Serotonin Transport by Cultured Bovine Aortic Endothelium

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ABSTRACT
Endothelial cells isolated from bovine aortas without prior treatment with enzymes were cultured in RPMI 1640 medium containing 17% fetal calf serum and antibiotics. The endothelial cells at confluency (7 days) were similar to endothelium in situ or to freshly isolated endothelial cells from blood vessels as seen by light, scanning, and electron microscopy. Cultured and freshly isolated endothelial cells exposed to labeled serotonin, even in the presence of iproniazid \((5 \times 10^{-4} \text{M})\), took up approximately 125 and 250 pmoles \(^{14}\text{C}-\text{serotonin/mg protein, respectively}, in 3 hours. Imipramine \((10^{-4} \text{M})\) reduced uptake for both cell groups. Cold \((4^\circ \text{C})\) and metabolic inhibitors sharply reduced serotonin uptake by both freshly isolated and cultured endothelial cells. Ouabain \((10^{-5} \text{M})\) almost completely blocked serotonin transport. Six analogues of serotonin at concentrations ten times above experimental serotonin concentrations did not affect serotonin transport in the cultured endothelial cells but did reduce it in the freshly isolated endothelial cells by 50%. The data on transport suggest that serotonin uptake is not unique to pulmonary endothelium, as has been suggested previously. In addition, using cultured endothelial cells to study serotonin transport is compatible with using other serotonin model systems such as platelets, lung, or brain. Lastly, serotonin uptake by endothelial cells may involve an active transport mechanism similar to that described for the pulmonary circulation, platelets, and insect salivary glands.

KEY WORDS
- gap junction
- iproniazid
- lung
- imipramine
- serotonin analogues
- ouabain
- platelets

Although the endothelial cells that line the heart and the blood vessels are the principal constituents of the microvasculature, they are probably the least characterized tissue in the cardiovascular system. Endothelium is significantly involved in lung lesions and shock, thrombogenesis, the synthesis of fibrinolysins, and the growth and metastasis of tumors. However, the most significant role of endothelial cells is in homeostasis: the cells act as a principal sensor of a servomechanism that links the blood with the vascularized tissue. Our interest is in the interaction between endothelial cells and platelets in nonhemostatic situations—more pointedly, in the mechanism of serotonin (5-hydroxytryptamine) transport from platelets to the pulmonary tissues. A number of investigators (1-4) have shown that serotonin is avidly taken up by the lung, and we have demonstrated (5) that 70% of the labeled serotonin released by platelets during one passage through a pump-perfused lung lobe is removed by the pulmonary circulation. We have also observed that when the in vivo and in vitro lung is malfunctioning, as measured by (1) increased venous resistance and pulmonary shunt, (2) decreased compliance, (3) delayed platelet transit times, and (4) platelet entrapment, no measurable amount of labeled serotonin from platelets is removed by the pulmonary circulation. The serotonin released by the platelets is picked up by the circulating platelets and not by the endothelium (5, 6).

The latter data, coupled with other experimental evidence on endothelial cell damage and circulation (7-12), suggest that pulmonary insufficiency may be tied to endothelial damage and that one manifestation of this pathology is impaired or eliminated transport of serotonin by endothelial cells. Strum and Junod (4) have demonstrated by autoradiography that most of the serotonin taken up by the lung remains within the endothelial cells; they have suggested that pulmonary endothelial cells have specific properties not shared by endothelial cells of other organs. These authors concluded, however, that “until isolated endothelial cells become available,” suppositions on the specificity of pulmonary endothelial cells for serotonin cannot be confirmed and other questions related to the transport of this amine cannot be answered.
Recently, a number of laboratories including our own (13-18) have reported on the successful culturing of endothelial cells. Our procedure for obtaining endothelial cells differs from most of the other methods in that we harvest cells for the primary inoculum from aortas without treating this tissue with a proteolytic enzyme; this approach provides certain advantages for control preparations (fresly isolated endothelial cells) that will be discussed subsequently. With the ability to amass isolated endothelial cells, we can investigate more directly than can be done by organ perfusion the transport of serotonin by endothelium. In this paper, we addressed ourselves to three questions. (1) Is serotonin uptake, as seen in lung circulation, unique to the pulmonary endothelium? (2) What are the characteristics of serotonin transport by endothelial cells? (3) Will cultured endothelial cells mimic the transport of serotonin observed in endothelium in situ and in freshly isolated endothelium?

**Methods**

**MATERIALS**

RPMI 1640 medium and fetal calf serum were purchased from Flow Laboratory. 14C-serotonin binoxalate from New England Nuclear Corp., nonlabeled serotonin from Calbiochem, and ouabain, iproniazid, metabolic inhibitors, and serotonin analogues from Sigma. Imipramine was donated by Ciba-Geigy. All other chemicals used were of reagent grade and dissolved in RPMI medium without fetal calf serum. Culture flasks and dishes were obtained from Falcon Plastics.

**CULTURES**

Neonatal calf aortas obtained immediately post-mortem and drained of blood were rinsed in Hank's balanced salt solution containing penicillin and streptomycin (100 IU/ml and 100 μg/ml, respectively). The open ends of each vessel were clamped with sterile hemostats, the intercostal arteries were closed with artery clamps, and the aorta was filled with Hank's balanced salt solution and incubated for 20 minutes at 37°C. The solution, which contained the endothelial cells, was then gently removed from the vessel, and the aorta was rinsed with the balanced salt solution to remove any remaining endothelial cells. The rinse plus the initial perfusion solution were transferred to sterile glass conical centrifuge tubes and centrifuged for 5 minutes at 1,720 g. The pellet was resuspended in 1.2 ml of RPMI 1640 medium containing penicillin (100 IU/ml), streptomycin (100 μg/ml), neomycin (200 μg/ml), and 17% fetal calf serum (v/v). The cells were counted in a hemocytometer and diluted to concentrations appropriate to the plating surface (25-cm² tissue culture flask 3.25 × 10⁶ cells/ml, 75 × 25-mm slide 4.875 × 10⁴ cells/ml, 11 × 22-mm coverslip or 902-mm² culture dish 1.25 × 10⁶ cells/ml). All plating densities were 13 cells/mm². Each culture vessel, slide, and coverslip was incubated at 37°C in a humidified atmosphere containing adequate CO₂ to maintain a pH of 6.8-7.4. The medium was exchanged for fresh RPMI medium at the end of 24 hours and thereafter every 2 days. After 7 days the cultures became confluent and were used for experiments.

**MICROSCOPY**

For light microscopic studies, monolayers of cultured cells were fixed in 2.5% glutaraldehyde in phosphate buffer for 90 minutes or in 10% neutral Formalin. The fixed cells were then treated with appropriate reagents for a histological survey; hematoxylin and eosin, Feulgen stain, periodic acid-Schiff reagent, Gomori-Takamatsu phosphoenolpyruvate, and silver nitrate.

For routine electron microscopic observations, endothelial cell monolayers in Falcon petri dishes (35 × 10 mm) were fixed in 2-3% glutaraldehyde in 0.1m cacodylate buffer, pH 7.4, postfixed in 1% osmium tetroxide in the same buffer, stained with aqueous 0.1% uranyl acetate, and dehydrated through a graded ethanol series. Toluidine blue O (0.5%) was included in the final 100% ethanol solution to facilitate subsequent observation and selection of cells for sectioning. Monolayers were embedded in Epon 812 after infiltration through mixtures of Epon and 100% ethanol containing toluidine blue O. The Falcon petri dish was pried away from the Epon block before polymerization was complete and while the Epon was still flexible. Areas selected for sectioning were cut out with a jeweler's saw and mounted with Epoxy cement onto a holder suitable for the microtome chuck. Sections, cut either parallel or oblique to the plane of growth, were stained with uranyl acetate and lead citrate and examined in a JEM-100B electron microscope.

**SEROTONIN UPTAKE**

Confluent monolayers were used in all experiments. 14C-serotonin in fresh medium was added directly to the flask to obtain a final concentration of 10⁻⁶M and a specific activity of 0.5 c/mmole. The endothelial cells were incubated with the monoamine oxidase inhibitor, iproniazid (final concentration 5 × 10⁻⁶M), for 30 minutes before the serotonin was added. This general procedure of incubating the endothelial cells with iproniazid was used as the first step in other experiments utilizing metabolic inhibitors. Imipramine, a serotonin transport inhibitor (3, 4), was added to the medium at a final concentration of 10⁻⁴M simultaneously with the iproniazid. Other metabolic inhibitors, at stated concentrations, were also added to the medium along with iproniazid. For experiments using serotonin analogues, the analogues (final concentration 10⁻⁴M) were added simultaneously with the 14C-serotonin following the 30-minute incubation with iproniazid. Except for the tests involving the effect of cold on serotonin transport, all experiments were performed at 37°C, and the reactions were stopped by chilling. The decanted medium, seven wash solutions, and the endothelial cells solubilized in 0.15M NaOH were counted separately using standard liquid scintillation procedures. Uptake of serotonin was calculated in picomoles and expressed as a function of endothelial cell protein. The method of Lowry et al. (19) was used for protein determinations. Cells for serotonin control experiments included freshly isolated aortic endothelium, autologous erythrocytes, or cultured fibroblasts. Parallel experiments were always performed with freshly isolated endothelial cells, which were treated as the principal controls, as well as with cultured endothelial cells. In reporting the data, however, either freshly isolated or...
cultured endothelial cell results are given to limit the number of graphs without limiting the evidence of endothelial cell activity.

Results

MICROSCOPY

The cultured endothelial cells generally attached to the substrate at an efficiency of 25%, and confluency was reached at the end of 7 days. Confluent cells were uniform in appearance (Fig. 1), and the stained cultured endothelial cells were in general morphologically similar to endothelium in situ or freshly isolated endothelial cells stained with the same reagents.

The ultrastructure of the endothelial cells cultured in our laboratory was similar to that reported for endothelial cells in situ. Cytoplasmic structures included endoplasmic reticulum, granules, lipid bodies, mitochondria, and an occasional Weibel-Palade body. Vesicles and a variety of intercellular membrane junctions (tight junctions and gap junctions) could be demonstrated (Fig. 2).

SEROTONIN TRANSPORT

In general, the cultured endothelial cells functioned in a manner comparable to that observed in the control freshly isolated endothelial cells. The optimal concentration of serotonin in the medium was $10^{-6}$M for both freshly isolated and cultured cells. Both groups concentrated serotonin (cultured cells $10^{-5}$M serotonin), but the magnitude of $^{14}$C-serotonin uptake by the freshly isolated endothelial cells was always slightly more than twice the uptake by the cultured endothelial cells; the relative inhibition by imipramine was similar in both groups. The freshly isolated cells appeared to be more sensitive to the analogues of serotonin than were the cultured endothelial cells.

Freshly Isolated and Cultured Endothelial Cells with Iproniazid.—Both groups of cells in the presence of the monoamine oxidase inhibitor rapidly removed $^{14}$C-serotonin from the medium, and 96% was recovered as serotonin. The only notable difference between the cultured and the freshly isolated cells was that the control cells took up more than two times the amount of $^{14}$C-serotonin (Fig. 3).

Cultured Endothelial Cells with Iproniazid and Imipramine.—Imipramine had a profound effect on $^{14}$C-serotonin uptake. At the end of 3 hours, serotonin uptake was reduced from 120 pmoles/mg protein to approximately 50 pmoles/mg protein (Fig. 4).

Fibroblast and Erythrocyte Uptake of $^{14}$C-Serotonin.—In these two experiments, no specific $^{14}$C-serotonin uptake was observed when fibroblasts or autologous erythrocytes were used. The amount of serotonin uptake was approximately the same with or without imipramine (Figs. 5 and 6).

Metabolic Inhibition.—Incubating cultured endothelial cells with $^{14}$C-serotonin at 4°C produced a sharp reduction in the uptake of the amine (Fig. 7).
Sodium azide and sodium fluoride (final concentrations $5 \times 10^{-3}$M) produced a 25% inhibition of $^{14}$C-serotonin uptake by the cultured cells. Inhibition of $^{14}$C-serotonin uptake was also observed in freshly isolated endothelial cells when they were treated with iodoacetate (final concentration $10^{-3}$M), and 2,4-dinitrophenol (final concentration $10^{-4}$M) almost completely blocked serotonin transport (Fig. 8). In addition, ouabain (final concentration $10^{-4}$M) almost completely blocked serotonin transport in freshly isolated endothelial cells (Fig. 9).

Analogue Effect on $^{14}$C-Serotonin Uptake.—Cultured aortic cells showed some degree of specificity for serotonin in that the transport of approximately 250 pmol/mg protein in 3 hours was not affected by even a tenfold increase in any of the analogues. However, in the freshly isolated endothelial cells, substrate competition was observed: tryptamine, tryptophan, and 5-hydroxyindole-acetic acid decreased $^{14}$C-serotonin uptake by 70%, and 5-methoxytryptamine, 5-hydroxytryptophan, and 5-methoxytryptamine produced a 50% inhibition of $^{14}$C-serotonin uptake.

Discussion

The present study was undertaken primarily to see if serotonin is unique to pulmonary endothelial cells and to compare the structure and activity of cultured endothelium with that of freshly isolated endothelium. In addition, we hoped that the studies would provide at least first-order explanations of serotonin transport by endothelial cells.

The process of isolating and culturing endothelial cells from bovine aortas as presented in this paper is very reliable, and the technique has no apparent effect on the structure of the cells or qualitatively on the transport of serotonin. The harvesting of cells from the aorta without the use of an enzyme has a distinct advantage over systems that use collagenase or trypsin in that the freshly isolated endothelial cells can be immediately used for control experiments without concern about the potential alteration of the cell membrane due to...
endothelium and serotonin

"C-serotonin (5-HT) uptake by isolated (solid circles), and cultured (open circles) endothelial cells in the presence of the monoamine oxidase inhibitor iproniazid (5 x 10^-5 M).

the enzyme treatment. Structurally, the cultured aortic endothelial cells showed the anatomic specializations that are seen in aortic endothelium in situ. Recently, Hüttner et al. (20) have demonstrated the presence of gap junctions in arterial endothelium. The presence of these low-resistance channels in aortic cultured endothelial cells constitutes another piece of morphological supportive evidence that the cultured cells are phenotypically similar to endothelium in situ, and the presence of gap junctions also suggests that the endothelium of the intima, and possibly that of the capillaries, should not be considered a passive layer of cells.

The overall kinetics of serotonin uptake by the freshly isolated and cultured aortic endothelial cells were quite similar except for quantity. In 13 hours, freshly isolated cells accumulated 250 pmoles serotonin/mg protein, whereas cultured cells took up about half as much. However, the increased uptake by the freshly isolated endothelial cells might be partly due to the uptake of a labeled metabolite of serotonin produced by erythrocytes in the perfusate. In both groups serotonin uptake plateaued in 2–2.5 hours; iproniazid had no effect on uptake, and imipramine produced the same relative degree of inhibition in both groups. Junod (3) has concluded from his studies in isolated rat lungs that the rate-limiting step in "C-serotonin uptake is its intracellular transport and not its subsequent metabolism. A similar mechanism might be operating with isolated and cultured endothelial cells. Serotonin uptake was dependent
$^{14}$C-serotonin (5-HT) uptake by autologous erythrocytes in the presence of iproniazid ($5 \times 10^{-6}$M) and imipramine ($10^{-6}$M) compared with that of freshly isolated endothelial cells. Solid circles = iproniazid alone, open circles = iproniazid and imipramine, solid lines = freshly isolated endothelial cells, and broken lines = red blood cells.

on external concentrations of the amine with $10^{-6}$M being the optimal final concentration (concentrations studied were between $10^{-5}$M and $10^{-8}$M). Two points cannot be explained by the present data: (1) the uptake of serotonin by freshly isolated endothelial cells is greater than that by cultured cells and (2) the analogues of serotonin only inhibit serotonin uptake in noncultured endothelial cells.

The data suggest that serotonin uptake may involve an active transport mechanism. Changing the external concentration of serotonin, inhibiting
ENDOTHELIUM AND SEROTONIN

the overall metabolism or aerobic respiration, and inhibiting membrane sodium-potassium adenosinetriphosphatase with ouabain all affected serotonin uptake. A similar model of uptake has been described by Okuda and Nemerson (21), who have characterized the transport of serotonin by platelets as a "pump-leak" system. However, it has yet to be determined whether a site specific for serotonin transport exists on the endothelial cell membrane or if the serotonin transport is coupled to sodium transport. In the pump-perfused rat lung, lowered sodium levels or increased potassium levels in the medium decrease 14C-serotonin uptake by the pulmonary circulation (3). It appears that the uptake of serotonin by endothelial cells is not limited to pulmonary endothelium since both freshly isolated and cultured aortic endothelial cells removed appreciable amounts of serotonin from the medium. (In a pilot experiment, a segment of aorta with intact endothelium, perfused with 14C-serotonin in the tissue culture media, removed serotonin; extrapolating the data, the removal by the intact intima approximated the amount observed for freshly isolated endothelial cells.) However, since the lung contains more serotonin than do most vascular organs such as the liver or the kidney, obviously some unique system prevails in the pulmonary tissue. A number of investigators have suggested that because of its strategic vascular position the lung may act as an excretory organ for excess serotonin that accumulates in the portal venous blood. A possible function for serotonin is related to its known vasoconstrictor effect. Serotonin may be taken up by the endothelial cells and "transported" to the adjacent smooth muscle to effect vasoconstriction. Experimental mammals infused with serotonin or humans with carcinoid syndrome show an increase in pulmonary hypertension and vascular resistance. It may be that serotonin affects the contractile proteins of the endothelial cells to produce intracellular motility. Since serotonin in certain vertebrates is taken up by an active transport mechanism and acts as a primary messenger to stimulate salivary secretion (22-24), it also may affect endothelial cells in a similar fashion. In the past few years a number of investigators have reported that endothelial cells in the lung play an active role in homeostasis, apart from gas exchange, by synthesizing, releasing, and activating peptides and proteins (25-30). Serotonin may stimulate the endothelial cells to secrete the specific enzymes involved in these and other homeostatic mechanisms.

In summary, these data support the view that possibly all endothelial cells can remove serotonin from a bathing medium and that a model using cultured endothelium to study the transport of serotonin is compatible with other systems used to study serotonin, such as platelets, lung, and brain. Lastly, the uptake of serotonin by endothelial cells is possibly by an active transport mechanism which has been observed indirectly in the pulmonary circulation and directly in other cell systems such as platelets and insect salivary glands.

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