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Neuroprotection of taurine against reactive oxygen species is associated with inhibiting NADPH oxidases

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ABSTRACT

It is well established that taurine shows potent protection against glutamate-induced injury to neurons in stroke. The neuroprotection may result from multiple mechanisms. Increasing evidences suggest that NADPH oxidases (Nox), the primary source of superoxide induced by N-methyl-D-aspartate (NMDA) receptor activation, are involved in the process of oxidative stress. We found that 100 μM NMDA induced oxidative stress by increasing the reactive oxygen species level, which contributed to the cell death, in vitro. Neuron cultures pretreated with 25 mM taurine showed lower percentage of death cells and declined reactive oxygen species level. Moreover, taurine attenuated Nox2/Nox4 protein expression and enzyme activity and declined intracellular calcium intensity during NMDA-induced neuron injury. Additionally, taurine also showed neuroprotection against H_2O_2-induced injury, accompanying with Nox inhibition. So, we suppose that protection of taurine against reactive oxygen species during NMDA-induced neuron injury is associated with Nox inhibition, probably in a calcium-dependent manner.

1. Introduction

Stroke is one of the most common diseases in the world (Iadecola and Anrather., 2011; Chen et al., 2011). The mechanisms underlying stroke damage are multifactorial, and excitotoxicity induced by excessive extracellular glutamate is a primary factor (Lai et al., 2011). Cell swelling-induced release of glutamate and reversal of the astrocytic glutamate transporter contribute to the ischemia-induced increases of extracellular glutamate (Rothstein et al., 1996; Seki et al., 1999). As the consequence, N-methyl-D-aspartate (NMDA) receptors are over-stimulated, resulting in source-specific excitotoxic Ca^{2+} entry (Hazell., 2007).

Oxidative stress has also been implicated in the pathogenesis of brain damage in ischemic stroke (Chen et al., 2011). Reactive oxygen species, including superoxide anions, hydroxyl radicals, hydrogen peroxide and single oxygen, are generated during normal cellular respiration and metabolic processes, physiologically serving as signaling molecules. But imbalance between the formation and elimination of reactive oxygen species leads to their accumulation within the cells, which induces oxidative damage. (Chen et al., 2011; Kahles and Brandes., 2013). Within recent years, NADPH oxidases (Nox) have been identified as the primary source of pathological reactive oxygen species, especially the superoxide anions, in the central neural system (Vallet et al., 2005; Bedard and Krause., 2007; Kahles and Brandes., 2013). Extensive activation of NMDA receptors can regulate Nox activity and leads to neuronal death (Brennan et al., 2009).

NADPH oxidases are a family of cytoplasmic enzymes transferring electrons across biological membranes. By far, seven members of Nox family enzymes have been identified, that is Nox1, Nox2 (gp91phox), Nox3, Nox4, Nox5, Duox1 and Duox2 (Bedard and Krause., 2007; Touyz et al., 2011). Ischemic stroke upregulates Nox2 and Nox4 mRNA level (Kahles and Brandes., 2013). Meanwhile, Nox2 deletion and Nox4 deletion are both found to exert neuroprotective effect against brain damage, significantly improve stroke outcome and decrease infarct volume (Suh et al., 2008; Kleinschnitz et al., 2010).

Taurine (2-aminoethanesulfonic acid) is one of the major intracellular free β-amino acids in most mammalian tissues. It has been implicated in several processes involving brain development, osmoregulation, membrane stabilization and neurotransmission (Huxtable, 1992; Sturman, 1993). Moreover, taurine is found to exert potent protections against glutamate-induced neurotoxicity

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and many possible mechanisms are suggested (Ye et al., 2013; Kumari et al., 2013). However, its neuroprotection against reactive oxygen species during excessive activation of NMDA receptors and the underlying mechanisms are not well known. In this study, NMDA was used to induce the excitotoxicity in cultured neurons to investigate the protective effects of taurine and determine the role of NADPH oxidases in this neuroprotection against oxidative stress.

2. Materials and methods

2.1. Regents and drugs

2-aminoethanesulfonic acid (Taurine), 2′,7′-Dichlorofluorescin diacetate (DCFH-DA), 4′-Hydroxy-3′-methoxyacetophenone (Apocynin), Beta-Nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt (NADPH-4Na), (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 2,7-Diamino-10-ethyl-9-phenyl-9,10-dihydrophenanthridine (Dihydroethidium DHE), 4-(6-Acetoxymethoxy-2,7-dichloro-3-oxo-9-xanthenyl)-4′-methyl-2,2′(ethylenedioxy)dianiline-N,N,N′,N′-tetraacetic acid tetrakis (acetoxymethyl) ester (Fluo-3AM) were purchased from Invitrogen (Carlsbad, CA, USA). Lactate dehydrogenase (LDH) release assay kit was purchased from Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). All cell culture medium and supplements were purchased from Gibco (Grand Island, NY, USA).

Product description of taurine.

Molecular Formula: $C_2H_7NO_3S$	pKa (-SO_3H): 1.51, 2
Molecular Weight: 125.1	pKa (-NH_3): 9.061, 8.742
CAS Number: 107-35-7	Synonym: 2-aminoethanesulfonic acid

2.2. Animals

The experimental protocol was approved by the Institutional Animal Care and Use Committee of Nanjing Medical University. 15-day fetal ICR mice (Model Animal Research Center of Nanjing University), were used for cell cultures in this study. These mice were bred at the Model Animal Research Center of Nanjing University.

2.3. Cell cultures

Primary neurons were isolated from E15 ICR mouse cortex and cultured on dishes coated with polyornithine (10 μg/ml) in Neurobasal medium containing 2% B27 supplement, as we have reported (Luo et al., 2010). The planting density was 1.5×10^5 cells/cm^2 for biochemical detection. Cultured neurons were identified at 10 days in vitro (DIV), and the proportion of β-III-tubulin$^+$ cells was ~93%. All cultures were maintained in an incubator (HERAcell 150, Thermo Fisher Scientific) with a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

2.4. Neurotoxicity measurement

LDH release was evaluated to assess the neurotoxicity of H_2O_2 or NMDA in cortical neurons and was measured with the cytotoxicity detection kit (Jiancheng, Nanjing, China). Results were expressed as the percentage of maximum LDH release obtained by lysing the cells in 1% Triton X-100.

2.5. Detection of intracellular reactive oxygen species level

The determination of intracellular oxidant production was based on the oxidation of DCFH-DA. DCFH-DA is membrane permeative and is trapped intracellularly following deacetylation. The resulting compound, DCFH, reacts with reactive oxygen species to produce the oxidized fluorescent form 2,7-dichlorofluorescein (DCF).

After 9 DIV, cells were incubated with DCFH-DA for 30 min, then with 100 μM NMDA for 3 h. The fluorescence was read at 488 nm for excitation and 525 nm for emission with the SpectraMax M2 Microplate Reader (Molecular Devices, USA). The cellular fluorescence intensity was expressed as the fold increase compared to the controls.

2.6. Analysis of intracellular superoxide anion

DHE is the chemically reduced form of the commonly used DNA dye ethidium bromide. DHE itself is blue fluorescent in cell cytoplasm, whereas the oxidized form ethidium is red fluorescent upon DNA intercalation. After 9 DIV, pretreated with taurine (25 mM) for 24 h, cells were incubated with antioxidant Trolox (100 μM) or Nox inhibitor apocynin (500 μM) for 30 min, and subsequently incubated with DHE (5 μM) for 15 min. Thereafter, 150 μl Hank's balanced salt solution (HBSS) was applied, and the fluorescence was read (absorption/emission: 396/580 nm) for 10 min as the baseline. After treatment with 100 μM NMDA, the fluorescence intensity was analyzed with the microplate reader per 5 min.

2.7. Detection of intracellular calcium

After 9 DIV, the neurons were pretreated with taurine (25 mM) for 24 h, and treated with antioxidant Trolox (100 μM) or Calcium inhibitor EGTA (1 mM) for 30 min. Then, Fluo-3AM (5 μM) was added to the cultures and further incubated for 35 min. After rinsing 2 times with HBSS to eliminate excessive dye, 150 μl HBSS was added to the cultures, and the fluorescence was read (absorption/emission: 488/538 nm) for 10 min as the baseline. After treatment with 100 μM NMDA, the change in fluorescence was analyzed with the microplate reader for 30 min.

2.8. Detection of NADPH oxidase activity

Cultured neurons were pretreated with 25 mM taurine for 24 h at 9 DIV before the experiment. 100 μM NMDA or H_2O_2 was added in HBSS to induced neuron injury. Apocynin, an NADPH oxidase inhibitor, was administered 30 min before the reading so that NADPH reduction could be attributed to the activity of the NADPH oxidase rather than other system. Then neurons were detached with cold fresh phosphate buffered solution (PBS) using the cell scraper. Cell suspension was centrifuged at 2500 g at 4 °C for 5 min. The resulting pellet was resuspended in 200 μl PBS and transferred to a 96 well plate, where 100 μl of each sample was transferred to each well. Immediately before the plate was read in the plate reader, 250 μM NADPH was added to each well, and the decrease in absorbance as NADPH was converted to NADP was measured at 340 nm for 10 min using a Spectra Max M2e plate reader. The raw data were plotted and a graph showing the gradient was used to calculate the initial rate of activity. Data was expressed in pM NADP /μg protein/min and was calculated using the equation $C = A/(E \times L)$ where C is the concentration of NADP reduced by the NADPH oxidase, A is the absorbance(the gradient