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Supplementary methods

Generation of plasmids

A fragment of eNOS cDNA encoding amino acids 401-934 was amplified by 35 cycles of PCR (the conditions were denaturation at 94^{o}C for 30 s, annealing at 66^{o}C for 1min and extension at 72^{o}C for 1 min) using oligonucleotides EcoeNOSsense401 (5'-TCTTCCggAATTCTgAAATCAACgTggCCgTgCT-3') and Xba1eNOSanti934 (5'-CTTCCCTCTAgATCAgTAgTACCggggCTggAgCAg-3') and plasmid pcDNA3-eNOS as a template.

A fragment of eNOS cDNA encoding amino acids 193-735 was amplified by 35 cycles of PCR (the conditions were denaturation at 94^{o}C for 30 s, annealing at 62^{o}C for 1min and extension at 72^{o}C for 1 min) using oligonucleotides EcoeNOSsense193(5'-TCTTCCggAATTCTCTgCAggTgTTCgATgCCCg-3') and XbaIeNOSanti735 (5'-CTTCCCTCTAgATCACTggCgCTTCCAgCTCCg-3') and plasmid pcDNA3-eNOS as a template

A fragment of eNOS cDNA encoding amino acids 735-934 was amplified by 35 cycles of PCR (the conditions were denaturation at 94^{o}C for 30 s, annealing at 66^{o}C for 1min and extension at 72^{o}C for 1 min) using oligonucleotides EcoeNOSsense735 (5'-TCTTCCggAATTCTTACCggCTgAgCgCCCAggCC-3') and XbaIeNOSanti934 (5'-CTTCCCTCTAgATCAgTAgTACCggggCTggAgCAg-3') and plasmid pcDNA3-eNOS as a template.

Amplified fragments were digested with EcoRI and XbaI and cloned into the corresponding site of p3xFlag-CMV7.1 (Sigma-Aldrich). The resulting plasmids, pFlag-eNOS (401-934) pFlag-eNOS (193-735) and pFlag-eNOS (735-934) encode Flag-tagged truncated proteins containing amino acids 401-934, 193-735 and 735-934, respectively, of human eNOS (numbering according to Genbank^{NM} Accession No. NM_000603).

The fidelity of all amplified products was confirmed by sequencing.

pFlag-eNOS (1-505) was created by restriction enzyme digestion of pcDNA3-eNOS with Hind III and Bgl II (Bgl II restriction site is located at nucleotides 1804-1809 of the eNOS cDNA, numbering according to Genbank^{NM} Accession No. NM_000603). The fragment released was cloned into the HindIII-BglII sites of plasmid pFlagCMV5b (Sigma-Aldrich).

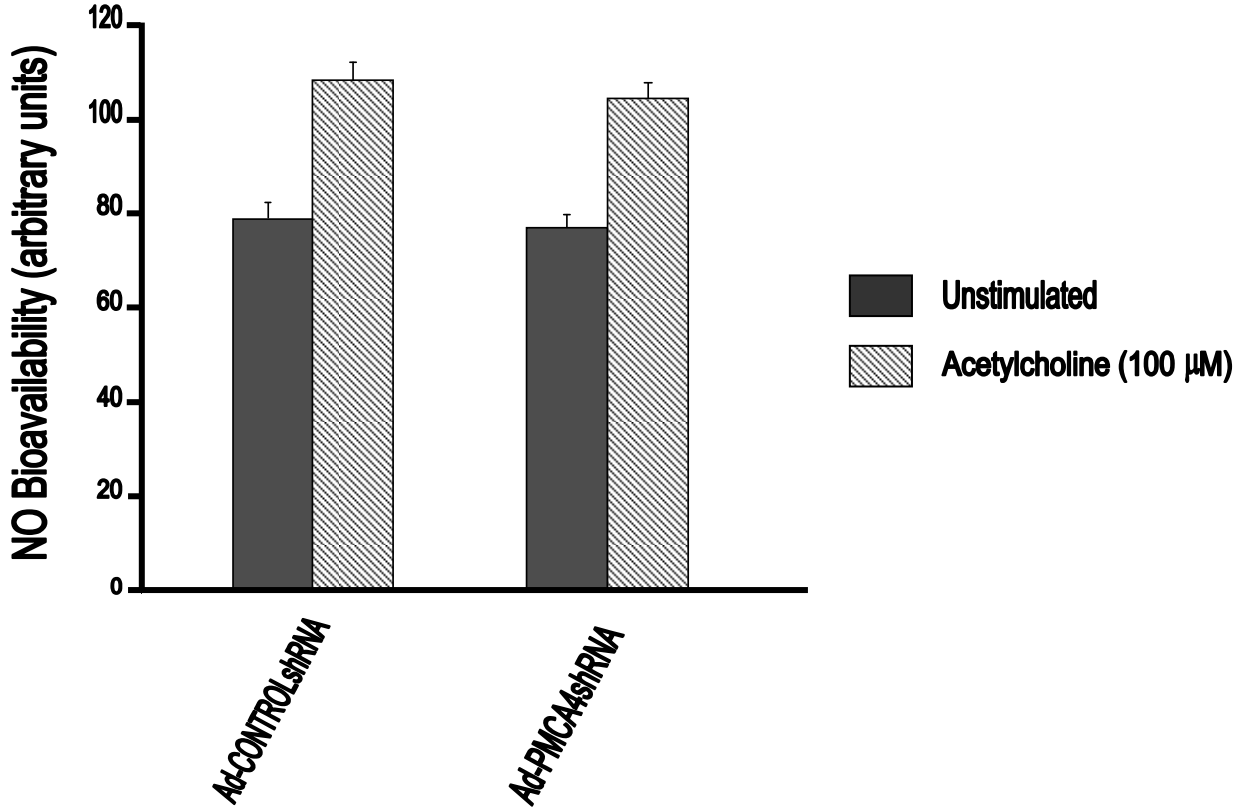
Western blot antibodies

Western blot membranes were incubated with a solution in TBS-T (TBS, 0.05% Tween 20) of the following antibodies; for Flag epitope detection with a 0.1% (v/v) solution of anti-Flag M2 peroxidase-conjugated monoclonal antibody (Sigma-Aldrich), for detection of all PMCA isoforms with a 0.25% (v/v) solution of the anti-PMCA 5F10 monoclonal antibody (Abcam), for the specific detection of PMCA1, 2 or 4 proteins with a 0.25% (v/v) solution of the corresponding isoform-specific rabbit polyclonal anti-PMCA antibody (Swant), for detection of eNOS with a 0.25% (v/v) solution of mouse monoclonal anti-eNOS (Zymed), or rabbit polyclonal anti-eNOS (Sigma), for detection of the phosphorylation status of residues Thr-495 and Ser-1177 of eNOS with a 0.25 (v/v) solution of a mouse phospho-specific anti-eNOS(pT495) or anti-eNOS(pS1177) antibody (BD Biosciences) respectively.

After antibody incubations, membranes were washed with TBS-T, incubated with appropriate secondary peroxidise-conjugated antibodies, and bound antibodies were detected by ECL as described[13].

Legend Supplementary figure S1

Supplementary figure S1. Knockdown of PMCA4 expression in endothelial cells has no effect on either basal or acetylcholine-induced NO production by the cells. HUVEC cells infected with Ad-PMCA4shRNA or Ad-CONTROLshRNA adenoviruses (MOI 25) were loaded with the NO-sensitive dye DAF-FM, and NO intracellular levels were determined in basal conditions or after treating cells with acetylcholine (100 μM) for 5 minutes.


NO Bioavailability (arbitrary units)
120
100
80
60
40
20
0
Ad-CONTROLshRNA
Ad-PMCA4shRNA
Unstimulated
Acetylcholine (100 µM)