

Hypoxia effects on cell volume and ion uptake of cerebral microvascular endothelial cells

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Brillault J, Lam TI, Rutkowsky JM, Foroutan S, O'Donnell ME. Hypoxia effects on cell volume and ion uptake of cerebral microvascular endothelial cells. *Am J Physiol Cell Physiol* 294: C88–C96, 2008. First published October 17, 2007; doi:10.1152/ajpcell.00148.2007.—Increased transport of Na across an intact blood-brain barrier (BBB) contributes to cerebral edema formation in ischemic stroke. Our previous studies have shown that ischemic factors stimulate activity of a luminal BBB Na-K-Cl cotransporter, and we have hypothesized that during ischemia, the cotransporter together with the abluminal Na/K pump mediates increased transport of Na from blood into the brain. However, it is possible that elevated Na-K-Cl cotransporter activity could also cause cell swelling if it outpaces ion efflux pathways. The present study was conducted to evaluate the effects of hypoxia on intracellular volume of BBB cells. Cerebral microvascular endothelial cell (CMEC) monolayers were exposed to varying levels of hypoxia for 1 to 5 h in an O₂-controlled glove box, and cell volume was assessed using 3-O-methyl-D-[³H]glucose and [¹⁴C]sucrose as markers of total and extracellular water space, respectively. Cells exposed to either 7.5%, 3%, or 1% O₂ showed gradual increases in volume (compared with 19% O₂ normoxic controls) that became significant after 3 or more hours. By ion chromatography methods, we also found that a 30-min exposure to 7.5% O₂ caused an increase in bumetanide-sensitive net Na uptake by the cells without increasing cell Na content. CMEC Na content was significantly increased, however, following 3 or more hours of exposure to 7.5% O₂. These findings are consistent with the hypothesis that during cerebral ischemia, the BBB Na-K-Cl cotransporter is stimulated to mediate transendothelial uptake of Na into the brain and that increased cotransporter activity also contributes to gradual swelling of the cells.

cotransport; brain microvessels; cerebral ischemia; cerebral edema; bumetanide

IN THE EARLY HOURS of ischemic stroke, brain edema forms in the presence of an intact blood-brain barrier (BBB) by a process involving net uptake of Na and water across the barrier from blood into the brain with barrier breakdown not occurring until 4–6 h after the onset of ischemia (4, 14, 18, 26, 32, 41). Previous studies have provided evidence that Na transporters in the luminal BBB membrane working in conjunction with abluminal Na/K pump and Cl efflux pathways play a major role in this process (41, 48). Ischemic conditions also stimulate astrocyte transporters, causing increased uptake of ions and water and swelling of the astrocytes (6, 14, 19, 25). However, because total brain Na and water increase in ischemia, astrocyte swelling cannot be explained by a simple uptake from the brain extracellular space. Rather, the astrocytes swell largely by taking up ions and water that cross the BBB from blood into the extracellular space of the brain. In this regard, increased

BBB secretion of Na and water into the brain during ischemia facilitates brain edema formation. Our previous studies have provided evidence that a Na-K-Cl cotransporter in the BBB luminal membrane is stimulated during ischemia to participate in the increased secretion of Na and water from blood into the brain that underlies cerebral edema formation. In immunoelectron microscopy studies, we found that the Na-K-Cl cotransporter is located predominantly in the luminal membrane of BBB endothelial cells in situ (37). We have also demonstrated that in the rat middle cerebral artery occlusion (MCAO) model of stroke, intravenous administration of the Na-K-Cl cotransport inhibitor bumetanide greatly reduces edema formation occurring in the early hours of stroke before BBB breakdown (37). In addition, studies evaluating Na-K-Cl cotransporter activity in cultured cerebral microvascular endothelial cells (CMEC) have revealed that the cotransporter is stimulated by hypoxia (12, 22), aglycemia (12), and arginine vasopressin (AVP) (34), three prominent factors present in cerebral ischemia. Together, these findings provide support for the hypothesis that the BBB Na-K-Cl cotransporter serves as a luminal Na and Cl influx pathway during ischemia-induced BBB Na and Cl secretion and subsequent edema formation.

The Na-K-Cl cotransporter functions not only in vectorial ion transport across epithelial cells but also in cell volume regulation of many cell types, including epithelia (15, 16, 40). Our previous studies have shown that the cotransporter of aortic endothelial cells mediates the regulatory volume increase, a restoration of intracellular volume that follows shrinkage of the cells in hyperosmotic media (33). Hyperosmotic media also stimulate activity of the CMEC Na-K-Cl cotransporter, suggesting a role for the cotransporter in the regulatory volume increase of BBB endothelial cells as well (36). We (33) have also shown that AVP and other factors that stimulate the aortic endothelial cell cotransporter can drive an increase in volume of those cells. In the case of BBB endothelial cells, we predict that stimulation of Na-K-Cl cotransporter activity by ischemic factors, when coupled to abluminal Na and Cl efflux pathways (e.g., Na/K pump and Cl channel), will result in Na, Cl, and water secretion across the BBB. However, if BBB Na-K-Cl cotransporter-mediated Na and Cl influx is not coupled to Na and Cl efflux pathways, increased cotransporter activity could cause swelling of the BBB endothelial cells in addition to or instead of secretion of Na and Cl across the barrier. The present study was conducted to evaluate the effects of ischemic factors, including hypoxia, aglycemia, and AVP, on intracellular volume of BBB endothelial cells to determine whether, and if so when, stimulation of the cotrans-

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porter might result in swelling of the cells. In these studies, we have also evaluated the effects of hypoxia on CMEC Na and K content and on Na-K-Cl cotransporter-mediated net Na uptake into the cells. We report here that CMEC intracellular volume gradually increases following exposure to hypoxia (at levels ranging from 7.5% to 1% O₂), becoming significant after 3 or more hours. We also present evidence that the increase in CMEC volume observed after 3 or more hours of hypoxia is accompanied by an increase in cell Na content and that while exposures of <3 h do not significantly increase cell volume, Na-K-Cl cotransporter-mediated net Na influx is significantly increased after a 30-min exposure to hypoxia. Finally, we report that the hypoxia-induced cell swelling observed after 5 h is significantly reduced by bumetanide inhibition of the Na-K-Cl cotransporter and also by the Na/H exchange inhibitor HOE-642. Together, these findings provide further support for the hypothesis that the BBB Na-K-Cl cotransporter is stimulated during the early stages of cerebral ischemia to participate in edema formation through increased secretion of Na and water from blood into the brain. They also indicate that the gradual cell swelling that occurs 3 or more hours after the onset of ischemia involves activity of both BBB Na-K-Cl cotransporter and Na/H exchanger.

MATERIALS AND METHODS

Cell culture. Bovine CMEC were cultured on rat tail collagen- and fibronectin-coated multiwell plates in Dulbecco's modified Eagle's medium (DMEM) in a 95% air-5% CO₂ atmosphere. DMEM growth medium contained 5 mM D-glucose, 1 mM Na-pyruvate, supplemented with 2 mM L-glutamine, 50 µg/ml gentamicin, 1 ng/ml basic fibroblast growth factor, 5% horse serum, and 5% calf serum. Growth medium was replaced with fresh medium every other day. Cells were refed with a 50:50 (vol/vol) mixture of fresh DMEM containing 10% fetal bovine serum (FBS) and astrocyte-conditioned medium (ACM) 24 to 48 h before each experiment. ACM was prepared as described previously (12, 36). CMEC used for this study were obtained from Cell Systems (Kirkland, WA). Astrocytes were isolated and cultured as described by us previously (36, 45).

Electron microscopy. Rat brains subjected to left MCAO for 90 min were fixed for 60 min using 4% paraformaldehyde plus 0.05% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed, then postfixed in 4% paraformaldehyde overnight, and subsequently subjected to freeze substitution as described previously (13). Tissues were embedded in lowiacryl resin and sectioned onto Butvar-coated grids, and then sections were immunolabeled with T4 antibody (monoclonal antibody that recognizes Na-K-Cl cotransport protein) and 15-nm gold particle-conjugated anti-mouse IgG as described by us previously (37). Sections were stained with uranyl acetate and lead citrate before being observed on a Philips 410 electron microscope.

Cell volume measurements. Intracellular volume was determined by radioisotopic evaluation of intracellular water space by a modification of previously described methods (3, 27). Confluent CMEC monolayers were placed in a hypoxia chamber (COY Laboratory Products, Grass Lake, MI) preset to 37°C and the desired O₂ level and 5% CO₂. The O₂ levels were reduced by displacing air in the chamber with N₂ (via O₂ sensor/regulator) and verified using a Corning dissolved O₂ sensor with Checkmate II meter (Corning, NY). O₂ levels ranged from the normoxic control of 19% O₂ (inclusion of 5% CO₂ reduces the O₂ level from 20% to 19%) down to 1% O₂. Growth medium in each well was immediately replaced with assay medium preequilibrated to the desired O₂ level. Assay medium used was DMEM with 5 mM glucose and 1 mM pyruvate, also containing 10 mM HEPES (DMEM HEPES). For experiments using glucose-free

medium, both glucose and pyruvate were omitted from the DMEM HEPES. CMEC were exposed to DMEM HEPES with the desired O₂ level for 1 to 5 h. The osmolarity of all assay media was 290 mosM (verified by osmometry). During the last 20 min of the hypoxia exposure period, 0.25 µCi/ml of 3-O-methyl-D-[³H]glucose (a marker of total water space, both intracellular and trapped extracellular) and [¹⁴C]sucrose (a marker of trapped extracellular water space) were added to the assay media. In some experiments, AVP, bumetanide, and/or HOE-642 were also present in the assay media, as indicated in the figure legends. The assay was terminated by washing monolayers with ice-cold isotonic MgCl₂. Cell-associated radioactivity was determined by liquid scintillation counting following extraction in 0.2% SDS. The total protein in each well was measured using the BCA assay (Pierce, Rockford, IL). Cell volume was calculated as total water space minus trapped extracellular water space (expressed as µl/mg of proteins). In initial experiments conducted to optimize these methods, cells were exposed to 3-O-methyl-D-[³H]glucose or [¹⁴C]sucrose over a time course of 1 to 45 min, and it was determined that for both markers, radioisotopic equilibration was complete by 10 min, whether in normoxic or hypoxic media (data not shown). In a few initial experiments we also evaluated intracellular volume using [¹⁴C]urea and [³H]inulin as markers of total water space and trapped extracellular space, respectively, and obtained the same results as in experiments using 3-O-methyl-D-[³H]glucose or [¹⁴C]sucrose (data not shown).

Na and K content assays. Confluent monolayers of CMEC grown on 12-well plates were placed in the hypoxia chamber preset to 37°C, 5% CO₂, and either 19% O₂ or 7.5% O₂. Growth medium in each well was immediately replaced with hypoxia treatment media (DMEM HEPES as described for cell volume assays) preequilibrated to the desired O₂ level. Cells were exposed to the treatment media for 30 min or 1, 3, or 5 h. For net Na influx assays (Fig. 5 experiments), bumetanide (10 µM) or vehicle was added to the hypoxia treatment media during the final 5 min of the 30-min or 5-h treatment time. In all cases, the assay was terminated by rapidly washing monolayers three times with ice-cold iso-osmotic glucose solution to remove all extracellular ions. Cells were lysed in 500 µl of deionized water for 1 h. The lysate was then analyzed for Na and K content by ion chromatography. For this, lysates were loaded on a Dionex DX500 chromatograph (Dionex, Sunnyvale, CA) equipped with a CS14 column for cation quantitation (Na and K). The total protein content in each well was measured using the BCA assay. Bumetanide-sensitive net Na uptake was determined as the slope of a plot of change in cell Na content occurring over the 5-min assay in the presence versus absence of bumetanide, as we have described previously (33).

Materials. DMEM and L-glutamine were purchased from GIBCO-BRL (Grand Island, NY), and gentamicin was from AG Scientific (San Diego, CA). FBS and calf serum were obtained from HyClone (Logan, UT), and horse serum was from Sigma (St. Louis, MO). Bumetanide was purchased from ICN Biomedicals (Costa Mesa, CA). 3-O-methyl-D-[³H]glucose was obtained from Amersham (Piscataway, NJ). [¹⁴C]Sucrose and [¹⁴C]inulin were from Perkin Elmer (Boston, MA), and [¹⁴C]urea was purchased from DuPont NEN (Boston, MA). T4 monoclonal antibody was obtained from the University of Iowa Developmental Studies Hybridoma Bank (Iowa City, IA), and secondary antibodies were from Zymed Laboratories (South San Francisco, CA). (Arg⁸)-vasopressin (AVP) was purchased from Peninsula Laboratories (a division of Bachem, San Carlos, CA). HOE-642 was a gift from Sanofi-Aventis Pharmaceuticals (Bridge-water, NJ).

Statistics. All values shown are means ± SE. In each experiment, three to four replicates were taken for each condition. The *n* values used for statistics represent the number of experiments conducted. Data shown were analyzed for significance using paired Student *t*-test or analysis of variance as indicated. Differences with *P* values <0.05 were considered statistically significant.

RESULTS

Luminal membrane distribution of the BBB Na-K-Cl cotransporter is not altered by ischemia. Our previous studies have demonstrated that the BBB Na-K-Cl cotransporter is found predominantly in the luminal membrane in situ, consistent with the hypothesis that the cotransporter, working with the abluminal Na/K pump, participates in secretion of Na and water across the BBB from blood into the brain during early stages of cerebral ischemia. However, nothing is known about the in situ distribution of the BBB Na-K-Cl cotransporter in the ischemic brain. Thus in these studies we used immunoelectron microscopy methods to assess the plasma membrane distribution of the BBB cotransporter in the ischemic brain. For this, rats were subjected to permanent left MCAO for 90 min as described by us previously (37). Brains were perfusion fixed and then sections were prepared for immunoelectron microscopy as described in MATERIALS AND METHODS. Na-K-Cl cotransporter protein in brain sections was immunolabeled using T4 monoclonal antibody as primary antibody (using two different T4 antibody dilutions) and 15-nm gold particle-conjugated IgG as secondary antibody. Representative immunoelectron micrographs of ischemic cortex are shown in Fig. 1A. In these images, which were taken from frontoparietal cortex of the ipsilateral hemisphere (the occluded, ischemic left hemisphere), it can be seen that the Na-K-Cl cotransporter protein is indeed present in the luminal membrane of BBB endothelial cells. Quantitation of gold particles in electron micrographs showed that the cotransporter is distributed ~80% in the luminal membrane (with 20% in the abluminal membrane) whether we examined BBB of ipsilateral ischemic cortex or contralateral control normoxic cortex, as shown in Fig. 1B. In these experiments, we examined ipsilateral and contralateral cortex of both male and female rats subjected to MCAO and found no gender differences with respect to Na-K-Cl cotransporter protein distribution in the BBB of either normoxic or ischemic brain. Thus the cotransporter appears to be prominent in the luminal BBB membrane, whether in male or female, normoxic or ischemic cortex.

Effects of hypoxia, aglycemia, and vasopressin on cerebral microvascular endothelial cell volume. Whereas the presence of Na-K-Cl cotransporter protein in the luminal BBB membrane of ischemic cortex is consistent with a role for the cotransporter in ischemia-induced secretion of Na, Cl, and water from blood into the brain during ischemic stroke, it is possible that increased cotransporter activity during ischemia leads to BBB endothelial cell swelling in addition to, or even instead of, vectorial transport of Na and Cl across the BBB. To test this, we began by evaluating the effects of hypoxia on intracellular volume of cultured CMEC. We determined intracellular volume by radioisotopic equilibration methods using 3-O-methyl-D-[³H]glucose as a marker of total water space (intracellular and trapped extracellular) and ¹⁴[C]sucrose as a marker of trapped extracellular water space as described in MATERIALS AND METHODS. CMEC monolayers were exposed to varying levels of hypoxia for 1 to 5 h in a hypoxia chamber with O₂ levels ranging from 19% control (normoxia) down to 1% O₂. As shown in Fig. 2A, exposing CMEC to 1% O₂, a severe level of hypoxia as can be found in the core of an ischemic zone, caused a gradual increase in intracellular volume above control nonischemic volume. Whereas there was a

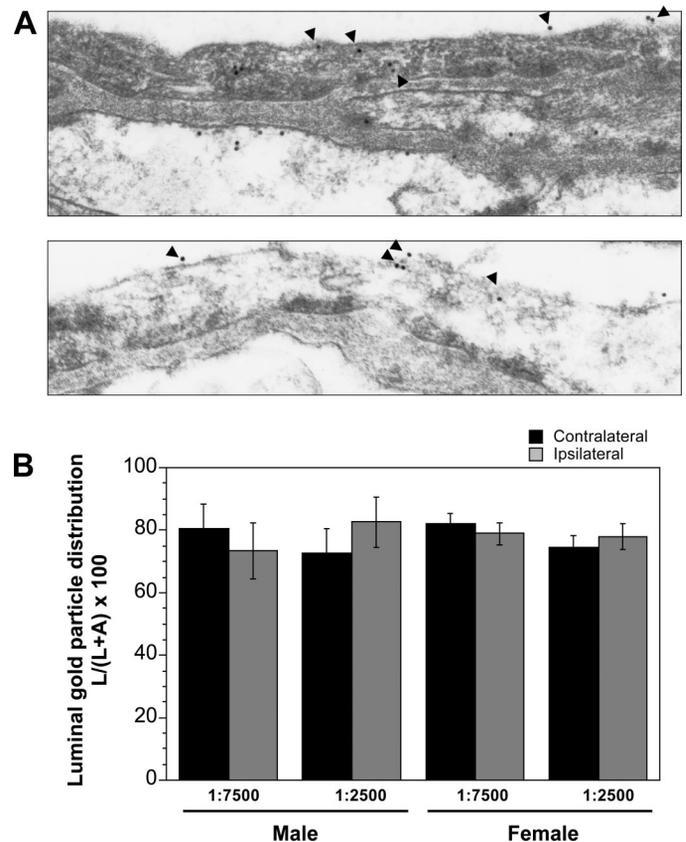


Fig. 1. Immunoelectron microscopy localization of Na-K-Cl cotransport protein in blood-brain barrier (BBB) of male and female ischemic rat brain. Rats were subjected to 90 min of left middle cerebral artery occlusion (MCAO), and then brains were perfusion fixed and prepared for immunoelectron microscopy as described in MATERIALS AND METHODS. Na-K-Cl cotransport protein was immunolabeled using T4 monoclonal antibody and 15-nm gold particle-conjugated secondary antibodies. A: representative immunoelectron micrographs of Na-K-Cl cotransporter distribution in plasma membrane of cerebral microvascular endothelial cell (CMEC) of ipsilateral (occluded) cortex of male rats. Top and bottom: micrographs from lateral and upper frontoparietal cortex regions, respectively. A T4 antibody dilution of 1:7,500 was used in both images. Arrowheads indicate the presence of gold particle-labeled cotransporter protein in the endothelial cells. In these images the microvessel lumen is at the top and perivascular astrocytic foot processes can be seen just below the basal lamina of the endothelium. Note that Na-K-Cl cotransporter protein is also present in the astrocytic foot process plasma membrane seen in the top micrograph. B: Na-K-Cl cotransporter distribution between luminal (L) and abluminal (A) microvascular endothelial membranes of ischemic (ipsilateral) and control (contralateral) rat frontoparietal cortex as determined by quantitation of gold particles in immunoelectron micrographs. Values shown are means \pm SE with *n* values of 7, 7, 27, and 23 for male 1:7,500, male 1:2,500, female 1:7,500, and female 1:2,500 contralateral microvessels, respectively, and *n* values of 8, 7, 19, and 14 for male 1:7,500, male 1:2,500, female 1:7,500, and female 1:2,500 ipsilateral microvessels, respectively. No significant differences were found among the values by ANOVA.

trend for volume to increase as early as 2 h, the increases became significant only after 4- and 5-h exposures, whether analyzed by ANOVA or Student's *t*-test. When CMEC were exposed to 3% O₂ (Fig. 2B), a gradual increase in cell volume also occurred, in this case reaching significance after 3 to 4 h, depending on the statistical analysis method (after 3 h by Student's *t*-test, 4 h by ANOVA). This is similar to what we have reported previously for CMEC exposed to 7.5% O₂, a moderate level of hypoxia as can be found in the penumbra of an ischemic zone (12) (these data are also included as part of

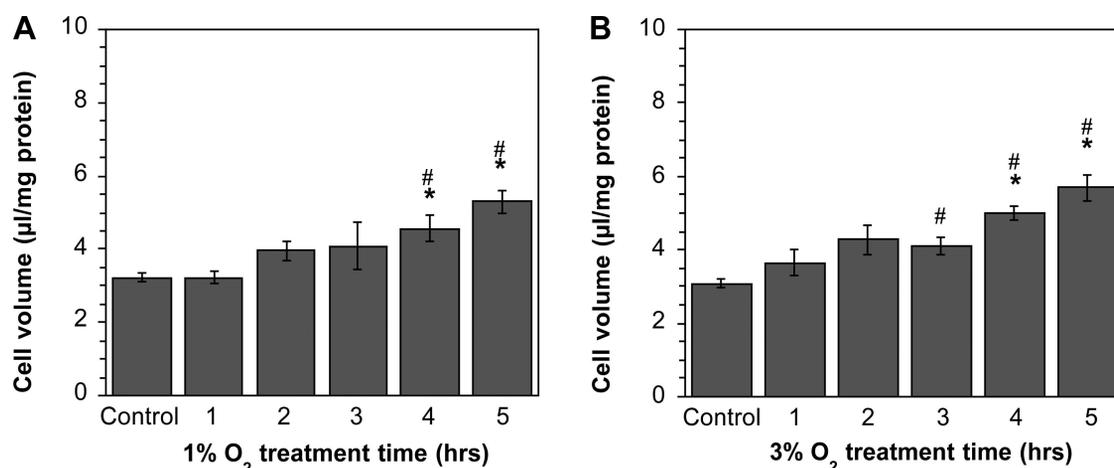


Fig. 2. Effect of hypoxia on CMEC intracellular volume. Confluent CMEC monolayers were subjected to 19% O₂ (control) or to 1% or 3% O₂ (A and B, respectively) for 1 to 5 h in glucose-containing DMEM HEPES medium at 37°C using a hypoxia chamber. Cell volume was subsequently assessed as described in MATERIALS AND METHODS. Data are means \pm SE of 3 experiments. *Significantly different from control by ANOVA, $P < 0.05$. #Significantly different from control by Student's paired t -test, $P < 0.05$.

Table 1 for reference). In the case of 7.5% O₂, the gradual increase in cell volume also reaches significance after 3 h by Student's t -test and 4 h by ANOVA.

The studies shown in Fig. 2 were done in DMEM HEPES containing 5 mM glucose (and also 1 mM pyruvate). Because cerebral ischemia generally involves reduction of both O₂ and glucose, we also determined whether CMEC might swell more quickly if exposed to a combination of hypoxia and aglycemia. In previous studies of the effects of aglycemia on CMEC Na-K-Cl cotransporter activity, we found that exposing the cells to medium lacking both glucose and pyruvate resulted in stimulation of cotransporter activity, whereas simply omitting glucose (and retaining pyruvate) did not (12). Thus, in the present studies, the aglycemic pretreatment and assay media lacked both glucose and pyruvate. As shown in Table 1, exposing CMEC to 1 to 5 h of aglycemia under hypoxic conditions (7.5% O₂) caused a gradual increase in intracellular volume that reached significance after 4 or 5 h of exposure by

Table 1. CMEC intracellular volume: effects of hypoxia in glucose- and pyruvate-containing and glucose- and pyruvate-free media

7.5% O ₂ Treatment, h	Intracellular Volume, µl/mg protein	
	+Glucose	-Glucose
Control	1.00 \pm 0.05	1.00 \pm 0.26
1	1.30 \pm 0.14	1.40 \pm 0.12
2	1.38 \pm 0.19	1.63 \pm 0.38
3	1.70 \pm 0.27	1.58 \pm 0.17
4	1.86 \pm 0.29*	1.79 \pm 0.14*
5	2.43 \pm 0.33*	1.81 \pm 0.36*

Cerebral microvascular endothelial cell (CMEC) monolayers were exposed to 19% O₂ (control, 20 min exposure) or to 7.5% O₂ in a hypoxia chamber for varying times in either glucose- and pyruvate-containing medium (+Glucose) or in glucose- and pyruvate-free medium (-Glucose), and intracellular volume was evaluated as described in MATERIALS AND METHODS. Data are means \pm SE of 4 separate experiments each for +glucose and -glucose conditions. +Glucose results have been published previously (12) and are provided here only for reference. *Values significantly different from their respective control values by ANOVA, $P < 0.05$. Student's t -test analysis of the data showed significant increases in volume also after 3 h for +glucose experiments ($P < 0.05$) but not for -glucose experiments.

ANOVA. In this case, Student's t -test did not reveal significant increases at times earlier than 4 h. The cell volume increase occurring in response to 7.5% O₂ and aglycemia was not significantly different from what we have observed previously for CMEC exposed to 7.5% O₂ in glucose- and pyruvate-containing media (12) (left column Table 1, shown here for comparison). In a few experiments we also tested the effects of hypoxia and aglycemia on CMEC intracellular volume using media lacking glucose but containing pyruvate and found similar results (data not shown). These findings suggest that the combination of hypoxia/aglycemia does not produce more rapid or greater increases in CMEC swelling than that observed with hypoxia in the presence of glucose.

Another prominent factor present during cerebral ischemia is AVP, released from extrahypothalamic nerve processes at the abluminal BBB surface (20, 29, 44). Previous studies have provided evidence that AVP contributes to cerebral edema formation during ischemia (9) and also can promote cerebral edema formation when administered to normal, nonischemic rat brain (10, 39). We have found previously that AVP is a potent and rapid stimulator of CMEC Na-K-Cl cotransporter activity (34) and that it can increase intracellular volume of aortic endothelial cells via increased cotransporter activity (33). Thus in the present study we conducted experiments to determine whether the effect of AVP on CMEC includes an increase in intracellular volume. Figure 3 shows that exposing CMEC to AVP (100 nM) for 5 h did not cause a significant increase in cell volume compared with CMEC exposed to vehicle (DMEM HEPES containing glucose and pyruvate) for 5 h, either in the presence of normoxia (19% O₂) or hypoxia (7.5% O₂). Evaluation of cell volume following 1- and 3-h exposures to AVP (100 nM) also revealed no significant changes. Relative to control, cell volumes were 0.97 ± 0.16 , 1.00 ± 0.13 , and 1.02 ± 0.13 after 1-, 3-, and 5-h exposures, respectively, to 7.5% O₂.

Effects of hypoxia on CMEC Na and K content and cotransporter-mediated net Na flux. To further evaluate the possibility that hypoxia stimulation of the BBB Na-K-Cl cotransporter causes swelling of the cells in addition to, or even instead of, secretion of Na and Cl into the brain, we examined the effects

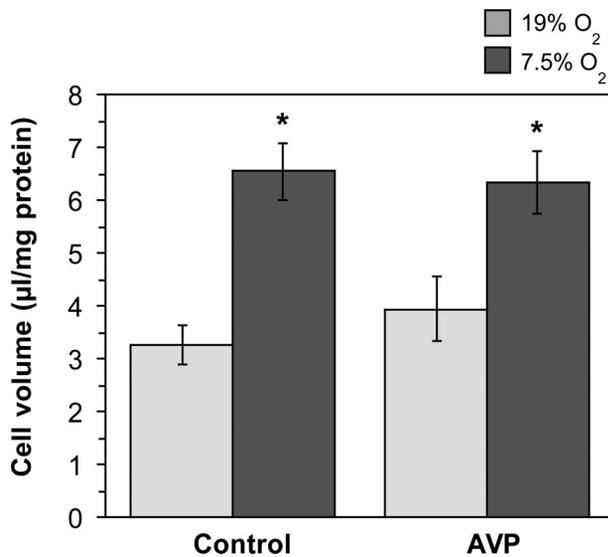


Fig. 3. Effect of arginine vasopressin (AVP) on CMEC intracellular volume. Confluent CMEC monolayers were exposed to AVP (100 nM) or vehicle (control) for 5 h in the presence of normoxia (19% O₂) or hypoxia (7.5% O₂) in glucose-containing DMEM HEPES as described in MATERIALS AND METHODS. Data are means \pm SE of 3 experiments with 3 replicates per experiment. *Significantly different from corresponding 19% O₂ condition, $P < 0.05$ by Students t -test.

of hypoxia on CMEC ion content. Here, we used ion chromatography methods to evaluate CMEC Na and K content after exposing the cells to 30 min to 5 h of 7.5% O₂ or 19% O₂ (control normoxia). As shown in Fig. 4A, we found that CMEC cell Na content was unchanged after 30 min or 1 h of hypoxia (7.5% O₂) compared with that of the normoxic control (19% O₂) but that it was significantly elevated after 3 or 5 h of exposure to 7.5% O₂ (1.82 \pm 0.22- and 1.94 \pm 0.16-fold increases, respectively). When we exposed CMEC to 7.5% O₂ for 5 h in the presence of bumetanide to inhibit Na-K-Cl cotransporter activity, we found that the hypoxia-induced increase in cell Na content was reduced by 42.2 \pm 8.8% ($n = 3$). This suggests that the cotransporter is responsible for at least a portion of the hypoxia-induced increase in Na content. This

issue is addressed further in the DISCUSSION. In each experiment we evaluated CMEC K content along with Na content (Fig. 4B). Here, we found that K content was unchanged following exposure to 7.5% O₂ for 30 min or 5 h. K content was also not altered following exposures of 1 or 3 h. Here, relative values for CMEC K content were 1.0 \pm 0.09 for normoxic control cells and 1.10 \pm 0.10, 0.99 \pm 0.03, 1.00 \pm 0.03, and 1.07 \pm 0.06 for 30 min and 1, 3, and 5 h of 7.5% O₂, respectively.

In these ion chromatography experiments we also evaluated Na-K-Cl cotransporter-mediated net Na uptake in CMEC exposed to hypoxia versus normoxia. Figure 5 shows that, in contrast to the slow onset hypoxia-induced elevation of CMEC Na content, the bumetanide-sensitive net Na uptake, i.e., Na-K-Cl cotransporter-mediated net influx of Na into the cells, was rapidly increased by hypoxia. Here we found that net Na influx was significantly increased (by 2.1-fold) after just 30 min of hypoxia (7.5% O₂). After 5 h of hypoxia, cotransporter-mediated net influx was 1.4-fold above control levels, although this was not found to be statistically different from control. These findings suggest that hypoxia stimulation of CMEC Na-K-Cl cotransporter-mediated net Na influx precedes hypoxia-induced elevation of Na content and swelling of the cells.

Bumetanide and HOE-642 effects on hypoxia-induced CMEC swelling. As an initial investigation of the extent to which the CMEC Na-K-Cl cotransporter contributes to hypoxia-induced swelling of the cells, we evaluated the effect of hypoxia on CMEC volume when cotransporter activity was inhibited by bumetanide. As shown in Fig. 6, when CMEC were exposed to 5 h of hypoxia (7.5% O₂) in the presence of bumetanide (10 μ M), the hypoxia-induced increase in intracellular volume was significantly reduced although not abolished. Previous studies have shown that Na/H exchange can contribute to ischemia-induced swelling of other cell types (38). Several studies have provided evidence that Na/H exchange is present in BBB endothelial cells (21, 43), and our own recent studies have found evidence that Na/H exchange activity in CMEC is stimulated by ischemic conditions (28). Thus, in the present study, we also evaluated the effect of the potent, highly selective Na/H exchange inhibitor HOE-642 (31, 42) on hy-

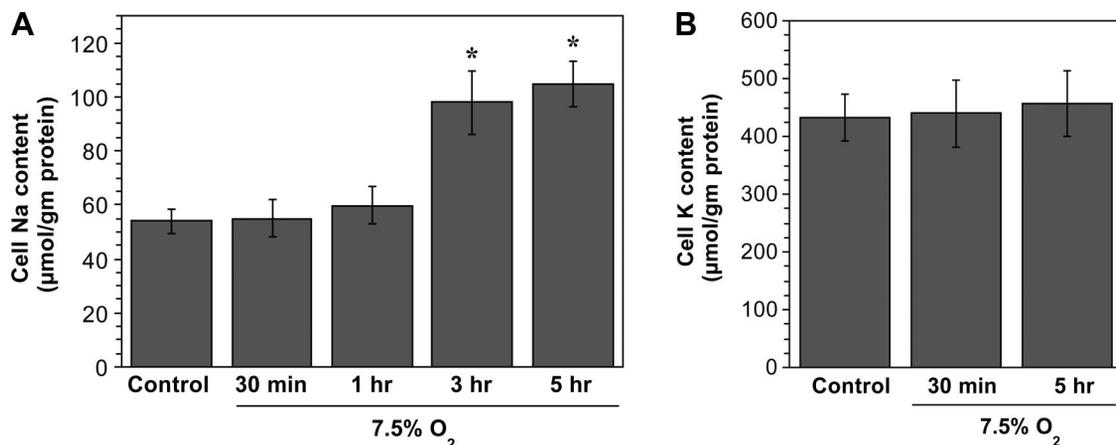


Fig. 4. Effect of hypoxia on CMEC intracellular Na and K contents. CMEC monolayers were subjected to normoxia or to hypoxia (7.5% O₂) for 30 min or 5 h in glucose-containing DMEM HEPES or to normoxia (control) for 5 h. Na and K cell contents (A and B, respectively) of the cells were then determined by ion chromatography as described in MATERIALS AND METHODS. Data are means \pm SE of 13, 10, 6.6 and 13 experiments for control (5 h normoxia), and 30 min, 1 h, 3 h, and 5 h 7.5% O₂, respectively for A; and 13, 11, and 12 experiments for control (5 h normoxia), 30 min 7.5% O₂, and 5 h 7.5% O₂, respectively for B. *Significantly different from control, $P < 0.05$ by ANOVA.

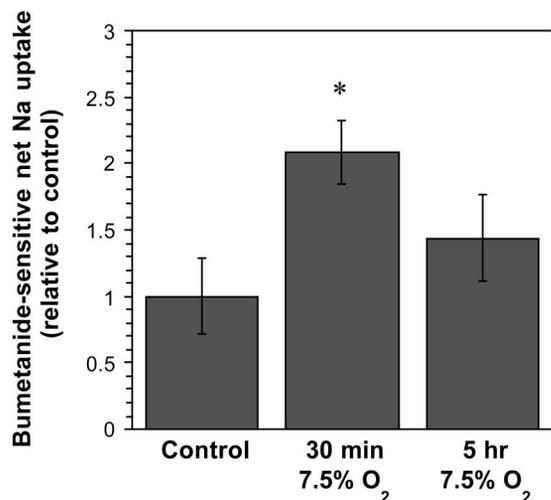


Fig. 5. Effect of hypoxia on Na-K-Cl cotransporter-mediated net Na uptake by CMEC. CMEC monolayers were subjected to normoxia or to hypoxia (7.5% O₂) for 30 min or 5 h in glucose-containing DMEM HEPES. To evaluate net Na influx, bumetanide (10 μM) or vehicle was added to the normoxic or hypoxic treatment medium during the final 5 min of the 30-min or 5-h treatment time. Cell Na content was then determined by ion chromatography, and bumetanide-sensitive net Na uptake was calculated as the rate of change of cell Na content occurring over the 5-min assay in the presence versus absence of bumetanide, as described in MATERIAL AND METHODS. Data are means ± SE of 4, 3, and 3 experiments for control (5 h normoxia), and 30 min and 5 h of hypoxia, respectively. *Significantly different from control, $P < 0.05$ by ANOVA.

poxia-induced swelling of CMEC. We found that, like bumetanide, HOE-642 (2 μM) significantly attenuated, but did not abolish, the increase in CMEC intracellular volume after 5 h of hypoxia (Fig. 6). In experiments using a higher dose of HOE-642 (200 μM), we did not find a greater reduction of the hypoxia-induced CMEC swelling. CMEC intracellular volume after 5 h of 7.5% O₂ in the presence of 200 μM HOE-642 was 4.77 ± 0.16 μl/mg protein (2 separate experiments each with 8–9 replicates) compared with 5.26 ± 0.105 μl/mg protein in the presence of 2 μM HOE-642 (Fig. 6). When the cells were exposed to 5 h of hypoxia in the combined presence of bumetanide and HOE-642 (2 or 200 μM), the hypoxia-induced increase in cell volume was abolished. These findings suggest that hypoxia-induced CMEC swelling is in some manner dependent on activity of both the Na-K-Cl cotransporter and Na/H exchanger.

DISCUSSION

Our previous studies have suggested that a Na-K-Cl cotransporter present in the luminal membrane of BBB endothelial cells is stimulated by ischemic factors to participate in brain edema formation during stroke. Whereas our findings have supported the hypothesis that during the early hours of ischemia, the luminal BBB Na-K-Cl cotransporter is functionally coupled to abluminal Na/K pump and an abluminal Cl efflux pathway such that Na (along with Cl and water) is secreted across the BBB from blood into the brain, we had not previously addressed the important question of whether, and under what ischemic conditions, the increased BBB Na-K-Cl cotransporter activity might also cause swelling of the endothelial cells. The results of the present study provide evidence that hypoxia increases BBB Na-K-Cl cotransporter-mediated Na

influx after just 30 min exposure, while also causing slower onset, gradual increases in cell Na content and intracellular volume that become significant by 3 or more hours exposure. Our study also shows that the increases in cell volume and Na content are attenuated by bumetanide. These findings are consistent with the hypothesis that the BBB cotransporter participates in ischemia-induced cerebral edema formation, first through increased secretion of Na, Cl, and water into the brain and later also through swelling of the BBB endothelial cells.

The findings of the present study reveal for the first time that Na-K-Cl cotransporter protein in ischemic rat brain is distributed predominantly in the luminal BBB, as it is in the healthy, nonischemic brain. We also show for the first time that there are no gender differences with respect to BBB distribution of the cotransporter, an important observation given our previous finding that estradiol reduces abundance of BBB Na-K-Cl cotransporter protein in CMEC (35). In the present study we did not evaluate whether the total amount of BBB Na-K-Cl cotransporter protein is altered by ischemia. However, in previous studies we found that moderate to severe hypoxia (7.5% O₂ for 30 to 80 min and 2% O₂ for 120 to 240 min, respectively) does not alter CMEC Na-K-Cl cotransporter protein abundance (12). Finally, whereas we have not yet evaluated BBB cotransporter distribution beyond 90 min of MCAO, the present findings show that during early stroke, when the majority of edema forms, the cotransporter has not changed its distribution from that observed in normoxic brains.

The present study also reveals that hypoxia stimulation of CMEC Na-K-Cl cotransporter activity is associated with gradual swelling of the cells that reaches statistical significance after 3 or more hours. In contrast to this, we found that

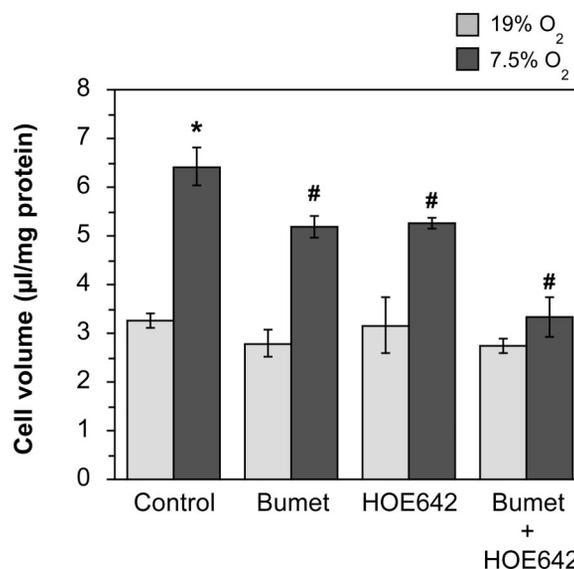


Fig. 6. Effects of bumetanide and HOE-642 on the hypoxia-induced increase in CMEC intracellular volume. Confluent CMEC monolayers were subjected to normoxia (19% O₂) or hypoxia (7.5% O₂) in the presence of bumetanide (0 or 10 μM) and/or HOE-642 (0 or 2 μM) for 5 h in glucose-containing media and intracellular volume assessed as described in MATERIALS AND METHODS. Data are means ± SE of 3 experiments. #Significantly different from normoxic control, $P < 0.05$ by ANOVA for Bumet and HOE-642, $P < 0.001$ by ANOVA for Bumet + HOE-642. *Significantly different from hypoxic (7.5% O₂) control, $P < 0.001$ by ANOVA.

aglycemia is without significant effect on CMEC intracellular volume. This is despite the fact that both aglycemia and hypoxia stimulate CMEC Na-K-Cl cotransporter activity, with the two having additive effects over the evaluated 30-min to 3-h exposure time (12). In these studies we did not test the effect of prolonged exposure to aglycemia on CMEC volume under normoxic conditions, although we did observe that a 20-min exposure of the cells to aglycemia alone did not cause cell swelling (data not shown). Whether longer exposures to aglycemic normoxic media might alter CMEC intracellular volume remains to be determined. Our studies also show that AVP, a third factor present during cerebral ischemia, is without effect on CMEC intracellular volume over exposures ranging from 30 min up to 5 h, whether in the presence or absence of hypoxia. This is despite the fact the AVP causes a rapid and sustained stimulation of CMEC Na-K-Cl cotransporter activity, i.e., activity is increased after 5 min as well as after 4, 18, and 36 h of exposure (34). These findings are in contrast to the observation that AVP stimulates Na-K-Cl cotransporter activity and increases intracellular volume of aortic endothelial cells (33). Thus our present findings suggest that of the three ischemic factors evaluated, only hypoxia induces CMEC swelling. Further studies are needed to determine whether other factors present during cerebral ischemia, e.g., endothelin, which has been shown to stimulate CMEC Na-K-Cl cotransporter activity (23, 24), contribute to BBB endothelial cell swelling during ischemia. Future studies will also need to fully address the mechanisms underlying hypoxia-induced swelling of the BBB endothelial cells.

Whereas our studies do not reveal significant CMEC swelling until 3 or more hours of hypoxia, whether moderate or severe, a previous electron microscopy study found cytoplasmic swelling in caudate putamen capillary endothelial cells after just 90 min of MCAO (30). It should be noted, however, that in that study little swelling occurred in capillary endothelial cells of the frontoparietal cortex. Because secretion across the BBB simply requires that luminal BBB Na and Cl influx is coupled to abluminal Na and Cl efflux, it is important to recognize that a modest degree of BBB endothelial swelling does not preclude the occurrence of secretion. A Na-K-Cl cotransporter-mediated cell volume increase may even precede activation of an abluminal ion efflux pathway, e.g., a swelling-activated Cl channel as has been reported to reside in CMEC (47). Similar phenomena have been described in a variety of Na- and Cl-transporting epithelia (2, 11, 40). With regard to maintaining coupling of Na-K-Cl cotransporter and Na/K pump during ischemia-induced Na and Cl secretion across the BBB, we have shown previously that Na/K pump activity and cell ATP levels do not fall for at least 4 h when CMEC are exposed to hypoxia (7.5% or 2% O₂) or combined hypoxia/aglycemia and that even after 24 h of 2% O₂ (with or without aglycemia), the CMEC ATP content remains at ~60% of normoxic levels (12). Furthermore, in those studies we found that hypoxia increases Na/K pump-mediated K influx in the absence, but not the presence, of bumetanide, suggesting functional coupling of cotransporter and pump (12). Collectively, these observations support the hypothesis that the increased BBB Na-K-Cl cotransporter activity occurring during ischemia contributes to Na and Cl secretion across the barrier in addition to promoting a slower onset cell swelling.

Our studies demonstrate that the hypoxia-induced increase in CMEC intracellular volume is associated with an increase in Na content of the cells. Thus, whereas Na content is not altered after 30 min or 1 h of hypoxia (7.5% O₂), it is significantly elevated after 3 and 5 h of hypoxia, consistent with the time course of cell swelling. Furthermore, although 30 min of hypoxia exposure does not change CMEC Na content, it does significantly increase Na-K-Cl cotransporter-mediated net Na uptake into the cells, as one would predict if the cotransporter participates in secretion across the BBB during the early stages of cerebral ischemia. The magnitude of bumetanide-sensitive net Na influx that we observed in CMEC under normoxic control conditions is somewhat lower than what we found previously in aortic endothelial cells (33). Whether this reflects a difference in cotransporter function in the BBB versus large vessel endothelial cells remains to be determined. Whereas the CMEC bumetanide-sensitive net Na influx is increased twofold by 30 min of exposure to hypoxia (7.5% O₂), it falls toward control levels after 5 h of hypoxia. This is consistent with our previous observation that Na-K-Cl cotransporter activity, evaluated by radioisotopic flux methods, is increased approximately twofold by 30 min of hypoxia (7.5% O₂) and is decreased back toward normoxic levels by 3 h (12). Unlike Na, in the present study, cell K content did not change following hypoxia exposures (7.5% O₂) of either 30 min or 5 h. This suggests that CMEC K influx occurring via Na-K-Cl cotransporter and Na/K pump is matched (i.e., recycled out of the cell) by K efflux pathways, which likely includes the luminal K channel described previously (46), and hypothesized to participate in absorption of K from the brain into blood across the BBB during stroke (1, 5). One might predict that measuring bumetanide-sensitive transendothelial Na flux across cultured CMEC monolayers would provide a direct measure of Na-K-Cl cotransporter participation in hypoxia-induced Na secretion across the cells. However, doing this requires use of CMEC monolayers with quite high resistances to reliably measure transcellular ion fluxes with relatively little paracellular flux background. Whereas quantitative transcellular ion flux measurements of some epithelial cultures grown on filters are readily evaluated (8, 17), this has proved to be more difficult with CMEC. Thus quantitative evaluation of transendothelial ion fluxes across cultured CMEC monolayers awaits optimization of existing CMEC *in vitro* BBB models.

Finally, our studies show that hypoxia-induced CMEC swelling is partially dependent on Na-K-Cl cotransporter activity. This is because bumetanide, at a dose that fully inhibits cotransporter activity, reduces but does not abolish, CMEC swelling observed after 5 h of hypoxia. Swelling also appears to be partially dependent on Na/H exchange activity because the Na/H exchange inhibitor HOE-642 also attenuates the hypoxia-induced CMEC volume increase. Bumetanide and HOE-642 in combination abolish CMEC swelling after 5 h of hypoxia, suggesting that together, the Na-K-Cl cotransporter and Na/H exchanger are in some manner responsible for hypoxia-induced increases in CMEC volume. Many factors can contribute to cell swelling during ischemia and thus, additional studies will be needed to clarify the events occurring in hypoxia that lead to the observed CMEC volume increases. It is noteworthy that there was a trend for smaller apparent increases in cell volume at lower O₂ levels. Thus, for example, volume was increased by 23% and 41% after 2 and 4 h,

respectively, at 1% O₂; by 39% and 62% after 2 and 4 h, respectively, at 3% O₂; and by 38% and 86% after 2 and 4 h, respectively at 7.5% O₂. This suggests that the mechanisms underlying the increase in volume are energy dependent. The consequences of BBB endothelial cell swelling during ischemia are not entirely known, although BBB endothelial cell swelling is predicted to decrease capillary lumen diameter, thereby further reducing blood flow during ischemic stroke and worsening edema and infarct. It should be noted that the time course of hypoxia-induced CMEC swelling observed here is consistent with the time course reported for disruption of the BBB in MCAO-induced ischemia (26, 32) and in in vitro experiments of CMEC monolayers (7). Whether increased CMEC intracellular volume is a contributing factor to BBB breakdown or is merely coincidental remains to be determined.

In summary, the present study provides evidence that hypoxia causes a rapid increase in CMEC Na-K-Cl cotransporter-mediated net Na influx and a slower onset, gradual increase in cell Na content and intracellular volume that is attenuated by bumetanide. These findings, together with the observation that Na-K-Cl cotransporter protein is present in luminal BBB membranes of ischemic brain, suggest that the Na-K-Cl cotransporter participates in both increased secretion of Na, Cl and water into the brain and swelling of the BBB endothelial cells during ischemic stroke.

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DISCLOSURES

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