**Supporting Information**

# Elevated Vascular Level of *ortho*-Tyrosine Contributes to the Impairment of Insulin-Induced Arterial Relaxation


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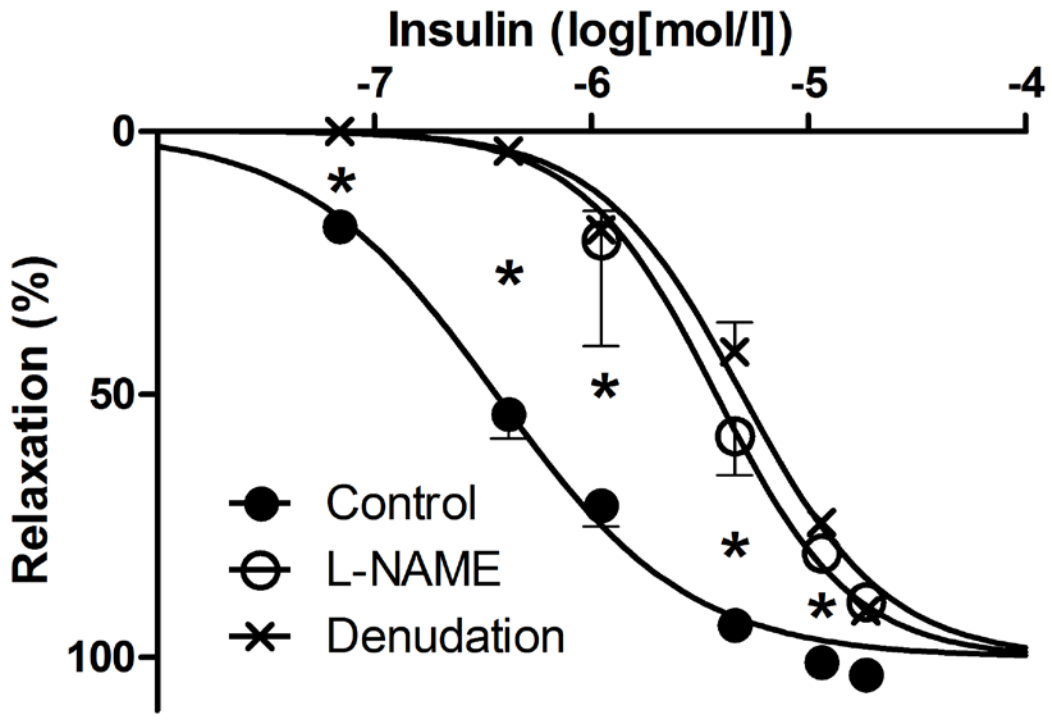
**Fig. 1S** Insulin-induced relaxation of femoral arteries in presence and absence of L-NAME or after endothelial denudation.




[Control: n = 4; L-NAME: n = 3; Denudation: n = 4]. Data are means ± SEM. (Two-Way ANOVA)
*$P < 0.05$

## Supplemental Methods

### Animals and tissue preparation

Animal experiments were carried out with the permission of the Animal Experiment Committee of the University of Pécs, Hungary. Male Sprague-Dawley rats (4-5 week-old, 90-120 g) were used in the ortho-tyrosine incorporation studies. Two-hours fasted rats were orally supplied by gavage with either 1.76 mg/die of o-Tyr (Sigma-Aldrich, St. Louis, MO, USA) dissolved in saline or vehicle (saline only) during six days per week for four weeks. At the end of four-week treatment, rats were sacrificed after anesthesia with ketamine, and HPLC (high-performance liquid chromatography) and vasomotor studies were performed.

The femoral arteries were removed, cleaned from connective tissue followed by their dissections into two sections. The proximal sections were immediately hydrolyzed for HPLC analyses. The distal parts were used for vasomotor studies.

### Assessment of oxidative status and tyrosine isomer incorporation in the vessel wall

Due to the fact that hydroxyl free radical has extremely short half-life and its detection is very limited [1]; we used an alternate approach to detect the stable end-product of oxidative reactions, o-Tyr, which is an isomer of the natural amino acid L-tyrosine [2–5]. The proximal sections of femoral arteries were hydrolyzed in well-closing, O-ring protected polypropylene tubes. Desferrioxamine and butylated hydroxytoluene (at final a concentration of 3.6 mM and 45 mM, respectively) were added to the samples to avoid a possible free radical formation during hydrolysis. Then 200 µl of 12N hydrochloric acid was added, and we performed an overnight acid hydrolysis of the proteins at 120°C. [4] The hydrolyzates were then filtered through a 0.2 µM filter (Millipore Co., Billerica, MA, USA), and 20 µL of the filtrate was injected onto the HPLC column of a Shimadzu Class LC-10 ADVP HPLC system (Shimadzu USA Manufacturing Inc., Canby, OR, USA) using a Rheodyne manual injector. Quantitative analysis of the amino acids was carried out upon their autofluorescence using a LiChroCHART 250-4 column (Merck KGaA, Darmstadt, Germany), in an isocratic run using aqueous solution containing 1% acetic acid and 1% sodium acetate as the mobile phase. The tyrosine isoforms were measured at 275 nm excitation and 305 nm emission wavelengths, so were the phenylalanine levels at 258 nm excitation and 288 nm emission wavelengths using a Shimadzu RF-10 AXL fluorescent detector (Shimadzu USA Manufacturing Inc., Canby, OR, USA) upon their autofluorescence. Therefore, no pre-column or post-column staining or derivatization was required [4,5]. The area under-the-curve (AUC) was determined for the amino acids, and exact concentrations were calculated using external standard calibration. In some cases the elution time of the substances was also verified by standard peak-addition method. The amino acid concentrations were corrected for phenylalanine concentrations.

**Assessment of vasomotor function**

The modified method described by Fésüs et al. [6] was used. The distal parts of the vessels were dissected into 2 mm long segments in ice-cold Krebs buffer, and rings were mounted on two stainless steel wires (40 μM in diameter) in a Danish Multimyograph Model 610M (DMT-USA Inc., Atlanta, GA, USA). Vessels were bathed at 37˚C in Krebs buffer (pH 7.4) containing (in mM) NaCl 119.0, KCl 4.7, $KH_2PO_4$ 1.2, $NaHCO_3$ 25.0, $Mg_2SO_4$ 1.2, glucose 11.1, $CaCl_2*2H_2O$ 1.6 and gassed with 5% $CO_2$ and 95% $O_2$. The resting tension/internal circumference relationship for each vessel was determined and then the internal circumference was set to 0.9 x L100, where L100 is the internal circumference of the vessel that would have *in vivo* when being relaxed under a transmural pressure of 100 mmHg. After this normalization procedure, vessels were allowed to stabilize for 30 min, then isometric tension was continuously recorded. Rings were pre-constricted with 100 nM epinephrine. After reaching a stabile contraction plateau, relaxant responses to increasing doses of insulin were assessed. The magnitude of relaxation caused insulin was expressed as the percentage of the contraction evoked by epinephrine which was taken 100%.

Insulin, epinephrine, and $Mg_2SO_4$ were obtained from Sigma-Aldrich (St. Louis, MO, USA). The NaCl, KCl, $KH_2PO_4$, $NaHCO_3$, $CaCl_2*2H_2O$, and glucose were purchased from Merck (Merck KGaA, Darmstadt, Germany).

**Cell culture**

Primary cultures of mouse endothelial cells (ECs) from endothelioma were purchased from LGC Promochem (Taddington, UK). ECs were grown in Dulbecco's modified Eagle medium (DMEM; Gibco, Csertex, Budapest, Hungary) supplemented with 10% Fetal Bovine Serum (Gibco) and 2% mixture of penicillin-streptomycin (Gibco) in a humidified incubator at 37°C and 5% CO2. The medium was changed every 2 days. ECs cultures were randomly assigned into three groups and were incubated in media containing 1) 400 μM p-Tyr (Control); 2) 400-400 μM p-Tyr and o-Tyr (o-Tyr) for 8 days. ECs were incubated with insulin (400 nM, 5 min) to assess the changes of its downstream effects on eNOS phosphorylation. At the end of experiments, ECs were scraped off mechanically and processed for further analyses.

**Assessment of ortho-tyrosine incorporation in endothelial cells**

The total protein-bound cellular tyrosine content of ECs was measured by a method described by Molnár et al. [4] After adding 200 μl of distilled water, samples were sonicated for 2 min with ultrasonic homogenizer to obtain cell lysates. After the addition of 100 μL of 60% trichloroacetic acid, samples were centrifuged (4000 rpm, 10 min) and the sediment was resuspended in 200 μL of 1% trichloroacetic acid. After resuspension, 100 μl of 60% trichloroacetic acid was added to the lysates followed by a second centrifugation (4000 rpm, 10 min) then previous steps were repeated once again. Finally, 4 μL of 400 mM desferrioxamine and 40 μL of 500 mM butylated

hydroxytoluene were added to the sediments to avoid a possible free radical formation during hydrolysis. Then 400 µL of 6N hydrochloric acid was added to the samples followed by processing steps for HPLC method as described above.

**Western blot analyses**

To assess eNOS phosphorylation, immunoblot analyses as we previously described were used.[7] ECs were solubilized in Tris-Triton extraction buffer [1 M Tris-HCl (pH 7.4), 1.15% Triton X-100, 500 mM EDTA, 200 mM EGTA supplemented with a mixture of protease and phosphatase inhibitors] on ice for 30 min. Cell lysates were centrifuged (13000 rpm, 10 min) then protein content of the supernatants was determined with the Bio-Rad protein assay kit (Hercules, CA, USA) using bovine serum albumin (BSA) as the standard. Equal amounts of proteins were resolved by SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA, USA). Equal protein loading was confirmed by Ponceau-S staining. Membranes were blocked in Tris base saline containing 0.1 v/v % Tween and 5 w/v % BSA (TBS-T-5% BSA) for 60 min at room temperature. The blots were then probed with primary antibody against phospho-(Ser1177)-eNOS (1:1000; Cell Signaling, Beverly, MA, USA) diluted in TBS-T-5% BSA for overnight at 4°C, followed by washing steps and incubation with secondary, HRP-conjugated anti-rabbit IgG antibody (1:2000; Cell Signaling) for 60 min at room temperature. Immunoblots were the visualized by enhanced chemiluminescence (ECL; Super-Signal West Pico, Thermo Fisher Scientific, MA, USA) and developed on X-ray films (Kodak XAR, Sigma-Aldrich). For densitometric analyses the Scion Image for Windows Software (Frederick, MD, USA) was used. Phospho-(Ser1177)-eNOS levels were corrected for total eNOS which was detected by reprobing the blots after stripping as described elsewhere [7].

**Statistical analyses**

Data are expressed as means ± SEM. All distributions were tested by Kolmogorov-Smirnov test. Statistical analyses were performed with unpaired t test and non-parametric tests as appropriate using GraphPad Prism5 (GraphPad Software Inc., La Jolla, CA, USA). Statistically significant differences were defined as $P \leq 0.05$.

**Supplemental References**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