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SEROTONIN INDUCED ACTIN POLYMERIZATION AND ASSOCIATION WITH CYTOSKELETAL ELEMENTS IN CULTURED BOVINE AORTIC ENDOTHELIUM

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SUMMARY: Serotonin, (5-hydroxytryptamine, 5-HT) binds to cultured endothelial cell stress fibers as identified by fluorescence microscopy and in vitro induces actin polymerization as measured by DNase 1 inhibition and differential centrifugation; the structural change in actin in the presence of 5-HT resembles actin paracrystals as visualized by negative staining under electron microscopy. These observations indicate that 5-HT, which is taken up by endothelium by a non-mediated diffusion, may interact directly with actin to affect cytoskeletal changes.

Serotonin, (5-hydroxytryptamine, 5-HT) is known to affect bovine aortic endothelial cell cytoskeleton, motility, and barrier function. We have reported that 5-HT inhibits significantly endothelial cell movement as measured by gold chloride phagokinetic tracking (Bottaro, et al. 1985), enhances the barrier to albumin in vitro (Bottaro, et al. 1986), and enhances the microvascular barrier in vivo and prevents the extravasation of erythrocytes in severely thrombocytopenic animals (Shepro, et al. 1984). Herman, et al. (1981) demonstrated that there was a decrease in stress fibers associated with motile endothelial cells. Bensch, et al. (1983) reported that endothelial proliferation in vitro is dependent on stress fibers. Bentzel, et al. (1981) reported that tight junctional permeability is dependent upon an actin filament system. Endothelial cell monolayers treated with agents that disrupt actin organization displayed increased macromolecular permeability, presumably through the loss of cell junctional integrity (Shasby, et al. 1983).

Small and Wurtman (1985), observed that actins isolated from both brain synaptosomes and skeletal muscle bind 5-HT in a highly specific and saturable manner. The authors suggest that through binding to actin, 5-HT may change the disposition of actin within cells and affect cell function.

In this communication, we report on 5-HT, its binding to endothelial cytoplasmic filaments in situ, decreasing the binding of actin to DNase 1, increasing the sedimentation of actin, and altering macromolecular actin structure as visualized by electron microscopy. These observations reported herein indicate that 5-HT binds to actin and induces its polymerization in vitro.

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These results also add further support to the premise that observed enhancement of the barrier observed with 5-HT treatment of endothelial cells may depend in part upon a direct action of this amine on actin.

MATERIALS AND METHODS

Indirect Immunofluorescence Localization of 5-HT

Bovine aortic endothelial cells were seeded onto 1.2 cm glass coverslips in limbro wells and allowed to reach 50 % confluency. This cell density was chosen because endothelial cells display a higher amount of stress fibers per cell at this density than at 100 % confluency. Cells were grown in DME supplemented with 10 % fetal calf serum, (FCS). At 50 % confluency, cells were removed from 10 % FCS, treated for 6 h in DME without FCS, washed 3 X with 37°C phosphate buffered saline, (PBS) and fixed in 4 % phosphate buffered formaldehyde for 15 min. After fixation, cells were permeabilized with 0.5 % Triton X-100 in PBS at 25°C for 15 min, washed 3 X in 25°C PBS and were inverted cell face down into 70 µl of 1:500 diluted rabbit anti-5-HT antiserum (Immunonuclear Co., Stillwater, MI.) with 1 % normal goat serum and 1 % bovine serum albumin added as blocking agents. The endothelial cells were incubated at 37°C for 1 h, washed 10 X with PBS and then incubated with 70 µl of rhodamine coupled goat anti-rabbit serum (Sigma Chemical, St. Louis, MI.) diluted 1:200 for 1 h at 37°C. After washing 10 X with PBS the monolayers on coverslips were mounted in 5 µl of PBS/glycerol, placed on a glass slide; the coverslips were then sealed with nail polish. Endothelial cells were examined and photographed with a Zeiss C-74 microscope adapted for rhodamine fluorescence. The stained cells were photographed with an Olympus OM-25 camera set-up on auto-exposure. Film speed was boosted to 1600 ASA with Diafine developer (Acufine, Inc., Chicago, IL.).

DNase 1 Inhibition Assay

DNase 1 inhibition was measured using a modification of the technique described by Blikstad, et al. (1978). Chicken skeletal muscle actin was purified according to Pardee and Spudich, (1982); protein purity was greater than 95 %. Aliquots of actin at 475 µg/ml were incubated with either G-actin buffer, G-actin buffer with 1 mM 5-HT, buffer with 1 nM 5-HT, buffer with 1 mM spermidine, or buffer with 1 nM spermidine for times between 0 and 20 min. (Spermidine was used in this assay and in the centrifugal assay to establish a baseline measurement for comparison with 5-HT as spermidine is known to induce *in vitro* actin polymerization, Oriol-Audit, (1978)). At each time point, a 10 µl aliquot was removed from the incubating sample and mixed with 20 µl of DNase 1 at 0.1 mg/ml. This mixture was vortexed for 2 s and then mixed with 3 ml of calf thymus DNA solution at 40 µg/ml. This cocktail was immediately transferred to a quartz cuvette and the change in absorbance at 260 nm recorded over 5 min. Active DNase 1 degrades DNA to its component nucleotides which absorb more strongly at 260 nm. DNase 1 activity of incubated samples was compared with activity of samples incubated with known amounts of G-actin. The activity of DNase 1 was expressed as a percentage of the uninhibited enzyme which is 100 % in the absence of G-actin. Since the G-actin monomer binds to DNase 1 on a 1:1 ratio, it is possible to use the inhibition of DNase 1 to determine the amount of G-actin in a sample.

Centrifugal Assay

Actin sedimentation was measured using a modification of the technique described by Cooper and Pollard (1982). Aliquots of actin at 475 µg/ml were incubated with test substances as described above. At time points between 0 and 20 min, 100 µl aliquots were removed and placed atop a 30 % sucrose cushion in G-buffer plus the pertinent test substances at the same concentration as in the sample. The tube with the sample was then centrifuged for 15 min at 15,000 g in a table top centrifuge. After centrifugation, the supernate was assayed for total protein according to Bradford (1976). The basis of this assay lies in the ability of the F-actin filaments to migrate readily in a centrifugal field, whereas G-actin will not. Decreases in total protein over time are evidence that

treatment has altered the actin during incubation from G-actin to a form more dense than the G-actin monomer.

Visualization of 5-HT Induced Actin Structures

Negative staining of actin forms induced by test agents was performed by a modification of the technique described by Cooper and Pollard (1982). Formvar coated grids were treated with bacitracin (15 μg/ml) to enhance adhesion of actin structures, (personal communication, Dr. David Begg, Boston, MA.). Aliquots of actin at 475 μg/ml were treated for 30 min with test agents as described. After incubation, the samples were diluted to 15 μg/ml, and 10 μl of the sample was applied to the grid. The grid was then dried by touching the grid edge with filter paper. Grids were then washed in distilled water and stained for 1 min with 1 % uranyl acetate. The grids were then rewashed in distilled water and then air dried. Samples were examined under transmission electron microscopy using a JEOL 100-B microscope (JEOL, Japan).

RESULTS

Anti-5-HT Immunofluorescence

Bovine aortic endothelial cells stained for the presence of 5-HT displayed fluorescence along cytoplasmic filaments that colocalized with actin stress fibers, Fig. 1. Controls for immunocytochemical specificity were conducted by incubating anti-5-HT antibody with the bovine serum albumin-5-HT

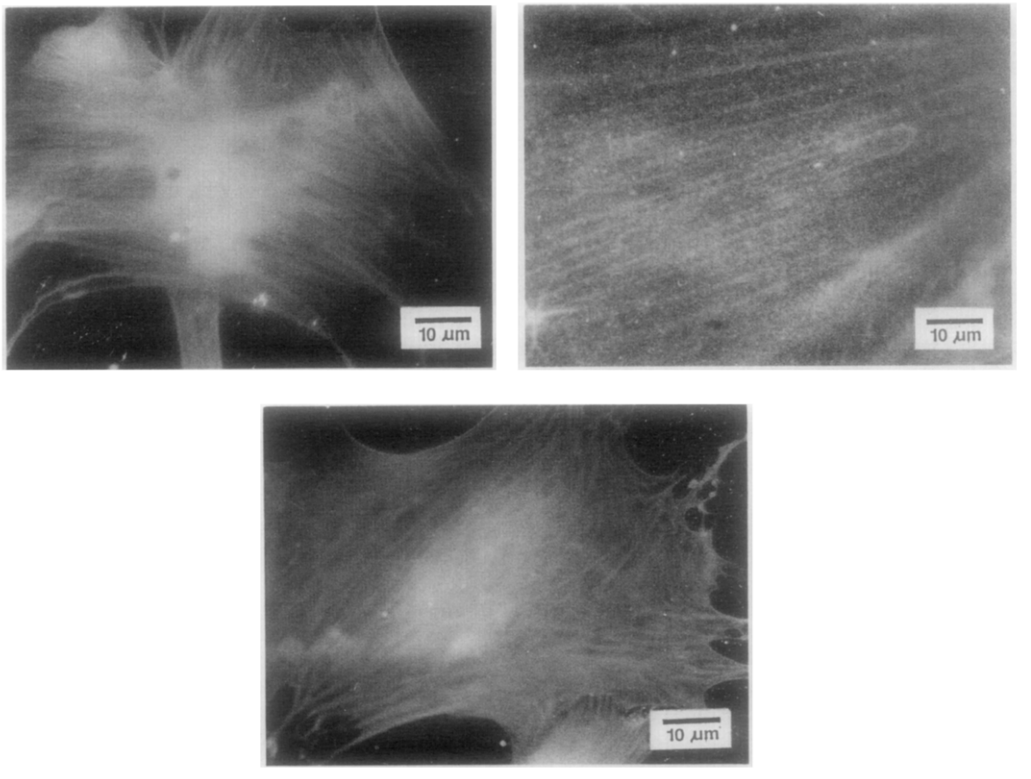

Fig. 1. Rhodamine immunofluorescence of endothelial cells stained for the presence of 5—HT.

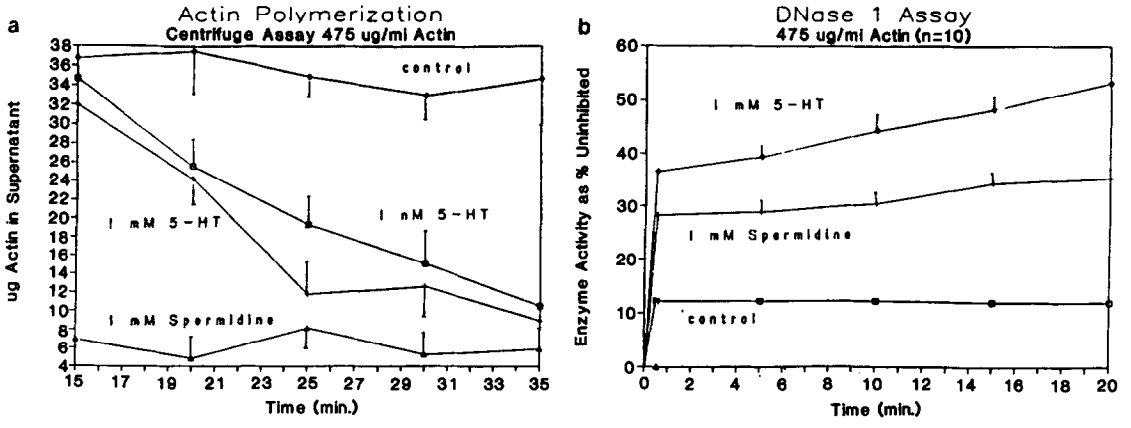

Fig. 2 a : Centrifugal assay results.
Fig.2 b : DNase 1 assay results.

conjugate used to raise the antibody (20 ul of 20 ug/ml). When cells were stained with this preparation in place of untreated antibody, no fluorescent staining was seen.

DNase 1 Inhibition

Incubation of actin with spermidine (used to test this assay) caused a rapid increase in DNase 1 activity over untreated G-actin, Fig. 2b. When 5-HT was used as an agonist at 1 mM concentration, increased DNase 1 activity was observed compared with controls, similar to the polyamine stimulation of activity.

Centrifugal Assay

Actin incubated with spermidine (1 mM) showed an increased rate of sedimentation, Fig. 2a. In a like manner, 5-HT increased the rate of actin sedimentation. These findings suggest that 5-HT induces formation of actin aggregates that are more physically dense than the actin G monomer.

Negative Staining

Actin structures (Fig. 3) assembled in the presence of spermidine show actin filaments similar to those assembled in the presence of high salt concentration. The actin structures formed following 5-HT treatment are short, interconnecting chains, they may be paracrystalline actin forms, but they are unlike spermidine induced filaments. However, both structures of actin assembled in the presence of 5-HT and spermidine are morphologically distinct from G-actin.

Discussion

There is a body of evidence that links the organizational state of the actin cytoskeleton to the ability of endothelial cells to maintain structural integrity and responsiveness as a selectively permeable barrier. Shasby, et al. (1983) demonstrated that endothelial monolayers treated with the microfilament

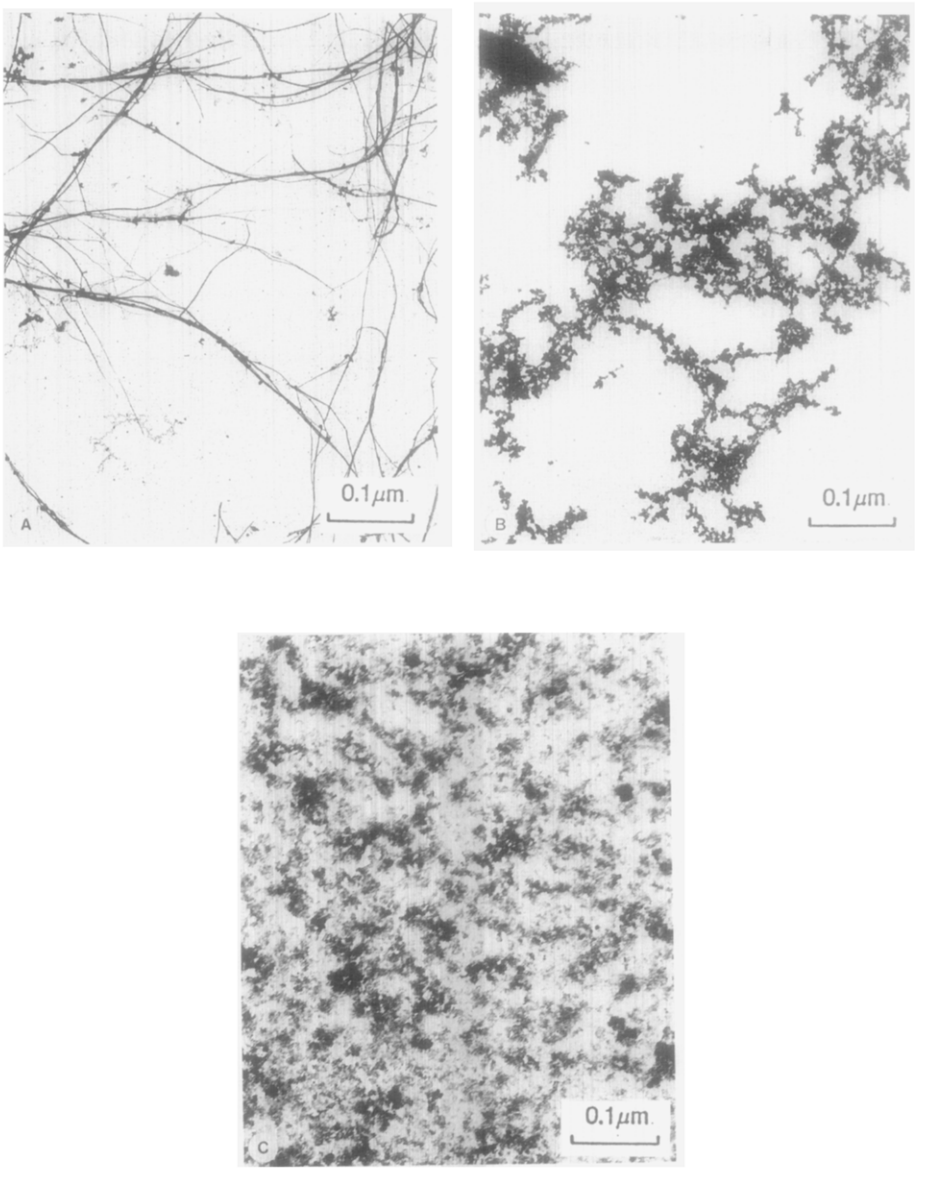

Fig. 3. Electron micrographs of negatively stained actin structures. A. Actin filaments assembled by 30 min incubation with Spermidine (1 mM). B. Actin 'paracrystals' assembled by 30 min. incubation with Serotonin (5-HT 1 mM). C. Control: untreated 'G'-actin.

toxin, cytochalasin B, which fragments and destabilizes actin filaments, displayed increased permeability, presumably through the effect of this agent on the cytoskeleton. Bentzel, et al. (1980) demonstrated that phalloidin, an agent which polymerizes and stabilizes actin filaments increased epithelial barrier function, presumably by an enhancing effect of this agent on the cytoskeleton, which may improve the intercellular junctional barrier. We have previously demonstrated that 5-HT (and other vasoactive amines) can modulate F-actin in

vitro, and barrier function in vitro and in vivo, (Welles, et al. 1985, Bottaro, et al. 1986, and Shepro, et al. 1984). Welles, et al. (1985) showed that cultured bovine aortic enothelial cells treated with 5-HT exhibited increased numbers of stress fibers per cell (81 %) over controls, with parallel increases in cell surface area. In addition, endothelial cells cultured on microcarrier beads, when treated with 5-HT impeded trypan blue-albumin diffusion by 43 % compared with controls (Bottaro, et al. 1986). In vivo, 5-HT administered intraperitoneally to severely thrombocytopenic animals, with 99 % of the platelets removed, prevented the extravasation of erythrocytes. Normal platelets avidly sequester 5-HT and possess a 5-HT pump/leak mechanism, and the released 5-HT is thought to help maintain microvascular integrity by its action on the cytoskeleton (Shepro, et al. 1984).

In this communication, data are provided to demonstrate directly 5-HT induced actin polymerization in vitro, and an association of internalized 5-HT with actin filaments in cultured bovine aortic endothelium. This direct action is particularly relevant in light of the numerous publications that report that endothelial cells avidly take-up 5-HT, and that not all endothelial cells have a membrane receptor for 5-HT, and that the amine can modulate metabolic and physiologic functions of these cells, (Shepro and D'Amore, 1982). The results presented in this article indicate that in cultured bovine aortic endothelium, that do not have a demonstrable receptor for 5-HT, 5-HT by binding directly to actin may alter its polymerization state. This in turn will affect cell shape and metabolism that are involved in maintenance and promotion of the endothelial cell barrier.

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