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Nitrate Resistance In Platelets From Patients With Stable Angina Pectoris

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Background—Hemodynamic resistance to nitrates has been previously documented in congestive heart failure. In patients with stable angina pectoris (SAP), we have observed a similar phenomenon: decreased platelet response to disaggregating effects of nitroglycerin (NTG) and sodium nitroprusside (SNP).

Methods and Results—In blood samples from normal subjects (n=32) and patients with SAP (n=56), we studied effects of NO donors (NTG and SNP) on ADP-induced platelet aggregation and on intraplatelet cGMP. NTG and SNP inhibited platelet aggregation in patients to lesser extents than in normal subjects ($P<0.01$). The cGMP-elevating efficacy of NTG and SNP was diminished in platelets from patients in comparison with those from normals ($P<0.001$). Inhibition of the anti-aggregatory effects of NTG and SNP by ODQ, a selective inhibitor of NO-stimulated guanylate cyclase, was significantly less pronounced in patients than in normal subjects. Content of O_2^- was higher in blood samples from patients than in those from normal subjects ($P<0.01$). In blood samples from patients with SAP, but not in normal subjects, the O_2^- scavenger superoxide dismutase (combined with catalase) suppressed platelet aggregation ($P<0.01$) and increased the extent of anti-aggregatory effect of SNP ($P<0.01$).

Conclusions—In patients with SAP, platelets are less responsive to the anti-aggregating and cGMP-stimulating effects of NO donors; this may reflect both reduction in guanylate cyclase sensitivity to NO and inactivation of the released NO by O_2^- . The implied impairment of anti-platelet efficacy of endogenous NO (in the form of EDRF) may contribute to platelet hyperaggregability associated with angina pectoris. (*Circulation*. 1999;100:129-134.)

Key Words: angina ■ platelet aggregation inhibitors ■ nitroglycerin

The organic nitrates such as nitroglycerin (NTG) are in widespread use for the treatment of both acute and chronic myocardial ischemia, as well as congestive heart failure. Until recently, it was assumed that their therapeutic efficacy was entirely secondary to relaxation of vascular smooth muscle.^{1,2} However, findings of several studies have indicated that pharmacological effects of nitrates include inhibition and reversal of platelet aggregation.^{3,4} The major limiting factor to the clinical utility of nitrates has been the induction of nitrate tolerance by virtue of chronic continuous nitrate therapy. This phenomenon has been documented at vascular⁵ and platelet levels.⁶ However, poor hemodynamic responsiveness to nitrates may also occur on a de novo basis (ie, independent of any prior nitrate therapy), particularly in patients with heart failure; this has been termed nitrate resistance.⁷ We have previously documented the occurrence of diminished anti-aggregatory effects of NTG and sodium nitroprusside (SNP) in platelet-rich plasma from patients with stable angina pectoris (SAP).^{8,9} However the extent and mechanism(s) of this phenomenon, which is apparently analogous to that of nitrate resistance in the vasculature, have not been investigated. Furthermore, we have documented in-

creased platelet aggregability in platelet-rich plasma obtained from patients with SAP.^{8,9} The relation between this hyperaggregability and reduced platelet response to NTG and other NO donors has not been examined.

The anti-aggregating effect of NTG and other nitrovasodilators is mediated via formation of NO, which activates platelet guanylate cyclase, leading to generation of cGMP [for review see 10]. Although the effects of NTG are mediated primarily by enzymatic thiol-dependent bioconversion to NO, SNP is a more direct NO donor.^{11,12} Therefore, reduced sensitivity to both NTG and SNP suggests reduction in responsiveness to NO. Furthermore, in our previous studies^{8,9} we observed a strong interrelationship between cGMP-stimulating and anti-aggregating effects of NTG and SNP: a decreased platelet sensitivity to the anti-aggregatory effects of NTG and SNP was associated with a decrease in intraplatelet cGMP accumulation in response to these NO donors. As the intracellular cGMP level reflects both generation of cGMP by guanylate cyclase and hydrolysis of cGMP by cyclic nucleotide phosphodiesterases (PDE), the input of both enzymes in the observed phenomenon needs to be investigated. Possible impairment of platelet guanylate cy-

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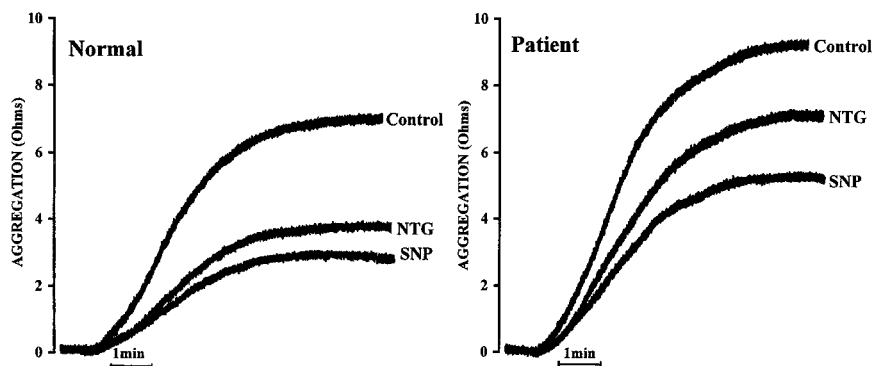


Figure 1. Representative tracings for inhibition of ADP ($1 \mu\text{mol/L}$)-induced aggregation by nitroglycerin ($100 \mu\text{mol/L}$, NTG) and sodium nitroprusside ($10 \mu\text{mol/L}$, SNP) in whole blood samples obtained from a normal subject and patient with stable angina pectoris.

class activity in patients with SAP has been tested in our previous study⁹; there were no indications of any dysfunction of the enzyme. However, the interaction of guanylate cyclase with NO and availability of NO for enzyme activation have not been examined. Regarding the latter issue, the decreased responsiveness of the platelet cGMP-system to NTG and SNP could be due to increased clearance of NO, by superoxide anion radical (O_2^-), the concentration of which is elevated in some cardiovascular disease states.¹³⁻¹⁵

This study was designed to investigate further the phenomenon of nitrate resistance in platelets. In blood samples obtained from normal subjects and patients with SAP, we studied the anti-aggregating and cGMP-elevating effects of NTG and SNP. We also assessed the influence of a PDE inhibitor (3-isobutyl-1-methyl-xanthine, IBMX), a selective inhibitor of NO-stimulated guanylate cyclase activity (1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one, ODQ). Possible interactions between O_2^- and responses to NO donors were studied via measurement of O_2^- content and by examination of effects of an O_2^- scavenger superoxide dismutase (SOD) on platelet responsiveness to NO donors.

Methods

Subjects

Studies were performed in the following groups: 1) normal subjects ($n=32$; 21 men and 11 women) aged 23 to 75 years, mean 48 years) not taking any medication affecting platelet aggregation; and 2) patients with SAP ($n=56$; 37 men and 19 women aged 34 to 76 years, mean 60 years) undergoing diagnostic cardiac catheterization and coronary angiography. In all cases at least one hemodynamically significant ($\geq 50\%$) stenosis was present in a major coronary artery; a background aspirin and nitrate medication profile was recorded at recruitment.

Numbers of subjects used in individual experiments are indicated below (see Results). In all cases, blood samples were withdrawn for in vitro platelet aggregation and intraplatelet cGMP assay. The protocol was approved by the Ethics of Research Committee of The Queen Elizabeth Hospital; written informed consent was obtained before study entry.

Blood Sampling and Preparation of Platelets

Blood samples from patients undergoing cardiac catheterization were withdrawn during the procedure via a femoral arterial sheath; blood was drawn from other patients and normal volunteers by venesection from an antecubital vein. It has been shown^{8,16} that there is no arteriovenous difference in platelet function. Blood was collected in plastic tubes containing 1:10 volume of acid citrate anticoagulant (2 parts of 0.1 mol/L citric acid to 3 parts of 0.1 mol/L trisodium citrate); acidified citrate was used in order to minimize deterioration

of platelet function during experiments.¹⁷ Blood was centrifuged at 250g for 10 minutes at room temperature to obtain platelet-rich plasma. Platelet-poor plasma was prepared by further centrifugation of the remaining blood at 2500g for 20 minutes. Platelet counts were performed on the STKS Coulter Counter (Coulter Electronics Inc) and the platelet-rich plasma was adjusted with platelet-poor plasma to a constant count of $250\,000/\mu\text{L}$.

Platelet Aggregation Studies

Aggregation in whole blood and platelet-rich plasma was examined using a dual-channel impedance aggregometer (Model 560, Chrono-Log). Tests were performed at 37°C and stirring speed of 900 rpm. Samples of blood or platelet-rich plasma were diluted 2-fold with normal saline (final volume 1 mL) and prewarmed for 5 minutes at 37°C . Aggregation was induced with adenosine 5'-diphosphate (ADP) (final concentration of $1 \mu\text{mol/L}$) in experiments with whole blood and $0.5 \mu\text{mol/L}$ ADP with platelet-rich plasma. Aggregation was monitored continually for 7 minutes, and responses were recorded (RO-3 Rikadenki chart recorder) for electrical impedance, in ohms. SNP and NTG (final concentration of 10 and $100 \mu\text{mol/L}$, respectively) were added to samples 1 minute before ADP. SOD and catalase (final concentration of 300 U/mL for both enzymes) were added immediately before NTG or SNP. 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ) ($1 \mu\text{mol/L}$) was added 5 minutes before NTG or SNP. The duration of incubations were estimated as those optimal in preliminary experiments (data not shown). In control tests, physiological saline was added in appropriate volumes. Inhibition of aggregation was evaluated as a percentage comparing the extent of maximal aggregation in the presence and absence of the anti-aggregatory agent studied. Representative aggregograms are shown in Figure 1.

cGMP Studies

Platelet-rich plasma (0.5 mL) was incubated at 37°C with SNP ($10 \mu\text{mol/L}$) for 2.5 minutes or with NTG ($100 \mu\text{mol/L}$) for 5 minutes. ODQ ($1 \mu\text{mol/L}$) and IBMX (0.5 mmol/L) were added to plasma 5 minutes before NTG or SNP. Intraplatelet cGMP content was assayed as described previously.⁸ Briefly, after incubation plasma was filtered through GF/C Glass Microfibre Filters (Whatman) for harvesting the platelets. Filters with absorbed platelets were rinsed with physiological saline and placed into 0.5 mL of 4 mmol/L EDTA for further extraction of cGMP in a boiling water bath for 5 minutes. After centrifugation of samples at 3000g for 10 minutes, cGMP concentration in supernatant was estimated using "cGMP [^{125}I] assay system" (Amersham). Results were expressed as pmol cGMP/ 10^9 platelets.

Chemiluminescence Assay of O_2^-

Detection of O_2^- in whole blood was performed using a chemiluminescence technique,¹⁸ with lucigenin as a probe for O_2^- . Blood samples were diluted 2-fold with normal saline (final volume 1 mL) and prewarmed for 5 minutes at 37°C before the addition of lucigenin (final concentration $125 \mu\text{mol/L}$). Chemiluminescence was monitored using a photoluminometer component of a dual-channel lumi-aggregometer (Model 560, Chrono-Log) equipped with a com-

Platelet Aggregation (Ohms) in Response to 1 $\mu\text{mol/L}$ ADP in Whole Blood

Subjects	Normals		Stable Angina	
	-ASA	-ASA	-ASA	+ASA
Men	7.8 \pm 0.7 (21)	12.9 \pm 1.6† (13)	11.0 \pm 1.1 (24)	
Women	9.8 \pm 0.6 (11)	13.9 \pm 1.8* (7)	12.8 \pm 1.5 (12)	

Samples from normal subjects and patients with stable angina pectoris receiving or not receiving aspirin (+/-ASA).

Number of subjects indicated in parentheses.

* $P < 0.05$ and † $P < 0.01$ for patients not receiving aspirin vs gender-matched normals.

puter interface system (Aggro/link, Chrono-Log) and 486DX IBM computer. Intensity of lucigenin chemiluminescence was expressed in millivolts. Specificity of the O_2^- detection was verified with SOD; addition of SOD (300 U/mL) instantly cancelled lucigenin signal. Coefficients of variation for replicate estimates were $< 15\%$.

Chemicals

ADP sodium salt, SNP, SOD (from bovine erythrocytes), catalase (from bovine liver), IBMX, and *bis*-N-methylacridinium nitrate (lucigenin) were obtained from Sigma (St.Louis, Mo). ODOG was obtained from Tocris Cookson Inc (St.Louis, Mo). NTG was purchased from Fisons (Thornleigh, NSW, Australia).

Data Analysis

Responses of platelets to anti-aggregating and cGMP-elevating effects of NTG and SNP were quantified on the basis of paired comparison as described previously.^{8,9} Inhibitory effects (percent) of anti-aggregating agents were normalized relative to extents of ADP-induced aggregation. Comparisons between normals and patients with SAP were made using ANOVA followed by 2-sided Dunnett's test (for multiple comparisons) or Student's non-paired *t* test as appropriate. Statistically significant difference was limited to $P < 0.05$. Results are expressed as mean \pm SEM.

Results

Platelet Responsiveness to ADP

Comparison of platelet responses to ADP in the various groups of individuals was potentially complicated by the gender-related differences and variable aspirin intake in patients group. Table 1 summarizes ADP responses from all of these subjects, with data expressed separately according to gender. Platelet aggregability toward ADP was greater in women than in men (ANOVA: $P < 0.01$ for normal subjects; $P < 0.05$ for all subjects). Platelet responses to ADP (1 $\mu\text{mol/L}$) were significantly greater in patients with SAP than in normals (ANOVA: $P < 0.01$), irrespective of subject gender; concomitant aspirin therapy was not a significant determinant of response to this concentration of ADP.

Analogous results were obtained with platelet-rich plasma. For example, in male subjects, extent of aggregation was 15.9 \pm 0.9 Ω in the control group (n=8), 20.8 \pm 2.8 Ω in patients with SAP who did not receive aspirin (n=6) and 18.4 \pm 2.4 Ω in patients who received aspirin (n=8); ANOVA: $P < 0.05$ for both groups of patients versus normals.

Inhibition of Platelet Aggregation by NTG and SNP

NTG and SNP inhibited platelet aggregation in whole blood samples from both normals and patients but to different

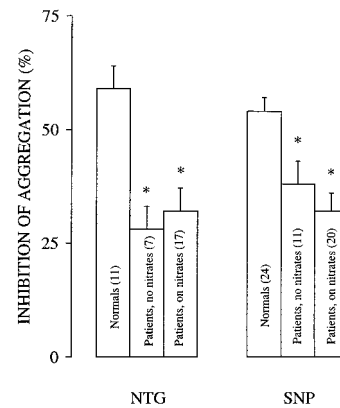


Figure 2. Inhibition of ADP-induced platelet aggregation by NTG (100 $\mu\text{mol/L}$) and SNP (10 $\mu\text{mol/L}$) in whole blood samples from normal subjects and patients with stable angina pectoris receiving or not receiving prophylactic nitrates. Number of subjects indicated in parentheses. * $P < 0.01$ for patients vs normals (unpaired analysis, ANOVA results shown in text).

extents. Representative aggregograms are shown in Figure 1. There were no differences between sexes and between patients receiving and not receiving aspirin regarding platelet responses to anti-aggregatory effects of NTG and SNP. These results were therefore pooled (Figure 2). There was a significant attenuation of platelet response to NTG (ANOVA: $P < 0.001$) and also to SNP (ANOVA: $P < 0.001$) in patients. Prior therapy with prophylactic nitrates was not a significant determinant of responsiveness to NTG or SNP in patients. There was no significant correlation between extent of fixed coronary artery disease and platelet responsiveness to NTG or SNP.

In platelet-rich plasma, anti-aggregatory effects of NTG and SNP in samples from patients with SAP were also less pronounced than in those from normal subjects, although this difference did not reach statistical significance. Specifically, NTG (100 $\mu\text{mol/L}$) and SNP (10 $\mu\text{mol/L}$) produced 77 \pm 8% and 81 \pm 9% inhibition of platelet aggregation, respectively, in samples from normal subjects (n=8), and 68 \pm 6% and 69 \pm 5% inhibition in patients (n=9).

Mechanisms of Nitrate Resistance

Taking into consideration the fundamental involvement of the cGMP system in the anti-aggregatory effects of nitrovasodilators, we assayed intraplatelet cGMP content after incubation of platelet-rich plasma with NTG and SNP (Figure 3). Basal cGMP concentrations in platelets from normal subjects and patients with SAP did not differ: 0.38 \pm 0.03 and 0.37 \pm 0.04 pmol cGMP/10⁹ platelets, respectively. There was, however, a significant attenuation of cGMP response to both NO donors in patients relative to normals (ANOVA: $P < 0.001$ for both NTG and SNP). For example, in platelets from normal subjects 10 $\mu\text{mol/L}$ SNP increased intraplatelet cGMP content 5.2-fold, whereas in platelets from anginal patients, this concentration of SNP produced only a 2.3-fold increase in cGMP. Prior NTG therapy was not a significant determinant of cGMP response in patients. We investigated whether the reduced accumulation of cGMP in response to NO donors in patients' platelets was a result of increased activity of PDE. Incubation of platelet-rich plasma with

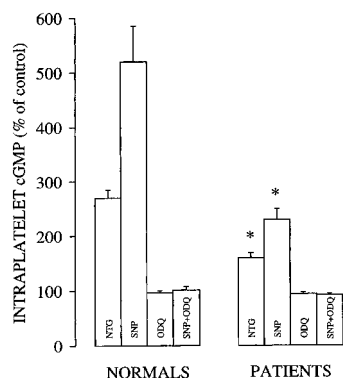


Figure 3. Effects of NTG (100 $\mu\text{mol/L}$), SNP (10 $\mu\text{mol/L}$), and ODQ (1 $\mu\text{mol/L}$) alone or together with SNP on cGMP content in platelets from normal subjects ($n=6$, basal cGMP content 0.38 ± 0.03 pmol/ 10^9 platelets) and patients with stable angina ($n=6$, basal cGMP content 0.37 ± 0.04 pmol/ 10^9 platelets). * $P < 0.001$ for patients vs normals (unpaired analysis, ANOVA results shown in text).

IBMX alone led to a significant increase ($210 \pm 24\%$ of baseline) in intraplatelet cGMP. However, when IBMX was added in combination with SNP, the SNP-dependent component of the total cGMP increase ($201 \pm 35\%$ of control) did not differ from the cGMP-elevating effect of SNP alone ($230 \pm 20\%$ of control). Thus, inhibition of PDE did not restore the impaired cGMP response to NO donor in platelets from anginal patients.

We explored the phenomenon of nitrate resistance further, examining the interaction of platelet guanylate cyclase with NO. We used ODQ, a compound that potently and selectively inhibits NO-stimulated guanylate cyclase activity.¹⁹ In our experiments, ODQ in a concentration of 1 $\mu\text{mol/L}$ abolished SNP-induced elevation of intraplatelet cGMP content with both normal subjects and patients (Figure 3). In this concentration, ODQ alone did not affect platelet aggregation response to ADP but reduced the anti-aggregatory effects of NTG and SNP in whole blood by $51 \pm 8\%$ and $58 \pm 9\%$, respectively ($P < 0.05$), in normals, but not in patients ($7 \pm 7\%$ and $2 \pm 7\%$, respectively). Similar results were observed in platelet-rich plasma. Thus, with both NO donors, effects of ODQ were significantly less pronounced in patients than in normal subjects. These results might possibly reflect a decreased NO-sensitivity of platelet guanylate cyclase in patients with SAP, or reduced availability of NO for activation of guanylate cyclase because of a clearance of NO by O_2^- in blood.²⁰ To examine the possible involvement of O_2^- in nitrate resistance at platelet level, we used SOD, a scavenger of O_2^- . In order to prevent any interference from H_2O_2 generated during the SOD-catalyzed dismutation of O_2^- , catalase was added. Equal amounts of both enzymes, 300 U/mL each, were used.²¹ As shown in Figure 4, SOD/catalase did not affect the extent of platelet aggregation in blood samples from normal subjects (extent of aggregation was $97 \pm 8\%$ of control, $n=18$) but inhibited the increased aggregation in patients' samples (aggregation was $74 \pm 4\%$, $n=20$, $P < 0.01$ versus control). There were no statistically significant effects of SOD/catalase on SNP-dependent inhibition of aggregation in normal subjects. However, in blood samples

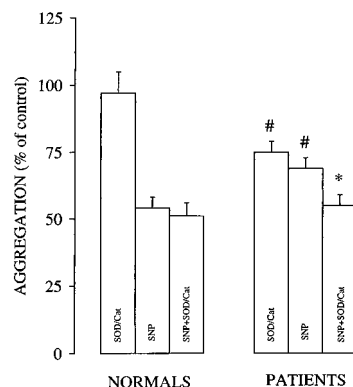


Figure 4. Platelet aggregation (% of control) in presence of superoxide dismutase plus catalase (300 U/mL each, SOD/Cat), SNP alone (10 $\mu\text{mol/L}$), and SNP in combination with SOD/Cat in whole blood samples from normal subjects ($n=18$) and patients with stable angina ($n=20$). * $P < 0.01$ vs no SOD (paired analysis); # $P < 0.01$ for patients vs normals (unpaired analysis).

from patients with SAP, addition of SOD/catalase increased the extent of anti-aggregatory effect of SNP; platelet aggregation decreased from $69 \pm 4\%$ of control to $55 \pm 4\%$ ($P < 0.01$). Although these results implied that O_2^- concentrations were increased in patients with SAP, in our further experiments we examined this possibility directly utilizing a chemiluminescence technique; lucigenin was used as a specific probe for O_2^- .¹⁸ There was a significantly ($P < 0.01$) higher O_2^- chemiluminescence signal in blood samples from patients (174 ± 37 mV, $n=15$) than in those from normal subjects (45 ± 11 mV, $n=6$).

Discussion

In the current study, platelets obtained from patients with SAP manifested increased aggregability with respect to normal subjects. Furthermore, the anti-aggregating and cGMP-stimulating effects of NTG and SNP were reduced in platelets from these patients relative to normal subjects, thus representing the phenomenon of nitrate resistance at the platelet level. This decrease in platelet responsiveness to NO donors may be attributed to reduction in platelet guanylate cyclase sensitivity to NO and to inactivation of the released NO by O_2^- .

We investigated whether the decreased platelet response to NTG and SNP in patients with SAP is associated with a defect in the NO/cGMP pathway (Figure 3). The intracellular cGMP system includes the enzymes responsible for cGMP generation (guanylate cyclase), decomposition (cyclic nucleotide phosphodiesterases), and signal transduction (cGMP-stimulated protein kinases).²² Previously, using the lipophilic analog of cGMP (db-cGMP), we have shown⁹ that the NO/cGMP pathway is intact distal to cGMP formation; the amount of cGMP generated in response to NO donor ultimately predetermines the extent of anti-aggregatory effect. As the current results show no evidence of phosphodiesterase dysfunction, attenuated platelet cGMP response to NTG and SNP suggests impairment at the site of guanylate cyclase. Our previous experiments have not detected any dysfunction of the enzyme; tests were performed in platelet cytosol fraction.⁹ However, dithiothreitol, a strong sulfhydryl-reducing agent,

normally used for the preparation of guanylate cyclase to prevent the oxidation of the enzyme,²² could obscure any preexisting impairment in SH-dependent enzyme sensitivity to NO induced by oxidative stress.²³ Therefore, in the current study, we examined the interaction of guanylate cyclase with NO donors in intact platelets. We used ODQ, a compound that inhibits activation of guanylate cyclase by NO, but does not affect basal activity of the enzyme.¹⁹ ODQ completely suppressed the cGMP-elevating effects of SNP in both normals and patients (Figure 3). In aggregation studies, the inhibition of the anti-aggregatory effects of NTG and SNP by ODQ was significantly less pronounced in patients than in normal subjects. These results imply a decrease in sensitivity of guanylate cyclase to NO in aggregating platelets of patients with SAP. It is possible that this impairment in the enzyme function could be caused by O_2^- . Indeed, O_2^- inhibits human platelet guanylate cyclase²⁴ and enhances platelet aggregation in vitro²¹ and in vivo, in the animal model.²⁵ Furthermore, increased O_2^- generation by neutrophils has been reported in patients with ischemic heart disease (stable and unstable angina)¹³ and myocardial infarction.^{14,15}

Inactivation of NO, both endogenous (EDRF) and exogenous (released from NO donors) by increased concentrations of O_2^- could be another detrimental factor. In the current study, we detected a 4-fold higher level of O_2^- in blood samples from patients with SAP, as compared with normal subjects. We attempted to reduce the concentration of O_2^- with SOD (in combination with catalase). Whereas in blood samples from normal subjects, addition of SOD did not affect aggregation, in samples from anginal patients SOD inhibited aggregation and enhanced anti-aggregatory efficacy of SNP (Figure 4). Although kinetics of O_2^- turnover and peroxynitrite formation were not measured in the current study, our findings imply that O_2^- can diminish platelet responsiveness to NO donors and, probably, contributes to the phenomenon of nitrate resistance at the platelet level.

Incomplete suppression of the anti-aggregating effects of NTG by ODQ observed even in blood samples from normal subjects suggests the existence of an additional, cGMP-independent component for the mechanism of NTG effect. This interesting observation is consistent with previous claims^{26,27} that the cellular effects of organic nitrates are not restricted to cGMP-dependent pathways. However, the precise mechanism(s) of the implied cGMP-independent effects are peripheral to the thrust of the current work.

The current study has several limitations. The results do not necessarily reflect accurately the extent of platelet resistance to NO (and NTG) in vivo. However, it is interesting to view these findings relative to the previous report by Folts and coworkers²⁸ that the anti-oxidant N-acetylcysteine potentiated responsiveness to NTG in reversing in situ platelet aggregation in the canine stenosed coronary artery. No precise correlation can yet be drawn between the currently defined phenomenon of NO resistance in platelets and either the originally designated condition of vasomotor resistance to NTG in patients with chronic cardiac failure or the phenomenon of impaired

endothelial function, with its associated reduction in NO-mediated responses to vasomotor stimuli.

Decreased platelet responsiveness to exogenous sources of NO implies diminution of responsiveness to endogenous NO (EDRF). This provides a potential basis not only for local or generalized increases in platelet aggregability associated with acute myocardial ischemia and/or acute redox stress; such changes could also be associated with further diminution in platelet responsiveness to organic nitrate therapy.

Acknowledgments

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