N-Acetylcysteine Potentiates Platelet Inhibition by Endothelium-Derived Relaxing Factor

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Recent evidence suggests that endothelium-derived relaxing factor exhibits properties of nitric oxide. Like nitric oxide, it inhibits platelet function and mediates its effects by elevating intracellular cyclic GMP. In this study we have investigated the role of reduced thiol in the mechanism of action of endothelium-derived relaxing factor on platelets. Bovine aortic endothelial cells were grown on microcarrier beads and pretreated with aspirin before use. Endothelial cells stimulated with bradykinin or exposed to stirred medium expressed a dose-dependent inhibition of platelet aggregation that was potentiated by the reduced thiol, N-acetylcysteine. Endothelial cell–mediated platelet inhibition was attenuated by methylene blue. Inhibition of platelet aggregation by endothelial cells was associated with a rise in platelet intracellular cyclic GMP, an effect that was enhanced by N-acetylcysteine. These data show that 1) the reduced thiol N-acetylcysteine potentiates platelet inhibition by endothelium-derived relaxing factor and 2) this effect is associated with increasing intracellular platelet cyclic GMP levels. (Circulation Research 1989;65:789–795)

Endothelium-derived relaxing factor (EDRF) is an endogenous vasodilator that has recently been characterized in detail. Bioassays linked to chemical, spectrophotometric, and chemiluminescent analyses have led to the conclusion that a highly labile humoral EDRF is indistinguishable from nitric oxide (NO).1,2 Both EDRF and NO are unstable,3,4 have similar half-lives,1,3,5 relax vascular smooth muscle cells by activating guanylate cyclase and raising cyclic GMP levels,5–11 and are inhibited by superoxide, hemoproteins, and methylene blue.1,4,12–14

Organic nitrates and other nitrosocompounds have been extensively studied as vasodilatory and antiplatelet agents,15–18 and their common mechanism of action is thought to result from the release of NO.8,9,12,19 The critical importance of thiol availability in mediating the effects of organic nitrates was first appreciated by Needleman et al.20 Furchgott and Zawadski6 and Ignarro and colleagues21,22 have since focused attention on the role of nitrosothiol intermediates, and they and others have demonstrated that the regulation of guanylate cyclase by NO depends upon the redox state of specific sulfhydryl groups of the enzyme.22–27 These in vitro observations have been demonstrated to have clinical relevance. Horowitz and colleagues28 showed that N-acetylcysteine (NAC) will potentiate vasodilation induced by nitroglycerin in patients, and we29 have shown that intravenous nitroglycerin will inhibit platelet aggregation ex vivo if adequate platelet thiol stores are maintained.

Recent studies suggest that EDRF can inhibit platelet aggregation30–34 and prevent platelet adhesion to endothelial surfaces.35,36 Radomski and colleagues32,35,36 have implicated NO that is generated from endothelium in both of these nonprostanoid platelet inhibitory functions, but the biochemical mechanism remains unknown. The critical importance of reduced thiol in the mechanism by which nitroglycerin inhibits platelet aggregation has recently been demonstrated by Loscalzo.37 He reported the formation of a nitrosothiol intermediate in the presence of nitroglycerin, the potency of a nitrosothiol adduct in inhibiting platelet aggregation, and the ability of reduced thiol to potentiate nitroglycerin-induced inhibition of platelet aggregation accompanied by increases in cyclic GMP. Subsequent confirmation of the clinical relevance of...
these findings and the mounting evidence for an endogenous antiplatelet nitrosocompound that activates guanylate cyclase have prompted our efforts to investigate the role of reduced thiol in the mechanism of action of EDRF.

**Materials and Methods**

### Chemicals and Solutions

ADP, trypan blue, bradykinin triacetate, methylene blue, hemoglobin (bovine), hematoxylin, eosin, acetylsalicylic acid, and norepinephrine hydrochloride were purchased from Sigma (St. Louis, Missouri). NAC was purchased from Mead Johnson (Evansville, Illinois). Radioimmunoassay kits for the determination of cyclic GMP were purchased from New England Nuclear (Boston, Massachusetts). Microcarrier beads for purposes of endothelial cell culture (Biosilon) were obtained from Vanguard International (Neptune, New Jersey). Krebs-Ringer bicarbonate solution consisted of (mM) NaCl 118.3, KCl 4.7, CaCl₂ 2.5, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25.0, calcium disodium acetate 0.026, and glucose 11.1. Dulbecco's Modified Eagle Medium (DMEM) consisted of (mM) CaCl₂ 1.8, Fe(NO₃)₉H₂O 357 (nM), KCl 5.5, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25.0, calcium disodium edetate 0.026, and d-glucose 11.1. The vessels were cleaned of adherent connective tissue and cut into 5-mm rings; the endothelium was removed by gentle rubbing with a watchmaker's forceps inserted into the lumen. The rings were then mounted on stirrups and connected to transducers (model FT03C Grass Instruments, Quincy, Massachusetts) by which changes in isometric tension were recorded.

### Endothelium-Dependent Relaxations

To study the effect of stirring (rpm) ECBs as occurs continuously in the aggregometer on EDRF release, mounted aortic rings were suspended in a small plastic chamber (2-ml volume, 0.75-cm radius), filled with oxygenated physiological saline solution, and fixed on a magnetic stirrer (model 120 MR, Fisher Thermix, Medford, Massachusetts). A magnetic stirring bar was added to the plastic chamber by which a maximum of 1,650 rpm could be generated. Sustained contractions were induced with norepinephrine, and the effect of stirring on endothelium-dependent relaxations was tested after the addition of ECBs. In some experiments bradykinin, methylene blue, or hemoglobin was added. Relaxations were expressed as percentage of tension remaining as compared with that at maximal contraction to norepinephrine.

### Microscopy and Studies of Endothelial Cell Viability

Assessments of cell growth and degree of cell confluence on microcarrier beads were routinely studied by both phase-contrast microscopy and light microscopy after staining with hematoxylin and eosin. Cell viability was determined at different time points during experiments by vital dye exclusion using trypan blue.
Stirring ECBs in both platelet cuvettes and the bioassay chamber produced a small time- and rpm-dependent detachment of endothelial cells from beads. At completion of experimental protocols at the maximum stirring rates used in this study, at least 70% ECBs remained confluent in both systems. Staining with trypan blue confirmed the viability of cells attached to microcarriers in both systems throughout the course of these experiments.

**Protein Determination**

After each experiment, 0.5 ml of 1N NaOH was added to one droplet of packed ECBs, and protein determination was performed by the method of Lowry and coworkers. Estimates of endothelial cell number per droplet of packed ECBs were made based on 86 μg protein per 10^6 endothelial cells, as determined in our laboratory.

**Cyclic Nucleotide Assays**

Measurements of cyclic GMP were performed by radioimmunoassay. Samples were prepared as described previously, and reactions were terminated by the addition of 0.5 ml of 10% trichloroacetic acid. Ether extractions of the supernatant were performed to remove trichloroacetic acid, and acetylation of samples with acetic anhydride was used to increase the sensitivity of the assay.

**Statistics**

Paired samples were compared by Student’s t test. The Wilcoxon rank-sum test was also used to analyze certain experiments. Values of p<0.05 were considered significant.

**Results**

**EDRF Release**

A bioassay system was used to document generation of EDRF by ECBs and designed to simulate conditions in the aggregometer in which platelets are stirred continuously. Norepinephrine (10^-6 M) was used to induce stable contractions in aortic rings, prepared as described in “Materials and Methods.” The addition of ECBs to the stirring chamber had no effect or induced small relaxations (less than 10% of the initial contraction). Bradykinin (10^-6 M) induced relaxations only in the presence of ECBs. This occurrence indicated that ECBs could produce EDRF. Stirring also induced relaxations in the presence of ECBs, and these relaxations could be enhanced by increasing rpm (Figure 1). Relaxations induced by stirring were inhibited by prior incubation with methylene blue (10^-5 M) for 45 minutes or reversed to contraction by the postrelaxation addition of methylene blue (10^-4 M) (Figure 1) or hemoglobin (5x10^-5 M). Taken together, these experiments suggest that stirring alone promotes the release of EDRF from ECBs.

**Inhibition of Platelet Aggregation and Potentiation by N-Acetylcysteine**

To demonstrate that ECB stirring promoted release of a platelet inhibitory factor, PRP was incubated in the presence of ECBs at increasing rpm and then aggregated with ADP at 900 rpm. Figure 2 shows increasing inhibition of platelet aggregation by ECBs as a function of increasing stirring frequency (rpm). Preincubation of PRP with 1.2 mM NAC for 4 minutes had no effect alone on aggregation but potentiated the inhibition of aggregation induced by stirred ECBs alone (or in the presence of bradyki-
Inhibition of Effects of EDRF on Platelet Aggregation

Increasing concentrations of stirred ECBs produced progressive inhibition of platelet aggregation (Figure 4). This effect was partly reversed by preincubation of PRP with methylene blue (Figure 5).

Effects of N-Acetylcysteine and Endothelial Cells on Levels of Platelet Cyclic GMP

As shown in Figure 6, the incubation of ECBs (induced to release EDRF with stirring) in the aggregometer with PRP for 3 minutes induced 1.6-fold elevations in intracellular platelet cyclic GMP compared with levels found in resting platelets ($p<0.01$). These elevations were enhanced 2.2-fold further by the presence of 1.2 mM NAC compared with those found in resting platelets ($p<0.05$). These elevations were enhanced 2.2-fold further by the presence of 1.2 mM NAC compared with those found in resting platelets ($p<0.05$) compared with platelets incubated with ECBs without NAC; $p<0.01$ compared with resting platelets or resting platelets incubated with 1.2 mM NAC without ECBs). Importantly,

platelets incubated with 1.2 mM NAC alone (i.e., without ECBs) failed to increase levels of cyclic GMP above those found in resting platelets ($p=NS$).
FIGURE 5. Bar chart showing attenuation of endothelium-dependent platelet inhibition by methylene blue. Platelet-rich plasma (diluted 1:5 with DMEM) was incubated with increasing numbers of endothelial cells on beads (ECs) for 3 minutes in the presence and absence of methylene blue. Then ECs were removed, and aggregation was induced with ADP at 37°C. The dose-dependent inhibition of platelet aggregation by ECs is depicted as the absolute change in aggregation rate in the presence of ECs (clear bars). Preincubation of platelet-rich plasma with methylene blue for 3 minutes resulted in attenuation of changes in aggregation rates induced by ECs (hatched bars) (n = 4–7 with six different platelet donors, mean ± SEM). *p < 0.05. (Methylene blue, at concentrations greater or equal to 10^-4 M, markedly attenuated platelet aggregation to ADP. At lesser concentrations [10^-5-10^-7 M], a variable degree of platelet inhibition was also noted. Therefore, in each experiment platelet aggregation was tested over this range of concentrations in order to determine the highest concentration of methylene blue that would not affect significantly the platelet aggregation response to ADP. In five experiments 10^-5 M methylene blue was used, and in two, 10^-7 M was used.)

FIGURE 6. Bar chart showing effect of N-acetylcysteine (NAC) and endothelial cells (ECs) on platelet cyclic GMP levels. Platelets (P) were incubated alone, with 1.2 mM NAC (P+NAC), with 4.4x10^5 ECs (P+EC), or with NAC and ECs (P+NAC+EC) for 3 minutes with stirring (at 650 rpm); then the platelets were removed and processed for cyclic GMP determinations as described in “Materials and Methods.” Each value represents the mean ± SEM of three to five experiments, each performed in duplicate. The presence of NAC had no significant effect on resting cyclic GMP levels in platelets, whereas stirred ECs increased cyclic GMP levels significantly compared with control (p < 0.02), and ECs plus NAC together further increased cyclic GMP levels significantly compared with control (p < 0.01) and compared with endothelial cells without NAC (p < 0.05).

may predispose to time-dependent production of antiplatelet prostaglandins by the endothelial cell; furthermore, with gradual release of reversible cyclooxygenase inhibitor from endothelial cell cyclooxygenase, such agents may become available to the platelet to inhibit directly its cyclooxygenase. 44 Thus, our study is of particular importance in that it demonstrates for the first time inhibition of human platelet aggregation by endothelial cells pretreated with an irreversible inhibitor of cyclooxygenase, and it also shows for the first time that this effect can be potentiated by a reduced thiol.

The mechanism by which reduced thiol contributes to EDRF-induced cyclic GMP elevation is complex. Sulfhydryl compounds have been shown to promote the generation of nitrite from organic nitrates and of nitric oxide from nitrosocompounds. 8,19,21,45 In this regard, thiol specificity may be important. 8,19,21 Sulfhydryl compounds, including N-acetylcysteine, can also react with NO to form S-nitrosothiols, which are potent activators of guanylate cyclase. 8,21,22 Furthermore, guanylate cyclase is regulated by the redox state of the vicinal dithiols at the enzyme active site, and the importance of other thiol groups for enzyme function has not been excluded. 22–27 In addition, reduced thiols stimulate partially purified soluble and particulate guanylate cyclase activated by NO and other nitrosocompounds, 8,22,23,26 whereas sulfhydryl oxidase and thiol alkylating agents rapidly inactivate guanylate cyclase. 22,23,26 Finally, in platelets, N-acetylcysteine potentiates inhibition of aggregation by nitroglycerin by a mechanism that involves the formation of a nitrosothiol intermediate, 37 a compound that strongly activates guanylate cyclase. 8 The likelihood that guanylate cyclase activity stimulated by EDRF is enhanced by the presence of NAC and is in part responsible for our findings is therefore supported by previous studies.

The biologic activity of NO results from its ability to stimulate cyclic GMP formation. 2,8,9,12,37 In the smooth muscle cell, this produces vasodilation and, in the platelet, attenuation of aggregation. 9,12 The importance of reduced sulfhydryl compounds in such a mechanism has also been demonstrated. 37,46 In this study, the further enhancement of cyclic GMP by ECs in the presence of NAC argues for the importance of this mechanism in the effect of EDRF on platelets, as well.

PRP is routinely stirred during studies of platelet aggregation. The addition of ECs in our system resulted in significant inhibition of platelet aggrega-
tion in the absence of an added promoter of EDRF release. This effect was shown to be dependent on stirring rate and, thus, not attributable to basal secretion of EDRF. Release of EDRF has previously been demonstrated as a response to flow, and shear force engendered by stirring may represent an in vitro model of this flow-dependent vasodilation.47 The mechanism by which the endothelium senses fluid forces and translates these to secretion of EDRF may involve a rise in intracellular calcium through pressure sensitive ionic channels5,48 or cell hyperpolarization associated with transmembrane fluxes of potassium ions,49,50 but the actual mechanism remains to be elucidated fully.52 Evidence for the free-radical nature of EDRF led investigators to study its pharmacology by use of various antioxidants and free-radical scavengers. Because of their reducing activity, thiols have been included in some of these studies, but with contradictory results. Thimerosal has been shown to induce endothelium-dependent relaxations53 and glutathione appeared to enhance the relative relaxation to acetylcholine in one study.54 While other thiols tested have failed to enhance vasodilation mediated by EDRF, studies have also frequently failed to demonstrate expected neutralization of EDRF by reduced thiol.5,53-55 Variations in methodology and the presence of experimental conditions conducive to formation of free radicals other than NO in these studies make extrapolation of these findings to the present study difficult. In summary, we have demonstrated that the reduced thiol NAC can potentiate platelet inhibition mediated by EDRF in association with increases in cyclic GMP. This finding provides new insight into the mechanism of action of EDRF and indirect evidence for the presence in vivo of NO or a chemically related nitrosoccompound involving a thiol adduct.

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