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Phosphatidylcholine-Specific Phospholipase C and ROS Were Involved in Chicken Blastodisc Differentiation to Vascular Endothelial Cells

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Abstract To find the key factors that were involved in the survival and vascular endothelial differentiation of chick blatodisc induced by fibroblast growth factor 2 (FGF-2), we built a chick vasculogenesis model in vitro. Subsequently, the activities of phosphatidylcholine-specific phospholipase C (PC-PLC), including Ca^{2+}-dependent and -independent PC-PLC, and the level of reactive oxygen species (ROS) were evaluated during the endothelial differentiation of chick blastodisc. The results showed that Ca^{2+}-indepentent PC-PLC underwent a remarkable increase in 24 h ($P < 0.01$), then it decreased gradually with the cell differentiation, while the Ca^{2+}-depentent PC-PLC was nearly not changed in the whole process. At the same time, ROS level dramatically decreased during the cell differentiation. To understand the role of PC-PLC and how it performs its function in the vascular endothelial differentiation induced by FGF-2, we suppressed PC-PLC activity by its specific inhibitor D609 (tricyclodecan-9-yl potassium xanthate) at 24 h during the cell differentiation. As a result, the cell differentiation could not progress and the intracellular level of ROS was elevated. The data suggested that PC-PLC and ROS were involved in chicken blastodisc differentiation to vascular endothelial cells. PC-PLC was an important factor in the blastodisc cell survival and differentiation, and it might perform its function associated with ROS. J. Cell. Biochem. 102: 421–428, 2007.

Key words: phosphatidylcholine-specific phospholipase C; vasculogenesis; reactive oxygen species; FGF-2; chicken; blastodiscs

INTRODUCTION

Earlier works provided descriptions of the vasculogenic process as it occurs in frogs, fish, xenopus, and avians in vivo [Stockard, 1915; Clark, 1918; Sabin, 1920; Slack et al., 1987]. But in vivo experiments have been hampered by difficulties in accessing the embryo prior to the establishment of the blood island and by the limited number of cells present at this stage of development. In 1992, the unincubated quail blastodiscs of stage X–XII were cultured and induced to differentiate to vascular endothelial cells by fibroblast growth factor 2 (FGF-2) in vitro [Flamme and Risau, 1992]. But, so far, there has not been any report about chicken vasculogenesis induction in vitro. The chick is a commercially important species, understanding the chicken vasculogenesis mechanism will promote the progress in poultry husbandry.

Vasculogenesis is an important event in blood island formation. The abilities to image blood vessels using antibodies and targeted gene deletion, greatly facilitated the characterization of cell behaviors that underlie vasculogenesis, and facilitated in vivo experimentation aimed at identifying the molecules that regulate

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vasculogenesis. The molecules include transcription factors, GATA-1, -2, SCL/tal-1, and Lmo2 [Minko et al., 2003], as well as several kinds of growth factors and the corresponding receptors, such as BMP-1, VEGF, FGF, and flk-1 [Krah et al., 1994; Flamme et al., 1995; Bautch et al., 2000; Moser and Patterson, 2005]. But the key factors that are involved in the intracellular signal transduction pathways in blastodisc vasculogenesis were not well known. Recently, we found that suppressing phosphatidylcholine-specific phospholipase C (PC-PLC) induced neuronal differentiation in mesenchymal stem cells [Wang et al., 2004]. So we deduced that PC-PLC might be an important factor affecting differentiation of stem cell. Chicken blastodisc cells were a kind of stem cells, whether PC-PLC participated in blastodisc cell differentiation was not clear. Furthermore, it was found that PC-PLC worked in VEC and vascular smooth muscle cell (VSMC) proliferation [Inui et al., 1994; Zhao et al., 2005]. The data suggested that PC-PLC acted as an important factor in vascular cell lineage. Moreover, PC-PLC was also found in the bull and rabbit sperm [Hinkovska-Galchev and Srivastava, 1992]. These results showed that PC-PLC was expressed both in spermic cells and adult cells, indicating that it might act as an important factor in animal development and vascular cell differentiation. But whether and how PC-PLC participated in blastodisc vasculogenesis was unknown.

Reactive oxygen species (ROS) affected multiple physiological processes in early development from oocyte maturation to fertilization, embryo development, and pregnancy [Shibata et al., 2003; Agarwal et al., 2005]. ROS also acted as signaling molecules that were involved in signal transduction cascades of numerous growth factor pathways [Sauer and Wartenberg, 2005], and regulated biological effects such as apoptosis, proliferation, and differentiation of vascular endothelial cells [Ruiz-Gines et al., 2000; Zhao et al., 2005; Su et al., 2006]. But whether ROS participate in blastodisc vasculogenesis was not known.

In previous reports, suppressing PC-PLC by its inhibitor D609 strongly inhibited ROS production in LPS-activated microglia [Akundi et al., 2005], but restored Phorbol 12-myristate 13-acetate-initiated ROS formation in RAW264.7 macrophages pretreated with IFN-γ [Von Knethen and Brune, 2005]. The data suggested that PC-PLC might perform its function associated with ROS, but the effect of PC-PLC to ROS level was different in various cells. How PC-PLC performed its function in the vasculogenesis induced by FGF-2 was not clear.

To address these questions, we first set up a chick vasculogenesis model in vitro. Then, we examined the activity changes of PC-PLCs, including Ca^{2+}-dependent and -independent PC-PLC, during the endothelial differentiation of chick blatodiscs induced by FGF-2. At the same time, the levels of ROS were examined during the cell differentiation. To understand the role of PC-PLC and how it performs its function in the vasculogenesis induced by FGF-2, we suppressed PC-PLC activity by its specific inhibitor D609 at 24 h during the cell differentiation. Our data suggested that PC-PLC and ROS were involved in chicken blastodisc differentiation to vascular endothelial cells. PC-PLC was an important factor in the blastodisc cell survival and differentiation, and it might perform its function associated ROS.

MATERIALS AND METHODS

Reagents

Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco BRL Co., Grand Island, NY. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium), D609, L-α-phosphatidylcholine (from egg yolk) and 2′,7′-dichlorofluorescin (DCHF) were purchased from Sigma Co. Fetal bovine serum (FBS) was obtained from Hycolon Lab, Inc. FGF-2 was purchased form EssexBio Group, China. DiI-Ac-LDL was purchased from Invitrogen Co. All other reagents were ultrapure grade.

Cell Culture and Differentiation Induction

Blastodiscs were cultured according to the method described by Flamme [Flamme and Risau, 1992]. Briefly, Blastodiscs of stages X–XII (according to the method of Eyal-Giladi and Kochav [1976]) were removed from unincubated chick eggs in PBS and carefully cleaned of adhering yolk. They were then transferred to DMEM medium containing 10% fetal calf serum (FCS) and antibiotics and dissociated by pipetting gently. After resuspended in fresh medium, the cells were plated onto 48-well plates at a density of one blastodisc per well

and incubated with 100 ng/ml of FGF-2. The morphological changes of the cells were observed under the phase contrast microscope (Nikon, Japan). The medium was changed every 3 days and cells were cultured at 37°C in humified air with 5% CO_2.

Uptake of DiI-Ac-LDL

The identification of VECs was performed by analyzing the uptake of acetylated low-density lipoprotein (AcLDL), which is extensively used as a marker to identify VECs [Voyta et al., 1984]. The blastodiscs were cultured on glutin coated 48-well plates for 2, 3, 5, and 12 days respectively. The cells were then washed with DMEM medium, and cultured in the medium containing 10 μg/ml of DiI-Ac-LDL for 6 h. After washed once with DMEM medium, the cells were observed under laser scanning confocal microscope (Leica, Germany). The differentiation rate (%) = (the number of positively stained cells /the total number of cells) × 100. Two hundred cells for each sample were counted at least in random visual fields. The results presented are the mean ± SE derived from three independent experiments.

Analysis of PC-PLC Activity

For the preparation of enzymes, after harvested and washed, the cells (30 blastodiscs) were homogenized with ultrasonic (400 W, 12 min) in 1 ml buffer A (20 mM Tris–HCl, pH 7.0, 10 mM EGTA, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 0.34 mM sucrose) on ice. After centrifuged at 800 rpm, at 4°C for 15 min, the supernatant was centrifuged again at 100,000 rpm, at 4°C for 1 h, then, was used for PC-PLC activity assay [Wu et al., 1997]. The activity of PC-PLC in the cells was determined by the method published previously [Zhao et al., 2005]. In brief, we prepared the enzyme and used L-α-phosphatidylcholine as the substrate of PC-PLC. The optical density was measured at 660 nm (wavelength). Enzyme activity was expressed as nmol per min per mg of protein (nmol/min · mg).

Determination of Intracellular ROS Levels

Intracellular ROS levels were measured using a fluorescent probe, DCHF, which could be rapidly oxidized into the highly fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of intracellular ROS. This assay is a reliable method for the measurement of intracellular ROS [Suematsu et al., 2002]. The fluorescence was monitored using excitation and emission wavelengths of 485 and 530 nm, respectively. The amount of ROS was quantified as the relative fluorescence intensity of DCF per cell in the scan room. The ROI (region of interest) was randomly selected, then zoomed in same folders, the value of relative fluorescent intensity per cell equals to the total value of sample in scan zoom divided by the total number of cells (at least 200 cells) in the same zoom. This assay repeated three times. Here, a representative result from the three similar experiments was shown.

D609 Treatment

After the blastodisc cells were cultured for 24 h, the cells were treated with or without 40 μg/ml of D609 (dissolved in double distilled water) for 48 h. Then, the cell viability and ROS levels were analyzed respectively.

Cell Viability Assay

Cells were seeded onto 96-well plates and treated with 40 μg/ml of D609 for 48 h. The cell viability was determined by using the method of MTT assay described previously [Price and McMillan, 1990]. In brief, when cells were treated with D609 for 48 h, 20 μl of MTT (5 mg/ml) in PBS solution was added to the cell culture medium. Then the cells were further incubated for 4 h. The remaining supernatant was removed and 100 μl of DMSO was added to each well and mixed thoroughly to dissolve the formed crystal formazan. After 10 min of incubation to ensure all crystals were dissolved, the light absorption was measured at 570 nm using SpectraMAX 190 microplate spectrophotometer (GMI Co.).

Statistical Analysis

Data were expressed as mean ± SE and accompanied by the number of experiments performed independently. Statistical analysis was done by *t*-test, and differences at $P < 0.05$ were considered statistically significant.

RESULTS

Morphological Characterization of the Blastodiscs Cultured In vitro

Blastodiscs of stages X–XII were isolated from unincubated chick eggs and cultured in the presence of FGF-2 100 ng/ml. Then the

morphological changes of cultured chick blastodiscs were observed under the phase contrast microscope. The blastodiscs just isolated from preincubation chick eggs were almost clone spheres (Fig. 1A). These cells adhered to the wall of culture dishes after 12 h, and formed a monolayer of epithelioid cells within 2 days, which consisted of numerous small yolk granules (Fig. 1B). At 3rd day, the flat cells with lamellopodia and fine filopodia appeared under and beside the blood-like cells, which formed the blood island (Fig. 1C). The phenomenon mentioned above was in accordance with the previous report about the quail blastodisc vasculogenesis [Flamme and Risau, 1992]. Twelve days later, many capillary-like networks were observed (Fig. 1D).

FGF-2 Induced the Blastodisc Differentiation Into Vascular Endothelial Cells

To know whether FGF-2 can induce the cultured blastodisc differentiation into VECs, we analyzed the uptake of DiI-Ac-LDL by the cells treated with 100 ng/ml of FGF-2 at 2nd, 3rd, 5th, and 12th day, respectively. The uptake of DiI-Ac-LDL happened after 3 days and increased uptake was observed with the time progress. At 12th day, the percentage of cells that could take DiI-Ac-LDL was 67.5% (Fig. 2E), suggesting that the cultured blastodiscs could be induced to differentiate into VECs by FGF-2.

PC-PLC Activity Changes During the Endothelial Cell Differentiation of the Blastodiscs

To know whether and how PC-PLC participates in the endothelial differentiation of blastodiscs, the activities of PC-PLCs, including Ca^{2+}-dependent and -independent PC-PLC, were analyzed during this process. The activity of Ca^{2+}-indepentent PC-PLC was increased from 12 to 24 h, then, it decreased gradually, at 72 h the activity came back to the same level as that of 12 h. But Ca^{2+}-depentent PC-PLC was almost not changed in this process (Fig. 3).

Intracellular ROS Levels Decreased During the Endothelial Cell Differentiation of the Blastodiscs

To understand whether and how ROS changes in the endothelial differentiation of blastodiscs, we examined the levels of intracellular ROS during the process, as shown in Figure 4, in the undifferentiated blastodiscs, the relative fluorescent intensity of DCF was high at 12 h (Fig. 4A), but when the cells differentiated to VECs at 72 h, the fluorescent intensity decreased significantly (Fig. 4D) ($P < 0.01$). The results showed that ROS level dramatically decreased during the endothelial cell differentiation.

Suppressing PC-PLC Inhibited the Viability of the Blastodisc Cells

To investigate the role of PC-PLC in FGF-2-mediated blastodiscs survival, proliferation and differentiation to VECs, we suppressed PC-PLC with its specific inhibitor D609 at 24 h (Fig. 5A), when the activity of PC-PLC is highest. We examined the viabilities of the cells treated with D609 at 72 h. The results showed that, following the treatment with D609, the viability of the cells decreased in a dose-dependent manner (Fig. 5B) ($P < 0.01$).

Suppressing PC-PLC Increased the Level of ROS in the Blastodisc Cells

After the activity of PC-PLC was suppressed with D609 at 24 h, we examined the ROS level at 72 h. The results showed that, following the treatment with D609, the ROS level was increased remarkably (Fig. 5C–E) ($P < 0.01$).

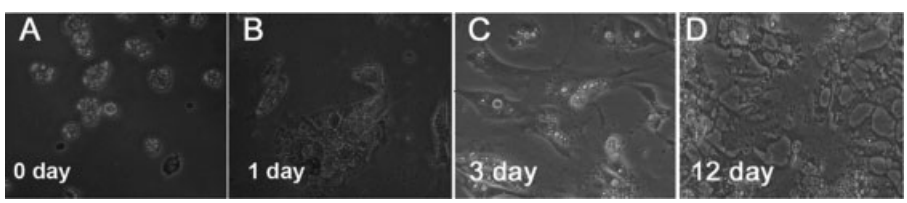

Fig. 1. The morphological changes of chick blastodiscs cultured in the presence of FGF-2 from 0 to 12 days. **A**: The cells just isolated from preincubation chick blastodisc. **B–D**: Cells cultured in the presence of 100 ng/ml of FGF-2 for 1, 3, and 12 days, respectively. After 1 day, cells already adhered to the wall of cell culture dishes. At 3rd day, cells presented endothelium-like morphology. At 12th day, capillary-like networks were formed (×200).

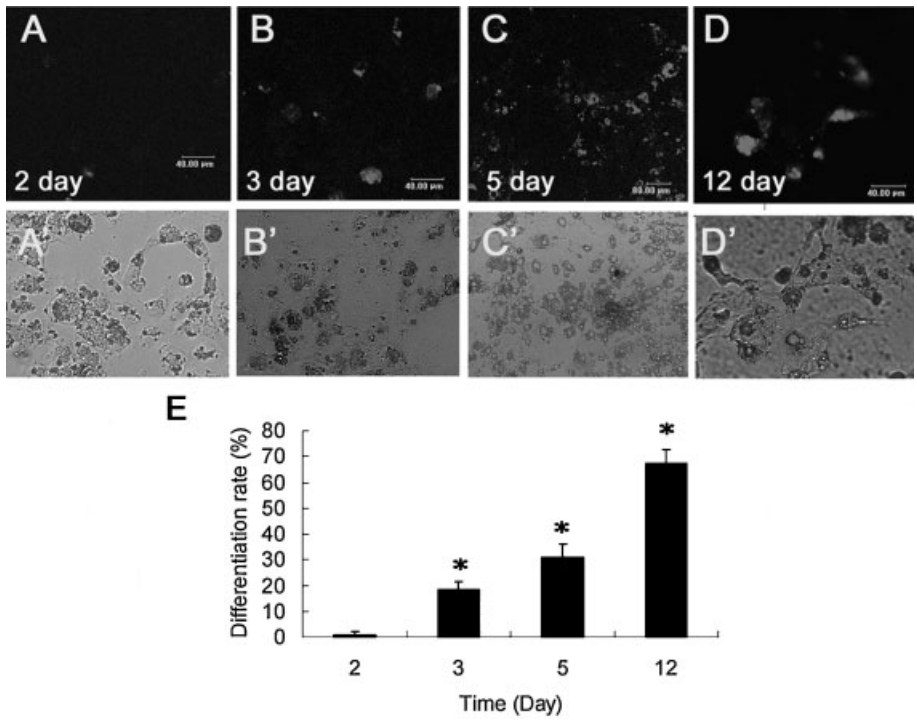

Fig. 2. Uptake of DiI-Ac-LDL. **A**–**D**: The uptake of DiI-Ac-LDL by the cells cultured in the presence of 100 ng/ml FGF-2 for 2, 3, 5, and 12 days, respectively. **A′**–**D′**: The cell photos corresponding to A, B, C, and D respectively. **E**: The differentiation rate of blastodisc cells induced by 100 ng/ml FGF-2 for 2, 3, 5, and 12 days, respectively. Uptake of DiI-Ac-LDL happened after 3 days and increased uptake was observed with the time progress (*$P < 0.01$ vs. the differentiation rate at 2nd day; n = 3).

DISCUSSION

Vasculogenesis is an important event not only in embryos but also in adults. There is only the unincubated quail blastodisc model in vitro. In this study, we first built a chick vasculogenesis model in vitro. In unincubated quail blastodisc model, the formation of capillary-like vascular networks from endothelial cells needs 4 weeks at least [Flamme and Risau, 1992]. In our present research, the capillary-like network formation just needed 12 days. The result showed that the vasculogenesis speed of chick embryo was different with that of quail embryo. This chicken blastodisc vasculogenesis model provides a quick and simple system to investigate vascular development mechanisms. Furthermore, it is convenient for us to get the blastodisc cells from chicken eggs, because they are much bigger than quail eggs.

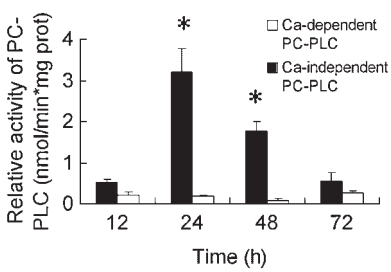

Fig. 3. The changes of PC-PLC activity during the blastodisc cell differentiation into VECs. The activity of Ca^{2+}-indepentent PC-PLC increased obviously before 24 h, then, it gradually decreased. Ca^{2+}-depentent PC-PLC was almost not changed in this process (*$P < 0.01$ vs. Ca^{2+}-indepentent PC-PLC activity at 12 h; n = 3).

The initial role of FGF-2 in vascular development appears to be in the induction of endothelial precursors, angioblasts [Kazemi et al., 2002]. In the previous report, FGF-2 can induce pluripotent cells of the unincubated quail blastodisc to differentiate into endothelial cells [Flamme and Risau, 1992]. In our present study, FGF-2 also can induce unincubated chick blastodisc differentiation into VECs in vitro. The data suggested that the FGF-2 function to induce endothelial precursor differentiation and vasculogenesis was universal to some extent.

Although, it has been known that some transcription factors [Minko et al., 2003], as

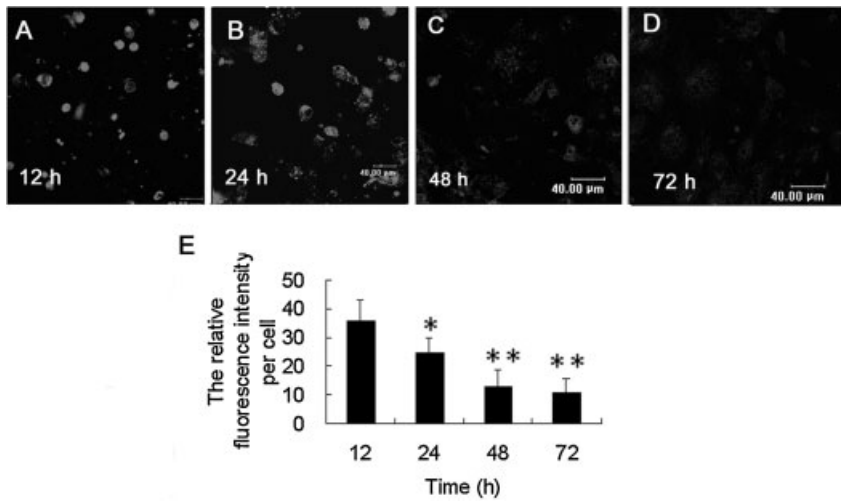

Fig. 4. The changes of intracellular ROS levels during the blastodisc cell differentiate into VECs. Fluorescent micrographs show the relative intensity of ROS at 12 h (**A**), 24 h (**B**), 48 h (**C**), and 72 h (**D**), respectively. **E**: The quantity of intracellular ROS levels at 12, 24, 48, and 72 h, respectively. The amount of ROS was represented by the relative fluorescence intensity of DCF, which was quantified by software of laser scanning confocal microscope (*$P < 0.05$, **$P < 0.01$ vs. ROS level at 12 h; n = 3).

well as several kinds of growth factors and the corresponding receptors [Krah et al., 1994; Flamme et al., 1995; Bautch et al., 2000; Moser and Patterson, 2005] are involved in vasculogenesis, the key factors that are involved in the intracellular signal transduction pathways in blastodisc vasculogenesis were not well known. It has been reported that both Ca^{2+}-dependent and -independent PC-PLC were involved in the mechanism of liver cancer cell proliferation and differentiation. The activation of Ca^{2+}-dependent PC-PLC was involved in rat hepatocarcinogenesis induced by DEN and that it played an important role in the phorbol ester-induced proliferation or retinoic acid-induced differentiation of liver cancer cells. There was a close relationship between Ca^{2+}-dependent PC-PLC activities and cellular DNA content, membranous gamma-glutamyltranspeptidase (gamma-GT), and tyrosine protein kinase. In contrast, Ca^{2+}-independent PC-PLC decreased during hepatocarcinogenesis and during CBRH-7919 cell proliferation induced by PMA but increased during RA-induced differentiation [Wu et al., 1997]. The data suggested that these two PC-PLCs had different roles in regulating liver cancer cell proliferation and differentiation. In our previous studies, we investigated the roles of these two PC-PLCs in human umbilical vascular endothelial cells (HUVECs). The results showed that Ca^{2+}-independent PC-PLC was likely more important than Ca^{2+}-dependent PC-PLC during HUVEC apoptosis [Zhao et al., 2005]. In this study, we found that Ca^{2+}-indepentent PC-PLC underwent a remarkable change with the cell differentiation, while the Ca^{2+}-depentent PC-PLC was nearly not changed in the whole process. The data suggested that Ca^{2+}-indepentent PC-PLC might be an important factor in FGF-2-mediated vasculogenesis of chick blatodiscs. But Ca^{2+}-depentent PC-PLC might be not involved in the vasculogenesis. The differences between the two isoforms in biochemical characteristics and physiological functions need to be investigated further.

It has been reported that ROS act as intracellular messengers in cell differentiation signaling pathways [Shibata et al., 2003]. But the change rules of ROS in the blastodisc cell differentiation to VECs are not known. In this study, the results showed that ROS level dramatically decreased during the endothelial cell differentiation. This finding first provided the evidence that ROS were implicated in the blastodisc cell differentiation to VECs, suggesting that ROS might be the downstream signal molecules in FGF-2-stimulated growth and differentiation signaling pathway during chick blatodisc vasculogenesis. Given recent studies on ROS, pulsechase exposure to low-level ROS enhances the differentiation of embryonic stem cells toward cardiomyogenic as well as vascular cell lineages [Sauer and Wartenberg, 2005]. Our

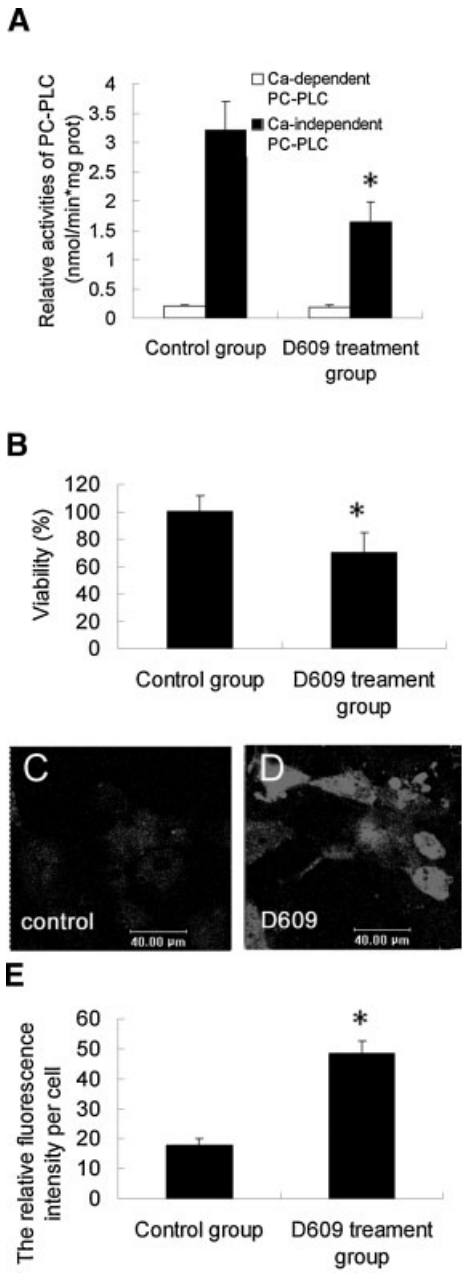

Fig. 5. Effects of PC-PLC on the blastodisc cell viability and ROS level in the cells. **A**: Effect of D609 on PC-PLC activity. The PC-PLC activity of the cells was measured at 1 h after start of the treatment with 40 μg/ml of D609. The activity of PC-PLC in the cells decreased significantly (*$P < 0.01$ vs. control; n = 3). **B**: Role of PC-PLC on the viability of cells. The viability of the cells treated with 40 μg/ml of D609 was measured at 72 h. The cell viability decreased obviously (*$P < 0.01$ vs. control; n = 3). **C–E**: Effect of PC-PLC on ROS level in the cells. Fluorescent micrograph shows the relative fluorescence intensity of DCF in control group (C) and D609 treatment group in which the cells were treated with D609 40 μg/ml (D). E: The quantity of intracellular ROS levels. The amount of ROS was represented by the relative fluorescence intensity of DCF, which was quantified by software of laser scanning confocal microscope (* $P < 0.01$ vs. control; n = 3).

findings are consistent with the report. These data suggested that the differentiation of pluripotent stem cells toward the vascular cell lineage might need the downregulation of ROS level. On the other hand, the differentiation of PC12 cells and neural stem cells toward the neuronal cell lineage requires upregulation of ROS level [Katoh et al., 1997; Suzukawa et al., 2000]. Taken together with previous reports, we deduced that ROS might play important roles in determining cell differentiation direction.

Previous reports showed that PC-PLC might perform its function associated with ROS, but the effect of PC-PLC to ROS level is different in various cells. Tricyclodecan-9-yl-xanthogenate (D609) has been known as a specific inhibitor of PC-PLC. It has been extensively studied in biological systems and exhibits different biological functions in various cells. According to the reports, D609 induced differentiation of marrow stromal cells. However, it promoted apoptosis of senescence VECs, neural stem cells and U937 human monocytic leukemia cells [Meng et al., 2004; Zhao et al., 2004; Wang et al., 2006]. Furthermore, it modulated neuronal cell death through a mechanism that was distinct from that involved in nonneuronal apoptosis [Li et al., 1998]. These activities have been attributed to the inhibitory effect of D609 on PC-PLC. In this research, our results showed that as PC-PLC activity was inhibited by D609 (Fig. 5A), the intracellular level of ROS increased (Fig. 5C–E) and the cell survival was blocked at the same time (Fig. 5B). As a result, the cell differentiation could not progress (data not shown). The data suggested that PC-PLC was an important factor in the blastodisc cell survival and differentiation, and it might perform its function associated with ROS.

In summary, FGF-2 could induce chick blastodisc cell differentiation into VECs effectively. PC-PLC and ROS were involved in chicken blastodisc differentiation to vascular endothelial cells. PC-PLC was an important factor in the blastodisc cell survival and differentiation, and it might perform its function associated with ROS.

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