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

To the Editor:

Reactive oxygen species (ROS) are a family of molecules that are involved in the modulation of arterial tone via rapid degradation of nitric oxide (NO).1 NADPH oxidase is a predominant cellular source of O2·−-producing enzymes.1 Four homologs of gp91phox (Nox 2) named Nox1, Nox3, Nox4, and Nox5 have been identified as components of nonphagocyte-type NADPH oxidase.2 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.3,4

X-Chronic 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 O2·−-generating enzymes.5 We speculated that in patients with X-CGD the reduction of oxidative stress could result in increased NO bioavailability and eventually enhanced arterial vasodilatation. To explore this hypothesis we studied 3 male patients (age 41±7.0 years) with hereditary deficiency of Nox2, in whom oxidative stress as well as flow-mediated vasodilatation (FMD) were determined. Twenty male healthy subjects (HS; age 38±6.0 years) were used as control.

None of the HS and patients had risk factors for atherosclerosis or previous cardiovascular disease.

HS had significantly higher oxidative stress, as assessed by 8-hydroxy-2′-deoxyguanosine (8-OHdG) serum levels (1.3±0.7 versus 0.40±0.10 ng/ml, P<0.001) and urinary isoprostanes (247±14 versus 50±42 pg/mg of creatinine, P<0.001) than Nox2-deficient patients. Consistently with the burst of ROS usually occurring during the reperfusion phase,6 after FMD HS showed an increase of oxidative stress that, however, was not detected in patients, suggesting that activation of Nox2 has a pivotal role in the ROS formation occurring during FMD (Figure, A and B).

Resting platelets of HS expressed iNOS; an upregulation of platelet iNOS was detected in Nox-2 deficient patients (Figure, C).

Despite that oxidative stress seems to be implicated in inhibiting NO-mediated human arterial dilatation, the role of NADPH oxidase has not been investigated.7 To explore this issue FMD was measured in Nox2-deficient patients and HS. Patients and HS had similar baseline brachial artery diameter (3.40±0.38 versus 3.66±0.10 mm, t test, P>0.05). FMD was higher and lasted longer in patients compared with HS (16.0±2.3% versus 10.6±1.5%, U test, P<0.001 and 17.3±2.8 minutes versus 5.6±1.5 minutes, t test, P=0.003, respectively) (Figure, D).

To investigate whether in patients NO was responsible for the prolonged arterial vasodilatation, the experiment was repeated after the iv injection of L-NAME, which fully blunted FMD (Figure, D). L-NAME infusion in HS elicited similar findings (not shown). No side effects were observed during the injection of L-NAME. Administration of a single dose of 0.4 mg nitroglycerin induced similar dilatation in patients and HS (not shown).

Even if FMD of each patient was analyzed by the same operator in 3 separate occasions and had an acceptable reproducibility (<3%), the small sample size and the large variability of the method8 limit definite conclusion. The fact that in patients platelet iNOS was upregulated could indirectly support the hypothesis that NADPH-generating O2·−attenuates eNOS expression,9 but other mechanism cannot be excluded. For instance, because of the short half-life of NO, persistent arterial dilatation could also depend on other mechanism including ROS interaction with vasoconstrictor molecules.10

In conclusion, this pilot study provides the first evidence that in human Nox2 activity may play an important role in enhancing systemic and local oxidative stress and modulating NO-mediated arterial dilatation. This finding may help to develop strategies that prevent ROS formation and ultimately arterial dysfunction.

Disclosures

None.

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References


Letter to the Editor
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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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
1:5000. 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:**


