Phospholipase C- γ 1 is required for cell survival in oxidative stress by protein kinase C

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Phospholipase C- γ 1 (PLC- γ 1) activation has been reported to enhance cell survival during the cellular response to oxidative stress. We studied the role of protein kinase C (PKC) pathways in mediating PLC- γ 1 survival signalling in oxidative stress by using mouse embryonic fibroblasts genetically deficient in PLC- γ 1 (*Plcg1*^{-/-}) and its wild type (*Plcg1*^{+/+}). PLC- γ 1 was activated by H₂O₂ treatment in a dose- and time-dependent manner. Activation of PKC was also markedly increased in both cell lines treated with H₂O₂ (1–5 mM), but with low doses (50–200 μ M), PKC activation was considerably decreased in *Plcg1*^{-/-} cells. After treatment with H₂O₂, PKC-dependent phosphorylation of Bcl-2 and cell viability of *Plcg1*^{-/-} cells decreased dramatically and caspase-3-like activity increased significantly compared with

that of the wild-type cells. Furthermore, pretreatment of *Plcg1*^{+/+} cells with PKC-specific inhibitor decreased levels of PKC-dependent Bcl-2 phosphorylation, enhanced caspase-3 activity and their sensitivity to H₂O₂. On the contrary, treatment of *Plcg1*^{-/-} cells with PKC-specific activator increased the Bcl-2 phosphorylation, decreased caspase-3 activity and improved their survival. These results suggest that PLC- γ 1 mediates survival signalling in oxidative-stress response by PKC-dependent phosphorylation of Bcl-2 and inhibition of caspase-3.

Key words: apoptosis, Bcl-2, caspase-3, phosphorylation, signal transduction.

INTRODUCTION

Many environmental stimuli including cytokines, UV radiation, chemotherapeutic agents and even growth factor generate high levels of reactive oxygen species that can perturb the normal redox balance and shift cells into a state of oxidative stress. When the stress is severe, survival is dependent on the ability of a cell to adapt to or resist the stress. Alternatively, cells may respond to the stress by undergoing apoptosis [1]. Among the main survival-signalling pathways and/or central mediators activated in response to oxidant injury are the extracellular signal-regulated kinase (ERK)-signalling cascades, p53 activation, phosphoinositide 3-kinase (PI-3K)/Akt pathway, nuclear factor κ B signalling system and the heat-shock response [1–6]. Also, growth factor receptors play an important role in initiating cellular responses to oxidative stress [7–10]. Certain proliferation-associated signalling pathways, including those leading to activation of ERK and Akt, are activated by oxidative stress in a growth-factor receptor-dependent manner [7]. Thus some signalling pathways involved in regulating proliferation also participate in the cellular response to oxidative stress.

Phospholipase C- γ 1 (PLC- γ 1) is known to be activated in response to growth-factor stimulation by a mechanism that relies on tyrosine phosphorylation and plays an important role in regulating cell proliferation and differentiation by the generation of the second messengers, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate, leading to the activation of protein kinase C (PKC) and increased levels of intracellular calcium, respectively

[11–14]. Recently, an anti-apoptotic role of PLC- γ 1 activation in oxidative stress has been reported in mouse embryo fibroblasts (MEF) [15]. The study demonstrated that PLC- γ 1 was tyrosine-phosphorylated following H₂O₂ treatment and PLC- γ 1 deficient MEF showed more sensitivity to H₂O₂ than wild types. However, the downstream targets of PLC- γ 1 activation that are involved in mediating stress resistance remain to be identified.

PKC is known as a key enzyme in several signal-transduction pathways and is involved in the regulation of numerous cellular functions [16]. Some PKC isoenzymes play an anti-apoptotic role under some stress applications by phosphorylation of Bcl-2 [17–19] and inhibition of caspase-3 activity [20–22]. Considering that PKC is a downstream of PLC- γ 1 in mitogenic signalling and is also activated in oxidative stress [23–25], we studied the role of PKC pathway in oxidative stress by using MEF that have been rendered deficient for PLC- γ 1 (*Plcg1*^{-/-}) by targeted disruption of both *plc- γ 1* alleles and its wild type (*Plcg1*^{+/+}) as a model system [26,27]. The results showed that PKC-dependent phosphorylation of Bcl-2 and caspase-3 inhibition are involved in PLC- γ 1 survival signalling following oxidative stress.

EXPERIMENTAL

Materials

Dulbecco's modified Eagle's medium and bovine foetal serum were from Gibco BRL (Gaithersburg, MD, U.S.A.). Antibodies

Abbreviations used: DAG, diacylglycerol; ERK, extracellular signal-regulated kinase; MEF, mouse embryonic fibroblasts; PI-3K, phosphoinositide 3-kinase; PKC, protein kinase C; PLC- γ 1, phospholipase C- γ 1; *Plcg1*^{-/-} and *Plcg1*^{+/+}, MEF genetically deficient in PLC- γ 1 and its wild type; PTK, protein tyrosine kinase.

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specific to PLC- γ 1, phosphotyrosine, Bcl-2, caspase-3 and ECL[®] Western-blotting detection system were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-phosphotyrosine agarose beads and Protein A–Sepharose resins were purchased from Sigma (St Louis, MD, U.S.A.). Wortmannin, chelerythrine chloride, Bryostat-1, U73122 and herbimycin A were from Calbiochem–Novabiochem (La Jolla, CA, U.S.A.). Caspase-3 substrate was from Biomol (Plymouth Meeting, PA, U.S.A.). [γ -³²P]ATP and [³²P]orthophosphoric acid were obtained from Beijing YaHui Biotech. *myo*-[2-³H]inositol was from Amersham Pharmacia Biotech (Piscataway, NJ, U.S.A.). The PKC assay system was purchased from Promega (Madison, WI, U.S.A., catalogue no. V7470). Extracellular signal-regulated kinases 1 and 2 (ERK1/ERK2) assay kit was from Cell Signaling Technology (Beverly, MA, U.S.A., catalogue no. 9800).

Cell culture and treatment

Immortalized *Plcg1*^{-/-} and *Plcg1*^{+/+} cells were established by Ji and co-workers [26,27]. Both cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) foetal bovine serum and 1% (v/v) antibiotics mixture in a humidified 95% air, 5% CO₂ atmosphere. Cells were stimulated by H₂O₂ at 37 °C with gentle stirring at various concentrations (0.05, 0.2, 0.5, 1 or 5 mM).

Immunoprecipitation and immunoblotting

Stimulated cells (1 × 10⁷ cells/ml) were lysed in ice-cold lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% (v/v) Nonidet P-40, 100 μ M Na₂VO₄, 2 mM PMSF, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin). Clear samples of lysates were obtained by centrifugation at 12000 *g* for 10 min at 4 °C. The supernatants were incubated sequentially (1 h for each incubation) with antibodies and Protein A–Sepharose at 4 °C. Whole-cell lysate or immunoprecipitates were analysed by SDS/PAGE and transferred electrophoretically to PVDF membranes. Blots were probed with the indicated antibodies and immunoreactive proteins were revealed by the ECL[®] system.

PLC assay

Cells cultured in 100 mm dishes were serum-starved for 12 h in inositol-free medium supplemented with *myo*-[2-³H]inositol (1 μ Ci/ml). After treatment with 20 nM LiCl for 30 min, cells were stimulated with H₂O₂. H₂O₂ stimulation was terminated by the addition of 50% (v/v) HClO₄ to a final concentration of 5% (v/v). After 30 min on ice, the extracts were centrifuged, diluted 1:4 with distilled water and applied to a Bio-Rad Dowex AG 1-X8 anion-exchange column. Total inositol phosphate was eluted from the column with a solution containing 1 M ammonium formate and 0.1 M formic acid, and quantified by liquid-scintillation counting.

PKC kinase assay

PKC assay was performed by using the PKC assay system according to the manufacturer's recommendations. Briefly, 5 × 10⁶–1 × 10⁷ stimulated cells were washed and homogenized by using a cold homogenizer in 0.5 ml of cold extraction buffer [25 mM Tris/HCl, pH 7.4; 0.5 mM EDTA; 0.5 mM EGTA; 0.05% (v/v) Triton X-100; 10 mM β -mercaptoethanol, supplemented with the protease inhibitors PMSF (0.5 mM), leupeptin (1 μ g/ml), aprotinin (1 μ g/ml)]. The lysates were centrifuged at 14000 *g* for 5 min and the supernatant was applied to a 1 ml

DEAE-cellulose anion-exchange chromatography column. Bound PKC was eluted batchwise with 5 ml of extraction buffer containing 200 mM NaCl. Then, 5 μ l of the eluate (enzyme sample) was added to the reactants containing 5 μ l of PKC coactivation buffer (five times) (1.25 mM EGTA, 2 mM CaCl₂, 0.5 mg/ml BSA), 5 μ l of activation buffer (five times) (1.6 mg/ml phosphatidylserine, 0.16 mg/ml DAG, 100 mM Tris/HCl, pH 7.5, 50 mM MgCl₂), 5 μ l biotinylated peptide substrate (Neurogranin, AAKIQAS*FRGHMARKK, where * indicates the biotinylated amino acid) and 5 μ l of [γ -³²P]ATP mix (0.5 mM ATP, 100 μ Ci/ml) per tube and incubated at 30 °C for 5 min. After termination by 7.5 M guanidinium chloride, 10 μ l of the reaction mixture was spotted on to SAM Biotin Capture Membrane (Promega). The membrane was washed with 2 M NaCl for less than 20 min and PKC activity was quantified by liquid-scintillation counting.

ERK1/ERK2 assay

ERK1/ERK2 was performed by using non-radioactive kits manufactured by Cell Signaling Technology according to the manufacturer's recommendations. Briefly, activated ERK was precipitated from cell lysates by using anti-phospho-ERK1/ERK2 (Thr²⁰²/Tyr²⁰⁴) antibody, and precipitates were incubated with a specific substrate Elk-1 and ATP. The reaction was terminated by adding boiling gel loading buffer. ERK activity was detected by immunoblotting the products of the kinase reaction with anti-phospho-Elk (Ser³⁸³) antibody.

Phosphorylation of Bcl-2

Cells were labelled with [³²P]orthophosphoric acid and Bcl-2 proteins were immunoprecipitated by using Bcl-2 antibody. Immunoprecipitates were analysed by SDS/PAGE and transferred to PVDF membranes, then exposed to Kodak X-Omat film for 16 h at -80 °C. The same blot was then used to perform immunoblotting analysis with the anti-Bcl-2 antibody.

Caspase-3 enzyme activity

For detection of caspase-3 activity, 1 × 10⁶ cells were lysed, followed by centrifugation. Caspase-3 activity was detected in the resulting supernatants by measuring the proteolytic cleavage of the fluorogenic substrate Asp-Glu-Val-Asp-7-amino-4-methyl coumarin and 7-amino-4-methyl coumarin as standard in assay buffer by using an excitation wavelength of 380 nm and an emission wavelength of 460 nm.

Cell viability analysis

Cells were stimulated by using various concentrations of H₂O₂. After 22 h, cell viability was determined by the Trypan Blue dye-exclusion method.

RESULTS

H₂O₂ stimulates PLC- γ 1 in *Plcg1*^{+/+} fibroblasts

To determine whether H₂O₂ activates PLC- γ 1 in *Plcg1*^{+/+} fibroblasts that express PLC- γ 1 but not PLC- γ 2 (neither of them expressed in *Plcg1*^{-/-}; results not shown), we first incubated cells with *myo*-[2-³H]inositol so as to label the inositol ring of PtdIns-4,5-P₂ and then treated the cells with various concentrations of H₂O₂ for 2 min. After treatment, we measured the total amount of ³H-labelled inositol phosphates to determine PLC- γ 1 activity. PLC- γ 1 activity started increasing when treated with 50 μ M H₂O₂ and was maximally stimulated with 1 mM H₂O₂ (Figure 1A). The time course of PLC- γ 1 activation was determined by

Figure 1 Dose- and time-dependent change in PLC- γ 1 activation by H₂O₂ in *Plcg1*^{+/+} MEF

(A) H₂O₂ dose response of PLC- γ 1 activity. *Plcg1*^{+/+} cells were labelled with 1 μ Ci/ml *myo*-[2-³H]inositol and incubated in the absence or presence of 5 μ M herbimycin A or 100 nM wortmannin for 30 min. They were then treated with various concentrations of H₂O₂ (0.05–5 mM) for 2 min. The radioactivity of total inositol phosphates fractionated from these cells was measured with a scintillation counter to determine PLC- γ 1 activity. (B) Time course of PLC- γ 1 activation. PLC- γ 1 activity was determined as described above. Untreated cells or cells pretreated with either 5 μ M herbimycin A or 100 nM wortmannin and then incubated with 200 μ M H₂O₂ for the above-mentioned periods of time were used. (C) Dose-dependence and (D) time course of PLC- γ 1 phosphorylation. PLC- γ 1 was immunoprecipitated from cell lysates prepared from cells as treated in (A) or (B). One half of the immunoprecipitate was immunoblotted with the same antibody and the other half with an anti-phosphotyrosine antibody. Data are the mean $\pm$ S.E. for three independent experiments. IP, immunoprecipitation; IB, immunoblot; α PLC- γ 1, anti-PLC- γ 1 antibody; α PY, anti-phosphotyrosine antibody.

treating the cells with 200 μ M H₂O₂ (Figure 1B). PLC- γ 1 activity started increasing 2 min after H₂O₂ treatment, reached a maximum after 5 min (3-fold increase over basal state) and decreased to basal levels after 20 min. Addition of 5 μ M herbimycin A, a specific protein tyrosine kinase (PTK) inhibitor, completely abolished the activation of PLC- γ 1, but pretreatment with 100 nM wortmannin, a PI-3K inhibitor, had no effect on PLC- γ 1 activation (Figures 1A and 1B).

Next, we tested whether H₂O₂ could induce tyrosine phosphorylation of PLC- γ 1. Immunoprecipitation of PLC- γ 1 after H₂O₂ treatment and subsequent Western blotting with an anti-phosphotyrosine antibody revealed that phosphorylation of PLC- γ 1 is initiated on treatment with 50 μ M H₂O₂ and reaches a maximum with 1 mM H₂O₂ (Figure 1C). Also, PLC- γ 1 phosphorylation started increasing after 2 min when treated with 200 μ M H₂O₂, was maximum after 5 min and remained at higher than basal levels after 20 min (Figure 1D). These results showed that PLC- γ 1 was phosphorylated and activated by H₂O₂ treatment in a dose- and time-dependent manner by a PTK-dependent and PI-3K-independent mechanism.

PKC is activated in a PLC- γ 1-dependent manner by treatment with low doses of H₂O₂

PLC- γ 1 may regulate PKC activity by DAG and Ca²⁺ [28]. In this study, we examined the role of PLC- γ 1 in the activation of PKC pathway in fibroblasts exposed to oxidative stress. Wild-type or PLC- γ 1-deficient cells were treated with various concentrations of H₂O₂, and the cell extract was subjected to kinase assay as described in the Experimental section. In wild-type cells, PKC activity increased rapidly and reached a maximum 10 min

after stimulation with 200 μ M H₂O₂, whereas activation of PKC in PLC- γ 1-deficient cells was completely abolished (Figure 2A). On the contrary, when cells were exposed to high doses of H₂O₂ (1–5 mM), the levels of PKC activity were about the same in both the *Plcg1*^{-/-} and *Plcg1*^{+/+} cells (Figure 2B). These observations showed that PKC activation in fibroblasts stimulated with low doses of H₂O₂ was dependent on PLC- γ 1.

H₂O₂-induced ERK1/ERK2 activation is PLC- γ 1-independent

ERK is a crucial kinase for cell survival in many cell types [1]. In the present study, we examined the effect of PLC- γ 1 and PKC on ERK1/ERK2 activation by treatment with H₂O₂. *Plcg1*^{-/-} and *Plcg1*^{+/+} cells were treated with various concentrations of H₂O₂ for 10 min, and the cell lysates were subjected to kinase assay as described in the Experimental section. ERK activity increased in a dose-dependent manner after stimulation with H₂O₂ in both cell lines and there was no difference between wild-type and PLC- γ 1-deficient cells (Figure 3). This implies that PLC- γ 1 was not required for H₂O₂-induced ERK activation in MEF.

PLC- γ 1 is involved in H₂O₂-induced Bcl-2 phosphorylation and negatively regulates caspase-3 activation

Bcl-2 plays an important role in cell survival in stress response, and phosphorylation of Bcl-2 at Ser⁷⁰ may be required for its full and potent anti-apoptotic function [17–19,29,30]. To determine the role of H₂O₂ in Bcl-2 phosphorylation and whether PLC- γ 1 is involved in this process, *Plcg1*^{-/-} and *Plcg1*^{+/+} cells were labelled with [³²P]orthophosphoric acid and then incubated with

Figure 2 PLC-γ1-dependent PKC activation by treatment with low doses of H₂O₂

(A) *Plcg1*^{+/+} and *Plcg1*^{-/-} MEF were treated with 200 μM H₂O₂ for the specified time, and cell extracts were subjected to PKC kinase assay. (B) *Plcg1*^{+/+} and *Plcg1*^{-/-} MEF were treated with the various doses of H₂O₂ for 10 min and cell extracts were subjected to PKC kinase assay. Data are the mean ± S.E. for three independent experiments.

200 μM H₂O₂ for 12 h. During this period, phosphorylation of Bcl-2 dramatically increased in *Plcg1*^{+/+} but not in *Plcg1*^{-/-} cells (Figure 4A).

Figure 4 Role of PLC-γ1 in Bcl-2 phosphorylation and caspase-3 activation by treatment with H₂O₂

(A) *Plcg1*^{+/+} and *Plcg1*^{-/-} MEF were labelled with [³²P]orthophosphoric acid and then incubated with 200 μM H₂O₂ for 12 h. Bcl-2 protein was analysed by autoradiography and immunoblotting as described in the Experimental section. (B) *Plcg1*^{+/+} and *Plcg1*^{-/-} MEF were treated with 200 μM H₂O₂ for 12 h and cell lysates were subjected to oxidative stress to determine caspase-3-like activity and to immunoblot with pro-caspase-3 antibody. Data are the mean ± S.E. for three independent experiments.

We further investigated the relationship between caspase-3 activation and PLC-γ1 in oxidative stress. Following stimulation with 200 μM H₂O₂ for 12 h, it was observed that caspase-3-like activity in *Plcg1*^{-/-} cells had increased considerably compared with that in wild types (Figure 4B). Western blot analysis of pro-

Figure 3 Effect of PLC-γ1 on ERK1/ERK2 activation by treatment with H₂O₂

Plcg1^{+/+} and *Plcg1*^{-/-} MEF were treated with various doses of H₂O₂ for 10 min and cell lysates were subjected to ERK1/ERK2 kinase assay as described in the Experimental section.

Figure 5 PLC- γ 1 protects MEF from H₂O₂-induced cell death by PKC-dependent phosphorylation of Bcl-2 and inhibition of caspase-3

Plcg1^{+/+} and *Plcg1*^{-/-} MEF, untreated or pretreated with either 150 nM Bryostatin-1, a PKC activator, or 5 μ M chelerythrine chloride, a PKC inhibitor, were incubated with 200 μ M H₂O₂ for 12 h. The activity of PKC (A), levels of Bcl-2 phosphorylation (B) and caspase-3 activation (C) were detected as described above. (D) *Plcg1*^{+/+} and *Plcg1*^{-/-} MEF untreated or pretreated either with 150 nM Bryostatin-1 or 5 μ M chelerythrine chloride were incubated with 200 μ M H₂O₂ for 22 h, and cell viability was determined by the Trypan Blue dye-exclusion method. Results shown are the mean $\pm$ S.E. for three independent experiments. Bryo, Bryostatin-1; Ch, chelerythrine chloride; Con, control.

caspase-3 also indicated that caspase-3 was cleaved into active form in *Plcg1*^{-/-} but little affected in *Plcg1*^{+/+} (Figure 4B). These results suggest that PLC- γ 1 plays an important role in the phosphorylation of Bcl-2 and inhibition of caspase-3 following oxidative stress.

Role of PKC

PKC plays an anti-apoptotic role in cellular response to some stress applications by phosphorylation of Bcl-2 and inhibition of caspase-3 activity [17–22]. To determine whether effects of PLC- γ 1 on Bcl-2 phosphorylation and caspase-3 activation in oxidative stress are PKC-dependent, we examined further the effects

of PKC-specific inhibitor or activator on PKC activation, Bcl-2 phosphorylation, caspase-3 activation and cell viability in *Plcg1*^{-/-} and *Plcg1*^{+/+} cells during oxidative stress. Results showed that pretreatment with 150 nM Bryostatin-1, a PKC activator, increased the activity of PKC, levels of Bcl-2 phosphorylation, cell viability and decreased caspase-3 activity in *Plcg1*^{-/-} cells following 200 μ M H₂O₂ treatment for 12 h (Figure 5). Meanwhile, pretreatment with 5 μ M chelerythrine chloride, a PKC inhibitor, decreased PKC activation, levels of Bcl-2 phosphorylation and cell viability and enhanced caspase-3 activity in *Plcg1*^{+/+} cells (Figure 5). These results suggest that PKC mediates PLC- γ 1 protection of fibroblasts from oxidative stress-induced apoptosis by phosphorylation of Bcl-2 and inhibition of caspase-3.

DISCUSSION

In the present study, we have demonstrated that PLC- γ 1 is activated in response to H₂O₂ treatment by a PTK-dependent mechanism. In addition, by using *Plcg1*^{-/-} MEF as a system in which no endogenous PLC- γ 1 is expressed, we have provided evidence that stimulation of PLC- γ 1 is required for cell survival in oxidative-stress response and that PKC-dependent Bcl-2 phosphorylation and caspase-3 inhibition may be the downstream targets of PLC- γ 1 in this process.

It is well established that tyrosine phosphorylation of PLC- γ 1 is necessary for its activation and that PLC- γ 1 undergoes phosphorylation on tyrosine residues in response to H₂O₂ treatment [15,31–33]. Our present study not only showed that PLC- γ 1 is phosphorylated following H₂O₂ treatment but also demonstrated that activities of PLC- γ 1 measured by the total amount of inositol phosphates increased in a dose- and time-dependent manner. On the other hand, the concentration of H₂O₂ employed (as low as 200 μ M) in this study was much lower than that of Qin et al. [31], Blake et al. [32] and Schieven et al. [33] (in excess of 1 mM, and usually between 5 and 10 mM) and is consistent with the recent study by Wang et al. [15] using the same cell line. Survival of MEF deficient in PLC- γ 1 function is severely compromised over the same dose response of H₂O₂ (Figure 5D). Such observations suggest that H₂O₂ is a strong activator of PLC- γ 1 and the role of PLC- γ 1 is protection of cells against H₂O₂-induced cell death.

The mechanisms leading to PLC- γ 1 phosphorylation in response to oxidant injury have been reported by Wang et al. [15]. They showed that H₂O₂-induced phosphorylation of PLC- γ 1 in MEF could be blocked by pharmacological inhibitors of Src-family tyrosine kinases or the epidermal growth factor receptor tyrosine kinase, but not by inhibitors of platelet-derived growth factor receptor or PI-3K. In the present study, we also found that PLC- γ 1 is stimulated in a tyrosine phosphorylation-dependent and PI-3K-independent manner following oxidative stress. However, we could not demonstrate which PTKs are involved in this process. It appears that PTKs such as epidermal growth factor receptor and Src-family tyrosine kinases may contribute to the activation of PLC- γ 1 in oxidative stress.

A major conclusion of the present study is that PLC- γ 1 plays a pro-survival role in the cellular response to acute oxidative stress by PKC. Consistent with our observations, Wang et al. [15] showed that pretreatment of *Plcg1*^{+/+} MEF with inhibitors of PKC increased their sensitivity to H₂O₂, whereas treatment of *Plcg1*^{-/-} MEF with agents capable of directly activating PKC significantly improved their survival. However, they did not demonstrate whether PKC is stimulated by H₂O₂ treatment and whether PLC- γ 1 is required for PKC activation in oxidative stress. Our results showed that PKC activity increased in a time-dependent manner after stimulation with 200 μ M H₂O₂ in wild-type cells, whereas the activation of PKC in PLC- γ 1-deficient cells was completely abolished (Figure 2A). On the contrary, when cells were exposed to high doses of H₂O₂ (1–5 mM), the levels of PKC activity were about the same in both the *Plcg1*^{-/-} and *Plcg1*^{+/+} cells (Figure 2B). These results strongly support the relevance of PKC activation and pro-survival role of PLC- γ 1 in oxidative-stress response, but raise the question as to why the increase of PKC activity following high doses of H₂O₂ treatment is PLC- γ 1-independent. It is possible that the mechanisms leading to the activation of PKC by a high dose of H₂O₂ are different from those initiated by low doses of H₂O₂. Several reports have demonstrated that PKC isoforms, including α , β I and γ of classical PKC, ϵ and δ of novel PKC and ζ of atypical PKC, are tyrosine phosphorylated and catalytically activated in a DAG-

independent manner in the H₂O₂-treated (5 mM) cells [34–36]. Therefore PKC isoforms can be activated by tyrosine phosphorylation in cells treated with high doses of H₂O₂ by using a mechanism unrelated to receptor-coupled hydrolysis of inositol phospholipids. Furthermore, the tyrosine phosphorylation sites of PKC δ in the 5 mM H₂O₂-treated cells are identified as Tyr-311, Tyr-322 and Tyr-512 [37] and this phosphorylation may facilitate the proteolytic activation of PKC δ by caspase-3 and promote apoptosis induced by anti-Fas-antibody and radiation [38,39]. These findings are consistent with our result that both *Plcg1*^{-/-} and *Plcg1*^{+/+} MEF died rapidly on stimulation with high doses (5 mM) of H₂O₂ (results not shown).

The role of PKC in cell death and apoptosis is well documented (for a review see [40]). Promoting cell survival or apoptosis depends on specific PKC isoforms, the cell type, the state of activation of the cell, phase of the cell cycle and the nature of the agent used [40]. Our results demonstrated that PLC- γ 1-dependent PKC activation in MEF is required for cell survival during oxidative stress and that PKC-dependent phosphorylation of Bcl-2 and inhibition of caspase-3 activity may be the downstream targets of PLC- γ 1 survival signalling pathway. However, it remains to be determined which PKC isoenzyme(s) is involved in the regulation of PLC- γ 1 survival signalling during the cellular response to oxidative stress.

We thank Professor Y. Cheng and T. M. Gao for a critical reading of this manuscript. This work was supported by grants from the National Natural Sciences Foundation of China (no. 39870381) and the Natural Sciences Foundation of Guangdong Province (no. 980213).

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Received 3 September 2001/7 January 2002; accepted 31 January 2002