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Neuroscience Letters 389 (2005) 133–136
Neuroscience Letters
www.elsevier.com/locate/neulet

Increased susceptibility to oxidative stress in scrapie-infected neuroblastoma cells is associated with intracellular iron status

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Received 18 April 2005; received in revised form 20 July 2005; accepted 20 July 2005

Abstract

The molecular mechanism of neurodegeneration in prion diseases remains largely uncertain, but one of the features of infected cells is higher sensitivity to induced oxidative stress. In this study, we have investigated the role of iron in hydrogen peroxide (H_2O_2)-induced toxicity in scrapie-infected mouse neuroblastoma N2a (ScN2a) cells. ScN2a cells were significantly more susceptible to H_2O_2 toxicity than N2a cells as revealed by cell viability (MTT) assay. After 2 h exposure, significant decrease in cell viability in ScN2a cells was observed at low concentrations of extracellular H_2O_2 (5–10 μM), whereas N2a cells were not affected. The increased H_2O_2 toxicity in ScN2a cells may be related to intracellular iron status since ferrous iron (Fe^{2+}) chelator 2,2′-bipyridyl (BIP) prevented H_2O_2-induced decrease in cell viability. Further, the level of calcein-sensitive labile iron pool (LIP) was significantly increased in ScN2a cells after H_2O_2 treatment. Finally, the production of reactive oxygen species (ROS) was inhibited by 30% by iron chelators desferrioxamine (DFO) and BIP in ScN2a cells, whereas no significant effect of iron chelators on basal ROS production was observed in N2a cells. This study indicates that cellular resistance to oxidative stress in ScN2a cells is associated with intracellular status of reactive iron.

Keywords: Prion disease; Oxidative stress; Cell viability; Labile iron pool

Prion diseases are fatal neurodegenerative disorders that include Creutzfeldt–Jakob disease, Gerstmann–Sträussler–Scheinker disease, kuru and fatal familial insomnia in humans, scrapie in sheep and goat, and bovine spongiform encephalopathy in cattle. Characteristic for prion diseases is the conformational change of the cellular prion protein (PrP^C) into a pathological and infectious isoform (PrP^{Sc}). Although the precise function of PrP^C is still largely unknown, a possible role for PrP^C in modulating cellular oxidative stress has been suggested. It has been shown that the activity of copper–zinc superoxide dismutase (Cu/Zn-SOD) is reduced in PrP knockout mice ($PrP^{0/0}$), and neuronal cells lacking PrP^C are more sensitive to oxidative stress and undergo cell death more readily than wild-type cells [2]. Further, $PrP^{0/0}$ neurons have reduced glutathione reductase activity and increased susceptibility to hydrogen peroxide (H_2O_2) toxicity [13]. Studies using scrapie-infected hypothalamic GT1 cells have shown that scrapie infection renders neuronal cells more susceptible to oxidative stress and impairs their free radical metabolism, as evidenced by a higher sensitivity to induced oxidative stress, an increase in lipid peroxidation and a reduction in the activities of the glutathione-dependent and SOD antioxidant systems over non-infected cells [10]. Reduced Cu/Zn-SOD and glutathione peroxidase activities along with elevated levels of nitric oxide and superoxide anions ($O_2^{\bullet -}$) have been detected in the brains of scrapie-infected mice [14], suggesting that oxidative stress partly contributes to neurodegeneration in scrapie infection.

Redox-active transition metals have been implicated in human neurodegenerative diseases due to their ability to generate reactive oxygen species (ROS). In the brains of scrapie-infected mice, it has been demonstrated that the oxidative impairment and the presence of oxidative stress is associated with changes in metal levels [9,14]. We have recently shown that the treatment of scrapie-infected mouse neuroblastoma

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0304-3940/$ – see front matter © 2005 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.neulet.2005.07.032

N2a (ScN2a) cells with iron results in a rapid and significant ROS formation accompanied by a decrease in cell viability [6]. To better understand a possible involvement of iron in oxidative stress in scrapie-infected cells, we studied the relationship between H_2O_2-induced toxicity and intracellular iron status.

ScN2a cells were generated as previously described [3] and together with non-infected N2a cells were generously provided by Dr. Stanley B. Prusiner. The cells were maintained at 37 °C under an atmosphere of 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) with Glutamax II and 4.5 g/l D-glucose supplemented with 5% fetal bovine serum (FCS), 100 U/ml penicillin and 100 mg/ml streptomycin. The ScN2a cells were routinely checked for their scrapie infection as described by Östlund et al. [11]. For iron chelator treatments, cells were treated when indicated with 100 μM ferrous iron (Fe^{2+}) chelator 2,2′-bipyridyl (BIP), 100 μM ferric iron (Fe^{3+}) iron chelator desferrioxamine (DFO) or 100 μM ferrozine.

Cell viability was assessed by using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5,diphenyl-tetrazolium bromide (MTT) assay [8]. Briefly, around 10,000 cells per well were plated in 96-well microtiter plates with 100 μl of medium. Next day, the medium was changed and 10 μl of MTT (5 mg/ml stock in PBS) was added to each well for 1 h at 37 °C. One hundred microliters of solubilization solution containing 20% SDS/50% DMF of pH 4.7 was added and absorption readings were performed at 540 nm with reference at 690 nm.

The cytosolic labile iron pool (LIP) was determined as described [4]. Briefly, 4×10^6 cells were loaded with 100 nM calcein-AM (Molecular Probes) in PBS at 37 °C for 15 min. After washing non-internalized calcein, the cells were transferred to a stirred cuvette, and the basal calcein fluorescence was recorded (excitation 488 nm, emission 517 nm). The fluorescence of the calcein–iron complex was de-quenched by the addition of 100 μM salicylaldehyde isonicotinoyl hydrazone (SIH), and the increase in fluorescence was monitored until a steady signal was obtained (<5 min).

Intracellular ROS levels were measured by incubating 500,000 cells with 800 nM 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA, Molecular Probes) for 15 min. After washing the cells twice with PBS, 2′,7′-dichlorofluorescein (DCF) fluorescence was measured at 488 (excitation) and 520 nm (emission).

For Western blotting analysis, indicated amounts of total cell extracts prepared in Triton X-100 lysis buffer (20 mM Tris–HCl, pH 8.0, 137 mM NaCl, 1% (v/v) Triton X-100, 10% (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 μg/ml leupeptin) were resolved by SDS–PAGE, electrotransferred onto a nitrocellulose membrane, and probed with anti-transferrin receptor 1 antibodies at 1:1000 and anti-tubulin antibodies at 1:1000 to verify even protein loading. Densitometric analysis was performed using the Image Gauge software (Fuji).

We first measured cell viability by using a MTT assay after treatment of cells with H_2O_2 for 2 h. ScN2a cells were significantly more sensitive to the H_2O_2 toxicity than N2a cells as shown in a dose–response curve (Fig. 1A). Analysis of H_2O_2 toxicity revealed that ScN2a cells are susceptible to H_2O_2-induced oxidative stress at low H_2O_2 concentrations, 5–10 μM (Fig. 1B, grey bars), whereas no significant reduction in cell viability was measured in N2a cells at 10 μM concentration of H_2O_2 (Fig. 1B, white bars).

Since iron is involved in free radical generation through Fenton reaction, we studied whether iron chelators affect H_2O_2-induced toxicity in ScN2a cells. Treatment of cells with highly permeant Fe^{2+} chelator BIP prevented H_2O_2-induced reduction in cell viability in ScN2a cells (Fig. 1B, grey bars), whereas no significant effect on cell viability by BIP was observed in N2a cells (Fig. 1B, white bars). Fe^{3+} chelator DFO did not affect H_2O_2-induced toxicity in N2a or ScN2a cells (Fig. 1B).

After finding that Fe^{2+} chelator BIP prevented H_2O_2-induced reduction in cell viability in ScN2a cells, we measured LIP levels in cells treated with H_2O_2. We have demonstrated earlier that ScN2a cells contain two-fold lower LIP than N2a cells [5]. Treatment of cells with 50 μM H_2O_2 resulted in a rapid and significant increase in LIP in ScN2a cells (Fig. 2A, grey bars), whereas LIP level was

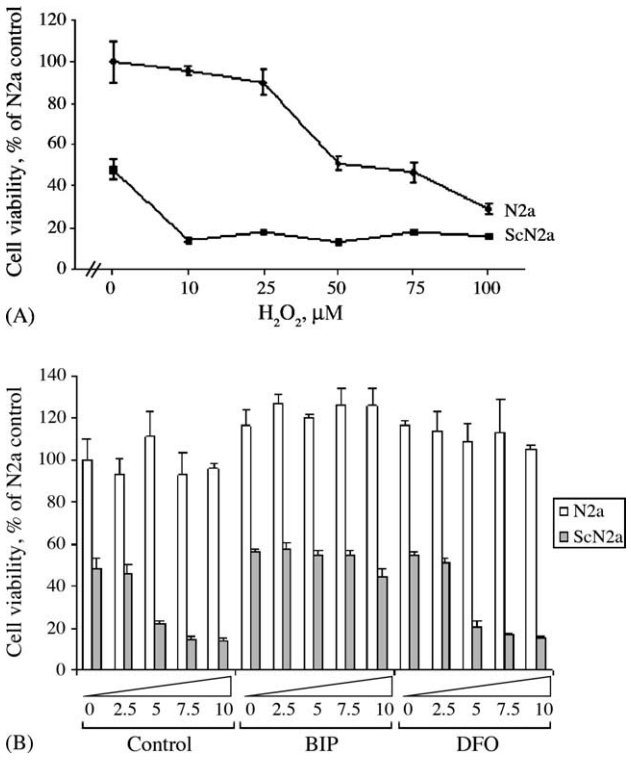

Fig. 1. Increased susceptibility to H_2O_2 in ScN2a cells. (A) Cell viability was measured in cells treated with H_2O_2 at the concentration range 0–100 μM for 2 h. (B) Cell viability in the absence or presence of Fe^{2+} chelator BIP (100 μM) or Fe^{3+} chelator DFO (100 μM) in cells treated with H_2O_2 at the concentration range 0–10 μM for 2 h. Means of four independent experiments.