Participation of Endothelial Cells in the Protein C–Protein S Anticoagulant Pathway: The Synthesis and Release of Protein S

David Stern,* Jerry Brett,‡ Kevin Harris,* and Peter Nawroth*

*Thrombosis Research, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma 73104; and ‡Department of Pathology, College of Physicians and Surgeons, Columbia Presbyterian Medical Center, New York, New York 10032

Abstract. The protein C–protein S anticoagulant pathway is closely linked to the endothelium. In this paper the synthesis and release of the vitamin K–dependent coagulation factor protein S is demonstrated. Western blotting, after SDS PAGE of Triton X-100 extracts of bovine aortic endothelial cells grown in serum-free medium, demonstrated the presence of protein S. A single major band was observed at M_r ~75,000, closely migrating with protein S purified from plasma absent from cells treated with cycloheximide. Metabolic labeling of endothelial cells with [^{35}S]methionine confirmed *de novo* synthesis of protein S. Using a radioimmunoassay, endothelium was found to release 180 fmol/10^5 cells per 24 h and contain 44 fmol/10^5 cells of protein S antigen. Protein S released from endothelium was functionally active and could promote activated protein C–mediated factor V_a inactivation on the endothelial cell surface. Warfarin decreased secretion of protein S antigen by >90% and increased intracellular accumulation by almost twofold. Morphological studies demonstrated intracellular protein S was in the Golgi complex, concentrated at the *trans* face, rough endoplasmic reticulum, lysosomes, and in vesicles at the periphery. In contrast, protein S was not found in vascular fibroblasts or smooth muscle cells. A pool of intracellular protein S could be released rapidly by the calcium ionophore A23187 (5 μM). This effect was dependent on the presence of calcium in the culture medium and could be blocked by $LaCl_3$, which suggests that cytosolic calcium flux may be responsible for protein S release. These results demonstrate that endothelial cells, but not the subendothelial cells of the vessel wall, can synthesize and release protein S, which indicates a new mechanism by which the inner lining of the vessel wall can contribute to the prevention of thrombotic events.

PROTEIN S is a regulatory vitamin K–dependent plasma protein, which is an essential component of the protein C anticoagulant pathway (46). Protein S functions as a non-enzymatic cofactor which promotes binding of the enzyme-activated protein C to membrane surfaces (46, 47). Once bound to a cellular or phospholipid surface, activated protein C can effectively exert its anticoagulant function, inactivating the cofactors, factors V_a and $VIII_a$, essential for the reactions that lead to clot formation (18, 40, 47). The clinical significance of protein S is emphasized by the thrombotic diathesis observed in kindreds deficient in this coagulation factor (5, 7, 37).

Recent studies have indicated that the protein C pathway is closely associated with the vessel wall. The activation of protein C by thrombin is promoted by the presence of the endothelial cell membrane protein thrombomodulin (10). Assembly of functional activated protein C–protein S complex also occurs effectively on the inner surface of the vessel wall (4). Formation of this complex requires binding of protein S, which allows specific activated protein C–endothelial cell interaction to occur. Furthermore, cell-bound protein S can be cleared from the cell surface by endocytosis and degraded by a lysosomal-dependent mechanism (40). These findings suggest that endothelium is physiologically important in protein S function and metabolism. In terms of protein S synthesis, clinical studies have demonstrated that although the level of the other vitamin K–dependent coagulation factors is decreased by 50% in patients with liver disease, protein S is decreased by only 25% (2). The existence of extrahepatic sites of protein S synthesis could explain these findings.

The close relationship of protein S to endothelium led us to examine if protein S is synthesized by endothelium. The results of our study indicate that cultured bovine aortic endothelial cells do synthesize and release functional protein S. Endogenous protein S is released either constitutively or in response to stimulation of endothelium by an agent that induces cytosolic calcium flux. Synthesis and release of protein S by endothelium is thus a new addition to the list of mechanisms through which the vessel wall can regulate coagulant events.

Materials and Methods

Cell Culture

Bovine aortic endothelial cells were isolated from calf aortas as described by Schwartz (36) and were grown in minimum essential medium that contained penicillin-streptomycin (50 U/ml – 50 µg/ml) and fetal bovine serum (10%; HyClone Laboratories, Logan, UT). Cells were separated for subculture nonenzymatically with Dulbecco's phosphate-buffered saline (PBS) (calcium- and magnesium-free) that contained 10 mM sucrose and 1 mM EDTA. For experiments, cells (passages 4–10) from different aortas were grown to confluence in 0.79-cm^2 wells (1.1–1.5 × 10^5 cells/cm^2). Cultures were characterized as endothelial by morphological criteria (36), the formation of a cobblestone-like monolayer with contact inhibition at confluence, and the presence of von Willebrand factor antigen using indirect immunofluorescence (20).

24 h after endothelial cells achieved confluence they were washed with Hanks' balanced salt solution (HBSS)[1] that contained 10 mg/ml dextran sulfate to elute surface bound protein S (40) and then placed in serum-free medium (minimum essential medium that contained penicillin-streptomycin [50 U/ml – 50 µg/ml], 10 mM Hepes [pH 7.4], 20 µg/ml transferrin [Sigma Chemical Co., St. Louis, MO], 10 µg/ml insulin [Sigma Chemical Co.], and 5 mg/ml bovine serum albumin [BSA, Sigma Chemical Co.]). Experiments were then done as described below.

Coagulation Factors and Assays

All purified coagulation factors were of bovine origin. Purification of protein S was done as described (45). Radiolabeling of protein S was accomplished by the lactoperoxidase method (8), using Enzymobeads according to the manufacturer's instructions. The reaction was done at room temperature for 15 min by incubating Enzymobeads (50 µl; Bio-Rad Laboratories, Richmond, CA), protein S (40 µl; 29 µg), Na^{125}I (2 mCi), and 2% glucose (20 µl). Free iodine was separated from protein S by gel filtration using a column (1 × 20 cm) of Sephadex G-25. The specific radioactivity of ^{125}I-protein S was 9,000–12,000 cpm/ng (corresponding to ~0.1 mol ^{125}I per mol of protein S) over five radioiodinations. Radioiodinated protein S co-migrated with unlabeled material on SDS PAGE.

Protein C was purified as described previously (44). Protein C was activated by incubation with 5% thrombin (16) (wt/wt) for 3 h at 37°C in 1 mM Tris (pH 7.4), 0.1 M NaCl. The reaction mixture was adjusted to pH 6.5 with 4-morpholinoethanesulfonic acid and chromatographed on QAE-Sephadex (0.6 × 5 cm) equilibrated with 5 mM 4-morpholinoethanesulfonic acid, 0.1 M NaCl. Activated protein C was eluted using a 0.1–0.6 M linear salt gradient (5 ml/reservoir). Factor V$_a$ was purified by previously described methods (3), and the preparations used in this study were recombined from isolated subunits in the presence of 10 mM CaCl$_2$ overnight at 4°C. Factors IX, X, and prothrombin were purified as described (14, 15, 28). Factor VII was generously provided by Dr. W. Kisiel (Department of Pathology, University of New Mexico, Albuquerque).

Rabbit antisera to protein S were prepared as described by Vaitukaitis (43), and the IgG was isolated by chromatography on DE52 (17). Affinity purified anti–protein S antibody was prepared using protein S immobilized on affigel 15 (2 mg of protein S/ml of resin; coupling was done by the manufacturer's protocol). Total IgG (10 ml) was applied to the protein S–affigel 15 column (0.6 × 15 cm) in 0.02 M Tris (pH 7.4) – 0.14 M NaCl, followed by extensive washing of the column in 20 vol of the same buffer. Antibody to protein S was eluted using 0.2 M glycine (pH 2.5), and then the IgG (1 mg) was dialyzed versus 0.02 M Tris (pH 7.4) – 0.14 M NaCl. This antibody did not react with the other vitamin K–dependent coagulation factors, factors VII, IX, X, prothrombin, and protein C in the radioimmunoassay described below. Western blotting, done as described below, after SDS PAGE of bovine plasma, demonstrated only a single band that co-migrated with purified protein S (data not shown). Monospecific rabbit antiserum to protein C was prepared as described above for protein S, and antiserum to prothrombin was generously provided by Dr. E. W. Davie (Department of Biochemistry, University of Washington, Seattle). Affinity purified goat anti–rabbit IgG (Pel-Freeze Biologicals, Rogers, AR) was radiolabeled by the solid state lactoperoxidase technique as described above for protein S.

Western blotting of endothelial cells for protein S was done after extensive washing of cells with HBSS. Cells were then incubated for 20 min at 25°C with 0.02 M Tris (pH 7.4), 0.1 M NaCl that contained 1% Nonidet P-40, 10 mM EDTA, 2 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co.), and 0.3 mM leupeptin (Boehringer Mannheim Diagnostics, Inc., Houston, TX). Samples were then prepared for electrophoresis (reduced SDS PAGE, 7.5%) by the

1. *Abbreviation used in this paper:* HBSS, Hanks' balanced salt solution.

method of Laemmli (24). Material from the gel was electrophoretically transferred to nitrocellulose paper, 0.45-µm pore size (Schleicher & Schuell, Inc., Keene, NH), by a modification of the method of Towbin et al. (42). Electrophoretic transfer was done for 12 h at 8.5°C and at constant power (13 W). Excess binding sites on the nitrocellulose membrane were blocked by a 2-h incubation at 37°C with 0.05 M Tris (pH 7.4) - 0.1 M NaCl that contained Carnation non-fat dry milk (22.7 g/8 oz of buffer) (21). Fresh buffer that contained milk and affinity purified anti–protein S (10 µg/ml) or other antibodies (900 µg/ml) was added for 2 more h at 37°C. Nitrocellulose membranes were then washed over 1 h with four changes of 0.05 M Tris (pH 7.4), 0.15 M NaCl, 3 mM EDTA, and 0.05% Tween. Finally, buffer that contained milk and ^{125}I-affinity purified anti–rabbit IgG (1.3 × 10^5 cpm/ml) was added for 2 h at 37°C. Blots were washed as described above, dried, and 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, which included 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), were run simultaneously.

Endogenous labeling of endothelial cell protein S was done by maintaining cultures for 60 h in methionine-free growth medium (Gibco, Grand Island, NY) that contained penicillin-streptomycin (50 U/ml–50 µg/ml), 10 mM Hepes (pH 7.4), 20 µg/ml transferrin, 10 µg/ml insulin, and 5 mg/ml BSA supplemented with [^{35}S]methionine (20 µCi/ml; 1,340 Ci/mmol Amersham Corp., Arlington Heights, IL). Culture supernatants were harvested and inhibitors were added: EDTA (1 mM), phenylmethylsulfonyl fluoride (2 mM), leupeptin (0.3 mM). After centrifugation at 10^4 g for 20 min, protein A–Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) was added (30% by volume) to the supernatants for 30 min at room temperature. The mixture was again centrifuged (10^3 g for 5 min) and either affinity purified rabbit anti–bovine protein S (10 µg/ml) or rabbit anti–bovine protein C (20 µg/ml) was added for 18 h at 4°C. The next morning, protein A–Sepharose (20% by volume) was added for 30 min at room temperature and the mixture was centrifuged (10^3 g for 5 min). After centrifugation, the pellet was washed three times (20 min/wash) with 50 mM Tris/HCl (pH 7.4), 0.1 M NaCl, and finally resuspended in 0.1 ml of 5% SDS, 25 mM Tris/HCl (pH 6.8), 10% 2-mercaptoethanol. The resuspended pellet was incubated at 100°C for 3 min and then samples were prepared for SDS PAGE (7.5%) by the method of Laemmli (24). Autoradiography was done as described above. Molecular weights were interpolated from semilogarithmic plots based on the migration of standard proteins (same as described above) run simultaneously.

Radioimmunoassay for protein S antigen was done by the general method described by Suzuki and Thompson (41) using staphylococcal protein A (Enzyme Center, Boston, MA) in place of a second antibody. 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 for protein S determination were concentrated ~10-fold before assays. Competition studies using this assay indicated no inhibition of ^{125}I-protein S–antibody binding in the presence of factors VII, IX, X, protein C, or prothrombin when each was added at a concentration of 100 µg/ml. Radioimmunoassays were also done which compared the inhibition of ^{125}I-protein S–antibody binding by purified protein S, normal bovine plasma, and bovine plasma from animals treated with dicoumarol. Dicoumarol plasma was prepared from cows treated with dicoumarol (1 g/d) for 10 d (30). Functional levels of factor X and protein S were <8% and <5%, respectively, based on coagulant assays (1, 7). The antibody to bovine protein S recognized protein S in the plasma of dicoumarol-treated cows as well as fully γ-carboxylated protein S since inhibition of ^{125}I-protein S–antibody binding by dilutions of dicoumarol-treated and normal plasma occurred in parallel and with the same slope. Hence, this assay could be used in the experiments where warfarin is added to the culture medium.

Before experiments were done to assess release of protein S from endothelium, monolayers were rinsed briefly with dextran sulfate (10 mg/ml)-containing buffer. Dextran sulfate has been shown to remove surface-bound protein S (40), although it did not affect the viability of the monolayer based on trypan blue exclusion (>90%) or morphological criteria.

Activated protein C–protein S-mediated factor V$_a$ inactivation on the endothelial cell surface was done as described previously (40). First endothelial cell monolayers were washed with dextran sulfate (10 mg/ml)-containing HBSS and then 0.35 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 37°C, and at the indicated times factor V$_a$ (80 nM) and activated protein C (1 nM) were added. Where indicated, affinity purified antibody to protein S (50 µg/ml) was also present. The reaction mixture was incubated at room temperature with constant gentle mixing, and one 25-µl aliquot was removed from each well at 10, 30, 40, 60, 90, and 120 s of

incubation. Samples were immediately assayed in a one-stage clotting assay (22) by adding 25 µl of diluted sample to 25 µl buffer (0.05 M Tris [pH 7.5], 0.1 M NaCl, 1 mg/ml BSA), 50 µl rabbit brain thromboplastin (Dade, Puerto Rico), and 50 µl $CaCl_2$ (25 mM). Finally, 50 µl of factor V–deficient human plasma (30) was added and the clotting time determined. Standard curves were constructed using purified factor V_a and all clotting times were done in duplicate or triplicate. The rate of factor V_a inactivation was determined from the slope of the linear initial portion of a plot of factor V_a activity versus incubation time and generally included the 10-, 30-, 40-, and 60-s points. In the presence of exogenous protein S (5 nM), the maximal rate of factor V_a inactivation was 0.19 nM/s (0.03 pmol/10^5 cells).

Ionophore-induced Release of Protein S from Endothelial Cells

After 5 d in serum-free medium, endothelial cells were rinsed with HBSS that contained dextran sulfate (10 mg/ml) and then 0.35 ml of 10 mM Hepes (pH 7.45), 137 mM NaCl, 4 mM KCl, 11 mM glucose, 3 mM $CaCl_2$ was added along with the indicated concentration of ionophore. Ionophore A23187 (Cal-biochem-Behring Corp., La Jolla, CA) was prepared as a 1 mM stock solution in absolute ethanol, ionomycin (Calbiochem-Behring Corp.) was prepared as a stock solution of 1 mM in acetone, and valinomycin (Calbiochem-Behring Corp.) was prepared as a stock solution of 2 mM in acetone. In each case the ionophore was diluted such that the final concentration of organic solvent was ≤0.01%. This concentration of ethanol or acetone had no effect on endothelial cell protein S release in the absence of ionophore. After addition of an ionophore to cell cultures, samples were obtained at the indicated times for determination of protein S antigen by radioimmunoassay. Only one sample was removed from each well.

Immunolocalization of Protein S

Immunofluorescence studies were done as follows: cell monolayers were grown on coverslips, washed extensively with HBSS, fixed for 1-2 min with paraform-aldehyde (3.5%) in PBS-0.1% Nonidet P-40, washed in PBS, and fixed for an additional 5 min with 3.5% paraformaldehyde. Washed coverslips were incu-bated with affinity purified rabbit antibody to bovine protein S, and sites of binding of primary antibody were visualized with fluorescein-conjugated goat anti–rabbit immunoglobulin (Cappel Laboratories, Cochranville, PA). Stained coverslips were mounted in Gelvatol that contained 1 mg/ml p-phenylenedia-mine and examined in a Leitz Dialux 20 microscope with a 2.4 Ploempak filter block and water immersion fluorite objectives and recorded on Kodak Tri-X film. Controls included substitution of preimmune rabbit serum for specific immunoglobulin and exposure to secondary (fluorochromed) antibody alone. Monolayers not permeabilized with aid of detergent also failed to stain (these monolayers were washed extensively resulting in removal of any surface-bound protein S).

Monolayers grown in 35-mm dishes were used for electron microscopic studies. Monolayers were fixed for 1-2 min with 2% paraformaldehyde in PBS, permeabilized for 1 min with 3.5% paraformaldehyde in PBS that contained 0.1% Saponin, washed briefly in PBS, and fixed further for 10 min with 3.5% paraformaldehyde. All solutions contained 3% sucrose. After fixation, mono-layers were incubated with affinity purified rabbit anti–protein S IgG diluted in PBS that contained 0.1% BSA overnight at 4°C. Sites of primary antibody were visualized with biotinylated goat anti–rabbit immunoglobulin and horse-radish peroxidase–conjugated avidin (Vectastain, Vector Laboratories, Burlin-game, CA). The enzyme was revealed with 0.01% hydrogen peroxide and 0.5% diaminobenzidine in 0.2 M Tris buffer pH 7.4. After reaction product had been developed, monolayers were postfixed for 30 min with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, with 3% sucrose and again for 30 min in 2% OsO_4 in cacodylate buffer for 30 min, block-stained in 0.25% aqueous uranyl acetate for 1 h, dehydrated, and embedded flat in Epon 812. Thin-sections (cut with the DuPont-Sorvall MT6000 microtome) were examined in a Philips 200 electron microscope. Inclusion of glutaraldehyde even at 0.1% in the first fixation and permeabilization steps sharply reduced antibody binding or pene-tration even after blockade of free aldehyde.

Results

Confluent endothelial cells incubated for 6 d in serum-free medium contained protein S, as shown by Western blotting of cell extracts after reduced SDS PAGE in which a double antibody technique was used to visualize protein S (Fig. 1). Autoradiograms of the Western blots demonstrate a single
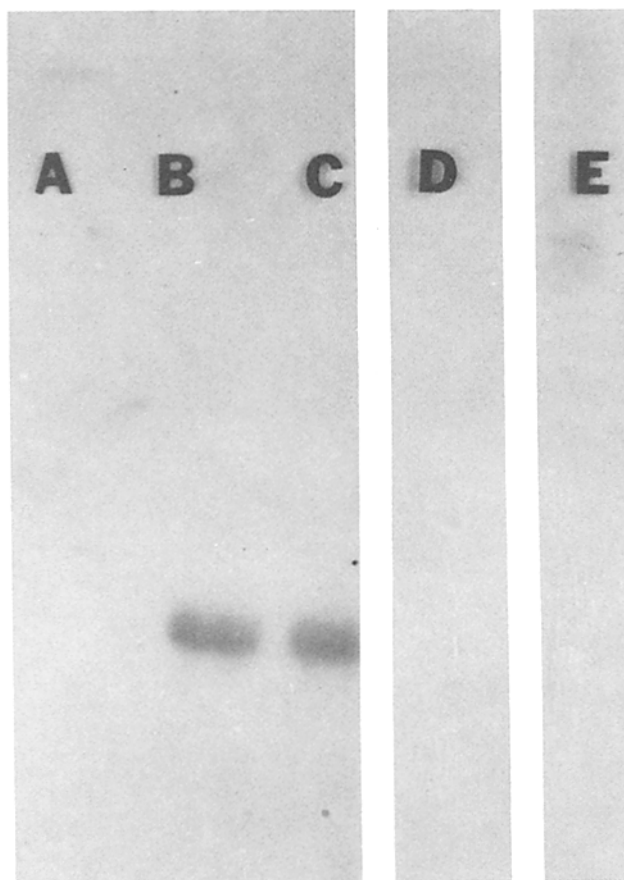

Figure 1. Western blotting of endothelial cell extracts. Confluent endothelial cell monolayers (passage 1) were washed with HBSS and maintained in serum-free medium for 4 d. Then either cycloheximide (2 µg/ml) was added or no further addition was made for the following 48 h. Cells were washed with HBSS and solubilized with Nonidet P-40 (1%). After reduced SDS PAGE (7.5%), Western blotting was done and immunoreactive material was visualized using first a mon-ospecific rabbit antibody to the indicated coagulation factor and second an affinity purified ^{125}I-goat anti–rabbit IgG. Details of the procedure are described under Materials and Methods. Lane A, endothelial cells incubated with cycloheximide; affinity purified an-tibody to protein S used in the first stage; lane B, endothelial cells incubated without cycloheximide; affinity purified antibody to pro-tein S used in first stage; lane C, purified protein S from plasma; affinity purified antibody to protein S used in the first stage; lane D, endothelial cells incubated without cycloheximide; anti–protein C IgG used in the first stage; lane E, endothelial cells incubated without cycloheximide; anti–prothrombin IgG used in the first stage.

major band, M_r ~75,000, which co-migrates with protein S purified from bovine plasma (Fig. 1, lanes B and C). To determine whether endothelial cell protein synthesis was re-quired to generate this protein S, cycloheximide (2 µg/ml) was added to cultures for 48 h. Although this dose of cyclo-heximide did not reduce cell viability in the cultures, based on trypan blue exclusion and morphology of the monolayers, protein S was no longer apparent on the autoradiograms (Fig. 1, lane A). The possibility that endothelial cells could synthe-size protein S de novo led us to examine cultures for the presence of protein C and prothrombin antigen. Substitution of monospecific rabbit IgG directed against protein C or prothrombin for the anti–protein S antibody did not reveal

any band on the autoradiograms even after a 10-fold concentration of the samples (Fig. 1, lanes D and E). To confirm the Western blotting results, endothelial cells were labeled with [³⁵S]methionine and supernatants were subjected to immunoprecipitation (Fig. 2). Autoradiograms demonstrated a sin-
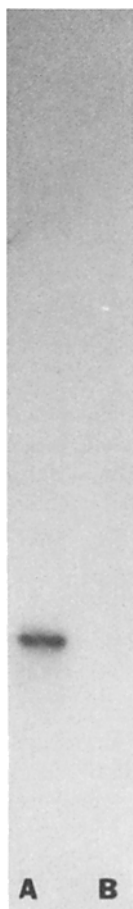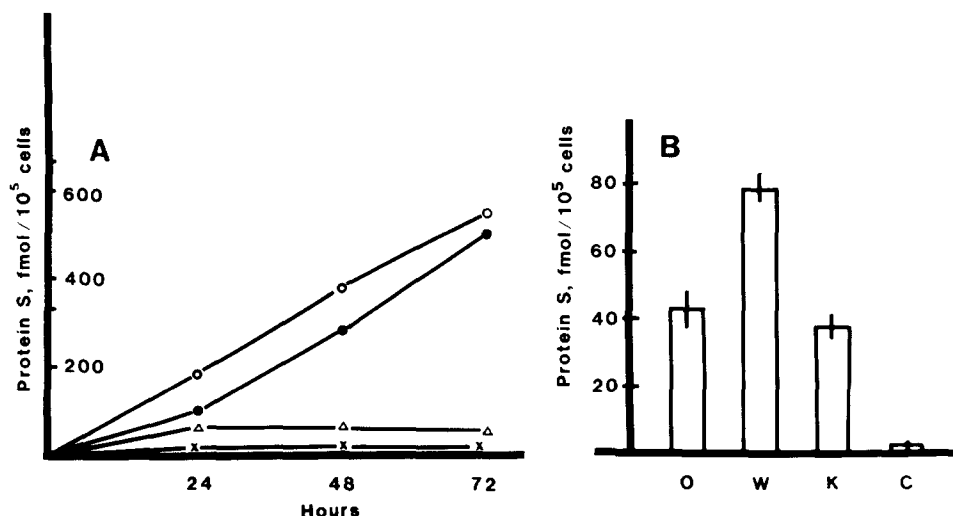

Figure 2. Autoradiogram of gel that contained immunoprecipitated [³⁵S]methionine-labeled proteins released into the medium by bovine aortic endothelial cells. Monolayers were incubated in [³⁵S]methionine-containing medium for 60 h and immunoprecipitated with antibody to protein S (lane A) or protein C (lane B). Immunoprecipitates were subjected to reduced SDS PAGE (7.5%) and autoradiography was done. Details of the procedure are described under Materials and Methods.

gle major band, M_r ~74,000, when immunoprecipitation used antibody to protein S (lane A) but no bands when antibody to protein C, another vitamin K–dependent coagulation protein, was used (lane B). These findings indicate that cultured bovine aortic endothelial cells synthesize the coagulation factor protein S.

Since endothelial cell protein S is physiologically significant in hemostasis only after release from the intracellular pool, secretion of protein S antigen was studied (Fig. 3). After endothelial cells were maintained in serum-free medium for 3 d, protein S was eluted from the cell surface using dextran sulfate (10 mg/ml) (40), and monolayers were then incubated in serum-free medium. Protein S was steadily released into the culture fluid (Fig. 3A). Supplementation of culture medium with vitamin K (25 µg/ml) led to an increase in protein S release with a small decrease in the pool of intracellular protein S (Fig. 3B). The warfarin derivative 3(α-acetonylben-zyl)-4-hydroxycoumarin (1 µg/ml), in contrast, dramatically decreased protein S release with an increase in the intracellular pool. These results are consistent with previous studies showing that defective γ-carboxylation of vitamin K–dependent coagulation proteins leads to defective cellular release (11, 29). Addition of cycloheximide to cultures decreased both protein S release and the amount of intracellular protein S, as predicted from the results shown in Fig. 1.

Activated protein C and protein S can interact on the surface of bovine aortic endothelial cells, which results in considerable acceleration of activated protein C–mediated factor V_a inactivation (40). This led us to study the functional significance of protein S released from endothelium in terms of an increase in the rate of factor V_a inactivation (Fig. 4). After 3 d in serum-free medium, surface-bound protein S was eluted and endothelial cells were incubated in serum-free medium for the times indicated in Fig. 4. The capacity of the endothelial cell surface to accelerate activated protein C–dependent factor V_a inactivation was then assessed using low

Figure 3. Protein S released from and remaining associated with endothelial cells. (*A*) Released protein S. Confluent endothelial cell monolayers (passage 2) maintained in serum-free medium were washed with dextran sulfate (10 mg/ml)-containing HBSS and then incubated in serum-free medium alone (●), or supplemented with either cycloheximide (2 µg/ml) (△), vitamin K (25 µg/ml, Aqua Mephyton, Merck, Sharp and Dohme, West Point, PA) (O), or the warfarin derivative 3(α-acetonyl benzyl)-4-hydroxycoumarin (1 µg/ml, Sigma Chemical Co.) (×). Aliquots of supernatant were taken at the indicated times and assayed for protein S antigen. The

mean of duplicates is shown. (*B*) Intracellular protein S. After removal of the culture medium at 72 h (see *A* above), monolayers were washed once in dextran sulfate (10 mg/ml)-containing HBSS and solubilized with 1% Nonidet P-40. The radioimmunoassay for protein S antigen was then done. O, endothelial cells maintained in serum-free medium. W, endothelial cells maintained in serum-free medium that contained the warfarin derivative used in *A* (1 µg/ml). K, endothelial cells maintained in serum-free medium that contained vitamin K (25 µg/ml). C, endothelial cells maintained in serum-free medium that contained cycloheximide. The mean and SEM are shown (*n* = 7). In both *A* and *B* above, details of the experimental procedure are described under Materials and Methods.

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concentrations of activated protein C to minimize protein S–independent factor V_a inactivation. During the incubation period, the capacity of the endothelial cell surface to accelerate the rate of factor V_a inactivation rose. This was due to the
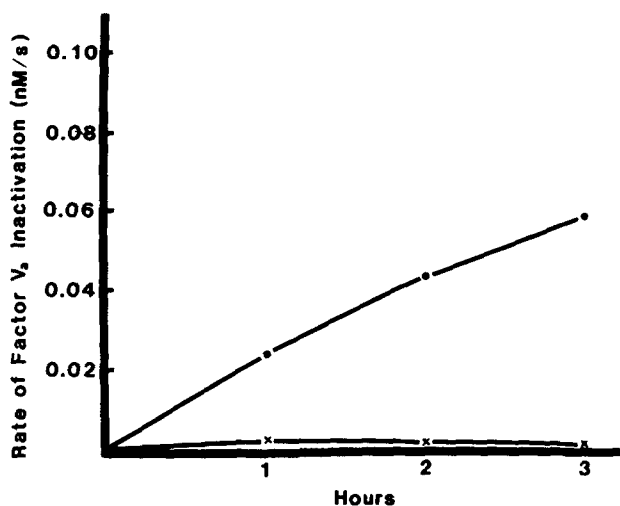

Figure 4. Promotion of activated protein C–mediated factor V_a inactivation by protein S from endothelial cells. Confluent endothelial cell monolayers (passage 1) maintained in serum-free medium for 3 d were washed with dextran sulfate (10 mg/ml)-containing HBSS and incubated in 10 mM Hepes (pH 7.45) that contained 137 mM NaCl, 4 mM KCl, 11 mM glucose, 3 mM $CaCl_2$, 1 mg/ml BSA (●). Where specified, affinity purified anti–protein S IgG was present (50 μg/ml) (×). At the indicated times factor V_a (80 nM) and activated protein C (1 nM) were added, and the rate of factor V_a inactivation was assessed. Nonimmune IgG (50 μg/ml) had no effect on the rate of factor V_a inactivation. The mean of duplicates is shown and details of the experimental procedure are described under Materials and Methods.

presence of endothelial cell protein S, as indicated by its decrease to baseline in cultures that contained affinity purified anti–protein S IgG. Cultures maintained in the presence of 3(α-acetonyl benzyl)-4-hydroxycoumarin did not show enhanced capacity of the endothelial cell surface to accelerate the rates of activated protein C–dependent factor V_a inactivation (data not shown). Thus, protein S derived from endothelial cells maintained in normal growth medium can associate with the cell surface and, in the presence of activated protein C, promote factor V_a inactivation.

To complement the biochemical studies presented thus far, intracellular sites of protein S were visualized immunocytologically (Figs. 5 and 6). Immunofluorescence microscopy showed endothelial cell protein S to be concentrated in the Golgi complex with some additional fine punctate deposits in the peripheral cytoplasm of many of the cells (Fig. 5a). After addition of cycloheximide to cultures, immunofluorescent staining of intracellular protein S was greatly diminished (Fig. 5b). In contrast to endothelium, vascular smooth muscle cells and fibroblasts had almost no immunocytologically demonstrable antigen (data not shown) consistent with their much smaller protein S content. At the resolution of the electron microscope, protein S, demonstrable by immunoperoxidase staining, was identified intralumenally in cisterns of rough endoplasmic reticulum (Fig. 6a), in occasional distal stacks at the concave trans face of the Golgi apparatus (Fig. 6b), and in adjacent vesicles. Protein S was also evident within 30–120-nm vesicles at the periphery, near the cell cortex, some of these in apparent process of fusion (Fig. 6c).

The presence of protein S within endothelium suggested the possibility of a mechanism for its rapid release in response to perturbations. By analogy with the von Willebrand factor (16, 26), we considered whether elevation of endothelial cell cytosolic calcium might mediate protein S release. Incubation
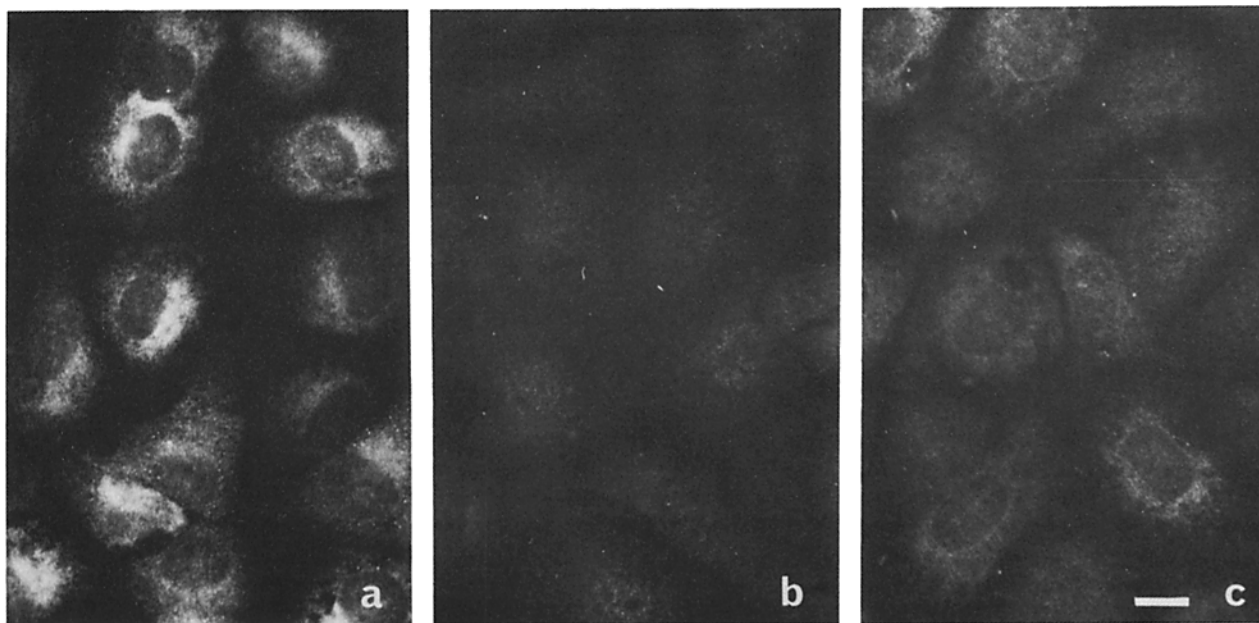

Figure 5. Immunofluorescent localization of protein S in cultured bovine aortic endothelial cells. (a) In normal, untreated cells there is a finely punctate to granular endoplasmic distribution, and an apparent concentration in the juxtanuclear Golgi region. (b) Little or no staining is observed in cells that have been treated with 2 μg/ml cycloheximide for 48 h. (c) Monolayers treated with the calcium ionophore A23187 (5 μM) demonstrate a reduction of fluorescence intensity, especially in the perinuclear area corresponding to the Golgi region. Bar, 10 μm.
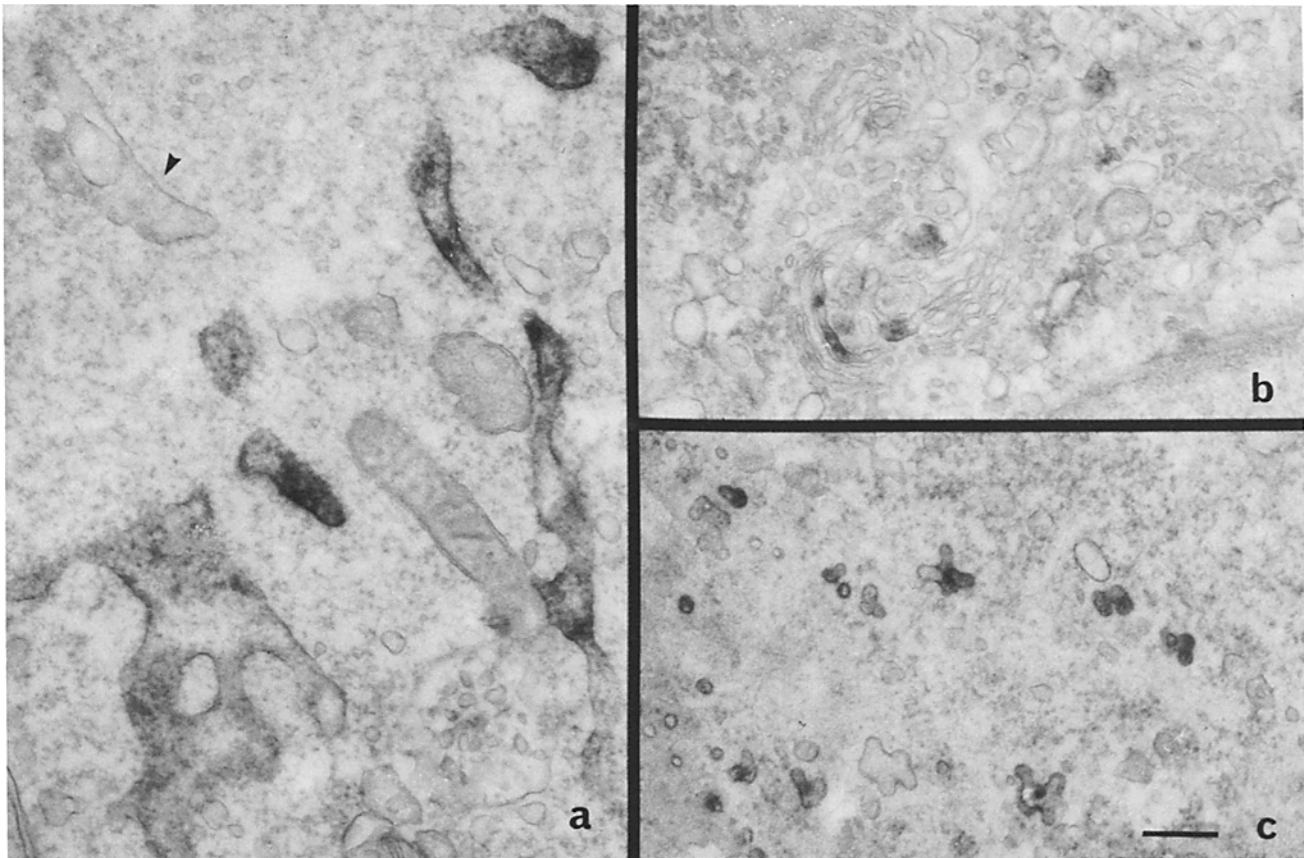
Figure 6. Immunolocalization of protein S in endothelial cells in the electron microscope visualized with indirect biotin-avidin-horseradish peroxidase method (see Materials and Methods). Many cisternae of endoplasmic reticulum contain reaction product, i.e., protein S, but some are not stained (arrowhead, *a*). Protein S is also localized within a few lamellar stacks at the concave *trans* face of the Golgi complex, and in small vesicles associated with them (*b*). In the subcortical endoplasm of most cells there are many small (60–100 nm) horseradish-peroxidase-positive (i.e., protein S–containing) vesicles, some of them in process of fusion (*c*). Bar, 300 nm.
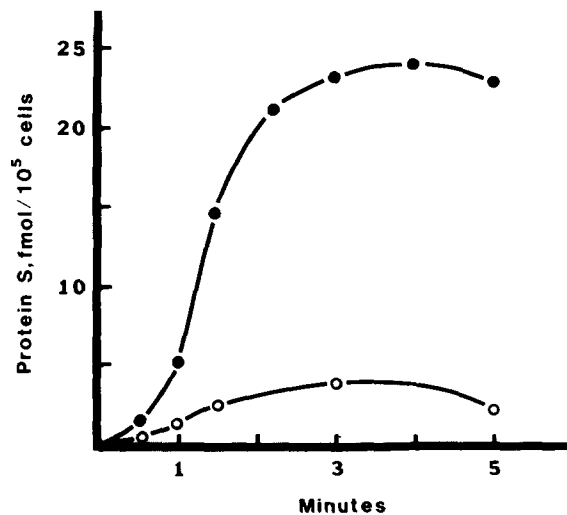

Figure 7. Time course of ionophore A23187–induced protein S release from endothelial cells. Confluent endothelial cell monolayers (passage 2) maintained in serum-free medium for 3 d were washed with dextran sulfate (10 mg/ml)-containing HBSS and then 0.35 ml of 10 mM Hepes (pH 7.45) that contained 137 mM NaCl, 4 mM KCl, 11 mM glucose, 3 mM CaCl₂, 1 mg/ml BSA was added in the presence (●) or absence (○) of ionophore A23187 (5 µM). At the indicated times aliquots of supernatant were removed for protein S radioimmunoassay. The mean of duplicates is shown.

*Table I. The Effect of Ionophores on Endothelial Cell Protein S Release**

Ionophore added	Final concentration	Protein S released
	μM	$fmol/10^5$ cells
Ionophore A23187	5	26 ± 5
	1	20 ± 4
Ionomycin	10	19 ± 4
Valinomycin	10	2 ± 1
No addition	0	3 ± 1

* Confluent endothelial cell monolayers (passage 3) maintained in serum-free medium for 3 d were washed with dextran sulfate (10 mg/ml)-containing HBSS and then 0.35 ml of 10 mM Hepes (pH 7.45) that contained 137 mM NaCl, 4 mM KCl, 11 mM glucose, 3 mM CaCl₂, 1 mg/ml BSA was added alone or in the presence of the indicated concentration of ionophore. Aliquots of supernatant were assayed for protein S antigen after 5 min and the mean and SEM are shown (*n* = 11).

of endothelium with low concentrations of the calcium ionophore A23187 (4) resulted in rapid release of protein S antigen into the culture medium (Fig. 7, Table I). In contrast to A23187-induced release of the von Willebrand factor (26), which continues beyond 30 min, protein S secretion is maximal by 5 min. Approximately 50% of total intracellular protein S is in this rapidly releasable pool. Consistent with these findings, protein S immunofluorescence studies demonstrated an apparent reduction in fluorescence intensity after exposure of endothelium to A23187 (Fig. 5 c). Similar to the

*Table II. Calcium Dependence of Ionophore A23187-induced Endothelial Cell Protein S Release** *

Ionophore A23187 (5 μM)	Other addition	Protein S released
		fmol/10^5 cells
+	0	5 ± 1
+	CaCl$_2$ (0.01 mM)	6 ± 1
+	CaCl$_2$ (0.1 mM)	10 ± 2
+	CaCl$_2$ (3.0 mM)	23 ± 4
+	CaCl$_2$ (3.0 mM) + LaCl$_3$ (10 μM)	6 ± 1
0	0	4 ± 1

* Confluent endothelial cell monolayers (passage 3) maintained in serum-free medium for 3 d were washed with dextran sulfate (10 mg/ml)-containing HBSS and then 0.35 ml of 10 mM Hepes (pH 7.45) that contained 137 mM NaCl, 4 mM KCl, 11 mM glucose was added alone, or in the presence of the ionophore A23187 (5 μM) and the indicated amount of calcium chloride or lanthanum chloride. Aliquots of supernatant were assayed for protein S antigen after 5 min and the mean and SEM are shown ($n = 8$).

release of von Willebrand factor from endothelium (16, 26), protein S release is dependent on the presence of calcium in the medium (Table II). Inclusion of lanthanum chloride (10 μM), an antagonist of calcium influx (34, 38) also prevents A23187-stimulated release of protein S (Table II). Further evidence that A23187-induced release of protein S was a specific effect mediated by calcium is suggested by the finding that ionomycin, another calcium ionophore (23), but not valinomycin, a potassium ionophore (33), induced protein S secretion (Table I).

Discussion

The first suggestion that endothelium might elaborate functional protein S occurred during studies of assembly of activated protein C–protein S on the endothelial cell surface (40). During prolonged incubation of endothelial cells in serum-free medium, the rate of activated protein C–mediated factor V$_a$ inactivation steadily increased in the absence of exogenous protein S. When subsequent experiments showed that this acceleration of factor V$_a$ inactivation could be blocked by antibody to protein S, this suggested production of protein S by endothelium. The results presented in this paper demonstrate that protein S is synthesized by cultured bovine aortic endothelial cells. The subcellular distribution of protein S antigen is like that of other proteins produced and secreted by cells. Thus, in addition to promoting the formation and function of activated protein C, endothelial cells also synthesize the cofactor, protein S, necessary for expression of activated protein C anticoagulant activity. For an average person having a blood volume of 5 liters and $\sim 10^{12}$ endothelial cells (49), the rates of synthesis observed in this study would result in the production of 10 μg/ml per d of protein S. The total plasma concentration of protein S, 20 μg/ml (2), may thus be accounted for at least in part by endothelial cell–derived protein S. Preliminary results have indicated that bovine adrenal capillary and human umbilical vein endothelial cells also synthesize protein S, which suggests that this may be a general property of endothelial cells. Studies by Fair and colleagues (13) have also demonstrated that human endothelial cells synthesize protein S. The rates of protein S synthesis by these different types of endothelium, however, remain to be determined.

Traditionally, hepatocytes have been felt to be the major source of vitamin K–dependent coagulation proteins, which include protein S (11, 12, 16). The synthesis of protein S by endothelium, coupled with the previous demonstration of factor VII synthesis by monocyte (6, 31), indicates that extrahepatic sites may be involved in controlling the plasma levels of these vitamin K–dependent proteins. At least two separate results suggest that endothelium contains the vitamin K–dependent carboxylase necessary for the posttranslational modifications required for expression of protein S function. First, protein S synthesized by endothelium is functionally active (Fig. 4). Second, a vitamin K antagonist blocks secretion (Fig. 3), and the protein found in the presence of the antagonist lacks functional activity. In this context the presence of a vitamin K–dependent carboxylase system in the vessel wall may explain the presence of γ-carboxyglutamic-containing proteins in calcified atherosclerotic plaques (25).

Release of plasminogen activators (27) and prostacyclin (48) have been considered integral components of the anticoagulant nature of the vessel wall. Rapid release of protein S from endothelium would provide another mechanism by which the vessel wall can potentially respond to perturbation with an increase in its antithrombotic potential. Although physiological effectors of protein S release remain to be elucidated, the link between intracellular calcium flux and secretion of protein S, as suggested by the ionomycin and A23187 data, implies a similarity to the mechanism of von Willebrand factor release from endothelium (16, 26). In the latter case, elevation of cytosolic calcium directly by A23187 or indirectly by thrombin, via phospholipid methylation, leads to von Willebrand factor secretion (16). Studies are underway to compare the mechanism of protein S release with this model. The presence of protein S within endothelium suggests that it may be involved in other activated protein C–mediated cellular phenomena, such as activated protein C–mediated alteration of endothelial cell fibrinolytic activity (19, 35).

Synthesis of protein S by endothelium indicates another aspect of the protein C anticoagulant pathway in which the vessel wall plays a central role. This represents a final step in a protein S–endothelial cell cycle which is quite unique in coagulation: protein S can be synthesized and released by endothelium, it can function on the endothelial cell surface, and finally be cleared from the cell surface by an endocytotic mechanism that results in its degradation (39).

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