Inhibition of NAD(P)H oxidase attenuates aggregation of platelets from high-risk cardiac patients with aspirin resistance

Gyorgyi Stef¹*, Anna Csiszar²*, Zhao Xiangmin², Peter Ferdinandy³, Zoltan Ungvari², Gabor Veress¹

¹State Hospital for Cardiology, Balatonfured 8230, Hungary
²Department of Physiology, New York Medical College, Valhalla, New York 10595, USA
³Cardiovascular Research Group, Department of Biochemistry, University of Szeged, Szeged, Hungary

*These authors contributed equally to this manuscript

Correspondence: Zoltan Ungvari, e-mail: zoltan_ungvari@nymc.edu

Abstract:
Up to one-third of serious vascular events in high-risk patients is attributable to a failure of aspirin (ASA) to suppress platelet aggregation. We hypothesized that inhibition of NAD(P)H oxidase may inhibit aggregation of platelets from ASA-resistant (ASA-R) patients. Thus, platelet-rich plasma was isolated from ASA-sensitive (ASA-S) and ASA-R patients (aspirin resistance was defined as higher than expected aggregation to collagen and epinephrine [≥ 40%] after chronic oral treatment with 100 mg/day ASA). Aggregation to adenosine diphosphate (ADP) (5 and 10 μmol/l), collagen (2 μg/ml) and epinephrine (10 μmol/l) was measured by optical aggregometry. Maximal aggregation of ASA-R platelets to collagen and epinephrine was significantly decreased by DPI and apocynin, whereas they had no effect in ASA-S platelets. Maximal aggregation to ADP was unaffected by NAD(P)H oxidase inhibition in either group. In ASA-R platelets both NADPH-driven O₂⁻ production (lucigenin chemiluminescence assay) and expression of gp91phox and p67phox subunits of the NADPH oxidase (Western blotting) tended to increase. Collectively, inhibition of NAD(P)H oxidase effectively suppressed collagen and epinephrine-induced aggregation of platelets from ASA-R patients, which may represent a novel pharmacological target for cardioprotection in high-risk cardiac patients.

Key words:
oxidative stress, thrombocyte, NADPH oxidase, thrombosis, coronary artery disease, myocardial infarction

Introduction

There is growing evidence that platelets play an important role in atherogenesis, plaque formation and plaque rupture. In clinical practice, inhibition of platelet activity with aspirin (ASA) significantly reduces the odds of serious atherothrombotic vascular events and death in high-risk patients [1, 2, 27]. However, in many patients ASA is not effective. Among many possible causes of ASA treatment failures, aspirin resistance (ASA-R) emerges as a major therapeutic challenge [36, 41, 46]. Clinical studies showed that
there was a significant correlation between ASA-R (diagnosed as insufficient inhibition of platelet aggregation by aspirin) and myocardial infarction (MI), or cerebrovascular accident in patients with stable cardiovascular disease [25, 26]. Yet, the etiology of ASA-R is poorly understood. Because ASA non-responder status may contribute to failure of ASA therapy in the secondary prevention of cardiovascular and cerebrovascular incidents in as many as 30–40% of patients, it is important to identify alternative pharmacological treatments that effectively inhibit aggregation of ASA-R platelets [1, 2, 24].

In the last decade, numerous studies have revealed a central role for NAD(P)H oxidases in cardiovascular pathophysiology (reviewed in [23]). It is significant that recent studies demonstrated that functional NAD(P)H oxidase(s) were also expressed in platelets [38]. Indeed, upon stimulation platelets were shown to generate reactive oxygen species (ROS), which could be prevented by inhibitors of the NAD(P)H oxidase [12, 34, 38]. Importantly, there is increasing evidence that NAD(P)H oxidase(s) play an important role in platelet aggregation [3, 4, 10, 19].

On the basis of the aforementioned studies, we hypothesized that inhibition of NAD(P)H oxidase is able to inhibit aggregation of platelets in high-risk, ASA-R cardiovascular patients. To test the hypothesis, platelet-rich plasma was isolated from ASA sensitive (ASA-S) and ASA-R patients (aspirin resistance was defined as higher than expected aggregation to collagen and epinephrine [≥40%] after oral treatment with 100 mg/day ASA as reported earlier [39], following the guidelines of the Hungarian Society of Cardiology. We have been using this criterion in our institution for several years to adjust the ASA dosage in our high-risk patient population for the prevention of secondary cardiac events. Because ADP aggregation in human platelets is largely mediated by ASA-insensitive pathways, we did not consider ADP-induced aggregation as an inclusion criterion.

All drugs and chemicals were purchased from Sigma Chemical Company (St. Louis, MO).

**Materials and Methods**

**Patient selection**

Patients hospitalized after (MI) or with stable or unstable angina pectoris, peripheral artery disease and/or scheduled for cardiac catheterization as potential candidates for percutaneous coronary intervention (PCI) were eligible for the study if they were undergoing chronic treatment with ASA (≥100 mg/day). We did not include: patients with acute MI according to the American Heart Association/American College of Cardiology criteria, patients with HIV, patients with chronic oral anticoagulation, and patients with contraindication to aspirin. All patients gave written informed consent, and our institutional ethics committee approved the study.

**Study protocol and platelet function assays**

After inclusion in the study, blood was drawn for platelet function assays with tubes that contained 3.8% sodium-citrate. Platelet function was evaluated ex vivo by optical aggregometry (TX-4 aggregometer, CARAT Ltd., Budapest, Hungary) with 2 concentrations of ADP (5 and 10 μmol/l), collagen (2 μg/ml) and epinephrine (10 μmol/l) in the absence and presence of DPI (1 μmol/l), apocynin (3 × 10⁻⁴ mol/l) [5, 6] or acetylsalicylic acid (100 μmol/l) according to the protocols described previously by Ungvari et al. [45] and Stef et al. [39], with platelet-rich plasma adjusted to 275 to 325 × 10³ platelets/μl. The coefficient of variation of this optical aggregometry assay is <6% after stimulation with ADP [39].

Different institutions use various criteria to diagnose aspirin resistance. In the present study, aspirin resistance was defined as higher than expected aggregation to collagen and epinephrine [≥40%] after oral treatment with 100 mg/day ASA as reported earlier [39], following the guidelines of the Hungarian Society of Cardiology. We have been using this criterion in our institution for several years to adjust the ASA dosage in our high-risk patient population for the prevention of secondary cardiac events. Because ADP aggregation in human platelets is largely mediated by ASA-insensitive pathways, we did not consider ADP-induced aggregation as an inclusion criterion.

**Measurement of NADPH oxidase activity**

NADPH oxidase activity was measured in homogenates of platelets by the lucigenin chemiluminescence (5 μmol/l) method [13, 15, 44] after the addition of 10⁻⁴ mol/l NADPH as reported previously [15]. Samples used for these measurement were from patients (n = 5 in both groups) matched for major cardiovascular risk factors (i.e. no diabetes, no smoking).

**Western blotting**

Platelets from ASA-S and ASA-R patients were washed three times, pelleted and snap-frozen in liquid
nitrogen. The samples were homogenized by sonication and proteins were extracted as described earlier [15, 16]. After determination of protein concentration, samples were boiled in Laemmli buffer for 5 min, electrophoresed on 10% SDS-PAGE gel and transferred to Hybond-P (Amersham-Life Science, Arlington Heights, IL) membrane at 1 mA/cm² for 60 min with a semidy blotting system (Biorad). The membranes were blocked in Tris-buffered-saline (TBS) containing 5% nonfat milk and 0.05% Tween 20 (overnight, at 4°C). Primary antibodies directed against cyclooxygenase (COX)-1 and the gp91phox and p67phox subunits of the NAD(P)H oxidase (Santa Cruz Biotechnology Inc.) were added to the membrane for 1 h at room temperature. Anti-β-actin (Abcam) was used for normalization purposes. The membranes were developed with ECL-Plus (Amersham) and analyzed with densitometry.

**Statistical analysis**

For all statistical analyses, we used the GraphPad Prism software package, version 3.02. Discrete variables are reported as counts (percentages) and continuous variables as the mean ± SD or SEM, as indicated. We tested differences between groups with the χ² test for discrete variables and with one-way ANOVA followed by a Scheffé test for continuous variables.

**Results**

The present study included 50 high-risk cardiac patients. Table 1 shows the baseline demographic and clinical characteristics of the study cohort.

**Optical aggregometry**

Aspirin resistance was defined as higher than expected aggregation to collagen and epinephrine [≥ 40%]. Using this criterion 28% of our patients were diagnosed as ASA-R. ADP-induced platelet aggregation was only slightly elevated in ASA-R patients. Maximal aggregation to 5 μmol/l ADP was not affected significantly by apocynin or DPI (Fig. 1A). Similar results were obtained using 10 μmol/l ADP as well (Fig. 1B). In contrast, maximal aggregation of ASA-R platelets to collagen was significantly decreased by both apocynin and DPI, whereas NAD(P)H oxidase inhibitors did not affect responses of ASA-S platelets (Fig. 1C). Apocynin and DPI also significantly attenuated maximal aggregation of ASA-R platelets to epinephrine (Fig. 1D). ASA-R patients had a significantly higher level of platelet inhibition than ASA-S patients (Fig. 1D). There was a correlation between the magnitudes of collagen- and epinephrine-induced aggregation, whereas collagen-induced aggregation did not correlate with ADP-induced responses. By all platelet function tests, we did not find any significant correlation between age, plasma triglyceride and cholesterol concentration or any other variables shown in Table 1 and platelet aggregability.
In each case, we have also performed experiments to verify that ASA-R is not due to incomplete blockade of COX (e.g. due to patient non-compliance or sub-optimal ASA dosage). We found that in vitro administration of ASA did not alter significantly collagen-induced aggregation of platelets in either group (Fig. 2). Similarly, in vitro ASA administration did not affect significantly epinephrine- and ADP-induced aggregation (data not shown). In control experiments in vitro administration of ASA substantially reduced collagen- and epinephrine-induced aggregation (by 51% and 59%, respectively; p < 0.01; n = 7) of platelets from young, healthy subjects, who were not treated with ASA.

Expression of NADPH oxidase subunits

Using Western blotting we were able to detect the expression of gp91phox and p67phox subunits of the NADPH oxidase in human platelets. Our results show that expression of both subunits was increased in ASA-R platelets as compared to that in ASA-S ones (Fig. 3B, C). We did not detect significant differences in COX-1 expression between the two groups (data not shown).

Discussion

Our study investigated the antiplatelet effects of NAD(P)H oxidase inhibition in a cohort of 50 high-risk cardiac patients. The major finding of this study is that NAD(P)H oxidase inhibitors, such as apocynin attenuate collagen- and epinephrine-induced aggrega-
tion of platelets from high-risk cardiac patients presenting with ASA-R (Fig. 1C, D).

ASA-R is significantly associated with an increased risk of death, MI or cerebrovascular accident compared with ASA-S patients [1, 2, 24]. There are several studies reporting that approximately one quarter of ASA users with coronary artery disease are resistant to antiplatelet effects of aspirin [1]. Our data showing that 28% of high-risk cardiac patients are ASA-R are in complete agreement with these results. There are many controversies regarding the diagnosis of ASA-R [27]. In the present study, ASA-R was defined as higher than expected aggregation to collagen and epinephrine (> 40%) after oral treatment with 100 mg/day ASA, following our institutional guidelines. We have been using this criterion in our institution for several years to adjust the aspirin dosage of our high-risk patient population for the prevention of secondary cardiac events.

Recent studies suggest that ASA-R may promote plaque-associated thrombus formation in patients with coronary artery and cerebrovascular disease. In ASA-R patients, an increased platelet activity also results in the release of prothrombotic and proinflammatory microvesicles (platelet microparticles, PMPs) and likely promotes the development of atherosclerosis. Our study suggests that ASA non-responders might obtain significant benefits from NAD(P)H
oxidase inhibitors with respect to cardiovascular events.

The pathological mechanisms underlying ASA-R are likely multifactorial [18]. Previous studies suggested that cigarette smoking, increased levels of norepinephrine due to extended periods of mental stress, increased platelet sensitivity to collagen and/or increased oxidative stress-related increased COX-independent isoprostane formation [14] that might contribute to clinical ASA-R [27]. It was also proposed that hypercholesterolemia altered platelet function [40, 42] and that aspirin might not be cardioprotective in patients with hyperlipidemia [21], however, our data did not reveal a correlation between plasma lipids and platelet aggregability (Tab. 1).

There have been reports that failure of aspirin therapy might also be a consequence of reduced bioavailability of ASA (due to inadequate intake of aspirin/poor compliance, inadequate dose of aspirin and/or reduced absorption or increased metabolism of aspirin) [27]. In addition, ASA has only a 20-min half-life in the circulation and in cases of increased turnover of platelets (e.g. after coronary artery bypass surgery) newly formed platelets may be unexposed to ASA during the 24-h dose interval. In our studies, ex vivo treatment of platelets with ASA did not decrease further collagen and epinephrine-induced aggregation (Fig. 2). These results confirm that the chronic ASA treatment of the patients provided a steady state inhibition in the entire platelet population produced during ASA therapy and that clinical ASA-R in our cohort is not due to patient incompliance or decreased oral bioavailability of ASA [20].

The mechanisms by which NAD(P)H oxidase inhibition exerts its anti-platelet effects in ASA-R are not completely understood [3, 4, 10, 19]. The findings that inhibition of NAD(P)H oxidase (Fig. 1C, D) and scavenging of O$_2^-$ and H$_2$O$_2$ [Ungvari Z., unpublished observation, 2006] decreased ASA-insensitive collagen- and epinephrine-induced platelet aggregation suggest that NAD(P)H oxidase-derived ROS are involved in regulation of platelet aggregation in high-risk, ASA-R patients. There are also data demonstrating that vitamin E can potentiate the antiplatelet activity of ASA by inhibiting the early events of platelet activation pathways induced by collagen [8]. Indeed, NADPH oxidase activity (Fig. 3A) and expression (Fig. 3B, C) were higher in platelets of ASA-R patients than in ASA-S ones.

Despite the recent surge in interest in vascular NAD(P)H oxidases, the role of NAD(P)H oxidase-derived ROS in platelet function has not been clearly elucidated. Earlier it has been suggested that NAD(P)H oxidase activity in platelet samples is due to possible contamination of the preparation by monocytes. However, several lines of evidence suggest that platelets express functionally active NAD(P)H oxidases. In our studies, we were unable to detect leukocyte markers (i.e. CD-14, ED-1) in the platelet preparation used (data not shown). Recent studies demonstrated that thrombin-induced ROS production might be inhibited by DPI and apocynin [3, 4, 37]. Also, in patients with inherited gp91$^{phox}$ deficiency, collagen-, thrombin-, and arachidonic acid-stimulated platelets showed a significantly decreased O$_2^-$ generation [33]. The findings that apocynin and a superoxide dismutase mimic can inhibit collagen-induced aggregation of platelets, as well as collagen-induced release of thromboxane B$_2$ suggest a key role of platelet NAD(P)H oxidase-derived ROS in the regulation of thromboxane receptor-mediated aggregation of platelets [10]. Accordingly, apocynin potently inhibited arachidonic acid-induced aggregation of bovine platelets [20]. Apocynin was also shown to inhibit thrombin-induced platelet aggregation [3, 4, 10], which also depends on arachidonic acid metabolism, and to inhibit the formation of TXA$_2$ in macrophages in a concentration-dependent manner [19]. This mechanism of action also explains why ADP-induced platelet responses were not significantly attenuated by apocynin or DPI (Fig. 1A, B), viz. while collagen and epinephrine-induced aggregation depends on the COX/TXA$_2$ synthase axis, this pathway plays a lesser role in ADP-induced signaling. Because there are reports suggesting ASA-R formation of isoprostanes and/or TXA$_2$ in patients with severe unstable angina [11, 35], it would be interesting to determine whether inhibition of NAD(P)H oxidase decreases production of these thromboxane receptor agonists [28] in platelets of ASA-R high-risk cardiac patients. Recent data suggest that the inhibitory effect of apocynin on platelet aggregation seems to be independent of the nitric oxide/cyclic guanosine monophosphate (NO/cGMP) pathway [3, 4]. We also found that inhibitory effect of apocynin on platelet aggregation in ASA-R platelets was unaffected by nitrate treatment (57% of ASA-R patients were treated with NO donors; Tab. 1). Our recent study showed that the polyphenol resveratrol also inhibited aggregation of ASA-R platelets [39], likely
due to the ability of polyphenols to inhibit NAD(P)H oxidase-dependent ROS generation [32]. Collectively, our data suggest that an up-regulation/increased activity of NAD(P)H oxidase may contribute to the development of ASA-R in high-risk cardiac patients.

At present, it is completely unknown whether systemic inhibition of NAD(P)H oxidase activity would affect progression of cardiovascular diseases in ASA-R patients. Although previous observational and epideimiologic studies have suggested that dietary supplementation with antioxidants or a diet rich in antioxidants might interfere with formation of atherosclerotic lesions, two large randomized clinical trials have shown no benefit when vitamin E was given to patients after myocardial infarction or in those with vascular disease or diabetics with a high-risk coronary artery disease (CAD) profile [7, 9, 47]. The use of vitamin E (administered with vitamin C and beta-carotene) was also the subject of a large (> 20,000 participants) trial of patients at risk for CAD and with CAD [29]. In that study, vitamin E had no effect on the end points of cardiovascular death, cardiovascular events, stroke, or revascularization, considered alone or in combination. On the basis of these results, the American College of Cardiology considers the issue still unresolved and currently does not recommend that patients take vitamin C or E supplements for the express purpose of preventing or treating CAD. Currently the view emerges that vitamin E is not the best antioxidant agent to block cellular free radical signaling. Vitamin E goes directly to the lipid membranes and protects them from peroxidation. However, ROS likely act as signaling molecules/second messengers primarily in cytosolic/sub-sarcolemmal microdomains. Thus, drugs that directly inhibit ROS producing enzymes (such as NAD(P)H oxidases) are expected to have superior efficacy in disrupting ROS-induced cellular signaling pathways.

The mechanisms that regulate NAD(P)H oxidase activity in platelets are not well understood. Previous studies have shown that vascular NAD(P)H oxidases can be induced (via a protein kinase C-dependent pathway) by high plasma levels of angiotensin II [23], inflammatory cytokines, including tumor necrosis factor-α (TNF-α) [17, 31, 43] and endothelin, by water soluble components of cigarette smoke [30] and by metabolic diseases (including diabetes, hypercholes- terolemia and hyperhomocysteinemia [43]). One can speculate that these conditions will also up-regulate NAD(P)H oxidase function in platelets [17, 23, 31]. Importantly, angiotensin II [23], TNF-α and endothelin have been implicated in the pathogenesis of heart failure and CAD. Although the treatment of the patients with ACE inhibitors likely lowered ACE-derived circulating angiotensin II levels, the possibility that increased plasma levels of TNF-α [17, 31] or endothelin contribute to an increased NAD(P)H oxidase activity and ASA-R in platelets warrants further studies.

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