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IN SITU IODINATION OF ANGIOTENSIN-CONVERTING ENZYME AND OTHER PULMONARY ENDOTHELIAL MEMBRANE PROTEINS*

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Abstract—Biochemical responses of endothelial cells in culture to pharmacological or physiological stimuli are often extrapolated to define the behavior of the vascular endothelium *in vivo*. However, culture conditions cannot recreate the environment of endothelial cells *in vivo*. To compare cell functions *in vivo* and *in vitro*, we iodinated endothelial membrane proteins of both the perfused rabbit lung and cultured rabbit lung endothelial cells. Endothelial cell protein ^{125}I-labeling in the perfused intact lung was catalyzed by lactoperoxidase and glucose oxidase immobilized on 3–10 μm polyacrylamide beads (Enzymobeads, Bio-Rad). Changes in 5-hydroxytryptamine uptake, angiotensin converting enzyme activity and perfusion pressure made before, during and/or after iodination were small, suggesting that the procedure does not grossly injure the lung. As confirmed by tissue autoradiography, iodination was confined to the vascular space. A subcellular "membrane" fraction of the whole homogenate was enriched for several iodinated proteins. Lectin binding further purified a library of putative iodinated endothelial membranes proteins, one of which was angiotensin-converting enzyme as shown by immunoprecipitation with goat anti-rabbit antibody to angiotensin-converting enzyme. Iodinated proteins of similar molecular weights were also isolated from cultured rabbit lung endothelium iodinated under the same conditions, thus confirming the endothelial lineage of proteins iodinated in the intact lung. We conclude that this technique labels endothelial surface proteins in the intact lung without causing observable tissue injury and thus should be valuable in the study of the physiology and pathophysiology of the vascular lining *in vivo*.

Endothelial cells in culture can express surface antigens that would, *in vivo*, confer important hemostatic, inflammatory and immunological functions upon the vascular endothelium [1, 2]. For example, initiation of inflammatory reactions by leukocyte adherence, intravascular coagulation and presentation of antigen to T lymphocytes is associated with synthesis and surface expression of specific proteins *in vitro* [3, 4]. Although appearance of an antigen linked to endothelial leukocyte adherence has been documented *in vivo* [5], most evidence for such modulation of endothelial activity is based on experiments with cultured endothelial cells, often derived from large resistance vessels. Certainly, tissue culture systems cannot recreate all components of the vascular microenvironment that share in regulating endothelial function *in vivo*, including the sheer stress of blood flow, nutrient supply, adjacent basal lamina and the proximity of other cell types [1]. Furthermore, there is evidence of functional and morphological heterogeneity among endothelial cells of different organs and between large and small vessel endothelium [6–10]. Therefore, it is not surprising that endothelium can respond differently in tissue culture than in the intact animal [11–13].

To circumvent difficulties associated with *in vitro* systems, we chose to study responses of lung endothelial membrane proteins *in vivo*. Accordingly, we developed a method to iodinate vascular cell membrane proteins of the intact rabbit lung using lactoperoxidase and glucose oxidase immobolized on polyacrylamide beads 3–10 μm in diameter (Enzymobeads). Since these beads are small enough to reach the microvasculature, yet large enough to be restricted to the vascular space, iodination occurs only in the lumen. This technique has allowed us to document the presence of several vascular, iodinated proteins, including angiotensin-converting enzyme (ACE§), which are specific to the endothelial membrane.

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§ Abbreviations: ACE, angiotensin-converting enzyme; BPAP, benzoyl-phenylalanyl-alanyl-proline; BPhe, benzoyl-phenylalanine; Con A, concanavalin A; 5-HT, 5-hydroxytryptamine; DiI-Ac-LDL, di-iodoindocarbocyanine acetylated low density lipoprotein; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; LFA, *Limax flavus* agglutinin; RCA, *Ricinus communis* agglutinin I; SBA, soybean agglutinin; SDS, sodium dodecyl sulfate; and WGA, wheat germ agglutinin.

MATERIALS AND METHODS

Materials. Rabbits were purchased from Millbrook Farms (Amherst, MA), Enzymobeads from Bio-Rad (Richmond, CA) and dextran (mol. wt 60,000–90,000) from the United States Biochemical Corp. (Cleveland, OH). Dulbecco's Modified Eagle's Medium (DMEM) and bovine fetal calf serum (FCS) were purchased from the Grand Island Biological Co. (Grand Island, NY). All other tissue culture supplies were from CoStar (Cambridge, MA). [^{3}H]Benzoyl-phenylalanyl-alanyl-proline ([^{3}H]BPAP, 20 Ci/mmol) was from the Ventrex Co. (Portland, ME) and X-omat film and Triton X-100 were from Kodak (Rochester, NY). Unlabeled BPAP was from Vega Biochemicals (Tuscon, AZ). Biotinylated lectins, agarose linked wheat germ agglutinin and avidin linked agarose beads were from E-Y Laboratories (San Mateo, CA). Protein A-sepharose CL-4B was obtained from Pharmacia (Uppsala, Sweden). Normal goat serum was from ICN Immunobiologicals (Lisle, IL). HEPES was purchased from the Calbiochem-Behring Corp. (La Jolla, CA). [^{14}C]5-HT (57 mCi/mmol) and Na ^{125}I were from the Amersham Corp. (Arlington Heights, IL). Other chemicals were supplied by the Sigma Chemical Co. (St. Louis, MO). Goat anti-rabbit angiotensin-converting enzyme antibody was a gift of Dr. Richard Soffer, Cornell University Medical School [14].

Iodination of rabbit lung in situ. Six rabbit lungs were prepared for *in situ* perfusion by methods in routine use in this laboratory [15]. New Zealand male rabbits (2–3 kg) were heparinized (1000 units/kg, i.v.) and anesthetized with urethane (100 mg/kg) and allobarbital (400 mg/kg) administered i.v. The thoracic cavity was exposed via a median sternotomy and the trachea, pulmonary artery and left atrium were cannulated. Each cannula was connected to pressure transducers, the output of which was recorded continuously on a Grass model 7WC polygraph recorder. Lungs were statically inflated with room air at a constant pressure of 5 mm Hg. The height of the left atrial cannula was adjusted to maintain left atrial pressure at 5 mm Hg (zero reference was at the level of the pulmonary artery). Lungs were perfused with a peristaltic pump via the pulmonary artery with Krebs–bicarbonate buffer (Na^+, 144.3 mM; K^+, 5.9 mM; Ca^{2+}, 2.5 mM; Mg^{2+}, 1.2 mM; Cl^-, 128.0 mM; PO_4^{3-}, 1.2 mM; SO_4^{2-}, 2.3 mM; HCO_3, 26.2 mM; dextrose, 11.1 mM; 3% 60,000–90,000 mol. wt dextran) prewarmed to 38° and aerated with 95% O_2, 5% CO_2.

To remove most blood cells from the lung, lungs were perfused for 30 min (flow rate, 10 ml/min) with Krebs-bicarbonate medium. Iodination was carried out by recirculating 50 ml of perfusion medium (flow rate, 10 ml/min; 38°), containing four vials of Enzymobeads, 1.5 mCi Na^{125}I, 11.1 mM β-D-glucose and 0.1 μM NaI in Krebs-bicarbonate buffer. Enzymobeads were added immediately before recirculation. Iodination was allowed to proceed for 20 min after which lungs were again perfused (non-recirculating) with Krebs-bicarbonate buffer (flow rate, 10 ml/min) for about 30 min until radioactivity in the perfusate reached a constant low level. The left lower lobe of the lung was clamped and removed for biochemical studies. The remaining lobes were perfused with fixative (2% glutaraldehyde, 1% paraforamldehyde, 0.1 M sodium cacodylate, 4% polyvinylpyrrolidone) for 15 min, excised and immersed in fixative.

Determination of lung metabolic function. To determine whether iodination caused lung damage, in six additional experiments we measured metabolic functions of the pulmonary endothelium as reflected both by hydrolysis of the angiotensin-converting enzyme substrate, BPAP, and removal of 5-HT. In these experiments the conditions of iodination were mimicked except that Na^{125}I was omitted. Perfused lungs were prepared as described [15]. 5-HT removal and BPAP hydrolysis were determined by the bolus injection multiple indicator dilution technique described previously [15, 16]. Substrates were injected through a latex sleeve proximal to the pulmonary artery cannula. For each experiment, two bolus injections containing [^{3}H]BPAP (0.012 nmol, 0.25 μCi), [^{14}C]5-HT (3.0 nmol, 0.15 μCi) and normal saline (total volume, 0.3 ml) were made, one before and one after lung iodination. Immediately upon injection, left atrial outflow was diverted to a fraction collector and samples were collected at 2-sec intervals for 40 sec. Samples collected prior to the emergence of radioactivity were mixed with aliquots of the injectate (10 μl) to serve as reference standards [15, 16].

Aliquots of each collected sample were assayed for total ^{3}H and ^{14}C (Packard liquid scintillation spectrometer). The extent of [^{3}H]BPAP metabolism to [^{3}H]benzoyl-phenylalanine ([^{3}H]BPhe) was determined after acidification (0.1 ml of 1 N HCl) of an aliquot of each sample (1 ml), extraction with toluene (1 ml), and assay of radioactivity in aqueous and solvent phases (see Ref. 16 for details). The major metabolite of 5-HT, 5-hydroxyindole acetic acid, was not measured since it does not appear during the 40 sec of collection [16]. For each effluent sample, percent [^{3}H]BPAP metabolism = [1 − ([^{3}H]BPhe/[^{3}H]total)] × 100 where [^{3}H]BPhe is the concentration of BPhe and [^{3}H]total is the concentration of total tritium. Because hydrolysis of BPAP is confined to the vascular space, the concentration of total tritium (associated with both BPAP and its hydrolytic product, BPhe) is used as an intravascular reference against which to compare loss of the parent molecule during a single transpulmonary transit [15]. Similarly, percent removal of [^{14}C]5-HT for each sample = [1 − ([^{14}C]5-HT/[^{3}H]total)] × 100 where [^{14}C]5-HT is the concentration of 5-HT and [^{3}H]total is the concentration of total tritium, again used as intravascular reference. Removal of [^{14}C]5-HT or metabolism of [^{3}H]BPAP is reported as the integral of percent removal or metabolism values up to the peak of the total [^{3}H] outflow curve [15, 16].

Tissue autoradiography. Sections (1 × 3 mm) cut from fixed portions of each radioiodinated lung were washed twice in 0.2 M sodium cacodylate buffer (pH 7.4) and once in veronal acetate buffer (0.125 M), each for 5 min before osmification (2% osmium tetroxide) in veronal acetate buffer for 1 hr. Tissues were washed in distilled water, incubated in uranyl acetate (2%) for 1 hr, and dehydrated by

successive 10-min washes in ethanol (one 70%, one 95% and three 100% ethanol). Tissue was embedded in EPON resin, sectioned, and processed for autoradiography.

Autoradiography of proteins isolated from iodinated rabbit lungs. The portion of lung removed before fixation was weighed, minced and homogenized in 5 vol. of homogenization buffer (10 mM HEPES, 250 mM sucrose, 3 mM EDTA, 1 mM phenylmethyl sulfonyl fluoride, 0.1 μM NaI) at 4° using an Ultra-Turrax homogenizer. The homogenate was centrifuged (16,000 *g*) for 15 min and the pellet was designated P1. The supernatant fraction was recentrifuged (140,000 *g*) for 90 min, and the pellet containing the lung membrane fraction (P2) was resuspended in homogenization buffer (1–4 ml). The supernatant fraction was designated S. Cells that emerged from the lung (primarily blood cells 10^6–10^7 cpm/mg protein) into the recirculating medium during iodination and into the perfusate following the iodination were collected by centrifugation (800 *g*) and resuspended in homogenization buffer (~1 ml) and an equal volume of solubilization buffer A (1% SDS, 5% Triton X-100 in phosphate-buffered saline). To compare the spectrum of proteins iodinated in whole lung homogenates with lung fractions S, P1 and P2 and effluent cells, aliquots of each (S, ~90 μg protein, 30,000 cpm; P1, ~180 μg protein, 10,600 cpm; P2, 170 μg protein, 11,000 cpm; blood cells (BC), 6 μg protein, 30,000 cpm; whole homogenates (WH), ~20 μg protein, ~8,000 cpm) were solubilized by solubilization buffer B [final concentration in samples = 3% (w/v) sodium dodecyl sulfate, 1.2% (v/v) β-mercaptoethanol, 2 M urea, 2 mM EDTA, 166 mM Tris–HCl, pH 6.8] followed by heating to 80° for 15 min and applied to 5–15% SDS polyacrylamide gels with 3% stacking gels [17]. The protein profiles were visualized by autoradiography, for which gels were exposed to Kodak X-omat film at −70° with an intensifying screen.

Lectin affinity of iodinated proteins in membrane (P2) and effluent cell fractions. In five out of six iodination experiments, aliquots of P2 fraction from prelabeled lung were solubilized in equal volumes of solubilization buffer A and centrifuged in an Eppendorf centrifuge (13,750 rpm, 1 min). In the experiment shown (see Fig. 3, B and C, and Fig. 5), portions of the supernatant fraction containing membrane fraction (P2) proteins (120 μl, ~0.25 mg protein, 60,000 cpm), solubilized radiolabeled blood cell suspension (80 μl, ~0.07 mg protein, 170,000 cpm) or solubilized endothelial cell suspension (80 μl; ~20 μg protein, 60,000 cpm) were mixed with biotinylated Con A, LFA, RCA, SBA (30 μl, 10 μl and 20 μl for P2 fraction, blood cell or cultured endothelial cell incubates, respectively, equivalent to 0.1 to 0.2 mg lectin per mg protein for P2 and BC fractions and 1 mg lectin per mg protein for endothelial cell samples. In subsequent experiments, we used 0.5 to 1 mg lectin/mg protein for P2 and BC fractions with similar results) or agarose linked WGA, (90 μl, 30 μl and 60 μl for P2 fraction, blood cell and cultured endothelial cell incubates respectively). After incubation for 90 min at room temperature, the suspensions were centrifuged in an Eppendorf centrifuge (13,750 rpm for 1 min), and the avidin linked agarose (60 μl, 20 μl or 40 μl for P2, blood cell and cultured endothelial cell incubates respectively) was added to pellets (except for the WGA incubate). To assess background binding of proteins in the lung membrane fraction to avidin beads, samples containing avidin beads but no lectin were included in each experiment (designated control lanes in Fig. 3, B and C). After 90 min at room temperature, the suspensions were centrifuged in an Eppendorf centrifuge (13,750 rpm for 1 min) and supernatant fractions were discarded. The pellets were washed twice by centrifugation and resuspension in Tris buffer (500 μl, pH 6.8, containing 1.25% Triton X-100 and 0.25% SDS). The pellets were resuspended in solubilization buffer B (30 μl), and the proteins were identified by polyacrylamide gel electrophoresis (PAGE) and autoradiography. The molecular weights are mean values from the four experiments; when proteins of the same molecular weight were bound by more than one lectin, they were included in the same mean value.

Immunoprecipitation of ACE from iodinated rabbit lungs. In two of the five iodination experiments, an aliquot of the lung membrane fraction (P2) (900 μl, ~4 mg protein) was made 0.5% (v/v) with respect to Nonidet P-40 (NP-40) by addition of 100 μl of 5% NP-40 in 10 mM Tris–HCl, 500 mM NaCl (pH 7.4). The mixture was agitated, incubated on ice (3 hr), and centrifuged (13,750 rpm in an Eppendorf centrifuge, 1 min). Dilutions of the solubilized protein (1:15, 1:45) were made in Tris Buffer (10 mM Tris, 500 mM NaCl, pH 7.4) and the final concentration of NP-40 in each solution was adjusted to 0.1%. Antibody to ACE (10 μl) or normal goat serum (20 μl) was complexed to Protein A-Sepharose CL-4B by mixing with 500-μl aliquots of Protein A-Sepharose CL-4B (2 mg/ml of immunoprecipitation buffer containing 10 mM Tris, 500 mM NaCl, 0.1% NP-40). The mixtures were agitated (24 hr, room temperature), and the Protein A-sepharose-antibody conjugates were washed three times by centrifugation and resuspension in immunoprecipitation buffer. Equivalent amounts of immune or nonimmune immunoglobulin are complexed to the Sepharose under these conditions. Duplicate portions of the solubilized lung membrane fraction (1:15 or 1:45) (500 μl) were mixed with Protein A-Sepharose conjugated to either anti-ACE antibody or normal goat immunoglobulin and agitated at room temperature. After 2 hr, the suspensions were centrifuged, the supernatant fractions were removed, and the pellets were washed three times by centrifugation and resuspension in immunoprecipitation buffer (500 μl). The pellets and portions of the supernatant fractions (25 μl) were solubilized in solubilization buffer B and applied to SDS-polyacrylamide gels. Iodinated proteins were visualized by autoradiography.

Isolation and iodination of rabbit lung endothelial cells. Endothelial cells were isolated from rabbit peripheral lung segments (six to eight segments, each 0.5 g in weight) by the fluorescence-sorting technique described by Voyta *et al.* [18] and Gerritsen *et al.* [19] which relies on specific endothelial cell uptake of DiI-labeled acetylated low density lipoprotein (DiI-AC-LDL). One passage after cell sorting, the cells

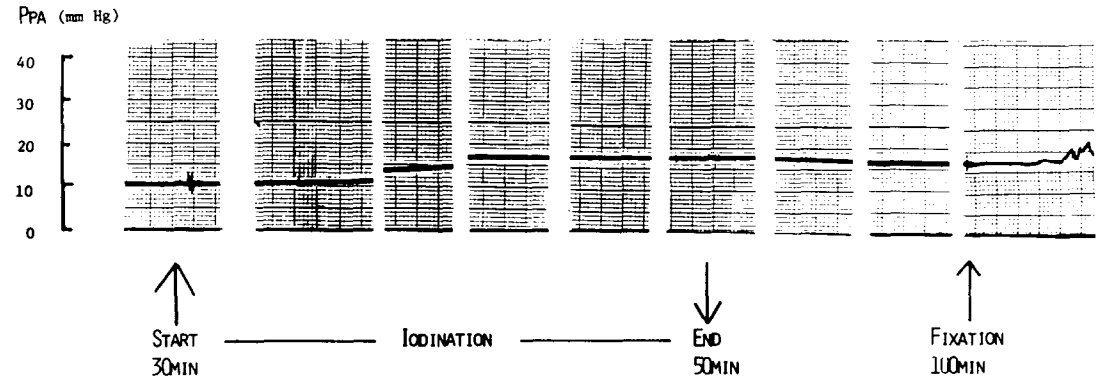

Fig. 1. Pulmonary artery pressure (Ppa) recorded before, during, and after iodination of the rabbit pulmonary vasculature.

were again sorted, yielding a more homogeneous endothelial cell population. Cells isolated in this manner exhibit few Weibel–Palade bodies, tight junctions and express ACE on their surface [20]. The cells were routinely not used beyond the tenth passage after the second sort and were monitored for surface ACE expression and DiI-AcLDL uptake every third passage. To iodinate membrane proteins of cultured cells, a solution of the same composition as that used for iodination of the lung *in situ* (2 ml Krebs-bicarbonate buffer containing 3% dextran, 0.3 vial Enzymobeads, 11.1 mM β-D-glucose, 1 μM NaI and 0.06 mCi $Na^{125}I$) was added to each of two 35-mm diameter dishes of cultured endothelial cells. After 20 min of incubation at room temperature with frequent agitation, the iodination solution was removed, and each dish of cells was washed three times with PBS (1 ml). In one experiment, cells were directly solubilized in equal volumes of solubilization buffer and homogenization buffer, and the proteins were analyzed by SDS-PAGE and autoradiography. In a second experiment, iodinated cells were scraped into homogenization buffer, homogenized using an Ultra-Turrax, and centrifuged as described for the lung tissue to yield a membrane fraction analogous to P2. This fraction was suspended in homogenization buffer (1 ml) and an equal volume of solubilization buffer. Lectin affinity purification experiments were performed as described above using either the whole solubilized cell extract or the solubilized membrane fraction.

RESULTS

A modest increase in pulmonary artery pressures (Ppa) occurred during the iodination reaction (from 7 ± 1 mm Hg to 18 ± 4 mm Hg; (mean ± SEM, N = 5). In some cases, Ppa increased during the recirculation period and decreased towards baseline during subsequent single pass wash out (Fig. 1).

During the study of lung metabolic function before and after simulated radioiodination, Ppa increased from a mean of 9 ± 1 mm Hg before, to 16 ± 2 mm Hg after, iodination (Table 1). Although 5-HT removal was decreased significantly after iodination (81 ± 1 to 63 ± 5%; $P \leq 0.05$), ACE activity (BPAP hydrolysis) was not affected.

Tissue autoradiography was used to localize covalently bound ^{125}I in the lung. In representative autoradiographs of lung sections (Fig. 2, A–C), silver grains representing ^{125}I were localized in the lumen of vascular segments, around Enzymobeads, on blood elements, some of which appeared to have engulfed Enzymobeads and, often, on endothelial cells. The decoration of endothelium was evident both in the absence (A) and presence (B) of Enzymobeads. Silver grains were occasionally seen outside the vascular space (possibly iodinated albumin, see below).

To identify iodinated proteins specific to endothelial cells, the spectrum of iodinated proteins in the radiolabeled whole lung homogenate was compared to that of fractionated lung components and effluent cells. Autoradiography of an SDS-poly-

Table 1. Effect of iodination reaction on pulmonary metabolic function

	Ppa (mm Hg)	BPAP hydrolysis (%)	5-HT removal (%)
Before iodination	9 ± 1	81 ± 3	81 ± 1
After iodination	16 ± 2*	78 ± 2	63 ± 5*

Lung metabolic function was measured by techniques described in Materials and Methods. Values are means ± SEM (N = 6).

* Significantly different from value observed before iodination (unpaired *t*-test), $P \leq 0.05$.

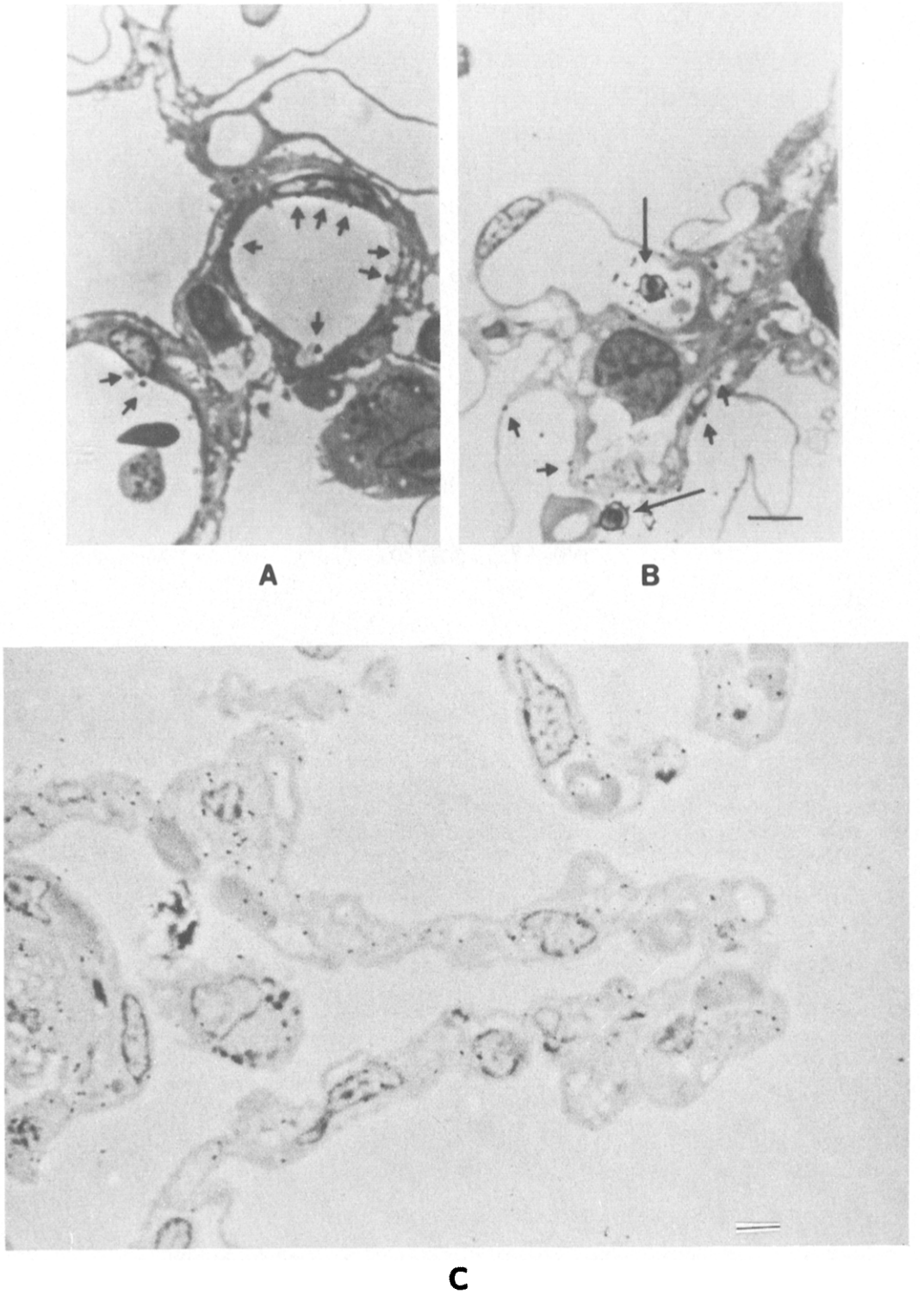

Fig. 2. Autoradiogram of lung tissue after iodination *in situ*. Iodination (small arrows) is shown in the absence (panel A) and in the presence (panel B) of observable beads (large arrows). Panel C shows a longitudinal section of iodinated capillary. Iodinated lung tissue was prepared for autoradiography as described in Materials and Methods. Bar = 10 μm.

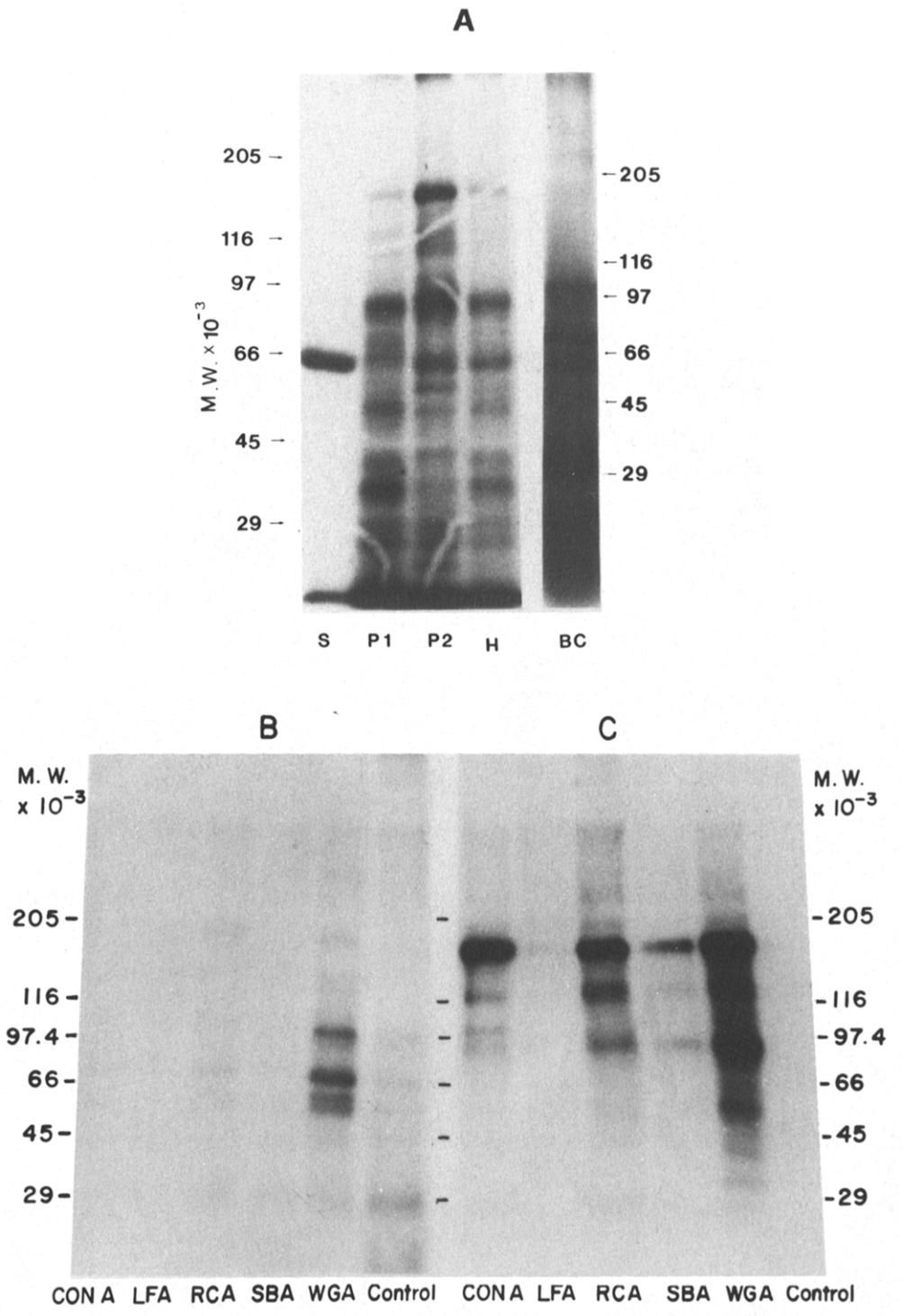

Fig. 3. Autoradiograms of proteins isolated from iodinated rabbit lungs. (A) Radioiodinated proteins in whole lung homogenate, H, fractions S, P1 and P2 and lung effluent (mainly blood) cells, BC. (B) Radioiodinated proteins from effluent blood cells isolated by binding to concanavalin A (Con A), *Limax flavus* agglutinin (LFA), *Ricinus communis* agglutinin I (RCA), soybean agglutinin (SBA), and wheat germ agglutinin (WGA). (C) Radioiodinated proteins from lung membrane fraction (P2) isolated by binding to Con A, LFA, RCA, SBA and WGA.

acrylamide gel (Fig. 3A), representative of results of four experiments, shows that the final supernatant fraction of homogenized tissue (S) contained primarily one iodinated protein at 66 kD (possibly albumin), that was enriched compared to the homogenate (H). The spectrum of iodinated polypeptides in P1 was similar to the whole homogenate. However, the membrane fraction, P2, was enriched in iodinated proteins of molecular weights 170 kD (mean, 160 kD; range, 155–170; N = 5), 120 kD (mean, 120 kD; range 115–135 kD; N = 5) and to a lesser extent 60 kD (mean, 65 kD, range 60–70 kD; N = 5) and 25 kD (mean 28 kD; range 26–30 kD; N = 5). The effluent cells shared 90 kD and 60 kD proteins with the homogenate and/or other fractions and were enriched for primarily lower molecular weight components. There were no radiolabeled proteins of molecular weight greater than 97 kD associated with effluent blood cells.

To further select for specific iodinated endothelial membrane glycoproteins, lectin affinity purification was employed in five experiments. Iodinated proteins isolated by lectin affinity from the lung membrane fraction, P2 (Fig. 3C) and effluent cells (Fig. 3B) were compared. Con A, RCA and WGA agglutinins bound a 175 kD (mean, 180 kD; range, 170–195; N = 15) iodinated protein from the P2 fraction leading to dramatic enrichment of this protein (Fig. 3C). SBA agglutinin bound this protein to a lesser extent (Fig. 3C), while LFA bound very little of any iodinated proteins (Fig. 3C). Con A, RCA, SBA and WGA also bound components at 135 kD (mean, 135; range 120–155; N = 14) and at 90 kD (mean, 100 kD; range 85–115 kD; N = 15). In two experiments, Con A, RCA and WGA also bound iodinated proteins of higher molecular weight, 230 kD (mean, 230 kD; range 210–250 kD; N = 5) and 200 kD (mean, 200 kD; range 195–210; N = 5). Several iodinated lower molecular weight proteins were also seen; however, these were difficult to distinguish from effluent cell components (Fig. 3, B and C). The background binding of proteins to avidin beads was more pronounced in the effluent cells than in the P2 fraction (Fig. 3, B and C, control lanes). Nevertheless, although WGA, RCA and Con A bound a 175–180 kD iodinated component from effluent cells (Fig. 3B), less was evident than in the P2 fraction, and it was not enriched compared with other lectin-purified effluent cell proteins. Likewise, WGA bound 125 and 135 kD proteins from the effluent cells (Fig. 3B) but these were not enriched relative to the other proteins bound by this lectin. However, in this experiment, WGA did bind a 90 kD protein from effluent cells leading to a relative enrichment of this component that may be shared with proteins (mean 100 kD) in the WGA, RCA and Con A affinity

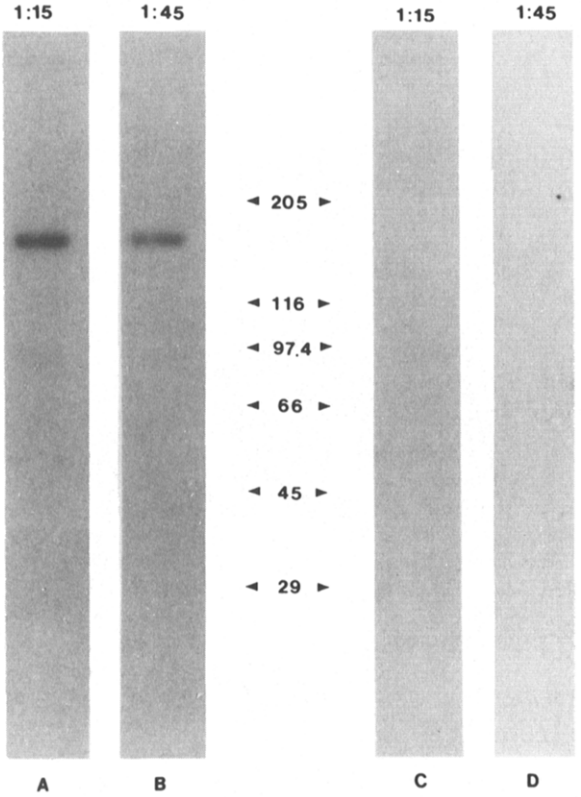

Fig. 4. Autoradiograms of iodinated proteins from lung membrane fraction immunoprecipitated with antibody to ACE. Radioiodinated proteins in lung membrane fraction (P2), diluted 1:15 or 1:45, were immunoprecipitated with either immune sera (A and B) containing antibody to ACE or non-immune sera (C and D), as described in Materials and Methods.

purifications of the lung membrane fraction, P2. In contrast to P2 proteins, WGA affinity purification of effluent cell proteins led to an enrichment of 66 kD and 58 kD components that were also present, but not enriched, in the Con A, RCA and SBA purifications. These data suggest that at least the 175 kD and 135 kD and possibly the 90 kD iodinated proteins are endothelial specific membrane glycoproteins.

Iodinated components in the lung membrane fraction (P2) were further characterized by immunoprecipitation with antibody to ACE (Fig. 4). A concentration-dependent immunoprecipitation of a 155 kD (mean, 165; range 155–170; N = 2) protein was observed. A 50 kD component was also present at the higher concentration of P2 protein; however, this was a non-specific effect since the latter component was also seen in the precipitation with non-immune serum.

To compare endothelial protein iodination *in vivo* and *in vitro*, endothelial cells from rabbit lung were isolated, growth and purified by fluorescence activated cell sorting of a mixture of lung cells stained with dil acetylated low density lipoproteins [18, 20]. Fluorescence micrographs of these labeled cells show elongated, attenuated cells that grow in a monolayer [20]. The purified endothelial cells also express ACE as determined by immunofluorescence [20].

The spectrum of lectin affinity purified iodinated proteins of the purified population of the cultured rabbit lung endothelium was compared to those seen in the lung membrane fraction (P2) (Fig. 5). In two experiments using either the solubilized whole cell extract or a solubilized cell membrane fraction, ConA, RCA and WGA bound proteins of 130 (mean, 125 kD; range 115–135; N = 6) and 180 kD (mean, 170 kD; range 160–180 kD; N = 5) and 100 kD (mean 100 kD; range 97–100 kD; N = 6) as well as other proteins (68 and 40 kD). LFA and SBA did not appear to bind significant amounts of protein.

DISCUSSION

These experiments demonstrate that pulmonary endothelial membrane proteins of the intact rabbit lung can be labeled without causing observable or measurable lung injury. Furthermore, endothelial cell membranes from the intact lung and tissue culture have membrane proteins of the same molecular weight in common. Our tissue autoradiograms (Fig. 2, A–C) also indicate that the Enzymobeads used to catalyze the iodination were small enough (3–10 μm) to reach the microvasculature, suggesting that a portion of the population of labeled proteins is of microvascular origin. Finally, one of the labeled endothelial proteins from whole lung vasculature is angiotensin-converting enzyme (ACE), a known endothelial protein.

Since we intend to use this method to detect changes in endothelial membrane protein expression under defined conditions of activation or injury, we had first to document the stability of the organ preparation under control conditions. Injury during the iodination protocol could result from both the H_2O_2 generated during the reaction and the physical presence of the polyacrylamide Enzymobeads. However,

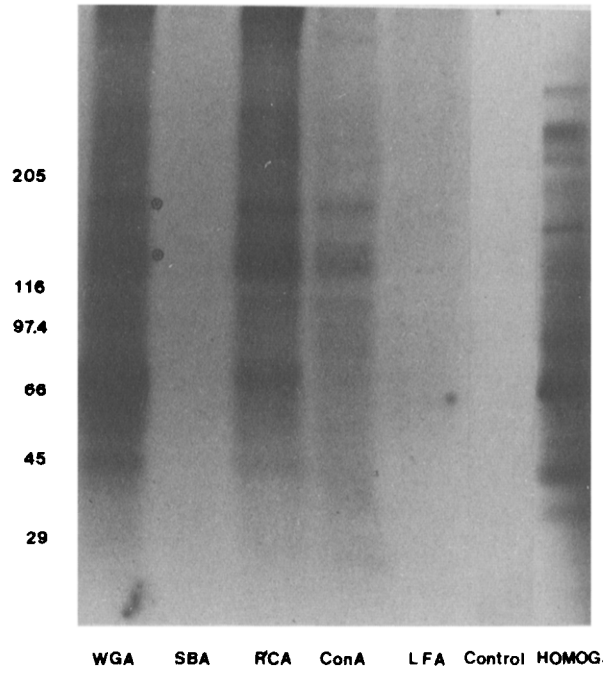

Fig. 5. Autoradiograms of radioiodinated proteins isolated from iodinated rabbit lung endothelial cells in culture. Cells were iodinated and assayed for lectin affinity, as described in Materials and Methods. Radioiodinated proteins from iodinated cells were isolated by binding to Con A, LFA, RCA, SBA, and WGA and whole cell homogenate.

the absence of visible edema and only minimal increases in Ppa during the course of reaction (Table 1) suggest that the iodination procedure did not cause acute lung injury. Furthermore, while 5-HT removal decreased modestly (18%) as a result of the protocol, ACE activity was unaffected (Table 1). If the endothelium had been injured, dramatic decreases in both functions would be expected. For example, in contrast to the mild consequences of iodination we report here, a prior study from this laboratory [21] showed that xanthine oxidase-induced injury causes substantial edema in perfused lungs (mean weight gain ~30 g), large increases in perfusion pressure (30–40 mm Hg), and a 50% decrease in 5-HT removal. These data therefore strongly suggest that, although the *in situ* iodination did not cause acute lung injury, more subtle forms of injury may have occurred. The absence of acute lung injury suggests that enzymatically generated H_2O_2 is present at low enough local concentrations to be relatively innocuous or to be scavenged effectively by endogenous defense mechanisms. Therefore, Enzymobeads probably caused only a minor physical barrier to flow in the lung, which moderately increased Ppa. This was partially reversed when the reactants were washed out, causing Ppa to decrease (Fig. 1). The residual elevation in Ppa may reflect beads that remain in the vasculature despite perfusion following iodination (see Fig. 2). Indeed, decreased 5-HT removal is consistent with the modest loss of perfused vascular surface during the iodination despite the absence of specific endothelial injury [22].

The endothelial membrane origin of the 180, 135 and 100 kD proteins was supported by several criteria. Labeling was seen primarily in the intravascular space (Fig. 2), implying that iodination was confined to the endothelium, blood cells and soluble intraluminal proteins. The membrane fractionation step yielding P2, which gives rise to insoluble proteins, yielded a membrane fraction enriched in four proteins (i.e. 170, 120, 60 and 25 kD). Further fractionation of the P2 proteins by lectin affinity also selected for membrane proteins by preferential binding of glycoproteins (i.e. 230, 200, 170 and 135 and, perhaps, 100 kD). Differential binding of iodinated proteins to lectins was observed. Blood cell proteins possibly contaminating P2 were revealed by analysis of labeled blood cell proteins (Fig. 3) in the effluent cell homogenate before and after lectin purification. For example, the 100 kD protein bound by Con A, RCA and WGA from the lung membrane fraction (P2) may be the same as the 90 kD protein of WGA from the effluent cells and, therefore, may not be specific to endothelium. Alternatively, proteins such as this may appear in the effluent cell fraction as a result of endothelial protein shedding. The use of two-dimensional gel electrophoresis should identify more accurately iodinated proteins from the endothelial surface or blood cells. It is important to note that experiments were attempted in which either equal amounts of radioactivity or protein from each tissue fraction were applied to the gels (or lectins). However, differences in the specific activities resulted in gels that did not adequately represent the spectrum of proteins in each fraction. In addition, since highly glycosylated glycoproteins are not acid precipitable, we could not rely on this method of normalizing amounts of the fractions used for experiments. Hence, we used amounts of tissue fractions that optimized visualization of radioactive bands within each fraction in order to compare the relative intensities of radioactive proteins within any fraction with those in other fractions.

Additional evidence for the endothelial origin of 180, 135 and 100 kD proteins was obtained when analyzing radioiodinated proteins from a purified population of rabbit lung endothelial cells, established to be endothelial by morphology, expression of membrane ACE, and uptake of dil LDL [18, 20, 23–25]. When these cultured cells were labeled under the same conditions used for the iodination *in situ*, proteins of the anticipated molecular weight (170, 130 and 100 kD) were iodinated. The ~10-fold higher specific activity of the iodinated cells as compared to the P2 fraction may account for the apparent increase in the number of radioactive proteins associated with this fraction.

Finally, immunoprecipitation demonstrated that ACE was radioiodinated in the intact lung (Fig. 4). The radioiodinated peptide specifically immunoprecipitated by the ACE antibody had a molecular weight of 155–170 kD. Purified rabbit ACE has been reported to be 129–150 kD or higher, depending on the method of determination. Variability in the estimate is known to be due to the high carbohydrate content of the enzyme [26, 27]. Within lung, ACE is localized to vascular endothelium, plasma and activated macrophages [27]. Our studies were performed in blood-free lungs, eliminating the possibility that the labeled ACE was from plasma. It is also unlikely that the labeled ACE originated from pulmonary intravascular, alveolar, or interstitial resident macrophages in the lung. Pulmonary intravascular macrophages are found primarily in ruminants, not rodents [28]. Interstitial macrophages lie in connective tissue beds near lymphatics, beyond the endothelial basement membrane, and alveolar macrophages line alveoli [29]. Because iodination was confined to the intravascular space, the reactive iodine species did not have access to alveolar or interstitial resident macrophages. In addition, circulating monocytes are not thought to express ACE activity [27]. Finally, only 3–9% of total lung cells in mammals are macrophages, while 30–45% are endothelial [29]. Therefore, inaccessibility of reactive iodine to the subendothelial structures and relative cell number make it most likely that radioiodinated ACE is of endothelial origin.

In conclusion, iodination of pulmonary vasculature and subsequent protein isolation steps presented in this report allow identification of endothelial cell membrane proteins that are expressed *in vivo*. This technique can be exploited to characterize intravascular endothelial proteins from different organs, endothelial membrane responses to pharmacological and physiological perturbation of intact organs, and differences in endothelial membrane reactivity *in vivo* and *in vitro*. For example, lung ACE activity and turnover *in vivo* are sensitive to drugs and hormones, such as bleomycin and phorbol esters [30]. Such treatments may affect labeling of ACE by altering either the amount of ACE

expressed or its accessibility to iodination. Finally, this method should prove valuable in the study of disease-induced modification of endothelial membrane components that have been implicated in the pathogenesis of atherogenesis [31], the adult respiratory distress syndrome [32] and metastatic tumor diseases [33].

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