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Reactive oxygen species are involved in lysophosphatidic acid-induced apoptosis in rat cerebellar granule cells

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Abstract—Lysophosphatidic acid (LPA) induced apoptosis in primary rat cerebellar granule cells, which was characterized morphologically by chromatin condensation and the formation of apoptotic bodies. With redox-sensitive fluorescence probes DCFH-DA and DHR123, the formation of endogenous reactive oxygen species (ROS) inside cells during the apoptosis process was monitored by laser confocal scanning microscopy (LCSM). Pretreatment with the antioxidant tetramethylpyrazine (TMP) could effectively inhibit the formation of endogenous ROS and protect neurons from apoptosis. The results suggest that ROS might be involved in LPA-induced apoptosis in neurons.

Keywords: Lysophosphatidic acid; cerebellar granule cells; apoptosis; reactive oxygen species.

INTRODUCTION

Lysophosphatidic acid (LPA) is a bioactive phospholipid controlling numerous celluar responses through the activation of specific G-protein coupled transmembrane receptors. LPA and LPA receptors are enriched in the brain [1]. LPA has multiple effects on cells of the brain, including LPA-mediated stimulation of neurite retraction of neuroblastoma and PC12 cells, stimulation of astrocyte proliferation, and increased permeability of culture brain endothelial cells [2–5]. The expression of LPA receptor is developmentally regulated in the brain, and expression is markedly increased in association with embryonic programmed cell death of cerebral cortex [6]. The increased LPA in the cerebral spinal fluid (CSF) following hemorrhagic brain injury may lead to an increase in glutamate excitotoxicity via effects on astrocytes and neurons [7]. Treatment of hippocampal neurons with 10 μM LPA results

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in a large and extended increase in neuronal [Ca^{2+}] [8]. Steiner *et al.* thought that LPA elicits a unique response in primary hippocampal neurons and sympathetic neuron-like cells, PC12 cells differentiated with nerve growth factor: LPA is cytotoxic [9]. But, Swarthout and Walling suggested that LPA might promote other types of cell survival by preventing apoptosis as reviewed in reference [10].

In order to illuminate this controversy, we examine the effect of LPA on primary cultures of cerebellar granule cells and indicate that LPA induced neuronal apoptosis, depending on the concentration of LPA in the study. We have also found for the first time that formation of endogenous reactive oxygen species (ROS) are involved in LPA-induced neuronal apoptosis.

MATERIALS AND METHODS

Materials

Seven-day-old Wistar rats were purchased from the Experimental Animal Center of Peking University Medical School, China. Cell culture plastic ware was purchased from Corning Costar (Action, MA, USA). Dulbecco's modified Eagle medium (DMEM), cell culture supplements, fetal bovine serum and trypsin (1 : 250) were products of Gibco BRL (Grand Island, NY, USA). Thiazolyl blue (MTT), bovine serum albumin (BSA), poly-L-lysine and lysophosphatidic acid (LPA) were purchased from Sigma (St. Louis, MO, USA). The redox-sensitive fluorescence probes dihydrorhodamine 123 (DHR123) and 2′,7′-dichlorofluorescein diacetate (DCFH-DA) were purchased from Molecular Probes (Eugene, OR, USA). Tetramethylpyrazine (TMP) was a generous gift from Beijing Fourth Pharmaceutical Factory (Beijing, China). Other reagents were made in China.

Cell culture

The primary cultures of rat cerebellar granule cells were prepared following procedures decribed previously [11]. Briefly, cerebella from 7-day-old Wistar rats were dissected out, rinsed with HBSS, and dissociated by mild trysinization. Cells were plated on poly-L-lysine-coated 35 mm Petri dishes at 4×10^6 cells/2 ml or 96-well plates at 1×10^5 cells/100 μl. Culture medium consisted of DMEM supplemented with KCl (19.6 mM), glutamine (2 mM), HEPES (10 mM) and fetal bovine serum (10%, v/v). Cells were maintained at 37°C in a humidified 5% CO_2-95% air atmosphere. Experiments were carried out 48 h after plating. In some experiments, TMP was added to cells 15 min before the addition of LPA.

Assessment of cell viability

The viability of cells was assessed by MTT assay [12]. MTT can be reduced to formazan by mitochondrial respiratory enzymes, and the amount of formazan formed was related accurately with the cell viability. Cells cultured in 96-well

plates were treated with LPA for 24 h, then 10 μl of the MTT solution (5 mg/ml in PBS) was added to each well and incubated at 37°C for 30 min. Then 100 μl of solubilization solution (50% dimethylformamide, 20% sodium dodecyl sulfate, 1 % acetyl acid, pH 3.5) was added to the wells, followed by mixing for 10 min. The absorption at 570 nm was measured by a Bio-Rad 3550 microplate reader.

DNA analysis of flow cytometry

Flow cytometry DNA analysis was performed in order to evaluate the percentage of apoptotic cells whose DNA content was lower than that of diploid cells. As generally accepted, apoptotic cells can be recognized by their diminishing stainability with DNA specific fluorochromes, which is due to DNA degradation and subsequent leakage from the cell [13]. The method for DNA labelling was performed as previously described [14]. Briefly, culture cells were prepared as a single cell suspension in 200 μl PBS, fixed with 2 ml of ice cold 70% ethanol, maintained at 4°C overnight. The cells were harvested by centrifuged 200 $\times$ g for 10 min, resuspended in 500 μl PBS supplemented with RNase (100 μg/ml), incubated at 37°C for 30 min, and stained with 50 μg/ml propidium iodide (PI) at 4°C for 30 min. The red fluorescence of individual cell was measured with FACScan (Becton Dickenson, Mountain View, CA) and the number of apoptotic cells were counted by software provided by the manufacturer.

Morphological observation

The ultrastructure of cells was observed by transmission electron microscopy [15]. Briefly, cells were fixed with 2.5% glutaraldehyde at 4°C for 1 h and post-fixed with 1% OsO_4 at 4°C for 1 h, dehydrated through a series of graded ethanol solutions, and embedded in resin. Ultrathin sections of samples were stained with uranyl acetate/lead citrate and observed with a transmission electron microscopy.

Determination of intracellular ROS

The levels of cytosolic ROS were determined by laser confocal scanning microscopy (LCSM) using DCFH-DA as a peroxide-sensitive fluorescence probe. DCFH-DA is a non-fluorescent compound that can permeate cells freely [16]. When inside cells, it is hydrolyzed to DCFH and is trapped inside cells. Upon oxidation by the cytosolic ROS (namely hydrogen peroxide and other peroxides), it is converted to the fluorescent compound 2′,7′-dichlorofluorescein (DCF), which can be detected by LCSM. Oxidation of DCFH-DA is relatively specific for the detection of cytosolic ROS.

Cells were treated with 50 μM LPA for 4 h, washed twice with phenol red-free DMEM containing 0.2% BSA, and loaded with 10 μM DCFH-DA at 37°C for 60 min. After being washed twice, cells were observed by a Bio-Rad MRC 1024MP laser confocal scanning microscope with excitation set at 488 nm and emission at 520 nm.

DHR123 was used as a fluorescence probe to measure the ROS production in the mitochondria, namely superoxide, hydrogen peroxide and peroxynitrite [17]. Briefly, after exposure to 50 μM LPA for 2 h, cells were washed and then loaded with 2 μM DHR123 at 37°C for 45 min. After being washed twice, cells were observed by a Bio-Rad MRC 1024MP laser confocal scanning microscope with excitation set 488 nm and emission at 520 nm.

RESULTS

LPA induces neuronal injury in rat cerebellar granule cells

Results of MTT assay indicated that LPA induced dose-dependent cell death in primary cultures of rat cerebellar granule cells. After exposure to 50 μM LPA for 24 h, the MTT reduction decreased to 54.8 ± 11.5% of normal cells (Fig. 1). Pretreatment with TMP effectively prevented cells from death (Table 1).

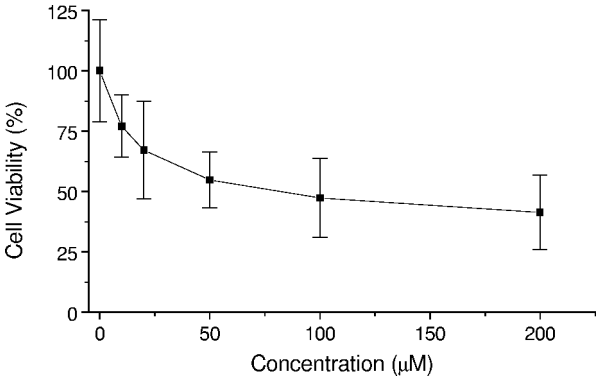
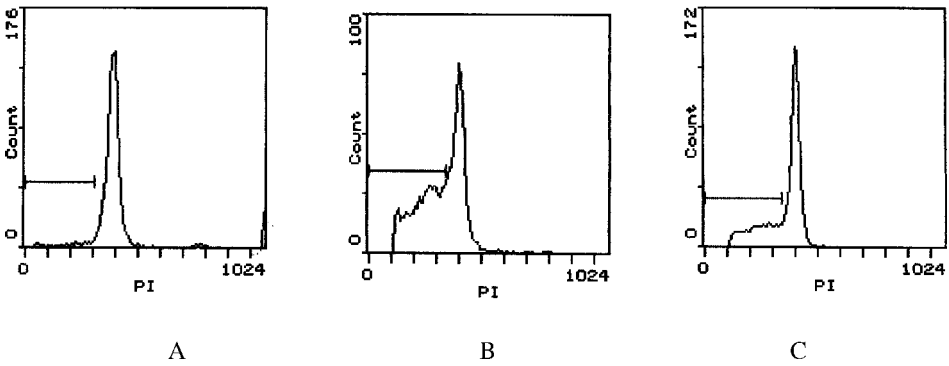


Figure 1. Dose-dependent course of cell death in rat cerebellar granule cells induced by LPA. Rat cerebellar granule cells were treated with 50 μM LPA for indicated dose, and the cell viability was assessed by MTT assay. Data are mean ± SD of 8 experiments.

Table 1.
Effects of the antioxidant tetramethylpyrazine on neuronal apoptosis induced by LPA

	Normal cells	50 μM of LPA	100 μM of TMP + 50 μM of LPA
Cell viability (%)	100 ± 21.12	54.8 ± 11.5*	84.7 ± 8.8**
Apoptotic cells (%)	8.4 ± 3.3	40.5 ± 2.3*	16.7 ± 5.8**

Cerebellar granule cells were incubated with 50 μM of LPA for 24 h and the cell viability was assessed by MTT assay. The number of apoptotic cells was assessed by flow cytometry. Data are mean ± SD of 8 samples. * $p < 0.05$ in comparison with normal cells; ** $p < 0.05$ in comparison with cells treated with 50 μM of LPA.



A B C

Figure 2. Typical histograms of apoptotic cells detected by flow cytometry. A, normal cells; B, cells treated with 50 μM of LPA for 24 h; C, cells pretreatment with 100 μM of TMP and then treated with 50 μM of LPA for 24 h.

LPA induces apoptosis in rat cerebellar granule cells

The percentages of normal (diploid and supradiploid DNA content) and apoptotic cells (subdiploid DNA content) determined by flow cytometry are shown in Fig. 2. Pretreatment with TMP effectively attenuated apoptosis as shown in Table 1.

Morphological observation of LPA-treated cells by transmission electron microscopy showed condensed chromatin and fragmented nuclei (Fig. 3b), which were well-accepted criteria of apoptosis, suggesting that LPA induces apoptosis in neuronal cells. In cells pretreated with TMP, LPA exposure did not cause significant alteration of the nuclear morphology (Fig. 3c).

LPA induces endogenous ROS formation

Endogenous ROS could be detected by LCSM using redox-sensitive fluorescence probes DCFH-DA and DHR123. Exposure of cells to LPA caused increase in the cytosolic ROS level as confirmed by the increasing of DCF fluorescence (Fig. 4b). Similarly, Rh123 fluorescence also increased significantly after the addition of LPA, suggesting that LPA exposure also caused the accumulation of intramitochondrial ROS (Fig. 5b). In cells pretreated with antioxidant TMP, the cytosolic and intramitochondrial ROS formation was suppressed significantly (Fig. 4c and Fig. 5c).

DISCUSSION

The LPA-mediated induction of neuronal death is a novel finding, particularly in view of the lack of toxicity of higher concentration of LPA in other cell system, e.g. the highest concentration of LPA tested were not cytolytic for glomerular mesangial cells (0.1 mM) [18], vascular smooth muscle cells (0.1 mM) [19], and fibroblasts (1.0 mM) [20]. Obviously, the responses to LPA are cell type-specific. However,

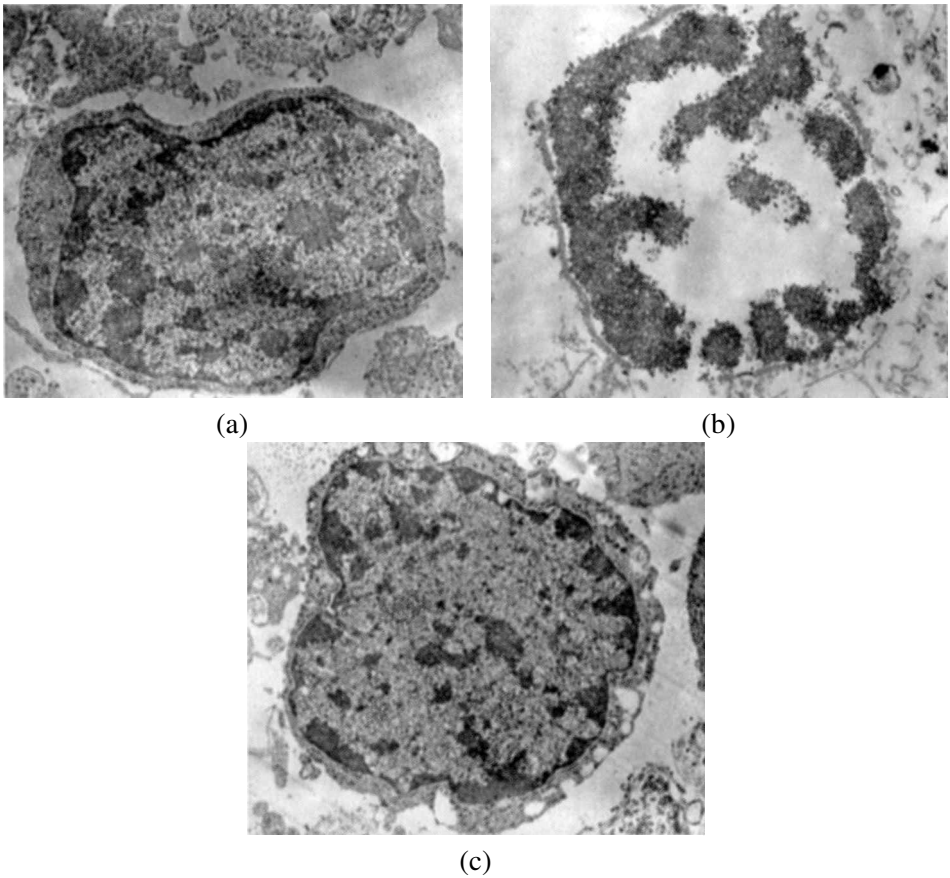


Figure 3. Nuclear morphology of rat cerebellar granule cells. Rat cerebellar granule cells were treated with 50 μM LPA and observed with a transmission electron microscopy. (a) Normal neuron; (b) a neuron treated with LPA 24 h, which shows condensed chromatin; (c) a neuron pretreated with 100 μM TMP for 24 h.

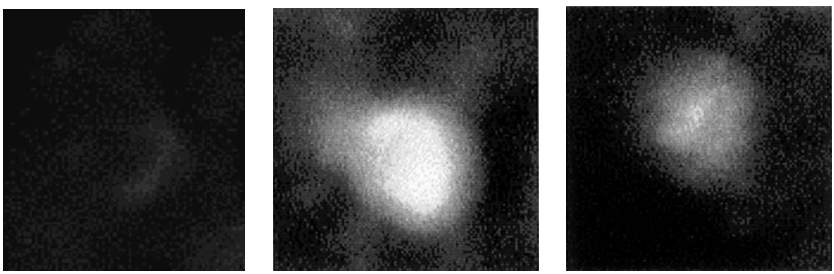
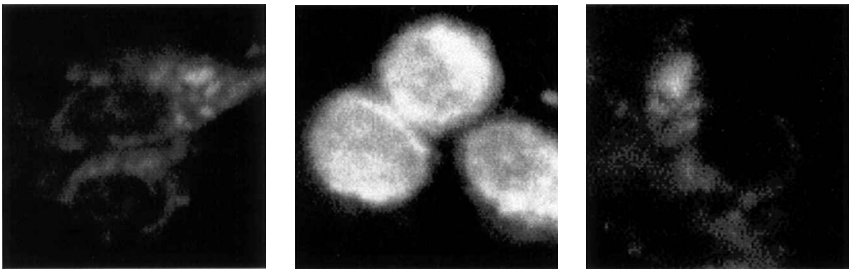


Figure 4. Detection of cytosolic ROS. Rat cerebellar granule cells wre treated with 100 μM LPA for 4 h, loaded with 10 μM DCFH-DA, and observed by laser confocal scanning microscopy. (a) Normal neuron; (b) a neuron treated with LPA for 4 h; (c) a neuron pretreated with 100 μM TMP, and treated with LPA for 4 h.



(a) (b) (c)

Figure 5. Detection of intramitochondrial ROS. Rat cerebellar granule cells were treated with 50 μM LPA for 2 h, loaded with 2 μM DHR123, and observed by laser confocal scanning microscopy. (a) Normal neuron; (b) a neuron treated with LPA for 2 h; (c) a neuron pretreated with 100 μM, and treated with LPA for 2 h.

the injurious mechanism of LPA on neurons is still unclear. In order to understand this injurious mechanism of LPA on neurons, we selected immature cultures of rat cerebellar granule cells (2 days *in vitro*) to exclude the involvement of excitatory amino acid receptors. The generation of endogenous ROS in neuronal cells upon exposure to LPA was monitored by a laser confocal scanning microscopy, which provides a direct and specific method for the detection of intracellular ROS.

In the present investigation, LPA showed potent dose-dependent neurotoxicity and the experimental results indicated that exposure of rat cerebellar granule cells to LPA induced oxidative stress, which was characterized by the formation of cytosolic and intra-mitochondial peroxides. LPA treatment resulted in apoptosis finally, which was characterized morphologically by chromatin condensation and by flow cytometry. The results provide direct evidence that oxidative stress induced by the generation of endogenous ROS is one of the important pathways leading to neuronal apoptosis induced by LPA.

Mitochondria are the major place where endogenous ROS were generated. Upon exposure to LPA for 2 h, the intra-mitochondrial peroxide level increased significantly. The intracellular peroxide level also increased markedly 4 h after the treatment with LPA. ROS can act as a trigger for apoptosis, as in the case of H_2O_2-induced. In this scenario, several mechanisms have been proposed including upregulation of the Fas/FasL system, perturbation of mitochondrial function resulting in cytochrome-c release, leading to caspase activation, and activation of transcription factors which might cause transcription of death promoting gene [21–24]. Conversely, with many triggers of apoptosis like death receptor ligation or overexpression of p53, intracellular ROS production is noted as an event that accompanies the apoptotic program [25]. But intracellular ROS production can also affect mitochondra, resulting in the release of cytochrome-c and subsequent caspase activation.

The present investigation first observed the generation of endogenous ROS in LPA-treated cerebellar granule cells, and proved that ROS are key mediators of LPA-induced neuronal apoptosis. Accordingly, some antioxidant that can diminish

LPA-induced endogenous ROS could also reduce neuronal apoptosis induced by LPA.

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