Platelet Nitric Oxide and Superoxide Release During the Development of Nitrate Tolerance

Effect of Supplemental Ascorbate

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Background—The therapeutic benefits that accompany the continuous administration of organic nitrates are attenuated by the development of tolerance to the compounds. Altered superoxide production and NO bioavailability have been implicated in contributing to the development of tolerance, an effect that may be ameliorated by the administration of antioxidants.

Methods and Results—We studied the effect of 3 days of continuous transdermal administration of nitroglycerin (NTG) (10 mg/24 hours) on platelet free radical (NO and superoxide anion [O$_2^-$] activity) with and without coadministration of supplemental ascorbate (2.4 g/24 hours). NAD(P)H oxidase activity, nitric oxide synthase (NOS) activity, and cyclic guanosine monophosphate (cGMP) content were also assessed. Radial artery pressure pulse waveforms were used to track the hemodynamic actions of NTG. Three days of NTG/placebo was associated with a significant increase in platelet NO and O$_2^-$ production from 1.0±1.7 to 2.52±0.88 pmol/10$^8$ platelets and 13.2±4.8 to 72.5±34.4 pmol/10$^8$ platelets, respectively (P<0.01 for both). These changes were accompanied by increased platelet NADH oxidase activity from 4.8 to 72.5 pmol O$_2^-$ min/mg protein and cGMP content from 0.60±0.10 to 0.89±0.16 pmol/10$^8$ platelets (P<0.05 for both). Administration of NTG/ascorbate attenuated both NO and O$_2^-$ release in platelets.

Conclusions—Three days of continuous transdermal administration of NTG was accompanied by increased platelet NO and O$_2^-$ production and NADH oxidase activity that was suppressed by coadministration of oral ascorbate. Although a significant degree of tolerance would be expected during continuous nitrate administration, a residual hemodynamic action could be identified by arterial pulse contour analysis. (Circulation. 2002;106:208-213.)

Key Words: nitroglycerin ■ platelets ■ nitric oxide

The therapeutic benefits of organic nitrates result from smooth muscle vasodilation in arteries and veins that accompanies administration of these agents.¹ Exogenous organic nitrates react with thiol groups in smooth muscle cells, resulting in denitration and oxidation of the thiols to disulphide.² The reaction facilitates the generation of the reactive intermediates NO and s-nitrosothiols that activate guanylate cyclase, enhance production of cGMP, and reduce available intracellular calcium to produce vasodilation.³ The rapid development of tolerance associated with continuous nitrate administration extends beyond their vascular actions, because abnormal platelet activation can also accompany nonintermittent prescription of these compounds.⁴,⁵

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Although experimental and clinical evidence implicates several mechanisms involved in the development of tolerance to organic nitrates,⁶⁻⁹ recent studies have highlighted an important role for enhanced superoxide production in contributing to the development of tolerance. It has been proposed that decreased bioavailability of NO, derived from both endogenous sources and NTG, occurs as a consequence of the interaction of NO with superoxide and contributes to the development of tolerance. Enhancing antioxidant status and the scavenging of O$_2^-$ with ascorbate has been shown not only to attenuate the development of hemodynamic tolerance⁴,⁵,¹² but also the platelet activation accompanying continuous nitrate administration.⁴,⁵ These indirect observations suggest that reactive oxygen species O$_2^-$ and NO influence cellular redox-sensitive signaling cascades that play an important role in blunting the therapeutic efficacy of organic nitrates. Because the fate of NO and O$_2^-$ are inextricably linked,¹³ the altered activity of the free radicals may provide a unifying mechanism to account for the abnormal platelet activation and diminished vascular actions found in the setting of nitrate tolerance.
Platelets, like endothelial cells, contain the l-arginine–NOS pathway and membrane-bound oxidases that use NAD(P)H as substrates.14,15 Platelets have long been used as an accessible alternative to vascular myocytes for functional studies,16,17 and, because they share similar contractile regulation systems with vascular smooth muscle,18,19 activity of the NO pathway and superoxide generation in platelets may relate to changes in smooth muscle tone. This compartmentalized approach permits study of platelet NO and O$_2^·$ activity and the relationship between platelet free radical activity and the development of vascular tolerance in response to continuous nitrate administration.

Methods

Participants and Procedures

Healthy male volunteers (n=10) aged 23 to 48 years were recruited for study. All subjects underwent a full history and examination that included an ECG. No subjects were taking any drugs or vitamin supplements before or at the time of the study. All subjects gave written informed consent for all procedures. The studies were approved by the local Ethical Committee of the Queen’s University of Belfast.

Subjects entered a placebo-controlled crossover trial with random allocation of treatments. Subjects refrained from consuming alcohol, tobacco, or caffeine for 12 hours before each study day. On the first study day, each subject had radial pressure pulse waveforms recorded and blood sampling for laboratory analysis. Subjects were randomized to receive 1.2 g ascorbate or lactose (placebo) in a 209 mg/mL Tyrode’s-HEPES buffer, and 10 µL lucigenin (1800 µmol/L) was added to produce a final concentration of 20 µmol/L. The sample was allowed to dark adapt at 20°C for 5 minutes before being inserted into a Sirius Luminometer (Berthold Detection Systems), and the luminescence was recorded. After a 3-second delay, the platelets were activated by the addition of 100 µL of phorbol 12-myristate 13-acetate (10 µmol/L). The assay was calibrated with xanthine/xanthine oxidase (0 to 400 pmol) to generate known amounts of O$_2^·$. Samples were also analyzed in the presence of L-NAME (1 mmol/L) to allow for nitric oxide scavenging of O$_2^·$, SOD (200 u/mL) to confirm the identity of O$_2^·$, and diphenyliodonium and quinacrine (100 µmol/L and 1.0 mmol/L) to inhibit the NAD(P)H oxidases producing O$_2^·$.

A subset of experiments (n=5), superoxide production was also examined in the presence of oxyturpinol (100 µmol/L), rotenone (100 µmol/L), or indomethacin (10 µmol/L) to assess the effects of xanthine oxidase, mitochondrial respiration, and cyclo-oxygenase activity on the production of O$_2^·$.

NADH/NADPH Oxidase Activity

NADH and NADPH oxidase activity was measured according to the method described by Rajagopalan et al.25 who demonstrated that this assay specifically measures NAD(P)H oxidase–generated superoxide. Briefly, platelets were homogenized by freeze and thawing, as described above. Homogenate 20 µL was added to 900 µL Tyrode’s HEPES–buffered saline containing 20 µmol/L lucigenin, and the sample tube was placed in the luminometer. Allowance was made for basal luminescence, and enzyme activity was calculated as pmol O$_2^·$ min/10$^8$ cell.

NO Synthase Activity

NO synthase activity was measured in platelets as described by Radomski et al.26 NOS activity was measured by quantifying the rate of formation of [14C]-citrulline from l-[U-14C] arginine. Results were expressed as pmol citrulline/min per mg protein.

cGMP

The platelet pellet was washed twice with PBS containing 5 mmol/L EDTA and 0.1 mmol/L isobutyl-methylxanthine. cGMP was extracted from platelets into ice-cold 10% (wt/vol) trichloroacetic acid (TCA). The extracted cGMP was dried on a rotary evaporator and measured using a commercially available enzyme immunoassay kit (Biotrak, Amersham Biotech).

Ascorbate

Ascorbate concentrations in plasma were measured using a HPLC method with electrochemical detection, as described by Speek et al.27

Data Analysis

Standard methods for the analysis of 2-period crossover trials were used.28 This technique provides a comparison of ascorbate and placebo effects adjusted for any period effects and also gives a test
for treatment by period interaction. The response variable for the analysis was taken as the change from baseline. Paired t tests were used to compare results at the end of each period with the respective baseline measures. Correlations between variables were sought using Pearson’s correlation coefficient. Results are expressed as mean 

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**Results**

**Hemodynamic Measurements**

No significant change in systolic, diastolic, or mean arterial pressure or heart rate occurred in response to 3 days of administration of NTG/placebo or NTG/ascorbate (Table 1). By contrast, a significant effect of NTG was apparent on baseline; **P<0.01 vs baseline.**

**NO Production**

**Measured Directly as NO**

NTG/placebo increased activated platelet NO release from 1.00±0.17 at baseline to 2.52±0.88 pmol/10⁸ platelets

\( P<0.01, \) Figure 2. NTG/ascorbate decreased NO release from 1.30±0.28 at baseline to 0.87±0.25 pmol/10⁸ platelets \( P<0.05. \) NO release differed significantly when the response to NTG/placebo was compared with NTG/ascorbate (+1.39±0.77 for NTG/placebo versus −0.42±0.22 for NTG/ascorbate, ANOVA treatment effect \( P<0.001).\)

**Measured Indirectly as Nitrite**

NTG/placebo increased platelet NO production, assessed as nitrite, from 37.4±19.1 at baseline to 62.5±25.7 pmol/10⁸ platelets \( P<0.01, \) Figure 3. NTG/ascorbate did not influence nitrite production \( 31.1±13.1 \) at baseline versus \( 26.9±9.4 \) pmol/10⁸ platelets, \( P=NS.\) The comparison of nitrite response to NTG/placebo and to NTG/ascorbate differed significantly (ANOVA treatment effect \( P<0.05).\) Nitrite concentration measured at each baseline was not different between groups. The overall correlation between platelet nitrite concentration and NO release was \( r=0.70 \) \( P<0.01 \) Figure 4).

**Superoxide Production**

NTG/placebo significantly increased \( \text{O}_2^- \) production from activated platelets from 13.2±4.8 at baseline to 72.5±34.4 pmol/min per 10⁸ platelets \( P<0.01, \) Figure 5. NTG/ascorbate decreased \( \text{O}_2^- \) production from 25.2±10.7 at baseline to 10.0±4.4 pmol/min per 10⁸ platelets \( P<0.05).\) The \( \text{O}_2^- \) response to NTG/placebo and to NTG/ascorbate differed significantly (ANOVA treatment effect \( P<0.001).\) Incorporation of \( \text{l-NAME} \) in platelet samples increased the amount of \( \text{O}_2^- \) detected by 43% and 44% at each baseline, 35% with NTG/ascorbate, and 76% NTG/placebo \( P<0.05 \) versus baseline and NTG/ascorbate. Incorporation of \( \text{DPI} \) and quinacrine completely abolished \( \text{O}_2^- \) production, whereas oxyurinol, rotenone, and indomethacin had no effect on \( \text{O}_2^- \) production.
NOS, NADH, and NADPH Oxidase Activity

No changes were found in platelet NOS activity in subjects after NTG/placebo or NTG/ascorbate. In platelet homogenates from individuals treated with NTG/placebo, addition of NADH increased O$_2^\cdot$ production from 47.9 ± 11.0 at baseline to 65.3 ± 13.6 pmol O$_2^\cdot$/min per mg protein (P<0.05, Figure 6), whereas no change was detected in samples from individuals treated with NTG/ascorbate (54.6 ± 16.8 versus 52.8 ± 16.7). No changes were evident in NADPH-dependent activity after treatment with either NTG/placebo or NTG/ascorbate. Incorporation of diphenyliodonium and quinacrine into reaction mixtures reduced enzyme activity (NADH and NADPH oxidase) by ~75%. Oxypurinol, rotenone, and indomethacin had no effect on NADH or NADPH oxidase activity.

cGMP

An increase in platelet cGMP content accompanied increased NO production. NTG/placebo increased cGMP from 0.69 ± 0.10 to 0.89 ± 0.16 pmol/10$^9$ platelets, P<0.05 (Figure 7). No change in platelet cGMP content was found after NTG/ascorbate.

Plasma Ascorbate

All individuals receiving NTG/ascorbate showed an increase in plasma ascorbate from 12.2 ± 0.7 to 21.7 ± 4.7 μg/mL, P<0.01. Treatment with NTG/placebo did not alter ascorbate levels compared with baseline values.

Discussion

This is the first study to examine tissue O$_2^\cdot$ production and endogenous L-arginine–NOS-derived NO, with and without dietary ascorbate supplementation, in response to clinically relevant doses of organic nitrates in humans. Three days of transdermal NTG resulted in increased platelet NO and O$_2^\cdot$ production, increased NADH oxidoreductase activity, and platelet cGMP content without alteration in NADPH oxidoreductase or NOS activity. Coadministration of ascorbate with NTG significantly decreased O$_2^\cdot$ and NO production, whereas NAD(P)H oxidoreductase, NOS activity, and platelet cGMP content remained unchanged. Although a significant degree of tolerance had undoubtedly occurred in response to continuous nitrate administration, a residual hemodynamic action of transdermal NTG could be identified by pulse contour analysis.

Recent attention has focused on the role of O$_2^\cdot$ and the ability of NTG to influence activity of vessel wall–associated NAD(P)H oxidoreductases that generate the superoxide anion. Dikalov et al$^{29}$ have shown in a rabbit model that increased O$_2^\cdot$ production induced by organic nitrates correlated with the development of tolerance and that supplemental ascorbate provided effective protection against nitrate-induced superoxide radical formation. We found transdermal NTG for 3 days was associated with a 450% increase in O$_2^\cdot$ when administered with placebo and a 60% decrease in O$_2^\cdot$.

Figure 3. Platelet nitrite at baseline and after 72 hours of nonintermittent transdermal nitroglycerin coadministered with placebo or ascorbic acid. Data are mean±SEM. **P<0.01 vs baseline.

Figure 4. Correlation between total nitrite and NO in platelets.

Figure 5. Platelet superoxide release at baseline and after 72 hours nonintermittent transdermal nitroglycerin coadministered with placebo or ascorbic acid. Data are mean±SEM. *P<0.05 vs baseline; **P<0.01 vs baseline.

Figure 6. Platelet NADH oxidase activity at baseline and after 72 hours of nonintermittent transdermal nitroglycerin coadministered with placebo or ascorbic acid. Data are mean±SEM. *P<0.05 vs baseline.
production when coadministered with ascorbate. Results of additional experiments excluded redox cycling of lucigenin as a source of superoxide and also excluded a role for xanthine oxidase, mitochondrial respiration, and cyclo-oxygenase activity in contributing to superoxide production.

The increase $O_2^-$ production and suppression of $O_2^-$ production with dietary ascorbate supplementation in platelets mirrors previously described findings in vascular smooth muscle in the setting of nitrate tolerance.30 Bassenge et al4 found oral ascorbate supplementation completely prevented the development of hemodynamic tolerance and upregulation of platelet activity in healthy volunteers. With the treatment and dosing regimes used in our subjects, a residual hemodynamic action of organic nitrates could be identified that was unaffected by oral ascorbate administration. We and other groups have previously shown arterial waveform analysis represents a particularly sensitive method to detect and monitor the hemodynamic action of organic nitrates in altering the geometry and distensibility of peripheral blood vessels.4,20,21,31,32 The residual hemodynamic effect detected by this methodology could not be identified by monitoring changes in heart rate or blood pressure in response to nitrate administration. The discrepancy between our findings and those of Bassenge et al4 may relate to differences in data acquisition and descriptive analysis of the morphological change in the pulse contour accompanying nitrate administration.

A novel finding was the demonstration of a concomitant increase in platelet NO ($\approx 150\%$) associated with enhanced $O_2^-$ production during continuous nitrate administration and decrease in NO ($\approx 33\%$) accompanying a reduction in $O_2^-$ production with ascorbate coadministration. The incorporation of L-NAME in platelet samples resulted in a significant increase in $O_2^-$ production after NTG/placebo compared with values recorded at baseline or after NTG/ascorbate. The findings are consistent with less scavenging of $O_2^-$ because of inhibition of NOS activity and release of NO. Freedman et al,22 using similar technology and methods of platelet activation, describe comparable platelet NO concentrations and report similar trends in platelet-derived NO in response to SOD and $O_2^-$ in response to NOS synthase inhibition. Measured nitrite provides an estimate of total NO production less that reacting with peptide and thiol groups.33 We calculated that $\approx 3\%$ of NO released was detected at the electrode surface and were able to demonstrate a high degree of correlation between direct and indirect methods of detection.

In animal models and tissue culture systems, stimulation and inhibition of NOS activity accompanying the administration of organic nitrates has been documented.34,35 We found no change in platelet NOS activity in the present studies. A decrease in platelet steady-state cGMP has been previously described in response to the continuous administration of organic nitrates.7,12 In the present studies, platelet cGMP concentration increased in tandem with NO release in response to continuous nitrate administration, whereas NO release was suppressed and cGMP concentration remained unchanged with coadministration of ascorbate. Although methodological considerations and differing treatment regimens may account for the discrepant findings, Martin et al36 have highlighted that platelet cGMP levels used either as an index of NTG effect or as a biochemical marker of tolerance require cautious interpretation.

Platelets exposed to clinically relevant concentrations of organic nitrates provide a convenient compartmentalized tissue model that permits study of NO/O$_2^-$ activity and interactions that have been implicated in contributing to the development of tolerance to the compounds. Increased production of $O_2^-$ and NO accompanied the continuous administration of NTG, and these changes were effectively suppressed by oral ascorbate supplementation. Additional studies are needed to firmly establish links between changes in platelet NO and $O_2^-$ production and hemodynamic tolerance to organic nitrates.

References


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