Endothelin-1 Inhibits NADPH Oxidase Activity in Human Abdominal Aortic Endothelial cells: a Novel Function of ET\textsubscript{B1} receptors

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Online Supplemental Methods

Cell Culture

Human abdominal aortic endothelial cells (HAAECs) (ATCC, Manassas, VA, USA) were cultured using pre-coated 0.1% porcine gelatin. Cells were grown at 37°C in a humidified incubator with 5% CO\textsubscript{2} in F-12K culture medium containing L-glutamine (2 Mm), sodium bicarbonate (1.5g/L), heparin (0.1 mg/ml), endothelial growth supplement (0.03 mg/ml), penicillin-streptomycin (100 U/mL), and 10% fetal bovine serum.

Effects of ET1 on superoxide generation in HAAECs

Superoxide generation in HAAECs was estimated as described earlier.\textsuperscript{1} Briefly, cells were incubated with nitric oxide inhibitor N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME, 1 mM, Sigma) one hour before the treatment with ET1 (10 nM) for 20 min (Assay Design). Cells were trypsinized and re-suspended in phenol red free HBSS (10\textsuperscript{6} cells/ml). Cells were then incubated with dihydroethidium (DHE) (10 µM) in phosphate-buffered saline at 37°C in the dark for 30 min in 5% CO\textsubscript{2} humidified chamber. Flow cytometry (FACScan, Becton Dickinson) was used to select a homogeneous population of 10,000 live cells. The geometric mean of ethidium fluorescence intensity (excitation 488 nm, emission 610 nm) in the population was used for analysis.
In a separate experiment, HAAECs were cultured using chamber slide and treated with ET1 (10 nM) for 20 min and 48 h separately in the presence and absence of L-NAME. Cells were then treated with DHE (10 µM) for 30 minutes at 37°C. Ethidium fluorescence images were examined by a fluorescence microscopy (Nikon TE2000-E). As a positive control, AngII (100 nM) was used to confirm that the HAAECs were responsive to Nox1 activator.

Effect of ET1 on intracellular ROS production in HAAECs

Intracellular ROS level was assayed as follows. Dishes of 60-70% confluent cells were washed with 1X HBSS lacking phenol red and then incubated in the dark for 10 minutes in 1X HBSS (Hanks' balanced salt solution) containing 5 µM 2',7'-dichlorofluorescein (DCFH-DA). Cells were treated with L-NAME one hour prior exposure to DCFH-DA. Following the DCFH-DA incubation, cells were treated with ET1 for 20 min. Fluorescent images were captured using a confocal microscope (Leica TCS SP2) equipped with a 40 x HCX objective. To avoid photooxidation of DCF, the fluorescence image was collected by a single rapid scan (8-line average, total scan time of 8.66 seconds).

Western blot analysis of Nox1 and Nox2 in HAAECs

The procedure for western blotting was described in our previous studies.²,³ Briefly, the equal amount of protein was loaded in 3% Tris-HCl gel followed by electronic transfer. After blocking with 3% milk (in TBS-T), the membranes were incubated with antibodies (diluted in 3% milk /TBS-T) against Nox1 (Santa Cruz, 1:750) and Nox2 (BD transduction laboratories Inc, 1:1000) at 4°C overnight. The membranes were incubated with HRP conjugated secondary anti-goat and anti-mouse antibodies (1:2000-1:5000), respectively, for 1 h at room temperature. Target protein expression was normalized with the expression of β-actin which was served as an internal control. For this purpose, a parallel gel with identical samples was run and the western blot analysis was performed with the β-actin monoclonal antibody (Sigma-Aldrich, 1:20000).

Effects of ET1 on NADPH oxidase activity and PCNA: role of ET_{B1} receptors

To test the role of ET_{B1} receptors in mediating the effect of ET1 on NADPH oxidase activity, ET_{B1} receptors were silenced by transfection of HAAECs with small interfering RNA (siRNA) against ET_{B1} receptors (ET_{B1}siRNA) before treatment with ET1. Briefly, cells were
grown in 100 mm Petri dishes until they reach 70 to 80% confluence. Cell transfection was carried out according to the manufacturer’s recommendations (Santa Cruz).

Human ET_{B1} (Accession number: NM_000115) siRNA was designed, synthesized and annealed by Santa Cruz (Cat# sc-39962, Santa Cruz). The scrambled siRNA (Cat#: sc-36869) was used as a control siRNA (ControlsiRNA). The ControlsiRNA was proved, by Santa Cruz, not to match any known gene sequence. In brief, cells were incubated with 60 pmol of ET_{B1}siRNA or ControlsiRNA mixed with Oligofectamine Reagent. After 8 hours of transfection, the medium was refreshed and the cells were then incubated with or without ET1 (10 nM) for 20 minutes. NADPH oxidase activity was measured using lucigenin as described previously.\(^4\) Proliferating cell nuclear antigen (PCNA) was measured by western blot using anti-PCNA antibody (Abcam 1:5000).

**Effect of RNAi inhibition of Nox1 on ET1-induced inhibition of NADPH oxidase activity**

To determine if Nox1 fully mediates ET1-induced inhibition in NADPH oxidase activity, Nox1 was silenced by transfection of HAAECs with Nox1siRNA before treatment with ET1. Human Nox1 (Accession number: NM_007052) siRNA was purchased from Santa Cruz (Cat# sc-43939). Briefly, cells were incubated with 60 pmol of ET_{B1}siRNA or ControlsiRNA for 8 hours. The medium was refreshed and the cells were then incubated with or without ET1 (10 nM) for 20 minutes. NADPH oxidase activity was measured using lucigenin.

**Effect of ET1 on intracellular nitric oxide (NO) levels**

Intracellular NO was measured as described earlier.\(^5\) Briefly, HAAECs were harvested with trypsin and resuspended in lack of phenol red HBSS (10^6 cells/mL). Cells were incubated with DAF-2DA (10 μM, Peninsula laboratory) in the presence of 3 mM L-arginine for 30 min at 37°C in the dark in 5% CO\(_2\) in humidified chamber. DAF-2DA was excited at a wavelength of 490 nm and the emitted fluorescence at a wavelength of 515 nm was measured using fluorescence micro plate reader (Synergy 2, BioTek).

Because NOS generates ROS instead of NO in the absence of L-arginine, we added an excess concentration of L-arginine (3 mM) to all solutions used for NO measurement, except for cells treated with L-NAME (0.1 mM) which was added during the last 30 min of the DAF-2DA
incubation period. NOS activity was measured in real time using the NO-specific fluorescence probe DAF-2DA in the presence of L-arginine as described by Govers et al.\textsuperscript{6}

**Intracellular Ca\textsuperscript{2+} Assay**

Intracellular calcium levels were measured as described previously.\textsuperscript{7} Subconfluent HAAECs were loaded with 5 μmol/L Fura-2-AM for 30 minutes at 37°C in Ringer buffer (150 mmol/L NaCl, 4 mmol/L KCl, 2 mmol/L CaCl\textsubscript{2}, 1 mmol/L MgCl\textsubscript{2}, 5.6 mmol/L glucose, and 5 mmol/L HEPES-NaOH, pH 7.4) containing 0.1% BSA, and cells were harvested by trypsinization. The cell density of the suspension was adjusted to 1×10\textsuperscript{6}/ml in Ringer buffer without BSA. Changes in the fluorescence emission were monitored using a fluorescence microplate reader at 37°C with excitation wavelengths at 340 and 380 nm and an emission wavelength at 500 nm.

$$[\text{Ca}^{2+}]_i = \frac{K_d \times ([F - F_{\text{min}}] / [F_{\text{max}} - F]) \times (S_f / S_b)}{F}$$

Intracellular Ca\textsuperscript{2+} concentration was calculated according to above equation where $K_d$ is 224 nmol/L, $F$ is the ratio of the fluorescence emission at 340 nm and at 380 nm, and $F_{\text{max}}$ and $F_{\text{min}}$ are obtained in the presence of 0.5% Triton X-100 and subsequently 37.5 mmol/L EGTA, respectively, at the end of each assay. $S_b$ and $S_f$ are emission values at 380 nm corresponding to $F_{\text{max}}$ and $F_{\text{min}}$, respectively.

**Effect of ET1 and AG-17 on phosphorylation of Pyk2 and Rac1: role of ETB1 receptors**

To determine the effect of ET1 on the activity of Pyk2 and Rac1, we measured the phosphorylated Pyk2 and Rac1 proteins in HAAECs treated with ET1 (10 nM), AG-17 (a Pyk2 inhibitor, 500 nM, Calbiochem), ET\textsubscript{B1}siRNA (60 pmol), and ET\textsubscript{B1}siRNA+ET1, respectively. AG-17 specifically inhibits phosphorylation of tyrosine 402 in pyk2 molecule. The phosphorylated Pyk2 was measured by western blot using phosphor-specific ant-Pyk2 antibody (Santa Cruz).

The endogenous GTP-associated form of Rac1 in HAAECs was detected using Rac/CDC42 Assay kit (Upstate Biotechnology) according to the manufacturer's instructions. The cells were lysed with lysis buffer provided by the manufacturer and supplemented with protease inhibitor mixture. The lysate was centrifuged at 13,000 rpm for 15 min at 4 °C. A total of four samples were prepared for each condition. Two equal aliquots of the lysate were
preloaded with either GTP\textsubscript{S} or GDP as controls. A third equal aliquot was not preloaded. All three aliquots were then shaken with glutathione S-transferase-PAK1-(14-325) bound to glutathione-agarose beads at 4 °C for 1 h. The fourth sample was used directly for western blot analysis of phosphorylated Rac1. Briefly, beads were pelleted by pulse centrifugation and washed with cold lysis buffer. The pellets were resuspended in 40 µl of SDS-PAGE loading buffer and subjected to western blotting. Rac1-GTP was visualized using an anti-Rac1 monoclonal antibody supplied with the kit.

To evaluate the effect of inhibition of Pyk2 on ET1-induced inhibition of NADPH oxidase, NADPH oxidase activity was measured in HAAECs treated with AG-17 (500 nM) followed by ET1.

**Effects of ET1 on AngII-induced NADPH oxidase activation and cell proliferation**

NADPH oxidase activity and PCNA expression were measured in HAAECs treated with ET1 (10 nM), AngII (100 nM), or ET1+AngII. In the latter, cells were treated with ET1 (20 min) followed by AngII.

**Statistical Analysis**

Data were analyzed using two-way ANOVA or \( t \)-test where appropriate. A value of \( p<0.05 \) was considered significant.

**References**


