

Different Degrees of NADPH Oxidase 2 Regulation and In Vivo Platelet Activation: Lesson From Chronic Granulomatous Disease

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Background—In vitro study showed that NADPH oxidase (NOx), the most important enzyme producing reactive oxygen species (ROS), plays a role in the process of platelet activation. However, it is unclear if changes in its activity affect platelet activation in vivo.

Methods and Results—In vivo and ex vivo experiments assessing platelet activation were investigated in healthy subjects, obese patients, and subjects with different low rates of NOx2 activity, namely X-linked chronic granulomatous disease (X-CGD) patients and X-CGD carriers. We included 27 X-CGD patients, 31 women carriers of hereditary deficiency of NOx2, 31 obese women, and 62 healthy subjects matched for sex and age. Plasma levels of soluble sCD40 L (sCD40L) and soluble P (sP)-selectin, 2 markers of in vivo platelet activation, were reduced in X-CGD patients (sCD40L=−55%; sP-selectin=−51%, $P<0.001$) and in X-CGD carriers (sCD40L=−41%; sP-selectin=−57%, $P<0.001$) compared with respective controls. Conversely, obese women, who disclosed NOx2 upregulation, had significantly higher plasma levels of sCD40L (+47%, $P<0.001$) and sP-selectin (+70%, $P<0.001$) compared with controls. Ex vivo study showed platelet isoprostane downexpression and enhanced platelet NO generation in both X-CGD patients and X-CGD carriers compared with controls; opposite findings were observed in obese patients. Correlation analysis showed that platelet NOx2 regulation was directly associated with plasma levels of sCD40L ($R=0.336$, $P<0.001$) and sP-selectin ($R=0.441$; $P<0.001$).

Conclusions—The study provides the first evidence that in vivo platelet activation is significantly and directly associated with NOx2 activity. Platelet NOx2 may be a novel target for platelet activation inhibition. (*J Am Heart Assoc.* 2014;3:e000920 doi: 10.1161/JAHA.114.000920)

Key Words: NOx2 • oxidative stress • platelet activation • X-CGD

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Reactive oxygen species (ROS) are chemically unstable molecules that rapidly react with other molecules, inducing the formation of oxidized products such as oxidized low-density lipoprotein, peroxynitrite, or protein adducts.^{1,2} ROS serve as second messengers at physiologic concentration and, as such, they behave as intracellular signals for cell activation.¹ Among the cells, platelets represent a typical example of ROS involvement in the activation process.³ Thus, on stimulation by common agonists, platelets produce several types of ROS such as superoxide anion (O_2^-) or hydrogen peroxide (H_2O_2), which in turn contribute to the propagation of platelet aggregation.⁴ There are several enzymatic pathways that elicit the formation of ROS into the cells, including NADPH oxidase (NOx), myeloperoxidase, xanthine oxidase, and uncoupled nitric oxide synthase. Amid these, experimental and clinical evidence focused on the role of NOx as the key enzyme in producing ROS and eventually eliciting platelet activation.^{5–7} In particular, Krotz et al^{8,9} demonstrated that NOx-dependent platelet O_2^- formation enhances platelet aggregation and platelet-dependent thrombosis. In accordance with this finding,

we observed that in subjects with hereditary deficiency of NOx2, the catalytic subunit of NOx, an impaired O₂⁻ formation occurred. Also, agonist-induced platelet aggregation was reduced, which further supports the role of O₂⁻ in eliciting platelet activation.⁶ It is unclear, however, if an interplay between NOx2 activity and platelet activation does exist in vivo. To explore this issue, we examined markers of in vivo platelet activation, such as soluble CD40L (sCD40L) and soluble P-selectin (sP-s), in subjects with different degrees of NOx2 activity including youths with X-linked chronic granulomatous disease (X-CGD), adult carriers of NOx2 hereditary deficiency, healthy subjects (HS), and obese subjects, who are known to disclose NOx2 upregulation.^{10–12}

Methods

Study Population

We conducted a multicentre study in collaboration with the Italian Primary Immunodeficiency Network. Among the 60 patients with CGD registered in the national database,¹³ 28 were not included in the study due to the presence of acute infections or critical physical conditions or unwillingness to participate in the study; 5 patients with p47^{phox} hereditary deficiency were excluded. The remaining 27, who were X-CGD patients were included; 31 age-matched HS were also included. Furthermore, among the female relatives of the 60 CGD patients registered in the national database,¹³ we studied 31 female carriers of X-CGD who were willing to participate in the study. The group of carriers was composed of 23 mothers, 3 grandmothers, and 5 sisters of X-CGD patients.

Diagnosis of X-CGD was performed as previously described.¹³ All X-CGD patients were under treatment with itraconazole, trimethoprim, and sulfamethoxazole.

Thirty-one women matched for age and atherosclerotic risk factors were screened from routine visits and used as controls. Further, we included 31 age-matched obese female subjects; body mass index (BMI) ≥ 30 kg/m² was used as a cut-off to define *obesity*. In 2 female subjects, aged 9 and 10 years, *obesity* was defined as a BMI ≥ 95 th percentile.¹⁴ Controls and obese patients were recruited from the outpatient clinic of our division at the “I Clinica Medica” of the Sapienza University of Rome. None of the patients included in the study had a clinical history complicated by thrombotic events and were taking antiplatelet drugs during the month before blood sampling.

HS matched for sex, age, and atherosclerotic risk factors were screened from routine visits. 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, deep venous thrombosis; were in treatment with statins or antioxidant vitamins; or were a current smoker.

The study was approved by the Ethical Committee of Sapienza University. Each subject enrolled gave informed consent to participate in the study.

Blood Sampling

After overnight fasting (12 hours) and supine rest for at least 10 minutes, blood samples were collected in vacutainers between 8 and 9 AM (Vacutainer Systems, Belliver Industrial Estate) and centrifuged at 300g for 10 minutes to obtain supernatant, which was stored at -80°C until use.

Total cholesterol was measured according to routine methods using an enzymatic colorimetric method on a Dimension RXL apparatus (Dade Behring AG).

Platelet Preparation

To obtain platelet-rich plasma, blood samples mixed with 3.8% NA citrate (ratio 9:1) were centrifuged for 15 minutes at 180g. To avoid leukocyte contamination, only the top 75% of the platelet-rich plasma was collected according to Pignatelli et al.⁶

Platelet pellets were obtained via centrifugation (10 minutes, 300g) of platelet-rich plasma. Acid citrate–dextrose (1:7 v/v) was added to avoid platelet activation during processing; samples were suspended in HEPES buffer in presence of 0.1% albumin, pH 7.35 (2×10^8 /mL). Supernatant was separated from cells and stored until analysis.

Platelet 8-Iso-Prostaglandin F2 α Assays

Concentration of 8-iso-prostaglandin (PG)F2 α in supernatant of platelet was measured by using a previously described and validated enzyme immunoassay method (Tema Ricerca)^{15,16} and expressed as pmol/L. Intra-assay and interassay coefficients of variation were 4.4% and 8.8% respectively.

Platelet Soluble NOx2-Derived Peptide

Platelet soluble NOx2-derived peptide (sNOx2-dp), a marker of NADPH oxidase activation, was detected in platelets supernatant by using the ELISA method as previously described by Pignatelli et al.¹⁷ The peptide was recognized by the specific monoclonal antibody against the amino acidic sequence (224 to 268) of the extramembrane portion of NOx2. Values were expressed as pg/mL, and intra-assay and interassay coefficients of variation were 5.2% and 6%, respectively, for serum and platelets.

Platelets NOx Measurement

A colorimetric assay kit (Tema Ricerca, Italy) was used to determine the nitric oxide metabolites nitrite and nitrate

(NOx) in the supernatant of platelet. Intra-assay and inter-assay coefficients of variation were 2.9% and 1.7%, respectively.

Plasma and Platelet Levels of sCD40L and sP-selectin

Plasma and platelet levels of sCD40L and sP-selectin were measured with use of a commercial immunoassay (Tema Ricerca). Intra-assay and interassay coefficients of variation were 5% and 7% for sCD40L and 4.3% and 6.1% for sP-selectin, respectively.

Statistical Analysis

Data are presented as mean±SD unless indicated otherwise. Categorical variables were reported as counts (percentage); independence of categorical variables was tested by using χ^2 test. The correlation analysis was carried out via Pearson correlation test. We performed an ANOVA to compare means across the groups; post-hoc analysis was performed with Tukey’s test. Results were further confirmed by nonparametric tests (Mann–Whitney for pairwise comparisons and Kruskal–Wallis test for all groups in 1 analysis). Statistical significance was defined at $P<0.05$. Statistical analysis was performed with SPSS 18.0 for Windows (SPSS Inc).

For sample size determination, on the basis of the data emerging from previous studies,^{10,11} we computed the minimum sample size with respect to a 2-sample Student *t* test, considering (1) relevant difference for plasma CD40L variation to be detected between the X-CGD patients and controls; (2) standard deviations to be homogeneous between groups 16; and (3) type I error probability $\alpha=0.05$ and power

$1-\beta=0.90$. This resulted in a minimum sample size of 19 subjects for each group. Sample size calculations was performed using the software nQuery Advisor®, version 5.0 (Statistical Solutions, Saugus, MA).

Results

Clinical characteristics of the 5 groups, which include X-CGD patients, X-CGD carriers, children and adult controls, and obese subjects (n=151), were reported in Tables 1 and 2. No significant difference in medications was detected among the groups (Table 2).

There were no differences in terms of age, sex, and risk factors of atherosclerosis between X-CGD, X-CGD carriers, and the respective controls (Tables 1 and 2). As expected, BMI was significantly higher in obese subjects compared with the other groups (Table 2).

Compared with young HS, plasma levels of sCD40L and sP-selectin were reduced in X-CGD patients (−55%, $P<0.001$, and −51%, $P<0.001$, respectively) (Table 1 and Figure 1). Compared with adults controls, X-CGD carriers had lower plasma levels of sCD40L and sP-selectin (−46% and −57%, respectively, $P<0.001$) (Table 2 and Figure 1). Also, compared with controls, obese women had higher plasma levels of sCD40L and sP-selectin (+47% and +70%, respectively, $P<0.001$) (Table 2 and Figure 1).

A correlation analysis in the overall population showed that platelet sNOx2-dp correlated with platelet 8-iso-PGF2 α ($r=0.584$, $P<0.001$), platelet NOx ($r=-0.320$, $P<0.001$), plasma sP-selectin ($r=0.336$, $P<0.001$), sCD40L ($r=0.441$, $P<0.001$), total cholesterol ($r=0.275$, $P=0.001$), age ($r=0.176$,

Table 1. Clinical Characteristics of X-Chronic Granulomatous Disease (X-CGD) Patients and Controls

	X-CGD (n=27)	Controls (n=31)	P Value
Age, y	16.3±9.2	16.6±8.4	1.0
Gender	27 males	31 males	n.s.
Systolic blood pressure, mm Hg	110±11	108±8	1.0
Diastolic blood pressure, mm Hg	68±8	70±8	1.0
BMI	18.3±2.9	19.8±2.9	1.0
Total-cholesterol, mg/dL	128.8±20.7	138.0±15.6	1.0
Plasma sP-selectin, ng/mL	6.9±4.0	13.5±6.4	<0.001
Plasma sCD40L, ng/mL	14.4±9.0	33.2±15.6	<0.001
Platelet sP-selectin, ng/mL	10.4±3.7	14.2±2.6	<0.001
Platelet sCD40L, ng/mL	1.4±0.2	4.2±1.5	<0.001
Platelet sNOx2-dp, pg/mL	5.3±1.5	13.6±4.8	<0.001
Platelet 8-iso-PGF2 α , pmol/L	45.7±35.0	79.8±18.3	<0.001
Platelet NOx, μ mol/L	31.7±19.3	13.1±8.8	<0.001

BMI indicates body mass index; NOx, NADPH oxidase.

Table 2. Clinical Characteristics of X-Chronic Granulomatous Disease (X-CGD) Carriers, Controls and Obese Patients

	X-CGD Carriers (n=31)	P Value*	Controls (n=31)	P Value*	Obese Patients (n=31)
Age, y	41.6±14.3	1.0	41.2±14.3	1.0	42.6±13.7
Gender	31 females	n.s.	31 females	n.s.	31 females
Systolic blood pressure, mm Hg	116±6	1.0	117±7	0.05	120±5
Diastolic blood pressure, mm Hg	72±8	1.0	72±10	0.573	75±7
BMI	24.2±4.3	1.0	24.8±5.7	0.001	32.5±4.8
Total-cholesterol, mg/dL	199.8±79.7	1.0	196.7±88.7	0.076	244.9±81.3
Current smokers	11/31	1.0	11/31	1.0	10/31
Hypertension	4/31	1.0	4/31	0.919	5/31
Hypercholesterolemia	0/31	0.472	2/31	0.256	6/31
Type 2 diabetes mellitus	0/31	1.0	1/31	0.351	4/31
Plasma sP-selectin, ng/mL	13.9±6.8	<0.001	32.0±6.4	<0.001	54.5±25.0
Plasma sCD40L, ng/mL	34.5±10.4	<0.001	57.7±25.4	<0.001	84.9±28.0
Platelet sP-selectin, ng/mL	14.0±6.6	<0.001	23.2±6.4	<0.001	42.5±25.0
PlateletsCD40L, ng/mL	3.3±1.1	<0.001	6.1±1.7	<0.001	16.0±2.9
Platelet sNOx2-dp, pg/mL	6.9±3.5	0.04	13.6±9.3	<0.001	27.1±19.3
Platelet 8-iso-PGF2α, pmol/L	62.9±33.6	<0.001	114.5±50.2	<0.001	245.3±50
Platelet NOx, μmol/L	20.7±7.7	0.04	14.4±5.5	0.04	7.3±3.7
<i>Drugs</i>					
Statin	0/31	0.472	2/31	0.256	6/31
Angiotensin-converting enzyme inhibitors	4/31	1.0	4/31	1.0	5/31
Corticosteroid therapy	2/31	1.0	1/31	1.0	0/31
Methotrexate	1/31	1.0	1/31	1.0	0/31
Hydroxychloroquine	2/31	0.472	0/31	1.0	0/31

BMI indicates body mass index; NOx, NADPH oxidase.
*Compared with controls.

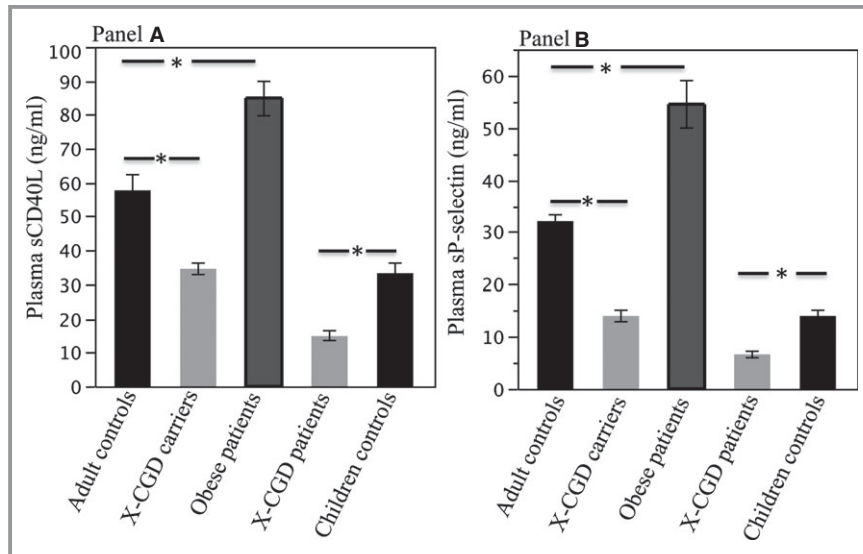


Figure 1. Plasma sCD40L (A), and plasma sP-selectin (B) levels in adult controls, X-CGD carriers, obese patients, X-CGD patients and children controls (* $P<0.001$, ** $P<0.05$). X-CGD indicates X-linked chronic granulomatous disease.

$P=0.031$), BMI ($r=0.393$, $P<0.001$), systolic blood pressure ($r=0.210$, $P=0.01$), and diastolic blood pressure ($r=0.204$, $P=0.01$).

Ex Vivo Study

In young subjects, X-CGD disclosed less oxidative stress, as assessed by platelet sNOx2-dp release and 8-iso-PGF2 α (-62% and -43% , respectively, $P<0.001$), compared with HS (Table 1 and Figure 2). Furthermore, NO generation, as assessed by platelet NOx, was significantly higher in X-CGD patients ($+140\%$, $P<0.001$) compared with young HS (Table 1 and Figure 2).

In adults, platelet sNOx2-dp release and 8-iso-PGF2 α were different among the 3 groups (Table 2 and Figure 3). Thus, compared with controls, X-CGD carriers had lower levels of

platelet sNOx2-dp (-50% , $P=0.04$) and 8-iso-PGF2 α (-46% , $P<0.001$) (Table 2 and Figure 3). Also, compared with controls, obese women had higher levels of platelet sNOx2-dp ($+99\%$, $P<0.001$) and 8-iso-PGF2 α ($+114\%$, $P<0.001$) (Table 2 and Figure 3A and 3B). Platelet NOx differed in the 3 groups. Thus, compared with controls, X-CGD carriers and obese patients had significantly higher ($+44\%$, $P=0.04$) and lower (-50% , $P=0.04$) levels of platelet NOx, respectively (Table 2 and Figure 3C).

Compared with young HS, levels of platelet sCD40L and sP-selectin were reduced in X-CGD patients (-67% and -27% , respectively, $P<0.001$) (Table 2 and Figure 4). Compared with adult controls, X-CGD carriers had lower levels of platelet sCD40L and sP-selectin (-40% , $P<0.001$, and -46% , $P<0.001$, respectively) (Table 2 and Figure 4). Also, compared with controls, obese women had higher levels of platelet

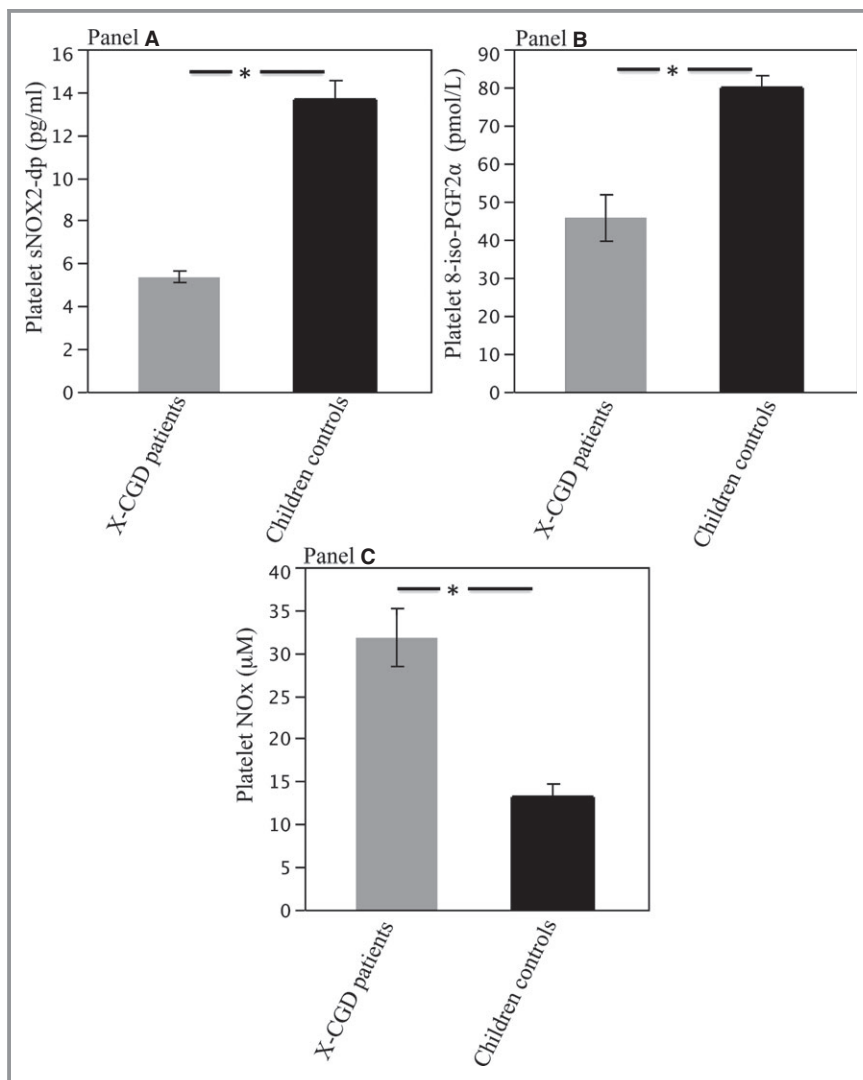


Figure 2. Platelet sNOx2-dp release (A), platelet 8-iso-PGF2 α formation (B) and platelet NOx production in X-CGD patients and children controls (* $P<0.001$, ** $P<0.05$). NOx indicates NADPH oxidase; X-CGD, X-linked chronic granulomatous disease.

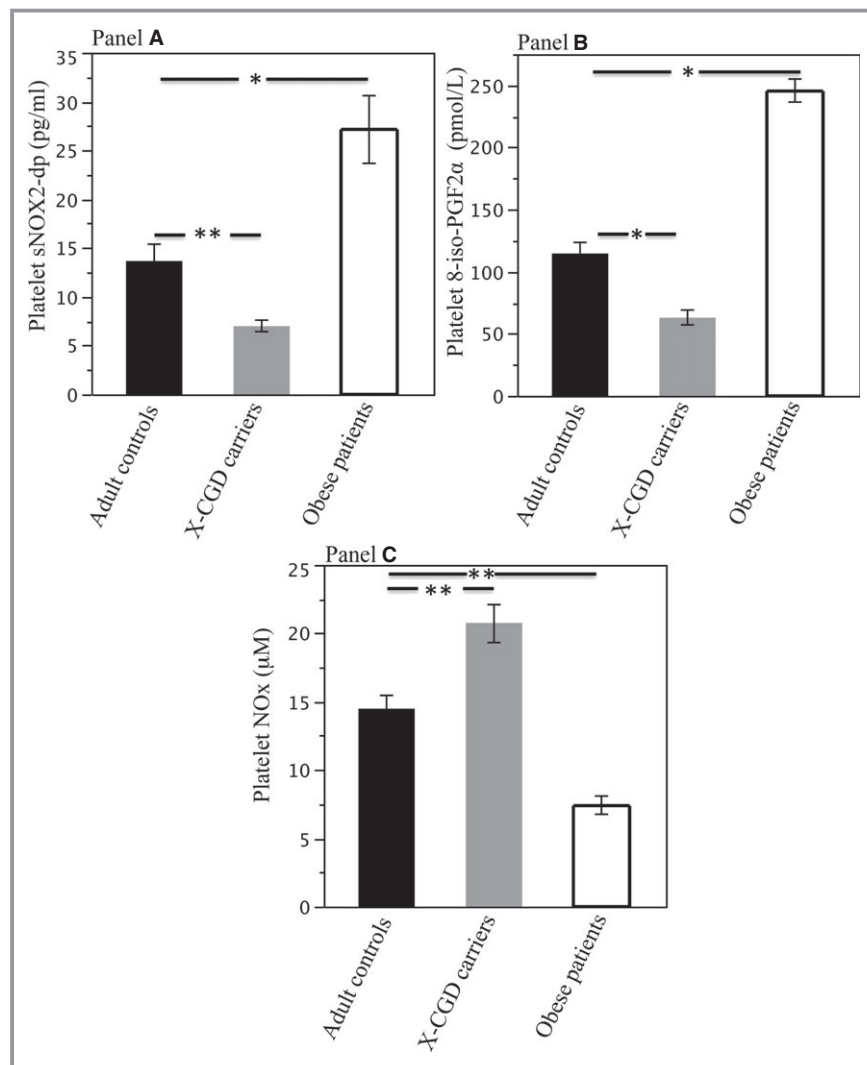


Figure 3. Platelet sNOx2-dp release (A), platelet 8-iso-PGF2 α formation (B) and platelet NOx production in adult controls, X-CGD carriers, and obese patients (* P <0.001, ** P <0.05). NOx indicates NADPH oxidase; X-CGD, X-linked chronic granulomatous disease.

sCD40L and sP-selectin (+162% and +83%, respectively, P <0.001).

Discussion

The present study reports the first evidence that NOx2 activity is significantly associated with platelet activation in vivo. In particular, we demonstrated that plasma levels of sCD40L and sP-selectin were reduced in subjects with X-CGD affected by complete or partial NOx2 deficiency and increased in obese subjects, who disclosed NOx2 upregulation. Experimental and clinical studies consistently showed that platelet NOx2 is implicated in platelet activation. In an experimental model of platelet-dependent thrombosis, Dayal et al⁵ demonstrated that aged animals are characterized by platelet H₂O₂, which was associated with higher tendency to thrombosis; such

prothrombotic change was significantly impaired by treatment with apocynin, which inhibits p47phox assembly to NOx2 on platelet surface.⁵ The interplay between platelet hyperactivity and NOx2 was corroborated by the fact that H₂O₂ overproduction was associated with enhanced levels of mRNA for the catalytic subunit NOx2 and the cytosolic subunit p47phox.⁵ Consistent with these findings, experimental research in animals demonstrated that apocynin also impairs platelet function in vivo and that NOx2 activation is therefore implicated in platelet activation. Thus, a molecular image of mice prone to atherosclerosis and treated with or without apocynin demonstrated enhanced platelet adhesion to atherosclerotic plaque in untreated animals, an effect that was dose-dependently abrogated in animals treated with apocynin.⁵ That NOx2 is relevant for platelet activation was also corroborated by investigating platelet activation in patients with X-CGD, whose platelets disclosed severe but not

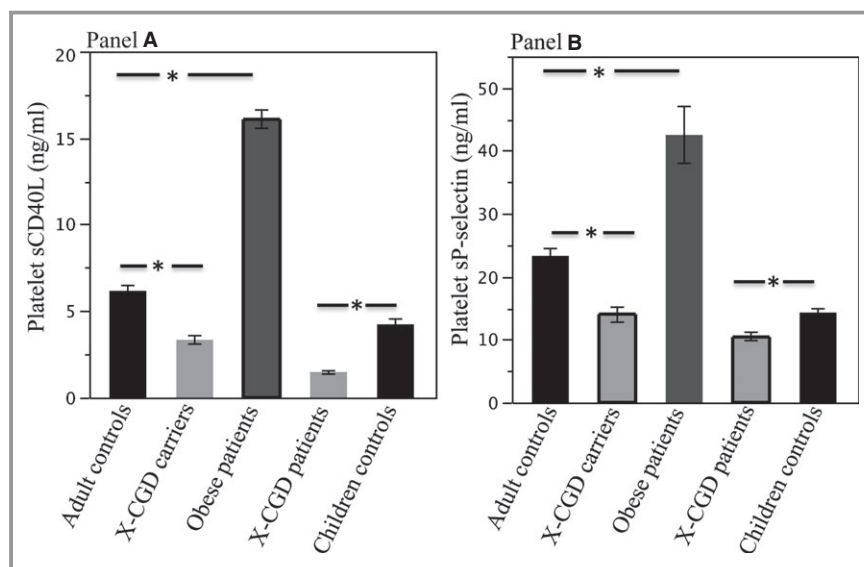


Figure 4. Platelet sCD40L (A), and platelet sP-selectin (B) levels in adult controls, X-CGD carriers, obese patients, X-CGD patients and children controls (* $P < 0.001$, ** $P < 0.05$). X-CGD indicates X-linked chronic granulomatous disease.

complete suppression of platelet O_2^- as a consequence of the hereditary deficiency of NOx2 along with impaired ex vivo aggregation.⁶ In particular, on activation, platelets from X-CGD patients disclosed impaired release of CD40L; lowered formation of isoprostanes, which are chemically stable eicosanoids with proaggregating property; and reduced platelet recruitment.⁶

The impact of NOx2 regulation on markers of in vivo platelet activation has never been explored. We decided to investigate in vivo platelet activation in X-CGD patients and in the X-CGD patients' mothers, who are carriers of NOx2 deficiency. Both represent a unique model of complete or partial deficiency of NOx2 and can give the opportunity to assess the interplay, if any, between the rate of NOx2 activity and in vivo platelet activation. To investigate this issue, we measured 2 biomarkers, namely sCD40L and sP-selectin, which are established markers of in vivo platelet activation.¹⁸ A significant reduction in both markers was detected in X-CGD and NOx2 carriers compared with their respective controls; however, no difference in the inhibition rate of the 2 biomarkers was detected between X-CGD patients and X-CGD carriers. This finding was corroborated by analysis of in vivo platelet activation in obese subjects who, conversely, disclosed NOx2 upregulation coincidentally with a significant increase of both sCD40L and sP-selectin. This is consistent with previous studies showing upregulation of NOx2 in patients with atherosclerotic risk factors such as those with hypercholesterolemia^{19,20} or diabetes.²¹

To substantiate these in vivo data, we performed ex vivo experiments in which the expression of CD40L and P-selectin was measured in subjects with different degrees of NOx2

regulation. Consistent with in vivo study, platelet expression of both CD40L and P-selectin was significantly reduced in both X-CGD and NOx2 deficiency carriers compared with the respective controls. Such reduction was coincident with impaired platelet isoprostane production, which is likely to reflect the reduced formation of ROS in X-CGD and NOx2 deficient carriers and with enhanced platelet NO generation.⁷ In contrast to these findings, obese patients disclosed significantly higher expression of platelet CD40L and P-selectin along with a higher production of platelet isoprostanes, further corroborating the concept of a direct interplay between NOx2 regulation and in vivo platelet activation.

There are at least 2 mechanisms that may potentially account for NOx2-dependent platelet activation. Thus, ROS generation by NOx2 rapidly inactivate NO, which is a powerful antiplatelet molecule.⁶ On the other hand, ROS generated by NOx2 may interact with arachidonic acid to give formation of isoprostanes, which are proaggregating molecules contributing to propagate platelet aggregation.²² Consistent with this, NOx2 downregulation was associated with impaired isoprostane formation and enhanced NO generation.

As a limitation of the study, we must take into account the possibility that some antibiotics such as beta-lactams²² may inhibit platelet activation; however, this potential bias can be reasonably excluded because inhibition of platelet activation was also detected in X-CGD carriers, who were not taking antibiotics.

The study has pharmacologic implications. Thus, the direct association between NOx2 activity and in vivo platelet activation suggests that downregulation of NOx2 activation may represent a novel tool to inhibit platelet activation.

This potentially interesting new therapeutic option should be, however, wisely considered as NOx2 has a key role in the innate immune system and its downregulation could be associated with a higher risk of infectious disease. Thus, the extent to which NOx2 may be reduced without interfering with the activity of innate immune system is a critical issue that should be taken into account. Some interesting clues on how developing a drug that inhibits platelet activation without interfering with the innate immune system are provided by the present study showing that female carriers of NOx2 hereditary deficiency have lower platelet activation compared with controls without suffering from bleeding complications or serious infections.¹¹ This would imply that up to 50% NOx2 activity lowering may be relatively well tolerated without serious clinical consequence. Inhibition of NOx2 activity without directly interfering with NOx2 may be another option. Thus, experimental studies with apocynin, which inhibits p47phox translocation to membrane NOx2, has been shown to reduce platelet activation in vivo and in vitro.^{5,17} However, apocynin has been studied only in experimental models of atherosclerosis so far.

Experimental and clinical studies have shown that oxidative stress is implicated in clotting system and platelet activation; therefore, its inhibition may reduce thrombosis-related vascular disease.²³ However, the results of interventional trials with vitamin E alone and/or in combination with other antioxidant vitamins provided inconclusive findings.²⁴ In addition to several factors that may have biased such results,²⁵ it may be possible that downstream inhibition of oxidant species, as exerted by antioxidant vitamins, does not represent an ideal approach to prevent oxidative stress-related cellular damage. The present study suggests that upstream inhibition of oxidative stress by targeting precise cellular oxidant pathways such as NOx2 or other prooxidant enzymatic pathways may represent an alternative option not only to inhibit platelet activation but also to retard atherosclerotic progression.

In conclusion, we demonstrate that NOx2-derived platelet ROS formation is implicated in platelet activation ex vivo and in vivo with a mechanism involving NO and isoprostane generation. Downregulation of NOx2 activation may represent a new tool for platelet activation inhibition.

Author Contributions

R. Carnevale and P. Pignatelli performed the experiments and conceived the study and designed research, analyzed and interpreted data, and wrote the manuscript; Sanguigni V., Plebani A., Rossi P., Pignata C., Martire B., Finocchi A., Pietrogrande MC., Martino S., Azzari C., Soresina A.R., Cirillo E., Martino F., N. patients enrollment; Loffredo L., analyzed and

interpreted data and wrote the manuscript; Violi F. conceived the study and designed research and wrote the manuscript.

Disclosures

None.

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