Neurochemical Research, Vol. 20, No. 12, 1995, pp. 1417–1427

Free Radical-Induced Endothelial Membrane Dysfunction at the Site of Blood-Brain Barrier: Relationship Between Lipid Peroxidation, Na,K-ATPase Activity, and ^{51}Cr Release

Danica B. Stanimirovic,[1,2] Josée Wong,[1] Rita Ball,[1] and Jon P. Durkin[1]

(Accepted August 28, 1995)

Na,K-ATPase activity, membrane lipid peroxidation (TBARM), and membrane 'leakiness' for small molecules were examined in rat cerebromicrovascular endothelial cells (RCEC) following exposure to hydrogen peroxide and xanthine/xanthine oxidase. Whereas short-term (15–30 min) exposure to either oxidant decreased ouabain-sensitive ^{86}Rb uptake and increased TBARM in a concentration-dependent fashion, significant release of ^{51}Cr (30–40%) from cells was observed only after one hour exposure to the oxidants. By comparison, much longer exposure times (i.e., 4 hours) were needed to induce significant lactate dehydrogenase release from oxidant-treated cells. The oxidant-evoked decrease in Na,K-ATPase activity and increases in TBARM and RCEC 'permeability' were abolished in the presence of the steroid antioxidants U-74500A and U-74389G (5–20 μM). Reduced glutathione (4 mM) partially attenuated oxidant-induced changes, whereas ascorbic acid (2 mM) and the disulfide bond-protecting agent, dithiothreitol (1 mM), were ineffective. These results suggest that the oxidant-induced loss of Na,K-ATPase activity in RCEC results primarily from changes in membrane lipids, and implicate both the inhibition of Na,K-ATPase and membrane lipid peroxidation in the mechanism responsible for the delayed free radical-induced increase in RCEC membrane 'permeability'.

KEY WORDS: Blood-brain barrier; endothelium; free radicals; Na,K-ATPase; lipid peroxidation; lazaroids.

INTRODUCTION

The unique properties of cerebral capillary endothelial cells (i.e., tight junctions, limited transcellular transport) selectively restrict the passage of water and nutrients between blood and brain, thus forming the blood-brain barrier (BBB) (1). Disruption of the BBB following cerebral ischemia/reperfusion results in the development of vasogenic brain edema (2). Free radicals are considered to be principal mediators of both reperfusion brain injury and vasogenic brain edema (3,4). During ischemia/reperfusion, free radicals can be released from ischemic brain tissue (3,4), infiltrating leukocytes (5–6), and endothelial cells themselves. Cerebral endothelial cells generate free radicals by a variety of mechanisms including activation of the arachidonic acid cascade (7–8) and/or the ischemia-induced conversion of xanthine dehydrogenase to xanthine oxidase (9–10).

Uncontrolled exposure of the cerebral microvascular bed to free radicals can lead to profound functional and/or structural changes in microvessel vasoreactivity and BBB permeability. For example, the generation of reactive oxygen intermediates on the brain surface by topical application of either arachidonic acid or xanthine/xanthine oxidase has been shown to alter the reac-

[1] Cellular Neurobiology Group, Institute for Biological Sciences, National Research Council of Canada, Ottawa, Canada K1A OR6.

[2] Address reprint requests to: Dr. Danica Stanimirovic, Cellular Neurobiology Group, Institute for Biological Sciences, National Research Council of Canada Montreal Road Campus, Bldg. M-54, Ottawa, ONT, Canada K1A OR6. Phone: (613)-993-3730 Fax: (613)-941-4475 E-mail: Danica@biologym54.lan.nrc.ca

0364-3190/95/1200-1417$07.50/0

tivity of cerebral vessels to physiological regulators (11), cause prolonged vasoconstriction (12), and to decrease oxygen consumption in vessel walls (13). More recently, it has been demonstrated that reactive oxygen species generated in peripheral endothelial cells can serve as intracellular messengers which increase both the expression of endothelial adhesion molecules and leukocyte recruitment (14). Under more severe conditions the exposure of the cerebral vasculature to oxidants has been shown to result in increased BBB permeability for macromolecules both in vitro (15) and in vivo (16).

Alterations in membrane function and/or integrity are a common outcome of the exposure of cells to free radicals (17–18). These alterations can affect the permeability of membranes for ions, the presentation and/or function of membrane proteins, and processes coupled to cell-cell communication (19). Lipid-dependent, membrane-attached enzymes rich in thiol groups, such as Na,K-ATPase (20–21), are particularly vulnerable targets for free radicals in the brain. It has been reported that cerebral microvessels are highly enriched in Na,K-ATPase, in terms of both cellular levels (22) and activity (23), relative to other brain compartments. Na,K-ATPase is distributed predominantly on the abluminal side of cerebral endothelium (24), and is considered to be a crucial enzyme in the regulation of water homeostasis and ion exchange at the site of BBB.

In this report, we demonstrate that free radicals cause a loss of in situ Na,K-ATPase activity in cerebral endothelial cells. This loss in sodium pump activity appears to be effected by membrane lipid peroxidation and is implicated in the free radical-induced increase in endothelial membrane permeability for small molecules.

EXPERIMENTAL PROCEDURES

Materials. All tissue culture media and supplements were obtained from Gibco BRL (Gaithersburg, MD). Rat tail collagen was purchased from Collaborative Biomedical Products (Bedford, MA). ^{86}Rb-chloride was obtained from New England Nuclear (DuPont Canada Inc., Mississauga, Ontario), and ^{51}Cr-sodium monochromate from ICN Pharmaceuticals (Costa Mesa, CA). All enzymes used in the study were purchased from Sigma Chemical Co. (St. Louis, MO). Lazaroids (U-74389G, U-74500A) were kindly provided by The UpJohn Company (Kalamazoo, MI). Primary and secondary antibodies used for immunocytochemistry were obtained from Accurate Chemical and Scientific Corp. (Westbury, NY).

Endothelial Cell Culture. Cultures of rat cerebromicrovascular endothelial cells (RCEC) were obtained by a modification of the method of Spatz et al., 1980 (25). Briefly, the brains from forty 2–4 days old Sprague-Dawley rats were removed under sterile conditions, freed from meninges and homogenized in a glass-teflon homogenizer (5–6 strokes). Microvessels were separated on a discontinuous sucrose gradient (26), washed thoroughly in sterile Ca^{2+} and Mg^{2+}-free Hank's balanced salt solution containing antibiotic/antimycotic (100 units/ml penicilin G, 100 μg/ml streptomycin sulfate, 0.25 μg/ml amphotericin B), and then passed sequentially through 112 μm and 20 μm Nitex nylon meshes. Microvessels retained on the 20 μm mesh were dissociated by a brief (10 min) exposure to type I collagenase (1 mg/ml, 37°C). The dissociated microvessel fragments were seeded in rat-tail collagen coated tissue culture flasks in medium 199 (containing Earle's salts, 25 mM Hepes, 4.35 g/l sodium bicarbonate and 3 mM L-glutamine) (M199) supplemented with 1% basal media Eagle's (BME) amino acids, 1% BME vitamins, antibiotic/antimycotic mixture (100 units/ml penicilin G, 100 μg/ml streptomycin sulfate, 0.25 μg/ml amphotericin B), 1% glucose, 0.05% peptone, and 20% fetal bovine serum (FBS), and grown to confluence in a humidified atmosphere of 5%CO_2/air at 37°C. Numerous colonies composed of migrating endothelial cells were observed between day 3–6. Individual colonies of endothelial cells were isolated using cloning rings (BELLCO Glass, Inc., Vineland, NJ) and 2–3 of these cloned colonies were pooled and propagated further. Cultures were routinely transferred at a 1:2 ratio and used for experiments up to the 15th passage.

^{86}Rb Uptake. ^{86}Rb uptake experiments were performed on confluent RCEC cultures grown in 24 well tissue culture plates as described by Dong et al. (27). Sodium pump (Na,K-ATPase activity) was defined as ouabain-sensitive ^{86}Rb uptake, whereas Na-K-2Cl cotransporter activity was measured as the component of total ^{86}Rb uptake sensitive to bumetanide. Briefly, assays were performed at 37°C in Krebs solution containing (in mM) 119 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 25 $NaHCO_3$, 2.5 $CaCl_2$, 1 $MgCl_2$, and 5.5 glucose at pH 7.4. The Krebs solution was saturated with 95% O_2/5% CO_2 prior to use. At the beginning of each experiment, the growth medium was removed and the cells washed and then preincubated in Krebs solution with or without ouabain and/or bumetanide for 30 min. Cultures were subsequently incubated with various oxidant [hydrogen peroxide (H_2O_2) and xanthine/xanthine oxidase (X/XO)]/antioxidant combinations for the specified times. All antioxidants used were dissolved in Hank's balanced salt solution (PBS) except for the 21-aminosteroids, U-74500A and U-74389G, which were solubilized in PBS containing 3% fatty acid-free bovine serum albumin (BSA). Antioxidants were added to the cells 30 min prior to the addition of oxidants. Control cultures were incubated with the corresponding drug solvents. 1 μCi/ml ^{86}Rb was then added to the cultures for 15 min at which time the uptake of radiolabel was stopped by rapidly washing the cells with ice-cold Krebs solution. Cells were subsequently lysed in 0.1% Triton X-100, small aliquots were removed from each well for the determination of protein content by the method of Lowry et al. (28), and the ^{86}Rb content of the lysates determined by scintillation counting.

Reduction of Nitro-Blue Tetrazolium. The reduction of nitroblue tetrazolium (NBT) to nitroblue formazan (NBF) was used as a measure of superoxide ($\cdot O_2^-$) radical production in RCEC exposed to either H_2O_2 or xanthine/xanthine oxidase (29). For the detection of NBF, RCEC were preincubated in PBS solution containing 1 mg/ml NBT for 15 min before being exposed to the oxidants for various periods. The intracellular density and distribution of NBF, the insoluble product of NBT reduction, was initially observed microscopically, and was subsequently quantified by scraping the cells from the dishes and extracting the intracellular NBF by dimethyl-formamide as previously described (30). The dimethyl formamide extracts were boiled for 10 min, centrifuged, and the concentration of NBF in the supernatants determined spectrophotometrically at 550 nm using a standard NBF curve. Both basal and oxidant-induced NBF formation was completely inhibited by superoxide dismutase (SOD), indicating that the superoxide anion was principally responsible for the intracellular reduction of NBT.

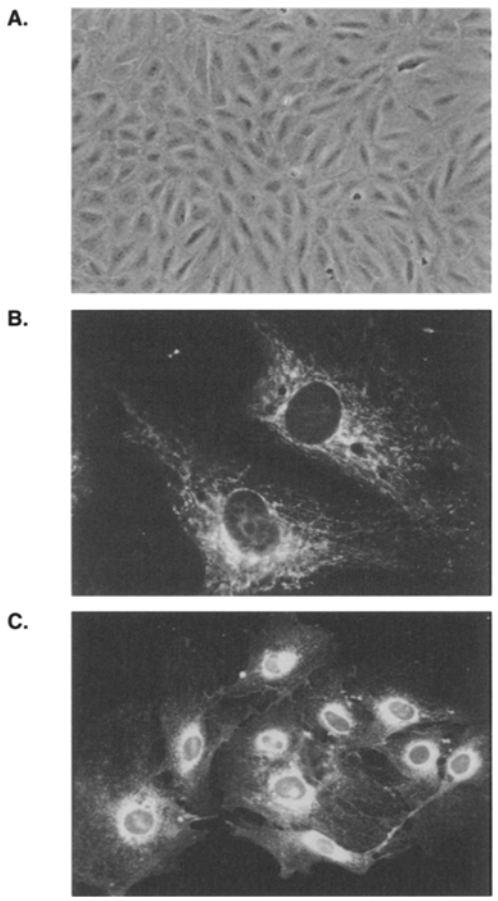

Fig. 1. A) Phase contrast micrograph (magnification × 40) of rat cerebromicrovascular endothelial cells (RCEC) in culture demonstrating a typical polygonal 'cobblestone' appearance. B) Fluorescence microscopy (magnification × 100) of immunocytochemical staining of RCEC for Factor VIII-related antigen. Cells display an intense perinuclear and cytoplasmic fibrillary type fluorescence. C) Binding of the rhodamine-labeled lectin concanavalin A to RCEC showing an intense perinuclear and cytoplasmic staining.

Lipid Peroxidation. Lipid peroxidation of RCEC membranes was estimated by the formation of thiobarbituric acid-reactive material (TBARM) using a modification of a previously described method (19,31). Briefly, cells were grown to confluence in 60 mm Petri dishes, washed, and exposed to oxidants in PBS for the indicated times. The reaction was stopped by washing, and the cells scraped from dishes and lysed in ice-cold hypotonic medium (1 mM $NaHCO_3$, 5 mM $MgCl_2$, and 100 μM phenylmethylsulphonyl fluoride-PMSF). Nuclei and unlysed cells were removed by centrifugation at 600 *g* for 5 min, and membranes in the post-nuclear supernatant were subsequently sedimented at 100,000 *g* for 10 min. The membranes were preincubated with cold TBA reagent (15% trichloroacetic acid, 0.1 M HCl, 0.75% thiobarbituric acid) for 30 min at 37°C and then heated at 95°C for 15 min in the presence of 50 μM desferoxamine to prevent heat-initiated iron-dependent peroxidation. Samples were cooled, centrifuged at 1,500 *g* for 15 min, and the concentrations of TBARM in the supernatants determined spectrophotometrically at 533 nm. Quantification of TBARM was based upon a molar extinction coefficient of 1.56×10^5 obtained from a standard curve of malondialdehyde bis(diethylacetal) generated in the concentration range of 0.1–100 μM.

^{51}Cr Release. Confluent RCEC, grown in 24 well tissue culture plates, were prelabeled with 0.3 μCi/well ^{51}Cr-sodium chromate overnight at 37°C in M199 containing 1% serum. Cells were then washed in PBS, preincubated for 1 hour in M199, and the medium then replaced with fresh oxidant-containing M199 medium. At the indicated times, the levels of ^{51}Cr released into the medium and that remaining in 0.1% Triton X-100-extracted cells was determined by scintillation counting. Labeling efficiency for ^{51}Cr was 15–20%. The release of ^{51}Cr into the medium was expressed as the percent of total activity [(supernatant × 100)/(supernatant + cells)]. In some experiments, lactate dehydrogenase (LDH) activity released from cells into the medium was determined, in parallel with ^{51}Cr release, using a Sigma LDH assay kit (No 340-LD).

Statistical Analysis. The indicated functional and biochemical parameters and cell viability were measured and compared in sister cultures. Each type of experiment was performed in triplicate or quadruplicate dishes and repeated in cells obtained from three different isolations. The results were statistically evaluated using one way analysis of variance (ANOVA) and Fisher's protected least significant difference test to compare the means. The mean values for Na,K-ATPase activity, lipid peroxidation, and ^{51}Cr release were used in linear regression analysis. All statistical tests were done using computer software SSPS Ver 6.01 for personal computers.

RESULTS

Characterization of RCECs. Both primary and propagated (up to 15th passage) RCEC cultures expressed several key characteristics of the capillary endothelium in situ including close membrane contact and polygonal ''cobblestone'' appearance (Fig. 1A). The cells stained positively for Factor VIII-related antigen (Fig. 1B) and avidly bound the fluorescently labeled lectin concanavalin A (Fig. 1C). Moreover, the RCEC cultures exibited high levels of alkaline phosphatase and γ-glutamyl transpeptidase activity, two specific markers for cerebral endothelium (data not shown). The purity of the cell cultures was estimated to be >95% as determined by the extent of Factor VIII-related antigen staining and the lack of immunocytochemical staining for smooth muscle cell actin and tropomyosin, and the astrocyte-specific marker, glial fibrillary acidic protein (GFAP) (data not shown).

Na,K-ATPase and Na-K-2Cl Cotransporter Activities in RCEC. ^{86}Rb was used as a tracer to study potassium transport into RCEC since both ions have been shown to be transported into cells in an equivalent manner (32). In these experiments, sodium pump (Na,K-ATPase) activity was defined as the portion of total ^{86}Rb uptake that could be inhibited by ouabain. The addition of ouabain to RCEC cultures resulted in a concentration dependent inhibition of total ^{86}Rb uptake (Fig. 2A) with maximal inhibition, about 50% of the total ^{86}Rb uptake,

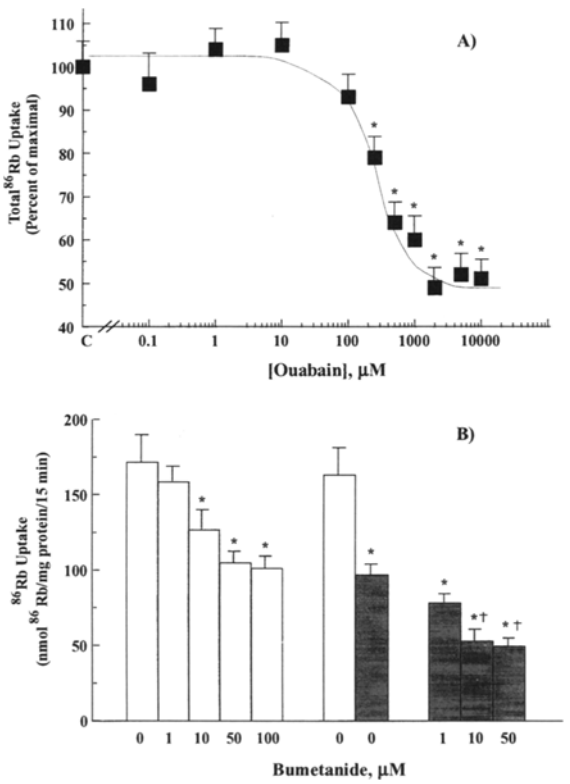

Fig. 2. Effects of ouabain and bumetanide on ^{86}Rb uptake into RCEC. A) Cells were preincubated with the indicated concentrations of ouabain for 30 min in Krebs solution, before the addition of 1 µCi/ml ^{86}Rb. B). Indicated concentrations of bumetanide were added to the cells alone (□) or together with 2 mM ouabain (▩) 30 min before the addition of 1 µCi/ml ^{86}Rb to cell cultures. The amount of ^{86}Rb uptake into the cells was measured over 15 min as described in the Experimental Procedures. The values represent the means ± S.E.M. of six replicate dishes and are representative of the results of 3 separate experiments. *-Indicates significant difference ($P < 0.01$, ANOVA) as compared to the control value(s); †-Indicates significant difference ($P < 0.01$, ANOVA) as compared to ouabain alone.

occurring at 1 mM ouabain. Consequently, 2 mM ouabain was used in all subsequent experiments to determine Na,K-ATPase activity in RCEC cells. The ouabain-sensitive ^{86}Rb uptake observed in RCEC (i.e., 50%) was considerably higher than the 20–30% typically reported in other cells types (27). Thus, it would appear that high sodium pump activity is a characteristic feature of RCEC, a conclusion supported by the previous finding that isolated cerebral microvessels are highly enriched in Na,K-ATPase relative to brain tissue at large (22).

The loop-diuretic bumetanide was used to determine the contribution of the Na-K-2Cl co-transporter to the total ^{86}Rb uptake effected in RCEC cells. This co-transporter is driven by a Na,K-ATPase-generated sodium gradient and has been shown in several 'barrier' cell types other than RCEC to play an important role in transcellular solute transport and cell volume regulation (27, 32). Bumetanide was found to inhibit ^{86}Rb uptake in RCEC in a concentration-dependent manner to a maximum of 40% of the total cellular ^{86}Rb accumulation (Fig. 2B). Bumetanide was also observed to inhibit ouabain-insensitive ^{86}Rb-uptake by 40% (Fig. 2B). Thus, the relative contributions of Na,K-ATPase and the Na-K-2Cl cotransporter to potassium influx in RCEC were about 50% and 40%, respectively, leaving other routes of entry (i.e., ouabain-insensitive and bumetanide-resistant) to account for the remaining 10% of total ^{86}Rb uptake.

'Oxidative Stress' in RCEC. In order to generate 'oxidative stress' in RCEC, cultures were exposed to either hydrogen peroxide (H_2O_2) or xanthine/xanthine oxidase (X/XO). The intracellular reduction of nitro-blue tetrazolium (NBT) to the insoluble product nitro-blue formazan (NBF), was used as a measure of superoxide radical formation induced by either of these oxidants (29). Both H_2O_2 and X/XO induced a 4.5–5.0-fold increase in intracellular NBT reduction within 60 min of their addition to the cells (Fig. 3B & C). A predominantly perinuclear accumulation of insoluble formazan was observed in cells exposed to 1 mM H_2O_2, whereas an intense and diffuse pattern of formazan precipitation, accompanied by cell shrinkage, was observed in cells treated with 100 µM/0.1u X/XO (Fig. 3B & C). The increases in formazan accumulation by H_2O_2 and X/XO were completely ablated by the presence of 200 u/ml catalase or 250 u/ml SOD, respectively (Fig. 3B & C). The data in Figure 3C also indicates that at submaximal concentrations of SOD (100 u/ml), 200 units of catalase caused a further reduction in X/XO-induced formazan accumulation suggesting that excess H_2O_2 formed during superoxide anion dismutation by SOD contributed to formazan formation.

Effects of Oxidants on Ouabain-Sensitive ^{86}Rb Uptake in RCEC. The effect of free radicals on in situ Na,K-ATPase activity was examined by measuring the impact of H_2O_2 or X/XO on ouabain-sensitive ^{86}Rb uptake in RCEC. A brief (15 min) exposure of RCEC to H_2O_2 caused a concentration-dependent decrease in Na,K-ATPase activity with half-maximal inhibition occurring at 1 mM H_2O_2 (Fig. 4A). Catalase completely reversed the H_2O_2-induced inhibition of Na,K-ATPase activity in RCEC (Fig. 4A). Similarly, the addition of increasing concentrations of xanthine oxidase in the presence of 100 µM xanthine caused a significant concentration-dependent inhibition of Na,K-ATPase activity in RCEC (Fig. 4B), which was completely reversed in the presence of exogenous SOD (Fig. 4B). These data

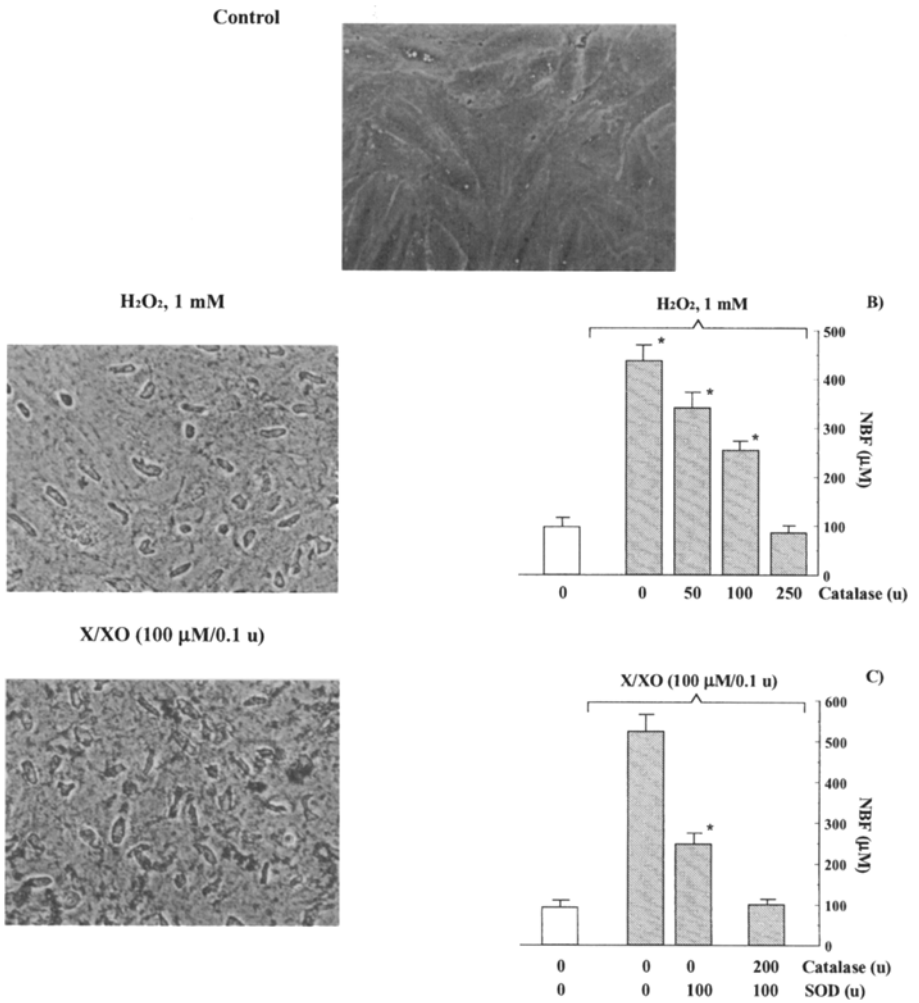

Fig. 3. Effects of H_2O_2 and X/XO on intracellular nitro-blue tetrazolium (NBT) reduction in RCEC. A) Phase contrast micrograph (× 40) of nitroblue formazan (NBF) precipitates in control RBEC culture after 60 min of incubation with NBT-containing solution. B) Phase contrast micrograph (× 100) of NBF precipitates and the amounts of NBF formed in control RCEC (□) or RCEC exposed to 1 mM H_2O_2 (▨) for 60 min in the absence or presence of 200 u/ml catalase. C) Phase contrast micrograph (× 40) and the amounts of NBF formed in RCEC exposed to 100 μM/0.1u X/XO (▨) in the absence or presence of the indicated concentrations of catalase and/or SOD. The values represent the means ± S.E.M. of four replicate dishes and are representative of the results of 3 separate experiments. *-indicates significant difference ($P < 0.01$, ANOVA) as compared to control levels.

indicate that H_2O_2 and X/XO had a profound inhibitory effect on membrane-associated Na,K-ATPase activity at concentrations shown to generate superoxide anion and other NBT-reducing reactive intermediates.

Effects of Oxidants on Membrane Lipid Peroxidation in RCEC. The extent of lipid peroxidation in control and oxidant-treated RCEC was estimated from the ability of oxidants to generate thiobarbituric acid-reactive material (TBARM) in RCEC membranes in situ. The exposure of RCEC to either H_2O_2 or X/XO resulted in a time- and concentration-dependent increase in TBARM accumulation in RCEC membranes (Fig. 5A & B). The fact that catalase and SOD effectively inhibited H_2O_2- and X/XO-induced membrane lipid peroxidation, respectively, when added to cells in situ (Fig. 5A & B) indicated that the increase in TBARM accumulation observed in RCEC membranes occurred in vivo and was not an artifact of the in vitro TBARM assay.

Effects of Oxidants on ^{51}Cr and LDH Release from RCEC. In order to determine the extent to which free radicals affected RCEC viability and membrane permeability, ^{51}Cr release and lactate dehydrogenase (LDH) release measurements were made. It has been demonstrated that the release of ^{51}Cr from ^{51}Cr-loaded periph-

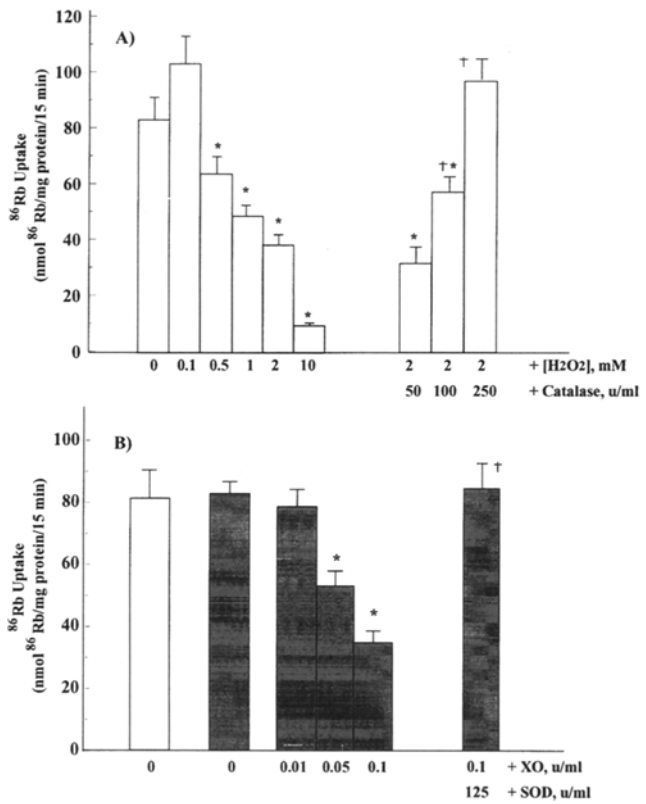

Fig. 4. Effects of H_2O_2 and X/XO on the Na,K -ATPase activity in RCEC. Ouabain-sensitive ^{86}Rb uptake into the cells was measured during the last 15 min of a 30 min exposure to oxidants as described in the Experimental Procedures. A) Indicated concentrations of catalase were added to the cells 30 min before the addition of H_2O_2. B) Cells were supplemented with either buffer (□) or 100 μM xanthine (▩) before the addition of the indicated concentrations of XO. SOD was added to the cells 30 min before the addition of XO. The values represent the means ± S.E.M. of six replicate dishes and are representative of the results of 3 separate experiments. *-indicates significant difference ($P < 0.01$, ANOVA) as compared to the control value(s); †-indicates significant difference ($P < 0.01$, ANOVA) as compared to oxidants (H_2O_2 or X/XO) alone.

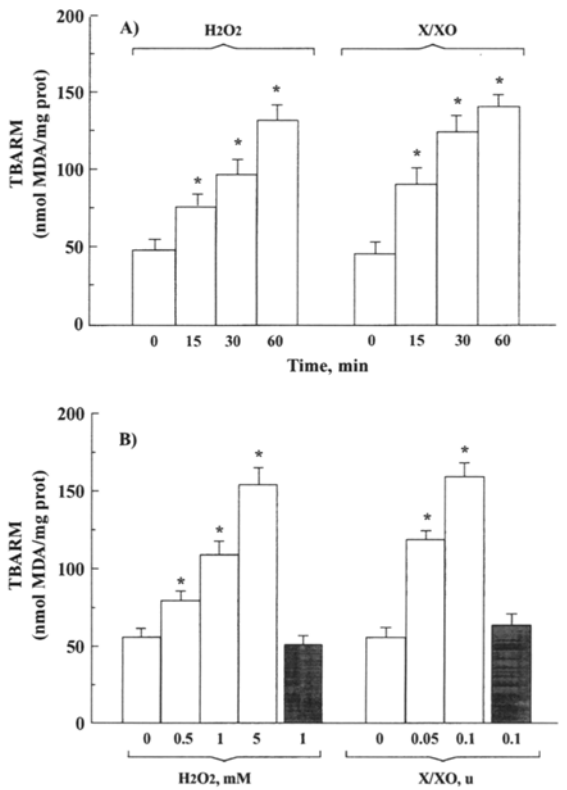

Fig. 5. Effects of H_2O_2 and X/XO on lipid peroxidation of RCEC membranes. Membranes were prepared as described in the Experimental Procedures. Lipid peroxidation of the membranes was determined as the amount of thiobarbituric acid-reactive material (TBARM) accumulated in vivo. A) Cells were exposed to 1 mM H_2O_2 or (100 μM/0.1 u) X/XO for the indicated periods of time. B) Cells were exposed to the indicated concentrations of H_2O_2, or 100 μM xanthine plus the indicated concentrations of XO (□) for 30 min. 200 u/ml catalase or 250 u/ml SOD (▩) were added to the cells 30 min prior to the addition of H_2O_2 or X/XO, respectively. The values represent the means ± S.E.M. of four replicate dishes and are representative of the results of 3 separate experiments. *-indicates significant difference ($P < 0.01$, ANOVA) as compared to the control value(s).

eral (33) and cerebral endothelial (34) cells is a measure of cell membrane "leakiness" to small molecules of less than 1 kD molecular weight. By contrast, the release of significant levels of the relatively large (134 kD) cytoplasmic enzyme, LDH, into the medium was indicative of a major breach in cell membrane integrity (33).

The addition of 1 mM H_2O_2 to the medium induced a 3.7-fold increase in ^{51}Cr release from RCEC within 60 min without a concomitant loss in cellular LDH activity (Fig. 6A & B). However, significant release of LDH from cells was observed 4 hours post-H_2O_2 treatment, indicating that prolonged exposure to the oxidant would indeed induce a major loss in membrane integrity. The H_2O_2-induced release of ^{51}Cr was prevented by the presence of catalase in the medium (Fig. 6A). Similarly, an increase in ^{51}Cr release from RCEC was observed between 30–60 min after the addition of 100 μM xanthine/0.1u XO, whereas a significant loss of LDH from cells was not detectable until the 2–4 hours after treatment (Fig. 6C & D). X/XO-induced ^{51}Cr release from RCEC was prevented by the presence of SOD (Fig. 6C). Significantly, neither H_2O_2 nor X/XO effected ^{51}Cr release from RCEC at earlier times (i.e., 15 min or 30 min); times at which the same concentrations of oxidants significantly reduced Na,K-ATPase activity and increased peroxidation of cellular membranes.

We have previously demonstrated that a loss in cell viability, measured by LDH release and trypan blue exclusion, is manifested by the release of >80% of the

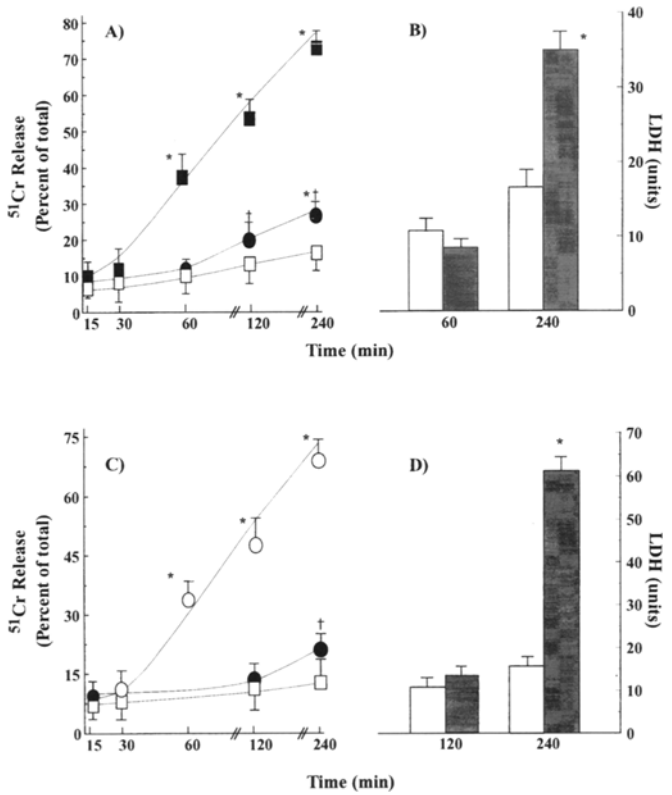

Fig. 6. Effects of H_2O_2 and X/XO on ^{51}Cr and LDH releases from RCEC. Cells were preloaded with 3 μCi/ml ^{51}Cr overnight in 1% serum-containing medium, and subsequently exposed to oxidants for the indicated periods of time. Amounts of radiolabel or LDH activity released into the medium were determined as described in the Experimental Procedures. A) Spontaneous (□) or 1 mM H_2O_2-induced ^{51}Cr release from RCEC in the absence (■) or presence (●) of 200 u/ml catalase was measured at indicated times. B) LDH release in control (□) or 1 mM H_2O_2-treated (▨) RCEC cultures. C) Spontaneous (□) and (100 μM/0.1u) X/XO-induced ^{51}Cr release from RCEC in the absence (○) or presence of 250 u of SOD (●). D) LDH release in control (□) and (100 μM/0.1u) X/XO-treated (▨) RCEC cultures. Catalase or SOD were added to the cultures 30 min prior to the addition of oxidants. The values represent the means ± S.E.M. of six replicate dishes and are representative of the results of 3 separate experiments. *-indicates significant difference ($P < 0.01$, ANOVA) as compared to the control value(s); †-indicates significant difference ($P < 0.01$, ANOVA) as compared to oxidant (H_2O_2 or X/XO) alone.

total cellular ^{51}Cr in ^{51}Cr-loaded cells (35). Thus, the loss of ~40% of the total cellular ^{51}Cr without detectable LDH release in RBEC subjected to a 60 min oxidant exposure (Fig. 6) likely reflected subtle oxidant-induced membrane injury resulting in a selective increase in membrane 'permeability' for small molecules. We have previously shown that low levels of ^{51}Cr release from human cerebral capillary endothelial cells without the accompanying loss of cellular viability can be induced by receptor-agonist interaction(s) or by stimulating protein kinase C (35), and is competitively inhibited by the specific receptor antagonist(s) (35), a process known as receptor-mediated permeabilization.

Effects of Antioxidants on Free Radical-Induced Changes in Na,K-ATPase Activity and Membrane Integrity in RCEC. In order to gain a clearer understanding of the mechanism by which free radicals caused a loss in Na,K-ATPase activity in RCEC, we compared the ability of lipophilic, membrane-targeting antioxidants and hydrophilic antioxidants to affect the changes in sodium pump activity, membrane lipid peroxidation, and membrane leakiness induced by H_2O_2 or X/XO. The lipophilic 21-aminosteroid derivatives, U-74500A and U-74389G, have been shown to block lipid peroxidation by inserting into membranes and chelating iron (36). Both of these compounds, in a concentration-dependent fashion, prevented the H_2O_2- and X/XO-induced loss in Na,K-ATPase activity, the concomitant increase in membrane lipid peroxidation, and the temporally delayed release of ^{51}Cr from RCEC monolayers (Table 1). By contrast, the hydrophilic antioxidant ascorbic acid (2 mM) had no effect on H_2O_2- or X/XO-induced changes in Na,K-ATPase activity, lipid peroxidation, and ^{51}Cr release in RCEC (Table I). Reduced glutathione (GSH, 4

Table I. Effects of Various Antioxidants on H_2O_2- or X/XO-Induced Changes in Na,K-ATPase Activity, Membrane Lipid Peroxidation, and ^{51}Cr Release Form RCEC

	Na,K-ATPase 30 min	TBARM 30 min	^{51}Cr Release 60 min
Control	64.6 ± 5.9	58.09 ± 4.08	4.78 ± 0.41
H_2O_2, 1 mM	34.0 ± 6.1[a]	126.93 ± 8.72[a]	43.74 ± 4.22[a]
+U-74500A, 5 μM	49.6 ± 4.8[a,b]	87.45 ± 4.71[a,b]	28.90 ± 0.98[a,b]
+U-74500A, 20 μM	66.8 ± 7.1[b]	62.78 ± 7.61[b]	6.21 ± 0.37[b]
+U-74389G, 5 μM	45.6 ± 5.1[a,b]	82.68 ± 6.23[a,b]	19.17 ± 0.67[a,b]
+U-74389G, 20 μM	68.3 ± 4.9[b]	61.31 ± 5.75[b]	9.08 ± 0.55[a,b]
+VIT C, 2 mM	33.2 ± 2.3[a]	123.23 ± 11.87	42.23 ± 4.12[a]
+GSH, 4 mM	44.6 ± 4.1[a,b]	107.38 ± 7.14[a,b]	40.87 ± 3.54[a]
+DTT, 1 mM	30.6 ± 5.1[a]	119.61 ± 9.12[a]	41.32 ± 2.56[a]
X/XO (100 μM/0.1 u)	21.4 ± 3.3[a]	174.58 ± 12.42[a]	34.87 ± 6.36[a]
+U-74500A, 20 μM	54.9 ± 6.2[b]	58.95 ± 6.37[b]	5.43 ± 0.32[b]
+U-74389G, 20 μM	49.4 ± 3.8[a,b]	79.12 ± 3.48[a,b]	4.13 ± 0.19[b]
+VIT C, 2 mM	21.3 ± 1.8[a]	153.12 ± 13.82[a]	39.15 ± 3.12[a]
+ GSH, 4 mM	29.1 ± 3.1[a,b]	134.14 ± 12.3[a,b]	30.14 ± 3.68[a]
+ DTT, 1 mM	21.6 ± 2.2[a]	192.67 ± 14.54[a]	36.76 ± 2.91[a]

Values are given as means ± S.E.M. for 6 replicate dishes in one representative out of three experiments with similar results. Na,K-ATPase activity is expressed in nmol ^{86}Rb/mg protein/15 min, thiobarbituric acid-reactive material (TBARM) as nmol malondialdehyde/mg protein, and ^{51}Cr release as percent of release of total radiolabel incorporated into the cells. [a]-indicates significant difference ($P<0.01$, ANOVA) as compared to control value(s); [b]-indicates significant difference ($P<0.01$, ANOVA) as compared to H_2O_2 or X/XO alone.

Table II. Characteristics of Linear Regression Between Each Two of Three Parameters Examined in This Study (i.e., Na,K-ATPase, TBARM, and ^{51}Cr release), and Multiple Regression in Which ^{51}Cr Release Was Treated as the Dependent, and Na,K-ATPase and TBARM as the Independent Variables

	^{51}Cr release		Na,K-ATPase	
	R	P<	R	P<
Na,K-ATPase	−0.853	$2.33*e^{-7}$	—	—
TBARM	0.814	$2.32*e^{-6}$	−0.931	$1.13*e^{-10}$
Multiple	0.848	$1.45*e^{-8}$		

The linear regression analysis was performed using the corresponding mean values for each parameter in variously treated control and experimental groups (N=20; values from Table I, and control values not shown in the Table I), utilizing SPSS for Windows (Ver. 6.0.1) computer software. Columns depict dependent variable(s), whereas rows show independent variables used for regression analysis. R - correlation coefficient; P< - indicates significance at P values lower than those shown in the Table.

mM), an endogenous antioxidant that acts both as a substrate for the H_2O_2-neutralizing enzyme, glutathione peroxidase, and directly as a thiol-reducing agent (37), had only marginal effects on the membrane lipid peroxidation and Na,K-ATPase activity changes induced by both oxidants (Table I). In conjunction with the observation that the powerful reducing and SH-group protecting agent, dithiothreitol (DTT) (38) was ineffective against any of the H_2O_2- or X/XO-induced alterations measured in RCEC (Table I), the results indicate that the free radical-mediated loss of Na,K-ATPase activity in RCEC did not result from the oxidation of critical thiol groups present on the enzyme, and was likely due to the peroxidation of membrane lipids essential for enzymatic activity.

The results from Table I were subjected to linear regression analysis and the correlation between the various parameters examined. The analysis demonstrated that a highly significant correlation existed between any two of the three experimental parameters measured (Table II). Moreover, the results of multiple regression analysis, in which changes in ^{51}Cr release were treated as the dependent variable, indicated a strong statistical link between changes in lipid peroxidation/Na,K-ATPase activity and the release of ^{51}Cr (Table II). This statistical link reinforces the possibility of a functional connection between free radical induced changes in membrane lipids and sodium pump activity and membrane permeability for small molecules in RCEC subjected to oxidative stress.

DISCUSSION

The observation that oxidants such as hydrogen peroxide and xanthine/xanthine oxidase are potent inhibitors of ouabain-sensitive ^{86}Rb uptake in cerebral endothelial cells is the first demonstration of free radical-

mediated inhibition of functional Na,K-ATPase in cerebromicrovascular endothelial cells in situ. The results are consistent with previous studies indicating that free radicals and free fatty acids inhibit Na,K-ATPase in brain (8,21,40) and in isolated cerebral capillaries (41). It has also been determined that inhibition of brain Na,K-ATPase activity occurs during ischemia (42) and that longterm changes in Na,K-ATPase kinetic properties persist upon restoration of blood flow after cerebral ischemia (21,42-43). The abrupt development of brain swelling following reperfusion has been reported to coincide with a drop in tissue Na,K-ATPase activity (42,44).

The free radical-induced loss in RCEC sodium pump activity observed in this study did not result from a widespread loss in cell membrane integrity, since almost complete inhibition of the pump occurred within 15 min of oxidant addition, much earlier than the time needed (i.e., 1–4 hrs) to initiate detectable increases in LDH release from RCEC. Furthermore, reduction in Na,K-ATPase activity and increase in membrane lipid peroxidation were also observed before any appreciable loss of ^{51}Cr from RCEC. Alterations in membrane bilayer composition/fluidity due to lipid peroxidation have been shown to compromise functions of membrane receptors (19) and the activity of membrane-attached enzymes, including Na,K-ATPase (20). The observation that lipid peroxidation and Na,K-ATPase inhibition were rapid and concomitant events in oxidant-challenged RBEC suggested that alterations in membrane lipid structure and function may have been responsible for the loss of sodium pump function in these cells. This suggestion was supported by the finding that the lipophilic antioxidants 21-aminosteroids, U-74500A and U-74389G, effectively blocked both oxidant-induced lipid peroxidation of RCEC membranes and the loss of Na,K-ATPase activity. These compounds have previously been shown to inhibit iron-dependent lipid peroxidation (36), to reduce arachidonic acid-induced vasogenic brain edema (45), to improve outcome of global cerebral ischemia (46), and to protect the blood brain barrier following subarachnoidal hemorrhage (47). Furthermore, direct oxidation of vulnerable thiol moieties on the enzyme did not appear responsible for the loss of enzyme activity since both glutathione and DTT were unable to restore oxidant-inhibited Na,K-ATPase activity. The results strongly suggest that free radical-mediated Na,K-ATPase inhibition in RCEC was primarily caused by oxidant-induced changes in the membrane lipid environment and not by the direct oxidation of the enzymes thiol-groups by free radicals. The highly significant negative correlation observed between changes in Na,K-ATPase activity and lipid peroxidation in membranes during RCEC exposure to oxidants supports this contention. Previous studies have shown that lipoxidation of isolated synaptosomal membranes results in the loss of Na,K-ATPase activity (20), and that free radical-induced oxidation of polyunsaturated free fatty acids in vitro can be reversed by active oxygen scavenging agents superoxide dismutase, D-mannitol, and beta-carotene (48). Thus, the preservation of cerebral endothelial membrane lipid composition may be crucial for sustaining effective homeostatic responses at the site of BBB.

Since BBB function depends on the integrity and performance of cerebral endothelial plasma membrane and membrane-linked proteins, it was important to determine the impact of oxidant-induced RCEC membrane lipid peroxidation and Na,K-ATPase inhibition on the 'barrier' properties of these cells. Significant increases in RCEC 'permeability' for small molecules were temporally delayed relative to membrane lipid peroxidation and Na,K-ATPase inhibition in oxidant challenged RBEC. The protection of Na,K-ATPase by potent inhibitors of lipid peroxidation also effectively averted subsequent oxidant-evoked increases in ^{51}Cr release. The highly significant correlation between 'permeability' changes and changes in Na,K-ATPase and lipid peroxidation ($R = 0.848$) suggested that oxidant-induced leakiness of cerebral endothelial cell membranes for small molecules was the result of a series of cellular events which impaired the ability of RCEC membranes to maintain a barrier, a process in which Na,K-ATPase is intrinsically involved.

Na,K-ATPase plays a central role in ion transport and water homeostasis at the site of BBB by directly regulating intracellular ion concentrations, and by indirectly providing the electrochemical gradients used by other transporters (49–50). Vasogenic brain edema, a prominent feature of reperfusion brain injury, results from the structural and/or functional 'disruption' of the BBB (2–4) in which the loss of cerebral microvessel Na,K-ATPase activity may play a significant role. Reperfusion brain injury is also characterized by enhanced production of reactive oxygen species to which cerebral endothelial cells may be extensively exposed. Free radical-induced functional and/or structural breaches of the BBB, similar to the ones described in this in vitro model of BBB, have been proposed to be the principal cause of reperfusion brain edema in vivo (2–4). Thus, preventing injury to those cerebral endothelial cell compartments closely linked to the development of edema and sensitive to free radical damage, such as membrane lipids and Na,K-ATPase, may provide novel modalities for effective anti-edema therapies.

REFERENCES

1. Rapoport, S. I. 1976. Blood-Brain Barrier *in* Physiology and Medicine. Raven Press. New York.
2. Chan, P. H., Schmidley, J. W., Fishman, R. A., and Longar, S. M. 1984. Brain injury, edema and vascular permeability changes induced by oxygen-derived free radicals. Neurology 34:315–320.
3. Hall, E. D., and Braughler, J. M. 1989. Central nervous system trauma and stroke II. Physiological and pharmacological evidence for involvement of oxygen radicals and lipid peroxidation. Free Radical Biol. & Med. 6:303–313.
4. Watson, D. B., and Ginsberg, D. M. 1989. Ischemic injury in the brain: role of oxygen radical mediated processes. Ann. New York Acad. Sci. 559:269–281.
5. Hill, M. E., Bird, I. N., Daniels, R. H., Elmore, M. A., and Finnen, M. J. 1994. Endothelial cell-associated platelet-activating factor primes neutrophils for enhanced superoxide production and arachidonic acid release during adhesion to but not transmigration across IL-1β-treated endothelial monolayers. J. Immunol. 153: 3673–3683.
6. Matsuo, Y., Onodera, H., Shiga, Y., Nakamura, M., Ninomiya, M., Kihara, T., and Kogure, K. 1994. Correlation between myeloperoxidase-quantified neutrophil accumulation and ischemic brain injury in rat: effects of neutrophil depletion. Stroke 25:1469–1475.
7. McCord, J. M., Omar, B. A., and Rusell, W. J. 1989. Sources of oxygen-derived radicals in ischemia-reperfusion. Pages 1113-1118, *in* Hayashi, O., Niki, E., Kondo, M., and Yoshikawa, T. (eds.), Medical, Biochemical, and Chemical Aspects of Free Radicals, Elsevier Sci. Publishers B. V., Amsterdam.
8. Chan, P. H., Fishman, R. A., Longar, S., Chen, S., and Yu, A. 1985. Cellular and molecular effects of polyunsaturated fatty acids in brain ischemia and injury. Prog. Brain Res. 63:227–235.
9. Kinuta, Y., Kimura, M., Itokawa, Y., Ishikawa, M., and Kikuchi, H. 1989. Changes in xanthine oxidase in ischemic rat brain. J. Neurosur. 71:417–420.
10. Parks, D. A., and Granger, D. N. 1983. Ischemia-induced vascular changes: role of xanthine oxidase and hydroxyl radicals. Am. J. Physiol. 245:285–289.
11. Wei, E. P., Christman, C. W., Kontos, H. A., and Povlishock, J. T. 1985. Effects of oxygen radicals on cerebral arterioles. Am. J. Physiol. 248:H157–H162.
12. Macdonald, R. L., and Weir, B. K. 1994. Cerebral vasospasm and free radicals. Free Radical Biol. & Med. 16:633–643.
13. Kontos, H. A., and Wei, E. P. 1986. Superoxide production in experimental brain injury. J. Neurosur. 64:803–807.
14. Weber, C., Erl, W., Pietsch, A., Strobel, M., Ziegler-Heitbrock, H. W. L., and Weber, P. C. 1994. Antioxidants inhibit monocyte adhesion by suppressing nuclear factor-κB mobilization and induction of vascular cell adhesion molecule-1 in endothelial cells stimulated to generate radicals. Artherioscler. Throm. 14:1665–1673.
15. Kempski, O., Villacara, A., Spatz, M., Dodson, R. F., Corn, C., Merkel, N., and Bembry, J. 1987. Cerebromicrovascular permeability: in-vitro studies. Acta Neuropath. 74:329–334.
16. Wei, E. P., Ellison, M. D., Kontos, H. A., and Povlishock, J. T. 1986. O_2 radicals in arachidonate-induced increased blood-brain barrier permeability to proteins. Am. J. Physiol. 251:H693–H699.
17. Flohe, L. and Giertz, H. 1987. Free radicals, membrane damage, and cell-mediated cytolysis. Br. J. Cancer 55:30–48.
18. Richter, C. 1993. Pro-oxidants and mitochondrial Ca^{2+} their relationship to apoptosis and oncogenesis. FEBS Lett. 325:104–107.
19. Villacara, A., Kumami, K., Yamamoto, T., Mrsulja, B. B., and Spatz, M. 1989. Ischemic modification of cerebrocortical membranes: 5-hydroxytryptamine receptors, fluidity, and inducible in vitro peroxidation. J. Neurochem. 53:595–601.
20. Sun, Y. A. 1972. The effect of lipoxidation on synaptosomal (Na^+, K^+)-ATPase isolated from cerebral cortex of squirel monkey. Biochem. Biophys. Acta 266:350–360.
21. Enseleit, W. H., Domer, F. R., Jarrott, D. M., and Baricos, W. H. 1984. Cerebral phospholipid content and Na^+, K^+-ATPase activity during ischemia and postischemic reperfusion in the Mongolian gerbil. J. Neurochem. 43:320–327.
22. Zlokovic, B. V., Wang, L., Mackic, J. B., Saraj, A. J., McComb, J. G., and McDonough, A. 1993. Expression of Na,K-ATPase at the blood-brain interface. Pages 55–60, *in* Drewes, L. R. and Betz, A. L., (eds.), Frontiers in Cerebral Vascular Biology: Transport and Its Regulation, Plenum Press, New York and London.
23. Djuricic, B. M., and Mrsulja, B. B. 1977. Enzymatic activity of the brain: microvessels v.s. total brain homogenate. Brain Res. 138:561–564.
24. Betz, A. L., Firth, J. A., and Goldstein, G. W. 1980. Polarity of the blood brain barrier: distribution of enzymes between luminal and antiluminal membranes of brain capillary endothelial cells. Brain Res. 192:17–28.
25. Spatz, M., Bembry, J., Dodson, R. F., Hervonen, H., and Murray, M. R. 1980. Endothelial cell cultures derived from isolated cerebral microvessels. Brain Res. 199:577–582.
26. Mrsulja, B. B., Mrsulja, B. J., Fujimoto, T., Klatzo, I., and Spatz, M. 1976. Isolation of brain capillaries: a simplified technique. Brain Res. 110:361–365.
27. Dong, J., Delamere, N. A., and Coca-Prados, M. 1994. Inhibition of Na^+-K^+-ATPase activates Na^+-K^+-$2Cl^-$ cotransporter activity in cultured ciliary epithelium. Am. J. Physiol. 266:C198–C205.
28. Lowry, O. H., Rosebrough, N. U., Farr, A. L., and Randall, R. J. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265–275.
29. Auclair, C. and Voisin, E. 1985. Nitroblue tetrazolium reduction, Pages 123–132, *in* Greenwald, R. A., (ed.), Handbook of Methods For Oxygen Radical Research, CRC Press Inc., Boca Raton.
30. Chan, P. H., Chen, S. F., and Yu, A. C. H. 1988. Induction of intracellular superoxide radical formation by arachidonic acid and by polyunsaturated fatty acids in primary astrocytic cultures. J. Neurochem. 50:1185–1193.
31. Rehncrona, S., Smith, D. D. S., Akesson, B., Westenberg, E., and Siesjo, B. K. 1980. Peroxidative changes in brain cortical fatty acids and phospholipids as characterized during Fe^{++}-and ascorbic acid-stimulated lipid peroxidation in vitro. J. Neurochem. 34: 1630–1638.
32. Geck, P. and Heinz, E. 1986. The Na-K-2Cl cotransporter system. J. Membrane Biol. 91:97–105.
33. Chopra, J., Joist, H. J., and Webster, R. O. 1987. Loss of ^{51}chromium, lactate dehydrogenase, and ^{111}indium as indicators of endothelial cell injury. Lab. Invest. 57:578–584.
34. Stanimirovic, D. B., McCarron R. M., Bertrand, N., and Spatz, M. 1993. Endothelins release ^{51}Cr from cultured human cerebromicrovascular endothelium. Biochem. Biophys. Res. Comm. 191:1–8.
35. Stanimirovic, D. B., Bertrand, N., McCarron, R. M., Uematsu, S., and Spatz, M. 1994. Arachidonic acid release and permeability changes induced by endothelins in human cerebromicrovascular endothelium. Acta Neurochir. (Suppl. 60):71–75.
36. Braughler, J. M., Pregenzer, J. F., Chase, R. L., Dunkan, L. A., Jacobsen, E. J., and McCall, J. M. 1987. Novel 21-amino steroids as potent inhibitors of iron-dependent lipid peroxidation. J. Biol. Chem. 262:10438–10440.
37. Meister, A., and Anderson, M. E. 1983. Glutathione. Annu. Rev. Biochem. 52:711–760.
38. Cleland, W. W. 1964. Dithiothreitol, a new protective reagent for SH groups. Biochemistry 3:480–485.
39. Huang, W., Wang, Y., Askari, A., Zolotarjova, N., Ganjeizadeh, M. 1994. Different sensitivities of the Na^+/K^+-ATPase isoforms to oxidants. Biochim Biophys Acta 1190:108–114.
40. Hexum, T. D., and Fried, R. 1979. Effects of superoxide and radicals on transport (Na-K) adenosine triphosphatase and protection by superoxide dismutase. Neurochem. Res. 4:73–82.

41. Lo, W. D., and Betz, A. L. 1986. Oxygen free-radical reduction of brain capillary rubidium uptake. J. Neurochem. 46:394–398.
42. Mrsulja, B. B., Micic, D. V., and Stefanovich, V. 1985. Propentofylline and postischemic brain edema: relation to Na,K-ATPase activity. Drug Develop. Res. 6:339–344.
43. Stojanovic, T., and Mrsulja, B. B. 1988. Alterations in synaptosomal membrane Na,K-ATPase of the gerbil cortex and hippocampus following reversible brain ischemia. Metab. Brain Dis. 3: 265–272.
44. Djuricic, B., Micic, D. V., and Mrsulja, B. B. 1984. Phase recognition of edema caused by ischemia. Pages 491–498 *in* Go K. A. and Baethmann A. (eds.), Recent Progress in the Study and Therapy of Brain Edema, Plenum Press, New York.
45. Hall, E. D., and Travis, A. M. 1988. Inhibition of arachidonic acid-induced vasogenic brain edema by the non-glucocorticoid 21-aminosteroid U-74006F. Brain Res., 451:350–352.
46. Hall, E. D., Pazara, K. E., and Braughler, J. M. 1986. 21-aminosteroid lipid peroxidation inhibitor U-74006F protects against cerebral ischemia in gerbils. Stroke 19:997–1002.
47. Zuccarello, M., and Anderson, D. K. 1989. Protective effect of a 21-aminosteroid on the blood-brain barrier following subarachnoid hemmorhage in rats. Stroke 20:367–371.
48. Fukui, Y., Morita, M., Oskada, K., Makayama, T., Matsuoka, M., Hirota, R., Iizumi, H., and Ueda, T. 1995. Visible light-sensitized oxidation of arachidonic acid in the presence of inperatorin. J. Nutr. Sci. Vitamin. 41:387–393.
49. Keep, R. F., Xiang, J., and Betz, A. L. 1993. Potassium transport at the blood-brain and blood-CSF barriers, Pages 43–54, *in* Drewes, L. R. and Betz, A. L., (eds.), Frontiers in Cerebral Vascular Biology: Transport and Its Regulation, Plenum Press, New York and London.
50. Stahl, W. L. 1986. The Na,K-ATPase of nervous tissue. Neurochem. Int. 8:449–476.