Anoxia-Reoxygenation Enhances Platelet Thromboxane A2 Production via Reactive Oxygen Species–Generated NOX2 Effect in Patients Undergoing Elective Percutaneous Coronary Intervention

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Objective—Platelets undergoing anoxia-reoxygenation (AR) simultaneously increase reactive oxygen species (ROS) and thromboxane (Tx) B2. Our aim was to assess whether there is an interplay between activation of NOX2, the catalytic subunit of NADPH oxidase, and platelet TxB2 in vitro and in vivo.

Methods and Results—Platelets that underwent AR had enhanced ROS. This was associated with NOX2 activation and was inhibited by incubation with NOX2-blocking peptide. AR was associated with TxB2 and isoprostane production, which were inhibited by NOX2-blocking peptide, vitamin C, and the inhibitor of phospholipase A2. Platelet incubation with 100 μmol/L aspirin fully prevented AR-induced TxA2 but did not affect isoprostane production. We included 56 aspirin-treated patients undergoing elective percutaneous coronary intervention (PCI) who were randomly allocated to receive either placebo or intravenous infusion of 1 g of vitamin C. Blood TxB2, isoprostanes, and soluble NOX2-derived peptide, a marker of systemic NADPH oxidase activation, significantly increased at 60 and 120 minutes after PCI in placebo-treated but not in vitamin C-treated patients.

Conclusion—AR is associated with overproduction of platelet TxB2 and isoprostanes, which is dependent on NOX2-dependent ROS generation. Low doses of aspirin are unable to prevent TxB2 formation in patients who undergo PCI. (Arterioscler Thromb Vasc Biol. 2011;31:1766-1771.)

Key Words: aspirin ■ thromboxanes ■ NOX2 ■ vitamin C ■ isoprostanes

Experimental models of ischemia-reperfusion are associated with a burst of reactive oxygen species (ROS), which may have deleterious effect in the coronary circulation.1,2 Accordingly, percutaneous coronary intervention (PCI), a typical in vivo model of ischemia-reperfusion, has been associated with overproduction of isoprostanes, which are markers of oxidative stress, as well as with impaired microcirculatory flow.3 Furthermore, in vitro experiments of anoxia-reoxygenation (AR) demonstrated enhanced platelet activation via formation of thromboxane (Tx) A2; this may negatively influence the circulatory flow by predisposing to thrombotic complication.4 AR-induced platelet TxB2 formation follows arachidonic acid release by platelet membrane via ROS-induced phospholipase A2 (PLA2) activation.5 However, the mechanism accounting for ROS formation in platelets undergoing AR is still undefined.

NADPH oxidase, the most important cellular producer of ROS, is present not only in the immune innate cells but also in vessel walls and platelets.6 Platelets express NOX2, the catalytic subunit of NADPH oxidase, along with the other cytosolic subunits.7,8 Genetic deficiency of NOX2 is associated with low/absent platelet ROS formation and platelet dysfunction, thus suggesting that NOX2 activation is implicated in platelet activation and ROS formation.9

We hypothesized that AR could enhance platelet TxB2 formation via NOX2 activation and that antioxidants are able to prevent it. To this end, we analyzed in vitro whether NOX2 plays a role in the ROS formation elicited by AR and whether there is an interplay between NOX2 activation and platelet TxB2. Furthermore, we investigated whether ROS generation stems from NOX2 activation and its interplay with platelet TxB2 in patients who underwent PCI.

Methods

Anoxia Reoxygenation Protocol
Five nonsmoking, healthy volunteers, recruited from the transfusion center (3 males and 2 females, mean age 62±3 years), who had not ingested any drug known to interfere with platelet function in the previous 15 days were enrolled in the study after providing informed consent. All subjects had fasted for 12 hours before venipuncture, and blood samples were drawn between 8:00 and 9:00 AM.

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To obtain platelet-rich plasma, citrated samples were centrifuged for 15 minutes at 180g. To avoid leukocyte contamination, only the top 75% of the platelet-rich plasma was collected. Platelets were pelleted and suspended in 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid buffer, pH 7.4 (2\times10^4 platelets/mL, unless otherwise specified).

Platelets were incubated with the NOX2-blocking peptide gp91ds-tat (50 \mu mol/L) or with control peptide tat (50 \mu mol/L) or vitamin C (50 \mu mol/L) or superoxide dismutase (polyethylene glycol-superoxide dismutase [SOD], 300 \mu mol/L) or Aspirin (Acetylsalicylic Acid: ASA, 100 \mu mol/L) or the selective inhibitor of the cytosolic PLA2 arachidonyl trifluoromethyl ketone (14 \mu mol/L) or allopurinol (10 \mu mol/L, an inhibitor of xanthine oxidase) or 5,8,11,14 eicosatetraynoic acid (ETYA, 10 \mu mol/L, an inhibitor of lipooxygenase). Appropriate solvents were used as control. Samples were purged with nitrogen gas (20 minutes), and then platelets were reoxygenated. Samples exposed to a gentle stream of room air were used as control. The intraassay coefficient of variation was 7%, and the interassay coefficient of variation was 6%.

**Flow Cytometric Analysis of Platelet ROS Formation**

To detect ROS, 2',7'-dichlorofluorescein-diacetate was added to platelet suspension (final concentration, 5 \mu mol/L) immediately after the anoxia phase. Fluorescence intensity was analyzed by flow cytometry. For every histogram, 50,000 platelets were counted. The fluorescent signal generated by the probe was expressed as mean fluorescence intensity. The lower limit of detection of ROS was 1.5 (mean fluorescence intensity). Intra- and interassay coefficients of variation were 4% and 6%, respectively.

**Platelet TxB2, and 8-Isoprostaglandin F2α**

Platelet TxB2 and isoprostane 8-iso-prostaglandin F2α (8-iso-PGF2α) were measured in free cell supernatants by a commercially available immunoassay (Amersham Pharmacia). Values are expressed as pmol/L. Intra- and interassay coefficients of variation were 4.0% and 3.6% for TxB2, respectively, and 4.5% and 5.1% for 8-iso-PGF2α.

**Platelet NADPH Oxidase Activation**

We analyzed p47phox translocation from platelet cytosol to membranes according to Fortuno et al\textsuperscript{10} as a direct index of NADPH oxidase activation. Membrane and cytoplasmic proteins extraction was performed using the ProteoJET Membrane Protein Extraction Kit (Fermentas International Inc.). The extraction was performed in 3 steps: platelet harvesting and permeabilization, extraction of cytoplasmic proteins, and extraction of membrane proteins.

Proteins (130 \mu g/lane, estimated by the Bradford assay) were solubilized in a 2X Laemmli sample buffer and loaded in a denaturing SDS10% polyacrylamide gel. As a positive control, polymorphonuclear leukocytes from controls were used.\textsuperscript{3} Western blot analysis was performed using monoclonal anti-p47phox (2 \mu g/ mL) (overnight incubation at 4°C) and goat anti-mouse IgG1 (horseradish peroxidase) for 2 hours. Immune complexes were detected by enhanced chemiluminescence. The developed spots were calculated by densitometric analysis on an NIHimage 1.62f analyzer, and the values were expressed as arbitrary units.

**Soluble NOX2-Derived Peptide Levels**

Extracellular levels of soluble NOX2-derived peptide (sNOX2-dp), a marker of NADPH oxidase activation, were detected by the ELISA method as previously described by Pignatelli et al\textsuperscript{11} in both supernatant from AR-activated platelets and patients’ serum.

The peptide was recognized by the specific mononclonal antibody against the amino acid sequence (224 to 268) of the extra membrane portion of NOX2 that was released in the medium on cell activation. Activation of platelets, leukocytes, and monocytes accounts for 90% of circulating sNOX2-dp.\textsuperscript{11} Values were expressed as pg/mL, and intraassay and interassay coefficients of variation were 5.2% and 6%, respectively.

**Platelet Aggregation**

Platelet aggregation induced by AR was measured as light transmission difference between platelet-rich plasma and platelet-poor plasma, as described previously.\textsuperscript{3}

**Interventional Study**

The study design, as well as coronary procedures of intervention and patients’ characteristics, has been described previously.\textsuperscript{3} Briefly, 56 patients (47 men and 9 women, mean age 67 [50 to 84] years) were enrolled in the study. Informed consent was obtained from each participating subject, and the ethics committee of the Sapienza University of Rome approved the study protocol.

The study was designed as a prospective, single-center, placebo-controlled, randomized study aimed at evaluating the effects of vitamin C infusion on indexes of platelet activation and oxidative stress after PCI. All eligible patients were randomly assigned in a 1:1 manner to receive ascorbic acid solution (1 g of vitamin C diluted in 250 mL of isotonic saline infused at 16.6 mg/minute [4.2 mL/minute] during the 1 hour before PCI) or placebo (isotonic saline infused at 4.2 mL/minute longer than 1 hour before PCI).

All participants were studied after a 12-hour fast. Blood samples were collected at baseline (before the start of procedure) and at 60 and 120 minutes after PCI.

To ensure blind analysis, blood tubes were identified by a numeric code and sent by the cardiologists to the laboratory for analytic testing. The randomization list was unveiled only on conclusion of the analytic phase.

**Blood Levels of 8-IsopGF2α, 8-Hydroxy-2-Deoxyguanosine, TxB2, and sNOX2-dp**

Blood samples were immediately centrifuged at 300g for 20 minutes at 4°C, and the supernatant was collected and stored at \(-80°C\) for batch analysis.

Serum levels of 8-hydroxy-2-deoxyguanosine (8OH-dG) and plasma levels of 8-iso-PGF2α were measured using commercially available immunoassays (both by Tema Ricercare Srl, Bologna, Italy). 8OH-dG and 8-iso-PGF2α intraassay coefficients were 2.1% and 4.5%, respectively. 8OH-dG and 8-iso-PGF2α interassay coefficients of variation were 4.5% and 5.1%, respectively.

Serum TxB2 was measured by a commercially available immunoassay (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom). Intra- and interassay coefficients of variation were 4.0% and 3.6%, respectively.

Serum sNOX2-dp were measured as reported above according to Pignatelli et al.\textsuperscript{11}

**Statistical analysis**

Differences between percentages were assessed by the \(\chi^2\) test or the Fisher exact test. The unpaired Student t test and Pearson product moment correlation analysis were used for normally distributed continuous variables. Appropriate nonparametric tests (Mann-Whitney U test and Spearman rank correlation test \(R_s\)) were used for all the other variables. The differences between baseline and posttreatment values were analyzed with the Wilcoxon signed-rank test. Interventional study data were also analyzed for the assessment of treatment effect on blood levels of 8-iso-PGF2α, 8OH-dG, TxB2, and sNOX2-dp, performing a repeated-measures ANOVA with between-subject factor (treatment group) and within-subject factor (time at 3 levels: at baseline [before the start of procedure] and at 60 and 120 minutes after the end of PCI) analysis.

Data are presented as mean (1 SD) or as median and interquartile range (25th and 75th percentiles). Only probability values <0.05 were regarded as statistically significant. All tests were 2-tailed.

With regard to the in vitro study, the minimum sample size was computed considering (1) relevant difference to be detected in
ROS levels $\geq 7.6$ mol/mean fluorescence, (2) $SD=3.7$ mol/mean fluorescence, and (3) type I error probability $\alpha=0.05$ and power $1-\beta=0.90$. This resulted in $n=5$.

All statistical analyses were performed using computer software packages (Statistica, version 7, StatSoft Inc, Tulsa, OK, and Statistical Package for the Social Sciences, version 16.0, SPSS Inc, Chicago, IL).

Results

In Vitro Study

Platelets that underwent AR showed enhanced formation of ROS that was associated with sNOX2-dp release in the medium (Figure 1A and 1B). AR-induced ROS production was inhibited by platelet incubation with NOX2-blocking peptide (Figure 1A). Further data in support of AR-induced NOX2 activation were outlined by (1) reduction of sNOX2-dp in the medium in samples incubated with a specific NOX2 inhibitor, and (2) enhanced translocation of p47phox to cell membrane (Figure 1B and 1C).

Platelet incubation with 2 antioxidants (SOD and vitamin C), an inhibitor of PLA2 (arachidonyl trifluoromethyl ketone), and, to a lesser extent, aspirin significantly reduced platelet ROS, p47phox translocation to platelet membrane, and sNOX2-dp release (Figure 1).

Platelets that underwent AR showed enhanced formation of TxB2, which was significantly reduced by NOX2-blocking peptide, the inhibitor of PLA2, and the 2 antioxidants SOD...
and vitamin C (Figure 2A). In vitro inhibition of cyclooxygenase 1 by 100 μmol/L aspirin fully prevented AR-induced platelet TxB₂ (Figure 2A). A partial restoration of TxB₂ together with AR-induced platelet aggregation, was detected in aspirin-treated platelets incubated with 1% to 10% nonaspirinated autologous platelets (Figure 3).

AR was also associated with an increase of platelet isoprostane formation (Figure 2B). This change was scarcely influenced by aspirin but was markedly inhibited by the NOX2-blocking peptide, the inhibitor of PLA₂, and the antioxidants SOD and vitamin C.

Neither ROS nor platelet eicosanoids were affected by platelet incubation with allopurinol or ETYA, which are inhibitors of xanthine-xanthine oxidase and lipoxygenase, respectively (not shown).

**Interventional Study**

Baseline serum TxB₂, 8OH-dG, 8-iso-PGF₂α, and sNOX₂-dp were similar in patients allocated to receive either placebo or vitamin C infusion (Figure 4). Univariate linear regression analysis showed a direct correlation between serum TxB₂ and sNOX₂-dp levels (Rₛ=0.52, P<0.0001). This correlation was observed at each study time (baseline: Rₛ=0.47, P=0.00039; 60 minutes: Rₛ=0.67, P<0.0001; 120 minutes: Rₛ=0.49, P=0.0001).

TxB₂, 8OH-dG, 8-iso-PGF₂α, and sNOX₂-dp significantly increased at 60 and 120 minutes after PCI in placebo-treated patients. Conversely, vitamin C infusion resulted in a significant decrease of 8OH-dG, 8-iso-PGF₂α, and sNOX₂-dp at 60 and 120 minutes after PCI, whereas TxB₂ was unmodified from baseline (Figure 4).

To further analyze intergroup and between–treatment group differences, we performed an ANOVA for repeated measures. Thus, we found a significant effect of the interaction between time and group, showing a significant effect of the 2 different treatments on TxB₂ (F[2, 108]=4.18, P=0.01782), 8OH-dG (F[2, 108]=25.27, P<0.0001), 8-iso-PGF₂α (F[2, 108]=3.78, P=0.029), and sNOX₂-dp (F[2, 108]=5.84, P=0.004).

![Figure 3](image3.png) **Figure 3.** TxB₂ formation (A) and platelet aggregation (B) detected in aspirin-treated platelets (PLT-ASA) incubated with 1% to 10% nonaspirinated autologous platelets (PLT) that underwent AR (20 minutes). Data are expressed as mean±SD. *P<0.001.

![Figure 4](image4.png) **Figure 4.** Blood TxB₂ (A), 8OH-dG (B), 8-iso-PGF₂α (C), and sNOX₂-dp (D) levels before and after placebo or vitamin C infusion at baseline (T0) and at 60 (T60) and 120 (T120) minutes after PCI completion. *P<0.001, **P<0.05. Data are expressed as mean±SD.
The results reported here provide evidence that in vitro AR is associated with platelet production of TXB₂ and isoprostanes via NOX2-dependent ROS formation. The increase of the 2 eicosanoids along with NOX2 activation in patients who underwent PCI demonstrates that a similar phenomenon occurs in vivo after ischemia-reperfusion of coronary vessels.

In vitro models of AR are usually associated with a burst of ROS. Although the role of NADPH oxidase in the platelet production of ROS by agonists has been defined, its role in platelets that undergo AR is still unclear. In this study, we showed that AR is associated with burst of ROS that originates from platelet NOX2 activation. These findings are consistent with previous reports showing that AR is associated with NADPH oxidase activation. Oxidative stress elicited by NOX2 activation greatly influenced the production of the 2 platelet eicosanoids, namely TXB₂ and isoprostane, which were in fact increased by AR but were blunted in platelets incubated with NOX2-blocking peptide.

The release of arachidonic acid by ROS from platelet membrane was likely to play a pivotal role, as both TXB₂ and isoprostanes derive from enzymatic and nonenzymatic oxidation of arachidonic acid, respectively. Consistent with this suggestion is the fact that an inhibitor of PLA₂ prevented AR-induced platelet isoprostane and TXB₂ formation. Of note, 2 ROS scavengers, such as SOD and vitamin C, inhibited not only AR-induced isoprostane and TXB₂ production but also AR-induced NOX2 activation. This may be explained by the interruption of a positive feedback between ROS-induced arachidonic acid release by PLA₂ activation and arachidonic acid-induced ROS generation by activation of p47phox, a subunit of NADPH oxidase. Accordingly, aspirin inhibited NOX2-induced ROS formation, which is consistent with previous data demonstrating that TXA₂ activates NADPH oxidase.

Then we investigated whether an interplay between platelet eicosanoid generation and NOX2 activation occurs also in vivo. Using a recently developed assay that measures the activation of NOX2 by circulating blood cells, including platelets, we observed that NOX2 was activated as early as 60 minutes after PCI in patients treated with placebo. This activation was associated with an increase of markers of oxidative stress, such as 8-iso-PGF2α and 8OH-dG.

The detection of increased serum TXB₂ in placebo-treated patients simultaneously with the increase of ROS as early as 60 minutes after PCI was an unexpected finding. This change was seen in patients on chronic treatment with low doses of aspirin, indicating that aspirin was unable to prevent platelet TXB₂ activation.

Although this finding is in agreement with the in vitro study showing that AR is associated with TXB₂ formation, the in vivo inability of low doses of aspirin to prevent TXB₂ formation may be only a matter of speculation. We hypothesize that low doses of aspirin were unable to induce a full acetylation of cyclooxygenase 1, thus allowing arachidonic acid released by ROS to be converted to TXB₂. The plausibility of this hypothesis may be supported by an in vitro experiment in which incomplete acetylation of cyclooxygenase 1 was associated with AR-induced platelet TXB₂ formation. Accelerated turnover of platelets seems to be less sensitive to low doses of aspirin, but it remains to be established whether this occurs in patients who undergo PCI.

Of note, in the intravenous vitamin C–treated group, NOX2 activation and TXB₂ formation were prevented, reinforcing the concept that ROS generated by NOX2 are implicated in the TXB₂ synthesis in patients who underwent PCI.

The results reported here have several clinical implications and limitations. AR is associated in vitro and in vivo with enhanced formation of 2 platelet eicosanoids with proaggregating property, namely TXB₂ and isoprostanes. Platelet TXB₂ formation after PCI despite concomitant low doses of aspirin suggests incomplete inhibition of platelet TXB₂, which could favor post-PCI thrombotic complications. Future studies should investigate whether a different therapeutic approach with aspirin may prevent PCI-induced platelet TXB₂ formation. sNOX2-dp reflects activation of blood cells, including leukocytes, monocytes, and platelets; it is possible that sNOX2-dp elevation after PCI stems not only from platelets but also from other blood cells. Although the ischemia was relatively brief, a short phase of ischemia is sufficient to enhance ROS formation in the coronary tree. The increase of isoprostanes was detected only in plasma; therefore, we cannot be certain that this change occurs also in platelets. Vitamin C infusion may be a simple and cheap approach to prevent PCI-induced platelet TXB₂, but the small sample size and the post hoc analysis do not permit any conclusion on its clinical value.

In conclusion, the results reported here provide evidence that AR is associated in vitro and in vivo with NOX2-dependent ROS generation and platelet TXB₂ formation. In patients undergoing elective PCI, low doses of aspirin are unable to prevent platelet TXB₂, suggesting incomplete cyclooxygenase 1 acetylation. The capability of intravenous vitamin C to blunt platelet TXA₂ suggests that oxidative stress also plays a major role in enhancing platelet activation in vivo.

Disclosures

None.

References

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