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Hereditary Deficiency of gp91^{phox} Is Associated With Enhanced Arterial Dilatation

Results of a Multicenter Study

Francesco Violi, MD; Valerio Sanguigni, MD; Roberto Carnevale, PhD; Alessandro Plebani, MD; Paolo Rossi, MD; Andrea Finocchi, MD; Claudio Pignata, MD; Domenico De Mattia, MD; Baldassarre Martire, MD; Maria Cristina Pietrogrande, MD; Silvana Martino, MD; Eleonora Gambineri, MD; Anna Rosa Soresina, MD; Pasquale Pignatelli, MD; Francesco Martino, MD; Stefania Basili, MD; Lorenzo Loffredo, MD

Background—NADPH oxidase is believed to modulate arterial tone, but its role in humans is still unclear. The objective of this study was to evaluate whether NADPH oxidase is involved in flow-mediated arterial dilation (FMD).

Methods and Results—Twenty-five patients with hereditary deficiency of gp91^{phox}, the catalytic core of NADPH oxidase, (X-CGD), 25 healthy subjects, and 25 obese patients matched for sex and age were recruited. FMD, platelet gp91^{phox}, serum levels of nitrite and nitrate as markers of nitric oxide generation, oxidized low-density lipoprotein, and urinary excretion of isoprostanes as markers of oxidative stress were determined. Platelet gp91^{phox} expression was downregulated in X-CGD patients (1.0 ± 0.8 mean fluorescence; $P < 0.001$) and upregulated in obese patients (4.1 ± 2.2 mean fluorescence; $P = 0.01$) compared with healthy subjects (2.9 ± 1.7 mean fluorescence). Urinary excretion of isoprostanes was reduced in X-CGD patients (41.7 ± 33.3 pg/mg creatinine; $P = 0.04$) and increased in obese patients (154.4 ± 91 pg/mg creatinine; $P < 0.001$) compared with healthy subjects (69.5 ± 52.4 pg/mg creatinine). Obese patients had higher serum oxidized low-density lipoprotein than healthy subjects (35.3 ± 6.7 versus 24.8 ± 9.8 U/L; $P < 0.001$) and X-CGD patients (28.5 ± 7.2 U/L; $P < 0.001$). X-CGD patients had significantly higher FMD ($14.7 \pm 5.9\%$) compared with healthy subjects ($7.9 \pm 2.5\%$; $P < 0.001$); obese patients had lower FMD ($5.3 \pm 3.0\%$; $P = 0.028$) compared with healthy subjects. Serum nitrite and nitrate levels were significantly higher in patients with X-CGD (36.0 ± 10.8 $\mu\text{mol/L}$; $P = 0.016$) and lower in obese patients (9.3 ± 11.0 $\mu\text{mol/L}$; $P = 0.001$) compared with healthy subjects (27.1 ± 19.1 $\mu\text{mol/L}$). Serum nitrite and nitrate levels significantly correlated with FMD ($R_s = 0.403$, $P < 0.001$) and platelet gp91^{phox} ($R_s = -0.515$, $P < 0.001$). FMD inversely correlated with platelet gp91^{phox} ($R_s = -0.502$, $P < 0.001$) and isoprostanes ($R_s = -0.513$, $P < 0.001$).

Conclusion—This study provides the first evidence that, in humans, gp91^{phox} is implicated in the modulation of arterial tone. (*Circulation*. 2009;120:1616-1622.)

Key Words: atherosclerosis ■ gp91^{phox} protein, human ■ oxidative stress

Endothelial dysfunction is a hallmark of early atherosclerosis and predicts cardiovascular events in high-risk cohorts, including the elderly and patients with hypertension, coronary heart disease, or peripheral arterial disease.¹⁻⁴ The mechanism accounting for endothelial dysfunction is not completely understood. Oxidative stress is believed to play an important role in that it can influence activity and generation of nitric oxide (NO), a potent vasodilator molecule produced by

endothelial cells.⁵ Several reactive oxidative species (ROS)-generating enzymes, including myeloperoxidase, xanthine oxidase, and NADPH oxidase, may be implicated in arterial dysfunction.⁶ Accordingly, experimental studies performed in animal models suggest a pivotal role of NADPH oxidase in modulating arterial tone.⁷⁻⁹ In particular, overexpression of gp91^{phox}, the catalytic subunit of NADPH oxidase (Nox2), potentiates the hemodynamic response to angiotensin II.⁷

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Table. Clinical Characteristics of X-CGD Patients, Healthy Subjects, and Obese Patients

	X-CGD Patients (n=25)	P*	HS (n=25)	P*	Obese Patients (n=25)
Age, y	16.6±9.5	...	16.6±9.5	...	16.6±9.5
Men, n	25	...	25	...	25
Systolic blood pressure, mm Hg	109.8±12.4	0.352	107.4±8.7	0.002	115.8±9.7
Diastolic blood pressure, mm Hg	68.0±9.2	0.064	72.4±7.5	0.698	71.6±10.0
Serum total cholesterol, mg/dL	126.7±20.0	0.483	133.5±13.8	<0.001	197.2±55.5
Serum fasting blood glucose, mg/dL	76.7±8.0	0.98	79.9±7.0	0.421	81.4±7.9
BMI, kg/m ²	18.1±2.9	0.052	19.6±3.0	<0.001	25.9±3.8
Serum total protein, g/dL	7.1±0.9	0.529	7.3±0.5	0.637	7.2±0.9
Serum albumin, g/dL	4.5±0.4	0.101	4.7±0.3	0.196	4.5±0.6
Urinary isoprostanes, pg/mg creatinine	41.7±33.3	0.04	69.5±52.4	<0.001	154.4±91
Serum ox-LDL, U/L	28.5±7.2	0.122	24.8±9.8	<0.001	35.3±6.7
Serum CRP, mg/L†	2.0 (1.1–2.0)	0.332	1.4 (0.6–2.0)	0.937	1.4 (0.7–2.0)
Platelet gp91 ^{phox} , mean fluorescence	1.0±0.8	<0.001	2.9±1.7	0.010	4.1±2.2
Serum NOx, μmol/L	36.0±10.8	0.016	27.1±19.1	0.001	9.3±11.0
IMT, mm	0.41±0.09	<0.001	0.50±0.10	0.004	0.58±0.16
FMD, %	14.7±5.9	<0.001	7.9±2.5	0.028	5.3±3.0

BMI indicates body mass index; CRP, C-reactive protein.

*Linear mixed-effects models.

†Expressed as median (interquartile range).

Clinical Perspective on p 1622

However, a critical issue of these experimental studies is their transferability to the comprehension of human atherosclerosis. In other words, it remains to be clarified whether ROS-generating pathways have some role in the process of human arterial dysfunction.

Chronic granulomatous disease (CGD) is a very rare genetic disorder (1 in 1 000 000) characterized by life-threatening infectious diseases resulting from defective activity of the innate immune system caused by functional deficiency of NADPH oxidase subunits.¹⁰ Among the NADPH oxidase subunits, the functional deficiency of gp91^{phox} (Nox2) is the most frequent hereditary disorder (X-CGD).¹⁰

In a pilot study performed on 3 patients affected by gp91^{phox} genetic deficiency, we demonstrated that gp91^{phox} activity may play an important role in enhancing systemic and local oxidative stress and modulating flow-mediated dilation (FMD)¹¹; the latter maximally reflects NO production by endothelial cells.¹² Nonetheless, the association reported in that study was not conclusive because of the small sample size and the lack of correction for major atherosclerotic risk factors or other confounding variables. Thus, a multicenter study enrolling a larger number of X-CGD patients recruited by various Italian institutions was designed with the ultimate goal of evaluating the involvement of gp91^{phox} with FMD.

Methods

Study Population

We conducted a multicenter study in collaboration with the Italian Primary Immunodeficiency Network. Of the 60 patients with CGD registered in the national database,¹⁰ 35 were not included in the study because of the presence of acute infections, critical physical conditions, hereditary NADPH oxidase deficiency unrelated to gp91^{phox}, or unwillingness to participate in the study. The remaining

25 patients who were gp91^{phox} deficient and willing to participate in the study were included.

Diagnosis of X-CGD was performed as previously described.¹⁰ All X-CGD patients were receiving itraconazole, trimethoprim, and sulfamethoxazole.

Unpublished observations obtained by our department showed that X-CGD patients had serum cholesterol levels ≈20% lower than healthy subjects. Because this could represent a confounding factor, 25 control subjects matched for serum cholesterol, sex, and age were identified and included in the study. Twenty-five patients affected by obesity who were matched for gender and age were randomly collected from the pediatric clinic and from the outpatient clinic of our division at the "I Clinica Medica" of the Sapienza University of Rome (the Table). Control populations were screened from routine visits. For subjects <20 years of age, obesity was defined according to body mass index–age–growth charts, which identify obesity as a body mass index ≥95th percentile.¹³ Adult patients with a body mass index >30 kg/m² were considered obese.¹⁴

Subjects were excluded from the study if they had liver insufficiency, serious renal disorders (serum creatinine >2.8 mg/dL), cancer, myocardial infarction, unstable angina, acute cerebrovascular disease, or deep venous thrombosis; if they were being treated with statins or antioxidant vitamins; or if they were current smokers. The study was approved by the ethics committee. Each subject enrolled gave informed consent to participate in the study. A substudy of X-CGD patients (n=7), healthy subjects (n=7), and obese patients (n=7) who were matched for age was performed to investigate platelet formation of NO and isoprostanes.

Blood Sampling

Between 8 and 9 AM, subjects underwent routine biochemical evaluations, including fasting total cholesterol and glucose. After an overnight fast (12 hours) and supine rest for at least 10 minutes, blood samples were collected in Vacutainers (Vacutainer Systems, Belliver Industrial Estate, Plymouth, UK) and centrifuged at 300g for 10 minutes to obtain supernatant, which was stored at –80°C until use. Total cholesterol was measured with a routine enzymatic colorimetric method on a Dimension RXL apparatus (Dade Behring AG, Ziegelbrücke, Switzerland).

Urinary Collection

Morning spot urine samples were collected from all participants between 7 and 9 AM. Morning urine collected for F₂-isoprostanes was stored in 10-mL aliquots at -80°C until analysis.

Urinary 8-Iso-Prostaglandin F_{2α} Assays

Urinary 8-iso-prostaglandin F_{2α} (8-iso-PGF_{2α}) was measured by a previously described and validated enzyme immunoassay method (Cayman Chemical, Ann Arbor, Mich)^{15,16} and expressed as picograms per milligram of creatinine. Intra-assay and interassay coefficients of variation were 2.1% and 4.5%, respectively.

Oxidized Low-Density Lipoprotein and C-Reactive Protein

Serum levels of oxidized low-density lipoprotein (ox-LDL) and C-reactive protein were measured by commercially available immunoassays (Tema Ricerca, Bologna, Italy). Intra-assay and interassay coefficients of variation were 4.0% and 8.3% for ox-LDL and 9.5% and 9.0% for C-reactive protein.

Nitrite and Nitrate Measurement

A colorimetric assay kit (Tema Ricerca) was used to determine the NO metabolites nitrite and nitrate (NOx) in the serum and supernatant of platelet-rich plasma (platelets=3×10⁸/mL) activated with collagen (7 μg/mL) at 37°C for 15 minutes as previously described.¹⁷ Intra-assay and interassay coefficients of variation were 2.9% and 1.7%, respectively.

Platelet gp91^{phox} Expression

Platelet gp91^{phox} expression was analyzed as previously described.¹⁸ Briefly, blood samples were incubated with the unconjugated antibody anti-gp91^{phox}, followed by an FITC-labeled donkey anti-goat immunoglobulin G secondary antibody. For platelet detection, the monoclonal antibody CD61-PE was used.

Samples were analyzed on an Epics XL-MCL cytometer (Coulter Electronics, Hialeah, Florida) equipped with an argon laser at 488 nm. FITC was detected at 825 to 850 nm, PE at 875 to 900 nm. Analysis was stopped automatically after the measurement of 50 000 events. Platelet gp91^{phox} was expressed as mean fluorescence. Intra-assay and interassay coefficients of variation were 1.0% and 0.2%, respectively.

Platelet 8-Iso-PGF_{2α} Assay

Citrated blood samples (ratio of blood to sodium citrate, 9:1) were centrifuged for 15 minutes at 180g to obtain platelet-rich plasma. Platelet-rich plasma was then centrifuged for 20 minutes at 300g to obtain washed platelets, and the pellets were suspended in Tyrode buffer to obtain a final platelet concentration of 3×10⁸/mL.

Platelet suspensions were incubated with or without collagen (7 μg/mL) for 15 minutes at 37°C. After incubation, platelets were pelleted 3 minutes at 300g with acid citrate dextrose.

Supernatants from stimulated and unstimulated platelets were acidified to pH 3 with 1 mol/L HCl; 20 μg internal standard (8-iso-PGF_{2α}) was added. After addition of the internal standard, the mixture was vortexed and applied to a C₁₈ Sep-Pak column (Waters Corp, Milford, Mass) preconditioned with 5 mL methanol and 5 mL water (pH 3). The sample and subsequent solvents were eluted through the Sep-Pak column with a 10-mL plastic syringe. The column was then washed sequentially with 10 mL water (pH 3), 10 mL acetonitrile/water (15:85 vol/vol), and 10 mL heptane. The isoprostanes were then eluted with 10 mL ethyl acetate/heptane (50:50 vol/vol).¹⁹ The eluted samples were completely evaporated under ultrahigh purity (99.9%). The samples, previously dissolved with 10 μL acetonitrile, were derivatized at various times (2 to 24 hours) with 40 μL *N*,*o*-Bis(trimethylsilyl)trifluoroacetamide with trimethylchlorosilane.

The 8-iso-PGF_{2α} in derivatized samples was analyzed by a PerkinElmer gas chromatograph (PerkinElmer, Waltham, Mass) equipped with a flame ionization detector with a SPB5 wide-bore column (Supelco, Bellefonte, Pa). Detector responses for 8-iso-prostaglandin and internal standard (8-iso-PGF_{2α}) were calculated by use of an area integrator LC100 (PerkinElmer).

The carrier gas, helium, was set to a flow rate of 1 mL/min. Derivatized samples (0.5 μL) were injected into the gas chromatograph injection port. The column temperature was maintained at 170°C for 2 minutes, increased to 275°C at 15°C/min, and then held at 275°C for 11 minutes. Finally, the temperature was raised to 300°C at 25°C/min and held for 10 minutes. 8-Iso-PGF_{2α} content was expressed as picomole per liter.

FMD and Carotid Intima-Media Thickness

Ultrasound assessment of FMD was investigated according to the recently reported guidelines²⁰ as previously described.²¹ The coefficient of variation for FMD measurements, obtained on 3 separate occasions, was 12.5%.

Longitudinal ultrasonographic scans of the carotid artery were obtained on the same day as the studies of the brachial artery reactivity and included the evaluation of the right and left common carotid arteries 1 cm proximal to the carotid bulb. Three measurements of intima-media thickness (IMT) were obtained from the right and left carotid arteries, respectively, and were averaged to determine the mean IMT for both sides combined. The coefficient of variation for IMT measurements, obtained on 3 separate occasions, was 4.90%. FMD and IMT were performed with a 7.5-MHz linear-array transducer ultrasound system (Sonomed, Lake Success, NY).

Statistical Analysis

We used linear mixed-effects models to compare means across groups because the subjects in the study were matched by age and sex. We used subject-specific random intercepts that were assumed to arise from Gaussian distributions, with clusters of random effects identified by the matched triplets (X-CGD, healthy subjects, obese). The group indicators were included as fixed effects. Results were further confirmed by nonparametric tests with the rank transformation.

Data are presented as mean±SD unless indicated otherwise. The correlation analysis was carried out by Spearman rank correlation test. Statistical significance was defined at *P*<0.05. Statistical analysis was performed with SPSS 13.0 for Windows (SPSS Inc, Chicago, Ill).

Sample Size Determination

On the basis of data from a pilot study,¹¹ we computed the minimum sample size with respect to a 2-sample Student *t* test, considering a clinically relevant difference for FMD variation to be detected between the X-CGD patients and control subjects |δ|≥5%, SDs that were homogeneous between groups (SDs=3%), and type I error probability of α=0.05 and power 1-β=0.90. This resulted in a minimum sample size of 9 subjects for each group. Sample size calculations were performed with the nQuery Advisor software, version 5.0, (Statistical Solutions, Saugus, Mass).

Results

Platelet expression of gp91^{phox} and urinary excretion of 8-iso-PGF_{2α} were downregulated in X-CGD patients (*P*<0.001 and *P*=0.04) and increased in obese patients (*P*=0.01 and *P*<0.001) compared with healthy subjects (the Table). On the other hand, obese patients had higher serum ox-LDL than healthy subjects and X-CGD patients (*P*<0.001), and X-CGD patients and healthy subjects had similar values of serum ox-LDL (the Table). Furthermore, X-CGD patients had significantly higher FMD (*P*<0.001) and serum levels of NOx (*P*=0.016), markers of NO generation,²¹ compared with healthy subjects (Table 1). Conversely, obese patients had lower FMD (*P*=0.028) and serum NOx levels (*P*=0.001) compared with healthy subjects (the Table). IMT was significantly lower in patients with X-CGD (*P*<0.001) but significantly higher in obese individuals (*P*=0.004) compared with healthy subjects (the Table).

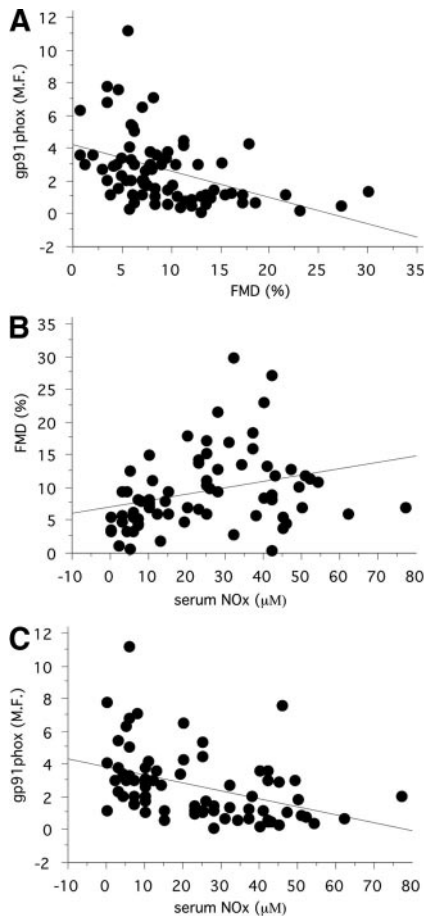


Figure 1. Linear regression analysis between FMD and gp91^{phox} (A), FMD and serum NOx gp91^{phox} (B), and gp91^{phox} and serum NOx (C). The straight line represents the least-squares regression line.

No difference was found among the brachial resting vessel size of the 3 groups (X-CGD, 2.90 mm [median] [interquartile range 2.48 to 3.60]; healthy subjects, 3.27 mm [interquartile range, 2.69 to 3.75]; obese patients, 3.30 mm [interquartile range, 2.90 to 4.0]). However, we stratified patients according to the median value observed in healthy subjects (≥ 3.27 mm) and found that X-CGD patients with basal brachial artery diameter >3.27 mm still showed significantly higher values of FMD ($F=13.0$, $P<0.001$) than healthy subjects and obese patients ($12.3\pm 1.1\%$ versus $7.2\pm 17\%$ versus $5.4\pm 3.6\%$, respectively).

Correlation analysis carried out by Spearman test showed that FMD inversely correlated with platelet gp91^{phox} expression ($R_s=-0.502$, $P<0.001$; Figure 1A) and urinary excretion of 8-iso-PGF_{2 α} ($R_s=-0.513$, $P<0.001$). Moreover, FMD directly correlated with serum NOx levels ($R_s=0.403$, $P<0.001$; Figure 1B). The latter, in turn, significantly correlated with platelet gp91^{phox} ($R_s=-0.515$, $P<0.001$; Figure 1C). IMT significantly correlated with FMD ($R_s=-0.303$, $P=0.008$), platelet gp91^{phox} ($R_s=0.398$, $P<0.001$), NOx ($R_s=-0.433$, $P<0.001$), and urinary excretion of 8-iso-PGF_{2 α} ($R_s=0.334$, $P=0.003$).

Finally, a substudy of age-matched X-CGD patients ($n=7$), healthy subjects ($n=7$), and obese patients ($n=7$) was performed to investigate platelet formation of NO and isopros-

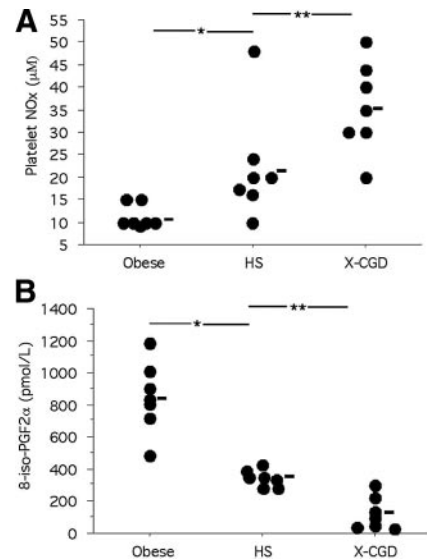


Figure 2. NOx generation (A) and isoprostane formation (B) in platelets stimulated with collagen (7 $\mu\text{g/mL}$) in X-CGD patients, healthy subjects (HS), and obese patients. Data are presented as box plots. * $P<0.005$; ** $P<0.05$.

tanes. The clinical characteristics of enrolled subjects did not differ from those of each subgroup study. After platelet stimulation with collagen, patients with X-CGD had significantly higher NO platelet production (35.5 ± 10.0 $\mu\text{mol/L}$, $P<0.05$), whereas obese individuals had lower NO production compared with healthy subjects (11.2 ± 2.5 $\mu\text{mol/L}$, $P<0.001$; Figure 2A). Moreover, 8-iso-PGF_{2 α} formation in platelets stimulated with collagen was lower in X-CGD patients (124.4 ± 104.8 pmol/L; $P<0.05$) and higher in obese patients (851.8 ± 222.8 pmol/L; $P<0.001$) compared with healthy subjects (373.4 ± 59.2 pmol/L; Figure 2B).

Discussion

This study provides the first evidence of an increased FMD in patients with hereditary deficiency of gp91^{phox}, suggesting a role for this ROS-generating pathway in modulating arterial tone. We also show that gp91^{phox} is relevant for the isoprostane formation, given that both systemic and cellular formation of isoprostanes is significantly reduced in gp91^{phox}-deficient patients.

Because NADPH oxidase is the most important cellular producer of superoxide anion,²² we investigated its role in the formation of oxidant species such as isoprostanes and ox-LDL. We found that only urinary excretion of isoprostanes was significantly reduced in X-CGD patients compared with healthy subjects. Isoprostanes are chemically stable free-radical catalyzed products of arachidonic acid.²³ So far, the ROS-generating pathway eliciting isoprostane formation has not been fully clarified. Here, we provide evidence that gp91^{phox} activation may play a pivotal role in the formation of isoprostanes because patients with gp91^{phox} knockout had significantly reduced urinary excretion of isoprostanes. Monocytes and platelets express gp91^{phox}^{24,25} and produce isoprostanes.²⁶ Therefore, it is conceivable that the reduced formation of isoprostanes reflects functional deficiency of

gp91^{phox} in these cells. Consistent with this suggestion was the significant reduced production of platelet isoprostanes from patients with hereditary gp91^{phox} deficiency. The contribution of gp91^{phox} by the cells from the artery wall is more complicated because they produce superoxide anion not only by gp91^{phox} but also by other NADPH oxidase homologs.²² Recent studies demonstrated, in fact, the existence of several NADPH oxidase homologues such as NOX1, NOX4, and NOX5 in the cells of the artery wall, including endothelial cells, smooth muscle cells, and adventitia cells.²²

The presence of normal ox-LDL levels in X-CGD suggests that other ROS-generating enzymes⁶ are involved in generating this marker of oxidative stress. An implication of this finding is that the increase in ox-LDL in obese patients should be attributed to other ROS-generating enzymes, which is in agreement with our previously published observations of a significant association between serum ox-LDL and myeloperoxidase, which suggested a role for this enzyme in generating ox-LDL *in vivo*.²⁷ Hence, in patients at risk of cardiovascular disease such as those with obesity, the coexistence of high values of different markers of oxidative stress suggests that >1 ROS-generating pathway is upregulated.

Several experimental studies have shown a pivotal role of NADPH oxidase in modulating arterial tone.⁷⁻⁹ In an animal model of NADPH oxidase knockout, an increased arterial dilation compared with wild-type animals was observed.^{8,9,28} Furthermore, individuals with impaired dilation show an overexpression of the NADPH oxidase subunit p47^{phox} in endothelial cells,²⁹ but a cause-effect relationship between FMD and NADPH oxidase activation was never demonstrated. The results achieved in our human knockout model of NADPH oxidase are consistent with animals studies in that X-CGD patients had a higher FMD compared with healthy subjects.

FMD is prevalently dependent on NO release from endothelium,¹² as also suggested by the significant correlation between FMD and plasma nitroso compounds.³⁰ Oxidative stress seems to play a pivotal role in modulating FMD, likely through altered NO bioavailability and biosynthesis.²¹ Accordingly, oxidative stress and FMD were inversely related in patients with or without metabolic syndrome, and a 1-time infusion of an antioxidant such as vitamin C was associated with rapid restoration of arterial function.³¹ In this study, NOx serum levels were higher in X-CGD patients and lower in obese individuals compared with healthy subjects. The direct correlation between NOx and FMD suggests a role for NO in the enhanced arterial dilatation observed in X-CGD patients. This suggestion is corroborated by the finding of a lower NOx production in obese patients who had, in accordance with a previous report,³⁰ reduced FMD. We recognize, however, that determination of NOx may be an unreliable method to measure NO generation. This may depend on the fact that NOx is influenced by many endogenous and exogenous factors, including dietary nitrate uptake, inhalation of atmospheric nitrogen oxides, salivary formation, and renal function.³² Even if we cannot exclude that such factors may have influenced NOx serum levels, platelet production of NO paralleled NOx in serum; thus, platelet generation of NOx

was increased in X-CGD, suggesting that cellular production of NO was upregulated.

A potentially intriguing observation is the striking difference in FMD between healthy subjects (8%) and X-CGD patients (15%). It is possible that during the evolution phase of human beings, maximization of innate immune defense mechanisms against infectious disease needed "upregulation" of NADPH oxidase. Because NADPH oxidase is also expressed in the vascular wall, this could result in higher vascular oxidative stress and ensuing lowering of the arterial relaxation capability.

IMT is a noninvasive diagnostic measure of atherosclerosis that correlates with histology and predicts cardiovascular events, including myocardial infarction and stroke.³³ IMT is already increased in children with cardiovascular risk factors, suggesting that it may be a good tool to measure premature atherosclerosis.³⁴ Previous studies have shown a direct correlation between oxidative stress and IMT, suggesting a role for oxidative stress in eliciting arterial damage.^{35,36} Indirect evidence in support of the role of NADPH oxidase in enhancing IMT has also been reported. In fact, in asymptomatic subjects free of overt atherosclerosis, phagocyte production of superoxide anion significantly correlated with IMT,³⁵ but direct evidence of NADPH activation was not provided. In our X-CGD population, in whom gp91^{phox} was functionally deficient, we observed a significant decrease in IMT compared with healthy subjects. However, these data should be considered cautiously because the difference was very small and may not firmly reflect a slower atherosclerotic progression in X-CGD patients.

This study has potential limitations. In particular, for ethical reasons, we did not have the possibility of discriminating whether the enhanced arterial dilatation was dependent on endothelium. Therefore, the role of NO in enhancing arterial dilatation was not fully elucidated. However, FMD is mostly dependent on endothelial release of NO.¹² As a result, it is possible that enhanced NO generation and/or bioactivity resulting from low oxidative stress may be responsible for the enhanced arterial dilatation of X-CGD patients. This hypothesis is consistent with our previous report in which the administration of *N*-nitro-*L*-arginine methyl ester, an inhibitor of endothelial NO synthase, to adult X-CGD patients abolished the increase in FMD.¹¹ Reduced isoprostane formation could be an alternative mechanism because isoprostanes are vasoconstrictor molecules,²³ although it has recently been argued that isoprostanes may have such property *in vivo*.³⁷

Despite the increase in NO generation, X-CGD patients had normal arterial diameter and blood pressure. A possible interpretation of this finding is that a resting increase in NO (33% versus control) was insufficient to change such parameters. Thus, it is of note that in the group with obesity and high blood pressure, NO generation was reduced by 65% compared with control subjects.

Even if in obese patients gp91^{phox} upregulation could contribute to lower FMD, it is possible that other mechanisms may contribute to lowering FMD in this setting. For instance, ox-LDL, which was not influenced by gp91^{phox}, could play a role because its increase may directly affect NO³⁸ and

isoprostanes³⁹; however, the relationship between ox-LDL and FMD is still unclear.^{40–42}

Finally, we must take into account the possibility that some antibiotics may favorably affect FMD either directly, as in the case of azithromycin,⁴³ or indirectly through reduced serum cholesterol levels.^{44,45} Reduced serum cholesterol levels, in fact, are known to inversely correlate with FMD,¹⁸ but this potential bias was eliminated in our study by the inclusion of healthy subjects with comparable serum cholesterol. Moreover, no data on the effects of the antibiotics used by our X-CGD patients (itraconazole, trimethoprim, and sulfamethoxazole) on FMD have been reported so far.

Conclusion

In this human knockout model of NADPH oxidase, arterial dilatation was enhanced, thus providing the first evidence that this ROS-generating pathway is implicated in modulating arterial tone.

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Disclosures

None.

References

- Yeboah J, Crouse JR, Hsu FC, Burke GL, Herrington DM. Brachial flow-mediated dilation predicts incident cardiovascular events in older adults: the Cardiovascular Health Study. *Circulation*. 2007;115:2390–2397.
- Perticone F, Ceravolo R, Pujia A, Ventura G, Iacopino S, Scozzafava A, Ferraro A, Chello M, Mastroberoberto P, Verdecchia P, Schillaci G. Prognostic significance of endothelial dysfunction in hypertensive patients. *Circulation*. 2001;104:191–196.
- Heitzer T, Schlinzig T, Krohn K, Meinertz T, Münzel T. Endothelial dysfunction, oxidative stress, and risk of cardiovascular events in patients with coronary artery disease. *Circulation*. 2001;104:2673–2678.
- Gokce N, Keaney JF Jr, Hunter LM, Watkins MT, Nedeljkovic ZS, Menzoian JO, Vita JA. Predictive value of noninvasively determined endothelial dysfunction for long-term cardiovascular events in patients with peripheral vascular disease. *J Am Coll Cardiol*. 2003;41:1769–1775.
- Cooke JP. Flow, NO, and atherogenesis. *Proc Natl Acad Sci U S A*. 2003;100:768–770.
- Förstermann U. Oxidative stress in vascular disease: causes, defense mechanisms and potential therapies. *Nat Clin Pract Cardiovasc Med*. 2008;5:338–349.
- Bendall JK, Rinze R, Adlam D, Tatham AL, de Bono J, Wilson N, Volpi E, Channon KM. Endothelial Nox2 overexpression potentiates vascular oxidative stress and hemodynamic response to angiotensin II: studies in endothelial-targeted Nox2 transgenic mice. *Circ Res*. 2007;100:1016–1025.
- Oelze M, Warnholtz A, Faulhaber J, Wenzel P, Kleschyov AL, Coldewey M, Hink U, Pongs O, Fleming I, Wassmann S, Meinertz T, Ehmke H, Daiber A, Münzel T. NADPH oxidase accounts for enhanced superoxide production and impaired endothelium-dependent smooth muscle relaxation in BKbeta1-/- mice. *Arterioscler Thromb Vasc Biol*. 2006;26:1753–1759.
- Jung O, Schreiber JG, Geiger H, Pedrazzini T, Busse R, Brandes RP. gp91^{phox}-containing NADPH oxidase mediates endothelial dysfunction in renovascular hypertension. *Circulation*. 2004;109:1795–1801.
- Martire B, Rondelli R, Soresina A, Pignata C, Broccoletti F, Finocchi A, Rossi P, Gattorno M, Rabusin A, Azzari C, Dellepiane RM, Pietrogrande MC, Trizzino A, Di Bartolomeo P, Martino S, Carpino L, Cossu F, Locatelli F, Maccario R, Pierani P, Putti MC, Stabile A, Notarangelo LD, Ugazio AG, Plebani A, De Mattia D, for IPINET. Clinical features, long-term follow-up and outcome of a large cohort of patients with Chronic Granulomatous Disease: an Italian multicenter study. *Clin Immunol*. 2008;126:155–164.
- Violi F, Sanguigni V, Loffredo L, Carnevale R, Buchetti B, Finocchi A, Tesaro M, Rossi P, Pignatelli P. Nox2 is determinant for ischemia-induced oxidative stress and arterial vasodilatation: a pilot study in patients with hereditary Nox2 deficiency. *Arterioscler Thromb Vasc Biol*. 2006;26:e131–e132.
- Joannides R, Haefeli WE, Linder L, Richard V, Bakkali EH, Thuillex C, Lüscher TF. Nitric oxide is responsible for flow-dependent dilatation of human peripheral conduit arteries in vivo. *Circulation*. 1995;91:1314–1319.
- Cole TJ, Bellizzi MC, Flegal KM, Dietz WH. Establishing a standard definition for child overweight and overweight worldwide: international survey. *BMJ*. 2000;320:1240–3.
- Overweight, obesity, and health risk: National Task Force on the Prevention and Treatment of Obesity. *Arch Intern Med*. 2000;160:898–904.
- Wang Z, Ciabattini G, Crémion C, Lawson J, Fitzgerald GA, Patrono C, MacLouf J. Immunological characterization of urinary 8-epi-prostaglandin F2 alpha excretion in man. *J Pharmacol Exp Ther*. 1995;275:94–100.
- Hoffman SW, Roof RL, Stein DG. A reliable and sensitive enzyme immunoassay method for measuring 8-isoprostaglandin F2 alpha: a marker for lipid peroxidation after experimental brain injury. *J Neurosci Methods*. 1996;68:133–136.
- Pignatelli P, Di Santo S, Buchetti B, Sanguigni V, Brunelli A, Violi F. Polyphenols enhance platelet nitric oxide by inhibiting protein kinase C-dependent NADPH oxidase activation: effect on platelet recruitment. *FASEB J*. 2006;20:1082–1089.
- Martino F, Loffredo L, Carnevale R, Sanguigni V, Martino E, Catasca E, Zanoni C, Pignatelli P, Violi F. Oxidative stress is associated with arterial dysfunction and enhanced intima-media thickness in children with hypercholesterolemia: potential role of NADPH oxidase. *Pediatrics*. 2008;122:e648–e655.
- Lee CY, Huang SH, Jenner AM, Halliwell B. Measurement of F2-isoprostanes, hydroxyeicosatetraenoic products, and oxysterols from a single plasma sample. *Free Radic Biol Med*. 2008;44:1314–1322.
- Corretti MC, Anderson TJ, Benjamin EJ, Celermajer D, Charbonneau F, Creager MA, Deanfield J, Drexler H, Gerhard-Herman M, Herrington D, Vallance P, Vita J, Vogel R. Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery: a report of the International Brachial Artery Reactivity Task Force. *J Am Coll Cardiol*. 2002;39:257–265.
- Loffredo L, Marzocchia A, Pignatelli P, Andreozzi P, Borgia MC, Cangemi R, Chiarotti F, Violi F. Oxidative-stress-mediated arterial dysfunction in patients with peripheral arterial disease. *Eur Heart J*. 2007;28:608–612.
- Cave AC, Brewer AC, Narayanapanicker A, Ray R, Grieve DJ, Walker S, Shah AM. NADPH oxidases in cardiovascular health and disease. *Antioxid Redox Signal*. 2006;8:691–728.
- Praticò D. Prostanoid and isoprostanoid pathways in atherogenesis. *Atherosclerosis*. 2008;201:8–16.
- Cathcart MK. Regulation of superoxide anion production by NADPH oxidase in monocytes/macrophages: contributions to atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2004;24:23–28.
- Pignatelli P, Sanguigni V, Lenti L, Ferro D, Finocchi A, Rossi P, Violi F. gp91^{phox}-Dependent expression of platelet CD40 ligand. *Circulation*. 2004;110:1326–1329.
- Patrono C, FitzGerald GA. Isoprostanes: potential markers of oxidant stress in atherothrombotic disease. *Arterioscler Thromb Vasc Biol*. 1997;17:2309–2315.
- Pignatelli P, Loffredo L, Martino F, Catasca E, Carnevale R, Zanoni C, Del Ben M, Antonini R, Basili S, Violi F. Myeloperoxidase overexpression in children with hypercholesterolemia. *Atherosclerosis*. 2009;205:239–243.
- Wolford RM, Stokes KY, Granger DN. CD4+ T lymphocytes mediate hypercholesterolemia-induced endothelial dysfunction via a NAD(P)H

- oxidase-dependent mechanism. *Am J Physiol Heart Circ Physiol*. 2008;294:H2619–H2626.
29. Donato AJ, Eskurza I, Silver AE, Levy AS, Pierce GL, Gates PE, Seals DR. Direct evidence of endothelial oxidative stress with aging in humans: relation to impaired endothelium-dependent dilation and upregulation of nuclear factor-kappaB. *Circ Res*. 2007;100:1659–1666.
 30. Heiss C, Lauer T, Dejam A, Kleinbongard P, Hamada S, Rassaf T, Matern S, Feelisch M, Kelm M. Plasma nitroso compounds are decreased in patients with endothelial dysfunction. *J Am Coll Cardiol*. 2006;47:573–579.
 31. Cangemi R, Angelico F, Loffredo L, Del Ben M, Pignatelli P, Martini A, Violi F. Oxidative stress-mediated arterial dysfunction in patients with metabolic syndrome: effect of ascorbic acid. *Free Radic Biol Med*. 2007;43:853–859.
 32. Szabó C, Day BJ, Salzman AL. Evaluation of the relative contribution of nitric oxide and peroxynitrite to the suppression of mitochondrial respiration in immunostimulated macrophages using a manganese mesoporphyrin superoxide dismutase mimetic and peroxynitrite scavenger. *FEBS Lett*. 1996;381:82–86.
 33. Lorenz MW, Markus HS, Bots ML, Rosvall M, Sitzer M. Prediction of clinical cardiovascular events with carotid intima-media thickness: a systematic review and meta-analysis. *Circulation*. 2007;115:459–467.
 34. Järvisalo MJ, Jartti L, Näntö-Salonen K, Irjala K, Rönnemaa T, Hartiala JJ, Celermajer DS, Raitakari OT. Increased aortic intima-media thickness: a marker of preclinical atherosclerosis in high-risk children. *Circulation*. 2001;104:2943–2947.
 35. Zalba G, Beloqui O, San José G, Moreno MU, Fortuño A, Díez J. NADPH oxidase-dependent superoxide production is associated with carotid intima-media thickness in subjects free of clinical atherosclerotic disease. *Arterioscler Thromb Vasc Biol*. 2005;25:1452–1457.
 36. Ashfaq S, Abramson JL, Jones DP, Rhodes SD, Weintraub WS, Hooper WC, Vaccarino V, Harrison DG, Quyyumi AA. The relationship between plasma levels of oxidized and reduced thiols and early atherosclerosis in healthy adults. *J Am Coll Cardiol*. 2006;47:1005–1011.
 37. Greaves K, Dixon SR, Coker IO, Mallet AI, Avkiran M, Shattock MJ, Fejka MJ, O'Neill WW, Senior R, Redwood S, Marber MS. Influence of isoprostane F2alpha-III on reflow after myocardial infarction. *Eur Heart J*. 2004;25:847–853.
 38. Chikani G, Zhu W, Smart EJ. Lipids: potential regulators of nitric oxide generation. *Am J Physiol Endocrinol Metab*. 2004;287:E386–E389.
 39. Liu ML, Ylitalo K, Salonen R, Salonen JT, Taskinen MR. Circulating oxidized low-density lipoprotein and its association with carotid intima-media thickness in asymptomatic members of familial combined hyperlipidemia families. *Arterioscler Thromb Vasc Biol*. 2004;24:1492–1497.
 40. van der Zwan LP, Teerlink T, Dekker JM, Henry RM, Stehouwer CD, Jakobs C, Heine RJ, Scheffer PG. Circulating oxidized LDL: determinants and association with brachial flow-mediated dilation. *J Lipid Res*. 2009;50:342–349.
 41. Järvisalo MJ, Lehtimäki T, Raitakari OT. Determinants of arterial nitrate-mediated dilatation in children: role of oxidized low-density lipoprotein, endothelial function, and carotid intima-media thickness. *Circulation*. 2004;109:2885–2889.
 42. Skyrme-Jones RA, O'Brien RC, Luo M, Meredith IT. Endothelial vasodilator function is related to low-density lipoprotein particle size and low-density lipoprotein vitamin E content in type 1 diabetes. *J Am Coll Cardiol*. 2000;35:292–299.
 43. Parchure N, Zouridakis EG, Kaski JC. Effect of azithromycin treatment on endothelial function in patients with coronary artery disease and evidence of *Chlamydia pneumoniae* infection. *Circulation*. 2002;105:1298–1303.
 44. Jenkins DJ, Kendall CW, Hamidi M, Vidgen E, Faulkner D, Parker T, Irani N, Wolever TM, Fong I, Kopplin P, Connelly PW, Onderdonk A, Rao AV. Effect of antibiotics as cholesterol-lowering agents. *Metabolism*. 2005;54:103–112.
 45. Schneider B, Gerdson R, Plat J, Dullens S, Björkhem I, Diczfalusy U, Neuvonen PJ, Bieber T, von Bergmann K, Lütjohann D. Effects of high-dose itraconazole treatment on lipoproteins in men. *Int J Clin Pharmacol Ther*. 2007;45:377–384.

CLINICAL PERSPECTIVE



Arterial dysfunction is a hallmark of early atherosclerosis and predicts cardiovascular events. The mechanisms accounting for arterial dysfunction in humans are still unclear. NADPH oxidase is an enzyme that plays a major role in producing reactive oxidant species into the cells. Reactive oxidant species formation is associated with inhibition of nitric oxide activity and/or biosynthesis and may have deleterious effects in arterial function. We conducted a multicenter study to measure flow-mediated dilation in 25 patients with chronic granulomatous disease (X-linked CGD) that is characterized by hereditary deficiency of gp91^{phox}, the catalytic subunits of NADPH oxidase. Compared with 25 healthy subjects and 25 obese patients, X-CGD patients had lower oxidative stress, as assessed by urinary excretion of isoprostanes, and higher flow-mediated dilation. Gp91^{phox} expression increased progressively from X-CGD to obese patients, who showed lower flow-mediated dilation compared with the other 2 groups. In the entire cohort, gp91^{phox} expression inversely correlated with flow-mediated dilation. In a subgroup study, platelet formation of nitric oxide was also determined. This analysis showed a progressive decrease in nitric oxide from X-CGD to obese patients. Together, these findings suggest that vascular tone is modulated by NADPH oxidase—generating reactive oxidant species with a mechanism involving nitric oxide generation. Systemic and cellular measurement of NADPH oxidase expression could have a relevant impact on our understanding of the role of reactive oxidant species in the initiation and progression of atherosclerosis. This could be a useful tool for exploring whether inhibition of NADPH oxidase is able to halt the atherosclerotic process and its clinical sequelae.