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SCIENCE DIRECT

Free Radical Biology & Medicine 39 (2005) 345 – 354

www.elsevier.com/locate/freeradbiomed

Original Contribution

Activation of c-Jun-N-terminal kinase is required for apoptosis triggered by glutathione disulfide in neuroblastoma cells

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Received 7 December 2004; revised 18 March 2005; accepted 18 March 2005
Available online 7 April 2005

Abstract

Changes in intracellular redox status are crucial events that trigger downstream proliferation or death responses through activation of specific signaling pathways. Moreover, cell responses to oxidative challenge may depend on the pattern of redox-sensitive molecular factors. The stress-activated protein kinases c-Jun-N-terminal kinase (JNK) and p38 MAP kinase ($p38^{MAPK}$) are implicated in different forms of apoptotic neuronal cell death. Here, we investigated the effects, on neuroblastoma cells, of the prooxidant molecule GSSG, which we previously demonstrated to be an efficient proapoptotic compound able to activate the $p38^{MAPK}$ death pathway in promonocytic cells. We found that neuroblastoma cells are not prone to GSSG-induced apoptosis, although the treatment slightly induced growth arrest through the accumulation of p53 and its downstream target gene, p21. However, GSSG treatment became cytotoxic when cells were previously depleted of intracellular GSH content. Under this condition, apoptosis was triggered by an increased production of superoxide that led to a specific activation of the JNK-dependent pathway. The involvement of superoxide and JNK was demonstrated by cell death inhibition in experiments carried out in the presence of Cu,Zn superoxide dismutase or with specific inhibitors of JNK activity. Our data give support to the studies that indicate preferential requirements for the involvement of stress-activated kinases in apoptotic neuronal cells.

Keywords: Apoptosis; MAP kinase pathway; Neuroblastoma; Glutathione; ROS; Oxidative stress

Introduction

Reactive oxygen species (ROS) have been traditionally considered to be damaging to cells. However, a large number of evidence has shown that ROS may also function as important activators of key physiological processes playing a vital role in the cell signaling network [1–3]. The ROS-mediated signaling network relies on a dynamic regulation of the oxidized/reduced form of redox-sensitive proteins containing critical cysteines. In this context, glutathione, the most abundant low molecular weight thiol within the cell [4], represents the main buffer system regulating the redox state of these proteins [5].

In the last few years, a direct link between mitogen-activated protein (MAP) kinases signaling network and ROS production has been suggested. In fact, it has been found that redox-sensitive proteins, such as thioredoxin and glutathione *S*-transferase (GST), can directly bind and inactivate apoptosis signal-regulating kinase 1 (ASK1) and JNK1, respectively [6,7]. Under redox unbalance GST and thioredoxin are induced to dissociate, due to the oxidation of specific cysteine residues, thus leading to the activation of their MAP kinase partners. As a result, ASK1 and JNK1 are able to autoactivate and transduce the signal by phospho-activating downstream effectors. This sequence of events represents the switch able to transform a specific redox change (thiol/disulfide) in a phosphorylative potential that may act in a broad range within the cell.

Abbreviations: GSH, reduced glutathione; GSSG, glutathione disulfide; ROS, reactive oxygen species; MAPK, mitogen-activated protein kinase; ASK1, apoptosis signal-regulating kinase; JNK, c-Jun-NH_2-terminal kinase; Cu,Zn SOD, copper, zinc superoxide dismutase; BSO, D,L-buthionine *S,R*-sulfoximine; DCF-DA, 2′-7′-dichlorodihydrofluorescein diacetate; hSOD, SH-SY5Y cells transfected with the human Cu,Zn SOD gene.

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doi:10.1016/j.freeradbiomed.2005.03.022

Previously, we demonstrated that exogenous GSSG, a membrane-impermeable oxidizing molecule, was able to induce, in U937 promonocytic cells, a rapid alteration of intracellular GSH levels by shifting the equilibrium toward the formation of mixed disulfides with protein thiols [8]. This redox unbalance triggered an apoptotic response by the activation of the canonical mitochondrial pathway, which was preceded by dissociation of the thioredoxin/ASK1 complex and phospho-activation of the p38 MAP kinase ($p38^{MAPK}$) pathway. We postulated that such responses could be mediated by specific trans-membrane proteins rich in cysteine residues, which transduce the oxidizing power of GSSG across the plasma membrane through a thiol/disulfide exchange. Since the activation of ASK1/$p38^{MAPK}$ pathway is also involved in cell response mediated by the engagement of ligands to cysteine-rich death receptors [i.e., those belonging to tumor necrosis factor (TNF) superfamily], we hypothesized that the transduction of oxidizing power could also occur through these trans-membrane proteins. This hypothesis was supported by the evidences that leukemia cells express TNF superfamily-related death receptors [i.e., TNF-related apoptosis-inducing ligand (TRAIL) receptors] at high levels on membrane surfaces, and GSSG was not toxic when added to differentiated macrophages or lymphocytes, although high concentrations were used.

In order to verify whether the cell response to GSSG treatment was hystotype-dependent, we determined the effects of GSSG on neuroblastoma cells. In particular, we found that these cells were resistant to GSSG unless depleted of glutatione content. Focusing on the MAPK signaling pathway activated by GSSG treatment under glutatione deprivation, we found that the predominant MAP kinase pathway to be induced was that mediated by JNK. The results may provide a better understanding about the contribution of cell-specific redox-sensitive proteins in the different cellular responses to oxidative stimuli.

Materials and methods

Materials

Oxidized glutathione (GSSG) was from Roche Molecular Biochemicals (Monza, Italy). D,L-Buthionine-*S,R* sulfoximine (BSO), propidium iodide, protease inhibitor cocktail, IGEPAL CA-630, and anti-p53 (clone BP53-12) and anti-actin (clone AC-40) monoclonal antibodies were obtained from Sigma (St. Louis, MO). Anti-p21(clone C-19) polyclonal antibody, monoclonal anti-p-JNK (G-7), and anti p-c-Jun (KM-1) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Polyclonal anti-phospho-p38 MAP kinase (Thr180/Tyr182) was from Cell Signaling Technology-New England BioLabs (Beverly, MA). Hoechst 33342, C_5-maleimide Alexa Fluor and 2′,7′-dichlorofluorescein diacetate (DCF-DA) were from Molecular Probes (Eugene, OR). IgG (H+L)-HRP-conjugated goat anti-mouse and anti-rabbit secondary antibodies were from Bio-Rad Lab. (Hercules, CA). SuperSignal substrate chemiluminescent reagent was from Pierce (Rockford, IL). All other chemicals were obtained from Merck (Darmstadt, Germany).

Cell cultures

Human neuroblastoma cells SH-SY5Y were purchased from the European Collection of Cell Culture and grown in Dulbecco's modified Eagle's/F12 medium supplemented with 15% fetal calf serum, at 37°C in an atmosphere of 5% CO_2 in air. A monoclonal cell line transfected with human wild-type Cu,Zn superoxide dismutase (hSOD cells) was obtained as previously described [9]. Cells were routinely trypsinized and plated at 30×10^5/75-cm^2 flasks. Cell viability was assessed by trypan blue exclusion.

Treatments

GSSG solution was prepared by dissolving the purified compound in PBS (phosphate-buffered saline, 10 mM phosphate buffer, 2.7 mM KCl, 137 mM NaCl, pH 7.4), buffered at pH 5.5 with NaOH, and sterilized by filtration. GSSG was used at concentration of 1 mM at 37°C in medium supplemented with serum on the basis of previous results obtained with promonocytic cells [8]. Treatment with BSO was performed at a concentration of 1 mM for 6 h before the addition of GSSG and maintained in culture media throughout the experiments. Catalase or Cu,Zn superoxide dismutase (Cu,Zn SOD) were added concomitantly with GSSG and used at a concentration of 1 μM. Treatments with the cell-permeable JNK inhibitor I and II (SP600125) or $p38^{MAPK}$ inhibitor SB203580 (Calbiochem, La Jolla, CA) were performed at a concentration of 10 μM because lower concentrations did not show significant inhibition and higher concentrations were toxic. All the compounds were added concomitantly with BSO and maintained throughout the experiments.

Detection of intracellular ROS

For detection of intracellular ROS, cells were incubated with 50 μM DCF-DA for 30 min at 37°C, scraped, washed, and resuspended in ice-cold PBS. The fluorescent signals derived by reaction of DCF with ROS, of more than 10,000 cells from each sample, were analyzed by recording FL-1 fluorescence by the FACScalibur system. Prior to data collection, propidium iodide was added to the samples for gating out dead cells. Experiments were repeated at least three times with similar results. The data are given as one representative histogram.

Detection of exofacial sulfhydryls

For detection of external plasma membrane thiols, cells were incubated with 10 μM Alexa Fluor C_5-maleimide for