Effect of nitroxyl on human platelets function

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Summary
There is growing body of evidence on the role of nitric oxide (NO) in human platelet physiology regulation. Recently, interest has developed in the functional role of an alternative redox form of NO, namely nitroxyl (HNO/NO2-), because it is formed by a number of diverse biochemical reactions. The aim of the present study was to comparatively analyze the effect of HNO and NO on several functional parameters of human platelets. For this purpose, sodium trioxodinitrate (Angel’s salt, AS) and sodium nitroprusside (SNP) were used as HNO and NO releasers, respectively. Both AS and SNP significantly inhibited platelet aggregation and ATP release induced by different agonists and adrenalone. AS or SNP did not modify the expression of platelet glycoproteins (Ib, IIb-IIIa, la-lla, IV), whereas they substantially decreased the levels of CD62P, CD63 and of PAC-1 (a platelet activated glycoprotein IIb/IIIa epitope) after the stimulation with ADP. AS and SNP significantly increased cGMP accumulation in a 1H-[1,2,4]oxadiazolo[4,3-a] quinoxalin-1-one (ODQ)-sensitive manner. However, while L-cysteine reduced the effect of AS, it increased the effect of SNP on this parameter. Accordingly, a differential effect of L-cysteine was observed on the antiaggregatory effect of both compounds. In summary, these results indicate that HNO is an effective inhibitor of human platelet aggregation.

Keywords
Nitroxyl, nitric oxide, platelet physiology

Introduction
There is a growing body of evidence on the role of NO in human platelet physiology regulation. In 1981, for the first time, it was described that nitrovasodilators and NO inhibit platelet aggregation probably through the stimulation of a soluble guanylyl cyclase and by increasing cGMP levels (1). The vascular endothelium and platelets synthesize NO from the terminal guanidino nitrogen atom(s) of L-arginine in a reaction catalyzed by NO synthase (NOS) in a Ca2+-dependent process (2, 3). An increase of NO levels exhibits antithrombotic properties by influencing the response of circulating platelets (4).

Recently, interest has developed in the functional roles of an alternative redox form of NO, namely nitroxyl (HNO/NO2-), because it is formed by a number of diverse biochemical reactions. Specifically, several authors have suggested that NOS produces nitroxyl rather than NO under certain conditions, particularly at low cofactor concentrations (5, 6), although this hypothesis has been challenged (7). Nitroxyl may also be produced from the oxidation of azide by peroxidase (8), by the decomposition of S-nitrosothiols in the presence of thiol (9), by the decomposition of peroxynitrite (10), and by the reduction of NO by ferrocychrome c (11).

Nitroxyl is the only electron reduction product of NO. The uncertainty on physicochemical relevant parameters (redox potential, pKa) during the past decades has led to misinterpretation of the distinct effects of NO and HNO/NO2- (12). More recently, orthogonal effects for both species on postischemic myocardial injury (13), and on cardiac inotropic and lusitropic action on failing hearts (14) have been described. Distinct chemical reactivity of both redox partners against hemeproteins (15), and thiol compounds (16) has also been shown and explains the molecular basis of their differential biological action. Furthermore, different mechanisms of action have been postulated for both species. While NO is known to act via a soluble guanylyl cyclase activation, it was suggested that nitroxyl activates a calcitonin gene-related peptide (CGRP) that results in cAMP accumulation (17). This means that even parallel effects can not be simply explained by the intracellular interconversion of both species. In 2001, Mondoro et al. compared the effects of NO on platelets from nor-
mal individuals and from patients with sickle cell disease by using what they mistakenly considered three NO-generating substances: sodium 2-(N,N-diethylamino)-diazenolate-2-oxide, S-nitrosocysteine and sodium trioxodinitrate (18). Soon after it was synthesized for the first time, it was reported that the sodium trioxodinitrate, formerly regarded as the sodium salt of the nitrohydroxaminic acid, decomposed into nitrous acid (HNO$_2$) and a species of formula NOH, “the aldehyde of nitrous acid” (19). In 1975, it was demonstrated by kinetic measurements that AS is an effective HNO releaser at 4 < pH < 8.5 ($t_{1/2}=2.5$ min), and only forms NO below pH 3 (20). Recently, the mechanism of aerobic Angelí’s salt (AS) decomposition at physiological pH was further explained (21).

In the present work, we comparatively investigated the effect of Angelí’s salt and SNP on several human platelet parameters, pointing both to aspects of the regulation of platelet physiology and to the growing area of the pharmacological potentiality of nitroxyl.

Materials and methods

Materials

Thrombin (Thr) was from Biopool, Ventura, CA, USA; adrenaline (ADR) was purchased from Diagnostica Stago, France; SNP, ADP, ATP, luciferin-luciferase (LL), arachidonic acid (AA), 3-isobutyl-1-methylxanthine (IBMX), and 1H-[1,2,4]oxadiazolo [4,3-a] quinoxalin-1-one (ODQ) were obtained from Sigma Chemical Co, St Louis, USA. Collagen was obtained from Horm, Germany. Isolow Buffer was from Coulter Corporation, USA. KT5720 was obtained from Biolog Life Science Institute (CA, USA) while KT5823 was purchased from Calbiochem (CA, USA). KT5720 and KT5823 were dissolved in dimethylsulfoxide to produce a stock solution (1 mM). On use, the stock solution was dissolved in Tyrode’s buffer giving a dimethylsulfoxide concentration no higher than 0.5% for final drug concentrations.

Washed platelets (WP)

A suspension of platelets was obtained from PRP by washing it twice in modified Tyrode’s buffer (138 mM NaCl, 2.9 mM KCl, 12 mM NaHCO$_3$, 0.4 mM NaH$_2$PO$_4$, 0.1% glucose, 0.35% bovine serum albumin (BSA), pH 6.5) with 50 ng/ml PGE$_1$ (23). The final resuspension was performed in modified Tyrode’s buffer, pH 7.3 with 1 mM CaCl$_2$. All manipulations were completed within 4 h of collection.

Functional studies

Platelet aggregation studies were performed with 450 µl of PRP or WP at 37°C with constant stirring in an optical aggregometer (Chronolog, Havertown, PA, USA) (24). To avoid decomposition of AS, freshly prepared solutions were stored on ice in the dark.

Figure 1: Effect of AS (panel A) and of SNP (panel B) on human platelet aggregation induced 2.5 µM ADP. After 2 min of incubation, both compounds significantly inhibited this parameter in a dose-dependent manner, with a threshold concentration of 1 µM. Data (% of aggregation 5 min after adding ADP) are the mean ± SEM of three independent experiments performed in triplicate. *p < 0.05, **p < 0.01 by Dunnett’s test.
until ready to be added to the platelets, usually no more than 10 min later.

To assess ATP-release from dense granules, the luciferin-luciferase assay in PRP was performed (25). Samples were incubated with buffer, AS, or SNP (both 3 μM) for 2 min at 37°C. Then, ATP-release was induced by ADP or AA. ATP-release was calculated by comparing the peak luminescence recorded from the sample against an ATP standard.

**Flow cytometry**

Flow cytometry was carried out with a FACSCalibur (Becton-Dickinson San Jose, CA, USA) flow cytometer, equipped with an air-cooled, argon-ion laser and operated at 15 mW power at a wave length of 488 nm. The instrument was calibrated for fluorescence and light scattering daily by using 2 μm Calibrite beads (Becton-Dickinson San Jose, CA, USA). Fluorescence intensity was measured by using a 530/30 band-pass filter of FL1 detector. Light scattering and fluorescence channels were set at logarithmic gain, and at least 10000 events were collected for each sample. After data gathering, a gate was set around the platelets for analysis of particle forward and right-angle light scattering and fluorescence. Data were analyzed on a Macintosh computer with CellQuest software. A negative control sample was stained with an isotype-matched non-specific monoclonal antibody.

Changes in the expression of platelet membrane markers were analyzed by MoAb binding to antigens that were present on resting or activated platelets with the mean fluorescence intensities (MFI) (26, 27).

To investigate the effect of HNO and NO on constitutive platelet glycoprotein expression, PRP was incubated with or without AS or SNP (10 μM) for 2 min; then 5 μl- aliquots were added to tubes containing 50 μl of Isoflow with saturating concentration of different anti-glycoproteins antibodies (CD42b, CD41, CD61, CD29, CD36). After 40 min of incubation at room temperature in the dark, the samples were diluted by adding 450 μl of Isoflow buffer and analyzed by flow cytometry. A similar assay was performed with WP using thrombin 0.5 U/ml as the agonist.

**Cyclic GMP levels assessment**

Platelets were incubated in 500 μl of buffer containing 140 mM NaCl, 5 mM KCl, 2.5 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, 10 mM Tris base (adjusted to pH 7.4 with HCl) with 0.5 mM IBMX for 30 min in the presence or absence of AS or SNP. After centrifuging for 10 min at 900 g, pellets were resuspended in distilled water and boiled for 2 min. Cyclic GMP content was measured in the supernatants by RIA as previously described (28). For this purpose, aliquots of samples or standards were acetylated with acetic anhydride/triethylamine. The acetylated samples and the standard curve were mixed with [125I]cyclic GMP (15,000–20,000 cpm, specific activity 140 mCi/mmol) and a rabbit antiserum (Chemicon, Temecula, CA, USA) diluted 1:150 and incubated overnight at 4°C. The antibody complex was precipitated with ethanol at 4°C using 2% bovine serum albumin as a carrier, centrifuged at 2,000 x g for 30 min, and separated by aspiring supernatants. The range of the standard curve was 10 – 5000 fmol of cGMP.
Results

Figure 1 depicts the effects of AS and SNP (0.5 – 40 µM) on human platelet aggregation induced by 2.5 µM ADP. AS and SNP significantly decreased this parameter, with a threshold concentration of 1 µM for both compounds. No antiaggregatory effect was observed (data not shown) when a solution of 3 µM AS was decomposed at 37°C (pH 7.4) for 60 min, and then added to a platelet suspension and preincubated for 2 min prior to adding ADP, or when platelets were preincubated in the presence of an equivalent concentration of sodium nitrite (NaNO₂). The inhibitory effect of AS and SNP was already evident at 2 min of incubation in the presence of ADP, whereas after 60 min of preincubation of the platelets with 3 µM AS prior to the addition of ADP, the inhibitory effect was not evident (Fig. 2).

To further examine the effect of AS on platelet aggregation, it was assessed in the presence of AA, ADR, Col and Thr. In the case of thrombin, WP were used. AS and SNP (both 3 µM) significantly inhibited platelet aggregation induced by 0.5 mM AA, 10 µM ADR, 1 µg/ml Col, and 0.5 U/ml Thr (Fig. 3).

After 2 min of incubation, ATP-release, triggered by ADP or AA, was completely inhibited by 3 µM AS or SNP as shown in figure 4, but not by 3 µM NaNO₂ (data not shown).

Flow cytometry studies were performed to evaluate the effect of AS on glycoprotein expression. There were no significant differences in the mean fluorescence intensity of CD42b (Ib), CD41 (Ila), CD61 (IIIa) CD29 (Ia) or CD36 (GP IV) among PRP incubated for 2 min with Tyrode buffer, AS, or SNP (10 µM) as shown in Table 1. Table 2 summarizes the effect of AS or SNP on CD62p, CD63, and PAC-1 levels in PRP incubated with ADP. Both compounds significantly inhibited the expression of these activation markers. Isotypic controls did not change after the stimulation with ADP in the presence or absence of AS or SNP.

Figure 5 shows the effect of AS or SNP (10 µM) on PRP cGMP accumulation in the presence of IBMX. Although SNP significantly decreased this parameter, with a threshold concentration of 10 µM, the inhibitory effect of AS and SNP was already evident (Fig. 2).

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Table 1: Effect of AS or SNP on platelet membrane glycoproteins (CD42b, CD41, CD61, CD29 and CD36). PRP was incubated for 2 min with or without both compounds (10 µM), and glycoprotein levels were assessed by flow cytometry, as described in Materials and Methods. These parameters were unchanged in the presence of AS or SNP. Values represent the MFI ± SD (n = 5).

Table 2: Effect of AS or SNP on CD62p, CD63 and PAC-1 levels. Platelets were incubated for 2 min in the presence of AS or SNP (10 µM), and then 2.5 µM ADP was added. The levels of these activation markers were assessed by flow cytometry as described in Materials and Methods. AS and SNP significantly reduced these parameters. Values represent the MFI ± SD (n = 5).
Bermejo et al.: Effect of nitroxyl on platelets

was more effective than AS, both compounds significantly increased this parameter. However, while an inhibitor of soluble guanylyl cyclase (ODQ) significantly decreased the effect of AS and SNP, the addition of 3 mM L-cysteine diminished the inhibition induced by SNP. Data (% of aggregation with respect to the maximal aggregation induced by ADP in control conditions, 5 min after adding the agonist) are the mean ± SEM (n= 10): a: p < 0.01 vs AS; b: p < 0.01 vs SNP; c: p < 0.01 vs AS plus cysteine; d: p < 0.05 vs AS plus ODQ; e: p < 0.01 vs SNP plus cysteine, f: p < 0.05 vs SNP plus ODQ, by Tukey’s test.

Discussion

These results suggest that nitroxyl derived from AS effectively inhibited human platelet aggregation in a rapid and concentration-dependent manner. In platelets incubated with AS a complete inhibition of ATP-release triggered by ADP or AA was observed. Furthermore, the activation marker (CD62P, CD63, and PAC-1) levels post-stimulation with ADP and SNP whereas KT5823 was ineffective. Data (% of aggregation with respect to the maximal aggregation induced by Thr in control conditions, 5 min after adding the agonist) are the mean ± SEM (n= 10): a: p < 0.01 as compared to AS; b: p < 0.01 vs SNP, by Tukey’s test.

Several reports point to a crucial role for NO in regulating platelet physiology (2–4). However, scientists are becoming aware that the effects elicited by the NO group can be better appreciated by recognizing the complex chemistry of this diatomic entity, dictated by the redox interconversion of the radical NO, the cation nitrosonium (NO$^+$), and the anion nitroxyl (HNO/NO$^-$). Several observations support the belief that HNO, and not
AS, is the chemical entity promoting the antiaggregatory effect as described herein:

- the time course of the AS effect is compatible with its half life as a HNO donor ($t_{1/2} = 2.5$ min) (22),
- after a 60 min-period of incubation, decomposed AS did not affect platelet aggregation and
- L-cysteine significantly prevented AS-mediated effect, as described in other systems (29, 30). Moreover, although it was postulated that HNO could cause loss of cell viability (31), the fact that the antiaggregatory effect of AS disappeared after a 60-min period of platelet preincubation, led us to discard irreversible or toxic effects of AS on human platelets.

In human platelets, both SNP and AS significantly increased cGMP accumulation in the presence of IBMX. Although the activation of a soluble guanylyl cyclase by NO is well-known, a similar effect for nitroxyl is subject to debate. A number of the effects of AS were shown to be cGMP independent (32), and several authors reported that NO is the only nitrogen monoxide redox form capable of directly activating soluble guanylyl cyclase (33, 34). In contrast, it was demonstrated that HNO elicits vasorelaxation in rabbit aorta and bovine intrapulmonary artery (35) and enhances human neutrophils migration (36) through a guanylyl cyclase-dependent pathway. Furthermore, the addition of a phosphodiesterase inhibitor enhances relaxation of urethral smooth muscle induced by AS, whereas guanylyl cyclase inhibitors almost abolished it (37). In human platelets, ODQ reduced the response of AS, supporting the belief that the effect of HNO is, at least in part, mediated through a cGMP-dependent mechanism. Although there is no ready explanation for the discrepancy in the link between cGMP and nitroxyl, it seems that cellular-type differences may account for it.

The effects of HNO have usually been explained by its conversion to NO (12). This hypothesis would have AS reproducing the behavior and all the effects elicited by NO. However, even though similar responses were elicited by NO and HNO in human platelets, the mechanism of action of these compounds seems to differ in some aspects. For example, although ODQ blocked the effect of both compounds on cGMP accumulation and on platelet aggregation, L-cysteine significantly inhibited the increase in cGMP levels induced by AS, but it increased the response provoked by SNP. As for platelet aggregation, L-cysteine partly blocked the effect of AS and increased the inhibitory effect of SNP. As already mentioned, the ability of L-cysteine to specifically block the effect of HNO was clearly demonstrated in a number of studies (29, 30). Indeed, L-cysteine was used to discriminate between NO and HNO by bioassay (30). The results obtained with SNP on human platelets are compatible with the evidence that L-cysteine increases the response of NO in several systems. In this sense, it was shown that L-cysteine enhances the relaxation of aortic rings induced by SNP and that this amino acid reduces the response provoked by AS (38). Taken together, these results confirm that the effects of nitroxyl cannot be merely attributed to its conversion to NO.

The fact that HNO and NO decreased human platelet levels of PAC-1, a monoclonal antibody used to measure the expression of the fibrinogen binding site to its platelet receptor, could suggest that the antiaggregatory effect of both compounds may be mediated through a signaling pathway that inactivates GPIIb-IIIa, as suggested by other groups (39, 40).

The inhibitory effects of NO on platelet activation have been considered to be due to activation of the soluble guanylyl cyclase causing production of cGMP and subsequent activation of PKG. However, elevation of cGMP levels could lead to an increase in cAMP (41, 42), causing activation of PKA. In addition, it was shown that PKG plays a biphasic role in platelet activation (43). Our results suggest that PKA, but not PKG, is involved in the inhibition of the thrombin-induced WP aggregation provoked by AS and SNP. Reflecting this, Jensen et al. (44) showed that PKA mediates inhibition of the thrombin-induced platelet shape change by NO, while Li et al. (45) have demonstrated a predominant role for PKA in cGMP-induced platelet inhibition. This conclusion does not exclude the possibility that PKG may be involved in the inhibition of platelet aggregation induced by other agonists. In fact, Jang et al. (46) reported that an inhibitor of PKA as well as a PKG inhibitor (to a lesser degree) partly reverses the effect of SNP on collagen induced platelet aggregation. However, PKG knockout or inhibitors have no effect on platelet aggregation induced by high concentrations of collagen (47). Thus, the role of PKG on platelet activation is still controversial and merits further investigation.

As mentioned before, several studies (6, 7) have shown that HNO can be synthesized by NOS isoforms in vitro. It remains unknown whether the HNO signaling observed in the present study can occur endogenously, or indeed, whether HNO is generated in vivo. This deficit is caused largely by the current lack of an assay for detecting HNO in vivo directly or even indirectly, although current efforts may provide methods to obtain such data in the future.

Despite the wide range of therapeutic options available for the whole spectrum of ischemic events, there is still a need for more efficacious and safe compounds. Present results support that HNO exhibits significant platelet aggregation inhibitory properties. Thus, regardless of whether its endogenous synthesis is ultimately confirmed or not, nitroxyl could be a new therapeutic strategy designed to inhibit platelet aggregation in atherothrombotic syndromes.

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