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 \([\text{O}_2^-]\)) 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 \([\text{O}_2^-]\) production from 1.0±1.7 to 2.52±0.88 pmol/10^9 platelets and 13.2±4.8 to 72.5±34.4 pmol/10^9 platelets, respectively (\(P<0.01\) for both). These changes were accompanied by increased platelet NADH oxidase activity from 47.9±11.0 to 65.3±13.6 pmol \([\text{O}_2^-]\) min/mg protein and cGMP content from 0.60±0.10 to 0.89±0.16 pmol/10^9 platelets (\(P<0.05\) for both). Administration of NTG/ascorbate attenuated both NO and \([\text{O}_2^-]\) release in platelets.

Conclusions—Three days of continuous transdermal administration of NTG was accompanied by increased platelet NO and \([\text{O}_2^-]\) 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,\(^1,6-9\) 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 \([\text{O}_2^-]\) with ascorbate has been shown not only to attenuate the development of hemodynamic tolerance\(^4,5,12\) but also the platelet activation accompanying continuous nitrate administration.\(^4,5\) These indirect observations suggest that reactive oxygen species \([\text{O}_2^-]\) 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 \([\text{O}_2^-]\) 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.\textsuperscript{14,15} Platelets have long been used as an accessible alternative to vascular myocytes for functional studies,\textsuperscript{16,17} and, because they share similar contractile regulation systems with vascular smooth muscle,\textsuperscript{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\textsubscript{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 double-blind fashion. Nitro-Dur transdermal nitroglycerin patches (0.4 mg/h) were applied over the deltoid area and replaced at 12 hourly intervals. Each subject received medication and wore the patches for 3 consecutive days. Subjects then returned to the department, and study protocol was repeated in an identical fashion to baseline studies. After a 2-week washout period, subjects again attended the department for repeat baseline hemodynamic studies and blood sampling. Subjects then received the oral medication not administered previously and had Nitro-Dur patches reapplied for 3 days and hemodynamic measurements and blood sampling repeated as described above.

**Hemodynamic Measurements**

Radial artery pressure pulse waveforms were recorded by an acoustic transducer using the principle of applanation tonometry (HDI pulse-wave CR-2000; Hypertension Diagnostics Inc), as previously described.\textsuperscript{20}

Noninvasive radial artery waveforms were recorded for 30 seconds for each subject in the supine position. Blood pressure waveforms were digitized at 200 samples/second, and those with a correlation coefficient of <0.95 were discarded. A printout of an average waveform representative of the 30-second data set was analyzed quantitatively using a modification of the method described by Imhof et al.\textsuperscript{21} An A/B ratio was calculated by measuring peak and trough heights on the average waveform generated from the pulse waveform analysis. A was measured as the height of the systolic peak and B as the height of the trough preceding the dicrotic notch. From analysis of these and other data sets, recorded in response to the acute and chronic administration of NTG, we have found the modified A/B ratio to represent the most sensitive means to quantify a hemodynamic action of organic nitrates.

**Measurement of NO Release**

Gel-filtered platelets were made up to 3×600 μL aliquots. NO release was measured using a NO-selective microelectrode (ami NO-700, Innovative Instruments Inc) and an amplifier (inNO meter, Innovative Instruments). Platelet NO production was quantified as the integrated signal detected by the microelectrode after platelet activation, as previously described.\textsuperscript{22} The electrode was calibrated by producing standardized concentrations of NO in 0.5% (wt/vol) KI in 0.1 mol/L H\textsubscript{2}SO\textsubscript{4} from NaN\textsubscript{3} standards. NO release was quantitated as the current detected at the electrode after platelet activation at 37°C in the presence of 2 mmol/L Ca\textsuperscript{2+}, 2 mmol/L Mg\textsuperscript{2+}, 5 mmol/L ADP, and 300 μg/ml fibrinogen. NO release was calculated as pmol/10\textsuperscript{7} platelets. NO production was also measured indirectly by measuring nitrite content in the supernatant. Nitrite was measured in 0.5% (wt/vol) KI in 0.1 mol/L H\textsubscript{2}SO\textsubscript{4} using the NO-specific electrode as described above. Nitrite was expressed as nmol/10\textsuperscript{7} platelets.

**Measurement of Superoxide Production**

Released O\textsubscript{2}– was measured by enhanced chemiluminescence detection using lucigenin as a luminescent substrate, as described by Gyllenhammar\textsuperscript{23} and modified by Skatchkov et al.\textsuperscript{24} Briefly, GFPs were made up to a volume of 900 μL in 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 Lumimeter (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\textsubscript{2}–. Samples were also analyzed in the presence of L-NAME (1 mmol/L) to allow for nitric oxide scavenging of O\textsubscript{2}–. SOD (200 u/mL) was added to produce a final concentration of 200 μmol/L to confirm the identity of O\textsubscript{2}– and diphenyliodonium and quinacrine (100 μmol/L) and 1.0 mmol/L to inhibit the NAD(P)H oxidases producing O\textsubscript{2}–. In a subset of experiments (n=5), superoxide production was also examined in the presence of oxypranol (100 μmol/L), rotenone (100 μmol/L), or indoethacin (10 μmol/L) to assess the effects of xanthine oxidase, mitochondrial respiration, and cyclo-oxygenase activity on the production of O\textsubscript{2}–.

**NADH/NADPH Oxidase Activity**

NADH and NADPH oxidase activity was measured according to the method described by Rajagopalan et al.\textsuperscript{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 lumimeter. Allowance was made for basal luminescence, and enzyme activity was calculated as pmol O\textsubscript{2}–/min per mg protein. Samples were also assayed in the presence of diphenyliodonium and quinacrine (100 μmol/L and 1.0 mmol/L, respectively) to inhibit the NAD(P)H oxidases producing O\textsubscript{2}–.

**NO Synthase Activity**

NO synthase activity was measured in platelets as described by Radomski et al.\textsuperscript{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 isobutyly-methylxanithine. 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.\textsuperscript{27}

**Data Analysis**

Standard methods for the analysis of 2-period crossover trials were used.\textsuperscript{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 ±SEM. **P<0.01 vs baseline.

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 pulse waveform morphology manifest as a significant increase in the A/B ratio (Figure 1). The ratio increased from 2.5±0.1 to 3.4±0.3 with NTG administration and from 2.6±0.2 to 3.9±0.4 with coadministration of ascorbate (P<0.01 versus respective baselines). No significant difference in response was apparent when comparing the effect of NTG alone and NTG with oral ascorbate supplementation (ANOVA treatment effect P=0.86).

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/10^8 platelets (P<0.01, Figure 2). NTG/ascorbate decreased NO release from 1.30±0.28 at baseline to 0.87±0.25 pmol/10^8 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/10^8 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/10^8 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 O_2^- production from activated platelets from 13.2±4.8 at baseline to 72.5±34.4 pmol/min per 10^8 platelets (P<0.01, Figure 5). NTG/ascorbate decreased O_2^- production from 25.2±10.7 at baseline to 10.0±4.4 pmol/min per 10^8 platelets (P<0.05). The O_2^- response to NTG/placebo and to NTG/ascorbate differed significantly (ANOVA treatment effect P<0.001). Incorporation of l-NAME in platelet samples increased the amount of 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 DPI and quinacrine completely abolished O_2^- production, whereas oxyquinol, rotenone, and indomethacin had no effect on O_2^- production.

Figure 1. Representative change in the radial artery pulse contour morphology before (A) and after (B) 3 days of NTG/placebo in 1 volunteer. Plots of change in the A/B ratio (C) in response to 3 days of NTG/placebo and NTG/ascorbate in the study subjects. All values are mean±SEM. **P<0.01 vs baseline.

Figure 2. Platelet NO production 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; **P<0.01 vs baseline.
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 $\text{O}_2^\cdot$ production from 47.9/11006/11006 to 65.3/11006/11006 pmol O2/11006/11006/min per mg protein ($P<0.05$, Figure 6), whereas no change was detected in samples from individuals treated with NTG/ascorbate (54.6/11006/11006 versus 52.8/11006/11006). 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 0.69/11006/11006 to 0.89/11006/11006 pmol/109 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 12.2/11006/11006 to 21.71/11006/11006/4.7/9262/4.7/9262 g/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 $\text{O}_2^\cdot$ 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 $\text{O}_2^\cdot$ production, increased NADH oxidoreductase activity, and platelet cGMP content without alteration in NADPH oxidoreductase or NOS activity. Coadministration of ascorbate with NTG significantly decreased $\text{O}_2^\cdot$ 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 $\text{O}_2^\cdot$ and the ability of NTG to influence activity of vessel wall–associated NAD(P)H oxidoreductases that generate the superoxide anion. Dikalov et al have shown in a rabbit model that increased $\text{O}_2^\cdot$ 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 $\text{O}_2^\cdot$ when administered with placebo and a 60% decrease in $\text{O}_2^\cdot$.
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 calcu-


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