Erythropoietin increases endothelial biosynthesis of tetrahydrobiopterin by activation of protein kinase B\textalpha/Akt1

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Expanded Materials and Methods

Experimental Animals: Male C57BL/6J (wild-type) mice, heterozygous Akt1 (Akt1+/-) mice, homozygous Akt1 (Akt1-/-) mice (C57BL/6J-Akt1tm1Mbb), and homozygous eNOS (eNOS-/-) mice (C57BL/6J-Nos3tm1Unc) were obtained from Jackson Laboratory (Bar Harbor, ME). EPO-transgenic mice were provided by Dr. M. Gassmann (Vetsuisse Faculty, Zürich, Switzerland). Mice were maintained on standard chow with free access to drinking water. Housing facilities and all experimental protocols were approved by the Institutional Animal Care and Use Committee of the Mayo Clinic and comply with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

In-Vitro Studies: Wild-type mice were euthanized (pentobarbital, 60 mg/kg body weight, i.p.). Whole aortas and lungs were carefully harvested and dissected free from connective tissue in cold (4°C) modified Krebs-Ringer bicarbonate solution (in mmol/L: NaCl 118.6; KCl 4.7; CaCl2 2.5; MgSO4 1.2; KH2PO4 1.2; NaHCO3 25.1; glucose 10.1; EDTA 0.026). Intraluminal blood was rinsed out and arteries were cut in 4-mm-long rings. Rings were randomly used and were incubated with EPO (recombinant human EPO alpha; Amgen, Thousand Oaks, CA) at various concentrations of 1-50 U/mL in minimal essential medium (MEM; containing 0.1% BSA, 100 U/ml penicillin, and 100 µg/ml streptomycin, Gibco) for 18 hours at 37°C in a CO2 incubator (5% CO2, Forma Scientific). In separate experiments, aortas were preincubated either with 10 mmol/L 2,4-diamino-6-hydroxypyrimidine, 1 µmol/L wortmannin, 5 µmol/L parthenolide, 3 µmol/L chelerythrine chloride (all Sigma), 5 µmol/L AG490 (Biomol), or 5 µmol/L 4,5,6,7-tetrabromobenzotriazole (TBB, Calbiochem) for 1 hour prior incubation to and for the duration of EPO incubation. After incubation, aortic rings were collected for assays. All inhibitors were
prepared as stock solutions using DMSO and diluted in phosphate buffered saline (PBS, Gibco). EPO was diluted in PBS prior experiments.

**In-Vivo Studies:** Wild-type, Akt1+/-, Akt1-/-, and eNOS-/- mice were randomly distributed to a control group (PBS, Gibco) and an EPO group (recombinant human EPO alpha, 1000 U/kg body weight, biweekly, s.c.). The dose of EPO was selected based on previous pharmacokinetic studies in mice.6,7 After 14 days of treatment the animals were euthanized (pentobarbital, 60 mg/kg, i.p.) and aortas and lungs were harvested. In separate experiments, wild-type mice were treated for 3 days with PBS or EPO (1000 U/kg body weight, s.c.) once daily.

**Systolic Blood Pressure:** Mice were trained for blood pressure measurement as described8 and systolic blood pressure (SBP) was recorded in quiescent mice by a tail-cuff method (Harvard Apparatus Ltd., Kent, England) before and on fourteenth day of treatment.6

**Blood Cell Count:** Mice were anesthetized in a bell jar containing isoflurane 1% and blood was drawn by the orbital venous sinus punch and transferred to EDTA containing tubes (Microtainer®; Becton Dickinson, Franklin Lakes, NJ). Blood cell counts were performed with ABAXIS VetScan HM2™ Hematology System (Union City, CA).

**Measurements of BH4 and 7,8-Dihydrobiopterin Levels:** Fresh aortas were homogenized in buffer containing 50 mmol/L Tris (pH 7.4), 1 mmol/L dithiothreitol, and 1 mmol/L EDTA at 4°C and were centrifuged. Biopterin levels were determined after differential oxidation in acid (which converts both BH4 and 7,8-dihydrobiopterin (7,8-BH2) to biopterin) and base (which converts only 7,8-BH2 to biopterin) conditions by reverse-phase HPLC (Beckman Coulter; Fullerton, CA) in 5% methanol/95% water and fluorescence detection (Jasco; Japan) as described
previously.\textsuperscript{9} Data were collected and analyzed by 32 Karat\textsuperscript{TM} chromatography software (Beckman Coulter) and normalized against tissue protein levels. BH\textsubscript{4} content was calculated from the difference in biopterin levels after acid and base oxidations.

**Measurement of GTPCH I Enzyme Activity:** Tissue supernatant homogenates were filtered using a Sephadex G25M column (Amersham; Piscataway, NJ) to remove endogenous neopterin, BH\textsubscript{4}, and phenylalanine. GTPCH I enzymatic activity was assayed using reverse-phase HPLC method by measurements of neopterin, which derived from dihydroneopterin triphosphate after oxidation and phosphate treatment.\textsuperscript{10} The results were normalized against tissue protein levels.

**Western Blot Analysis:** Aortas and lungs were homogenized on ice in lysis buffer containing 50 mmol/L NaCl, 50 mmol/L NaF, 50 mmol/L Na\textsubscript{4}P\textsubscript{2}O\textsubscript{7}, 5 mmol/L EDTA, 5 mmol/L EGTA, 0.1 mmol/L Na\textsubscript{3}VO\textsubscript{4}, 1\% Triton X-100, 10 mmol/L HEPES, and mammalian protease inhibitor cocktail (Sigma), and centrifuged. Equal amounts of protein (100 µg) were separated by SDS-PAGE and transferred to nitrocellulose membrane (Amersham), after which the membranes were probed using primary antibodies against eNOS, Ser\textsuperscript{1177}-phosphorylated eNOS (Transduction Labs), Akt1, Akt2, Ser\textsuperscript{473}-phosphorylated Akt1 (Upstate), GTPCH I\textsuperscript{10}, EPO-R (Santa Cruz), JAK2, Tyr\textsuperscript{1007/1008}-phosphorylated JAK2 (Cell Signaling). As a loading control, blots were rehybridized with monoclonal anti-\textbeta-actin (Sigma).\textsuperscript{9} Membranes were then incubated for 1 hour with horseradish peroxidase conjugated anti-IgG antibodies and visualized using enhanced chemiluminescence detection (Amersham). Densitometry was carried out using NIH-Image\textsuperscript{®} (Scion-Corp., Frederick, MD).
Calculations and Statistical Analysis: All results are expressed as means ± SEM and “n” indicates the number of animals from which tissues were harvested. Single values were compared by one-way ANOVA with Bonferroni's correction for multiple comparisons. For simple comparisons between two groups, an unpaired Student's t-test was used where appropriate. A value of P<0.05 was considered significant.

References


