Norepinephrine Down-Regulates the Activity of Protein S on Endothelial Cells

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Abstract. The adrenergic agonist norepinephrine is shown to stimulate endothelium to induce protein S release and degradation, leading to diminished anti-coagulant activity and to down-regulation of protein S cell surface-binding sites. Norepinephrine-induced release of intracellular protein S was blocked by the α_1-adrenergic antagonist prazosin (10^{-7} M) but not by the α-adrenergic antagonist propranolol (10^{-6} M) or the α_2-adrenergic antagonist yohimbine (10^{-5} M) indicating that this response resulted from the specific interaction of norepinephrine with a class of α_1-adrenergic receptors not previously observed on endothelium. Attenuation of norepinephrine-induced release of protein S by pertussis toxin in association with the ADP-ribosylation of a 41,000-D membrane protein indicates that this intracellular transduction pathway involves a regulatory G protein. The observation that protein S was released from endothelium in response to maneuvers which elevate intracellular calcium or activate protein kinase C suggests that the response may be mediated via intermediates generated through the hydrolysis of phosphoinositides. Morphologic studies were consistent with a mechanism in which norepinephrine causes exocytosis of vesicles containing protein S. In addition to release of protein S, norepinephrine also induced loss of endothelial cell protein S-binding sites, thereby blocking effective activated protein C-protein S-mediated factor Va inactivation on the cell surface. Norepinephrine-mediated endothelial cell stimulation thus results in loss of intracellular protein S and suppression of cell surface-binding sites, modulating the anti-coagulant protein C pathway on the vessel wall. These studies define a new relationship between an anti-coagulant mechanism and the autonomic nervous system, and indicate a potential role for an heretofore unrecognized class of α_1-adrenergic receptors in the regulation of endothelial cell physiology.

THE regulatory role of the endothelial cell in coagulation involves both control of receptor expression on the cell surface and release of hemostatically active products which play a role in anticoagulant and procoagulant mechanisms. These considerations indicate that a potentially important link between coagulation and environmental stimuli could involve modulation of endothelial cell coagulant properties by circulating mediators. The protein C pathway is an anticoagulant mechanism integrally involved in the defense against thrombosis and closely linked to endothelium in terms of its function (9). Endothelium provides receptors promoting initiation (9) and propagation (36) of this anticoagulant mechanism. Propagation involves the binding of protein S to the bovine endothelial cell surface which facilitates formation of the activated protein C/protein S complex (36). In addition to provision of receptors, endothelium also synthesizes and releases the vitamin K-dependent cofactor, protein S (12, 35). Modulation of endothelial cell-protein S interaction, induced by a mediator of the host response which redirects intracellular regulatory pathways, could thus impair function of this antithrombotic system.

Stimulation of the autonomic nervous system has long been associated with a prethrombotic state. Muller-Berghaus et al. (27, 28) observed that the generalized Schwartzman reaction, which includes prominent thrombotic pathology, could be prevented by α-adrenergic blockade. Catecholamines acting via an α-adrenergic mechanism have been shown to induce capillary thrombosis in multiple organs (23). To explain this apparent relationship between catecholamine stimulation and thrombosis, we tested the hypothesis that α-adrenergic stimulation of the vessel wall results in modulation of endothelial cell coagulant properties in addition to changes in vasomotor tone (24).

The results of our studies of the endothelial cell-protein S interaction indicate that norpinephrine interacts with previously undescribed endothelial cell α_1-adrenergic receptors which are coupled to release and inactivation of intracellular

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protein S by a GTP-binding protein. Since norepinephrine is also shown to decrease expression of protein S–binding sites on the endothelial cell surface, the vessel wall is rendered deficient not only in endogenous protein S, but also in its ability to interact with exogenous protein S. This effect of norepinephrine modulating function of the anticoagulant protein C pathway defines a new relationship between a vessel wall anticoagulant property and the autonomic nervous system, and indicates an intracellular mechanism by which coagulation can respond to environmental stimuli.

Materials and Methods

Cell Culture

Bovine aortic endothelial cells were isolated from calf aortas and cultured in minimal essential medium (M.A. Bioproducts, Walkersville, MD) supplemented with penicillin-streptomycin (50 U/ml–50 μg/ml; M.A. Bioproducts), glutamine (1%; M.A. Bioproducts) and FCS (10%; Hyclone, Sterile Systems, Logan, UT) as described previously (30). For these studies, growth medium was supplemented with vitamin K_1 (10 μg/ml) (Aqua MEPHYTON; Merck, Sharp and Dohme, West Point, PA) three to 4 d before experiments were carried out. Cultures were characterized as endothelial based on morphologic criteria (30) and immunofluorescence for von Willebrand Factor and protein S antigens (15, 35) and a functional assay for thrombomodulin (10). Cells were separated for subculture nonenzymatically with Dulbecco's PBS (calcium and magnesium-free) that contained 10 mM sucrose and 1 mM EDTA. For experiments, cells from different aortas were grown to confluence in 10, 2, or 0.32 cm^2 wells (1.1–1.5 × 10^5 cells/cm^2). To study release of intracellular protein S from endothelium and binding of exogenous [^{125}I]protein S to monolayers, it was necessary to elute protein S already on the cell surface prior to carrying out experiments. The following protocol resulted in complete elution of protein S from the cell surface. 48 h before experiments, cells were washed four times in serum-free medium (minimal essential medium containing penicillin-streptomycin [50 U/ml–50 μg/ml], Hepes [10 mM], transferrin [20 μg/ml] [Sigma Chemical Co., St. Louis, MO], insulin [10 μg/ml; Sigma Chemical Co.], vitamin K_1 [10 μg/ml], BSA [5 mg/ml; Sigma Chemical Co.]) and then maintained in this serum-free medium. Just before carrying out a study, cells were washed four times over a period of 1 h with serum-free medium (37°C) to promote dissociation of surface-bound protein S. In pilot studies, endothelium was then treated with the same buffer containing dextran sulfate (10 mg/ml) for 5 min at 23°C (this treatment has been previously shown to elute cell-bound protein S [36]), the eluate was concentrated 20-fold (Speed Vac Concentrator, E. Savant, Farmingdale, NY) and assayed for protein S antigen in the radioimmunoassay described below. The endothelial cell eluate had no detectable protein S antigen, corresponding to less than 20 fmole of protein S antigen per 10^6 cells. This amount of protein S would not interfere with [^{125}I]protein S endothelial cell–binding studies or protein S release experiments. Similar complete elution of protein S by this washing procedure was observed when [^{125}I]protein S was added to cultures before the first wash.

After the washing procedure, experiments were carried out in serum-free medium by the procedures described below using cells from passage 1 to 5. Cells which had been maintained in culture for long periods showed decreased responsiveness to norepinephrine and decreased amounts of intracellular protein S before stimulation.

Human umbilical vein endothelial cells were grown as described (16).

Coagulation Factors and Assays

All purified coagulation factors were of bovine origin. Purification of protein S was carried out as described (42) and protein S was radiolabeled by the lactoperoxidase method using Enzymobeads (Bio-Rad Laboratories, Richmond, CA) as previously described (35). Radioiodinated protein S comigrated with unlabeled material on SDS-PAGE and the specific radioactivity was 8,000–12,000 cpm/ng (corresponding to 0.2–0.3 mol ^{125}I per mole of protein S). Preparation of a monospecific rabbit anti–bovine protein S antiserum was done by standard methods (14) as described previously (35). Affinity purified antibody to protein S was prepared using a protein S-affigel column as described previously (35) and the radioimmunoassay for protein S was carried out by the previously described protocol (33). The limit of detection in this assay was 100 pM protein S antigen, which corresponded to 80% binding on the standard curve. When necessary, samples were concentrated (Speed Vac Concentrator, Savant, Farmingdale, NY) before assay.

Protein C was purified and activated as described previously (43). Activated protein C was stored at 4°C and used within 48 h of its preparation. Factor Va was also purified as described previously (8) and the preparation used in this study were recombined from isolated subunits in the presence of 10 mM $CaCl_2$, overnight at 4°C. Activated protein C/protein S-mediated Factor Va inactivation over endothelial cell monolayers was carried out by previously described methods (36). Monolayers (2 cm^2/well) were prepared with serum-free medium described above, and then 0.5 ml of 10 mM Hepes (pH 7.45) that contained 137 mM NaCl, 4 mM KCl, 11 mM glucose, 3 mM $CaCl_2$ and 1 mg/ml BSA was added. Monolayers were incubated at room temperature in the presence of protein S (60 nM or as indicated), Factor Va (80 nM) and activated protein C (1 nM). An aliquot (25 μl) was removed from each well at 10, 30, 60, 120, and 180 s of incubation and assayed immediately in a one-stage clotting assay (19) using Factor V-deficient human plasma (2) as described previously (34). The rate of Factor Va inactivation was determined from the slope of the linear initial portion of a plot of Factor Va activity vs. incubation time. Protein S functional activity on phospholipids was determined using a one-stage Factor Xa coagulant assay and barium adsorbed plasma by the method of Walker (43) as modified by others (5, 27). This assay measures activated protein C prolongation of the Factor Xa coagulant assay which is dependent on protein S. Factor Xa (100 U/mg) was prepared as described previously (37) and the barium adsorbed plasma was supplemented with purified prothrombin (13 U/mg) (25) to a final concentration of 0.1 mg/ml. Activated protein C was prepared as described above. When the anticoagulant activity of endothelial cell protein S was tested, endothelium was incubated in buffer containing 0.01% BSA (to facilitate subsequent concentration) and then exposed to norepinephrine. Next, samples were concentrated and assayed as described above. Control samples consisted of protein S added to the same volume of culture medium and treated identically.

Western blotting of protein S released from endothelium following treatment of cultures with norepinephrine (see below) was carried out as follows: protease inhibitors (2 mM PMSF and 0.3 mM leupeptin) and 5 mM EDTA were added to releasates and samples were then immediately prepared for reduced SDS-PAGE by the method of Laemmeli (22). Western blotting was carried out by a modification of the method of Towbin et al. (39) as described previously (35). After electrophoretic transfer of proteins to the nitrocellulose membrane, excess binding sites on the membrane were blocked (17) and blots were reacted sequentially with affinity-purified rabbit anti–bovine protein S IgG (10 μg/ml) and [^{125}I]purified anti–rabbit IgG (1.3 × 10^5 cpm/ml). This protocol has been described in detail previously (35). Dried blots were subjected to autoradiography at −80°C using Kodak X-Omat (XAR 5) film (Eastman Kodak Co., Rochester, NY) and a cronex intensifying screen (DuPont Co., Wilmington, DE). Standard proteins were run simultaneously for molecular weight determination: myosin heavy chain (M_r 200,000), phosphorylase B (M_r 97,400), BSA (M_r 68,000), ovalbumin (M_r 43,000), and α-chymotrypsin (M_r 25,700) (Bethesda Research Laboratories, Bethesda, MD). Controls in which antibody to protein S was omitted and only [^{125}I]anti–rabbit IgG was incubated with nitrocellulose membranes with immobilized proteins demonstrated no bands (Fig. 6, lane *E*).

Radioligand-binding studies were carried out as described previously (36). In brief, monolayers (0.32 cm^2/well) were prepared for experiments as described above, and then serum-free medium (50 μl) containing [^{125}I]protein S alone (total binding) or in the presence of an 100-fold excess of unlabeled protein S (nonspecific binding) was added for 90 min at 2°C. Assays were terminated by three rapid washes at 2°C with the above incubation buffer (0.1 ml/wash) and monolayers were eluted with dextran sulfate (10 mg/ml). Data from binding experiments were fit to the equilibrium-binding equation described by Klotz and Hunston (21) assuming a one-site model as described previously for [^{125}I]protein S-endothelial cell binding. A nonlinear least squares program (generously provided by Dr. Greg Reinhart, University of Oklahoma, Norman, OK) was used to obtain the binding parameters. A plot of residuals vs. free radioligand for the binding data shown in Fig. 7 *B* indicated that no systematic error was involved in fitting the binding to the model used (data not shown). ^{125}I-protein S-endothelial cell–binding studies were not carried out on cells in more than passage 5 in culture since cells in later passages and sprouting endothelial cells (31) showed variably decreased binding.

Release of Protein S from Endothelium

Confluent endothelial cell monolayers (10.0 cm^2/well) were washed as described above and equilibrated with serum-free medium consisting of mini-

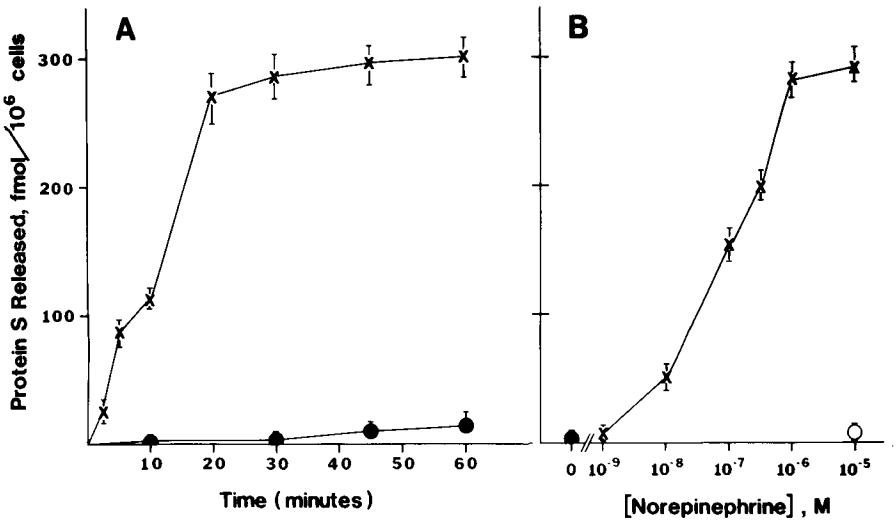

Figure 1. Norepinephrine induced release of endothelial cell protein S. (*A*) Time course. Confluent monolayers of endothelium (passage 2) were prepared as described under Materials and Methods and incubated at 37°C with serum-free medium alone (●) or serum-free medium containing norepinephrine (X) (10^{-5} M). At the indicated times, aliquots of culture supernatant were withdrawn, concentrated if required, and assayed for protein S antigen. Data shown represent protein S antigen released per 10^6 cells (the mean and SEM) vs. incubation time. Maximal protein S release (60 min) represented 80% of the total intracellular protein S (380 ± 40 fmole/10^6 cells). The latter number was obtained by assaying detergent extracts of untreated control cultures after eluting surface bound protein S. Details of experimental procedures are described under Materials and Methods. (*B*) Dose dependence. Confluent monolayers of endothelium (passage 3) were prepared as described under Materials and Methods, and incubated at 37°C for 60 min with serum-free medium alone (●), serum-free medium containing the indicated concentration of the (γ) stereoisomer of norepinephrine (X), or serum-free medium containing the indicated concentration of (d) stereoisomer of norepinephrine (○). Protein S antigen in the supernatant (mean ±SEM) is plotted vs. the added concentration of norepinephrine.

mum essential medium, 10 mM Hepes (pH 7.4) and 5 mg/ml BSA (1.0 ml). Then antagonists, either yohimbine (Squibb), prazosin (Squibb) or propranolol (Sigma Chemical Co.), were added and followed 5 min later by norepinephrine (Sigma Chemical Co.; the designation norepinephrine represents the [γ] stereoisomer). Where indicated, (*d*) norepinephrine was used in place of [γ] norepinephrine. When experiments were carried out with pertussis toxin (List Biologicals, Campbell, CA), the latter was added to the serum-containing growth medium 24 h before norepinephrine and cells were maintained in complete medium until norepinephrine was added in serum-free medium. After the addition of norepinephrine, cultures were incubated for the indicated times at 37°C and samples of supernatant were obtained and tested for protein S antigen (using the radioimmunoassay) or functional activity (using the coagulant assay) as described in the above section. Studies with the phorbol ester 12-O-tetradecanoyl-phorbol-13-acetate (Consolidated Midland, Brewster, NY) or 1-oleoyl-2-acetyl-rac-glycerol (Sigma Chemical Co.) were carried out by the same protocol except they were added to cultures at the indicated final concentrations in place of norepinephrine. The same protocol was also followed when the calcium ionophores A23187 (Calbiochem-Behring, La Jolla, CA) and Ionomycin (Calbiochem-Behring) were added to cultures. Ionophores were prepared as described previously (35). The final concentration of organic solvent was less than or equal to 0.01%. Total cell-associated protein S was determined by solubilizing endothelium for 20 min at 25°C in 0.5 ml of 0.02 M Tris (pH 7.4), 0.1 M NaCl that contained Nonidet P-40 (1%), EDTA (5 mM), PMSF (2 mM) and leupeptin (0.3 mM).

Immunolocalization of Protein S

Immunofluorescent localization of protein S was performed as previously described (35). Cells were prepared for immunoelectron microscopy as outlined by Dunphy et al. (7) with the following modification: monolayers grown in 35-mm dishes were fixed in 2% paraformaldehyde and 0.05% glutaraldehyde in PBS for 15 min, scraped from the dish with a rubber policeman, and fixation continued for an additional 30 min. Cell pellets were subsequently washed in PBS containing 50 mM NH_4Cl, 0.05% Saponin and 0.1% BSA three times (10 min/wash), dehydrated in a graded series of ethanol solutions, embedded in Lowicryl and U.V. polymerized at −20°C for 24 h followed by an additional 48 h polymerization at room temperature. Thin sections were collected on formvar-coated nickel grids, washed in deionized water and incubated with 1% normal sheep serum in 0.1% Tris buffer containing 0.01% Tween 20 for 15 min at 37°C. After this blocking step, sections were incubated with affinity purified rabbit anti-protein S in the above buffer for 1 h at 37°C and binding sites of anti-protein S IgG were displayed with 15-nm gold-conjugated goat anti-rabbit immunoglobulin (Janssen Pharmaceutica, Beerse, Belgium). After labeling with second antibody, grids were washed in water, stained with aqueous uranyl acetate and lead citrate, and viewed in a Philips 300 electron microscope. Sections incubated with gold-conjugated anti-rabbit immunoglobin alone or with preimmine serum followed by gold-conjugated anti-rabbit immunoglobulin did not stain.

ADP Ribosylation of G Proteins by Pertussis Toxin

Endothelial cells from control cultures or those pretreated with pertussis toxin were harvested in sucrose (250 nM), PMSF (0.1 mM), EDTA (10 mM), Tris (50 mM) buffer and homogenized with a Teflon-coated pestle. The pellet sedimenting after centrifugation at 40,000 *g* for 45 min was resuspended at a concentration of 1.5 mg/ml in the above buffer and stored at −80°C until use. ADP-ribosylation was performed as described previously (4) with minor modifications. Pertussis toxin was activated by incubation with 10 mM DTT for 10 min at 30°C. Endothelial cell membranes (25 μg) were incubated in buffer (0.1 ml) containing 50 mM K_3PO_4, 10 U aprotinin, 0.2 mM GTP, 20 mM thymidine, 5 mM ADP-ribose, 20 mM arginine, 1 mg/ml dimyristoyl phosphatidylcholine, 0.1% Lubrol, 10 μM [^{32}P]-NAD (18–54 Ci/mmol) and 2.5 μg pertussis toxin for 20 min at 30°C. The reaction was terminated by the addition of 20 μl of 10% SDS. Samples were treated with N-ethylmaleimide as described by Sternweis and Robishaw (38). Electrophoresis was performed on slab gels (resolving gel, 9%; stacking gel, 5% acrylamide) at 300 V for 3 h. The gels were subjected to autoradiography at −80°C using Kodak XRP-5 film and the concentration of G protein was calculated from the specific activity of the [^{32}P]NAD and the number of counts in the 41,000-D band cut from the gel.

Results

Incubation of endothelial cell monolayers with norepinephrine led to a time-dependent release of protein S into the culture medium (Fig. 1 *A*). Norepinephrine-induced release of protein S reached an apparent maximum after 20 min and the amount of protein S released in the supernatant was ~65–80% of the total cell-associated protein S as determined by radioimmunoassay. Protein S released from endothelium in response to norepinephrine probably originated in an intracellular pool since monolayers were grown in serum-free medium and were carefully prepared for studies in a manner which eluted previously bound protein S (see Materials and Methods). Further support for this hypothesis comes from immunofluorescence studies using affinity

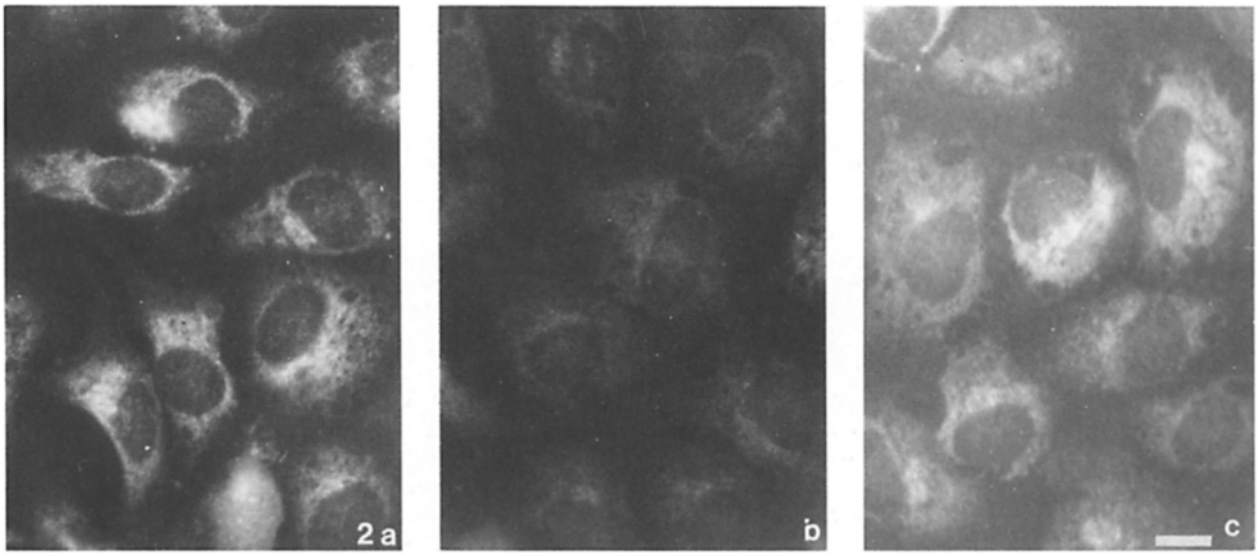

Figure 2. Immunofluorescent localization of protein S in endothelium: the effect of norepinephrine. Control monolayers (*a*) display dense cytoplasmic staining for protein S in the perinuclear Golgi region and also a finely punctate endoplasmic distribution. Treatment of endothelial cells with norepinephrine (10^{-5} M) (*b*) results in markedly reduced endoplasmic staining and the virtual loss of juxtanuclear staining. Incubation of monolayers with norepinephrine in the presence of prazosin (10^{-6} M) (*c*) effectively prevents loss of the cytoplasmic staining for protein S. Bar, 10 μm.

purified anti-bovine protein S IgG (Fig. 2). Before norepinephrine treatment, monolayers display a punctate endoplasmic distribution of protein S which is concentrated in the perinuclear Golgi region (Fig. 2 *a*). After exposure to norepinephrine (10^{-5}–10^{-6} M) for 30–60 min, discrete cytoplasmic staining is markedly reduced and the intense staining for protein S in the perinuclear region is virtually extinguished (Fig. 2 *b*). This loss of cytoplasmic staining of protein S is paralleled by an increase of protein S (as measured in culture supernatants by radioimmunoassay and in cells by immunofluorescence microscopy) relative to the total intracellular pool is somewhat variable, the response of isolates from a single aorta is quite constant over one to five passages in culture.

Norepinephrine-stimulated protein S release was maximal by 10^{-5} M and half-maximal at 10^{-7} M (Fig. 1 *B*). This effect of norepinephrine was mediated exclusively by an β_1-adrenergic mechanism. The β-adrenergic agonist isoproterenol did not stimulate protein S release and the α-adrenergic antagonist propranolol (10^{-6} M) (Fig. 3, *III*) did not inhibit the effect of norepinephrine on endothelial cell protein S. Absence of a β-adrenergic effect modulating protein S release indicates that the response to norepinephrine probably does not result from the intracellular accumulation of cAMP, a known consequence of α-adrenergic stimulation (4). Stimulation of protein S release by norepinephrine was inhibited by α-adrenergic antagonists with a hierarchy indicating α_1-adrenergic receptor subselectivity (Fig. 3, *IV–V*). Norepinephrine-induced protein S release was completely blocked by a low concentration of the α_1-adrenergic antagonist prazosin (10^{-7} M) but not by the α_2-adrenergic antagonist yohimbine (10^{-5} M). Stereoselectivity for the γ-isomer of norepinephrine also was observed, as d-norepinephrine (up to 10^{-5} M) had no effect on protein S release (Fig. 1 *B*, *o*). These data indicate that norepinephrine stimulates protein S release through an interaction with a heretofore unrecognized class of α_1-adrenergic receptors on endothelium. Studies with bovine endothelial cells and immunofluorescence to assess von Willebrand factor failed to show a change after exposure of cultures to norepinephrine (data not shown). Furthermore, norepinephrine did not stimulate re-

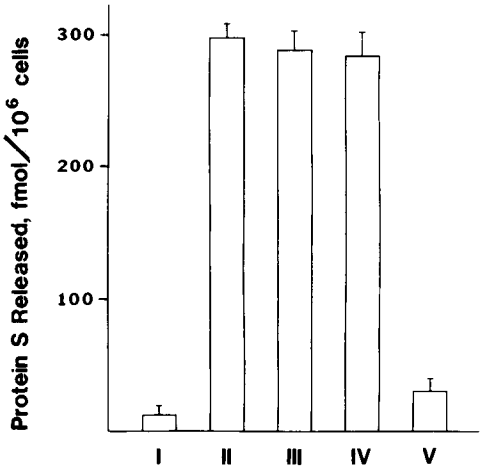

Figure 3. Norepinephrine-induced protein S release: effect of antagonists. (*A*) Confluent monolayers of endothelium (passage 1) were prepared for studies as described under Materials and Methods, and incubated at 37°C with serum-free medium alone (*I–II*) or serum-free medium containing either propranolol (10^{-6} M) (*III*), yohimbine (10^{-5} M) (*IV*) or prazosin (10^{-7} M) (*V*) for 5 min. Then norepinephrine (10^{-6} M) was added (*II–V*) and all cultures were incubated for a further 60 min at 37°C. Aliquots of culture supernatant were assayed for protein S antigen and the mean ±SEM is shown. Neither propranolol, yohimbine nor prazosin alone had an effect on endothelial cell protein S release (data not shown).

*Table I. Effect of Norepinephrine on Release of von Willebrand Factor and Protein S by Cultured Human Endothelium**

Addition	Final concentration	von Willebrand factor released	Protein S released
		ng/10⁶ cells	*ng/10⁶ cells*
0	0	8.1 ± 2.6	4.1 ± 3.6
PMA	20 ng/ml	95.1 ± 1.6	ND
Norepinephrine	0.1 μM	8.0 ± 6.5	14.8 ± 4.7
Norepinephrine	1.0 μM	7.9 ± 2.7	ND

* Confluent monolayers of human endothelium were incubated at 37°C with serum-free medium alone (no addition) or in the presence of the indicated concentrations of either PMA (12-O-tetradecanoyl-phorbol-13-acetate) or norepinephrine. An aliquot of culture supernatant was then withdrawn and assayed for von Willebrand factor antigen (30) or human protein S antigen (Hessing et al., manuscript in preparation). The mean and SEM are shown. ND, not done.

lease of von Willebrand factor (Table I) from human endothelial cells although protein S release was observed. These studies indicate that release of protein S in response to norepinephrine is a specific response rather than a component of a generalized release reaction and occur in the human system as well.

To further characterize the morphological correlates of norepinephrine-induced protein S release, immunoelectron microscopic studies were carried out (Fig. 4). Using gold-conjugated antibody to rabbit immunoglobulin, the intracellular distribution of protein S was determined on sections of Lowicryl-embedded cell pellets. In an earlier study (35), a portion of the intracellular poor of protein S was found to be localized in a population of cytoplasmic vesicles in the Golgi region and at the cell periphery which are morphologically distinct from Weibel-Palade bodies. This contrasts with the intracellular distribution of von Willebrand Factor which is thought to be stored in Weibel-Palade bodies and released from them after stimulation (40). After incubation of endothelium with norepinephrine, the number of protein S positive vesicles in the cytoplasm is considerably decreased (Fig. 4) and there is a concomitant increase in the number of vesicles at the cell surface, presumably in the process of fusion. Additionally, extracellular protein S can be observed in close proximity to both the luminal and abluminal cell surfaces after norepinephrine stimulation (Fig. 4 *b*).

These data, indicating that norepinephrine-induced protein S release is a distinct pathway, led us to examine intracellular mechanisms involved in mediating this α_1-adrenergic response. There is compelling evidence implicating regulatory GTP-binding proteins in signal transduction at the α_1-adrenergic receptor (33). Certain G proteins are targets

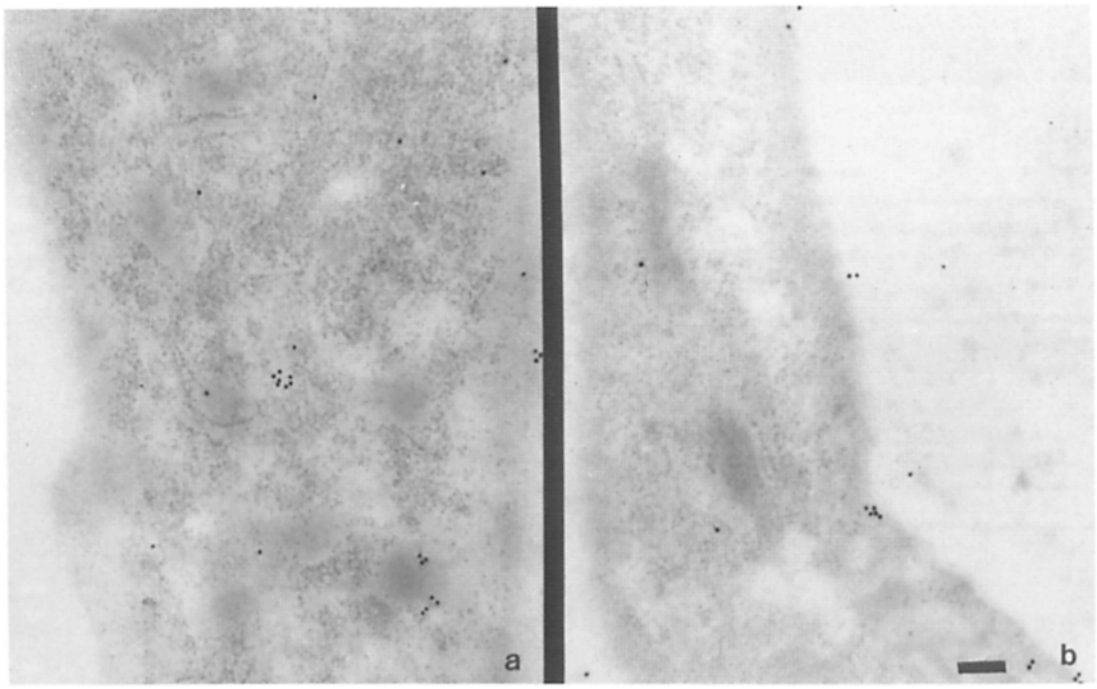

Figure 4. Immunoelectron microscopic localization of protein S in endothelium after norepinephrine stimulation. Control and norepinephrine-(10^{-5} M) stimulated monolayers were fixed and embedded in Lowicryl as described under Materials and Methods. Protein S was localized in sections using an affinity purified rabbit antibody to protein S and visualized with gold conjugated goat anti-rabbit antisera. Control endothelial cells (*a*) display gold particles in discrete cytoplasmic vesicles and in cisternae of the endoplasmic reticulum. Most sections show only an occasional vesicle fusing with the plasma membrane. After exposure to norepinephrine (10^{-5} M) (*b*) numerous cytoplasmic vesicles can be seen in the process of fusion with the plasma membrane and the number of cytoplasmic reactive vesicles is sharply decreased. Cells incubated with gold-conjugated secondary antibody alone did not stain (not shown). Bar, 100 nM.

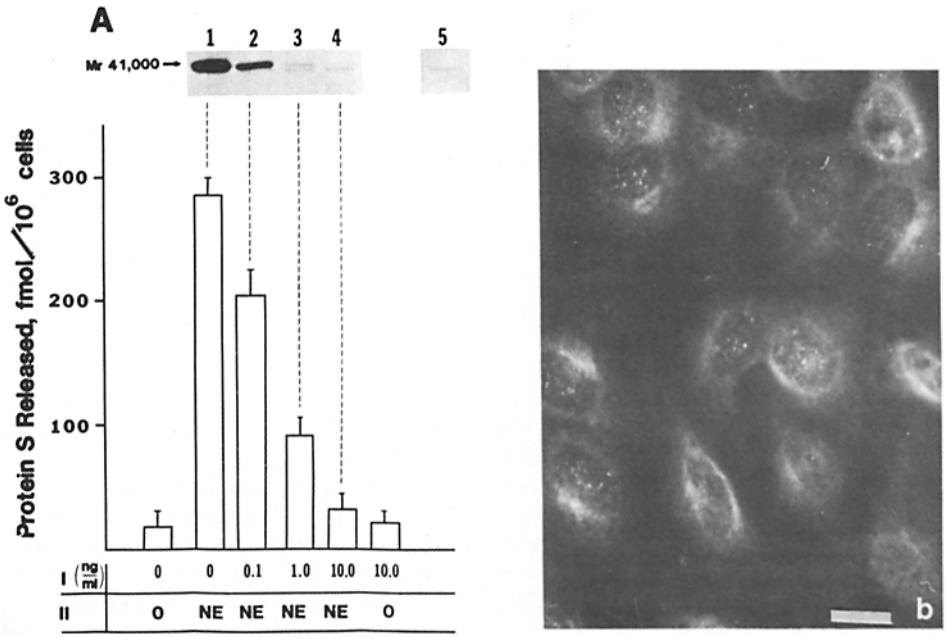

Figure 5. The effect of pertussis toxin on norepinephrine-stimulated release of endothelial cell protein S. (*A*) Correlation between inhibition of norepinephrine-stimulated protein S release and ADP-ribosylation of the 41,000-D protein. Confluent monolayers (passage 4) were incubated in the first stage (line I) with serum-containing medium alone (0) or in the presence of the indicated concentration of pertussis toxin for 24 h at 37°C. Cultures were then washed with buffer containing dextran sulfate and, in the second stage (line II), they were incubated at 37°C for 60 min with serum-free medium alone (0) or with norepinephrine at 10^{-6} M (*NE*). Aliquots of culture supernatant were withdrawn, concentrated if required, and assayed for protein S antigen. The mean and SEM are shown. (*Gel inset*) An autoradiogram of membrane proteins exposed to [^{32}P]-NAD in the presence (lanes *1–4*) or absence (lane *5*) of pertussis toxin from control cultures (lanes *1* and *5*) and cultures preincubated with pertussis toxin (lane *2*, 0.1 ng/ml; lane *3*, 1.0 ng/ml; lane *4*, 10 ng/ml) for 24 h. The 41,000-D membrane protein is specifically ADP-ribosylated by pertussis toxin and can be distinguished from a second slightly lower molecular weight band which is labeled in a pertussis toxin-independent fashion. In this experiment, the amount of G protein ADP-ribosylated by pertussis toxin in control cultures was 471 fmol/mg membrane protein. Pre-exposure to pertussis toxin (10 ng/ml) for 24 h resulted in ADP-ribosylation and inactivation of 98% of the pertussis toxin substrate. The bar shown between lanes *4* and *5* represents protein S released from cultures treated with pertussis toxin alone. (*B*) Immunofluorescence of endothelial cell protein S. Endothelial cell monolayers pre-incubated with pertussis toxin (10 ng/ml) in complete growth medium for 24 h before exposure to norepinephrine (10^{-5} M) display an immunofluorescent distribution of protein S that is indistinguishable from controls. (Compare with Fig. 2 *a*.) Bar, 10 μm.

for pertussis toxin-catalyzed ADP-ribosylation in which ADP-ribose is transferred to them from NAD (30). These G proteins, when ADP-ribosylated by pertussis toxin, lose their ability to couple receptors to their effector systems. The loss of a receptor function after exposure of cells to pertussis toxin constitutes strong evidence for the role of G proteins in the transduction of the signal from the receptor to the biochemical effector mechanism. Using this strategy, the effect of pertussis toxin on release of protein S from endothelium was examined (Fig. 5). Addition of pertussis toxin (10 ng/ml) to cultures for 24 h did not change levels of intracellular protein S or stimulate release of protein S antigen compared with untreated controls. However, norephinephrine-induced protein S release was inhibited progressively as the concentration of pertussis toxin in the pre-treatment culture medium was increased (Fig. 5 *A*). At a pertussis toxin concentration of 10 ng/ml, the effect of norepinephrine to release protein S was completely abolished. Pertussis toxin catalyzed the ADP-ribosylation of a 41,000-D protein in endothelial cell plasma membranes which comigrates on SDS–PAGE with the regulatory G protein (Fig. 5 *A*, *gel inset*). Pre-incubation of endothelial cell cultures with pertussis toxin results in ADP-ribosylation of this 41,000 D protein by endogenous, unlabeled cellular NAD to an extent dependent upon the concentration of pertussis toxin. Only substrate, not ADP-ribosylated by cellular NAD in membranes from these cells is available for in vitro ADP-ribosylation when subsequently exposed to pertussis toxin in the presence of exogenous [^{32}P]NAD. This inverse relationship between the extent of ADP-ribosylation of the G protein during the initial pretreatment interval and the amount of radioactivity that can be incorporated in the subsequent in vitro ADP-ribosylation reaction was used to determine that the concentration at which pertussis toxin achieved maximal ADP-ribosylation of the 41,000-D substrate with cellular NAD was 10 ng/ml (Fig. 5 *A*, *gel inset*). This concentration of pertussis toxin, which

*Table II. Phorbol Ester-Induced Release of Endothelial Cell Protein S**

Addition	Final concentration	Protein S released
		fmole/10^6 cells
Ionophore A23187	5 μM	300 ± 50
Ionomycin	10 μM	310 ± 40
12-O-tetradecanoyl-phorbol-13-acetate	16 nM	270 ± 50
12-O-tetradecanoyl-phorbol-13-acetate	1.6 nM	100 ± 20
1-oleoyl-2-acetyl-rac-glycerol	125 μM	240 ± 30
1-oleoyl-2-acetyl-rac-glycerol	2.5 μM	70 ± 20
None	0	20 ± 10

* Confluent monolayers of endothelium (passage 5) were prepared as described under Materials and Methods. Then, they were incubated at 37°C with serum-free medium alone (no addition) or in the presence of the indicated concentrations of either A23187, Ionomycin, 12-O-tetradecanoyl-phorbol-13-acetate or 1-oleoyl-2-rac-glycerol for 45 min. An aliquot of culture supernatant was then withdrawn and assayed for protein S antigen. The mean and SEM are shown.

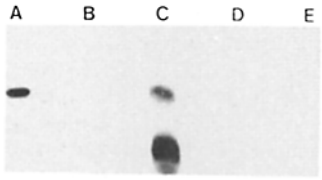

Figure 6. Western blotting of protein S released by endothelium following exposure to norepinephrine. Confluent monolayers of endothelium (passage 4) were prepared for experiments as described under Materials and Methods, and incubated at 37°C with serum-free medium containing norepinephrine (10^{-5} M) for 60 min. The culture medium was withdrawn, protease inhibitors were added and then samples were prepared for reduced SDS-PAGE (10%). After electrophoresis and Western blotting, immunoreactive material was visualized using first, affinity purified rabbit antibody to protein S (10 μg/ml) and second, an affinity purified [^{125}I]anti-rabbit IgG. Details of the procedure are described under Materials and Methods. (Lane *A*) Protein S purified from bovine plasma, was applied to gel (5 μg); (lane *B*) purified protein S (5 μg) was applied to the gel and excess protein S (500 μg/ml) was incubated with the blots simultaneously with the antibody to protein S; (lane *C*) protein S-containing releasate (50 μl) from norepinephrine treated endothelium was applied to the gel; (lane *D*) protein S-containing releasate (50 μl) from endothelium was applied to the gel and excess protein S (500 μg/ml) was incubated with the blots simultaneously with antibody to protein S; (lane *E*) protein S-containing releasate (50 μl) was applied to the gel and the antibody to protein S was omitted. The latter control indicates that second antibody ([^{125}I]anti-rabbit immunoglobulin) binding required the presence of the first antibody. When blots were exposed to the film for several additional days, weak higher molecular weight bands were also observed.

resulted in complete ADP-ribosylation, corresponds closely to the concentration necessary for maximal inhibition of norepinephrine-stimulated protein S release, implicating a G protein that is a substrate for pertussis toxin in α_1-adrenergic dependent release of protein S from endothelium. Consistent with this data, immunofluorescence studies for protein S in endothelial cells that were preincubated with Pertussis toxin (10 ng/ml) and stimulated with norepinephrine (10^{-5} M) showed intense staining and a cytoplasmic distribution of protein S (Fig. 5 *C*) quite similar to unstimulated controls (Fig. 2 *A*). This contrasts with the weak staining of control endothelium stimulated with norepinephrine (10^{-5} M) (Fig. 2 *B*).

α_1-Adrenergic catecholamines exert their effects in many tissues through the G protein-mediated hydrolysis of membrane phosphoinositides resulting in the generation of at least two intracellular second messengers (11). Inositol-triphosphate rapidly mobilizes calcium from an intracellular non-mitochondrial store while diacylglycerol activates the ubiquitous phospholipid-dependent, calcium-activated protein kinase C. A previous study (35) has demonstrated that maneuvers which increase cytosolic calcium, such as exposure of cultures to the calcium ionophores A23187 and ionomycin, mimic the effect of norepinephrine to release intracellular protein S from endothelium (see also Table II). In the present investigation, 12-O-tetradecanoyl-phorbol-13-acetate, a phorbol ester known to bind to and activate protein kinase C (20), was found to also induce release of protein S from endothelium (Table II). Furthermore, because 12-O-tetradecanoyl-phorbol-13-acetate potentially can activate a variety of cellular processes in addition to protein kinase C, 1-oleoyl-2-acetyl-rac-glycerol (a synthetic diglyceride capable of permeating the plasma membrane and activating protein kinase C in a more specific fashion) (18) was tested. 1-Oleoyl-2-acetyl-rac-glycerol also released protein S from endothelium (Table II). These results are compatible with release of protein S from endothelium via a mechanism involving intracellular processes activated by the intermediates generated through phosphoinositide hydrolysis.

We next assessed the significance of norepinephrine-induced perturbation of cellular physiology for the protein C anticoagulant mechanism. To examine the nature of protein S released from endothelium in response to norepinephrine, serum-free culture medium was concentrated and subjected to Western blotting with the anti-bovine protein S antibody (Fig. 6). Protein S purified from bovine plasma showed a single major band with M_r 76,000 (lane *A*), whose appearance could be blocked by the addition of excess free protein S to the reaction mixture (lane *B*). In contrast, the norepinephrine-induced endothelial cell releasate showed a range of more rapidly migrating material, M_r 33,000–52,000 with a major band M_r 43,000 (lane *C*). The material in these bands was immunoreactive with the anti-protein S antibody and the appearance of these bands was also blocked by adding excess free protein S (lane *D*). These more rapidly migrating bands probably represent cleaved forms of protein S and their presence suggests that protein S released in response to norepinephrine is proteolyzed. This appearance of multiple cleaved forms of endothelial cell protein S after exposure to norepinephrine contrasts with the previously described more homogeneous nature of protein S released from endothelium constitutively (36). This led us to examine the anticoagulant activity of protein S released from endothelium in response to norepinephrine. The endothelial cell releasate from 10^9 cells was pooled, concentrated and its functional activity, in terms of activated protein C cofactor activity compared to that of protein S purified from bovine plasma. Compared with protein S purified from plasma, nine-times more protein S released from endothelium was required to achieve the same anticoagulant effect: 70 nM protein S derived from plasma and 600 nM protein S from endothelial cell releasate resulted in a clotting time of 22 s. This result is consistent with the presence of functionally inactive cleaved forms of protein S in the endothelial cell releasate. Under the culture conditions used in this study, Fair et al. (12) has found that protein S from human endothelial cells has comparable anticoagulant activity to that of protein S purified from plasma, on a per weight basis. We observed similar results with protein S constitutively released from bovine endothelial cells (data not shown). Thus, endothelial cell protein S released in response to norepinephrine has decreased functional activity compared with protein S constitutively released by the cells.

The role of protein S in the protein C anticoagulant mechanism includes its function as a nonenzymatic cofactor for the binding of activated protein C to endothelium (36). Although initially we reasoned that release of protein S from endothelium would potentiate the protein C pathway by providing additional cofactor to facilitate interaction of activated C with cellular surfaces, the considerably decreased activity of the protein S released in response to norepinephrine suggested a different impact on the coagulation mechanism. Loss of endogenous protein S from endothelium with the elaboration of cleaved forms into the fluid phase would ap-

pear to locally compromise this anticoagulant mechanism. To further test this hypothesis, the binding and anticoagulant activity of protein S on norepinephrine-treated endothelium was studied (Fig. 7). Since assembly of functional activated protein C/protein S complex on the surface of bovine endothelial cells requires expression of specific cell surface binding sites, radioligand-binding studies were carried out with [^{125}I]protein S and norepinephrine-stimulated endothelium. To perform these binding studies, two issues had to be addressed. Since norepinephrine stimulates release and potential cleavage of protein S, this pool of endogenous protein S, which could occupy the receptors, had to be removed and cleavage of the [^{125}I]protein S added to endothelium had to be prevented. Removal of endogenous protein S was accomplished by extensive washing of the cultures as described under Materials and Methods. When norepinephrine-stimulated endothelium was incubated with [^{125}I]protein S for 60 min at 37°C, the precipitability of the tracer in trichloroacetic acid was decreased suggesting degradation had occurred. This led us to perform radioligand-binding studies at 2°C. At this lower temperature there was no evidence of tracer degradation based on precipitability of the tracer in trichloroacetic acid and identical migration on SDS-PAGE. Under these conditions, norepinephrine induced a dose-dependent decrease in [^{125}I]protein S-endothelial cell interaction (Fig. 7 *A*). The affinity of protein S for the endothelial cell-binding site is 12 nM. When the effect of norepinephrine on endothelial cell-protein S interaction was tested at a [^{125}I]protein S concentration of 60 nM (Fig. 7 *A*), a decrease in specific binding was observed. These results, which used a high ($5 \times K_d$) but subsaturating concentration of protein S, could reflect an effect of norepinephrine to decrease either the number or affinity of protein S binding sites. Experiments were carried out to distinguish between these possibilities. Protein S binding to endothelium was tested over a wide range of [^{125}I]protein S concentrations (including concentrations which resulted in saturation of protein S binding sites) (Fig. 7 *C*). When endothelium was exposed to norepinephrine at 10^{-7} M, there was no significant change in the affinity of binding (12 nM vs. 15 nM for control and norepinephrine-treated cultures, respectively), whereas a substantial decrease in the number of binding sites was observed (9.8×10^4 vs. 4.5×10^4 sites/cell for control and norepinephrine-treated cultures, respectively). Addition of high concentrations of norepinephrine (10^{-5} M) resulted in undetectable [^{125}I]protein S-endothelial-cell binding, presumably due to total loss of functional protein S-binding sites. We previously demonstrated that lack of binding of [^{125}I]protein S to norepinephrine-treated endothelium was not due to modification of the tracer by the stimulated endothelium, since there was no evidence of cleavage (see above). The integrity of the tracer was established by demonstrating that [^{125}I]protein S exposed to norepinephrine-treated cultures, when dialyzed extensively to remove catecholamine, bound effectively to fresh endothelial cell cultures (data not shown). Furthermore, endothelium treated with norepinephrine in the presence of prazosin (Fig. 7 *A*) maintained the ability to bind [^{125}I]protein S. Taken together, these data indicate that norepinephrine induces a decrease in the total number of binding sites available for exogenous [^{125}I]protein S through an α-adrenergic-dependent mechanism.

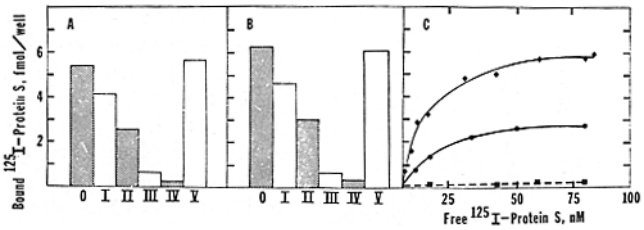

Figure 7. Norepinephrine-induced down-regulation of endothelial cell protein S binding sites. (*A*) Confluent monolayers of endothelium (passage 5) were incubated either in serum-free medium alone (0), in the presence of the indicated concentration of norepinephrine (I, 10^{-8} M; II, 10^{-7} M; III, 10^{-6} M; IV, 10^{-5} M) or norepinephrine (10^{-6} M) plus prazosin (10^{-7} M) (V) for 60 min at 37°C. Cultures were prepared for binding studies as described under Materials and Methods. Wells were then incubated with ^{125}I-protein S alone (60 nM) (total binding) or in the presence of unlabeled protein S (2 μM) (nonspecific binding) for 90 min at 2°C. Cultures were washed, solubilized, and counted. The mean of specific binding, the difference of total and nonspecific binding, is shown. In each case the SEM was less than 15%. (*B*) The [^{125}I]protein S-binding experiment in part A was repeated in the presence of activated protein C (1 nM), and the incubation time was 2 min at room temperature. The mean is shown and SEM was less than 15%. (*C*) Endothelial cell monolayers were incubated in serum-free medium alone (♦) or in the presence of norepinephrine (10^{-5} M [●] or 10^{-7} M [■]) for 90 min at 37°C. Binding of [^{125}I]protein S was then studied as described in A except that the indicated free concentration of [^{125}I]protein S was present. Specific binding is plotted vs. free [^{125}I]protein S. Data were analyzed by the nonlinear least squares program and the curve (−) indicates the best fit line. Nonspecific binding accounted for 15–22% of the total binding. Parameters of [^{125}I]protein S-endothelial cell binding in the absence of norepinephrine were: K_d, 12 ± 2 nM and $9.8 \pm 1.7 \times 10^4$ molecules found per cell at saturation. In the presence of norepinephrine (10^{-7} M), the K_d was about the same, 15 nM, but the number of sites was clearly decreased, 4.5×10^4 sites/cell.

Since the functional unit of the protein C pathway is the activated protein C/protein S complex, it was important to understand the effect of norepinephrine on binding of [^{125}I]protein S to endothelium in the presence of activated protein C (Fig. 7 *B*). In a previous study (36), we observed that activated protein C enhanced the affinity of protein S for the endothelial cell surface without changing the total number of binding sites. In the presence of 1 nM activated protein C, the K_d of [^{125}I]protein S for endothelial cell-binding sites falls from 12 to 0.2 nM. Thus, 60 nM [^{125}I]protein S, in the presence of activated protein C (1 nM), is in excess of the concentration necessary to saturate protein S-binding sites. In the presence of activated protein C (Fig. 7 *B*), a decrement in protein S binding to endothelium incubated with norepinephrine was seen, analogous to that observed in the absence of activated protein C. Since cell surface protein S-binding sites facilitate assembly of activated protein C/protein S complex formation on bovine endothelium (36), we expected that the loss of these sites on norepinephrine-stimulated cells would be paralleled by inhibition of Factor Va inactivation. To carry out Factor Va inactivation studies, conditions under which degradation of protein S would not occur had to be identified (as described above for binding studies). Incubation of [^{125}I]protein S with norepinephrine-stimulated en-

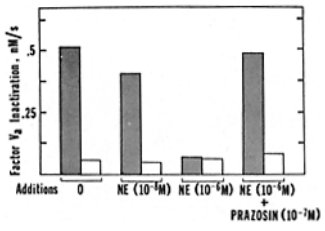

Figure 8. Norepinephrine-induced attenuation of endothelial cell-dependent Factor Va inactivation. Endothelium (passage 4) was incubated either in serum-free medium alone (○), or in the presence of norepinephrine (*NE*) and/or prazosin at the indicated concentration for 60 min at 37°C. Then, endothelium was pre-incubated for 2 min at room temperature in the presence (cross-hatched bars) or absence (open bars) of protein S (60 nM) and activated protein C (1 nM) followed by addition of Factor Va. Aliquots were removed to determine the rate of Factor Va inactivation. Details of experimental procedure are described under Materials and Methods. The mean is shown and SEM was less than 15% in each case.

dothelium for 120 s at room temperature, the time required for Factor Va inactivation studies, did not lead to cleavage or degradation of the tracer. Thus, under these conditions changes in the rate of Factor Va inactivation are not due to degradation of protein S. Factor Va inactivation rates on norepinephrine-stimulated endothelium decreased in a dose-dependent manner and this effect of norepinephrine was blocked by prazosin (Fig. 8). As noted in a previous study (36), rapid Factor Va inactivation on bovine endothelium in the presence of low levels of activated protein C (as in Fig. 8) requires protein S. Exposure of endothelium to norepinephrine blocks the protein S-mediated enhancement of Factor Va inactivation (compare hatched and open bars in Fig. 8). The conditions for the functional protein S assay in Fig. 8 and the [^{125}I]protein S–binding assay (Fig. 7 *B*) are identical, allowing for a close comparison of these experiments. Taken together, the data support the concept that cell-bound protein S plays a central role in mediating endothelial cell–dependent Factor Va inactivation and that this anticoagulant mechanism does not function effectively after endothelium is exposed to norepinephrine.

Discussion

Evidence for an association between the autonomic nervous system and thrombosis has been demonstrated in previous animal experiments (23, 24, 28, 29). The data presented here demonstrate that norepinephrine can stimulate α_1-adrenergic receptors on the endothelial cell surface resulting in release of cleaved protein S from endothelium and decrease of protein S cell surface–binding sites involved in assembly of the activated protein C/protein S complex on endothelium.

Previous studies have established the presence of α-adrenergic receptors (33) as well as a α-adrenergic responsive adenylate cyclase activity (4) in endothelium, although the role of this receptor in endothelial cell physiology is unclear. Other studies have shown that endothelium has an α_2-receptor which appears to modulate the contractile response of smooth muscle (1). The results of the present study indicate that cultured bovine aortic endothelial cells also possess an α_1-adrenergic catecholamine response. The full consequences of α_1-receptor stimulation for endothelial cell physiology is unclear at this time, but modulation of the protein S-endothelial cell interaction and alteration of the cytoskeleton (6, 44, and our unpublished observation; Brett et al., 1987) by norepinephrine are two documented expressions of α_1-adrenergic catecholamine activation. The α_1-adrenergic mechanism in endothelium shares certain fundamental properties with α_1-adrenergic receptor–stimulated protein S release involves a G protein that is a substrate for pertussis toxin (Fig. 5). Because several functionally and immunologically distinct G proteins with similar molecular masses can be ADP-ribosylated by pertussis toxin (32), the specific identity of the regulatory G protein lined to the endothelial cell α_1-adrenergic receptor is not yet known. However, the observation that maneuvers which elevate cytosolic calcium ion concentration or activate protein kinase C (Table I) mimic the response to norepinephrine suggests that the intracellular signaling mechanism activated by α_1-adrenergic receptors in endothelium involves G protein-mediated hydrolysis of phosphoinositides and generation of inositol-trisphosphate and diacylglycerol. Preliminary data, indicating that norepinephrine does stimulate metabolism of phosphoinositides in endothelium are consistent with this hypothesis. Increase in calcium ion concentration due to products of phosphoinositide hydrolysis may then promote exocytosis of protein S-containing vesicles. Although other endothelial cell products may be released by norepinephrine, this pathway appears to be relatively selective for protein S since von Willebrand Factor release was not observed. Stimulation of α_1-adrenergic receptors leading to protein S release thus constitutes a new type of endothelial cell release reaction.

During studies to define constitutive synthesis and release of protein S by bovine endothelium, the functional activity of endothelial cell protein S was evident (35). Our expectation was that norepinephrine-stimulated protein S release would lead to an outpouring of functional protein S into the intravascular space. Western blotting (Fig. 6) and functional studies of the endothelial cell releasate, however, indicate that protein S in culture supernatants is cleaved and has considerably attenuated anticoagulant activity. The mechanisms involved in norepinephrine-mediated cleavage of protein S are not yet clear, but modulation of cellular proteolytic activities probably plays a role. Previous studies have shown that quiescent endothelium binds and promotes the anticoagulant function of protein S (36). Furthermore, pilot experiments indicate that norepinephrine does not directly cleave protein S. The nature and mechanism of induction of this cellular proteolytic activity responsible for inactivation of protein S remains to be established. In this context, human platelets have recently been shown to bind and cleave protein S leading to its inactivation (27). A similar process may occur on norepinephrine-stimulated endothelium, although protein S released by endothelium appears to be more extensively degraded (Fig. 6) than protein S inactivated by platelets. In addition to inactivation of much of the protein S released by endothelium, after exposure to norepinephrine, protein S-endothelial cell binding was considerably decreased even when studied after elution of previously bound protein S and under conditions where cleavage of added protein S was not observed. Taken together, norepinephrine-induced modulation of endothelial cell mechanisms can effectively block the anticoagulant function of protein S in relation to the vessel wall. When these results are placed in the perspective of

previous observations concerning activation of the procoagulant Factor XII (26) and induction of stasis (24) during α-adrenergic stimulation, the thrombogenic potential of norepinephrine becomes apparent.

These studies define a new, potentially important relationship between the coagulation mechanism and the autonomic nervous system, and point to a possible role for α_1-adrenergic receptors in endothelial cell physiology. Norepinephrine-induced modulation of the endothelial cell-protein S interaction, both at the level of release of intracellular protein S and loss of functional cell surface receptors, represents a rapid mechanism through which the coagulation mechanism can respond to environmental stimuli. These observations in a cultured cell model may relate to the in vivo situation as suggested by the results of a pilot infusion study in calves. A calf infused with norepinephrine in the presence of the β-blocker propranolol showed a fivefold rise in plasma protein S antigen (after 1 h) compared with an animal treated with propranolol alone. Although the source of this protein S antigen is not clear, norepinephrine-induced release of endothelial cell protein S is certainly a possibility based on the studies described in this work.

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