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Effect of arachidonic acid on cultured cerebromicrovascular endothelium: permeability, lipid peroxidation and membrane "fluidity"

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Summary. The relationship of free arachidonic acid (AA) to cellular permeability, lipid peroxidation and physical state "fluidity" of the membrane was investigated in cultured endothelial cells (EC) dissociated from cerebral microvessels of rats. The results demonstrate that AA can induce a reversible alteration of endothelial permeability to trypan blue albumin (TBA). Exposure of EC to AA increases membrane "fluidity" as measured by fluorescence anisotropy using 1,6-diphenyl-1,3,5 hexatriene as a fluorescent probe. The AA modification of EC membrane "fluidity" is not associated with changes in EC permeability. Addition of AA and H_2O_2 to the incubation medium of EC leads to persistant alteration of EC permeability which can be prevented by catalase treatment. Both AA and H_2O_2 induce a greater formation of malondialdehyde, the product of lipid peroxidation, than AA alone. These findings strongly suggest that a release of AA either from the capillary or cellular membrane of the brain under a pathological condition may alone or through a peroxidative process alter the function of blood-brain barrier.

Key words: Cerebromicrovascular endothelium – Arachidonic acid – Lipid peroxidation

Tissue damage in the diseased brain, as in other organs, has been attributed to free radical formation, release of free fatty acids from phospholipids and lipid peroxidation of the membrane [1, 5, 8–10, 12, 15, 19, 26, 27]. The same processes have also been implicated in alterations of the blood-brain barrier (BBB) and induction of brain edema [6, 16, 24]. Recent in vitro studies of cultured cerebromicrovascular endothelium lend support to this concept by demonstrating an increased trypan blue albumin (TBA) permeability of endothelial cells (EC) exposed to free arachidonic acid (AA). Therefore, the aim of our present study was twofold: (1) to evaluate the effect of lipid peroxidation on the AA-inducible alteration of EC permeability and (2) to estimate the physical state "fluidity" of the membrane, since changes in lipid composition and/or peroxidation were described to affect the membrane "fluidity" [7, 20, 25].

This report clearly demonstrates that the AA-inducible alteration of EC permeability can be reversed in the absence of marked lipid peroxidation. The results also indicate that the observed changes in the physical state of membrane after exposure to AA are not associated with an attenuated permeability of EC.

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Experimental methods

EC cultures were derived from dissociated microvessels of rat brain [22]. The current experiments were conducted on propagated cells grown to confluence either on collagen-covered dextran microcarriers (cytodex 3) or as a monolayer. The respective procedures for each type of EC cultivation have been previously described in detail [22]. The cultured endothelium displayed the characteristic properties of cerebral endothelium observed in situ and in vitro: enzymatic markers, presence of Factor VIII antigen, membrane receptors and production of prostaglandins.

Permeability of EC

Effect of AA. Confluent EC-covered microcarriers and/or cell-free beads (empty microcarriers) were washed three times with phosphate-buffered saline (PBS). Aliquots of 0.5 ml of beads were resuspended in 3.0 ml PBS containing either AA (final conc 50 or 100 μM) or AA solvent solution (0.2% ethyl alcohol) pH 7.4. The samples were incubated at 37°C for 30 min prior to testing the permeability ("uptake") of EC.

Effect of AA and H_2O_2. The same aliquots of washed EC-covered beads and/or empty microcarriers were resuspended in PBS or

PBS with AA (final conc 50 or 100 μM) and incubated for 5 min prior to the addition of H_2O_2 (final conc 5 mM) for another 25 min at 37°C. Samples incubated with H_2O_2 alone served as controls.

Effect of catalase. Catalase in concentration of 20 or 40 U/ml was added to the PBS medium containing AA and H_2O_2. Specificity of the catalase effect on permeability of EC exposed to AA and H_2O_2 was assesed by addition of superoxide dismutase [SOD (60 U/ml)] instead of catalase to the medium containing AA and H_2O_2. Samples incubated in medium containing catalase or superoxide dismutase were used as respective controls.

Reversibility. The reversibility of the effect of AA either alone or with H_2O_2 on EC permeability was investigated in PBS-washed cells (three times) after incubation with these agents and prior to addition of TBA solution. The washed cell-covered beads were resuspended in PBS buffer containing fatty acid-free albumin (1 mg/ml), 0.25% glucose and 2.5 mM Hepes at pH 7.4.

Permeability test. EC permeability was evaluated by a modified technique of Boiadjeva et al. [2] described for EC obtained from large peripheral vessels and subsequently adapted for cerebral microvascular EC [13]. In brief, the assay medium was composed of a dialyzed solution of trypan blue (TB) with fatty acid-free albumin (0.2%) in PBS and 5 mM Hepes pH 7.4. Two hundred microliters of this solution was added to the incubated samples at 37°C. Aliquots (250 μl) of supernatant were recovered at 15 s and 1, 2, 3, 5, 10, 15 and 19 min and filtered through a nylon mesh [(HC 3-70) Tetco. Inc.]. Each sample of supernatant was mixed with 250 μl H_2O. The TB concentration in the supernatant was measured using a spectrophotometer at a wavelength of 580 nm.

Lipid peroxidation in vitro and membrane "fluidity"

The procedures for testing the effect of AA with and without H_2O_2 on the monolayer of EC were the same as those described for cell-covered microcarriers.

Lipid peroxidation in vitro. After incubation with the above agents, the washed monolayers of EC were harvested by scrapping in PBS and centrifuged at 1500 *g* for 10 min. The pellet was resuspended in 1 ml cold PBS and homogenized twice to a final volume of 1.5 ml. Each homogenated sample was divided into three equal aliquots. Two samples were used for estimation of lipid peroxidation while the third sample was used for protein determination by the Biorad method [3]. Lipid peroxidation was estimated by measuring the formation of thiobarbituric-reactive material (TBAR), i.e., malondialdehyde (MDA). The thiobarbituric acid assay is a simple and commonly used method for detection of tissue lipid peroxidation in vitro [4]. Thiobarbituric acid solution (0.375%) containing 20% trichloracetic acid and 0.25 M HCl was added to each of the two samples and incubated at 100°C for 15 min. Thereafter the samples were cooled and centrifuged at 1500 *g* for 10 min. The absorbance of TBAR material was measured in a spectrophotometer at 533 nm using MDA as standard.

The concentration of MDA was calculated using an extinction coefficient of 1.56×10^{-5} M, previously confirmed by a standard curve using 100 nM – 100 μM of malonaldehyde [bis (dimethyl acetal)].

Membrane "fluidity". The harvested washed cells were resuspended in cold 0.32 M sucrose (containing 3 mM Hepes) pH 7.4, homogenized and centrifuged at 1000 *g* for 15 min. The supernatant was centrifuged at 20000 *g* using an SW 50 rotor in a Beckman Ultracentrifuge for 30 min. The pellet was resuspended in 1 ml of cold 50 mM Tris buffer, pH 7.4 and rehomogenized. The homogenate was diluted with the same buffer to a volume of 2 – 5 ml to obtain a clear solution.

Membrane "fluidity" was determined by the polarization technique [17, 21] using 1,6-diphenyl-1,3,5 hexatriene (DPH) solubilized in tetrahydrofuran (5 mM stock solution). Actually, this method provides only information about the motility of DPH in the hydrophobic lipid region of the membrane and about the overall "fluidity" of the internal membrane. Mindful of this restriction, we used the detected changes in fluorescence anisotropy of DPH as an index of membrane "fluidity".

Cell membranes were mixed 1:1 with a diluted DPH (1:1000 in 50 mM Tris HCl) and incubated at 37°C for 30 min. Temperature-dependent fluorescence polarization was determined on cooled samples exposed to a wide range of temperatures (18°C – 40°C). The fluorescence polarization of the samples was measured in a Perkin-Elmer model MPF 44B fluorescence spectrophotometer equipped with two polarized filters. The samples were excited at 360 nm and emission was measured at 435 nm.

Fluorescence anisotropy of the samples was calculated as follows:

$$r = \frac{I^{\parallel} - I^{\perp}}{I^{\parallel} - 2I^{\perp}} = \text{fluorescence anisotropy}$$

where ($I^{\parallel}$), ($I^{\perp}$) are components of fluorescence emission intensity detected through analyzers oriented parallel ($I^{\parallel}$) and perpendicular ($I^{\perp}$) to the direction of polarization of the excitation light [21].

Statistical analysis of the data were carried out by Student's *t*-test to compare the experimental values obtained for endothelial lipid peroxidation in vitro and membrane "fluidity" with their respective control groups.

Light microscopy

Unstained endothelial-covered microcarriers were stained with TB to check the viability before and after incubation. Representative samples from each experimental group were photographed under light microscope.

Scanning electron microscopy

Phosphate (0.1 M) buffered 3% glutaraldehyde (pH 7.3) solution was used for fixation of endothelial-covered microcarriers. Samples were osmicated (osmium 1%) en bloc, stained in aqueous uranyl acetate (0.5%), dehydrated in ethanol and dried in CO_2. Afterward the cultures were mounted on carbon stubs, coated by cold sputtering with gold/palladium and examined in an AMRAY 1000 A scanning electron microscope.

Materials

TB was purchased from Gibco (Grand Island, NY), DPH, MDA and tetrahydrofuran from Aldrich Chemical Co. (Metuchen, NJ) while dextran microcarriers (Cytodex 3) from Pharmacia Fine Chemicals (Uppsala, Sweden). All other chemicals were obtained from Sigma Co. (St. Louis, MO).

Results

Permeability of EC

The exposure to both AA alone or with H_2O_2 led to increased permeability of EC to TBA. The cell-covered

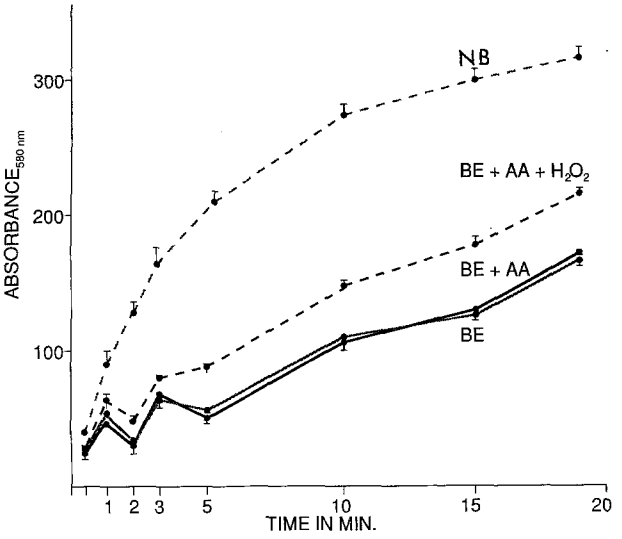

Fig. 1. Trypan blue (TB) absorption by microcarriers. *NB*: Naked beads; *BE*: beads covered by endothelial cells (EC) exposed to 0.2% ethyl alcohol; *BE + AA* = beads covered by EC exposed to arachidonic acid (AA) (final conc 100 μM); BE + AA + H_2O_2 = beads covered by EC exposed to AA (final conc 100 μM) and H_2O_2 (final conc 5 mM) in the incubating medium for 30 min at 37° C and rinsed with phosphate-buffered saline (PBS) prior to testing the permeability. Each point represents the mean ± SE values of 21–25 assays. Note: TB absorption of EC-covered beads rinsed after exposure to AA was not significantly different from the controls (rinsed BE)

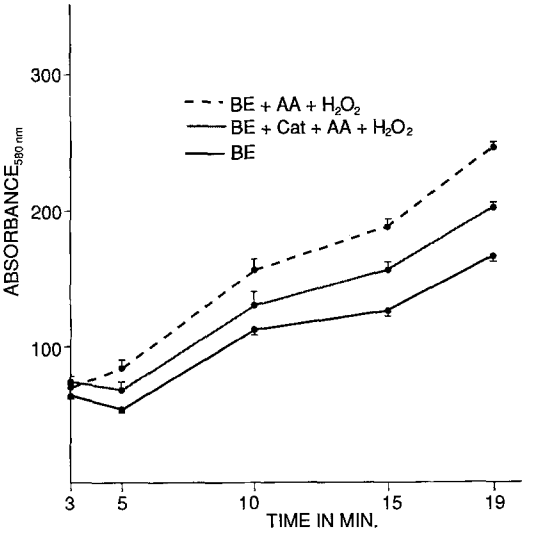

Fig. 2. TB absorption by EC-covered microcarriers (BE) incubated with AA and H_2O_2 in the presence or absence of catalase [Cat (40 U/ml)]; BE = controls exposed to AA solvent solution (0.2% ethyl alcohol). Each point represents the mean ± SE values of eight assays. Note: Catalase diminished the effect of AA + H_2O_2 on the endothelial permeability to TB

microcarriers incubated with either solvent solution (alcohol) of AA or H_2O_2 alone had no effect on EC permeability. The data for the AA-induced increase in EC "uptake" of TBA (shown in Fig. 1), were similar

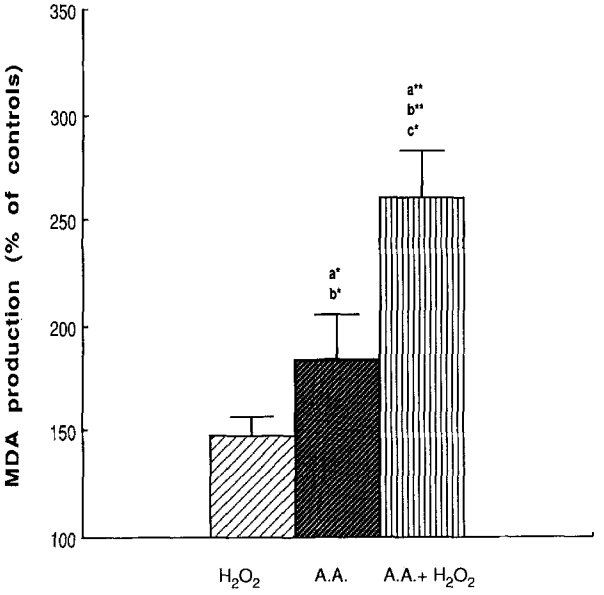

Fig. 3. Lipid peroxidation in vitro. The EC were incubated with either H_2O_2 or AA alone (final conc 5 mM and 100 μM, respectively) or with AA and H_2O_2 for 30 min at 37° C. The production of malondialdehyde (MDA) was estimated by thiobarbituric acid assay. The data are presented as percent of controls (100%). The control EC formed 2.55 nmol of MDA/mg protein. Each column represents the mean ± SE of three to four separate experiments. *: $p < 0.02$; **: $p < 0.006$; *a*: significant difference from control values; *b*: significant difference from H_2O_2 values; *c*: significant difference between AA + H_2O_2 and AA values

to previous observations, although the cells were incubated with albumin [14]. The presence of catalase (40 U/ml, but not 20 U/ml) in the incubating medium containing AA and H_2O_2 significantly diminished the alteration of EC permeability to TBA (Fig. 2). In contrast SOD failed to decrease the AA and H_2O_2 changes in EC permeability (data not shown).

The permeability of rinsed EC-covered beads after incubation with AA, contrary to those rinsed following exposure to AA with H_2O_2, was not significantly different from that of controls (Fig. 1). An increase in permeability of EC to TBA was also seen in H_2O_2-treated rinsed cells which were previously incubated with AA (data not shown).

Lipid peroxidation in vitro

A stimulation formated of TBAR material was seen in EC after AA alone and following AA with H_2O_2 treatment. As may be seen from Fig. 3, the TBAR production (expressed in percent of controls) in AA- and H_2O_2-treated cells was 2.6-fold greater than that of control and about 1.4-fold greater than in EC incubated with AA alone. A similar effect was seen in AA- and H_2O_2-treated cells rinsed after exposure to AA (data not shown). EC exposed to H_2O_2 alone showed

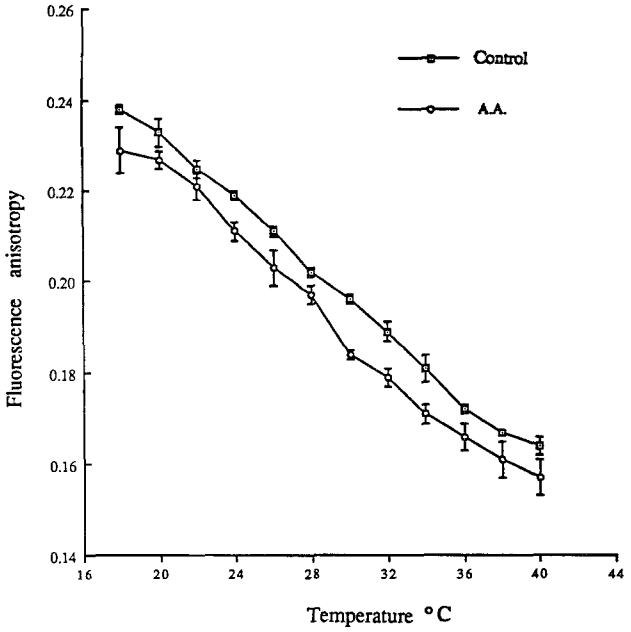

Fig. 4. Effect of AA on the physical state "fluidity" of EC membranes. The fluorescence anisotropy of 1,6-diphenyl-1,3,5 hexatriene (DPH) is significantly decreased in EC membranes treated with AA as compared to the fluorescence anisotropy of DPH in controls ($p < 0.001$ at 30° C; $p < 0.009$ at 32° C and $p < 0.03$ at 34° C). The data represent mean ± SE values of five to six separate experiments

a non-significant increase in the production of TBAR material.

Membrane "fluidity"

A temperature-dependent difference in fluorescence anisotropy was seen between the EC membranes exposed to AA and controls (Fig. 4). The fluorescence anisotropy of DPH was most significantly different at 30° C and 32° C in AA-treated cells ($r = 0.184 \pm 0.001$ and 0.179 ± 0.002, respectively) as compared with respective controls ($r = 0.196 \pm 0.001$ and 0.189 ± 0.002). The membranes obtained from EC incubated with either H_2O_2 alone or with AA and H_2O_2 showed no significant increase in fluorescence anisotropy to DPH (data not shown).

Light microscopy

The appearance of EC-covered microcarriers treated either with AA alone or with AA and H_2O_2 was not different from those of respective controls (Fig. 5a–c).

Scanning electron microscopy

Most of the microcarriers were completely covered by EC (Figs. 6a, c, d; 7a–d), while a few beads were only

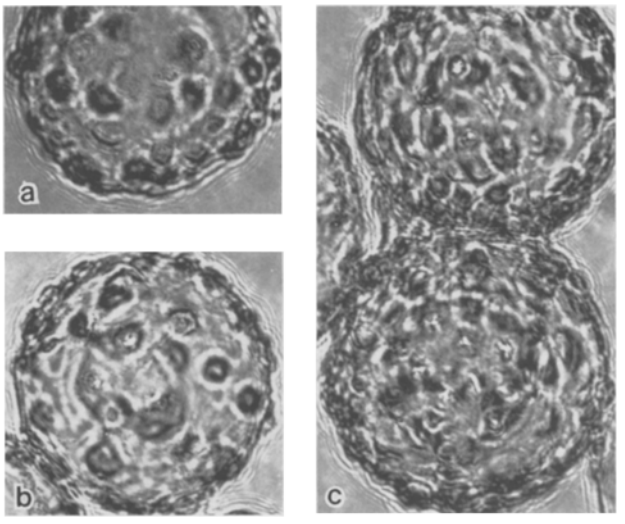

Fig. 5a–c. Light microscopy of unstained microcarriers covered by EC (BE). **a** BE rinsed with PBS after exposure to H_2O_2 for 30 min at 37° C; **b** BE rinsed with PBS after exposure to AA and H_2O_2, and **c** BE rinsed with PBS after exposure to AA for 30 min at 37° C. Note: The endothelial covered beads display a similar appearance irrespective of treatment. **a–c** ×400

partially covered with EC as exemplified in Fig. 6b. Moreover some of the beads appeared to contain more than one layer of cells (Figs. 6a, c, d; 7a–d). Careful examination of the samples revealed little difference in appearance between variously treated EC-covered beads. However, some of the EC after exposure to AA and H_2O_2 showed a fibrillary net-like surface and cellular retraction of the superficial layer as exemplified Fig. 7d. This pattern is distinctly different from other cells which show diffusely scattered pinpoint surface projections irrespective of the type of treatment (Fig. 7a–c).

Discussion

The results of this study indicate that the endothelial exposure to either AA alone or to AA with H_2O_2 can alter the permeability of these cells to TBA. Moreover the data clearly show that the AA-inducible change in EC permeability to TBA is reversible, since the TBA permeability of cells rinsed after exposure to AA was not different from that of controls. On the other hand, the increased endothelial permeability to TBA after exposure to AA and H_2O_2 could not be reversed by the same treatment. The changes induced by AA and H_2O_2 in EC permeability can be significantly prevented by the presence of catalase in the incubating medium. This observation is not surprising since catalase, a cytoplasmic heme-enzyme, catalyzes the divalent reduction of H_2O_2 to water.

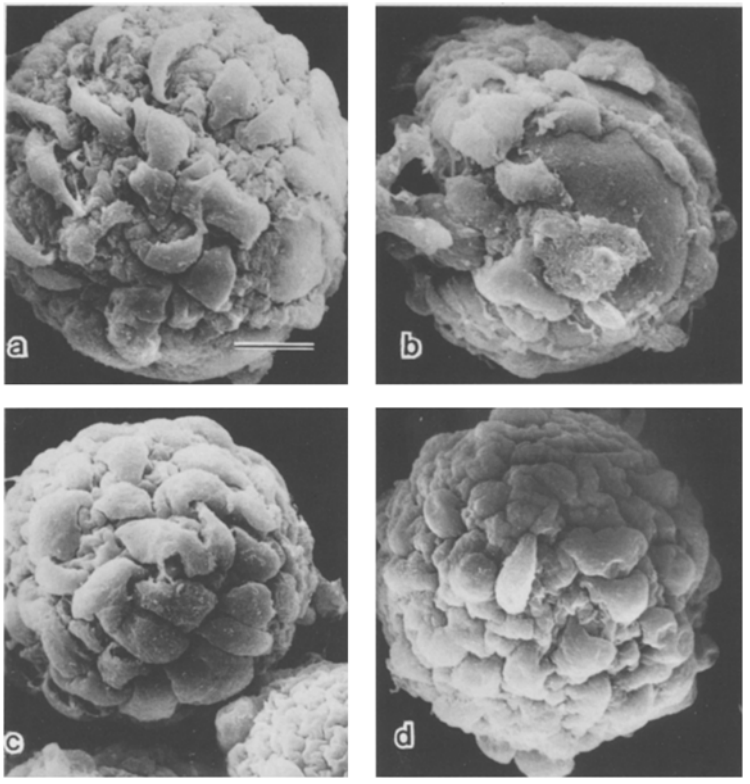

Fig. 6 a – d. Scanning electron microscopy of microcarriers covered by EC (BE). **a** Control BE rinsed with PBS after exposure to 0.2% ethyl alcohol; **b** BE rinsed with PBS after incubation with AA + H_2O_2; **c** BE rinsed with PBS after incubation with AA; **d** BE rinsed with PBS after incubation with H_2O_2. Note: There is little difference between the appearance of the differently treated BE. **b** A microcarrier partly covered by endothelial cells. Beads partially covered by endothelial cells were seen in each group prior to the individual treatment. **a – d** × 500

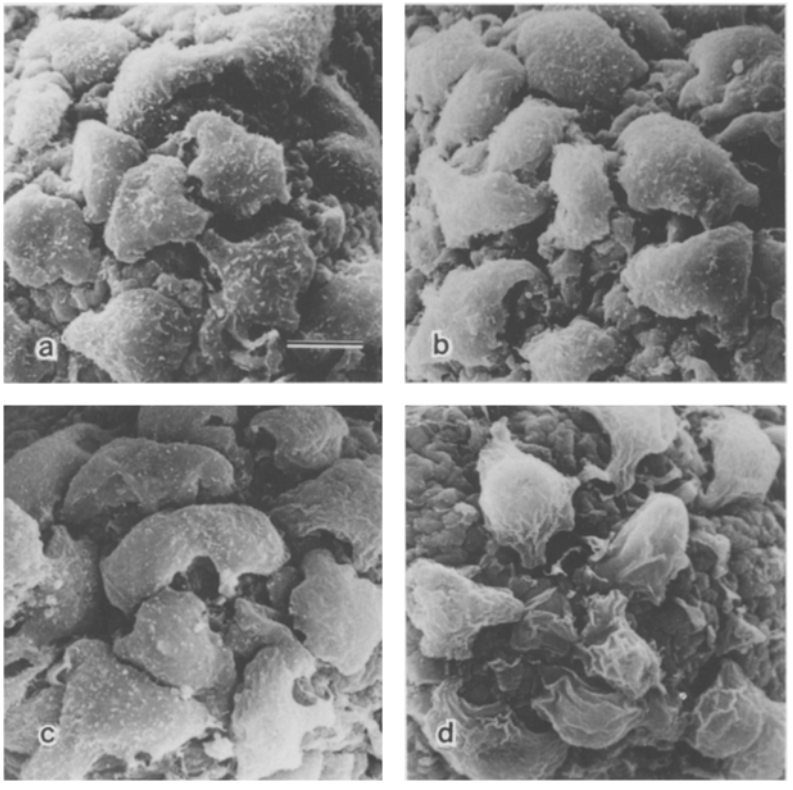

Fig. 7 a – d. Scanning electron microscopy of microcarriers covered by EC (BE). **a** BE treated with H_2O_2; **b** BE treated with AA, **c, d** BE treated with AA and H_2O_2. All the EC-covered beads were rinsed with PBS. Note: The cellular surface contains pin-point projections which are seen in each representative group of cells. **d** A fibrillary net-like cellular surface with apparent retraction (**c**) which is not seen in other cells from the same experimental group. **a – d** × 1000

AA is one of many agents which was shown to alter cerebral endothelial permeability in vivo [24] and in vitro [14] studies. Other free fatty acids were also shown to increase the passage of macromolecules across the cultured endothelium derived from peripheral vessels [11]. However, a reversible AA change in the endothelial permeability has not been previously observed.

Lately, we have shown that the AA-induced alteration of EC permeability is not mediated by cyclooxygenase, since neither indomethacin nor ibuprofen (inhibitors of cyclooxygenase) pretreatment prevented this change [14]. On the contrary, high concentration of indomethacin increased the endothelial "uptake" of TBA which was preventable by dexamethasone. It was, therefore, suggested that an excessive presence of either exogenous or endogenous free AA (not converted to cyclooxygenase products and/or not bound to albumin) may alter EC permeability.

Even though, the AA-inducible change in EC permeability is easily reversible, our studies clearly show that the exposure of EC to free AA is not completely inconsequential, since an alteration of fluorescence anisotropy and lipid peroxidation was detectable in the washed endothelial cell membranes.

It was suggested that AA as an amphipathic molecule could form miscelles which bind either to hydrophilic or hydrophobic substances which may alter membrane integrity [8, 24]. Free AA is also capable of intercalating into plasma membrane, where it remains an unsaturated fatty acid for a short period of time. The demonstrated decrease in fluorescence anisotropy found in the membranes of rinsed EC after EC incubation with AA is consistent with these concepts. Our findings are also in agreement with observations that a rise of unsaturated fatty acid in the membrane leads to a decrease in DPH fluorescence anisotropy and an increase in the "fluidity" of membrane [25]. However, the functional significance of AA-inducible alternation of membrane "fluidity" has to be clarified since a change in membrane "fluidity" may modify its function [18, 23].

The detected increased production of TBAR in rinsed and unrinsed EC after exposure to AA indicate that the free AA can serve as a substrate for lipid peroxidation which is greatly augmented by the presence of H_2O_2 in the environment (incubation medium). Moreover, the AA-inducible increase in the formation of TBAR strongly suggests that the EC are capable of generating reactive oxygen molecules needed for such a process [8].

These findings indicate that a transient EC exposure to free AA may alter the physical state of the membranes and lead to some increase in lipid peroxidation without causing persistant cellular damage. The H_2O_2-induced lipid peroxidation of EC without change in permeability suggest that the EC content of peroxidative substrate is insufficient for the reaction and/or that the EC possesses a mechanism which can inactivate H_2O_2. In contrast EC injury can be induced by exogenous treatment with AA and H_2O_2 as indicated by irreversible alteration of EC permeability, marked increase in lipid peroxidation and morphological changes in some cells. Thus, these results strongly suggest that a persistent endothelial damage does not occur unless an excess of AA (possibly also other free fatty acids) and reactive oxygen exist in the EC environment.

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