Antiplatelet Properties of Protein S-Nitrosothiols Derived From Nitric Oxide and Endothelium-Derived Relaxing Factor


5-Nitrosothiols may serve as carriers in the mechanism of action of endothelium-derived relaxing factor (EDRF) by stabilizing the labile nitric oxide (NO) radical from inactivation by reactive species in the physiological milieu and by delivering NO to the heme activator site of guanylyl cyclase. Low-molecular-weight thiols, such as cysteine and glutathione, form S-nitrosothiol adducts with vasodilatory and antiplatelet properties, and protein thiols can interact in the presence of NO and/or EDRF to form uniquely stable S-nitroso-proteins. We now show that the S-nitroso-proteins, S-nitroso-albumin, S-nitroso-tissue type plasminogen activator, and S-nitroso-cathepsin B, have potent antiplatelet effects with an IC50 of approximately 1.5 μM. In the dog, S-nitroso-albumin inhibits ex vivo platelet aggregation and significantly prolongs the template bleeding time from 2.15±0.13 (mean±SEM) to 9.70±1.24 minutes. The antiplatelet action of S-nitroso-proteins is associated with the stimulation of guanylyl cyclase and a significant decrease in fibrinogen binding to platelets. S-Nitroso-proteins undergo thiol-nitrosothiol exchange with low-molecular-weight thiols to form low-molecular-weight S-nitroso-thiols, and they also interact directly with the platelet surface, both of which processes facilitate generation of NO. These data suggest that S-nitroso-proteins are potent antiplatelet agents and may be intermediates in the antiplatelet mechanism of EDRF action. (Arteriosclerosis and Thrombosis 1993;13:791-799)

KEY WORDS • S-nitrosothiols • thionitrites • cyclic GMP • thiols • sulfhydryl groups

The vascular endothelium plays an important role in modulating platelet function. Aggregating platelets secrete serotonin and adenosine diphosphate that bind to receptors on endothelial cells and in turn stimulate the release of endothelium-derived relaxing factor (EDRF). Recent studies suggest that EDRF can prevent platelet aggregation to the endothelium and inhibit platelet aggregation. Chemical and spectrophotometric analyses have led investigators to conclude that at least one form of EDRF is the highly labile nitric oxide (NO) radical. S-Nitroso-proteins formed by thiol-nitrosothiol exchange with low-molecular-weight thiols to form low-molecular-weight S-nitroso-thiols, and they also interact directly with the platelet surface, both of which processes facilitate generation of NO. In light of the established antiplatelet properties of NO, we now examine the effects of S-nitroso-proteins on platelet function. In this study, we 1) show that S-nitroso-proteins inhibit platelet function, 2) investigate in detail the mechanism of platelet inhibition by these biological NO adducts, 3) demonstrate novel aspects of their biochemical metabolism, and 4) document these inhibitory mechanisms in vitro and in vivo.

Methods

Tissue type plasminogen activator (t-PA) was provided by Genentech, Inc., South San Francisco, Calif. Fatty acid-free bovine serum albumin (BSA), cathepsin B, adenosine 5'-diphosphate (ADP), iodoacetamide, 5,5'-dithio-ditolobenzilic acid (DTNB), methylene blue, Sepharose 2B-300, and acetylsalicylic acid were obtained from Sigma Chemical Co., St. Louis, Mo. Sodium nitrate was purchased from Fisher Scientific, Fairlawn, N.J. NO gas was obtained from Matheson Gas, Secaucus, N.J. Sulfanilamide and N-(1-naphthyl)-ethylenediamine were purchased from Aldrich Chemi-

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cal Co., Milwaukee, Wis. Human fibrinogen (plasminogen-free and von Willebrand factor free) was purchased from Enzyme Research Laboratories, South Bend, Ind. Sephadex G-25 was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Thrombin was purchased from ICN Immunobiologicals, Costa Mesa, Calif. Iodoacetamide was purchased from Pierce Chemical Company, Rockford, Ill. Soluble calf skin collagen was purchased from Worthington Biochemical Corp., Freehold, N.J. Microcarrier beads (Biosilon) for endothelial cell culture were obtained from Vanguard International, Neptune, N.J. Dulbecco's modified Eagle's medium was purchased from GIBCO Technologies, Grand Island, N.Y. Bleeding time templates were purchased from Organon Teknika Corp., Durham, N.C. Radioimmunoassay kits for cyclic guanosine monophosphate (GMP) were purchased from New England Nuclear, Boston. Phosphate-buffered saline (PBS), pH 7.4, consisted of 10 mM sodium phosphate and 150 mM NaCl. Tris-buffered saline, pH 7.4, consisted of 10 mM tris(hydroxymethyl)aminomethane and 150 mM NaCl. HEPES-buffered saline (HBS), pH 7.35, consisted of 140 mM NaCl, 6 mM HCl, 6 mM N-(2-hydroxyethyl)piperazine-N'-((2-ethanesulfonic acid), 2 mM Na2HPO4, 2 mM MgSO4, 0.1% dextrose, and 0.4% BSA.

Synthesis of S-Nitroso-proteins

We examined several thiol-containing proteins of diverse function: (bovine) serum albumin, the most abundant plasma protein and the major source of thiol reducing equivalents (Cys 34) in plasma; t-PA, an endothelial-derived serine protease possessing a single, free cysteine residue (Cys 83) in its epidermal growth factor domain; cathepsin B, a lysosomal cysteine protease; and plasma, estimated to contain 500 µM free protein-thiol, the predominant source of which is albumin (Cys 34). Two methods were used to S-nitrosate these proteins: 1) proteins were exposed to NO generated from equimolar NaNO2 in 0.5N HCl (acidified NaNO2) and 2) proteins were exposed to 5-nitrosate these proteins: 1) proteins were exposed to NO generated from equimolar NaNO2 in 0.5N HCl (acidified NaNO2) and 2) proteins were exposed to.
To measure the effects of S-nitroso-proteins on the dispersal of platelet aggregates, S-nitroso-proteins were added to PRP or GFPs after maximal aggregation was induced with 5 μM ADP (corresponding to approximately 60% light transmittance compared with PRP). Disaggregation was quantified by measuring the maximal rate and extent of decrease in light transmittance after the addition of S-nitroso-proteins.

To exclude irreversible damage to platelets exposed to S-nitrosated proteins, we examined platelet function after the removal of S-nitrosated protein by gel filtration. PRP was incubated in the presence of 14 μM S-nitroso-BSA for 10 minutes or 60 minutes. PRP exposed to BSA or S-nitroso-BSA was then gel filtered on a Sepharose-2B column, and the platelets were counted and adjusted to 150,000/μL by the addition of HBS. Aggregation was then induced with 5 μM ADP as above and found not to be significantly different (maximal rate of aggregation, 0.85±0.05% transmittance/sec versus 0.88±0.03% transmittance/sec, n=6, p=NS, for t=10 minutes; 0.80±0.02% transmittance/sec versus 0.79±0.03% transmittance/sec, n=3, p=NS, for t=60 minutes).

**Fibrinogen Binding**

Human fibrinogen (free of plasminogen and von Willebrand factor) was radioiodinated with Iodo-beads as previously described.23 Radioiodinated fibrinogen binding to GFPs stimulated with 5 μM ADP was performed as previously described.24 Specific binding in the presence or absence of 14 μM S-nitroso-BSA was determined over a range of fibrinogen concentrations from 0.25 to 2.0 μM. Nonspecific binding was determined by using a 15-fold excess of unlabeled fibrinogen and accounted for 15% of total binding.

**Cyclic Nucleotide Assays**

Intraplatelet cyclic GMP was measured by radioimmunoassay. ADP (5 μM) was added to 300 μL GFPs incubated in the presence of 14 μM BSA or S-nitroso-BSA. After 1 minute, 300 μL of 10% trichloroacetic acid was added. Trichloroacetic acid was removed by ether extraction, and samples were acetylated with acetic anhydride to increase the sensitivity of the assay.

**Bleeding Time Determination and Ex Vivo Aggregation**

Mongrel dogs of either sex weighing between 20 and 26 kg were anesthetized with 20 mg/kg body wt sodium thiopental (Boehringer Ingleheim, St. Joseph, Mo.), intubated with a cuffed endotracheal tube, and ventilated with a respirator (Drager AV, North American Drager, Telford, Pa.) with 50% O2 (22 breaths/min, 10–12 mL/kg body wt per stroke volume). Anesthesia was maintained throughout the experimental protocol with 1–2% halothane. Animals used in this study were maintained in accordance with the guidelines of the Committee on Animals of the institution, and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (Department of Health, Education, and Welfare publication No. [NIH] 78-23, revised 1978).

Venous and arterial access was achieved, and intravenous doses of BSA or S-nitroso-BSA were then infused. Ex vivo platelet aggregation (induced by 5 μM ADP or 0.1 mg/mL collagen) measurements were performed at baseline and 5 minutes after infusion of BSA or S-nitroso-BSA at 1, 10, or 50 nmol/kg body wt per minute for 5 minutes. Bleeding time measurements were performed at baseline and 5 minutes after bolus infusion of 183 nmol/kg body wt of BSA or S-nitroso-BSA using a template apparatus applied to the ventral aspect of the tongue to minimize temperature fluctuation.

**Protein Determinations**

Protein concentrations were determined by the method of Bradford.25

**Statistical Analysis**

 Determination of statistical significance was carried out by nonpaired t test or two-way analysis of variance followed by a Newman-Keuls comparison. Values of p<0.05 were considered significant.

**Results**

**Inhibition of Platelet Function by S-Nitroso-proteins**

The effects of S-nitroso-proteins were first examined using GFPs. S-Nitroso-BSA inhibited ADP-induced platelet aggregation in a dose-dependent manner (Figure 1) with an apparent IC50 of 1.5 μM. Similar IC50s were measured for aggregation induced by collagen (1.2 μM) and thrombin (1.3 μM). In control experiments, BSA alone had no significant effect on platelet aggregation over the range of concentrations tested. S-Nitroso-t-PA, S-nitroso-cathepsin B, and S-nitrosated plasma also inhibited platelet aggregation (Table 1). As shown in Table 1, S-nitroso-BSA, S-nitroso-t-PA, and S-nitroso-cathepsin B were essentially equipotent, with IC50s of approximately 1.5 μM. Quantification of S-nitrosothiol before and after trichloroacetic acid precipitation showed that approximately 95% S-nitrosothiol was protein bound.

S-Nitroso-proteins were also synthesized by exposure of the proteins to authentic EDRF (NO) from bovine aortic endothelial cells stimulated to secrete EDRF as described in “Methods.” S-Nitroso-proteins synthesized with EDRF also inhibited platelet aggregation (Figure 1). Neither BSA exposed to unstimulated endothelial cells nor PBS incubated with stimulated cells in the absence of BSA inhibited ADP-induced platelet aggregation in PRP. Pretreatment of endothelial cells with acetylcholine was performed in all cases to eliminate prostaglandin synthesis.

The platelet-inhibitory action of S-nitroso-proteins was also comparable in GFPs and whole-blood preparations. For example, the relative extent of aggregation compared with control for 1.4 μM S-nitroso-BSA was 0.54±0.04 (mean±SEM) in GFPs, 0.39±0.13 in PRP, and 0.64±0.03 in whole blood. Disaggregation of platelets by S-nitroso-proteins was also observed at equivalent concentrations as those inducing inhibition of platelet aggregation (data not shown).

**Time Dependence of Antiplatelet Effects of S-Nitroso-proteins**

The platelet-inhibitory effect of S-nitroso-proteins was time dependent. Increasing incubation times led to...
progressive increases in the degree of platelet inhibition (Figure 2). S-Nitroso-BSA (140 nM) completely inhibited platelet aggregation in PRP after 60 minutes of incubation, indicating inhibition of the primary phase of ADP-induced aggregation. This time dependence probably reflects the relative stability of the protein S-NO bond and the gradual, cumulative release of NO from the S-nitroso-protein source. Whereas S-nitroso-proteins have half-lives of approximately 24 hours in PBS at 37°C, they have half-lives on the order of only 60 minutes in plasma at 37°C. By comparison, the half-life of NO in vivo is approximately 0.1 second.

Transfer of NO From S-Nitroso-proteins to Low-Molecular-Weight Thiols

The stability of S-nitroso-proteins under physiological conditions, as well as their differential reactivity in PBS and plasma, begs the question of the mechanism by which these stable adducts release and deliver NO to the platelet in the aggregation experiments described here. To address this issue, we examined the effect of low-molecular-weight thiols on protein-bound S-nitrosothiol in a solution of S-nitroso-BSA. We incubated 50 μM S-nitroso-BSA with an equimolar concentration of reduced glutathione and measured the transfer of NO from BSA to glutathione over time by the Saville reaction after protein precipitation. As shown in Figure 3, approximately one half of the S-nitroso-BSA was converted to S-nitroso-glutathione after 10 minutes at 25°C. Comparable results were obtained with a cell-permeable, low-molecular-weight thiol, N-acetylcysteine. The functional correlate of this transfer was examined by showing that increasing concentrations of glutathione led to progressive inhibition of platelet aggregation in the presence of a subthreshold (under these conditions) concentration of S-nitroso-BSA (Figure 4). The implications of S-nitroso-glutathione-mediated platelet inhibition in these studies is further supported by the comparable potency of this compound synthesized de novo with acidified NaNO₂ (IC₅₀=20 μM).

The ability of low-molecular-weight thiols to enhance the inhibitory action of S-nitroso-proteins can account for the action of S-nitroso-BSA in PRP or whole blood; however, in a GFP system, some other mechanism of facilitated transfer of NO must be operative. The platelet surface is another source of reduced thiol equiva-
FIGURE 2. Effect of increasing incubation time on the degree of platelet inhibition by 140 nM S-nitroso- bovine serum albumin (BSA). Gel-filtered platelets were incubated with 140 nM S-nitroso-BSA for up to 1 hour, and platelet aggregation induced by 5 μM adenosine diphosphate in the presence of 0.1 mg/mL fibrinogen was determined at 1, 10, 30, 45, and 60 minutes during the incubation period as described in "Methods."

lents, and therefore, we tested the ability of GFPs in protein-free buffer (calcium-free Tyrode's-HEPES) to facilitate the removal of NO from 5-nitroso-BSA. GFPs were incubated with 132 μM S-nitroso-BSA, and the rate of loss of NO from BSA was determined by centrifuging the platelets from the incubation suspension over time, after which the S-nitroso-BSA content of the supernatant was measured by the Saville reaction.18 GFPs facilitated the release of NO from S-nitroso-BSA at a rate of 5.3±2.7 nmol/min per 10⁴ platelets (n=4); spontaneous release of NO from S-nitroso-BSA under these conditions was essentially undetectable (i.e., <0.1 nmol/min per 10⁴ platelets). These data support the hypothesis that the platelet surface can also catalyze the

FIGURE 3. Line plot showing the transfer of nitric oxide (NO) from S-nitroso-proteins to low-molecular-weight thiol. S-Nitroso-bovine serum albumin (BSA) (50 μM) was incubated with an equimolar concentration of reduced glutathione (GSH) at 25°C, and the distribution of NO between BSA (open circles) and GSH (closed circles) was determined over time by protein precipitation and the Saville reaction18 (n=2).

FIGURE 4. Semilog plot showing effect of increasing concentrations of reduced glutathione (GSH) on the extent of gel-filtered platelet (GFP) aggregation. S-Nitroso-bovine serum albumin (BSA) (15 μM) was incubated with increasing concentrations of GSH for 5 minutes and subsequently added to GFPs, in which the final concentration of S-nitroso-BSA was 5 μM. After 2 minutes the extent of aggregation induced by adenosine diphosphate was determined (open circles) in calcium-free Tyrode's-HEPES buffer containing 0.1 mg/mL fibrinogen and compared with that of S-nitroso-BSA (open circle on y axis) and GSH alone (open triangle).
release of NO from S-nitroso-proteins and thereby, possibly account for the antiplatelet action of these otherwise stable physiological adducts.

**Inhibition of S-Nitroso-protein Antiplatelet Action**

Blockade of protein thiols by carboxyamidation prevented S-nitrosothiol formation, as determined chemically by the method of Saville as well as spectroscopically. This modification rendered the proteins exposed to NO or EDRF incapable of inhibiting platelet aggregation (Figure 5).

**Effect of S-Nitroso-proteins on Platelet Cyclic GMP and Fibrinogen Binding**

Consistent with the mechanism of platelet inhibition by organic nitrates, low-molecular-weight S-nitrosothiols, and EDRF, the platelet-inhibitory effect of S-nitroso-proteins was abolished by methylene blue, an inhibitor of guanylyl cyclase (Figure 5). The importance of cyclic GMP in the mechanism of platelet inhibition by S-nitroso-proteins was confirmed by showing that S-nitroso-BSA (14 μM) induced an approximate twofold increase in intraplatelet cyclic GMP compared with basal levels at 1 minute after exposure (n=4; mean±SEM of 0.61±0.07 picomoles of cyclic GMP per 10^9 platelets compared with a basal level of 0.35±0.04; p=0.022). BSA alone had no effect on cyclic GMP levels.

To elucidate further the mechanism of platelet inhibition by S-nitroso-proteins, fibrinogen-binding studies were performed in GPs. Figure 6 shows that exposure of the platelets to S-nitroso-BSA led to a reduction in the number of platelet-bound fibrinogen molecules. S-Nitroso-BSA at a concentration of 14 μM, which inhibited platelet aggregation by 81%, caused a reduction in ADP-stimulated fibrinogen binding of 60% (54,000±12,000 fibrinogen molecules per platelet in the presence of BSA compared with 22,000±3,000 fibrinogen molecules per platelet in the presence of S-nitroso-BSA; mean±SEM of four experiments; BSA versus S-nitroso-BSA, p=0.04).

**Effects of S-Nitroso-proteins on Platelet Function In Vivo**

Finally, the biological/physiological relevance of S-nitroso-proteins was investigated in mongrel dogs by following the protocol described in "Methods." A dose-dependent inhibition of ex vivo aggregation was apparently induced by ADP (Figure 7) or collagen (data not shown) and was accompanied by a significant prolongation of the bleeding time (Table 2).

**Discussion**

The experiments presented here demonstrate that NO and EDRF will react with protein thiols to form S-nitroso-protein adducts that are potent platelet-inhibitory compounds in vitro, ex vivo, and in vivo. The mechanism by which exogenous and endogenous nitro(so) derivatives inhibit platelets parallels that for vascular smooth muscle: the free radical NO stimulates guanylyl cyclase by forming a nitrosyl-heme complex at the activator site of the enzyme. Mellion and co-workers extended these observations to the platelet, confirming that NO inhibits platelets by elevating cyclic GMP. Our laboratory showed that NO-mediated elevation of cyclic GMP results in a marked decrease in the number of fibrinogen molecules bound to the platelet, to an inhibition of intracellular calcium flux, and to an inhibition of platelet secretion. The mechanism by which cyclic GMP leads to these inhibitory effects remains to be defined but may involve protein phosphorylation events regulated by cyclic GMP or signal transduction events involving the phosphoinositol pathway. The characteristics of the platelet-inhibitory response for S-nitroso-proteins described in...
this study—namely, reversal by methylene blue, elevation of intraplatelet cyclic GMP, and corresponding diminution of fibrinogen binding to the platelet—suggest that S-nitroso-proteins act through a similar inhibitory mechanism as has been described for organic nitrates and related nitro(so) compounds, low-molecular-weight nitrosothiols, and EDRF. In view of the fact that NO is rapidly inactivated by molecular oxygen, superoxide anion, and heme as well as nonheme iron, it has been postulated that NO is stabilized by a carrier molecule that preserves its biological activity. Since Ignarro and coworkers demonstrated that nitro(so) compounds can react with reduced low-molecular-weight thiols such as cysteine and glutathione to form 5-nitrosothiols, evidence has mounted that supports the view that 5-nitrosothiols may serve such a carrier role as intermediates in the mechanism of nitrate and EDRF action. In platelets in particular, Loscalzo has shown that the reduced thiol N-acetylcysteine potentiates the antiplatelet effects of organic nitrates and that the incubation of nitroglycerin in PRP results in intraplatelet glutathione depletion coincident with the formation of S-nitrosothiols.

The role of reduced thiols in the action of EDRF is more controversial. Palmer and colleagues and Ignarro and coworkers have separately concluded that EDRF is chemically and spectrophotometrically identical to NO. However, it has recently been proposed that the vasodilatory properties of EDRF more closely resemble those of S-nitroso-cysteine than of NO, and the possibility that EDRF is released abluminally in the form of a nitroso derivative has been recently entertained. Notwithstanding the controversy over the chemical identity of EDRF, these reports, the findings that N-acetylcysteine potentiates the antiplatelet effects of EDRF and prolongs its half-life, and the present demonstration of S-nitrosothiol formation from endogenous NO, taken together, appear to support strongly the possibility of a role for reduced thiol in the metabolism of EDRF.
The biological importance of protein as a source of reduced thiol for reaction with NO remains to be determined. However, the remarkable prevalence of protein thiols (the most prevalent source of sulfhydryl groups in plasma and a rich source in the cell cytosol) and the highly specialized nature of this functional group suggest that the reaction with NO (through its oxidation to the reactive nitrosonium ion NO⁺ or related nitrondonium-generating species, N₂O₃) may be regulated in a manner other than simple diffusion limited. Several lines of evidence indirectly support the notion that the formation of protein S-nitrosothiols is of biological importance. First, Fung and coworkers have demonstrated that the thiol of albumin catalyzes the denitrogenation of nitroglycerin in plasma. Second, thiocyanates are proposed intermediates in the enzymatic denitrogenation of nitroglycerin by glutathione-S-transferase and glyceraldehyde-3-phosphate dehydrogenase. Third, hemoglobin has been shown to react by way of a sulfhydryl with a nitroso derivative of arylhydroxylamine metabolism. Fourth, Mordvintsev and coworkers have documented that platelet inhibition by iron dinitrosoyl complexes occurs in association with binding of these complexes to thiols on the platelet surface. Our findings that NO (or NO⁺) and EDRF (or a derivative thereof) will react with protein thiols to form stable S-nitroso-proteins suggest that proteins may need to be considered in the metabolism of EDRF. Several platelet proteins in particular, such as myosin, and one type of ADP receptor on the platelet, possess free thiols and are, therefore, potential thiol functional groups for reaction with endogenous NO (or NO⁺) by which platelet inhibition may theoretically result. The precise mechanism(s) by which NO, carried on proteins as a relatively stable S-nitrosothiol adduct, traverses the platelet membrane to enter the cell and thereupon activates guanylyl cyclase is as yet unknown. The potential for transfer of NO from an S-nitroso-protein to a low-molecular-weight thiol that can, in turn, enter the platelet or facilitate NO transfer and release at the platelet surface is supported by the data presented here and by recent data from our group. The greater nucleophilicity of low-molecular-weight thiols toward NO⁺ as a consequence of their much higher pKs compared with those for certain protein thiols, such as Cys 34 of serum albumin, favors the transfer of NO from protein thiol. In the case of GFPs, platelet surface-catalyzed release of NO must also be considered. In recent studies of nitro(so) vasodilator metabolism in smooth muscle, Fung and coworkers have reported preliminarily that S-nitrosotliol conversion to NO is mediated by the plasma membrane. These findings are now substantiated and expanded to the platelet by the data presented above and the data of Mordvintsev and colleagues. S-Nitroso-protein formation has pharmacological implications as well. The proteins selected in this study were simply chosen as model compounds for a plasma protein (BSA), an endothelium-derived enzyme (t-PA), and a cysteine protease (cathepsin B) that contain well-characterized, single, free thiols. The possibility that S-nitroso-protein adducts may be therapeutically useful as antiplatelet agents or vasodilators should be considered.

In summary, we have demonstrated that 1) NO and EDRF will react on exposure to sulfhydryl-containing proteins to form stable S-nitroso-proteins; 2) S-nitroso-proteins are potent platelet inhibitors in vitro, ex vivo, and in vivo; 3) the mechanism of platelet inhibition by S-nitroso-proteins, through increases in cyclic GMP, is similar to that of NO and related nitro(so) compounds; 4) NO is capable of being transferred from S-nitroso-proteins to low-molecular-weight thiols; and 5) the platelet surface is capable of facilitating the release of NO from S-nitroso-proteins. These data suggest that S-nitroso-proteins may serve as intermediates in the cellular metabolism of NO and raise the possibility that this posttranslational modification of protein structure may represent a novel type of cellular regulatory mechanism.

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References

Antiplatelet Effect of S-Nitroso-proteins


Antiplatelet properties of protein S-nitrosothiols derived from nitric oxide and endothelium-derived relaxing factor.
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