Inherited Human gp91phox Deficiency Is Associated With Impaired Isoprostane Formation and Platelet Dysfunction

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Object—Platelet isoprostane 8-ISO-prostaglandin F2α (8-iso-PGF2α), a proaggregating molecule, is believed to derive from nonenzymatic oxidation of arachidonic acid. We hypothesized that NADPH is implicated in isoprostane formation and platelet activation.

Methods and Results—We studied 8-iso-PGF2α in platelets from 8 male patients with hereditary deficiency of gp91phox, the catalytic subunit of NADPH oxidase, and 8 male controls. On stimulation, platelets from controls produced 8-iso-PGF2α, which was inhibited ~8% by aspirin and ~58% by a specific inhibitor of gp91phox. Platelets from patients with gp91phox hereditary deficiency had normal thromboxane A2 formation but marked 8-iso-PGF2α reduction compared with controls. In normal platelets incubated with a gp91phox inhibitor or with SQ29548, a thromboxane A2/isoprostane receptor inhibitor, platelet recruitment, an in vitro model of thrombus growth, was reduced by 44% and 64%, respectively; a lower effect (~17%) was seen with aspirin. Moreover, thrombus formation under shear stress (blood perfusion at the wall shear rate of 1500 s⁻¹) was reduced in samples in which isoprostane formation was inhibited by NADPH oxidase inhibitors. In gp91phox-deficient patients, agonist-induced platelet aggregation was within the normal range, whereas platelet recruitment was reduced compared with controls. Incubation of platelets from gp91phox-deficient patients with 8-iso-PGF2α dose-dependently (1 to 100 pmol/L) increased platelet recruitment by mobilizing platelet Ca²⁺ and activating gpIIb/IIIa; a further increase in platelet recruitment was detected by platelet coincubation with L-NAME, an inhibitor of NO synthase.

Conclusion—This study provides the first evidence that platelet 8-iso-PGF2α maximally derives from gp91phox activation and contributes to platelet recruitment via activation of gpIIb/IIIa. (Arterioscler Thromb Vase Biol. 2011;31:423-434.)

Key Words: platelets ■ reactive oxygen species ■ NADPH oxidase ■ recruitment ■ isoprostanes ■ oxidative stress

Platelets play a key role in the process of atherothrombosis via release of inflammatory and prothrombotic molecules. Thus, platelets produce oxygen free radicals (OFR) and molecules such as P-selectin and CD40L, which are implicated in the process of atherosclerosis. Platelets also release thromboxane (Tx) A₂, which is an aggregating substance formed via cyclooxygenase (COX)-1 activation and implicated in the thrombotic process. Thus, interventional trials with aspirin, which inhibits the COX-1 pathway, thus preventing Tx A₂ formation, showed a significant reduction of cardiovascular events in patients with cardiovascular diseases. In addition to Tx A₂, platelets release F₂-isoprostanes, in particular 8-iso-PGF2α, a chemically stable compound derived from enzymatic and nonenzymatic oxidation of arachidonic acid (AA). Platelet 8-iso-PGF2α seems to marginally stem from the COX-1 pathway; nonenzymatic AA interaction with OFR, such as superoxide anion (O₂⁻), would have a major role. However, it is still unclear how this occurs in platelets and, more in particular, which is the cellular O₂⁻ generating pathway involved in 8-iso-PGF2α formation. Platelets possess a complete armamentarium to produce O₂⁻ that could elicit 8-iso-PGF2α formation via nonenzymatic oxidation of AA. In particular, platelets possess all the subunits of the NADPH oxidase, the most important cellular producer of O₂⁻. Activation of platelet NADPH oxidase is crucial for O₂⁻ production, as shown by its complete suppression in case of NADPH oxidase functional deficiency. Regarding this, we have shown that platelets from patients with chronic granulomatous disease, a very rare illness (prevalence, 1:1 000 000) characterized by life-threatening infectious disease, have an almost complete suppression of platelet O₂⁻. This phenomenon was dependent...
on the hereditary deficiency of gp91phox, the catalytic subunit of NADPH oxidase.\textsuperscript{2,11,14} In a previous study conducted in patients who were genetically deficient for gp91phox, we have shown that urinary excretion of 8-iso-PGF\textsubscript{2\alpha} was reduced compared with controls.\textsuperscript{15} On the basis of this finding, we hypothesized that NADPH oxidase activation could have a role in platelet 8-iso-PGF\textsubscript{2\alpha} formation. To explore this hypothesis, we conducted a study in male patients with X-linked chronic granulomatous disease (X-CGD) to see the behavior of platelet 8-iso-PGF\textsubscript{2\alpha}.

### Materials and Methods

All materials were from Sigma Aldrich unless otherwise specified.

#### Subjects

We studied 8 recently identified male patients (aged 27±2.5 years) with hereditary deficiency of gp91phox that was diagnosed as previously described.\textsuperscript{5} X-CGD is a rare (prevalence, 1 in 1 000 000 individuals) primary immunodeficiency affecting the innate immunologic system and characterized by life-threatening bacterial and fungal infections.\textsuperscript{16} It is caused by mutations in any of the 4 genes encoding subunits of the O$_2$\textsuperscript{-}-generating NADPH oxidase, resulting in defective O$_2$\textsuperscript{-} generation and intracellular killing.\textsuperscript{13} None of X-CGD patients had a clinical history complicated by bleeding disorders. Eight healthy volunteers matched for sex, age, and risk factors of atherosclerosis were recruited from the same geographic area (Table).

### Platelet Preparation

To obtain platelet-rich plasma (PRP), samples were centrifuged for 15 minutes at 180g. To avoid leukocyte contamination, only the top 75% of the PRP was collected. Platelet pellets were suspended in HEPES buffer, pH 7.4 (2×10\textsuperscript{8} platelets/mL, unless otherwise noted).

#### Polymorphonuclear Leukocyte Preparation

Polymorphonuclear leukocytes (PMNs) were isolated from freshly taken EDTA blood from healthy volunteers by dextran-enhanced sedimentation of red blood cells, Ficoll-Histopaque density centrifugation, lysis of remaining erythrocytes with distilled water, and washing of cells with Hanks’ balanced salt solution in the absence of any divalent cations.

### Evaluation of OFR

Cell suspension was incubated with 2',7'-dichlorofluorescin diacetate (5 \muM) for 15 minutes at 37°C. After incubation, PMNs were treated with phorbol myristate acetate (PMA) (10 \muM), and platelets were activated with AA (1 mmol/L) in the presence or absence of the gp91phox-specific blocking peptide (gp91ds-tat, 50 \muM) or its control peptide tat (50 \muM).\textsuperscript{20} Basal OFR level in resting cells was expressed as mean fluorescence, and OFR production in stimulated cells was expressed as stimulation index (mean level of fluorescence in stimulated cells/mean level of fluorescence in unstimulated cells).

#### Platelet Aggregation

Platelet aggregation was induced by subthreshold (0.1 to 0.5 \muM) or threshold (2.0 \muM) doses of collagen (Mascia Brunelli) or AA (subthreshold=0.1 to 0.5 mmol/L; threshold=1.0 mmol/L) and measured as previously described\textsuperscript{21} in the presence or absence of 8-iso-PGF\textsubscript{2\alpha} (1 to 1000 \muM). Threshold concentration of the agonist was defined as the lowest concentration of the agonist that elicits >50% increase in light transmission difference between PRP and platelet-poor plasma 5 minutes after the addition of the agonist.\textsuperscript{22} A subthreshold concentration of agonist was defined as the highest concentration of the agonist that elicited <20% platelet aggregation.

### Platelet Recruitment

Platelet recruitment was performed with a method modified from that described by Kriszt et al.\textsuperscript{23} PRP samples were incubated (30 minutes. at 37°C) with or without NO synthase inhibitor nitro-L-arginine methyl ester (L-NAME, 100 \muM), gp91ds-tat (50 \muM), control peptide tat (50 \muM), aspirin (COX-1 inhibitor, 100 \muM), TXA\textsubscript{2} isoprostanes receptor inhibitor (SQ29548, 0.1 \muM/L), catalase (500 U/mL), superoxide dismutase (PEG-SOD), (300 U/mL), apocynin (100 \muM/L), 2,3-dimethoxy-1,4-naphthoquinone (DMNQ) (OFR generator, 100 \muM/L), the ADP scavenger apyrase (2 U/mL), the lipoxigenase (LOX) inhibitor 5,8,11,14-eicosatetraynoic acid (ETYA; 10 \muM), and the xanthine oxidase inhibitor allopurinol (10 \muM), before activation with collagen (2 \muG/mL) in the presence or absence of 8-iso-PGF\textsubscript{2\alpha} (1 to 1000 \muM) (Calbiochem). Collagen-induced platelet aggregation was measured for 10 minutes. Then, an equal portion of untreated platelets was added to each tube, which increased the density of the solution and hence caused a reduction in light transmission. Aggregation of the newly added platelet portion in PRP in the presence of an existing aggregate was then measured for 5 minutes and expressed as a percentage of the aggregation that had been initially reached.

Platelet recruitment was also induced by another method using the supernatant of activated platelets from healthy subjects and X-CGD as agonists. Briefly, collagen-induced platelet aggregation was stopped after 10 minutes, samples were immediately pelleted at 4°C, and the supernatant was used as agonist according to Valles et al.\textsuperscript{24} The concentration in the final suspensions was adjusted to 2×10\textsuperscript{8} platelets/mL. Aggregation was measured as increase in percentage of light transmission as reported above.

#### Platelet Thrombus Formation on Fibillar Collagen Type I on Blood Perfusion

**Thrombus Formation Ex Vivo**

A glass coverslip was coated with acid-insoluble type I collagen (Sigma-Aldrich, St. Louis, Mo) and assembled at the bottom of a rectangular flow chamber on the stage of an inverted fluorescence microscope equipped with a confocal module (TCS SP5, Leica, Milan, Italy). Platelets in whole blood anticoagulated with FPR-chloromethylketone (50 \muM/L) were rendered fluorescent with
10 μmol/L quinacrine hydrochloride (mepacrine) added before perfusion. A constant flow at wall shear rate of 1,500 s⁻¹ was controlled with aspirating syringe pump (New Era Pump System NE-1000, KF Technology, Rome, Italy). Images obtained through a HP PL APO ×20/0.70 objective (Leica) were digitized in real time with a video camera (QuickCam Coolered Mono, Q-Imaging, Surrey, British Columbia, Canada). Stacks of confocal z-sections were collected at defined time points.

**Thrombus Volume Measurement**

The area of the field of view seen through the ×20 objective and the video camera used was 207,025 μm². Total thrombus volume per field of view, reflecting the number of aggregated platelets, was calculated as the sum of the surface area covered by platelets in each individual confocal section multiplied by the z-axis interval (0.94 μm) between adjacent sections, as reported Savage et al.²³ The threshold between signal and image background was established using an unbiased algorithm. Image analysis was performed with Image J (http://rsbweb.nih.gov/ij/).

**Platelet and Serum TxA₂**

AA (1 mmol/L)-activated PRP samples were centrifuged, and platelet-poor plasma was stored at −80°C. Serum TxA₂ was evaluated in blood samples kept for 60 minutes at 37°C and centrifuged at 300 g before storage at −80°C. Platelet TxA₂ and serum TxA₂ were analyzed as previously described.²⁶,²⁷ Briefly, platelet TxA₂ and serum TxA₂ were measured with or without aspirin (100 μmol/L) by evaluating its stable metabolite TXB₂ by an enzyme immunoassay (EIA) commercial kit (Amersham Pharmacia, Biotech, Little Chalfont, United Kingdom) and expressed as pg/10⁸ cells or mg/mL, respectively. Intra- and interassay coefficients of variation for TxB₂ EIA kit were 4.0% and 3.6%, respectively.

**8-IsopGF2α Assays**

To evaluate platelet 8-IsopGF2α formation, PRP was centrifuged 20 minutes at 300g to concentrate platelets, and the pellet was suspended in Tyrode buffer (final platelet concentration of 2×10⁹/mL). Platelet suspensions were incubated with or without AA (1 mmol/L) in presence or absence of gp91ds-tat (50 μmol/L), control peptide tat (50 μmol/L), catalase (500 U/mL), superoxide dismutase (PEG-SOD, 300 U/mL), apocynin (100 μmol/L), allopurinol (10 μmol/L), ETYA (10 μmol/L), l-NAME (100 μmol/L), and ASA (100 μmol/L) for 15 minutes at 37°C. After incubation, platelets were pelleted 3 minutes at 300g. 8-iso-PGF2α content was measured by gas chromatography as previously described²⁸ and expressed in pmol/mL.

**Plasma 8-IsopGF2α Assays**

8-iso-PGF2α formation was also evaluated in the supernatant of samples from shear-induced thrombus formation. Whole blood samples were centrifuged (10 minutes, 300g), and the supernatant was treated as reported above and expressed as pmol/mL.

**Flow Cytometry Analysis of gpIIb/IIIa**

PAC1 is an antibody that recognizes an epitope on the glycoprotein IIb/IIIa (gpIIb/IIIa) of activated platelets, at or near the platelet fibrinogen receptor. PAC1 binding on platelet membrane was analyzed using the specific fluorescent isothiocyanate–labeled monoclonal antibody anti-PAC1 (BD International) as previously reported.²⁶ All assays included samples to which an irrelevant isotype-matched antibody (fluorescein isothiocyanate–labeled IgM) was added. To analyze PAC1 binding, PRP was incubated 30 minutes at 37°C with or without 8-iso-PGF2α (1 to 1000 pmol/mL) (Calbiochem), collagen (0.5 to 2.0 μg/mL), AA (0.1 to 1.0 mmol/L), or a combination thereof. In some experiments, platelets were also treated with the TxA₂/isoprostane receptor antagonist SQ29548 (0.1 μmol/L) or l-NAME (100 μmol/L). Fluorescence intensity was analyzed on an Epics XL-MCL Cytometer (Coulter Electronics) equipped with an argon laser at 488 nm/L. For every histogram, 5×10⁵ platelets were counted to evaluate the percentage of positive platelets. Antibody reactivity is reported as mean fluorescence intensity.

**Cytoplasmic Free Calcium Mobilization**

Cytoplasmic free calcium mobilization was evaluated according to Labiös et al.²⁹ Briefly, CD61PE (specific monoclonal antibody to platelet GPIIIa, Pharmingen) and 250 AM fluo-3-acetoxymethyl ester (FLUO 3-AM, calcium probe, Molecular Probes, Leiden, the Netherlands) were added to the sample. The basal fluorescence intensity corresponding to FLUO 3-AM (FL1 log) was measured for 15 seconds in the platelet population identified in the gate forward scatter log/side scatter log as CD61-positive events. Platelet stimulation with thrombin (0.1 U/mL) was used as positive control. Changes in fluorescence were evaluated in arbitrary units as fold increase in calcium mobilization. Samples were incubated for 30 minutes at 37°C with or without 8-iso-PGF2α (1 to 1000 pmol/mL) or collagen (0.5 to 2.0 μg/mL) and AA (0.1 to 1.0 mmol/L). In some experiments, platelets were also treated with SQ29548 (0.1 μmol/L) or l-NAME (100 μmol/mL). To further evaluate the role of calcium we incubated platelets with 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (200 μmol/L), a calcium chelator, before thrombin or 8-iso-PGF2α-induced platelet activation.

**Platelet Nitrite and Nitrate Measurement**

A colorimetric assay kit (Tema Ricerca) was used to determine the nitrite/nitrate metabolites (nitrite and nitrate; NOx) in the supernatant of recoupled platelet in presence or absence of l-NAME or gp91ds-tat (50 μmol/L) or control peptide tat (50 μmol/L) at 37°C for 15 minutes as previously described.³⁰ Intra- and interassay coefficients of variation were 2.9% and 1.7%, respectively.

**Membrane and Cytoplasmic Protein Extraction**

To analyze the specificity of gp91ds-tat in blocking platelets NADPH oxidase activation, the effect of this peptide was analyzed on the translocation of p47phox from cytosol to membranes in AA (1 mmol/L)-activated platelet according to Fortuito et al.³¹ Briefly, the extraction of membrane and cytoplasmic proteins was performed by using the ProteoJET Membrane Protein Extraction Kit (Fermentas International Inc.). The extraction was performed in 3 steps: cell harvesting and permeabilization, extraction of cytoplasmic proteins, and extraction of membrane proteins.

**Electrophoresis and Immunoblotting of Membrane and Cytoplasmic p47phox**

Equal amounts of protein (130 μg/lane) estimated by Bradford assay were solubilized in a 2× Laemmli sample buffer containing 2-mercaptoethanol and loaded in a denaturing SDS/10% polyacrylamide gel. Western blot analysis was performed with monoclonal anti-p47phox (2 μg/mL) incubated overnight at 4°C.

After incubation, the pure nitrocellulose membranes (0.45 μm) were washed and incubated with goat anti-mouse IgG–horseradish peroxidase for 2 hours. Immune complexes were detected by enhanced chemiluminescence. The developed spots were calculated by densitometric analysis on a NIH Image 1.62f analyzer, and the value was expressed in arbitrary units. Each sample was analyzed in triplicate.

**Urinary 8-IsopGF2α Assays**

Urinary 8-iso-PGF2α was measured by a previously described and validated EIA assay method.³² Ten milliliters of urine was extracted on a C-18 solid phase extraction column; the purification was tested for recovery by adding a radioactive tracer (tritiated 8-iso-PGF2α) (Cayman Chemical). The eluates were dried under nitrogen, recovered with 1 mL of buffer, and assayed in a PGF2α-III specific EIA kit (Cayman Chemical). Urinary 8-iso-PGF2α concentration was corrected for recovery and creatinine excretion and expressed as pg/mg of creatinine. Intra- and interassay coefficients of variation were 2.1% and 4.5%, respectively.

**ATP Release**

Platelet activation (collagen, 2 μg/mL) was stopped after 2 minutes by addition of formaldehyde/EDTA according to Costa and Murphy.³³ After centrifugation at 300g for 30 seconds, the ATP concen-
tration in the supernatant was measured on a LKB 1251 luminometer (Pharmacia, Uppsala, Sweden) by addition of luciferin (40 mg/mL) and luciferase (880 U/mL) (both by Chronolog Corp, Havertown, Pa) according to Holmsen et al.34

The results were expressed as /H9262 M/105 platelets, comparing the luminescence detected with that measured by a standard curve. The standard curve was obtained by evaluating the luminescence resulting by adding scalar concentrations of ATP (0.5, 1, and 2 /H9262 mol/L) to the buffer.

Statistical Analysis
Data are reported as mean±SD. The comparison between variables in the in vitro and ex vivo studies was made by the Student t test for paired and unpaired data. The data were also confirmed by nonparametric test. Bivariate analysis was performed with a Spearman correlation test. Significance was accepted at P<0.05.

Results
Clinical characteristics of the population studied are reported in the Table. Risk factors of atherosclerosis were similar in patients and controls. All subjects were nonsmoking.

OFR Production in PMNs and Platelets
For the purpose of assessing whether blocking gp91phox by its specific peptide, gp91ds-tat, inhibited the activity of NADPH oxidase, we stimulated PMNs and platelets from healthy subjects with PMA and AA, respectively, in the presence or absence of gp91ds-tat or the control peptide (tat). Treatment with gp91ds-tat resulted in 50% and 65% reduction of PMA-induced and AA-induced OFR production, respectively (Figure 1). No change of OFR production was observed in samples treated with the control peptide tat (data not shown).

Platelet Activation in Healthy Subjects and X-CGD Patients
Collagen (2 μg/mL)– and AA (1 mmol/L)–induced platelet aggregation were similar in healthy subjects and X-CGD patients. Platelet incubation with 8-iso-PGF2α (1 to 100 pmol/L) did not change platelet aggregation in either patients or healthy subjects (Figure 2A).

AA (1 mmol/L)–induced platelet activation elicited a similar amount of TxB2, the stable metabolite of TxA2, in healthy subjects and X-CGD patients. Aspirin reduced platelet TxB2 formation by more than 95% (P<0.001) in both healthy subjects and X-CGD patients; conversely, no change of platelet TxB2 was observed in samples treated with gp91ds-tat (Figure 2B).

AA-induced platelet 8-iso-PGF2α formation was significantly lower in X-CGD patients compared with healthy subjects (Figure 2C) (P<0.001). In platelets from healthy subjects, AA-induced platelet 8-iso-PGF2α formation was marginally reduced by aspirin (P=not significant [NS]), ETYA (P=NS), l-NAME (P=NS), and allopurinol (P=NS), whereas it was significantly inhibited by gp91ds-tat (P<0.001), catalase (P<0.001), PEG-SOD (P<0.001), and apocynin (P<0.001) (Figure 2C). Incubation of platelets with control peptide tat did not influence 8-iso-PGF2α formation (P=NS); gp91ds-tat and acetylsalicylic acid (ASA) did not modify 8-iso-PGF2α formation in X-CGD patients (Figure 2C).

Serum TxB2 was in the same range in healthy subjects and X-CGD patients (455.4±10.6 versus 445.0±11.6 ng/mL, respectively, P=NS) Consistent with platelet findings, systemic concentration of 8-iso-PGF2α, as assessed by urinary excretion of 8-iso-PGF2α, was depressed in X-CGD compared with healthy subjects (65±5.4 versus 132±30.5 pg/mg creatinine, respectively, P<0.001).

Platelets from healthy subjects and X-CGD released similar amounts of ATP on stimulation with collagen (0.86±0.10 and 0.82±0.06 μmol/L per 10^5 platelets, respectively, P=NS) (Figure 2D).

Platelet NOx in Healthy Subjects and X-CGD Patients
Platelet NOx was evaluated with or without l-NAME or gp91ds-tat in patients and healthy subjects (Figure 2E). Platelets from X-CGD produced a significantly higher
Figure 2. A, Platelet aggregation induced by collagen (2 μg/mL) or AA (1 mM) with or without 8-iso-PGF2α (100 pmol/L). B, TxB2 formation in AA (1 mM)–stimulated platelets with or without gp91ds-tat (50 μmol/L), ASA (100 μmol/L), or control tat peptide (50 μmol/L). C, 8-iso-PGF2α formation in AA (1 mM)–stimulated platelets with or without gp91ds-tat (50 μmol/L), control tat peptide (50 μmol/L), ASA (100 μmol/L), catalase (500 U/mL), PEG-SOD (300 U/mL), Apocynin (100 μM), Allopurinol (10 μM), ETYA (10 μM), L-NAME (100 μmol/L), or apocynin (100 μmol/L). D, ATP release in collagen-stimulated platelets from healthy subjects and X-CGD patient (P=NS). E, Platelet NOx production in agonist-stimulated platelets treated or not with the NO synthase inhibitor L-NAME (100 μmol/L), gp91ds-tat (50 μmol/L), or control tat peptide (50 μmol/L). Experiments were performed in samples from 8 healthy subjects and 8 X-CGD (*P<0.001, **P<0.05).
amount of NOx compared with healthy subjects (*P*<0.001). Incubation of platelets with l-NAME elicited a significant reduction of NOx, which was more marked in X-CGD patients (~72%, *P*<0.001) compared with healthy subjects (~55%, *P*<0.001). Incubation of platelets with gp91ds-tat enhanced NOx only in platelets from healthy subjects (*P*<0.05). Incubation of platelets with control peptide tat did not influence platelet NOx (Figure 2E).

**Platelet Activation by 8-Iso-PGF2α in Healthy Subjects**

8-iso-PGF2α increased platelet aggregation to >20% but <50% light transmission only at 100 pmol/L. Similarly, 8-iso-PGF2α dose-dependently increased gpIIb/IIIa activation and calcium mobilization (Figures 3 to 5). The higher 8-iso-PGF2α concentration of 1000 pmol/L was less effective compared with 100 pmol/L (not shown). When 100 pmol/L 8-iso-PGF2α was combined with a subthreshold concentration of agonists, platelet aggregation was above 50%, suggesting that 8-iso-PGF2α potentiated the aggregating property of collagen (*P*<0.001) and AA (*P*<0.001) (Figure 3A).

Representative tracings of platelet aggregation with a subthreshold concentration of agonists and 8-iso-PGF2α are reported in Figure 3C.

Similarly, 100 pmol/L 8-iso-PGF2α potentiated gpIIb/IIIa activation and calcium mobilization elicited by a subthreshold concentration of AA was unaffected by l-NAME (100 μmol/L) but significantly inhibited by SQ28548 (Figures 3A, 4A, and 4D).

**Platelet Activation by 8-iso-PGF2α in X-CGD**

The increase in platelet activation by 8-iso-PGF2α alone or in combination with a subthreshold concentration of agonists was observed also in X-CGD patients, in whom, however, the rate of platelet activation was lower than that observed with platelets from healthy subjects. Conversely, the increase in platelet aggregation (Figure 3B), gpIIb/IIIa activation (Figure 4B), and calcium mobilization (Figure 4E) by l-NAME was significantly increased in X-CGD compared with control.

**Platelet Recruitment and 8-iso-PGF2α**

Platelets from healthy subjects incubated with 8-iso-PGF2α (1 to 100 pmol/L) showed a dose-dependent increase in platelet recruitment (Figure 5A); treatment with 1000 pmol/L 8-iso-PGF2α did not increase platelet recruitment compared with control (not shown). 8-iso-PGF2α (100 pmol/L)-induced platelet recruitment was weakly enhanced by l-NAME (*P*<0.05) and significantly inhibited by SQ29548 (*P*<0.001) and apyrase (*P*<0.001) (Figure 5A). Incubation with SQ29548 and apyrase almost completely abolished 8-iso-PGF2α (100 pmol/L)-induced platelet recruitment (Figure 5A).

Platelet recruitment was almost completely absent in X-CGD patients (4±1% in X-CGD versus 50±3% in controls *P*<0.001) and was partially restored by platelet incuba-
Platelet recruitment was also analyzed by adding the supernatant of agonist-stimulated platelets from HS and X-CGD to platelets from HS or X-CGD. Although the supernatant of collagen-stimulated platelets from HS induced aggregation >50% when added to fresh platelets from both...
HS and X-CGD, the supernatant of collagen-stimulated platelets from X-CGD elicited aggregation >50% only if it was added with 8-iso-PGF2α (Figure 3A and 3B).

Effect of Aspirin, SQ29548, and Radical Scavenger on Platelet Function

As aspirin inhibits platelet TXA2 formation and SQ29548 antagonizes not only TXA2 but also isoprostanes, we investigated whether the 2 drugs affected recruitment of platelets from healthy subjects differently. Platelet recruitment was significantly inhibited by incubation of platelets with gp91ds-tat (P<0.001), catalase (P<0.001), the PEG-SOD (P<0.001), and apocynin (P<0.001) and significantly enhanced by 2,3-dimethoxy-1,4-naphthoquinone (DMNQ, 100 μmol/L), allopurinol (10 μmol/L), and ETYA (10 μmol/L) (*P<0.001, **P<0.05).

ASA pretreatment induced a lower inhibition (P<0.001) of platelet recruitment. The TxA2/isoprostane receptor inhibitor SQ29548 (P<0.001) significantly inhibited platelet recruitment in both controls and ASA-treated platelets (P<0.001) (Figure 5D). The LOX inhibitor ETYA and the xanthine oxidase inhibitor allopurinol only marginally affected platelet recruitment (P<0.05). Incubation of platelets with the control peptide tat did not influence platelet recruitment (Figure 5D).

Effect of gp91ds-tat and Apocynin on Platelet Thrombus Formation on a Collagen Type I Surface on Blood Perfusion at 1500 s⁻¹

Thrombus formation on blood perfusion over insoluble fibrillar collagen type I at the wall shear rate of 1500 s⁻¹ was studied on samples from HS. Thrombus volume increased progressively as a function of time. Using blood treated with gp91ds-tat, thrombus volume was reduced as compared with control at all time points and significantly reduced at 6 minutes. Similar findings were observed with apocynin, which, however, elicited a lower inhibitory effect as compared with gp91ds-tat (Figure 6A and 6B).
Thrombus formation was associated with isoprostane formation (350 ± 1100 pmol/L at 6 minutes) that was inhibited by treatment with gp91ds-tat (20 pmol/L at 6 minutes) and apocynin (15 pmol/L at 6 minutes).

Effect of gp91phox ds-tat on p47phox Translocation
To analyze the specificity of gp91 ds-tat in inhibiting platelet NADPH oxidase activation, we evaluated the effect of the translocation of p47phox from the cytosol to the membrane of platelets from healthy subjects. The peptide was described as able to block the binding of the cytosolic p47phox to the membrane subunit gp91phox. AA-induced p47phox translocation was inhibited by platelet preincubation with gp91ds-tat (0.8 fold increase versus 5.1 ± 0.2 fold increase, P<0.001) (Figure 6C and 6D). The preincubation of AA-stimulated platelets with the control peptide did not show any reduction of p47phox translocation (Figure 6C and 6D).

Discussion
This study provides evidence that platelet isoprostane 8-iso-PGF2α is formed via activation of gp91phox and contributes to the process of platelet recruitment by activating gpIIb/IIIa.

Previous studies have shown that on stimulation platelets release F2-isoprostanes, namely 8-iso-PGF2α, in a concentration depending on the type of agonist used but usually in the range of 100 to 500 pmol/L. 17,35,36 In our experiments, the

Figure 6. A, Volume of platelet thrombi forming onto fibrillar collagen type I on blood perfusion at the shear rate of 1500 s⁻¹ in samples from HS added with gp91ds-tat (50 μmol/L) or apocynin (100 μmol/L). Experiments were performed in samples from 3 healthy subjects (*P<0.05). B, Single-frame images of platelet thrombi formed on a collagen type I surface on blood perfusion at 1500 s⁻¹. Images were taken from real-time video recordings after 6 minutes of blood perfusion. Detector settings were kept constant throughout the experiment; thus, the intensity of light on a gray scale from 0 (black) to 255 (white) is proportional to the number of fluorescent platelets on the surface. The 3 bottom images are enlargements (scale bar=10 μm) of the corresponding top ones (scale bar=50 μm). The images at lower magnification show that thrombus formation on the surface is homogeneous. The enlarged images show the thrombi formed in the presence of gp91ds-tat or apocynin have a less compact structure than thrombi formed in control blood and are constituted by fewer platelets. C, Densitometric evaluation of the membrane p47phox content in platelets added with or without gp91ds-tat or tat before and after activation with AA in healthy subjects (n=5) (*P<0.001). D, A representative Western blot analysis of the membrane and cytosol 47phox content in a healthy subject.
concentration of 8-iso-PGF2α released by normal platelets on stimulation with agonist was on the same order of magnitude. In experiments conducted in platelets from healthy subjects treated with aspirin, we observed a reduction of 8-iso-PGF2α by 8%, which is consistent with previous data showing a marginal role for COX-1 in the formation of this isoprostanes. Conversely, a much higher inhibition (~58%) was observed with a specific inhibitor of gp91phox, suggesting that NADPH oxidase has a major role in 8-iso-PGF2α formation. To explore this hypothesis, platelet 8-iso-PGF2α was measured in platelets taken from patients with hereditary deficiency of gp91phox. We found that platelet 8-iso-PGF2α was significantly reduced in patients compared with controls, with a mean reduction of 75% (in the supernatant of AA-stimulated platelets), indicating that platelet generation of 8-iso-PGF2α is maximally dependent on nonenzymatic oxidation of AA via NADPH oxidase-generating O2−. The interplay between NADPH oxidase activation and F2-isoprostane formation was further corroborated by analysis of 8-iso-PGF2α urinary excretion, which reflects the systemic production of this isoprostane. Thus, compared with healthy subjects, X-CGD patients have lower urinary excretion of 8-iso-PGF2α, indicating that the low production of this isoprostane is likely to occur, not only in platelets but also in other cellular lines.

The simultaneous measurement of TXA2 allowed us to compare the behavior of these 2 eicosanoids in the population studied. It is of note that on agonist stimulation, normal platelets produced both TXA2 and 8-iso-PGF2α. Unlike the case with 8-iso-PGF2α, in X-CGD patients platelet formation of TXA2 was unaffected, indicating that the activation of COX-1 is not influenced by OFR formation. Together, these data show that on stimulation platelets produce 2 eicosanoids via different pathways as TXA2 derives exclusively from COX-1 activation, whereas 8-iso-PGF2α derives maximally from NADPH oxidase-dependent AA oxidation.

Although the role of TXA2 in the activation of platelets is well established, data concerning the role of 8-iso-PGF2α is less clear. Previous studies have consistently shown that 8-iso-PGF2α does not induce platelet aggregation; conversely, it elicits shape change and amplifies the platelet response to common agonists. However, this effect and other biological activities, including vasoconstriction, mitogenesis of smooth muscle cells, and proliferation of fibroblasts, have been questioned because they have been achieved with supraphysiologic concentrations of F2-isoprostane. In the present study, we could confirm that F2-isoprostanes actually amplify the platelet response to common agonists but at concentrations much lower than that previously used and in the same range produced by normal activated platelets.

To further explore the role of F2-isoprostane on platelet activation, we studied the effect of 8-iso-PGF2α in the process of platelet recruitment, which mimics in vivo accumulation of platelets at site of vascular injury; it is influenced by aggregating molecules, which propagate thrombus plug on release by platelets that originally initiated to aggregate. Among these molecules, OFR seem to have an important role, as suggested by the fact that OFR scavengers or inhibitors of O2− production reduced platelet recruitment.

Consistent with these findings, O2− scavenger, apocynin, and catalase inhibited platelet recruitment, whereas an OFR donor such as DNMQ enhanced it. We also evaluated the effect of a direct NADPH oxidase inhibitor peptide, namely gp91ds-tat: this peptide is able to block the binding of the cytosolic NADPH oxidase subunit p47phox to the membrane subunit gp91phox in neutrophils. Accordingly, gp91ds-tat prevented binding to platelet gp91phox and significantly inhibited both platelet recruitment and platelet 8-iso-PGF2α production, suggesting a potential role for 8-iso-PGF2α in the process of platelet propagation activation.

This hypothesis was strengthened by experiments performed in platelets from patients with X-CGD, in whom the reduced 8-iso-PGF2α production was associated with impaired platelet recruitment, and corroborated by a series of in vitro studies performed in normal platelets and platelets from X-CGD. In platelets from controls, a significant increase in platelet recruitment was observed when platelets were incubated with scalar concentration of 8-iso-PGF2α. Also, incubation of platelets from X-CGD patients with 8-iso-PGF2α resulted in a partial restoration of platelet recruitment. To investigate the mechanism through which 8-iso-PGF2α enhanced platelet recruitment, we focused our attention on the gpIIb/IIa, which has previously been shown to be activated by this isoprostane but at supraphysiologic concentrations. We could confirm that even low 8-iso-PGF2α concentration actually enhanced the activation of gpIIb/IIa. This effect seemed to be attributable to the mobilization of Ca2+ and Ca2+ mobilization induced by isoprostane incubation suggested a more complex scenario in such a phase of platelet activation. Thus, we hypothesized that the reduced formation of O2− could result in higher NO generation and eventually impaired platelet recruitment. In support of the validity of such hypothesis, we demonstrated that platelet generation of NO was higher in X-CGD patients than in controls and that the incubation of normal platelets with gp91ds-tat resulted in higher NO generation. Also, platelet incubation with l-NAME, an inhibitor of NO synthase, further enhanced recruitment of platelets incubated with isoprostanes with an effect that was much higher in X-CGD patients compared with healthy subjects. It is of note, however, that despite incubation with l-NAME and isoprostanes, platelet recruitment of X-CGD patients was not fully restored. This could be dependent on the fact that OFR production contributes to the expression of other molecules implicated in platelet activation, such as CD40L, which are lowered coincidentally with NADPH oxidase downregulation.

It is well established that Tx receptors are activated not only by TXA2 but also by F2-isoprostanes. We therefore investigated whether blockade of COX-1 or Tx receptors had a different impact in the process of platelet recruitment. The study showed that incubation with aspirin reduced platelet recruitment by 17%, an effect that was lower than that observed with SQ29548 (~64%), a specific inhibitor of...
The fact that aspirin inhibits TxA2, but scarcely influences isoprostane formation, whereas SQ29548 was likely to functionally blunt not only TxA2 but also 8-iso-PGF2α. In accordance with this hypothesis, SQ29548 prevented platelet activation elicited by isoprostanes.

The results of this study have implications and limitations. The fact that 8-iso-PGF2α promotes propagation of platelet activation at physiological concentration indicates that this isoprostane is a biologically active compound that may be relevant in the process of thrombus growth. The different chemical stability of TxA2 and 8-iso-PGF2α may suggest a different role for these 2 eicosanoids in the process of platelet activation. It is, therefore, conceivable that TxA2, because of its chemical instability, is a rapid and immediate agonist that is useful in the early phase of platelet activation. Conversely, 8-iso-PGF2α, because of its chemical stability, is an eicosanoid reservoir that may be useful in the late phase of platelet aggregation propagation. Such a hypothesis is in agreement with the observation that thrombus formation in blood perfused over fibrillar collagen type I at 1500 s⁻¹ was reduced in the presence of NADPH oxidase inhibitors that impaired isoprostane formation. Of note, impaired platelet isoprostane formation had no influence on platelet adhesion to collagen or the initial phase of aggregation, a finding that may explain the absence of bleeding in X-CGD patients. The fact that the Tx/isoprostane receptor inhibitor was more effective than aspirin in inhibiting platelet recruitment may have relevant pharmacological consequences, as it suggests that blocking TxA2/isoprostane receptors could more favorably influence thrombus formation and propagation. This was likely to occur in virtue of a “dual” antiplatelet effect, ie, antagonism of both TxA2 and 8-iso-PGF2α, but this hypothesis needs to be further investigated in other experimental models. A limitation of the study is that we focused our study on the analysis of platelet 8-iso-PGF2α, and we did not investigate whether other isoprostanes may have a role in the process of platelet recruitment. Further study should be done to analyze this issue.

In conclusion, this study shows that platelet formation of 8-iso-PGF2α is maximally dependent on nonenzymatic oxidation of AA via gp91phox-dependent OFR generation. The interplay between platelet formation of 8-iso-PGF2α and platelet recruitment via activation of gpIIb/IIIa may give new insight into the mechanism through which inflammation and oxidative stress contribute to platelet activation and thrombosis.

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Disclosures

None.

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