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Research report

Neuroprotective effect of *N*-acetyl cysteine on hypoxia-induced oxidative stress in primary hippocampal culture

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Abstract

Hippocampus has received a considerable attention in the recent past due to its role in a number of important functions such as learning and memory. The effect of hypoxia on neuronal cell injury especially on hippocampal cells is not well known. The aim of the present study was to characterize the biochemical changes in primary cultured hippocampal neurons during hypoxic exposure and the protective effect of *N*-acetyl cysteine on hypoxia-induced cytotoxicity. The hippocampal culture grown in 24-well plates was exposed to hypoxia for 3 h in a dessicator in 95% N_2, 5% CO_2 atmosphere at 37 °C. Later, the cells were allowed to recover for 1 h under normoxia. It was observed that there is an appreciable increase in cytotoxicity in cells exposed to hypoxia. Further, there was a significant decrease in mitochondrial membrane potential and appreciable increase in reactive oxygen species and single-strand DNA breaks in cells exposed to hypoxia compared to control. There is a significant fall in glutathione peroxidase, glutathione reductase, reduced glutathione levels, and nitric oxide in the cells exposed to hypoxia. Significant elevation in the intracellular calcium level in the cells on exposure to hypoxia was observed. Supplementation with NAC (50 μM) resulted in a significant cytoprotection, fall in ROS generation, and higher antioxidant levels similar to that of control cells. NAC also inhibited DNA strand breaks induced by hypoxia. The study indicates that NAC has significant neuroprotective activity during hypoxia in primary hippocampal culture.

Theme: Cellular and molecular biology
Topic: Tracing and imaging techniques

Keywords: Primary hippocampal culture; Oxidative stress; Antioxidant status; DNA fragmentation; Cytotoxicity; *N*-acetyl L-cysteine

1. Introduction

Reactive oxygen species (ROS) are by-products generated by cellular oxidative metabolism [22]. ROS include superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), nitric oxide (NO), etc. Some ROS have important functions as second messengers [15,29]. However, enhanced production of ROS overwhelms the antioxidant scavenging capacity, causing oxidative damage of DNA, lipids, and proteins [7] and leads to the development of cellular damage.

Hypoxia has been known to generate ROS production [1]. Of all the cells of the body, neurons are highly vulnerable to ROS damage. Mounting evidence has implicated the role of ROS in the neurodegenerative diseases like Parkinson's disease, Huntington's disease, and Alzheimer's disease [5,40]. Numerous reports have suggested that ROS may play an important role in the processes leading to neuronal

Abbreviations: ROS, Reactive Oxygen Species; $O_2^{\bullet -}$, Superoxide anion; H_2O_2, Hydrogen peroxide; NO, Nitric Oxide; NAC, *N*-Acetyl L Cysteine; DCFH-DA, Dicholorofluorescein Diacetate; GSH, Glutathione; PBS, Phosphate Buffered Saline; SDS, Sodium Dodecyl Sulphate; EDTA, Ethylene Diamine Tetra Acetic Acid; MMP, Mitochondrial Membrane Potential; LDH, Lactate Dehydrogenase; GPx, Glutahione peroxidase; GR, Gluthaione reductase; GSSG, Oxidised glutathione

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cell damage to hypoxia [36] but precise mechanism by which degeneration occurs is not fully known. Bossenmeyer et al. [6] had shown that cultured rat brain neurons on exposure to hypoxia/reoxygenation induce apoptosis. Accumulating evidence suggests that increased production of free radicals and modifications in detoxifying enzymes also leads to apoptosis in cultured rat forebrain neurons on exposure to transient hypoxia and can be prevented by antioxidants [26,27].

N-acetyl cysteine (NAC) is a potent antioxidant that has been shown to protect the brain from oxidative stress. In particular, NAC is known to increase the intracellular stores of glutathione thereby enhancing endogenous antioxidant levels. Murphy et al. [34] had shown that decreased cysteine uptake leads to depletion of glutathione levels. Lievre et al. [26] had shown that NAC has protective effects against oxidative stress when rat forebrain neurons were exposed to transient hypoxia.

Primary hippocampal culture is a very useful tool in elucidating the cellular and molecular mechanisms involved in various diseases, because these neurons are selectively vulnerable to the effect of hypoxia and ischemia both in vivo [4] and in vitro [2]. However, the biochemical events that account for the degeneration of hippocampal neurons during hypoxia remain poorly understood. In view of the above, the present study was undertaken to evaluate the biochemical changes associated with hypoxia and the neuroprotective effect of NAC in primary hippocampal cultures.

2. Materials and methods

2.1. Materials

Dulbecco's Modified Eagle's Medium, fetal bovine serum, poly-L-lysine, DCFH-DA, rhodamine 123, griess reagent, Fura 2 AM, Gelling agarose and propidium iodide, and *N*-acetyl cysteine were purchased from Sigma, USA. Glutathione peroxidase and glutathione reductase kits were procured from RANDOX, Germany.

2.2. Primary hippocampal culture

The Wistar rats were maintained with the highest standards of animal care and housing, according to the *National Institute of Health Guide for the Care and Use of Laboratory Animals*. Zero-day-old male Wistar rat pups (approximately 10 g of body weight) were decapitated and hippocampal neurons were isolated and cultured as previously described [41]. Briefly, the hippocampi were carefully collected, gently dispersed in culture medium and triturated with a pipette. Aliquots (300 μl) of dissociated cell suspension at a cell density of 10^2 to 10^3/ml in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum were plated in 24-well (Nunc) Tc plates precoated with 0.1 mg/ml of poly-L-lysine and incubated at 37 °C in a 5% CO_2 incubator for 8 days. The medium was replaced at an interval of every 2 days. After 8 days of culture in vitro, successful cultured neurons were selected for further studies (Fig. 1). In the hippocampal cell culture, the nerve and glial ratio is about 80 to 20.

2.3. Hypoxic exposure

The cell cultures were exposed to hypoxia for 3 h by transferring the culture plates to a humidified incubation chamber maintained at 37 °C and flushed with a gas mixture consisting of 95% N_2, 5% CO_2. Later, cultures were allowed to recover at 37 °C under normoxic conditions for 1 h. The controls were maintained under normoxic conditions throughout. The Po_2 levels in the extracellular medium collected just prior to the beginning and immediately after 3 h of hypoxic insult using IL-1312 Gas Analyzer (IL, SPA, USA). The Po_2 level in the culture medium under normoxic condition was found to be 165–167 mm Hg, and after 3 h of exposure to 95% N_2 and 5% CO_2, the reduction of the Po_2 was 83–85 mm Hg, indicating cells were exposed to reduced partial pressure of oxygen in the culture medium by about 50 ± 3% as compared to the control.

2.4. Supplementation of NAC

Earlier studies using different concentrations of NAC ranging from 10 to 100 μM concentrations has shown the optimum dose of NAC as 50 μM. NAC solution was prepared freshly in filter sterilized PBS before adding to the media. Since the optimum concentration of NAC was found to be 50 μM, all the experiments have been conducted in this concentration only.

2.5. Measurement of free radical production

The production of free radicals was determined by using DCFH-DA (2,7,dichlorofluorescein diacetate) as described

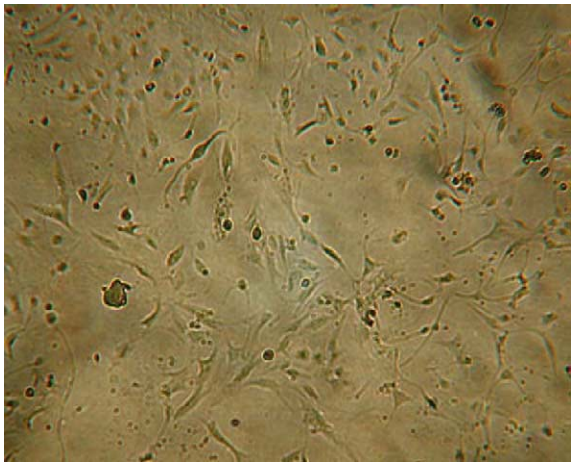

Fig. 1. Phase contrast micrograph of primary hippocampal cell culture.