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Phosphatidylcholine-specific phospholipase C, p53 and ROS in the association of apoptosis and senescence in vascular endothelial cells

Yizhe Cheng[a,b,1], Qitao Zhao[a,b,1], Xia Liu[a,b], Satohiko Araki[c], Shangli Zhang[a,b], Junying Miao[a,b,*]

[a] *Institute of Developmental Biology, School of Life Science, Shandong University, Jinan 250100, China*
[b] *The Key Laboratory of Experimental Teratology, Ministry of Education, Jinan 250012, China*
[c] *Sugashima Marine Biological Laboratory, School of Science, Nagoya University, Toba, Mie, 517-0004, Japan*

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Abstract **Previously, we found that phosphatidylcholine-specific phospholipase C (PC-PLC) participated in apoptosis signaling of vascular endothelial cells (VECs). Here, to explore whether PC-PLC is involved in the association of apoptosis and senescence in VECs, we analyzed p53 expression and intracellular reactive oxygen species (ROS) levels in young and senescent VECs before and after inhibiting PC-PLC activity. The results showed that suppressing PC-PLC inhibited apoptosis and the elevation of p53 expression induced by apoptosis in young cells, but not in senescent cells, and that inhibiting PC-PLC depressed intracellular ROS levels both in young and senescent cells. The data suggested that PC-PLC was involved in the association of apoptosis and senescence. Its function might be closely related to the level of p53 in VECs.**

Keywords: Phosphatidylcholine-specific phospholipase C; Senescence; Apoptosis; Vascular endothelial cells; p53; ROS

1. Introduction

Replicative senescence of human cells in primary culture is a widely accepted model for studying the molecular mechanisms of human aging. Many results showed that there are profound cell type-specific differences in the senescence program [1]. Therefore, it is necessary to select an appropriate model for understanding the senescence mechanism of certain histotypes.

It has been reported that apoptosis plays a key role for aging in vivo. There are many reports respectively directed to signaling pathways of apoptosis and senescence. The relationship between the two processes, however, is not well known. Unlike fibroblasts, vascular endothelial cells undergo aging associated with apoptosis in vitro. It provides a good model system to study the relationship between senescence and apoptosis in vascular aging at both cellular and molecular levels [2]. In this study, to find the key factors that associate senescence and apoptosis, we selected VECs as the most appropriate model.

PC-PLC, an important member of phospholipase C family, might be involved in age-related signal transduction in human lung fibroblasts and rat hepatocytes [3]. But it is not known whether and how PC-PLC changes in the senescence of VECs.

We have been studying the function of PC-PLC in apoptosis signaling of VECs. The results showed that PC-PLC was a key factor in apoptosis signal transduction pathways [4–10]. In this study, we focused on exploring whether PC-PLC is involved in the association of apoptosis and senescence in VECs.

In previous studies, we found that p53 played a key role in the regulation of VEC apoptosis [7–9,11,12]. To understand the mechanism by which PC-PLC regulates senescence and apoptosis in VECs, we investigated the relationship between PC-PLC and p53 during VEC aging.

Free radical theory of aging pointed out that reactive oxygen species (ROS) were major factors responsible for human aging. Moreover, ROS were implicated as potential modulators of apoptosis [13]. To understand the relationship between PC-PLC and ROS in the association of apoptosis and senescence of VECs, we examined the changes of ROS levels in young and senescent cells before and after the inhibition of PC-PLC activity.

2. Materials and methods

2.1. Cell culture

Human umbilical vein endothelial cells (HUVECs) were obtained as described previously [14]. The cells were cultured in MCDB131 medium (Sigma) supplemented with 10% fetal bovine serum (FBS), 70 ng ml^{-1} FGF and 100 μg ml^{-1}heparin.

2.2. PC-PLC activity assay

PC-PLC activity was detected as described by Wu et al. [15].

2.3. Apoptosis induction and inhibition

Rattlesnake venom can specifically trigger apoptosis in HUVECs [16]. D609, a specific inhibitor of PC-PLC, could suppress young VEC apoptosis induced by deprivation of FGF and serum [5,6]. In this study, we selected this model to study the relationship between senescence and apoptosis in HUVECs. When the cells reached sub-confluence, they were treated by the following three ways: (a) As a control group, cells were cultured in basal MCDB131 medium (without FGF and serum). (b) Cells were treated with rattlesnake venom of 2 μg ml^{-1} for 6 h. (c) Cells were incubated with rattlesnake venom of 2 μg ml^{-1} and D609 of 10 μg ml^{-1} for 6 h.

*Corresponding author. Address: Institute of Developmental Biology, School of Life Science, Shandong University, Jinan 250100, China. Fax: +86 531 88565610.
E-mail address: miaojy@sdu.edu.cn (J. Miao).

[1] These authors contributed equally to this work.

Abbreviations: HUVEC, human umbilical vein endothelial cell; PDL, population doubling level; PC-PLC, phosphatidylcholine-specific phospholipase C; ROS, reactive oxygen species; SA-β-Gal, senescence-associated β-galactosidase; VEC, vascular endothelial cell

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2.4. Analysis of nuclear fragmentation

The cells treated, in the three ways mentioned above (in Section 2.3), for 6 h were stained with acridinorange for 5 min, and observed under laser scanning confocal microscope (Zeiss, LSM510).

2.5. ROS assay

A fluorescent probe, 2′,7′-dichlorodihydrofluorescein (DCHF), which could be oxidized into 2′,7′-dichlorofluorescein (DCF) by the intracellular ROS while entering into the cell, was used for the assessment of intracellular ROS formation in cultured VECs. ROS assay was performed as described previously [17]. The levels of ROS in VECs were quantified using the software of Zeiss LSM510.

2.6. Western blot analysis

The Western blot analysis was performed as described previously [18]. The relative amount of proteins was analyzed by using Imagetool software.

2.7. Statistics analysis

The results were expressed as means ± S.E. Statistical analysis was performed by *t*-test, and differences at $P < 0.05$ were considered statistically significant.

3. Results

3.1. Identification of VEC senescence

VEC senescence is identified by the senescence-associated β-galactosidase (SA-β-Gal) as recognized in the art [19]. In this study, the activity of SA-β-Gal was examined in PDL 10, PDL 20 and PDL 36 cells respectively. As shown in Fig. 1A–C, SA-β-Gal activity was remarkably increased concomitant with the morphological changes of senescent cells. Based on these results, in the following experiments, we selected PDL 16 cells and PDL 36 cells as young and senescent representatives respectively.

3.2. The activity of PC-PLC remarkably decreased with VEC aging

To know whether and how PC-PLC changes with VEC senescence, we examined the activity of PC-PLC in PDL 16 and PDL 36 HUVECs respectively. The results showed that PC-PLC activity in PDL 36 HUVECs was much lower than that in PDL 16 HUVECs (Fig. 1D).

3.3. Suppressing PC-PLC inhibited apoptosis in PDL 16 cells but not in PDL 36 cells

Apoptotic body formation and the nuclear fragmentation are the typical characteristics of VEC apoptosis [6,8,9]. In this study, these morphological changes in VECs treated with rattlesnake venom were observed. The results showed that the apoptosis both in PDL 16 and PDL 36 VECs could be triggered by rattlesnake venom of 2 μg ml^{-1} (Fig. 2A(b) and (e); Fig. 2B(b) and (e)). In PDL 16 VECs, after the cells were exposed to D609 of 10 μg ml^{-1}for 6 h, the apoptosis was obviously suppressed and fewer apoptotic bodies were observed. In PDL 36 VECs, however, the apoptosis was not suppressed by D609 of 10 μg ml^{-1} (Fig. 2A(c) and (f); Fig. 2B(c) and (f)).

3.4. Suppressing PC-PLC inhibited p53 expression induced by apoptosis in PDL 16 cells but not in PDL 36 cells

To understand why inhibition of PC-PLC can not suppress the apoptosis in PDL 36 cells, we examined the expressions of p53 protein in PDL 16 and PDL 36 cells treated in the three

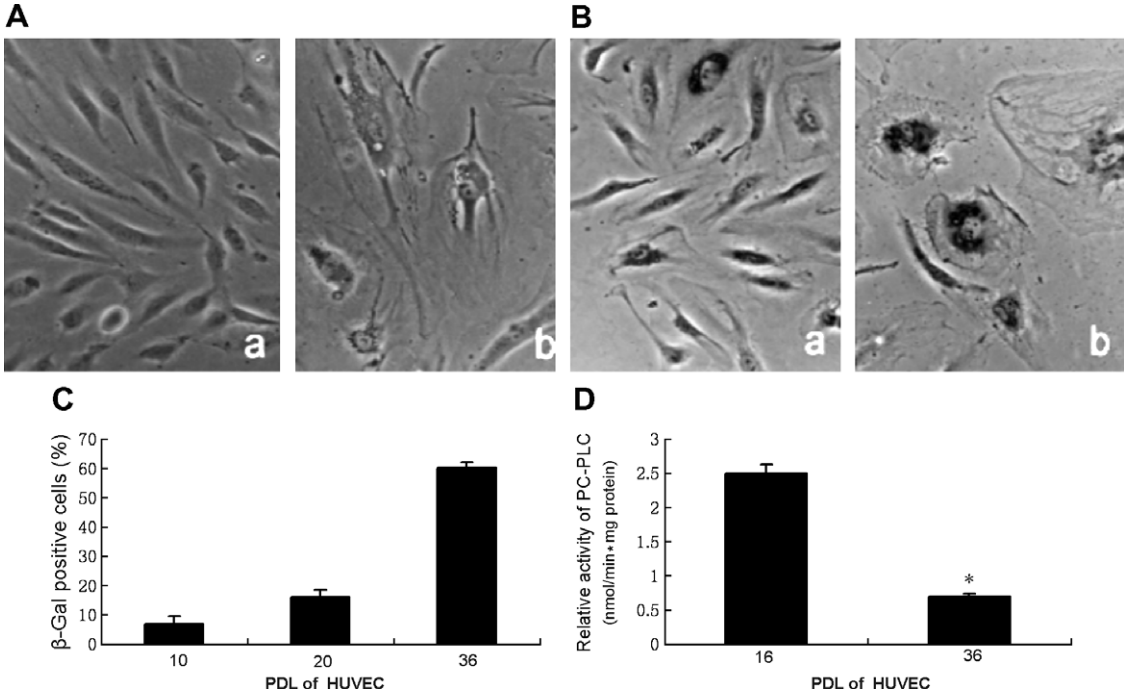

Fig. 1. (A) The morphological changes during VEC aging. PDL 36 cells (b) were bigger and more spread than PDL 16 cells (a) in morphology. (B) SA-β-Gal activity analysis. There were more β-Gal positive cells among PDL 36 cells (b) than those among PDL 16 cells (a). (C) The percentage changes of β-Gal positive cells with cell aging. The percentage of β-Gal positive cells among PDL 10, 20 and 36 cells amounted to 8%, 16% and 65% respectively, suggesting that the cell gradually became senescence. (D) The activity changes of PC-PLC during VEC aging. PC-PLC activity in PDL 16 cells was 2.5 nmol/min mg protein, whereas, it was 0.7 nmol/min mg protein in PDL 36 cells. The activity of PC-PLC decreased remarkably with VEC aging.*$P < 0.01$ ($n = 3$).