogen, Carlsbad, CA, USA). The sections were counterstained with hematoxylin for nuclear staining. The sections were then dehydrated through gradient ethanol, cleared in xylene and covered with coverglass in mounting medium. The photographs were reviewed under the Nikon T*i*-U microscope (Nikon instruments Inc, Lewisville, TX) with 60 x oil objective and the intensities were measured using Nikon T*i*-U microscope software.

Table S1 PCR programs used in the experiment

Gene name	Klotho	ET1	ETA	ETB	B-actin
Hot start	94°C/5min	94°C/5min	94°C/5min	94°C/5min	94°C/5min
Denature	94°C/45sec	94°C/45sec	94°C/45sec	94°C/45sec	94°C/45sec
Annealing	56°C/1min	55°C/45sec	53°C/1min	57°C/50sec	55°C/1min
Extension	72°C/1min	68°C/1min	72°C/1min	72°C/1min	72°C/1min
Final extension	72°C/10min	68°C/10min	72°C/10min	72°C/10min	72°C/10min

Table S2 Antibodies were used in the experiment

1st Ab	Source	Cat#	Species	dilution	2nd Ab (all the 2nd Abs from Santa Cruze)	2nd Ab dilution
Klotho	R&D	AF1819	goat	1:500	Donkey anti-goat-IgG-HRP	1:1000
ET1	abcam	Ab2786	mouse	1:250	Goat anti-mouse-IgG-HRP	1:1000
ETA	Alomone	AER-001	rabbit	1:200	Goat anti-rabbit-IgG-HRP	1:1000
ETB	Alomone	AER-002	rabbit	1:500	Goat anti-rabbit-IgG-HRP	1:1000

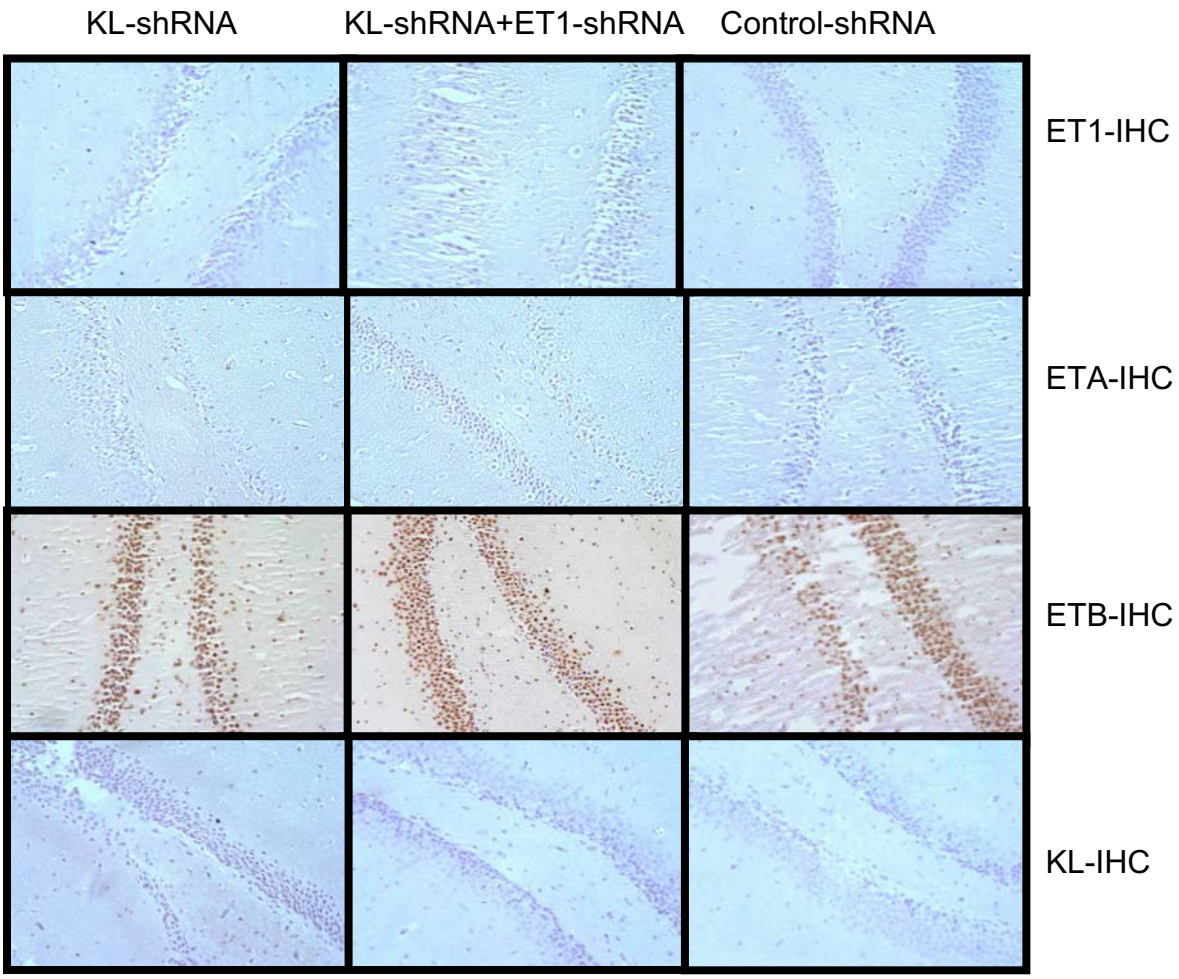

Figure S1. Immunohistochemical analysis of endothelin-1 (ET-1), ETA receptors, ETB receptors, and klotho in the brain hippocampus. ETB receptors expressed in the hippocampus which was not affected by KL-shRNA or ET1-shRNA. No staining was found for ET-1, ETA receptors, and klotho.

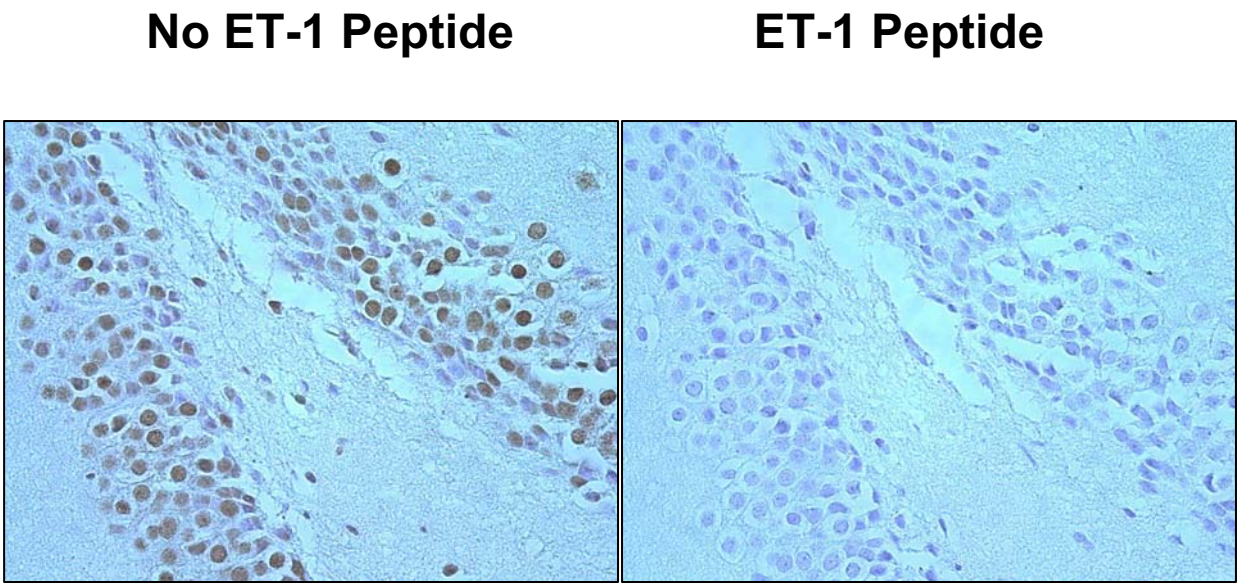

Figure S2. To confirm the specific staining of ETB receptors, we blocked ETB receptors using ETB-specific peptide. The data confirmed the specificity of the ETB staining.

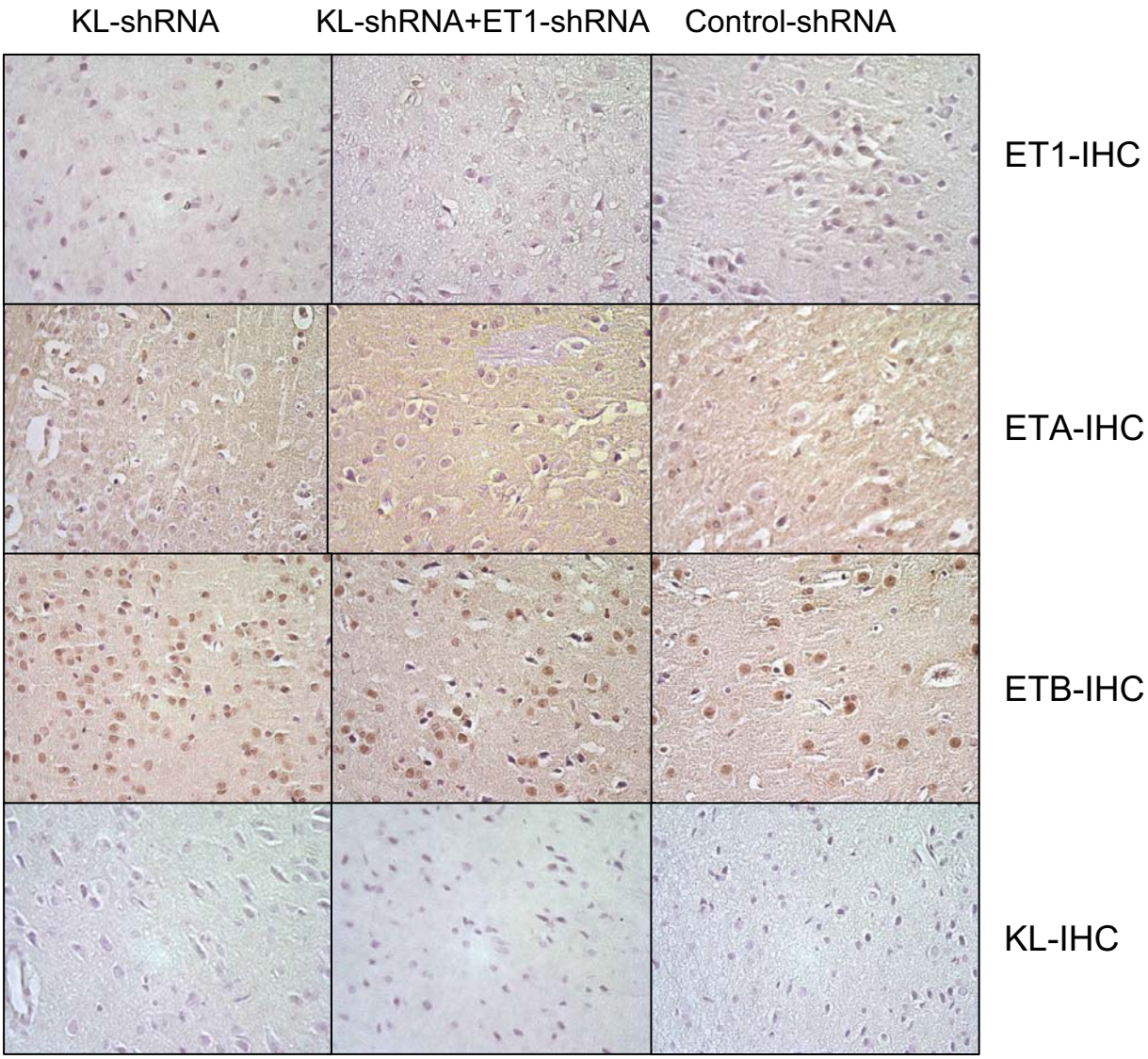

Figure S3. Immunohistochemical analysis of endothelin-1 (ET-1), ETA receptors, ETB receptors, and klotho in the brain hypothalamus. No staining was found for ET-1 and klotho. There were weak staining for ETA receptors and strong staining for ETB receptors in the hypothalamus in the hypothalamus, which were not affected by either KL-shRNA or ET1-shRNA.