



Protection against hypoxia-induced blood-brain barrier disruption: changes in intracellular calcium

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Brown, Rachel C., Karen S. Mark, Richard D. Egleton, and Thomas P. Davis. Protection against hypoxia-induced blood-brain barrier disruption: changes in intracellular calcium. *Am J Physiol Cell Physiol* 286: C1045–C1052, 2004. First published December 18, 2003; 10.1152/ajpcell.00360.2003.—Tissue damage after stroke is partly due to disruption of the blood-brain barrier (BBB). Little is known about the role of calcium in modulating BBB disruption. We investigated the effect of hypoxic and aglycemic stress on BBB function and intracellular calcium levels. Bovine brain microvessel endothelial cells were treated with A-23187 to increase intracellular calcium without hypoxia or treated with a calcium chelator (BAPTA) or calcium channel blockers (nifedipine or SKF-96365) and 6 h of hypoxia. A-23187 alone did not increase paracellular permeability. Hypoxia increased intracellular calcium, and hypoxia or hypoxia-aglycemia increased paracellular permeability. Treatment with nifedipine and SKF-96365 increased intracellular calcium under normoglycemic conditions, instead of blocking calcium influx, and was protective against hypoxia-induced BBB disruption under normoglycemia. Protection by nifedipine and SKF-96365 was not due to antioxidant properties of these compounds. These data indicate that increased intracellular calcium alone is not enough to disrupt the BBB. However, increased intracellular calcium after drug treatment and hypoxia suggests a potential mechanism for these drugs in BBB protection; nifedipine and SKF-96365 plus hypoxic stress may trigger calcium-mediated signaling cascades, altering BBB integrity.

nifedipine; SKF-96365; ischemia; permeability; fura 2

IONIC HOMEOSTASIS of the central nervous system (CNS) is critical for normal brain function and is dependent on the blood-brain barrier (BBB). This barrier, which isolates the brain from the peripheral circulation, is located at the level of the cerebral microvessel endothelial cells. The BBB is characterized by tight cell-cell contacts with limited paracellular diffusion and reduced fluid-phase endocytosis (56). The presence of specific endothelial cell transporters for ions, peptides, and nutrients allows for strict regulation of the CNS microenvironment. Disruption of the BBB occurs in a number of pathological conditions, including Alzheimer's disease, diabetes, multiple sclerosis, inflammatory pain, and stroke (1, 4, 28, 33, 39), and can contribute to CNS edema formation (45, 52).

The restrictive nature of the BBB is due to tight junctions (TJ) formed between adjacent endothelial cells in the brain capillaries (42). The TJ restricts ion flux and paracellular diffusion of macromolecules. A number of the proteins that compose TJ have been identified and extensively characterized, including the claudins (26, 54), occludin (25), and zonula occludens (ZO)-1, -2, and -3, which interact with claudins and

occludin (37, 53). Stabilization of TJ involves a complex network of the transmembrane proteins occludin and claudins, linked to the actin cytoskeleton by accessory ZO proteins, which mediate this linkage by binding the cytoplasmic tails of occludin and claudin to actin (32).

Calcium is critical to normal BBB function (13). Endothelial cells incubated in calcium-free medium had decreased trans-endothelial electrical resistance readings and increased monolayer paracellular permeability (58), indicating disruption of TJ. In brain microvessel endothelial cells in culture, hypoxia caused an increase in intracellular calcium (35, 41, 55), whereas blockade of calcium flux (1) or blockade of calcium-regulated signaling cascades (55) prevented hypoxia-induced disruption of BBB monolayer integrity.

Stroke is the third leading cause of death in the United States and is the leading cause of long-term disability, affecting ~500,000 patients every year (50). Although the neuronal damage associated with stroke is due in part to lack of oxygen and nutrients during an ischemic event, reperfusion, the restoration of oxygen supply, and subsequent production of free radicals are also major contributors in mediating neuronal damage and death (50). Ischemic damage to blood vessels, in particular to the brain microvessel endothelial cells that make up the BBB, results in deregulation of ion flux and increased ion permeability into the brain, leading to a net influx of solutes and water and subsequent edema formation. Several studies found increased paracellular permeability after hypoxic or ischemic insult (1, 5, 30), suggesting disruption of the TJ. Transient ischemia in rats increased extravasation of Evans blue albumin, a normally impermeable marker (5). This increase in paracellular permeability is correlated with the degree of tissue damage. A number of *in vitro* models have been used to investigate the effects of hypoxia and aglycemia on the BBB. Hypoxia and aglycemia increased the paracellular permeability of bovine brain microvessel endothelial cell (BBMEC) monolayers (1). Nifedipine (NIF), an L-type calcium channel antagonist, and SKF-96365 (SKF), an inhibitor of some store-operated calcium channels, blocked this increase in permeability, indicating a potential role for calcium in mediating some of the hypoxia- and/or aglycemia-induced alterations in BBB integrity. In the present study, we investigated the effect of the calcium channel blockers NIF and SKF on intracellular calcium levels, BBMEC monolayer permeability, and reactive oxygen species (ROS) generation to elucidate potential mechanisms by which calcium might be involved in mediating BBB integrity and function after a hypoxic and/or aglycemic insult.

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MATERIALS AND METHODS

In vitro BBB model. BBMEC were isolated from the gray matter of the cerebral cortex of fresh bovine brains obtained from the University of Arizona Meat Laboratory and cryopreserved as previously described (3, 49). Isolated cells were seeded onto collagen-fibronectin-coated Transwell filters or slides and grown in MEM-Ham's F-12 with 10% equine serum, 50 μ g/ml gentamicin, 2.5 μ g/ml amphotericin B, and 100 μ g/ml heparin. All BBMEC used for these studies were primary cultured cells from *passage 0*, which have been shown to maintain excellent BBB characteristics in vitro (2, 66). Cultures in Transwell filters were incubated with C6 astrocyte-conditioned medium in the basolateral chamber for 3 days before the start of experiments (14).

Calcium ionophore treatment and hypoxic stress. BBMEC monolayers were treated with 5 μ M A-23187, a calcium ionophore, for 6 h to increase levels of intracellular calcium. Some monolayers were pretreated before the addition of A-23187 for 30 min with 10 μ M BAPTA-AM (BAPTA), a cell-permeable calcium chelator, to inhibit the increase in intracellular calcium due to the calcium ionophore. Monolayer permeability was assayed as described below.

For hypoxic experiments, BBMEC monolayers were subjected to 6 h of hypoxia under different treatment conditions. Monolayers were incubated in normoglycemic RPMI 1640 (Life Technologies, Rockville, MD) and treated with 10 μ M BAPTA, 100 nM NIF, or 100 nM SKF; drugs were added 30 min before the onset of hypoxic stress. Other monolayers were subjected to hypoxic stress + aglycemia; these cells were incubated in modified RPMI 1640 without glucose (Life Technologies, Rockville, MD) and were treated with BAPTA, NIF, or SKF. Hypoxic stress was achieved by incubating the monolayers in a humidified, gas-controlled hypoxic workstation (Coy Laboratory Products, Grass Lake, MI) at 37°C with 99% N_2 -1% O_2 . At the end of the hypoxic period, the medium was removed and PO_2 levels were measured with a blood gas analyzer (ABL 505, Radiometer, Copenhagen, Denmark) to confirm hypoxia. PO_2 in medium from normoxic samples was 150.2 ± 4.5 mmHg; PO_2 in hypoxic medium was 44.1 ± 1.6 mmHg. Monolayers were assayed for changes in intracellular calcium levels or functional permeability (see below).

Measurement of intracellular calcium. To measure intracellular calcium, BBMEC were plated into 96-well plates. When confluent, cells were incubated in 5 μ M fura 2-AM alone or with BAPTA, NIF, or SKF for 30 min at 37°C. After the incubation period, fura 2-AM was removed, and medium containing appropriate drugs was added. Readings were taken at various times over a 6-h period of incubation as described below.

For changes in intracellular calcium levels over a hypoxic stress period, an initial reading was obtained, and plates were transferred to the hypoxic chamber for treatment. The microplate was covered with nonpermeable adhesive membrane during fluorescence readings to maintain hypoxic conditions. At each time point, the plate was covered with a nonpermeable adhesive membrane and removed from the hypoxic chamber for fluorescence readings. At the end of the reading, the plate was returned to the chamber and the adhesive membrane was removed; there was no reoxygenation of the medium or cells during the reading. Changes in intracellular calcium levels were determined by measuring fura 2 fluorescence at 340 nm excitation and 510 nm emission on a GENios microplate reader (Tecan, Research Triangle Park, NC). Calcium-insensitive fura 2 fluorescence was measured at 380 nm excitation and 510 nm emission, and results were expressed as percentage of *time 0* control ratio of fluorescence at 340 nm to fluorescence at 380 nm. Measurements were obtained as described above at various times over the 6-h hypoxic stress period. In a related experiment, cells were incubated with NIF and SKF, and basal fura 2-AM fluorescence was measured before the addition of 100 nM bradykinin. Changes in intracellular calcium fluorescence were measured as described above. After the time course of fura 2

fluorescence was plotted for each treatment, area under the curve (AUC) was determined, and values were statistically analyzed.

Permeability measurements. Permeability studies used [^{14}C]sucrose, a normally low-permeability marker, to determine paracellular flux across BBMEC monolayers. Apical-to-basolateral flux was determined by dividing picomoles of radioactive marker appearing in the receiver chamber by the time in minutes (1, 49). The apparent permeability coefficient (PC) was calculated as follows

$$PC = \text{flux}/(A * C_{D_0})$$

where flux is the slope of the line, A is the area of the membrane, and C_{D_0} is the initial donor concentration of radioactive marker.

Measurement of ROS. BBMEC were plated in 96-well plates and incubated with normoglycemic medium, aglycemic medium, or aglycemic medium + drug (100 nM NIF or 100 nM SKF). At 1 h before the end of the hypoxic stress, cells were incubated in 50 μ M 2',7'-dichlorofluorescein diacetate (Calbiochem, San Diego, CA), a fluorescent probe for measuring oxidative stress (12). Production of ROS was measured by obtaining fluorescence readings at 485 nm of excitation and 535 nm of emission on the GENios microplate reader after 6 h of normoxia, hypoxia, or hypoxia followed by 30 min of reoxygenation.

Statistics. Values are means \pm SE. Results were analyzed with one- or two-way ANOVA as appropriate and then with multiple pairwise comparisons with Tukey's test using Sigma Stat 2.03 (SPSS, Chicago, IL). Significance was defined as $P < 0.05$.

RESULTS

We used BBMEC monolayers as a model system to investigate the effects of increasing intracellular calcium levels or of hypoxic and/or aglycemic stress on BBB functional integrity. BBMEC were exposed to various treatment conditions before determination of intracellular calcium levels and monolayer paracellular permeability.

A-23187, a calcium ionophore, did not alter monolayer permeability. Incubation of BBMEC monolayers in 5 μ M A-23187 or 10 μ M BAPTA did not change [^{14}C]sucrose permeability compared with untreated control (Fig. 1). However, treatment with A-23187 + BAPTA significantly in-

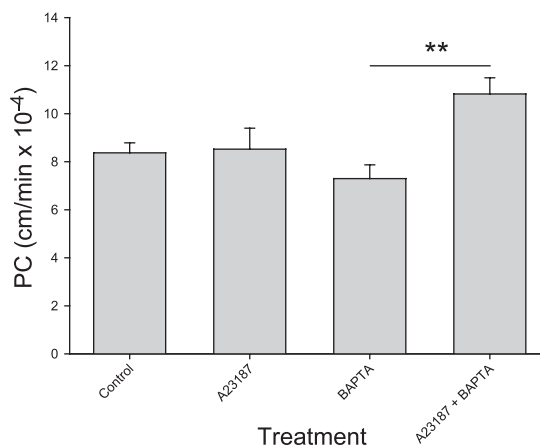


Fig. 1. A-23187 has no effect on bovine brain microvessel endothelial cell (BBMEC) monolayer permeability. BBMEC monolayers were incubated with 5 μ M A-23187 and/or 10 μ M BAPTA, and [^{14}C]sucrose permeability was assayed. Neither A-23187 nor BAPTA treatment affected BBMEC permeability. When monolayers were incubated with A-23187 + BAPTA, permeability was significantly increased compared with BAPTA treatment alone. PC, permeability coefficient. Values are means \pm SE; $n = 5-6$. ** $P = 0.01$.

creased BBMEC monolayer permeability compared with BAPTA alone ($F_{3,22} = 5.029$, $P = 0.01$).

Hypoxic stress increased intracellular calcium; treatment with NIF or SKF further increased levels of intracellular calcium over the course of hypoxic stress. BBMEC monolayers were exposed to 6 h of 1% oxygen in normoglycemic or aglycemic medium, and fura 2 fluorescence was measured as an indicator of changes in intracellular calcium. A characteristic pattern of changes in intracellular calcium in normoglycemic or aglycemic medium was observed (Fig. 2). During the 1st h of hypoxia, the stress appeared to induce long-term oscillations in intracellular calcium levels, with several apparent peaks. These initial oscillations appeared to subside, with intracellular calcium levels returning to near baseline (*time 0*) levels by 2 h. There was a subsequent increase in intracellular calcium at 4 h of hypoxic stress that returned to normal at 5 h and increased again at 6 h of hypoxic stress, perhaps as part of longer-term oscillatory behavior.

Using similar assay conditions, we examined the effects of BAPTA, NIF, and SKF, alone and in combination, on intracellular calcium levels. Data were expressed as percentage of *time 0* control samples, and the AUC was calculated and analyzed by two-way ANOVA (Fig. 3). Drug treatment had a significant effect on intracellular calcium levels ($F_{6,604} = 2.298$, $P = 0.033$); furthermore, there was a significant interaction between drug treatment and glycaemic condition ($F_{6,604} = 4.269$, $P < 0.001$). Under hypoxic-normoglycemic conditions, treatment with NIF and/or SKF increased levels of intracellular calcium, contrary to the predicted effects of these drugs as calcium channel blockers (Fig. 3A). Treatment with BAPTA decreased intracellular calcium levels, while treatment with BAPTA + NIF or SKF abolished the increase in intracellular calcium due to NIF or SKF alone. In contrast, under aglycemic conditions (Fig. 3B), treatment with the drugs alone after 6 h of hypoxic stress had no effect on calcium levels, but treatment with drug combinations (BAPTA + NIF or BAPTA + SKF) led to unexpectedly increased intracellular calcium.

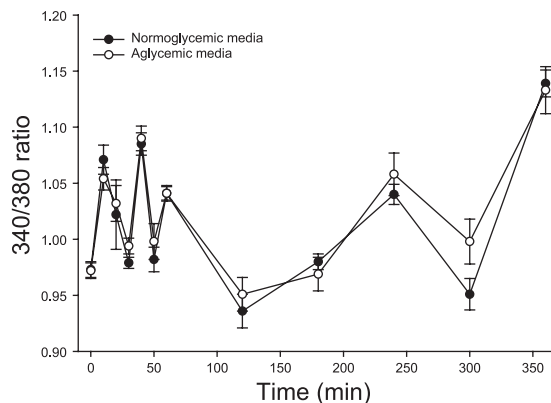


Fig. 2. Hypoxic stress causes a multiphasic increase in intracellular calcium in BBMEC. BBMEC were subjected to 6 h of hypoxic stress under normoglycemic or aglycemic conditions. Within the 1st h of hypoxic exposure, there were 3 transient peaks in intracellular calcium. Calcium levels then returned to baseline and, subsequently, increased at 4 and 6 h of hypoxic stress. There was no statistical difference in intracellular calcium between normoglycemic and aglycemic conditions. A representative trace is shown. Values are means \pm SE; $n = 8$. 340/380 ratio, ratio of fluorescence at 340 nm to fluorescence at 380 nm.

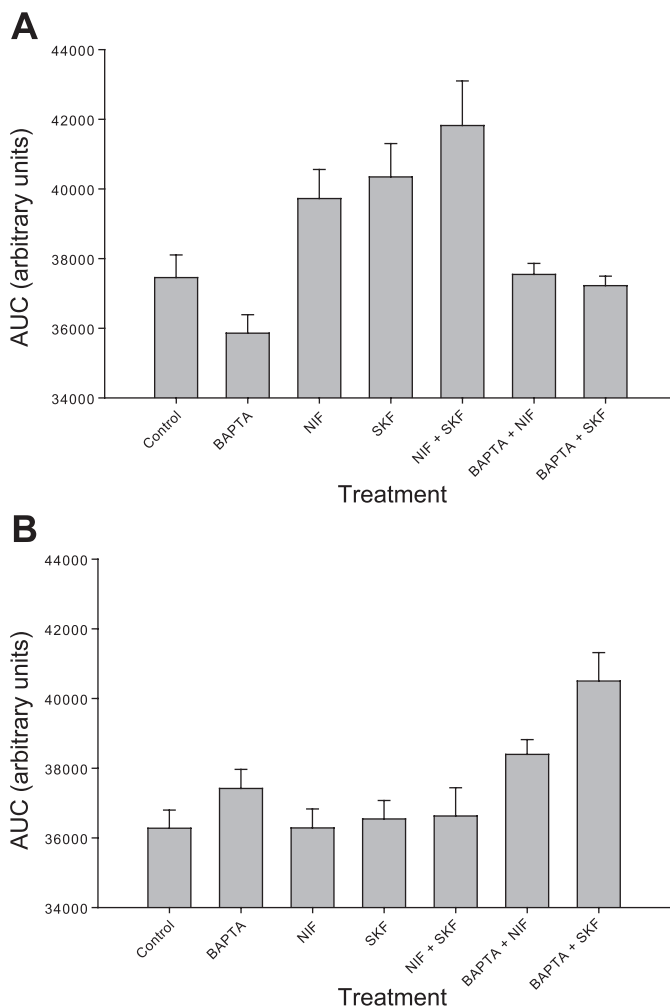


Fig. 3. Effect of BAPTA, nifedipine (NIF), and SKF-96365 (SKF) on intracellular calcium during 6 h of hypoxic stress. BBMEC were subjected to 6 h of hypoxia and incubated with 10 μ M BAPTA, 100 nM NIF, or 100 nM SKF, alone or in combination. There was a significant effect of drug treatment on intracellular calcium ($F_{6,604} = 2.298$, $P = 0.033$) and a significant interaction between drug treatment and glycaemic condition ($F_{6,604} = 4.269$, $P < 0.001$). A: under normoglycemic conditions, BAPTA decreased intracellular calcium. NIF and/or SKF increased the amount of intracellular calcium during the hypoxic stress. This effect of NIF and/or SKF was prevented by cotreatment with BAPTA. B: under aglycemic conditions, there was no significant effect of BAPTA, NIF, or SKF, alone or in combination, on intracellular calcium levels, although BAPTA + NIF and BAPTA + SKF did potentially increase intracellular calcium. Values are means \pm SE; $n = 6$ –20. AUC, area under the curve.

To determine whether NIF or SKF could actually block changes in intracellular calcium, fura 2 fluorescence was measured after stimulation of BBMEC with bradykinin, a peptide known to trigger an increase in intracellular calcium in endothelial cells (17). After bradykinin stimulation under normoxic conditions, only SKF decreased the amount of intracellular calcium over the period of exposure to the peptide ($F_{3,30} = 6.892$, $P = 0.001$; Fig. 4), demonstrating that SKF-sensitive calcium influx pathways do exist in these cells.

BAPTA, NIF, and SKF partially protected against hypoxia-induced BBMEC monolayer disruption after hypoxic stress. BBMEC monolayers were exposed to hypoxic stress, and paracellular permeability was measured using [^{14}C]sucrose.

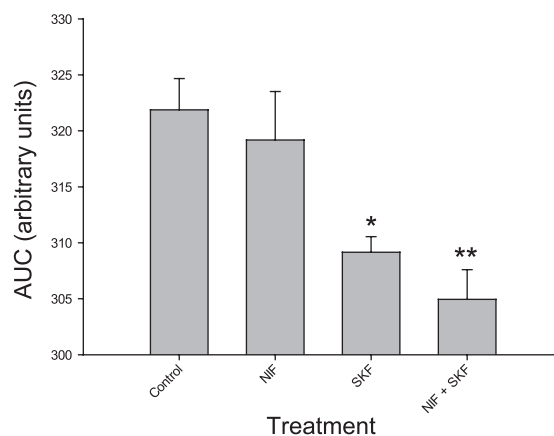


Fig. 4. SKF, but not NIF, prevents calcium influx after bradykinin stimulation. BBMEC were plated as described for hypoxic stress experiments. Cells were loaded with fura 2-AM and stimulated with 100 nM bradykinin. Intracellular calcium was measured for 6 h, and AUC was determined. One-way ANOVA indicated a significant blockade of bradykinin-stimulated calcium influx after SKF treatment ($F_{3,30} = 6.892$, $P = 0.001$) but not after NIF treatment. Values are means \pm SE; $n = 8-9$. * $P < 0.05$; ** $P < 0.01$ vs. control.

After 6 h of hypoxic stress, there was a significant effect of drug (BAPTA, NIF, or SKF) treatment ($F_{11,390} = 4.232$, $P < 0.001$) and of hypoxic stress ($F_{1,390} = 75.449$, $P < 0.001$) on BBB functional integrity. There was also a significant interaction between drug treatment and hypoxic stress ($F_{11,390} = 1.851$, $P = 0.044$). Under normoxic-normoglycemic conditions, treatment with BAPTA, NIF, or SKF significantly decreased BBMEC monolayer permeability (Fig. 5A). Hypoxic-normoglycemic stress increased BBMEC monolayer permeability in almost all conditions. Statistical analyses indicate that only treatment with BAPTA + NIF protected against hypoxia-induced monolayer disruption.

Under normoxic-aglycemic conditions, there was no effect of any of the drug treatments on BBMEC monolayer permeability (Fig. 5B). Hypoxia caused an increase in monolayer permeability in control monolayers. Treatment with NIF or SKF alone prevented the hypoxia-induced permeability increase, but BAPTA treatment alone had no effect on this hyperpermeability. Monolayers treated with BAPTA + NIF were protected against hypoxia-induced increases in permeability, but BAPTA + SKF was not protective.

Antioxidant effects of NIF and SKF. Since NIF and SKF did not appear to act as calcium channel blockers in our hypoxia experimental paradigm (Fig. 3), we investigated whether the protective actions of these drugs were due to antioxidant properties. Cells were incubated with normoglycemic medium, aglycemic medium, or aglycemic medium with NIF or SKF as described for the calcium assays and permeability studies. ROS were measured using 2',7'-dichlorofluorescein diacetate, a fluorescent probe that detects the presence of oxygen free radicals (12). BBMEC were subjected to 6 h of normoxia, 6 h of hypoxia, or 6 h of hypoxia followed by 30 min of reoxygenation in room air (Fig. 6). There was a significant effect of glucose and drug treatment ($F_{3,60} = 19.213$, $P < 0.001$) and of oxygen treatment ($F_{2,60} = 31.196$, $P < 0.001$) on ROS levels. Incubation in aglycemic medium alone increased ROS levels under any oxygen condition. ROS levels after 30 min of reoxygenation were significantly higher in all drug treatment groups than in the groups treated with 6 h of normoxia or 6 h

of hypoxia. No antioxidant effect was observed with NIF or SKF under any of the three oxygen conditions.

DISCUSSION

Ischemic stroke and hypoxic stress disrupt the BBB (18). We previously demonstrated that hypoxic (49) or hypoxic-aglycemic (1) stress can disrupt an in vitro BBB model system, providing an opportunity to investigate some of the cellular mechanisms underlying this disruption. Interestingly, two calcium channel blockers, NIF and SKF, can prevent BBB disruption in this model system (1). We chose to examine the role of intracellular calcium in modulating BBB functional perme-

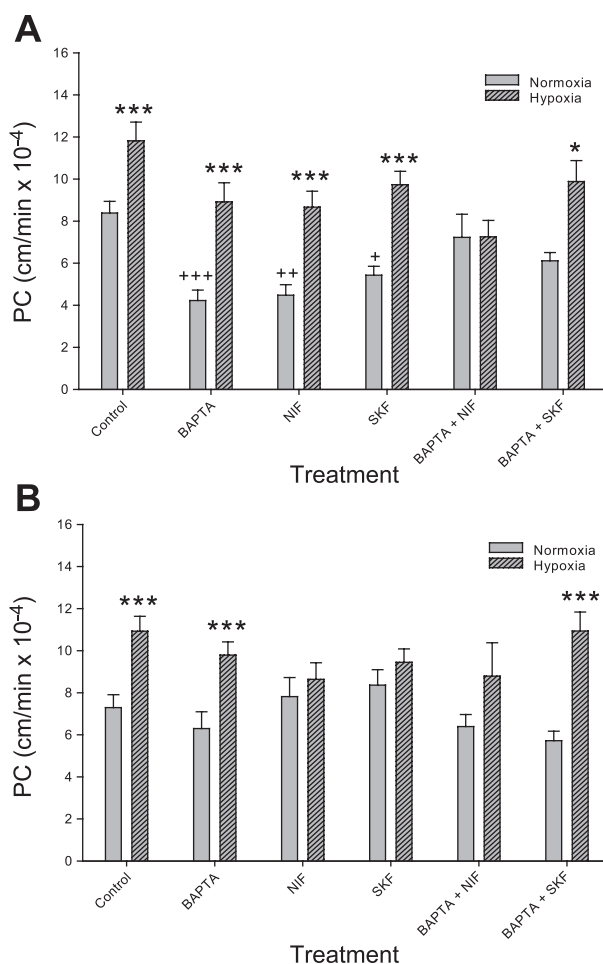


Fig. 5. BAPTA, NIF, and SKF partially protect BBMEC monolayer integrity. BBMEC monolayers were treated with 10 μ M BAPTA, 100 nM NIF, and 100 nM SKF, alone or in combination. After 6 h of hypoxic stress, monolayer permeability was assayed. Two-way ANOVA indicated a significant effect of drug treatment ($F_{11,390} = 4.232$, $P < 0.001$) and a significant effect of hypoxic stress ($F_{1,390} = 75.449$, $P < 0.001$), as well as a significant interaction between drug treatment and hypoxic stress ($F_{11,390} = 1.851$, $P = 0.044$). A: pairwise comparisons indicate that, under normoxic/normoglycemic conditions, BAPTA and NIF treatment significantly reduced monolayer permeability compared with normoxic control. Treatment with BAPTA, NIF, SKF, or BAPTA + SKF did not block the hypoxia-induced increase in monolayer permeability. Only BAPTA + NIF prevented monolayer disruption. B: in BBMEC subjected to aglycemic conditions, NIF and SKF prevented hypoxia-induced increases in permeability, as did BAPTA + NIF. Neither BAPTA alone nor BAPTA + SKF prevented this hypoxic effect. Values are means \pm SE; $n = 6-30$. *** $P < 0.001$ vs. normoxia. +++ $P < 0.001$; ++ $P < 0.01$; + $P < 0.05$; * $P < 0.05$ vs. normoxic control.

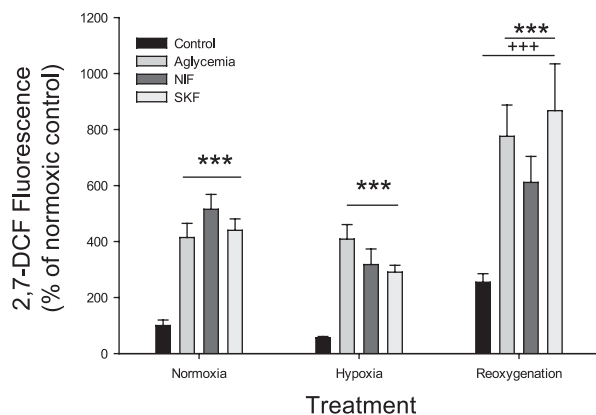


Fig. 6. NIF and SKF are not antioxidants in this system. BBMEC were plated and subjected to 6 h of hypoxic stress under normoglycemia, aglycemia, or aglycemia with NIF or SKF treatment. Production of reactive oxygen species (ROS) was measured using 2',7'-dichlorofluorescein diacetate (2,7-DCF). There was a significant effect of glucose and drug treatment ($F_{3,60} = 19.213$, $P < 0.001$) and a significant effect of oxygen ($F_{2,60} = 31.196$, $P < 0.001$). Aglycemic treatment alone increased ROS in BBMEC. A 30-min reoxygenation period after 6 h of hypoxic stress also caused a significant increase in ROS. Treatment with NIF or SKF did not prevent this reoxygenation-induced increase in ROS. Values are means \pm SE; $n = 10$ –12. *** $P < 0.001$ vs. normoglycemia (control). +++ $P < 0.001$ vs. normoxia.

ability and its potential involvement in mediating hypoxia-induced damage.

Although there is a large body of literature dealing with the role of calcium in modulating cell-cell adhesion through adherens junctions, much less is known about the role of calcium in modulating TJ function (13). We used a calcium ionophore to increase levels of intracellular calcium in our BBB model system and found that simply increasing intracellular calcium alone did not lead to increased paracellular permeability, indicating that high intracellular calcium levels alone, such as those seen after a stroke (43, 60) or hypoxic event (35, 41, 55), are not sufficient to alter TJ function. However, there is evidence that TJ components, such as ZO-1 (61, 69) and occludin (55), are sensitive to calcium levels. It remains to be seen how increased intracellular calcium alters TJ protein expression or function in this experimental paradigm.

We found that a 6-h hypoxic or hypoxic-aglycemic stress triggered a characteristic increase in intracellular calcium in BBB endothelial cells. Within the 1st h of exposure to the stressor, intracellular calcium levels displayed distinct oscillations with several peaks, perhaps representing the initial response of BBB endothelial cells to stress. This initial response was followed by increases in intracellular calcium at 4 and 6 h, which may reflect long-term slow calcium oscillatory behavior. There was no difference in the calcium response between cells incubated in aglycemic medium and those incubated in normoglycemic medium during hypoxic stress. However, the presence or absence of glucose did influence cellular responses to the calcium chelator (BAPTA) or calcium channel blockers (NIF or SKF). Under normoglycemic conditions, treatment with NIF and/or SKF increased intracellular calcium levels over the course of the hypoxic stress. This result is in direct opposition to the presumed mechanism of action of these drugs: NIF is an L-type calcium channel blocker, while SKF blocks some stores-operated calcium channels. Under aglycemic conditions, intracellular calcium levels were not signifi-

cantly altered, regardless of drug treatment. However, these cells do have some SKF-sensitive calcium channels, as indicated by the bradykinin experiment (Fig. 4), although these channels may not be involved in mediating hypoxic effects.

There is debate about the presence of L-type calcium channels on endothelial cells, a nonexcitable cell type. A few studies have demonstrated L-type channel currents in endothelial cells (64), and there is pharmacological evidence that these drugs prevent increases in intracellular calcium in endothelial cells (67) and protect against hypoxia-induced damage (1, 29); however, there is no convincing molecular evidence for the presence of these channels at the BBB. Therefore, we must consider the possibility that the protective effect of NIF seen in BBB models is mediated through an alternate mechanism. The present study and others have demonstrated that NIF has protective effects under hypoxic conditions. NIF can inhibit hypoxic damage by inhibiting protein kinase (PKC)- α (PKC- α) (29), but this does not correlate with the increase in intracellular calcium (potentially activating PKC- α) seen after treatment with NIF in this study and in others (8, 63).

SKF is considered to be a stores-operated calcium channel blocker, but its parent compound, SC-38249, can also inhibit extrusion of intracellular calcium via the plasma membrane Ca^{2+} -ATPase (PMCA) (16). SKF does block an increase in endothelial cell calcium after stimulation with bradykinin (22), as we have demonstrated in this study. However, other studies have demonstrated that treatment with SKF alone can trigger an increase in intracellular calcium that is mediated by release from intracellular, thapsigargin-sensitive stores (36) and by influx from the extracellular medium (38), suggesting that SKF may have dose- and cell system-specific effects on intracellular calcium.

What is the potential source of the signal triggering this increase in intracellular calcium under hypoxic-normoglycemic conditions? Why is such an increase not observed under hypoxic-aglycemic conditions? One potential trigger for intracellular calcium increases under these hypoxic-normoglycemic conditions is nitric oxide (NO) produced by calcium-dependent stimulation of endothelial NO synthase (eNOS) and released from surrounding endothelial cells. NO is produced by cerebral microvessel endothelial cells under hypoxic stress (48), which increases BBB endothelial cell permeability (10, 48); this effect is attenuated by inhibition of NOS (48). NO can also trigger an increase in intracellular calcium in endothelial cells (6, 9). Calcium is a major regulator of eNOS, a major source for NO production in these cells (23). Under our experimental conditions, hypoxic stress could trigger an initial increase in intracellular calcium that could activate eNOS to produce NO. This NO could act on the endothelial cell in a paracrine or autocrine fashion, thereby further increasing the level of intracellular calcium. The NO-mediated calcium increase would not be affected by NIF or SKF but would be subject to chelation by BAPTA, as seen in these studies (Fig. 3A). Furthermore, NIF treatment can upregulate eNOS protein levels (19) and stimulate endothelial NO production (19, 20, 24), further implicating NO in this experimental paradigm. SKF treatment has not been convincingly shown to stimulate NO production on its own (36), but it may inhibit the activity of the PMCA (16), thereby prolonging any increase in intracellular calcium. Under aglycemic conditions, energy-dependent mechanisms may be inhibited, leading to prevention of eNOS function, and, thus, may



prevent the potentially NO-induced increase in intracellular calcium.

There are other mechanisms by which endothelial cells can increase intracellular calcium levels via pathways distinct from store-operated or voltage-gated channels. Hypoxia triggers expression of endothelin-1 (ET-1) (31) and vascular endothelial growth factor (34, 44). ET-1 can increase intracellular calcium (59) via an inositol trisphosphate-sensitive pathway (70); this increase can be potentiated by NOS inhibitors or decreased by NO donors (15), indicating cross talk between these two pathways. NO can induce expression of vascular endothelial growth factor, which in turn activates eNOS (11, 51), feeding into the NO pathway described above. The BBB endothelial cell also expresses a large number of ion transporters, the function of which may be altered under pathological conditions. One of these, the Na^+/Ca^{2+} exchanger (NCX), can work in reverse (i.e., calcium influx coupled to sodium efflux) after pharmacological manipulation (21). Reversal of the NCX has been shown to be important in mediating spinal cord white matter injury after anoxia (46), and NCX inhibitors can protect against ischemic injury attributed to exchanger reversal (68). Furthermore, reversal of the NCX in endothelial cells has been linked to activation of eNOS and production of NO (57, 62). If the NCX in BBB endothelial cells is reversed under hypoxic conditions, leading to calcium overload inside the cells, this might also explain why NIF and SKF do not block the increase in intracellular calcium after hypoxic stress. The data presented in this study may also indicate a secondary effect of drug treatment on the activity or expression of ion transporters critical for the removal of intracellular calcium, such as the NCX or PMCA (65), the expressions of which can be directly modulated by changes in intracellular calcium (27, 40).

As previously demonstrated, NIF protects against hypoxia-induced BBB functional disruption, and we confirmed the protective effect of SKF (1). Interestingly, treatment with BAPTA, NIF, or SKF under normoxia-normoglycemia actually tightened the monolayer, perhaps implicating resting intracellular calcium levels in TJ function. Under normoxic-aglycemic conditions, there was no effect of drug treatment on monolayer permeability; however, NIF and SKF were able to completely prevent the hypoxia-induced increase in monolayer permeability under aglycemic conditions, while BAPTA had no protective effect. As with the A-23187 data, these results indicate that a dramatic alteration (an increase or a decrease) in intracellular calcium is not sufficient to disrupt or protect BBB monolayer functional integrity. Although other studies have proposed an antioxidant role for NIF (7, 47), we have ruled out the possibility that NIF or SKF is protective via an antioxidant mechanism in this system. As we have mentioned here, intracellular calcium levels are regulated by many cellular mechanisms, which makes it difficult to determine the exact mechanism by which calcium influences TJ and BBB monolayer permeability.

In conclusion, we examined the effects of BAPTA, NIF, and SKF treatment, alone or in combination, on BBB monolayer permeability, intracellular calcium levels, and ROS production after a hypoxic or hypoxic-aglycemic stress. We demonstrated an increase in intracellular calcium after NIF or SKF treatments under hypoxia-normoglycemia, in stark contrast to the presumed actions of these drugs to simply block calcium flux. The observed increase may reflect not a failure of the drugs to

block their respective targets, which still occurs after bradykinin treatment, but, rather, an unmasking of calcium released from a secondary source, which may be crucial for mediating NIF and SKF protection of monolayer integrity. What remains to be determined in this experimental paradigm is the role of calcium in modulating TJ protein expression or protein-protein interactions.

We envision several mechanisms by which calcium could be involved in regulating BBB TJ function: 1) a direct interaction between calcium and TJ proteins (i.e., occludin) in a manner similar to that occurring with E-cadherin at the adherens junction (54), 2) a mechanism by which alterations in intracellular calcium levels, and consequent activation of signal transduction cascades (i.e., calcium-calmodulin kinases or PKC), directly regulate expression of TJ protein genes, and/or 3) an indirect mechanism by which calcium affects other proteins, such as eNOS, ET-1, ion transporters, or ATPases, thereby affecting BBB integrity. Future studies are targeted at determining the effect of changes in intracellular calcium on TJ protein expression, elucidating the source of the increased intracellular calcium after NIF or SKF treatment, and investigating the signal transduction cascades triggered by the treatments described in these studies to clarify the role of calcium in TJ protein regulation and BBB function.

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GRANTS

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