Lysophosphatidic Acid Rescues Human Dental Pulp Cells from Ischemia-induced Apoptosis

Hongying Pan, MD, Li Cheng, PhD, Hui Yang, PhD, Wenling Zou, MD, Ran Cheng, PhD, and Tao Hu, PhD

Abstract

Introduction: Dental pulp is particularly susceptible to ischemic conditions (hypoxia and serum deprived) because it is commonly exposed to trauma, inflammation, chronic caries injury, and pulpitis. We investigated the apoptotic response of human dental pulp cells (HDPCs) to varying levels of oxygen and serum to mimic different degrees of ischemia, tested whether lysophosphatidic acid (LPA) could reverse ischemia-induced apoptosis, and investigated the possible mechanisms of LPA. **Methods:** HDPCs were cultured under conditions mimicking serum deprivation and ischemia for 2 days with or without LPA at 25 μg/mL. Flow cytometry and JC-1 fluorescence were used to detect any apoptotic change. Western blotting was used to measure the expression of the apoptosis regulators B-cell lymphoma 2 (Bcl-2) and Bax, focal adhesion kinase (FAK), Src, extracellular signal-regulated kinase (ERK), and Akt. **Results:** Flow cytometry and JC-1 immunofluorescence showed that ischemia could induce apoptosis of HDPCs in 2 days and treatment with LPA could reduce cell death significantly. To clarify the molecular mechanisms, Western blot results showed up-regulation of both pro-apoptotic Bax and antiapoptotic Bcl-2 during apoptosis. LPA functioned as an antiapoptotic cytokine by activation of the phosphorylation of FAK and ERK. No statistically significant difference was found in the activation levels of p-Src or p-Akt. **Conclusions:** A self-defense mechanism functioned during cell apoptosis. LPA could effectively rescue HDPCs from ischemia-induced apoptosis via regulation of Bax and Bcl-2 and the activation of phosphorylated FAK and phosphorylated ERK. LPA is a potent candidate for biological therapy of chronic pulpal inflammatory diseases. *(J Endod 2014;40:217–222)*

Key Words

Apoptosis, human dental pulp cells, ischemia, lysophosphatidic acid

The dental pulp cavity and root canal system are sensitive to injury because the only access to the vasculature is through the small opening at the apex, but the surrounding hard tissues lack elasticity. Inevitably, the dental pulp is trapped in ischemic conditions (hypoxia and serum deprivation) when encountering trauma, inflammation, long-term caries injury, or chronic pulpitis. Thus, apoptosis and repair might both occur in response to this injury.

In recent years, apoptosis, a key mediator of cell function under normal and pathological conditions, has been widely confirmed to play a key role in ischemia-induced bioreactions of mesenchymal stem cells, chronic lymphocytic leukemia cells, and cardiomyocytes (1–4). Furthermore, the repair process of human dental pulp cells (HDPCs) under ischemic conditions included the proliferation of side population cells, proangiogenic responses, enhanced expressions of STRO-1 and CD133 (stem cell markers), accelerated mineralization, and suppressed osteo-/odontogenic differentiation (4–8). However, the mode of apoptosis and survival during ischemic damage to HDPCs has not yet been investigated.

Lysophosphatidic acid (LPA), a serum-derived growth factor, has been characterized as a key mediator involved in several cellular functions, such as wound healing, cell survival, migration, differentiation, and actin fragment contraction in various cell lines (1–3, 9, 10). Interestingly, it appears to have both pro-/anti-inflammatory and pro-/antiapoptotic properties (11). In our previous studies, LPA effectively enhanced the repair process in HDPCs by regulating adhesion, migration, and differentiation (9, 10). Consequently, LPA has enormous potential for accelerating the repair of injuries.

Based on these findings, we hypothesized that apoptosis occurred when HDPCs were damaged by conditions mimicking ischemia and that LPA might play an antiapoptotic role, participating in the cells' autosurvival mechanism and repair processes. We performed this study to test this.

Materials and Methods

Cell Culture and Treatment

Impacted third molars were collected from 4 adults aged 18–25 years with informed consent according to the institutional standards (12). Dental pulp cells were isolated and cultured, and their identity was confirmed as reported (9, 10, 13). The cells were then cultured in Minimum Essential Medium Eagle Alpha Modification (Gibco, Grand Island, NY) with or without 10% fetal calf serum (Gibco, Logan, UT). Cells that had progressed through 3–6 passages were selected and treated with different conditions defined by restricted oxygen and/or serum levels (Table 1). For normal conditions, cells were incubated in a humidified atmosphere of 5% CO_2 in air at 37°C, whereas for ischemic conditions they were cultured in a hypoxic chamber under 0.5% oxygen and 5% CO_2 at 37°C (Modular Incubator

From the State Key Laboratory of Oral Diseases, West China Hospital of Stomatology, Sichuan University, Chengdu, Sichuan, China.

Address requests for reprints to Dr Ran Cheng, State Key Laboratory of Oral Diseases, West China Hospital of Stomatology, Sichuan University, No. 14, 3rd Section, South Renmin Road, Chengdu 610041, PR China. E-mail address: applecrbb@gmail.com

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TABLE 1. Simulated Conditions and Experimental Groups

Simulated conditions	Experiment groups	Concentrations of oxygen and serum
Negative control (normal)	1	20% O_2, 10% serum
Serum deprivation	2	20% O_2, 2% serum
	3	20% O_2, 0% serum
Positive control	4	0.5% O_2, 10% serum
Ischemia	5	0.5% O_2, 2% serum
	6	0.5% O_2, 2% serum with LPA
	7	0.5% O_2, 0% serum
	8	0.5% O_2, 0% serum with LPA

Chamber; Binder, Tuttlingen, Germany). Initial experiments were used to determine the optical time course and concentration of LPA (Sigma-Aldrich, St Louis, MO) by treating cells at 5, 10, 25, 50, and 100 μg/mL for 12, 24, 48, and 72 hours. Treatment with LPA at 25 μg/mL for 48 hours was finally chosen for modulating typical apoptosis. HDPCs in ischemic conditions were incubated with or without LPA (25 μg/mL) to investigate its potential protective function (Table 1).

Mitochondrial Membrane Potential Assay

To evaluate changes in mitochondrial membrane potential ($\triangle\Psi$m) of simulated cells, JC-1 immunofluorescence (C2006; Beyotime, Nantong, PR China) was used based on the manufacturer's instructions. Images were acquired using a fluorescence microscope (Leica DMI6000B; Leica Microsystems Wetzlar GmbH, Wetzlar, Germany).

Western Blot Analysis

For analyzing cellular protein levels, cell extracts were subjected to a standard Western blotting technique (2, 10). The antibodies used were against p-AKT, AKT, p-ERK, ERK, Bcl-2, Bax, p-FAK, FAK, p-SRC, SRC, and GAPDH (Epitomics, Burlingame, CA).

Flow Cytometry of Apoptosis

The annexin V-fluorescein isothiocyanate/propidium iodide apoptosis assay was conducted using a KeyGEN apoptosis detection kit (Nanjing KeyGEN Biotech Co, Ltd, Nanjing, PR China) following the manufacturer's protocols as reported (2, 13). Samples were detected by flow cytometry (FACScan; Beckman FC500, Beckman Coulter, Brea, CA) and analyzed with the CELLQuest software (BD Biosciences, Franklin Lakes, NJ). Fragments of dead cells and debris were gated out by forward- and side-scatter analysis.

Statistical Analysis

All experiments were performed at least 3 times. Data are expressed as the mean $\pm$ standard error of the mean. Differences were analyzed using analysis of variance and the Student t test. Significance was set at $P < .05$.

Results

Ischemic Trauma Induced Apoptosis in HDPCs

JC-1 fluorescence was used to detect the $\Delta\Psi$m value as an indicator of apoptosis. The results showed that serum deprivation (SD) alone could cause slight apoptosis, but hypoxia enhanced its lethal effects (Fig. 1*A*). This indicates that hypoxia plays a greater role as an apoptosis-predisposing factor than SD during ischemia-induced apoptosis. To investigate this phenomenon, we used flow cytometry to detect the quantitative apoptosis rate of HDPCs in different ischemic environments. The results confirmed that an ischemic microenvironment is a proapoptotic trauma to cells (Fig. 1*B*).

LPA Effectively Rescued Dental Pulp Cells from Ischemia-induced Apoptosis

LPA, a lysophospholipid, is generated by activated thrombocytes when damaged. To investigate the protective function of LPA, cells in ischemia were simulated with LPA (25 μg/mL). In JC-1 immunofluorescence red-stained cells increased quantitatively with LPA treatment, and the apoptosis rate decreased significantly as detected by flow cytometry (Fig. 2*A* and *B*). This revealed the protective function of LPA against apoptosis caused by ischemic trauma.

LPA Antagonized Apoptosis via the Activation of FAK and ERK

To clarify the mechanism by which LPA inhibited apoptosis, the phosphorylation levels of several signaling pathways, including those for FAK, Src, ERK, and PI3K/Akt, were tested. The levels of p-FAK and p-ERK increased during LPA treatment, whereas p-Src and p-Akt changed little (Fig. 3*A* and *B*). Thus, the FAK and ERK pathways may mediate LPA's antiapoptotic function.

Dynamic Regulation of Bax and Bcl-2 Was Involved in Auto- and Exogenous Defenses

Bax and Bcl-2 have pro- and antiapoptotic functions in cells, respectively. Western blotting was used to test changes in Bax and Bcl-2 expression levels. Bax was up-regulated during apoptosis and down-regulated with LPA treatment (Fig. 4). Surprisingly, the Bcl-2 expression levels also increased during apoptosis enhancement. When LPA was used to inhibit apoptosis, Bcl-2 increased in conditions of 0.5% O_2 plus 2% serum with LPA but decreased in conditions of 0.5% O_2 plus 0% serum with LPA (Fig. 4).

Discussion

Combined Effects of Hypoxia and SD Strongly Induced Apoptosis in HDPCs

In this study, a synergistic action of hypoxia and SD was responsible for significant apoptosis of HDPCs; however, cell survival was barely affected when they were administered alone. Thus, this combined effect might be critical to chronic pulpal injury including caries, pulpitis, and periapical inflammation. We suggest that there might be a threshold of hypoxia and SD in inducing apoptosis (ie, a proliferation/apoptosis switch controlled by the degree of ischemia). Thus, HDPCs undergo apoptosis when the microenvironment is below a certain level of oxygen or serum.