Protective Action of Indapamide, a Thiazide-Like Diuretic, on Ischemia-Induced Injury and Barrier Dysfunction in Mouse Brain Microvascular Endothelial Cells

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Abstract. The aim of the present study was to elucidate the effects of indapamide on ischemic damage to the blood-brain barrier (BBB) in vitro. The ischemia/reperfusion conditions employed here significantly decreased the viability of mouse brain capillary endothelial (MBEC4) cells, an effect ameliorated by indapamide. Ischemia increased the permeability of MBEC4 cells to two cellular transport markers, sodium fluorescein and Evan’s blue-albumin. Indapamide reduced the ischemia-induced hyperpermeability of cells. These results suggest that indapamide may have a protective role against ischemia-induced injury and dysfunction of the BBB.

Keywords: indapamide, ischemia, blood-brain barrier
NaH₂PO₄, 25 mM NaHCO₃, and 11 mM sucrose, pH 7.4) in an anoxic incubator replaced with 5% CO₂/95% N₂ for 10 h at 37°C (ischemia conditions). Subsequently, cells were incubated with serum-free DMEM in 5% CO₂/95% air at 37°C for 1 h (reperfusion conditions). As a control, cells were incubated with normal Krebs-Ringer buffer (143 mM NaCl, 4.7 mM KCl, 1.3 mM CaCl₂, 1.2 mM MgCl₂, 1.0 mM NaH₂PO₄, 25 mM NaHCO₃, and 11 mM D-glucose, pH 7.4) for 10 h and subsequently incubated with serum-free DMEM for 1 h in 5% CO₂/95% air at 37°C (normal conditions). To test whether indapamide protects against ischemia/reperfusion-induced cell death, cells were exposed to 10 – 50 µM of indapamide (Kyoto Pharmaceutical Industries, Kyoto) or vehicle (0.25% dimethyl Sulfoxide) during conditions of ischemia and reperfusion or normal conditions. Cell viability was measured by the WST-1 assay (Cell Counting Kit-8; Dojindo, Kumamoto).

Endothelial barrier function was evaluated by measuring permeability of cells to sodium fluorescein (Na-F) and Evan’s blue-albumin (EBA) as previously described (9). MBEC4 cells were grown on the collagen-coated membrane (1.1 cm², 0.4-µm pore size) of a Transwells™ insert (42,000 cells/cm², Corning) and subsequently exposed to the above-mentioned ischemia conditions for 7 h. As a control, MBEC4 cells were incubated with normal Krebs-Ringer buffer for 7 h (normal conditions). Cells were exposed to 10 – 100 µM of indapamide or vehicle during ischemia conditions or normal conditions. To initiate transport experiments, the medium was removed and cells were washed with normal Krebs-Ringer buffer. Krebs-Ringer buffer containing 100 µg/mL of Na-F (MW 376; Sigma, St. Louis, MO, USA) and 670 µg/mL EBA bound to 0.1% BSA (MW = 67 kDa) were loaded on to the luminal side of the insert. Samples were removed from the abluminal chamber at 30, 60, 90, and 120 min and immediately replaced with Krebs-Ringer buffer. The concentrations of Na-F and EBA were determined with a CytoFluor Series 4000 fluorescence multiwell plate reader [Ex(λ) 485 ± 10 nm and Em(λ) 530 ± 12.5 nm; PerSeptive Biosystems, Framingham, MA, USA] and an Opsys MR microplate reader (630 nm; DYNEX Technologies, Chantilly, VA, USA), respectively. The permeability coefficient and clearance were calculated according to the method of Dehouck et al. (10), as previously described (9).

Data are expressed as the mean ± S.E.M. Statistical analysis was performed using one-way analyses of variance (ANOVA) followed by Tukey-Kramer’s post-hoc test. The difference in means was considered to be significant when the P value was less than 0.05.

As shown in Fig. 1, the ischemia (10 h)/reperfusion (1 h) conditions significantly decreased the viability of MBEC4 cells grown on collagen-coated wells, by 26.5% of cells subjected to normal conditions. The effect of indapamide on ischemia/reperfusion-induced damage in MBEC4 cells was concentration-dependent: 20 – 50 µM indapamide improved recovery by 50% – 86%. These concentrations have no effect on cell viability under normal conditions. As shown in Fig. 2, a 7-h period of ischemia resulted in increased permeability of MBEC4 cells grown on collagen-coated membranes to Na-F (paracellular transport marker) and EBA (transendothelial transport marker). Following ischemia, the perme-
ability coefficients of Na-F and EBA were significantly increased to 223.7 ± 9.8% and 518.9 ± 42.5% of the vehicle under normal conditions, respectively. The viability of MBEC4 cells grown on membranes after a 7-h exposure to ischemic conditions and following termination of the permeability test (2 h) were 72.8 ± 3.6% and 71.3 ± 1.0% of cells subjected to normal conditions, respectively; there was no difference in cell viability before and after the permeability test. Following a 7-h exposure to indapamide (50 – 100 µM) under ischemic conditions, hyperpermeability of MBEC4 cells to Na-F and EBA was concentration-dependently reduced by 31.9% – 47.4% and 41.4% – 60.6% of the vehicle, respectively. This effect was not accompanied by a change in cell viability (vehicle: 72.8 ± 3.6%, 100 mM: 75.9 ± 2.3%). Under normal conditions, these concentrations of indapamide had no effect on the permeability coefficients of Na-F and EBA in MBEC4 cells. These findings suggest that indapamide may efficiently inhibit ischemia-induced hyperpermeability rather than inhibit...
ischemic cell death. MBEC4 cells that were grown on membranes exhibited a higher vulnerability to ischemic cytotoxicity and lower sensitivity to the cytoprotective action of indapamide than those grown on the smooth plastic surface of a well. Further experiments will be required to clarify these issues.

The in vitro ischemia/reperfusion conditions used in the present study significantly reduced the viability of MBEC4 cells, and this effect was ameliorated by indapamide. Ischemic conditions increased the permeability of MBEC4 cells to Na-F and EBA, but this hyperpermeability was reduced by indapamide.

Disruption of the BBB is a critical event during cerebral ischemia as it is followed by the passive diffusion of water, leading to vasogenic edema and secondary brain injury. Cerebral edema is a major and fatal complication of acute ischemic stroke. Free radical overproduction after brain ischemia and reperfusion contributes to brain edema and neuronal damage. Thus, an inhibition of free radical formation is likely to prevent the occurrence of brain edema and neuronal damage. Indapamide has an antioxidant effect and has the potential to scavenge oxygen free radicals and their derivatives. Boucher et al. reported that indapamide treatment has a protective effect on cardiac tissue during the early stages of postischemic reperfusion (6). The present findings suggest that indapamide may protect cerebral endothelial cells from ischemic damage due to antioxidation and/or free radical scavenging. This phenomenon may contribute, at least in part, to the mechanisms by which indapamide facilitated protection of perindopril against recurrent stroke in a recent clinical study (7).

Brain capillary endothelial cells form a metabolic and physical barrier that separates the periphery from the brain to maintain cerebral homeostasis. The lack of fenestrations and the presence of tight junctions differentiate brain microvessel endothelial cells from peripheral microvascular endothelium. While adherent junctions and other junctional proteins contribute to cell-to-cell contacts in the paracellular clef, tight junctions are critical for restricting paracellular diffusion in the cerebral microvasculature. Increased cerebrovascular permeability is a principal factor in the development of cerebral edema after brain ischemia. Several studies have shown a relationship between cyclic AMP (cAMP) levels and permeability of endothelial cell monolayers. For example, elevation of intracellular cAMP concentrations induces high transendothelial electrical resistance and increases P-glycoprotein function in brain capillary endothelial cells (11, 12). In addition, when bovine aortic endothelial cells were cultured in hypoxic conditions, cellular cAMP levels decreased and this phenomenon was associated with an increase in cellular permeability (13). Furthermore, a decrease in cAMP levels was detectable after brain microvascular endothelial cells were exposed to hypoxic conditions for 3 h (14). Moreover, indapamide was shown to augment cAMP production induced by forskolin, an adenylyl cyclase activator, but did not alter basal cAMP levels in cardiomyocytes alone (15). Therefore, indapamide may protect cerebral microvascular endothelial cells from ischemic dysfunction by increasing intracellular cAMP levels. The effects of indapamide on the expression of tight junction-related proteins and on intracellular messengers are now being investigated in rat brain primary cultured endothelial cells.

We present here in vitro evidence to suggest a possible protective action of indapamide against ischemia/reperfusion-induced injury and dysfunction of the BBB.

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