Blockade of peroxynitrite-mediated astrocyte death by manganese(III)-cyclam

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Abstract

Under glucose-deprived conditions, astrocytes rapidly underwent death due to their increased susceptibility to endogenously produced peroxynitrite (Gila 31, 155–164; J. Neuroimmunol. 112, 55–62; J. Neurochem. 74, 1989–1998). In the present study, the cell membrane-permeable synthetic superoxide dismutase (SOD) mimic cyclam manganese(III) 1,4,8,11-tetraazacyclodecane (Mn(III)-cyclam) completely inhibited the death of glucose-deprived immunostimulated astrocytes. However, the structurally related compounds Ni(II)-cyclam, Co(II)-cyclam, and H2-cyclam, which lacks metals, had no or a little cytoprotective effect. Of the cyclams used in this study, only Mn(III)-cyclam completely scavenged the peroxynitrite produced in glucose-deprived immunostimulated astrocytes and significantly blocked the depolarization of mitochondrial transmembrane potential in those cells. The present results suggest that cell membrane-permeable synthetic SOD mimetics such as Mn(III)-cyclam may be potential therapeutic agents for various diseases associated with the endogenous production of peroxynitrite.

Keywords: Mn(III)-cyclam; Immunostimulation; Glucose deprivation; Mitochondria transmembrane potential

1. Introduction

Cerebral ischemia is accompanied by a marked inflammatory reaction that is initiated by ischemia-induced expression of cytokines, intercellular adhesion molecules, and other inflammatory mediators including reactive oxygen and nitrogen species produced from inflammatory cells. In cerebral ischemic insult, inflammatory cells such as macrophages and lymphocytes infiltrate into the brain lesion and activate glial cells including astrocytes and microglia (Hallenbeck et al., 1986; Lees, 1993). The invading inflammatory cells and activated glial cells in turn react to disease through the secretion of various kinds of bioactive agents including nitric oxide (NO) (Hallenbeck et al., 1986; Lees, 1993).

NO has been implicated in a large number of pathologies (Bruhwyler et al., 1993). The cytotoxicity of NO is increased by reaction with superoxide anion (O2•−) to form the highly reactive peroxynitrite (ONOO−) (Beckman et al., 1990). Previously, we reported that immunostimulated astrocytes became highly vulnerable to glucose deprivation (Choi and Kim, 1998; Choi et al., 2000, 2001). We found that the augmented death was caused by the collapse of antioxidant system (e.g., depletion of reduced glutathione) and resultant increase of peroxynitrite level in glucose-deprived immunostimulated astrocytes (Ju et al., 2000).

In order to protect neuronal and glial injury during ischemic insult, much effort has been made by using antioxidants. On the same lines, various kinds of cell membrane-permeable superoxide dismutase (SOD) mimetics have been developed. Of synthetic SOD mimetics, metal-cyclam complexes that have the catalytic SOD activity by their included transition metal have been experimentally employed for the purposes of the detoxification of heavy metal and DNA modification (Zhang...
et al., 2001; Srivastava et al., 1990; Stuart et al., 2000; Burrows et al., 1996). In the present study, therefore, we tested whether low-molecular-weight antioxidants cyclams protected the astrocyte death caused by endogenously produced peroxynitrite.

2. Materials and methods

2.1. Astrocyte cultures, immunostimulation and glucose deprivation

Astrocytes were cultured from the prefrontal cortices of 2- to 5-day-old Sprague–Dawley rat pups as previously described (Choi and Kim, 1998). In our preliminary experiments, over 95% of the cells were immunoreactive for glial fibrillary acidic protein, an astrocyte-specific marker. After 8–10 days in culture, cells were pre-treated for 24–48 h with interferon-γ (IFN-γ, 100 U/ml) and lipopolysaccharides (LPS, 1 μg/ml). After immunostimulation, glucose deprivation was achieved by repeated rinse and incubation in glucose-free Dulbecco’s modified Eagle’s media (DMEM) that was not supplemented with serum, which interfered with the lactate dehydrogenase (LDH) assay (Choi et al., 2000, 2001).

2.2. Assessment of astrocyte injury or death

Astrocytes injury or death was assessed by measurement of the activity of LDH released into the bathing medium. LDH activity was measured by use with a microplate reader (SpectraMax 340pc, Molecular Devices). Cell viability was expressed as LDH activity (B–B U/ml). One B–B unit is defined as that amount of LDH that will reduce 4.8 × 10⁻⁴ μmol of pyruvate per minute at 25 °C.

2.3. Measurement of DCF fluorescence

For removing the residue of DMEM/F12 medium, plates were washed with PBS buffer. Cells were incubated with 2,7-dihydrodichlorofluorescein diacetate (DCF-DA, 30 μM) in PBS for 20 min and then rinsed with the same solution. For experiments, cells were exposed to glucose deprivation and SIN-1 (200 μM) for 3 h in the absence and presence of various kinds of cyclams. The fluorescence of DCF was measured at an excitation wavelength of 502 nm and emission wavelength of 529 nm at 37 °C, using a fluorescence microplate reader (SpectraMax GeminiXS, Molecular Devices). DCF-DA diffuses through cell membranes and is subsequently enzymatically deacetylated by intracellular esterases to the non-fluorescent DCF-H. Peroxynitrite effectively converts DCF-H to the highly fluorescent DCF (Possel et al., 1997). Fluorescence intensities were corrected for autofluorescence (i.e., fluorescence of cells not loaded with DCF-DA).

2.4. Measurements of mitochondrial transmembrane potential

The mitochondrial transmembrane potential (MTP) was measured according to the previous report by Reers et al. (1991) with minor modifications. In brief, astrocytes cultured on 24-well culture plates were loaded for 20 min at 37 °C with JC-1 (1.0 μg/ml) in culture medium. Depolarization of MTP was assessed by measuring the fluorescence intensities at emission wavelengths of 530 and 590 nm using a fluorescence microplate reader (SpectraMax GeminiXS, Molecular Devices). During the measurements, cells were protected from light and remained in a 5% CO₂/95% air chamber at 37 °C. Fluorescence intensity was acquired at indicated times for <2 s to minimize photobleaching and was corrected for autofluorescence. The intensity of autofluorescence (i.e., fluorescence of cells not loaded with JC-1) was unchanged during the whole experimental period. In control experiments, no photobleaching was observed during the whole period of fluorescence monitoring.

2.5. Materials

3-Morpholinosydnonimine (SIN-1) and LDH assay kits were purchased from Sigma Chemical Co (St. Louis, MO). DMEM and fetal bovine serum were purchased from Gibco BRL (Grand Island, NY). Cyclams were purchased from Mid-Century Chemicals (Posen, IL). JC-1 and DCF-DA were purchased from Molecular Probes (Eugene, OR).

3. Results

Neither immunostimulation for 1–2 days with IFN-γ (100 U/ml) and LPS (1 μg/ml) nor glucose deprivation for 12 h caused astrocyte death (Choi et al., 2000). But, a significant release of LDH was observed in IFN-γ/LPS-treated astrocytes for 4-h incubation with glucose-free DMEM. This LDH release was completely blocked by 10 μM Mn(III)-cyclam and partially by Ni(II)-cyclam, but not by Co(II)-cyclam and H₂-cyclam (Fig. 1A). The augmented death observed in glucose-deprived immunostimulated astrocytes was mimicked by the peroxynitrite-producing reagent SIN-1. The enhanced release of LDH in glucose-deprived SIN-1-treated astrocytes was also completely blocked by Mn(III)-cyclam, but partially by Ni(II)-cyclam, and not by other cyclams used (Fig. 1B). Maximal cytoprotective effects by Mn(III)- and Ni(II)-cyclams were obtained at 10 μM (data not shown). Of the concentrations (0.1–100 μM) used in this
study, Co(II)-cyclam and H2-cyclam did not alter the viability of glucose-deprived immunostimulated or SIN-1-treated astrocytes (data not shown).

Using a peroxynitrite-sensitive dye DCF-DA, we further found that Mn(III)-cyclam completely and Ni(II)-cyclam partially blocked the oxidation of DCF-H to DCF induced by SIN-1 in glucose-deprived astrocytes (Fig. 2). However, Co(II)-cyclam and H2-cyclam did not alter the oxidation of DCF-H. However, Ni(II)-cyclam was significantly less efficacious than Mn(III)-cyclam for preventing peroxynitrite-evoked cell death and scavenging peroxynitrite ($P < 0.05$, compared between the groups treated with Cyto/GD (Fig. 1A) or SIN-1/GD (Figs. 1A and 2) in the presence of Mn(III)-cyclam and Ni(II)-cyclam).

Pathologically formed oxidative stress causes cell death through mitochondrial dysfunction (Kroemer et al., 1997; Petit et al., 1997; Zamzami et al., 1995, 1997). Therefore, we further investigated whether cyclams could inhibit the MTP depolarization in glucose-deprived immunostimulated astrocytes. Neither immunostimulation nor glucose deprivation alone significantly altered the MTP (Fig. 3A). The marked decrease of the MTP in immunostimulated astrocytes was observed only after glucose deprivation (Fig. 3A). The peroxynitrite-releasing reagent SIN-1 rapidly depolarized the MTP in glucose-deprived astrocytes (Fig. 3B). Of cyclams used in the present study, only Mn(III)-cyclam blocked the MTP depolarization in immunostimulated or SIN-1-treated astrocytes deprived of glucose (Fig. 3A and B, respectively).

4. Discussion

Much interest has been drawn in small molecular weight transition metal complexes that catalyse the dismutation of superoxide anion. Some manganese complexes are able to catalyse the dismutation of superoxide anion. Some manganese complexes are able to catalyse the dismutation of superoxide anion.
superoxide anion and protect SOD-deficient organisms against oxidative stress (Chang and Kosman, 1989). Neutrophil-mediated human aortic endothelial cell injury in vitro is mediated by the superoxide anion and low-molecular-weight manganese-based SOD mimetics are effectively abrogating the cytotoxicity (Hardy et al., 1994; Riley et al., 1997). The mechanism by which manganese exerts the protective effect probably involves alternative oxidation and reduction of the metal complex.

The increased vulnerability of immunostimulated astrocytes to glucose deprivation was mainly caused by much accumulation of peroxynitrite secondary to complete depletion of intracellular antioxidant system including reduced glutathione, a well-known peroxynitrite scavenger (Choi et al., 2000, 2001; Ju et al., 2000). In our previous reports (Choi et al., 2000, 2001), porphyrin compounds such as Mn(III) tetrakis(N-methyl-4-pyridyl)porphyrin (MnTMPyP) and FeTMPyP also completely prevented the augmented death of glucose-deprived immunostimulated astrocytes by scavenging the endogenously produced peroxynitrite. Like porphyrin compounds MnTMPyP and FeTMPyP, we report here that another cell membrane-permeable and low-molecular-weight SOD mimetic Mn(III)-cyclam effectively prevented the enhanced death in glucose-deprived immunostimulated or SIN-1-treated astrocytes. Like Mn(III)-cyclam, Ni(II)-cyclam also significantly inhibited the augmented death in those cells. Previously, we reported a surprising property of Ni(II)-cyclam to strongly inhibit the free-radical autoxidation of aldehydes (Nam et al., 1996). To our knowledge, this is the first report showing that Ni(II)-cyclam may scavenge peroxynitrite in a cell system. Compared with Mn(III)-cyclam, however, Ni(II)-cyclam was less efficacious for preventing peroxynitrite-evoked cell death and scavenging peroxynitrite, as shown in Figs. 1 and 2 in this study. Depolarization of MTP has been established as one of the crucial sign of the cell death (Reichert et al., 2001; Nicholls and Ward, 2000; Choi et al., 2000). Obvious blockade of MTP depolarization was observed only by Mn(III)-cyclam, but not by Ni(II)-cyclam (shown in Fig. 3 in this study). The amount of peroxynitrite remaining in the presence of Ni(II)-cyclam might be sufficient to decrease the MTP in our experimental conditions. This result also indicate that Mn(III)-cyclam is a better scavenger against peroxynitrite than Ni(II)-cyclam.

Compared to natural cell-impermeable proteins such as SOD and catalase and, Mn(III)-cyclam was found to rapidly enter the immunostimulated astrocytes and effectively scavenge the ONOO⁻ produced endogenously in those cells. In several respects, cyclam compounds are thought to be better agents for clinical uses than porphyrins. While cyclam compounds such as Mn(III)-cyclam and Ni(II)-cyclam are colorless, porphyrin compounds such as MnTMPyP and FeTMPyP are strongly yellowish and turns out the whole body and body fluid yellowish. Unlike porphyrin compounds,

Fig. 3. Mn(III)-cyclam prevents the MTP depolarization in glucose-deprived immunostimulated or SIN-1-treated astrocytes. (A) Immunostimulated astrocytes were loaded with JC-1 and then deprived of glucose in the absence and presence of various kinds of cyclams (10 μM for each). Fluorescence intensities were acquired at indicated times after starting glucose deprivation. Because of photobleaching, fluorescence intensities were measured only at indicated times after starting glucose deprivation. Data were expressed as the mean ± S.E.M. of the ratios of aggregate fluorescence (F590) to monomer fluorescence (F530), N = 4 (A) and 5 (B).
cyclams are low-molecular-weight and may permeate better through the blood–brain-barrier. Taken together, the present results indicate that some cell membrane-permeable synthetic SOD mimetics might serve as therapeutically potential agents for inflammatory reaction-associated ischemic injury.

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