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 Mr ~75,000, closely migrating with protein S purified from plasma absent from cells treated with cycloheximide. Metabolic labeling of endothelial cells with [35S]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 Va 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 LaCl₃, 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 Va and VIIIa, 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 Schmid et al. (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-cm² wells (1.1-1.5 x 10⁵ cells/cm²). 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 antiserum using indirect immunofluorescence (20).

Coagulation Factors and Assays

All purified coagulation factors were of bovine origin. Purification of protein S was described as above for protein S. Radioiodination 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 μg; 29 μg NaCl (2 mM)), and 2% glucose (20 μl). Free iodine was separated from protein S by gel filtration using a column (1 x 20 cm) of Sephadex G-25. The specific radioactivity of ¹²⁵I-protein S was 9,000–12,000 cpm/pg (corresponding to ~0.1 mol ¹²⁵I/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-morpholinooctanesulfonic acid and chromatographed on QAE-Sephadex (0.6 x 5 cm) equilibrated with 5 mM 4-morpholinooctanesulfonic 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 ar 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 CaCl₂ 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 antiserum to protein S was prepared as described by Vairaktaris (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 x 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 radioiodolated 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 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 3 h at 85°C and at constant power (13 W). Excess binding sites on the nitrocellulose membrane were blocked by a 2-h incubation of Tris (pH 7.4) - 0.02 M Tris (pH 7.4) - 0.14 M NaCl - 0.01% Carnation fat-free 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 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, 0.05% Tween. Finally, buffer that contained milk and affinity purified anti-rabbit IgG (1.3 x 10⁶ 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, 200,000), phosphorylase B (M, 97,400), BSA (M, 68,000), ovalbumin (M, 43,000), and α-chymotrypsin (M, 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 [³⁵S]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 10³ 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 (10³ g for 5 min) and either affinity purified rabbit anti-bovine protein S (10 μg/ml) or rabbit anti- bovine protein S (20 μg/ml) was added for 18 h at 4°C. The next day, protein A-Sepharose (20% by volume) was added in this assay was 100 pM protein S antigen, which corresponded to 80% binding to the monoclonal antibody. 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 ¹²⁵I-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 ¹²⁵I-protein S–antibody binding to 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) (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 ¹²⁵I-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 HBSS. 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 Va 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 CaCl₂, and 1 mg/ml BSA was added. Monolayers were incubated at 37°C, and at the indicated times factor Va (80 nM) and activated protein C (1 μM) 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

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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 CaCl₂ (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 and all clotting times were done in duplicate or triplicate. The rate of factor Vₐ inactivation was determined from the slope of the linear initial portion of a plot of factor Vₐ 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 Vₐ inactivation was 0.19 nM/s (0.03 pmol/10³ 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 CaCl₂ was added along with the indicated concentration of ionophore. Ionophore A23187 (Calbiochem-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 paraformaldehyde (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 incubated 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-phenylenediamine 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 (fluorocepham) 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, monolayers were incubated with affinity purified rabbit anti-protein S IgG diluted (these monolayers were washed extensively resulting in removal of any surface-bound protein S).

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).

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 major band, Mₐ ~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 required to generate this protein S, cycloheximide (2 μg/ml) was added to cultures for 48 h. Although this dose of cycloheximide 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 synthesize 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 $[^{35}S]$methionine and supernatants were subjected to immunoprecipitation (Fig. 2). Autoradiograms demonstrated a single major band, $M_r \sim 74,000$, 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 $\mu$g/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(a-acetonylbenzyl)-4-hydroxycoumarin (1 $\mu$g/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 $\gamma$-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 Va 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 Va 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 Va inactivation was then assessed using low 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 $\mu$g/ml). K, endothelial cells maintained in serum-free medium that contained vitamin K (25 $\mu$g/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.
concentrations of activated protein C to minimize protein S-independent factor Va inactivation. During the incubation period, the capacity of the endothelial cell surface to accelerate the rate of factor Va inactivation rose. This was due to the 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(a-acetonyl benzyl)-4-hydroxycoumarin did not show enhanced capacity of the endothelial cell surface to accelerate the rates of activated protein C–dependent factor Va 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 Va 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 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/10⁶ 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. 5c). Similar to the
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 Va inactivation steadily increased in the absence of exogenous protein S. When subsequent experiments showed that this acceleration of factor Va 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 ~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 endocytic mechanism that results in its degradation (39).

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