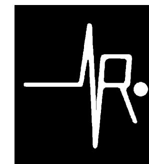
Available online at www.sciencedirect.com

SCIENCE DIRECT®

Free Radical Biology & Medicine 38 (2005) 1057–1072



www.elsevier.com/locate/freeradbiomed

Original Contribution

Regulation of apoptosis by glutathione redox state in PC12 cells exposed simultaneously to iron and ascorbic acid

Mayo Hiroi[a], Tohru Ogihara[a,*], Kazuya Hirano[a], Masashi Hasegawa[a], Takao Morinobu[a], Hiroshi Tamai[a], Etsuo Niki[b]

[a]*Division of Neonatology, Department of Pediatrics, Osaka Medical College, 2-7, Daigaku-machi, Takatsuki, Osaka 569-8686, Japan*
[b]*Human Stress Signal Research Center, National Institute of Advanced Industrial Science and Technology, 1-8-31 Midorigaoka, Ikeda, Osaka 563-8577, Japan*

Received 21 May 2004; revised 4 January 2005; accepted 4 January 2005
Available online 29 January 2005

Abstract

We previously reported that the levels of non-protein-bound iron (NPBI) and ascorbic acid (AA) are markedly increased in the cerebrospinal fluid of infants with perinatal asphyxia. The present study showed that $FeSO_4$ and AA synergistically induced apoptosis of PC12 cells, which was prevented by α-tocopherol and glutathione (GSH) ethyl ester. Markers of free radical damage, such as *ortho*-tyrosine, *meta*-tyrosine, and $F_{2\alpha}$-isoprostane, showed a gradual increase. AA and ferrous NPBI disappeared rapidly from the culture medium, but exposure for only a few hours was sufficient to trigger apoptosis. Intracellular GSH decreased progressively along with a concomitant increase of glutathione disulfide (GSSG). The baseline half-cell reduction potential (E_{hc}) for GSSG, $2H^+$/2GSH couple was −246 mV and an E_{hc} of −200 mV was the critical level to switch on apoptosis, although some cells escaped this fate by transient increase of intracellular GSH. Once E_{hc} reached around −165 mV (81 mV oxidation from the baseline), all cells lost the ability to maintain an adequate intracellular GSH level and subsequently underwent apoptosis. These findings at least partly explain the mechanism of Fe–AA cytotoxicity, in that ferrous iron catalyzes hydroxyl radical generation and induces lipid peroxidation, after which subsequent depletion of GSH raises E_{hc} to the critical level for triggering or potentiating the apoptotic cascade.
© 2005 Elsevier Inc. All rights reserved.

Keywords: Iron; Ascorbic acid; Free radicals; Apoptosis; Glutathione; Reduction potential

Introduction

The most advanced obstetric and perinatal management still cannot reverse brain damage in newborn infants with hypoxic ischemic encephalopathy (HIE) after perinatal asphyxia, so HIE remains one of the major causes of cerebral palsy, epilepsy, and other neurological disabilities.

Together with glutamate excitotoxicity, free radicals are the most widely accepted active species responsible for neuronal damage in HIE [1,2]. Among various possible mechanisms of free radical production, transition metal-catalyzed reactions may be one of the most important [3]. When transition metals like iron or copper are present in a non-protein-bound "free" form, such metals can convert less reactive compounds to more reactive species. Thus, the human body is normally prepared to sequester these metal ions to be incorporated into specific metal-binding proteins.

An experimental study using brain homogenates revealed that non-protein-bound iron (NPBI) is released from the storage protein (ferritin) by acidosis and anoxia [4]. Studies using animal models of hypoxia–ischemia have shown an increase of NPBI in the brain [5], as well as

Abbreviations: AA, ascorbic acid; AO, acridine orange; BSO, buthionine sulfoximine; CAT, catalase; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EB, ethidium bromide; E_{hc}, half-cell reduction potential; GPx, glutathione peroxidase; GSH, glutathione; GSSG, glutathione disulfide; GSHE, glutathione reduced ethyl ester; HIE, hypoxic ischemic encephalopathy; LDH, lactate dehydrogenase; NEM, *N*-ethylmaleimide; NPBI, non-protein-bound iron; OPA, *ortho*-phthalaldehyde; PHGPx, phospholipid hydroperoxide glutathione peroxidase; SOD, superoxide dismutase; TOC, *RRR*-α-tocopherol; VAD-FMK, *Z*-Val-Ala-Asp-fluoromethyl ketone.

* Corresponding author. Fax: +72 684 6554.
E-mail address: ped025@poh.osaka-med.ac.jp (T. Ogihara).

0891-5849/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.freeradbiomed.2005.01.001

demonstrating a protective effect of iron chelators [6]. In human infants with severe perinatal asphyxia, a close association between a raised plasma concentration of NPBI and an adverse outcome has been reported [7]. We also found that NPBI was increased in the cerebrospinal fluid of HIE infants, along with concomitant elevation of ascorbic acid (AA), compared to nonasphyxiated infants [8]. Furthermore, we found a significant increase of markers of free radical damage, such as *ortho*-tyrosine, *meta*-tyrosine, and $F_{2\alpha}$-isoprostane.

NPBI may promote the generation of highly damaging radical species, especially when reduced to its ferrous state by biological reducing agents like AA or superoxide. In fact, a mixture of Fe and AA has long been used as a radical initiator for in vitro studies. A number of in vitro studies have shown that either Fe or AA can induce apoptosis, but there have only been a few reports on synergistic cytotoxicity of Fe and AA resulting in apoptosis of spinal motoneurons [9], hepatoma cells [10], and PC12 cells [11]. Some of these studies also suggested a possible protective role for glutathione (GSH). It is well known that GSH depletion induces apoptotic death of various types of cells. Because the intracellular GSH concentration is far higher than that of most other redox couples, GSH is considered to be almost the sole determinant of cellular redox environment [12]. Recently, it was suggested that the cellular redox environment governed by the redox state of the glutathione disulfide (GSSG)/2GSH couple may be one of the final determinants for the execution of apoptosis [12–14].

Based on the assumption that free iron is reduced by AA and then catalyzes free radical reactions that may cause neuronal damage in HIE, we conducted a series of in vitro studies using PC12 cells to investigate the cellular mechanisms of neuronal damage mediated by Fe plus AA. PC12 is a cell line derived from a rat pheochromocytoma, which synthesizes dopamine, expresses functional NMDA receptors [15], and differentiates into sympathetic neuron-like cells with neurite extensions after exposure to nerve growth factor [16]. Therefore, PC12 cells have been widely used as a model for investigating the mechanisms of neuronal apoptosis. We specifically focused on the association between the cellular redox environment determined by the intracellular GSH status and cell viability.

Materials and methods

Materials

RPMI 1640 medium, penicillin–streptomycin liquid, and L-glutamine were obtained from Invitrogen (Carlsbad, CA, USA). Horse serum and fetal calf serum were obtained from ICN Pharmaceuticals (Costa Mesa, CA, USA). Superoxide dismutase (SOD), catalase (CAT), GSH, glutathione reduced ethyl ester (GSHE), ethidium bromide (EB), acridine orange (AO), buthionine sulfoximine (BSO), selenocystamine dihydride, *ortho*-tyrosine, *meta*-tyrosine, d_4-*para*-tyrosine, and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). The iron standard solution for atomic absorption spectroscopy and Triton X-100 were obtained from Nacalai Tesque (Kyoto, Japan). Z-Val-Ala-Asp-fluoromethyl ketone (VAD-FMK) was purchased from MBL (Nagoya, Japan), 2-thiobarbituric acid was obtained from Merck TGaK (Darmstadt, Germany), and *RRR*-α-tocopherol (TOC) was donated by Eisai Co., Ltd. (Tokyo, Japan). All other reagents were obtained from Wako Pure Chemical Industries (Osaka, Japan).

Cell culture

PC12 cells were obtained from Dainippon Pharmaceutical Co. (Osaka, Japan) and were plated on collagen-coated dishes (Asahi Techno Glass, Tokyo, Japan) at a density of 2×10^6 cells per 60-mm dish or 5×10^5 cells per well on 12-well plates. Cells were grown at 37°C under a humidified atmosphere of 5% CO_2 and 95% air in RPMI 1640 medium containing 10% heat-inactivated horse serum, 5% fetal calf serum, 50 units/ml penicillin G, and 0.1 mg/ml streptomycin sulfate. The medium was changed every 3 days. Before the experiments were performed, cells were cultured for 48 h until just before confluence. Then, various agents were added just before the addition of $FeSO_4$ and/or AA.

Assay of cell viability by measurement of lactate dehydrogenase efflux

Cytotoxicity was estimated by spectrophotometrically measuring the leakage of lactate dehydrogenase (LDH) into the culture medium with an LDH assay kit (Roche, Tokyo, Japan). The supernatant was used to measure LDH release, whereas the intracellular LDH content was determined after lysing the cells with 0.5% Triton X-100. Results were expressed as a percentage of the total LDH content (supernatant LDH/supernatant + intracellular LDH).

Detection of apoptosis

The TUNEL assay was performed with an ApopTag Peroxidase Kit according to the manufacturer's protocol (Intergen, New York, NY, USA). Evaluation of apoptosis was also done by examining the differential uptake of two fluorescent DNA-binding dyes, AO and EB. AO is taken up by all cells and stains the nuclei bright green, whereas EB is taken up only by cells that have lost membrane integrity and stains the nuclei bright orange. These dyes can be used to distinguish between the early and the late stages of apoptosis, because normal cells have bright green chromatin, early apoptotic cells show condensed or fragmented bright green chromatin, late apoptotic cells have condensed or fragmented bright orange chromatin, and necrotic cells

Download English Version:

https://daneshyari.com/en/article/10739890

Download Persian Version:

https://daneshyari.com/article/10739890

Daneshyari.com