SEX-SPECIFIC eNOS ACTIVITY AND FUNCTION IN HUMAN ENDOTHELIAL CELLS

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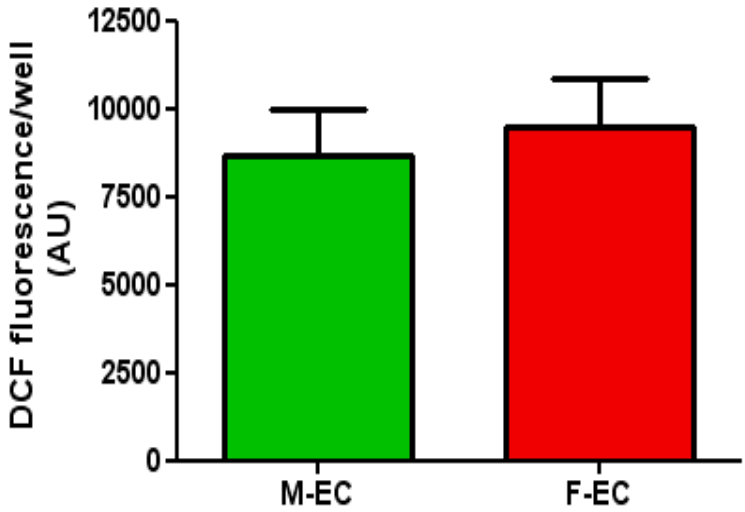

Fig. S1. ROS content was evaluated by measuring cell-associated fluorescence (expressed as arbitrary unit, AU) as described in the Supplementary Methods. p = 0.683, n=13.

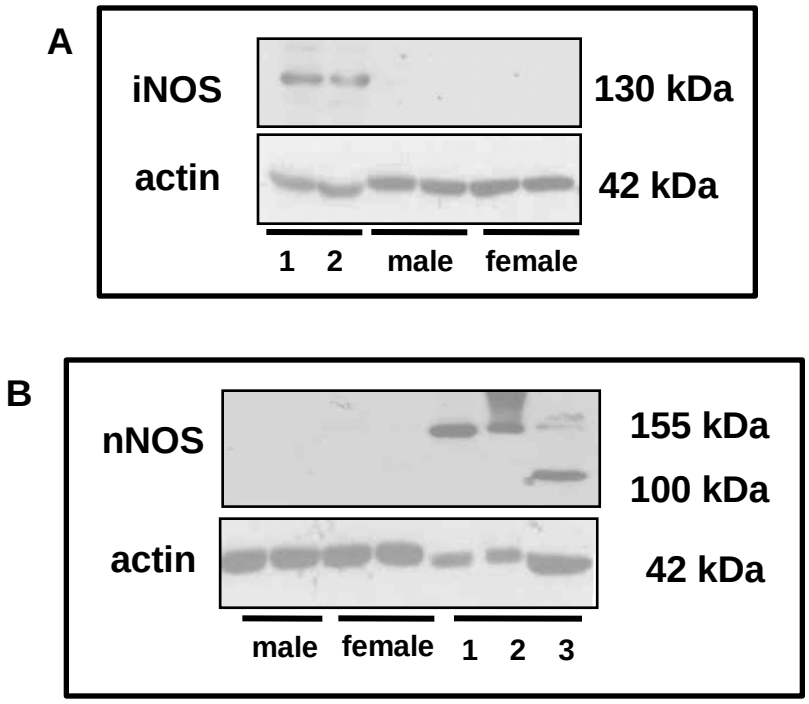

Fig. S2 (**A**) A representative immunoblotting showing total iNOS protein expression in 2 independent male and female EC lysates (20 µg/lane). Lanes 1 and 2: mouse macrophages stimulated with LPS as described in the Supplementary Methods. β-actin was used as a loading control.

Fig. S2 (**B**) A representative immunoblotting showing total nNOS protein expression in 2 independent male and female EC lysates (20 µg/lane). Lane 1: mouse brain cortex (7,5 µg/lane); lane 2: rat hippocampus (7,5 µg/lane); lane 3: human glioblastoma (U87MG cell line, 20 µg/lane). β-actin was used as a loading control.

In mouse and rat homogenates, the expected 155 kDa band was clearly detectable. In human U87MG cells, a weak band was present at 155 kDa whereas a 100 kDa band was evident. The expression of nNOS in U87MG cells has been reported in the literature[1] but the corresponding MW has not been shown. However, a nNOS isoform corresponding to a 100 kDa band has been described in human cells[2]**.** Nevertheless, neither the 155 nor the 100 kDa bands were detectable in EC lysates.

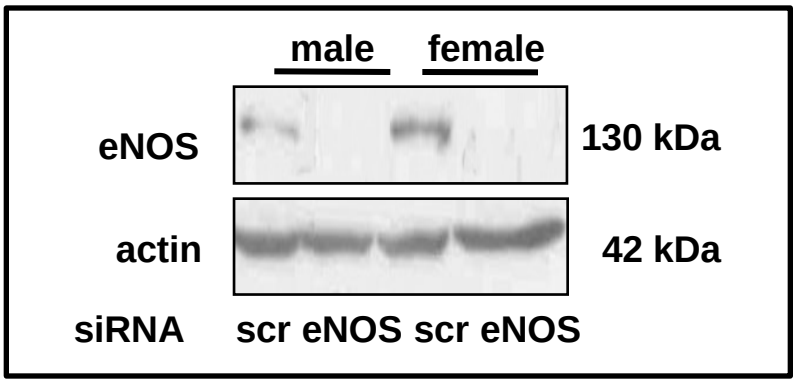

Fig. S3. A representative immunoblotting showing total eNOS protein expression in male and female EC lysates prepared 48h after transfection with scrambled (scr) or eNOS siRNA. β-actin was used as a loading control.

Supplementary Methods

Determination of Reactive Oxygen Species (ROS). Male and female HUVECs were plated at a density of $1.5x10^4$ cells/well in black 96-well microplates, and loaded for 30 min at 37°C in the dark with the fluorescent dye 5(6)-Carboxy-2'7'-dichlorofluorescein diacetate (CM-DCFA, 10 μM) in HBSS buffer (Hepes 25 mM pH 7.4, NaCl 120 mM, KCl 5.4 mM, $CaCl_2$ 1.8 mM, $NaHCO_3$ 25 mM, glucose 15 mM) containing 1% FBS[3]. Cell-associated fluorescence was assessed by means of a multiplate reader with excitation and emission wavelengths of 485 nm and 530 nm, respectively (Victor™, PerkinElmer).

Immunoblotting. Western blots for neuronal and inducible NOSs (nNOS and iNOS, respectively) were carried out on total EC lysates prepared in Laemmli sample buffer containing 1 mM sodium orthovanadate. Equal amounts of proteins (20 μg/lane) were separated by 8% SDS-PAGE, and then transferred onto nitrocellulose membranes following standard procedures. Membranes were blocked for 1 h with 5% milk in Tris-buffered saline containing 0.05% Tween-20 (TBS-T), and probed overnight at 4°C with the following primary antibodies diluted as indicated: mouse monoclonals anti-nNOS (BD Transduction Laboratories, cat #610309, 1:1.000 in 5% milk in TBS-T) and anti-β-actin (Sigma Aldrich, cat #A2228, 1:1.000 in 5% milk in TBS-T), and rabbit monoclonal anti-iNOS (Abcam, cat #178945, 1:1.000 in 5% milk in TBS-T). After incubation with the appropriate HPR-conjugated secondary antibody (1:10.000 in 5% milk in TBS-T), immunoreactive bands were visualized by chemiluminescence (LiteAblot Turbo, EuroClone). Full length blots are shown in Figures 2 and 3. Positive controls are: mouse macrophages stimulated for 24 h with lipopolysaccharide (LPS, 1 μg/ml) (kindly provided by Silvia Franchi, Dept of Pharmacological and Biomolecular Sciences, Università degli Studi di Milano, Italy) for iNOS; a mouse brain cortex homogenate (a kind gift of Irene Corradini, Dept of Medical Biotechnology and Translational Medicine, Università degli Studi di Milano, Italy), a rat hippocampus homogenate (kindly provided by Raffaella Molteni, Dept of Medical Biotechnology and Translational Medicine, Università degli Studi di Milano, Italy), and a human glioblastoma U87MG cell lysate (prepared in our

laboratory) for nNOS. Anti-iNOS and anti-nNOS antibodies were a kind gift of Cristiana Perrotta, Dept of Biomedical and Clinical Sciences "Luigi Sacco", Università degli Studi di Milano, Italy.

References

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