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NITRIC OXIDE PLAYS A DUAL ROLE IN THE OXIDATIVE INJURY OF CULTURED RAT MICROGLIA BUT NOT ASTROGLIA

J.-Y. WANG, [a] **C.-T. LEE** [b] **AND J.-Y. WANG** [c*]

[a] *Graduate Institute of Medical Sciences and Department of Physiology, College of Medicine, Taipei Medical University, Taipei, Taiwan*

[b] *Department of Nursing, Hsin-Sheng College of Medical Care and Management, Taoyuan, Taiwan*

[c] *Department of Neurology, School of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan*

Abstract—Nitric oxide (NO) and oxidative stress caused by reactive oxygen species (ROS) accumulation are two important factors that lead to the progression of human neurological diseases. NO can be detrimental or protective to neurons under oxidative toxicity; however, in the case of brain exposure to oxidative stress, in addition to neurons, the existence of glia may also be disturbed by toxic ROS. The influence NO will have on ROS-mediated glial injury remains unclear. Here, we examined the effects of NO on cell viability under oxidative stress induced by hydrogen peroxide (H_2O_2) in rat primary mixed glia cultures, as well as pure astroglia and microglia cultures. We found that in mixed glia cultures, both H_2O_2 and NO donor S-nitroso-N-acetyl-D, L-penicillamine (SNAP) elicited cell death in a concentration-dependent manner. Combinations of H_2O_2 and SNAP at sublytic concentrations were sufficient to damage mixed glia, and sublytic concentrations of SNAP could reduce the insults that resulted from toxic H_2O_2. Furthermore, in microglia or astroglia, sublytic concentrations of H_2O_2 were toxic when combined with SNAP, and the potency was increased with an increased SNAP concentration. In microglia but not astroglia, a toxic H_2O_2-induced apoptotic injury was attenuated by a sublytic level of SNAP. H_2O_2 at toxic levels activated p38 mitogen-activated protein kinases (MAPK) and p53 pathways and increased DNA double strand breaks (DSBs) in microglia, whereas the rescue exerted by sublytic SNAP against toxic H_2O_2 occurred via the activation of both Akt and extracellular-signal-regulated kinase (ERK) cascades and decreased DNA DSBs. Moreover, a sublytic concentration of SNAP induced both heat shock protein 70 and heme oxygenase-1, which may be involved in decreasing the susceptibility of microglia to H_2O_2 toxicity. These results suggest that NO exhibits a concentration-dependent dual action of weakening or enhancing oxidative injury in mixed glia, particularly microglia.

Key words: nitric oxide, H_2O_2, oxidative stress, microglia, astroglia, apoptosis.

INTRODUCTION

Nitric oxide (NO) acts as a gaseous signaling molecule and participates in various physiological and pathological processes in the central nervous system (CNS) (Calabrese et al., 2007). The brain has the highest concentration of NO among different tissues, and NO critically manages broad aspects of brain function, including synaptic plasticity, neurodevelopment and neuronal cell death (Centonze et al., 2003; Chung et al., 2005; Contestabile, 2012). NO can be produced extensively in the brain because all CNS cells have the ability to synthesize NO via NO synthase (NOS), which comprises three main isoforms, termed neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS) (Paakkari and Lindsberg, 1995). At low concentrations, NO plays a role in neurotransmission and vasodilation, whereas at higher levels, it is important in immunity and inflammation (Faraci and Brian, 1994; Liu et al., 2002). The imbalance of NO metabolism within the CNS can lead to pathogenic conditions. In particular, activated glial cells, such as astroglia and microglia, can produce toxic amounts of NO via iNOS expression, which leads to neuronal cell injury in many oxidative stress-associated pathological states, including cerebral stroke, trauma and several neurodegenerative disorders (Chung et al., 2005). However, the role of NO released by glia, which leads to gliotoxicity (i.e., glial toxicity) during the progression of these brain diseases, has not been clearly defined.

When the brain is exposed to oxidative stress, NO easily interacts with reactive oxygen species (ROS),

*Corresponding author. Present address: Department of Neurology, College of Medicine, Kaohsiung Medical University, No. 100, Shih-Chuan 1st Road, Sanmin District, Kaohsiung 80708, Taiwan. Tel: +886-7-3121101-5092-432; fax: +886-7-3222461.

E-mail address: jizyuhwang@cc.kmu.edu.tw (J.-Y. Wang).

Abbreviations: CNS, central nervous system; DAPI, 4,6-diamidin-2-phenylindol; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; DSBs, double strand breaks; EDTA, ethylenediaminetetraacetic acid; eNOS, endothelial NOS; ERK, extracellular-signal-regulated kinase; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GFAP, glial fibrillary acid protein; H_2O_2, hydrogen peroxide; HO-1, heme oxygenase-1; HSP70, heat shock protein 70; IgG, immunoglobulin G; iNOS, inducible NOS; JNK, c-Jun N-terminal kinase; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinases; MTT, 3-(4,5-dimethylthianol-2-yl)-2,5-diphenyltetrazolium bromide; NeuN, neuronal nuclear antigen; NF-κB, nuclear factor-kappa B; NO, nitric oxide; NOS, nitric oxide synthase; OD, optical density; PBS, phosphate-buffered saline; PFTα, pifithrin-α; RNS, reactive nitrogen species; ROS, reactive oxygen species; SNAP, S-nitroso-N-acetyl-D,L-penicillamine; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.

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thereby forming reactive nitrogen species (RNS) (Beckman and Koppenol, 1996). Many of the harmful effects of NO are more likely mediated by its oxidation products rather than NO itself, although NO is considered potentially toxic (Beckman and Koppenol, 1996). For example, peroxynitrite ($ONOO^-$) generated from the reaction of NO with superoxide anion ($^{\cdot}O_2^-$) is capable of initiating further protein oxidation and nitration, which ultimately induces apoptosis in primary neurons (Estevez et al., 1998; Wang et al., 2003). Several lines of evidence suggest that NO enhances oxidative damage evoked by hydrogen peroxide (H_2O_2) in vascular and rat hepatoma cells (Ioannidis and de Groot, 1993; Zhou et al., 2013); however, NO is protective against ROS-mediated cell death, as evidenced by NO-mediated suppression of heart hypoxia/reoxygenation or liver ischemia–reperfusion injury (Korge et al., 2008; Kuo et al., 2013). In neurons, NO can be either protective or detrimental to ROS toxicity, which depends on several critical factors, including the source (e.g., neuronal, glial or endothelial NOS), concentration and oxidation–reduction status of NO, the type, time length and severity of the ROS insult, and the use of experimental animal species/cell types (Mohanakumar et al., 1998; Wei et al., 2000; Calabrese et al., 2007; Allen et al., 2009; Godinez-Rubi et al., 2013). Nevertheless, to our knowledge, the effects of NO on oxidative toxicity in glial cells remain unclear.

In response to inflammatory signals, glial cells are rapidly activated and secrete toxic substances, such as proinflammatory cytokines, NO and ROS, to kill foreign pathogens, including bacteria (Pender and Rist, 2001). However, during conditions of gross neuronal injury and chronic neuroinflammation, over-activated glia may persistently release lethal amounts of ROS and NO, which re-injures neurons and may also cause glial cell damage. Accordingly, similar to neurotoxicity, gliotoxicity is deeply influenced by interactions between NO and ROS and must be extensively studied.

In this study, we used primary cell cultures as well as controlled dosages of the ROS donor H_2O_2 and a pure NO donor S-nitroso-N-acetyl-D,L-penicillamine (SNAP) to investigate the effects of NO on H_2O_2 toxicity in mixed glia, astroglia- and microglia-enriched cultures. Also, whether NO intervenes in toxic H_2O_2-stimulated intracellular signaling cascades was examined. We determined that NO interacts with H_2O_2 in a concentration-dependent manner, thereby playing a dual role to either enhance or suppress oxidative injury in glial cultures. The rescue effect of sublytic levels of NO on H_2O_2-induced apoptosis occurred in microglia but not astroglia. Therefore, we have provided novel data that establish the precise correlation between H_2O_2 and NO in glia and highlight gliotoxicity as important in oxidative stress-associated brain diseases, including ischemic stroke and neurodegeneration.

EXPERIMENTAL PROCEDURES

Antibodies and reagents

Antibodies, including extracellular-signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), p38, p53, phospho-ERK (pERK), phospho-JNK (pJNK), phospho-p38 (pp38), phospho-p53 (p53), cleaved caspase-3 and heat shock protein 70 (HSP70), were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies specific for Akt and phospho-Akt (pAkt) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Neuronal nuclear antigen (NeuN), γH2AX and H2AX antibodies were obtained from Millipore (Billerica, MA, USA). Heme oxygenase-1 (HO-1) antibody was purchased from Enzo Life Sciences (Farmingdale, NY, USA). The CD11b antibody (clone OX-42) was purchased from Abcam (Cambridge, UK). Glial fibrillary acid protein (GFAP) and β-actin antibody were purchased from GeneTex (San Antonio, TX, USA). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit immunoglobulin G (IgG) antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). SNAP, 3-(4,5-dimethylthianol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), 4′,6-diamidino-2-phenylindole (DAPI) and the p53 inhibitor pifithrin-α (PFTα) were purchased from Sigma–Aldrich (Saint Louis, MO, USA). H_2O_2 was obtained from Merck KGaA (Darmstadt, Germany). Chemical inhibitors, including the PI3 Kinase inhibitor LY294002 (LY), the MEK1/2 inhibitor PD98059 (PD), the p38 mitogen-activated protein kinases (MAPK) inhibitor SB20358 (SB) and the HSP70 inhibitor VER-155008 (VER), were purchased from Selleck Chemicals (Houston, TX, USA). The HO inhibitor tin protoporphyrin IX (SnPPIX) was purchased from Frontier Scientific (Logan, UT, USA). All cell culture reagents were obtained from Gibco-BRL/Invitrogen (Carlsbad, CA, USA).

Primary cultures of rat mixed glia, microglia and astroglia

Glial cultures were prepared from the whole brains of 1-day-old neonatal Sprague-Dawley rats, based on modifications of the method previously described (Wang et al., 1998; Liu et al., 2001). In brief, the brain tissues were quickly removed from the crania and then collected in ice-cold Ca^{2+}/Mg^{2+} free-Hank's Balanced Salt Solution. After the removal of the cranial meninges and blood vessels, the brains were finely chopped with scissors. The cell aggregations were further dissociated using 0.25% trypsin/0.05% EDTA (10 min, 37 °C) and gentle trituration using a pipette. Following centrifugation (500 × *g*, 5 min), the brain cells were suspended in 10% fetal bovine serum (FBS)/Dulbecco's modified Eagle's medium (DMEM), seeded at a density of 5×10^5 cells/ml and incubated at 37 °C in a humidified atmosphere of 5% CO_2/95% air. To diminish neuronal cell populations, the culture media were frequently refreshed at an interval of 2 days until the cell composition contained < 5% neurons, determined by immunocytochemistry. Two to three weeks subsequent to initial plating, primary cultures of glia were used for a series of experiments. Our glia cultures consisted of approximately 3–5% neurons, 60–65% astroglia and 30–35% microglia. Other unstained cell types, such as oligodendrocytes, fibroblasts and smooth muscle cells, were < 2% of the total cells.