Nox2 Is Determinant for Ischemia-Induced Oxidative Stress and Arterial Vasodilatation: A Pilot Study in Patients With Hereditary Nox2 Deficiency

To the Editor:

Reactive oxidant species (ROS) are a family of molecules that are involved in the modulation of arterial tone via rapid degradation of nitric oxide (NO). NADPH oxidase is a predominant cellular source of O$_2^-$-producing enzymes. Four homologs of gp91phox (Nox 2) named Nox1, Nox3, Nox4, and Nox5 have been identified as components of nonphagocyte-type NADPH oxidase. Recent studies performed in Nox1 and Nox2 knock-out animals suggested that these Nox isoforms may be implicated in controlling vascular function via modulation of NO bioactivity.

X-chromosomal granulomatous disease (X-CGD) is a rare primary immunodeficiency affecting the innate immunologic system; it is caused by mutations in any of the 4 genes encoding subunits of the gp91phox-containing NADPH oxidase mediates endothelial dysfunction in renovascular hypertension. Circulation. 2004;109:1795–1801.


A, Urinary excretion of PGF2α-III in patients and controls before (T=0) and after 4 to 6 hours (T=1) and 24 hours (T=2) from postischemic phase. B, 8-hydroxy-2′-deoxyguanosine plasma levels in patients and controls before (T=0) and after 3 to 15 minutes of postischemic phase. C, Representative Western blot demonstrating the different expression of iNOS protein in resting platelets from two HS (a, b) and from 2 X-CGD patients (c, d) (upper line). β-actin staining of respective lines (lower line). D, Percent change in brachial artery diameter in response to release of 5 minutes of forearm occlusion in 3 patients with X-chronic granulomatous disease (X-CGD) with (●) and without (◆) N-nitro-L-arginine methyl ester infusion and in 3 of 20 healthy subjects, matched for sex and age (●) (*P<0.001).
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Arterioscler Thromb Vasc Biol. 2006;26:e131-e132
doi: 10.1161/01.ATV.0000229710.13054.2d
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/26/8/e131

Data Supplement (unedited) at:
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Nox2 IS DETERMINANT FOR ISCHEMIA-INDUCED OXIDATIVE STRESS AND ARTERIAL VASODILATATION. A PILOT STUDY IN PATIENTS WITH HEREDITARY Nox2 DEFICIENCY.

Material and Methods

8-hydroxy-2’-deoxyguanosine (8-OHdG) serum levels

Blood samples were taken before and after ischemia from the contralateral arm and centrifuged 2,000 rpm for 20 min at 4°C, and the supernatant was collected and stored at −80°C until measurement. Serum levels of 8-OHdG were analyzed using a competitive enzyme-linked immunosorbent assay (Bioxytech 8-OHdG-EIA, OXIS Health Products, Portland, Oregon). Intra- and inter-assay coefficients of variation were 2.1% and 4.5% respectively.

iNOS Protein Expression

The iNOS protein was analyzed by Western blot in samples from resting platelets from healthy subjects and X-CGD patients. Protein were separated in denaturing SDS 10% polyacrylamide gels. Equal amounts of protein (20µg/lane) estimated by bradford assay were loaded. To verify that equal amounts of proteins had been loaded, a parallel gel with identical samples was run and stainend with beta-actin to compare the intensities of the protein bands. The separated proteins were blotted into PVDF membrane (BioRad), Blocked 1 hour at room temperature with 5% nonfat milk in TBS-T (20nmol Tris-HCl, 137mmol NaCl, 0,1% Tween 20) (I). Western blot analysis was performed with a monoclonal antibody against iNOS protein (BD Trasduction Laboratories) and beta-actin monoclonal antibody (Novus Biologicals Litteltown) as control. Overnight incubation at 4°C with the primary antibody (1:250) was followed by one hour incubation with secondary antibody (horseradish peroxidase-conjugated anti mouse immunoglobulin antibody) (Amersham) diluted at
Specific iNOS protein was detected by enhanced chemoluminescence (ECL, Amersham). Prestained protein markers (BioRad) were used for molecular mass determination.

**Urinary PGF2α-III assays**

Urinary PGF2α-III was measured by previously described and validated EIA assay method (II,III). 10 mL urine aliquots were extracted on a C-18 SPE column; the purification was tested for recovery by adding a radioactive tracer (tritiated PGF2α-III) (Cayman chemical). The eluates were dried under nitrogen, recovered with 1mL of buffer, and assayed in a PGF2α–III specific EIA kit (Cayman chemical). PGF2α-III concentration was corrected for recovery and creatine excretion and expressed as pg/mg of creatinine. Intra- and inter-assay coefficient of variation were 4.8% and 11.0% respectively.

**Flow-mediated vasodilatation**

Ultrasound assessment of endothelial dependent and independent FMD of brachial artery was investigated according to the recently reported guidelines (IV). Briefly, the study was performed in a temperature-controlled room (22°C) with the subjects in a resting, supine state between the hours of 8 A.M. and 10 A.M.; brachial artery diameter was imaged using a 7.5-Mhz linear array transducer ultrasound system (Siemens) equipped with electronic callipers, vascular software for two-dimensional imaging, color and spectral Doppler and internal electrocardiogram; the brachial artery was imaged at a location 3-7 cm above the antecubital crease; to create a flow stimulus in the brachial artery, a sphygmomanometric cuff was placed on the forearm; the cuff was inflated at least 50 mmHg above systolic pressure to occlude artery inflow for 5 minutes; all vasodilatation measurements were made at the end of diastole; flow-mediated vasodilatation was expressed as a change in post-stimulus diameter evaluated as a percentage of the baseline diameter; in all subjects following a 25 minute rest period after hyperemia sub-lingual nitroglycerin tablets (0.4 mg) were
given to determine the measure of endothelium-independent vasodilatation. Measurements were obtained in three separate occasions with a mean difference < 3% in FMD over time.

To test the role of NO in our experimental conditions, FMD was reevaluated after i.v. injection of 66 µg/kg N-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NO synthase; infusion lasted 60 minutes or until systolic blood pressure increased 20 mm Hg (V).

**Statistical analysis**

Data were expressed as mean ± standard deviation; comparisons between groups were carried out by Student’s t-test and were replicated as appropriate with nonparametric tests (Mann-Whitney-U test in case of non-homogeneous variances as verified by Levene’s test).

**References:**


