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Oxygen Free-Radical Reduction of Brain Capillary Rubidium Uptake

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Abstract: Free radicals are proposed to play a role in the injury following cerebral ischemia in which cerebral edema is a prominent feature. To determine whether free radicals might alter the movement of ions and water across the blood–brain barrier, we examined their effect on brain capillary transport. Rat brain capillaries were isolated, incubated with a system that generates free radicals, and various capillary transport systems were studied. Rubidium uptake was reduced 74% whereas rubidium efflux, glucose transport, and capillary water space were unchanged. The results following the addition of radical scavengers indicated that hydrogen peroxide or a related free radical was the toxic species. These data suggest that free radicals can impair capillary endothelial cell mechanisms that help maintain homeostasis of electrolytes and water in brain. **Key Words:** Free radicals—Brain capillaries—Cerebral ischemia—Na^+,K^+-ATPase—Blood–brain barrier—Rubidium transport. **Lo W. D. and Betz A. L.** Oxygen free-radical reduction of brain capillary rubidium uptake. *J. Neurochem.* **46**, 394–398 (1986).

Free radicals are proposed as mediators of tissue injury, in response to diverse insults such as ischemia, radiation, oxygen toxicity, and aging (Miquel et al., 1977; Adams and Wardman, 1977; Fridovich, 1978; Del Maestro, 1980). In brain, free radicals appear to be produced during cerebral ischemia and reperfusion (Yamamoto et al., 1983; Watson et al., 1984); however, their role in the mediation or augmentation of ischemic brain damage has not been firmly established. One group of investigators reported that within hours after a free radical generating system was injected into cerebral parenchyma, cell necrosis, cerebral edema, and increased vascular permeability to Evans Blue dye were detected (Chan et al., 1983, 1984).

The brain capillary endothelial cell plays an important role in maintaining brain ion homeostasis (Bradbury, 1979; Goldstein and Betz, 1983). This cell may be particularly susceptible to attack by free radicals since brain capillaries are enriched in xanthine oxidase which, with a suitable substrate, will form free radicals (Betz, 1985). During cerebral ischemia it is possible brain endothelial cells produce free radicals that subsequently damage endothelial cell water and ion homeostatic mechanisms and exacerbate the changes in Na, K, and water seen in cytotoxic brain edema. To test a part of this proposed mechanism, we examined the effects of free radical species on transport properties of isolated brain capillaries.

MATERIALS AND METHODS

Male Sprague-Dawley rats were obtained from Harlan (Haslett, MI, U.S.A.). ^{86}Rubidium chloride, sp act 0.5–35 Ci/g (^{86}Rb); α-[1-^{14}C]methylaminoisobutyric acid, sp act 48.4 mCi/mmol ([^{14}C]meAIB); and methyl-D-glucose, 3-*O*-[*methyl*-^{3}H], sp act 79 Ci/mmol ([^{3}H]3-*O*-MG) were purchased from New England Nuclear (Boston, MA, U.S.A.). Acetaldehyde was obtained from Mallinkrodt (St. Louis, MO, U.S.A.). All other reagents, including xanthine oxidase type I, were purchased from Sigma (St. Louis, MO, U.S.A.).

Isolation of brain microvessels

Brain capillaries were prepared by homogenization, dextran gradient centrifugation, and glass bead filtration as previously described (Betz, 1983). The capillary pellet was then suspended in Dulbecco's phosphate-buffered saline with 1 m*M* $CaCl_2$, 5 m*M* glucose, and 1% bovine

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Abbreviations used: HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; meAIB, α-methylaminoisobutyric acid; 3-*O*-MG, 3-*O*-methyl-D-glucose; PBS, phosphate-buffered saline.

serum albumin (PBS). The suspension was kept on ice until use within 3 h of isolation. Aliquots for capillary protein content were washed free of albumin, dissolved in 0.5 *M* NaOH, and then protein was determined by the Biorad protein assay (Biorad Labs, Richmond, CA, U.S.A.).

Free radical generation

The commercially available xanthine oxidase suspension was dialyzed for 4 h against 1,000 volumes of PBS at 4°C, diluted in PBS to the desired concentration, and kept on ice. Substrates, inhibitors, and free radical scavengers were added just prior to use. Superoxide anion production was qualitatively assessed using the reduction of nitroblue tetrazolium to nitroforman by the superoxide anion. This reaction appeared equally effective with either hypoxanthine or acetaldehyde as substrates for xanthine oxidase. Acetaldehyde was glass-distilled prior to each experiment, and was kept in a glass-stoppered bottle at 4°C.

Uptake studies

A 0.1-ml aliquot of the xanthine oxidase suspension and a 0.1-ml aliquot of the capillary suspension were combined and preincubated at 37°C for various times. ^{86}Rb uptake was measured by addition of 0.05 ml of the isotope in PBS to the reaction mixture and then incubation of the suspension at 37°C for predetermined times. Uptake was terminated by trapping the capillaries on a nitrocellulose filter (1.2 μm pore size) and washing with an iced stop solution. For experiments with ^{86}Rb, the filters were prewashed with 1 *M* KCl. The stop solution consisted of 10 m*M* *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered saline, pH 7.4. Glucose uptake was measured by using [^{3}H]3-*O*-MG in Dulbecco's PBS with 1 m*M* $CaCl_2$, 1% bovine serum albumin, 5 m*M* pyruvate, and 5 m*M* unlabeled 3-*O*-MG. The capillary suspension was then incubated at 37°C for 30 s for glucose transport studies or 10 min for glucose-equilibrium distribution studies. The wash solution consisted of ice-cold HEPES-buffered saline with 10 μ*M* cytochalasin B added to reduce the loss of label during the wash procedure. L-Amino acid uptake was measured using [^{14}C]meAIB in the presence of 1 m*M* unlabeled meAIB as a carrier.

Capillaries trapped on filters were lysed with 0.5 ml distilled water and mixed with Safety-Solve scintillation fluid (RPI, Mount Prospect, IL, U.S.A.). Counting was performed using a Beckman LS-7500 scintillation counter. The cpm per sample was divided by the cpm of a known volume of isotope and then divided by the amount of protein per sample. Uptake of isotope was then expressed as the equivalent of radioisotope-containing incubation media taken up per milligram of capillary protein.

Efflux studies

Capillaries were incubated with ^{86}Rb for 2 h until ^{86}Rb uptake achieved equilibrium (Chaplin et al., 1981). The capillaries were briefly centrifuged in a microcentrifuge, the ^{86}Rb-containing supernatant was discarded, and the capillaries were resuspended in 1 ml of PBS alone or PBS containing 1 m*M* hypoxanthine and 0.1 U/ml of xanthine oxidase. Aliquots of 0.2 ml were sampled at 0, 10, 20, and 30 min. The rubidium content remaining within the cells was determined by filtration and washing with iced buffered saline as in the uptake studies.

Statistical analysis

All conditions were examined in triplicate. Individual results were compared to control using a one-tailed *t* test for paired samples.

RESULTS

Free radicals generated by xanthine oxidase and hypoxanthine or acetaldehyde inhibited ^{86}Rb uptake. This inhibition progressed over 20 min in a dose-related fashion with an increase in xanthine oxidase concentration (Fig. 1). There was no effect on rubidium uptake at xanthine oxidase concentrations ranging from 0.001 U/ml to 0.01 U/ml, but a gradual reduction in ouabain-sensitive uptake occurred at an enzyme concentration of 0.03–0.1 U/ml. Therefore, a concentration of 0.1 U/ml of xanthine oxidase was chosen for subsequent experiments. There was no effect of free radicals on ^{86}Rb uptake in the presence of ouabain. In mammalian cells, ^{86}Rb is transported in essentially the same manner as potassium (Vaughn and Cook, 1972). Since ouabain-sensitive potassium transport is mediated by Na^+,K^+-ATPase (Akera et al., 1969) ouabain-sensitive ^{86}Rb uptake can be used as a measure of Na^+,K^+-ATPase activity. Thus, the free radical reduction of brain capillary ^{86}Rb uptake appears due to inhibition of Na^+,K^+-ATPase activity. When acetaldehyde alone was added to the capillaries,

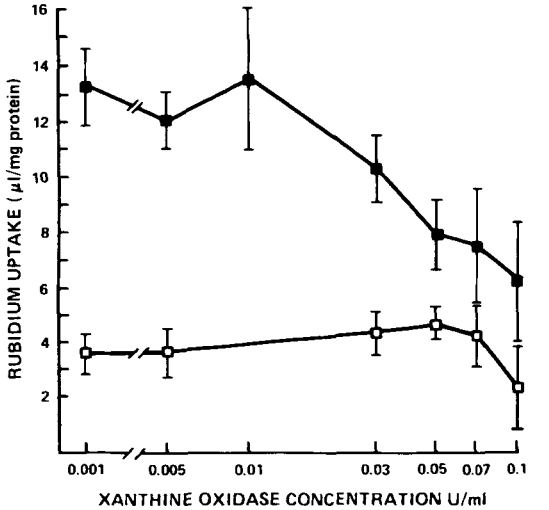

FIG. 1. Effect of increasing concentration of xanthine oxidase generated free radicals on total ^{86}Rb uptake. The upper curve (■) represents uptake with increasing xanthine oxidase concentration; the lower curve (□) represents uptake under the same conditions together with 5 m*M* ouabain. All samples contained 10 m*M* acetaldehyde and xanthine oxidase at concentrations of U/ml as shown, and were incubated for 1 h prior to examination of ^{86}Rb uptake over 20 min at 37°C.

TABLE 1. *Brain capillary rubidium uptake*

Conditions	Rubidium uptake (μl/mg protein/20 min)
Control (PBS buffer)	8.5 ± 2.1
Acetaldehyde (10 m*M*)	8.6 ± 1.8
Acetaldehyde (10 m*M*) + xanthine oxidase (0.1 U/ml)	2.6 ± 1.3[a]
Acetaldehyde (10 m*M*) + xanthine oxidase (0.1 U/ml) + allopurinol (3 m*M*)	7.9 ± 1.7

A 0.2-ml suspension of capillaries was preincubated in buffer containing the reagents listed above, for 2 h at 37°C. Capillary ^{86}Rb uptake over 20 min at 37°C was then measured as described. Results are the means ± SD of three determinations. In separate experiments xanthine oxidase alone at 0.1 U/ml had no effect on Rb uptake over 20 min incubation time.

[a] $p < 0.01$ when compared to control.

^{86}Rb uptake was unchanged (Table 1). The addition of allopurinol, a specific inhibitor of xanthine oxidase, prevented inhibition of rubidium uptake (Table 1). These results indicate that the inhibition of rubidium uptake was caused by xanthine oxidase generated free radicals and not by nonspecific effects of the free radical generating system.

If ^{86}Rb uptake in the presence of free radicals is reduced because Na^+,K^+-ATPase is inhibited, then the extracellular/intracellular Na^+ gradient should decrease. Small neutral amino acid transport as measured by [^{14}C]meAIB uptake is dependent on the Na^+ gradient (Betz and Goldstein, 1978), and therefore, [^{14}C]meAIB accumulation by brain capillaries serves as an indirect measure of the sodium gradient. As shown in Table 2, [^{14}C]meAIB uptake by isolated brain capillaries was reduced by preincubation with a free radical producing system. This suggests that the Na^+ gradient was reduced, consistent with the free radical inhibitory effect on ^{86}Rb uptake. As a reduction in ^{86}Rb uptake might reflect either nonspecific inhibition of all transport sytems or an enhanced efflux of ^{86}Rb, it was necessary to determine if other transport systems were affected and if cell membrane integrity was damaged. Neither the facilitated transport system for glucose (Table 2), the capillary water space (Betz et al., 1979) (Table 2), nor the rate of ^{86}Rb efflux from capillaries (Fig. 2) was altered by incubating with free radicals. Therefore, reduced ouabain-sensitive ^{86}Rb uptake indicates some specificity of free radicals for the Na^+,K^+-ATPase.

TABLE 2. *Capillary uptake of meAIB and 3-O-MG*

	Capillary uptake (μl/mg protein/time)		
	Control	Ouabain	Xanthine oxidase
meAIB (60 min)	11.2 ± 3.4	1.3 ± 0.6[a]	1.8 ± 1.0[b]
3-*O*-MG (30 s)	2.0 ± 0.4	—	1.5 ± 0.5
3-*O*-MG (10 min)	2.2 ± 0.7	—	1.9 ± 0.5

[^{14}C]meAIB uptake was measured in medium containing 1 m*M* hypoxanthine. Where indicated the concentration of ouabain was 5 m*M* and that of xanthine oxidase 0.1 U/ml. A 0.2-ml suspension of capillaries was preincubated at 37°C for 30 min with these compounds, then 0.05 ml of [^{14}C]meAIB in PBS was added, and the suspension incubated at 37°C for 60 min. [^{3}H]3-*O*-MG uptake was measured by incubating a 0.2-ml suspension of capillaries for 30 min at 37°C in medium containing 1 m*M* hypoxanthine either in the absence or presence of 0.1 U/ml of xanthine oxidase. [^{3}H]3-*O*-MG in 0.05 ml of PBS was added and incubation continued at 37°C. Uptake was measured over 30 s for transport studies, and equilibrium distribution of cell water, using [^{3}H]3-*O*-MG as a marker, was measured over 10 min. Results are the means ± SD of three determinations.

[a] $p < 0.005$ compared to control.

[b] $p < 0.02$ compared to control.

Since the superoxide anion produced by xanthine oxidase is converted to hydrogen peroxide and other radical species in biologic systems (Del Maestro, 1980), we used radical scavengers to determine which species inhibited transport. Catalase catalyzes the conversion of hydrogen peroxide to water and oxygen. The addition of catalase almost completely blocked the inhibition of ^{86}Rb uptake (Table 3). Superoxide dismutase, which decomposes superoxide to hydrogen peroxide and oxygen, was without effect (data not shown). Ethanol and mannitol, which should quench the hydroxyl radical (Dorfman and Adams, 1964), had no effect; neither did deferoxamine, which should inhibit iron-catalyzed formation of the hydroxyl and singlet oxygen radicals (Butler and Halliwell, 1982). Hydrogen peroxide at a concentration of 1 m*M* did not inhibit ^{86}Rb uptake (data not shown).

From these results it is unclear which free radical inhibited ^{86}Rb uptake although it appears to be a species related to hydrogen peroxide. The inconsistent pattern of protection by free radical scavengers is similar to the variable effect of scavengers evi-

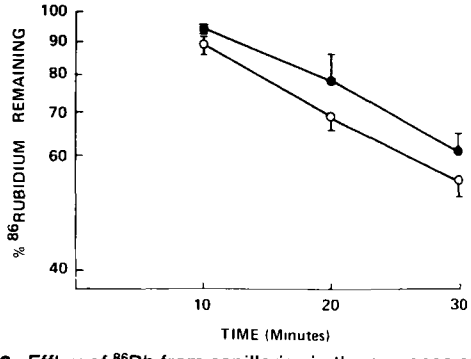

FIG. 2. Efflux of ^{86}Rb from capillaries in the presence of free radicals. Isolated capillaries were loaded with ^{86}Rb by incubation for 2 h at 37°C in media containing 1 m*M* hypoxanthine and 20 mCi/ml of ^{86}Rb. After rapid washing to remove extracellular ^{86}Rb, efflux at 37°C was studied either in the presence (●) or absence (○) of 0.1 U/ml of xanthine oxidase.

TABLE 3. *Capillary uptake of ^{86}Rb in the presence of free radical scavengers*

Condition	Rubidium uptake (μl/mg protein/20 min)
Control buffer	7.3 ± 1.7
Xanthine oxidase, 0.1 U/ml	1.9 ± 0.7[a]
XO + catalase, 10 μg/ml	6.1 ± 1.4
XO + ethanol 1% (vol/vol)	2.0 ± 0.7[a]
XO + mannitol, 50 m*M*	2.6 ± 1.1[b]
XO + deferoxamine mesylate, 10 μg/ml	2.6 ± 0.9[b]

Suspending buffer contained 10 m*M* acetaldehyde and additional reagents as listed above. A 0.2-ml suspension of capillaries was preincubated for 2 h at 37°C. ^{86}Rb uptake over 20 min at 37°C was then measured as described. Results are the means ± SD of three determinations. Superoxide dismutase at 50 μg/ml had no protective effect (data not shown). XO, Xanthine oxidase, 0.1 U/ml.

[a] $p < 0.005$ compared to control.

[b] $p < 0.01$ compared to control.

dent in other systems (Kellogg and Fridovich, 1977; Lynch and Fridovich, 1978; Del Maestro et al., 1980, 1981; Kono and Fridovich, 1982; Chan et al., 1982).

DISCUSSION

Brain edema is a prominent feature in cerebral ischemic injury (Adams and Victor, 1982). Since brain capillaries perform an important role in brain ion homeostasis, we examined whether free radical injury of the capillaries can be implicated in the formation of postischemic cytotoxic cerebral edema.

Our results show that when isolated brain capillaries are incubated with a free radical producing system, uptake of ^{86}Rb is inhibited. This effect appears to be the result of inhibition of Na^+,K^+-ATPase activity and not of a generalized reduction of cell membrane integrity or transport systems. The mechanism of free radical inhibition remains uncertain. One may speculate that free radicals attack double bonds in the lipid components of the membrane bilayer (Demopoulos et al., 1980). Altering the double bond would alter the structure of the lipid, thus affecting membrane bilayer fluidity and potentially affecting Na^+,K^+-ATPase function. Alternatively, free radicals could attack the protein structure of the Na^+,K^+-ATPase, resulting in alteration of transport. Finally free radicals might adversely affect energy metabolism of the cells which would cause a secondary decrease in Na^+,K^+-ATPase activity.

Other studies confirm that Na^+,K^+-ATPase is susceptible to attack by free radicals. Whole brain Na^+,K^+-ATPase activity was reduced following exposure to xanthine oxidase and xanthine similar to the system used in the experiements reported here (Hexum and Fried, 1979). In another study, incubation of bovine lenses in micromolar concentrations of hydrogen peroxide inhibited ^{86}Rb uptake without increasing membrane permeability (Garner et al., 1983). These same investigators observed that incubation of purified bovine lens or bovine kidney Na^+,K^+-ATPase with 1 m*M* hydrogen peroxide altered the kinetic properties of the enzyme (Garner et al., 1984).

There is also evidence to suggest that Na^+,K^+-ATPase in brain may be unusually sensitive to the effects of reperfusion injury. Although whole brain Na^+,K^+-ATPase was unaffected by up to 6 h of ischemia in the gerbil, on reperfusion, Na^+,K^+-ATPase declined to 42% of control and remained reduced for 5 h (Schwartz et al., 1976). In another study, Na^+,K^+-ATPase activity was inhibited during ischemia and recovered only after 1 h of reperfusion (Enseleit et al., 1984). Since it appears likely that free radicals are produced during ischemia and reperfusion (Yamamoto et al., 1983; Watson et al., 1984), our results suggest that these reactive species may be responsible for the observed decrease in Na^+,K^+-ATPase activity.

Taking all of these observations into consideration, a possible mechanism for the production of postischemic cerebral edema can be hypothesized. Following cerebral ischemia, brain tissue levels of hypoxanthine increase due to the breakdown of adenosine to hypoxanthine (Berne et al., 1974; Kleihues et al., 1974). During reperfusion, hypoxanthine from other regions may be transported to the brain capillary endothelial cells. These cells were recently shown to contain xanthine oxidase (Betz, 1985), which could produce free radicals on exposure to hypoxanthine. The free radicals produced by endothelial cells could then attack Na^+,K^+-ATPase, thereby impairing ion homeostasis in the brain capillary. We speculate that such damage could exacerbate the biochemical changes observed in cytotoxic brain edema.

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