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 chemoluminescent analyses have led to the conclusion that a highly labile humoral EDRF is indistinguishable from nitric oxide (NO). Both EDRF and NO are unstable, have similar half-lives, relax vascular smooth muscle cells by activating guanylate cyclase and raising cyclic GMP levels, 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 anti-platelet agents, and their common mechanism of action is thought to result from the release of NO. The critical importance of thiol availability in mediating the effects of organic nitrates was first appreciated by Needleman et al. and Furchgott and Zawadski and Ignarro and colleagues have demonstrated that the regulation of guanylate cyclase by NO depends upon the redox state of specific sulfhydryl groups of the enzyme. These in vitro observations have been demonstrated to have clinical relevance. Horowitz and colleagues showed that N-acetylcysteine (NAC) will potentiate vasodilation induced by nitroglycerin in patients, and we have shown that intravenous nitroglycerin will inhibit platelet aggregation in vivo if adequate platelet thiol stores are maintained.

Recent studies suggest that EDRF can inhibit platelet aggregation and prevent platelet adhesion to endothelial surfaces. Radomski and colleagues 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. 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\textsuperscript{29} 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

\textbf{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 Van-\textsuperscript{et al.} and colleagues.\textsuperscript{39} Microcarrier beads were anticoagulated with 13 mM sodium citrate.

\textbf{Preparation of Platelets}

Venous blood was obtained from volunteers and was anticoagulated with 13 mM sodium citrate. Volunteers had not consumed acetylsalicylic acid for at least 10 days. Platelet-rich plasma (PRP) was prepared by centrifugation at 80g for 10 minutes. Platelet-poor plasma was prepared by centrifugation at 800g for 10 minutes. Platelet counts were determined with a Coulter Counter (model Z, Coulter Electronics, Hialeah, Florida) and adjusted to 2.0×10\textsuperscript{8} platelets/ml by the addition of platelet-poor plasma.

\textbf{Preparation of Blood Vessels}

New Zealand White female rabbits weighing 3–4 kg were anesthetized with 30 mg/kg i.v. sodium pentobarbital. Descending thoracic aortae were isolated and placed immediately in a cold physiological salt solution (mM): NaCl 118.3, KCl 4.7, CaCl\textsubscript{2} 2.5, MgSO\textsubscript{4} 1.2, KH\textsubscript{2}PO\textsubscript{4} 1.2, NaHCO\textsubscript{3} 25.0, calcium disodium acetate 0.026, and glucose 11.1. Dulbecco’s Modified Eagle Medium (DMEM) consisted of (mM) CaCl\textsubscript{2} 1.8, Fe(NO\textsubscript{3})\textsubscript{3}·9H\textsubscript{2}O 357 (nM), KC\textsubscript{1} 5.5, MgSO\textsubscript{4} 1.7, NaHCO\textsubscript{3} 109, NaH\textsubscript{2}PO\textsubscript{4} 50.7, Na\textsubscript{2}PO\textsubscript{4} 1.0, glucose 30, phenol red 15 (mg/l), and sodium pyruvate 1.0. Phosphate-buffered saline, pH 7.4, consisted of 10 mM sodium phosphate and 150 mM NaCl.

\textbf{Microcarrier Endothelial Cell Culture}

Endothelial cells isolated from bovine aorta by established techniques\textsuperscript{38} were cultured on a microcarrier system of negatively charged spherical plastic beads (Biosilon), as previously described by Davies and colleagues.\textsuperscript{39}

\textbf{Preparation of Samples and Platelet Aggregation}

Before use, endothelial cells on microcarrier beads (ECBs) were exposed to 30 \mu M acetylsalicylic acid for 1 hour, washed three times, and resuspended in DMEM (without calf serum). ECBs were then drawn up and permitted to settle to a packed volume of approximately 350 \mu l in a 1-ml Eppendorf pipette tip that was cut 8 mm from its distal end and calculated to deliver 3,182±223 beads/droplet (42 \mu l) of packed ECBs. ECBs were added to 0.6 ml aliquots of PRP, previously diluted 1:5 with DMEM and stirred for 3 minutes in either a dual chamber aggregometer (Payton Associates, Buffalo, New York) or a PAP-4 aggregometer (Biodata, Hatboro, Pennsylvania) at 37° C. ECBs were then permitted to settle at unit gravity, and 500 \mu l PRP, free of ECBs, was removed for aggregation, which was induced within 2 minutes. Aggregation was initiated by addition of 15.5 \mu M ADP and monitored by a standard nephelometric technique\textsuperscript{40} in which changes in light transmittance were recorded as a function of time. Aggregation was quantitated by measuring the extent of change of light transmittance or the maximal rate of change of light transmittance.

\textbf{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).\textsuperscript{41} 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.

\textbf{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.42 Estimates of endothelial cell number per droplet of packed ECBs were made based on 86 μg protein per 10⁶ endothelial cells, as determined in our laboratory.

Cyclic Nucleotide Assays

Measurements of cyclic GMP were performed by radioimmunoassay. Samples were prepared as described previously,37 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 (10⁻⁴ 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 (10⁻⁴ M) for 45 minutes or reversed to contraction by the postrelaxation addition of methylene blue (10⁻⁴ M) (Figure 1) or hemoglobin (5×10⁻⁵ 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-
FIGURE 3. Bar chart showing inhibition of platelet aggregation by endothelial cells on beads (ECs) and N-acetylcysteine (NAC). Platelet-rich plasma (diluted 1:5 with DMEM) was incubated for 3 minutes with 1.2 mM NAC, 4.4 x 10^5 stirred ECs, or NAC + ECs; then ECs were removed, and aggregation was induced with ADP. The extent of aggregation was normalized to that in the absence of preincubation with ECs and NAC. NAC had no significant effect on the extent of aggregation; ECs attenuated the extent of aggregation by approximately 20% (p < 0.05 compared with control and with platelets incubated with NAC alone); ECs and NAC together attenuated the extent of aggregation by approximately 55% compared with control (p < 0.001) and by an additional 35% compared with that noted with ECs without NAC (p < 0.05). Each bar represents the mean ± SEM of 7–12 experiments with eight different platelet donors.

Inhibition of Effects of EDRF on Platelet Aggregation

Increasing concentrations of stirred ECs 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 ECs (induced to release EDRF with stirring) in the aggregometer with PRP for 3 minutes induced approximately 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 ECs without NAC; p < 0.01 compared with resting platelets or resting platelets incubated with 1.2 mM NAC without ECs). Importantly, platelets incubated with 1.2 mM NAC alone (i.e., without ECs) failed to increase levels of cyclic GMP above those found in resting platelets (p = NS).

Discussion

Inhibition of platelet aggregation by EDRF was first documented by Azuma and colleagues and has since been confirmed by others. Earlier studies, however, did not fully exclude the possibility that prostacyclin contributed to platelet inhibition. In these studies, investigators either failed to inhibit endothelial cell cyclooxygenase, added stabilizing concentrations of prostacyclin, or used reversible inhibitors of cyclooxygenase. Although the addition of stabilizing concentrations of prostacyclin has been argued to have no detectable effect on platelet function, in the presence of endothelial cells it might predispose to platelet inhibition by other endothelial products by a synergistic mechanism. In addition, the use of reversible inhibitors of endothelial cell cyclooxygenase...
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^-3 M methylene blue was used, and in two, 10^-7 M was used.)

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.

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. 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. In addition, reduced thiols stimulate partially purified soluble and particulate guanylate cyclase activated by NO and other nitrosocompounds, whereas sulfhydryl oxidase and thiol alkylating agents rapidly inactivate guanylate cyclase. Finally, in platelets, N-acetylcysteine potentiates inhibition of aggregation by nitroglycerin by a mechanism that involves the formation of a nitrosothiol intermediate, a compound that strongly activates guanylate cyclase. 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. In the smooth muscle cell, this produces vasodilation and, in the platelet, attenuation of aggregation. The importance of reduced sulfhydryl compounds in such a mechanism has also been demonstrated. In this study, the further enhancement of cyclic GMP by ECBs 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 ECBs 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. 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 channels or cell hyperpolarization associated with transmembrane fluxes of potassium ions, but the actual mechanism remains to be elucidated fully.

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 relaxations and glutathione appeared to enhance the relative relaxation to acetylcholine in one study. 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. 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 nitrated compound involving a thiol adduct.

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