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S-nitrosylation of tissue-type plasminogen activator confers vasodilatory and antiplatelet properties on the enzyme

(nitric oxide/*S*-nitrosothiol)

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ABSTRACT **Tissue-type plasminogen activator (t-PA) reacts upon exposure to endothelium-derived relaxing factor (EDRF) by way of the enzyme's single free sulfhydryl (Cys-83) to form a stable *S*-nitrosothiol protein adduct. S-nitrosylation endows t-PA with potent vasodilatory and antiplatelet properties that are accompanied by elevations in intracellular cyclic GMP analogous to those induced by low molecular weight (e.g., *S*-nitroso amino acid) *S*-nitrosothiols. Moreover, this chemical modification does not adversely affect the catalytic efficiency of t-PA, the fibrin stimulation of this activity, the binding of t-PA to fibrinogen, or the interaction of the enzyme with its physiologic serine protease inhibitor, plasminogen-activator inhibitor type I. The coupling of vasodilatory, antiplatelet, and fibrinolytic properties in one molecule makes the S-nitrosylated t-PA a unique molecular species and may provide insight into the mechanisms by which the endothelium maintains vessel patency. These data also suggest a pharmacologic approach to treatment of thromboocclusive disorders.**

The endothelium plays a central role in the mammalian hemostatic mechanism. The system is highly complex and involves secretion of specific products capable of dissolving fibrin clots, inhibiting platelet activation, and regulating vessel tone. Participation in fibrino(geno)lysis is achieved by elaboration of tissue-type plasminogen activator (t-PA), which converts the proenzyme plasminogen to the active serine protease plasmin (1). This action is localized to the fibrin clot—i.e., t-PA is relatively fibrin-specific compared with other plasminogen activators, owing to its greatly enhanced enzymatic activity in the presence of fibrin (1). The generation of plasmin also has secondary effects on platelet function (2), but t-PA itself neither directly inhibits platelets nor influences vessel tone (1, 3). These latter functions are executed by other molecules originating in endothelium, such as prostaglandin I_2 and endothelium-derived relaxing factor (EDRF), the latter having been identified as nitric oxide (NO) (4, 5) or a closely related *S*-nitrosothiol (6).

Efforts to improve the thrombolytic efficacy and pharmacological properties of plasminogen activators have taken several directions. In light of the role of platelets in clot formation and in reocclusive vascular events, one major focus has involved the use of ancillary antiplatelet therapy. Some success has been achieved with aspirin (7), and other benefits have been reported for several newer antiplatelet compounds (8). Attempts have also been made to improve the functional properties of the plasminogen activator itself through site-directed mutagenesis and synthesis of chimeric or hybrid molecules and biochemical conjugates (9, 10).

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We recently reported that several reduced thiol-containing proteins react readily upon exposure to oxides of nitrogen and EDRF to form stable *S*-nitrosothiols (11). Potent vasodilatory and antiplatelet properties, reminiscent of other low molecular weight S-nitro(so) compounds, were demonstrated for the exemplary compound *S*-nitroso-albumin (11). Here, we report the functional properties and biological activities of *S*-nitroso-t-PA (*S*-NO-t-PA). This work shows that the S-nitroso-derivatized protein retains the inherent, domain-specific properties of t-PA and, moreover, incorporates the vasodilatory and antiplatelet properties characteristic of EDRF.

MATERIALS AND METHODS

Materials. t-PA was kindly provided by Genentech, as primarily the single-chain form. Reactivated, purified plasminogen-activator inhibitor 1 (PAI-1) and a panel of six murine anti-t-PA monoclonal antibodies were kindly provided by Douglas E. Vaughan (Brockton/West Roxbury Veterans Administration Medical Center). Horseradish peroxidase-linked sheep antibodies against mouse immunoglobulins were purchased from Amersham. Sodium nitrite was purchased from Fisher Scientific. H-D-isoleucyl-L-prolyl-L-arginyl-*p*-nitroanilide (S2288) and H-D-valyl-L-leucyl-L-lysyl-*p*-nitroanilide (S2251) were purchased from KabiVitrum, Stockholm. Human fibrinogen purified free of plasminogen and von Willebrand factor was obtained from Enzyme Research Laboratories (South Bend, IN). Epinephrine, adenosine 5′-diphosphate (ADP), and iodoacetamide were purchased from Sigma. Bovine thrombin was obtained from ICN ImmunoBiologicals (Lisle, IL). Radioimmunoassay kits for the determination of cGMP and $Na^{15}NO_2$ were purchased from New England Nuclear.

Plasminogen Preparation. Glu-plasminogen was purified from fresh frozen plasma by a modification of the method of Deutsch and Mertz (12), as described (13).

Microcarrier Endothelial Cell Culture. Endothelial cells were isolated from bovine aorta by established techniques (14) and cultured on a microcarrier system of negatively charged, spherical plastic beads (Biosilon), according to the method of Davies *et al.* (15).

t-PA Derivatization. *Carboxyamidation.* The free thiol of t-PA was carboxyamidated by exposure of the enzyme to a 10-fold molar excess of iodoacetamide in the dark for 1 hr at 37°C in 10 mM Tris, pH 7.4/0.15 M NaCl (TBS). t-PA was then dialyzed extensively against 10 mM HCl to remove excess iodoacetamide. Carboxyamidated t-PA was used as a

Abbreviations: t-PA, tissue-type plasminogen activator; *S*-NO-t-PA, *S*-nitroso-t-PA; EDRF, endothelium-derived relaxing factor; PAI, plasminogen-activator inhibitor.

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control to compare the effects of t-PA lacking an available thiol with the effects of EDRF or NO.

S-nitrosylation (synthesis of S-NO-t-PA). t-PA was first dialyzed against a large excess of 10 mM HCl for 24 hr to remove excess L-arginine used to solubilize the protein. The enzyme was then allowed to react in the presence of equimolar $NaNO_2$ in 0.5 M HCl (acidified $NaNO_2$) for 30 min at 37°C. Solutions were titrated to pH 7.4 with equal volumes of 1.0 M NaOH and TBS/0.05 M L-arginine. Dilutions were then made as necessary in TBS. For comparative purposes and to illustrate its potential biological relevance, *S*-NO-t-PA was also synthesized with authentic EDRF in selected experiments. In this method, t-PA was incubated with bovine aortic endothelial cells stimulated by exposure to high shear forces (0.43 dyne/cm^2) to secrete EDRF, as we have previously described (11, 16, 17). Owing to the relative stability of the S—NO bond in *S*-NO-t-PA under physiologic conditions ($t_{1/2}$ > 24 hr in TBS at 25°C), samples were stored at pH 7.4 on ice throughout the course of the experiments.

Chemical and spectroscopic analysis. The formation of an S—NO bond and its stability were validated by four principal analytical methods. First, NO displaced from *S*-nitrosothiol groups with Hg^{2+} was assayed by diazotization of sulfanilamide and subsequent coupling to the chromophore *N*-(1-naphthyl)ethylenediamine (18). Second, the characteristic absorption spectrum of *S*-nitrosothiols in the range of 320–360 nm was monitored (11, 19). Third, ^{15}N NMR was used according to the method of Bonnett *et al.* (20): the chemical shift for RS-NO, referenced to a secondary $Na^{15}NO_2$ standard assigned at 587 ppm, is relatively specific in the range of 700–800 ppm. In these experiments, *S*-^{15}NO-t-PA was synthesized as described above using $Na^{15}NO_2$ and spectra were recorded with a Brucker 500 MHz spectrometer and Fourier transformed as described (11). Finally, the formation of an S—NO bond was verified by IR spectroscopy. In this method, *S*-NO-t-PA was desiccated under vacuum, and a Nujol mull of the sample was then prepared for Fourier transform IR spectroscopy (Nicolet ZDX Fourier transform IR spectrometer). The S—N bond absorbs in the range of 1150–1170 cm^{-1} (21).

Measurements of Enzymatic Functional Properties. *Fibrinogen binding.* The binding of t-PA and *S*-NO-t-PA to fibrinogen was measured using polystyrene microtiter wells (flat-bottom, high-binding 96-well EIA plates, Costar); wells were coated with fibrinogen (0.08 μg/μl), and the remaining polystyrene binding sites were blocked with 2% bovine serum albumin (13). This system has been characterized previously in detail: binding of t-PA is reversible and specific and saturates at 1500–3000 nM; at saturation 18 ng of t-PA is bound per well (0.05 mol of t-PA per mol of fibrinogen) with an estimated K_d of ≈350 nM (13).

Enzymatic activity. The amidolytic activities of t-PA and its S-nitrosylated derivative were measured using the relatively specific chromogenic substrate S2288 (22). Substrate hydrolysis was measured spectrophotometrically at 405 nm with a Gilford Response UV/visible spectrophotometer (CIBA–Corning, Oberlin, OH). Activity was measured at 25°C in TBS with substrate concentrations varying from 0.1 to 2.0 mM and a t-PA concentration of 100 nM. Kinetic parameters were determined from initial rates by analysis of double-reciprocal plots. Inhibition of the enzymatic activity of t-PA and *S*-NO-t-PA by PAI-1 was assessed at an enzyme concentration of 10 nM and a molar ratio of t-PA to active PAI-1 of 1.0. The degree of inhibition was determined by measuring the initial rates of enzyme activity in the presence and absence of the inhibitor.

In the coupled enzyme assay, t-PA and *S*-NO-t-PA activities were assayed using the native substrate, plasminogen, and the plasmin-selective chromogenic substrate, S2251 (22). In selected experiments, fibrinogen stimulation of enzymatic activity was also assessed at a fibrinogen concentration of 1 mg/ml. Substrate hydrolysis was measured spectrophotometrically with a Dynatech MR 5000 card reader (Dynatech) in TBS at 25°C. Initial reaction velocity in this coupled assay was determined from the slope of the plot of absorbance (at 405 nm)/time vs. time (22) for Glu-plasminogen concentrations ranging from 0.1 to 10 μM and an S2251 concentration of 0.8 mM. Kinetic parameters were determined from initial rates by double-reciprocal plot analysis.

Bioassays. *Platelet.* Platelet-rich plasma was prepared by differential centrifugation of venous blood of volunteers who had not consumed acetylsalicylic acid for at least 10 days (3, 9, 11). Platelets were gel-filtered on a 4 × 10-cm column of Sepharose 2B in Tyrode's Hepes buffer according to the method of Hawiger *et al.* (23) and suspended at a concentration of 1.5 × 10^8 per ml. Platelet aggregation was monitored by a standard nephelometric technique (24) in which 0.3-ml aliquots of gel-filtered platelets were incubated at 37°C and stirred at 1000 rpm in a PAP-4 aggregometer (Biodata, Hatsboro, PA). Gel-filtered platelets were preincubated with t-PA or *S*-NO-t-PA for up to 45 min and aggregations were induced with ADP (5 μM) or thrombin (0.025 unit/ml). Aggregations were quantified by measuring the maximal rate or extent of light transmittance and expressed as a normalized value relative to control aggregations.

Blood vessel. Deendothelialized vessel rings were prepared from descending thoracic aortae of New Zealand White rabbits (3–4 kg) according to a published protocol (17). The rings were suspended in glass chambers containing 7 ml of oxygenated Krebs's buffer and were connected to transducers (model FTO3C, Grass) (17). Sustained contractions were induced with 1 μM epinephrine, after which the effects of t-PA and *S*-NO-t-PA were tested. In certain experiments, a guanylate cyclase inhibitor, methylene blue, was preincubated with vessel rings for 15 min prior to initiation of contractions.

Cyclic Nucleotide Assays. cGMP was measured by radioimmunoassay. Gel-filtered platelets were preincubated for 180 sec with *S*-NO-t-PA and related controls. Reactions were terminated by the addition of 10% (wt/vol) trichloroacetic acid and samples were prepared as described (16).

Statistics. Statistical significance was analyzed using a nonpaired *t* test or two-way analysis of variance (ANOVA) followed by a Newman–Keul's comparison.

RESULTS

Chemical Synthesis and Spectroscopic Characterization. Of the several methods we have used in the synthesis of protein *S*-nitrosothiols (11), we have focused in the experiments presented here on exposure of t-PA to authentic EDRF and on treatment of t-PA with acidified nitrite to S-nitrosylate the enzyme. Inasmuch as the extraction of t-PA from tissue is carried out under similarly acidic conditions (25), its stability at low pH is evident. We further verified this stability by examining the effect of acid pretreatment (incubation in 0.5 M HCl) on the amidolytic activity of t-PA against the tripeptide substrate S2288. After 30 min of acid exposure, ≈85% of the enzymatic activity was retained. The reaction of equimolar amounts of $NaNO_2$ and t-PA is essentially complete after 30 min; the stoichiometry of *S*-NO-t-PA (mol/mol) is 0.9 ± 0.1 (n = 3) at the completion of the reaction, as determined by the method of Saville (18).

The formation of an *S*-nitrosothiol product is supported by additional spectroscopic evidence. Upon treatment with acidified nitrite, t-PA shows a UV/visible absorption maximum at 322 nm; other low molecular weight (26) and protein *S*-nitrosothiols exhibit absorption maxima in the 320–360 nm wavelength range (6, 11). IR spectroscopy revealed the presence of absorption bands at 1153 and 1167 cm^{-1}, char-

acteristic of the S—N bond (21). The ^{15}N NMR spectrum of t-PA treated with a molar equivalent of acidified $Na^{15}NO_2$ is essentially identical to that previously published for S-nitrosylated bovine serum albumin (11), consisting of a single resonance peak at 751 ppm. Owing to the comparatively limited aqueous solubility of t-PA, the signal-to-noise ratio of its spectrum is much poorer than that for albumin. The complete elimination of the signal upon addition of an excess of $HgCl_2$, however, confirms the presence and origin of the signal. The half-life of *S*-NO-t-PA at pH 7.4, 25°C, is ≈24 hr.

Comparison of t-PA and *S*-NO-t-PA. *Fibrin(ogen) binding.* The binding of t-PA to fibrin(ogen) accounts for the relative fibrin specificity of the enzyme compared with other plasminogen activators (1, 10). The affinity of t-PA for fibrin and fibrinogen is conferred by the kringle-2 domain of the molecule in conjunction with the finger-like domain (1, 10). Using established methods (13, 22), we examined the effect of S-nitrosylation on this functional property of the enzyme using surface-bound fibrinogen. The binding isotherms for t-PA and its S-nitrosylated derivative were not significantly different from one another by two-way ANOVA, and these data were therefore subjected to curve fitting to define the binding isotherm (Fig. 1). The estimated apparent K_d of *S*-NO-t-PA for surface-bound fibrinogen is ≈360 nM, which falls well within the reported range for t-PA binding (13, 22); the latter is also ≈360 nM in these experiments.

Enzymatic activity. The amidolytic activities of t-PA and *S*-NO-t-PA were first compared using the chromogenic substrate S2288 (13, 22). From a double-reciprocal plot analysis, it is evident that the kinetic parameters (K_m and V_{max}) and the catalytic efficiency (k_{cat}/K_m) of these molecules are essentially identical (Fig. 2*A*). The values of these kinetic constants are provided in Table 1.

The effect of S-nitrosylation on the ability of t-PA to activate its physiologic substrate, plasminogen, was assessed in the coupled enzyme assay in the presence and absence of fibrinogen. As seen in the Lineweaver–Burk plot (Fig. 2*B*) and from the derived kinetic parameters (Table 1), *S*-NO-t-PA has kinetic properties similar to those of native, unmodified t-PA.

Both fibrin and fibrinogen increase the rate of activation of plasminogen by t-PA. The enhanced enzymatic activity of t-PA is the result of its ability to bind directly to fibrin(ogen), which brings about a conformational change in either t-PA or plasminogen that promotes the interaction of enzyme with substrate (1, 27). The consequences of S-nitrosylation on these important functional properties of t-PA were, therefore, studied in a comparative analysis with t-PA, using the

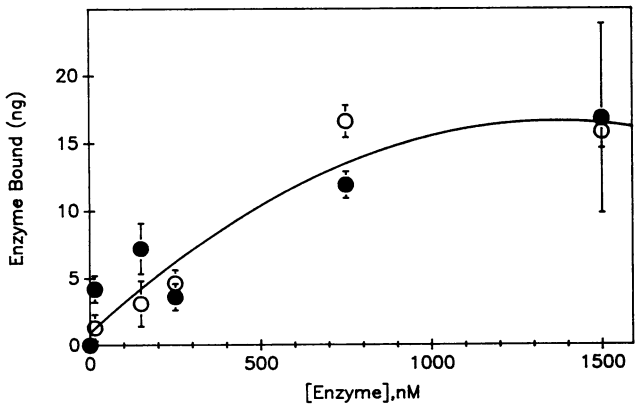

FIG. 1. Concentration-dependent binding of t-PA (○) and *S*-NO-t-PA (●) to fibrinogen-coated wells. Data have been fitted to a single binding isotherm because the individual binding isotherms for t-PA and its *S*-nitrosylated derivative were not significantly different by two-way ANOVA. Results are presented as mean ± SD (n = 4–8).

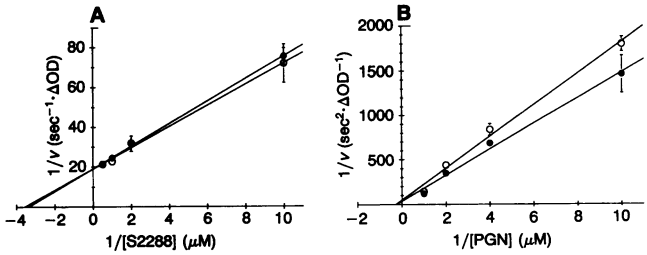

FIG. 2. Double-reciprocal plots for *S*-NO-t-PA and native t-PA. (*A*) Double-reciprocal plots of $1/v$ versus 1/[S] for t-PA (○) and *S*-NO-t-PA (●) were generated using the chromogenic substrate S2288 in TBS at 25°C. Results are expressed as mean ± SD (n = 3). These curves are not significantly different from one another by two-way ANOVA. (*B*) Curves for activation of Glu-plasminogen (PGN) (0.1–10 μM) by t-PA (○) and *S*-NO-t-PA (●) were generated using the plasmin-specific chromogenic substrate S2251 (0.8 mM). Results are expressed as mean ± SD (n = 3). The curves are significantly different from each other by ANOVA to $P < 0.01$. The k_{cat}/K_m ratio for *S*-NO-t-PA is 23% greater than that of native enzyme (Table 1).

coupled plasminogen–S2251 assay in the presence of fibrinogen. This analysis indicates that as a result of binding to fibrinogen, the enzymatic activity of *S*-NO-t-PA is enhanced (n = 9; mean ± SEM; 18.5 ± 1.7-fold increase over activity in the absence of fibrinogen). In the presence of physiologic (1 μM) plasminogen concentrations, the stimulation of *S*-NO-t-PA activity by fibrinogen is equivalent to that of unmodified t-PA; at lower plasminogen concentrations (0.1 μM), fibrinogen stimulation of *S*-NO-t-PA activity was 4.4-fold greater than that of t-PA (1 μM) ($P < 0.05$), representing an absolute increase in enzymatic activity of 39%.

t-PA is rapidly inhibited by its cognate serpin (serine protease inhibitor), PAI-1 (1, 10). By serving as a pseudosubstrate, PAI-1 reacts stoichiometrically with t-PA to form an inactive complex (1, 10). PAI-1 was equally effective at inhibiting the amidolytic activity of t-PA and *S*-NO-t-PA in the direct chromogenic assay with S2288 (n = 3; P nonsignificant). Thus, S-nitrosylation of t-PA does not appear to alter its interaction with PAI-1.

Vascular relaxation. The vasodilatory properties of *S*-NO-t-PA were examined in a vessel ring assay using endothelium-denuded rabbit aortic rings. As shown in the illustrative tracings of Fig. 3, *S*-NO-t-PA at pharmacologically relevant concentrations induced relaxations that were unmatched by equimolar amounts of t-PA or NO equivalents alone. Furthermore, as with other nitro(so) vasodilators, relaxations were attenuated by the guanylate cyclase inhibitor methylene blue. The effect of *S*-NO-t-PA on vessel relaxation is shown in Table 2 for four such experiments.

Platelet inhibition. The antiplatelet effects of *S*-NO-t-PA were studied using a gel-filtered platelet preparation. *S*-NO-t-PA preincubated with platelets for 10 min inhibited platelet aggregation in response to 5 μM ADP in a dose-dependent manner, with 50 ± 8% (mean ± SEM) inhibition of maximal rate and extent of aggregation (≡ IC_{50}) observed at 1.4 μM *S*-NO-t-PA (n = 4; $P < 0.001$ vs. control). This potency is

Table 1. Kinetic parameters of S2288 hydrolysis and Glu-plasminogen activation by t-PA and *S*-NO-t-PA

	K_m, μM	k_{cat}, sec^{-1}	k_{cat}/K_m, $sec^{-1}\cdot\mu M^{-1}$
S2288			
t-PA	280	0.52	0.0019
S-NO-t-PA	295	0.52	0.0019
Glu-plasminogen			
t-PA	3.5	0.200	0.056
S-NO-t-PA	3.8	0.262	0.069

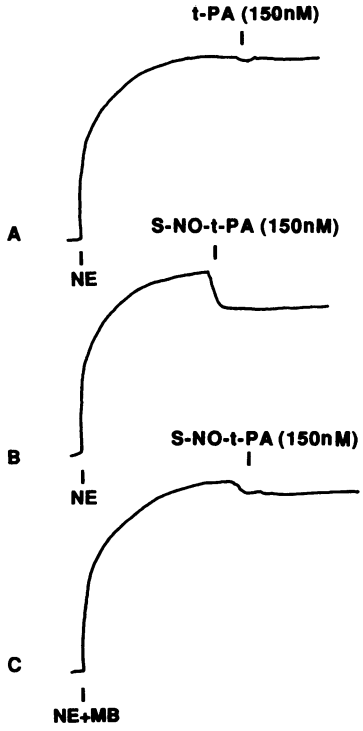

FIG. 3. *S*-NO-t-PA-induced vasorelaxation. Representative tracings comparing the effects of 150 nM t-PA (*A*), 150 nM *S*-NO-t-PA (*B*), and 150 nM *S*-NO-t-PA after vessel rings were pretreated with 10 μM methylene blue (MB) (*C*). The S-nitrosylated t-PA derivative was synthesized in acidified $NaNO_2$ as described in the text and then neutralized to pH 7.4 preceding addition to the bioassay (Kreb's buffer, pH 7.4, 37°C; 95% O_2/5% CO_2). NO equivalents (150 nM) generated from acidified $NaNO_2$ (150 nM) had no significant effect on vessel tone at this concentration (tracings not shown). Contractions were induced with norepinephrine (NE).

comparable to that for *S*-nitroso-L-cysteine, which has an IC_{50} of 4 μM in this system (data not shown). Inhibition of platelet aggregation induced by ADP (5 μM) or thrombin (0.024 unit/ml) was demonstrable at concentrations of *S*-NO-t-PA in the pharmacologically relevant range of 15–150 nM (n = 13–17; $P < 0.05$ vs. t-PA). By contrast, and as shown in the illustrative tracings of Fig. 4, platelet aggregation was not significantly affected by native t-PA in this concentration range. In further support of the potential biological relevance for RS-NO compounds and the comparable bioactivity of *S*-NO-t-PA irrespective of its method of synthesis, platelet aggregation was also significantly inhibited by *S*-NO-t-PA (333 nM) synthesized with authentic EDRF (data not shown).

cGMP. The antiplatelet actions of *S*-nitrosothiols are mediated by cGMP (11, 16). Consistent with this mechanism of platelet inhibition, *S*-NO-t-PA (9 μM) incubated with platelets for 180 sec induced a 290% increase in intracellular cGMP above the basal level (n = 4; mean ± SEM, 0.95 ± 0.045 pmol of cGMP per 10^8 platelets compared with basal level of 0.33 ± 0.055 pmol of cGMP per 10^8 platelets; $P < 0.01$); native t-PA had no such effect (0.51 ± 0.09 pmol of cGMP per 10^8 platelets; P nonsignificant). The increases in intracellular platelet cGMP induced by *S*-NO-t-PA were prevented by preincubation of platelets with the guanylate cyclase inhibitor methylene blue (10 μM) for 10 min (0.50 ± 0.095 pmol of cGMP per 10^8 platelets; $P < 0.01$ vs. *S*-NO-t-PA).

Table 2. Vessel relaxation

Test agent		% relaxation
t-PA	(150 nM)	3 ± 2
NO	(150 nM)	1 ± 1
S-NO-t-PA	(150 nM)	20 ± 3*

Values represent vessel relaxation (measured as a percentage of the maximal contraction induced by norepinephrine, reported as mean ± SEM, n = 4) induced by *S*-NO-t-PA, or by equimolar concentrations of NO equivalent (generated from $NaNO_2$), or by native t-PA.

*Relaxations in response to *S*-NO-t-PA were significantly greater than those induced by NO or t-PA ($P < 0.01$).

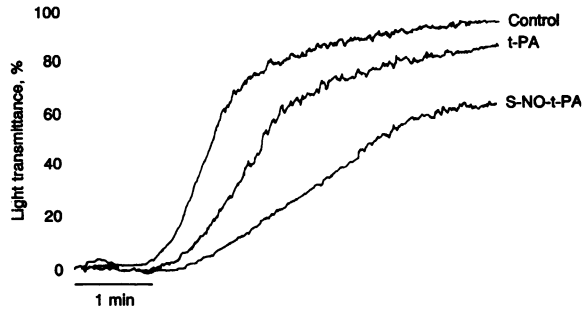

FIG. 4. Inhibition of platelet aggregation by *S*-NO-t-PA. *S*-NO-t-PA (150 nM) and related controls were incubated with gel-filtered platelets for 10 min prior to induction of platelet aggregation with thrombin (0.024 unit/ml). *S*-NO-t-PA was synthesized with $NaNO_2$ as described. In these experiments, NO equivalents generated from $NaNO_2$ had no significant effect on the extent of platelet aggregation at this concentration (tracing not shown). Similarly, native t-PA did not significantly affect the aggregation response (P nonsignificant). Comparable results were observed for *S*-NO-t-PA synthesized with EDRF (see *Materials and Methods*).

DISCUSSION

The chemical and spectroscopic data presented here confirm the formation of the S-nitroso derivative of t-PA upon its exposure to acidified nitrite or authentic EDRF. This modification confers cGMP-mediated antiplatelet and vasodilatory properties—with potency characteristic of other *S*-nitrosothiols—on t-PA without markedly altering its intrinsic functional properties, specifically its enzymatic activity, relative fibrin(ogen) specificity, and binding to PAI-1. The loci of proteolytic activity, as well as PAI-1 and fibrin(ogen) binding, reside in functional domains that are separate from the site of S-nitrosylation—the single free thiol, Cys-83, in the epidermal growth factor-like domain. Chemical modification of t-PA by NO does not adversely alter these functional properties of the enzyme residing in other domains. S-nitrosylation does appear to enhance slightly the catalytic efficiency of t-PA against plasminogen and to increase modestly its stimulation by fibrinogen. Sobel *et al.* (28) reported that a single cysteine-for-arginine substitution in the growth factor domain increases the specific activity of t-PA. Together, these results indicate that selective modification of the growth factor domain can lead to changes in enzyme activity.

The two molecular precursors of *S*-NO-t-PA (NO and t-PA) are among the products specifically secreted by the endothelium to counteract thrombogenic processes such as platelet activation, augmented local vasoconstriction, and activation of the coagulation system (29). t-PA converts plasminogen to plasmin on fibrin and platelet thrombi, which in turn induces fibrinolysis and platelet disaggregation (1, 3). Elaboration of EDRF (NO or RS-NO) reverses local vasospasm (30), directly inhibits platelet aggregation (16, 30, 31) and secretion (32), and promotes dispersal of platelet thrombi (33). Moreover, EDRF may inhibit PAI-1 release from platelets, thereby increasing t-PA activity (34, 35), and may act synergistically with t-PA to disaggregate platelets (36). The demonstration that exposure of t-PA to endothelial cells

stimulated to secrete EDRF leads to *S*-NO-t-PA formation raises intriguing possibilities regarding the biological role of this modified protein. Inasmuch as NO is highly labile and undergoes rapid inactivation in the plasma and cellular milieus, this reaction between NO and protein thiol can serve as a means of stabilizing NO and preserving its bioactivity, selectively delivering it to areas of thrombus formation at which sites endothelial damage or dysfunction most likely engenders a relative deficit of NO. In this regard, it is noteworthy that S-nitrosylation most likely involves the reaction of thiolate anion (RS^-) with nitrosonium ion (NO^+) (37, 38), implicating redox pathways in the potential biochemical mechanism(s) of *S*-nitroso-protein formation *in vivo*. Our results suggest, then, that the endothelium is capable of synthesizing a molecule with intrinsic vasodilatory, antiplatelet, and fibrinolytic activities, potentially enabling it to counteract the major thrombogenic mechanisms that may be active in the local vascular milieu.

In summary, the S-nitrosylated plasminogen activator *S*-NO-t-PA possesses properties that may facilitate dispersal of thrombi *in vivo*. These data provide insight into the endogenous mechanism(s) by which the endothelium maintains vessel patency and offer a pharmacologic approach to the dissolution of thrombi.

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