A role of phospholipase C isozymes in H_2O_2-induced oxidative stress of rat aortic smooth muscle cells

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A role of phospholipase C isozymes in H_2O_2-induced oxidative stress of rat aortic smooth muscle cells

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This certifies that the Master's Thesis of Juyong Lee is approved.

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Contents

List of Figures

ABSTRACT

A role of phospholipase C isozymes in H_2O_2-induced oxidative stress of rat aortic smooth muscle cells

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Backgroung Intracellular calcium ions ($[Ca^{2+}]_i$) have an important function in modulating the contractility of all kinds of muscle, including vascular smooth muscle cells. Reactive oxygen species (ROS) can induce Ca^{2+}-overload by directly affecting the Ca^{2+} handling proteins. Little is known that phospholipase C (PLC) isozymes are involved in the Ca^{2+}-overload induced by ROS in smooth muscle cells.

Methods and Results $[Ca^{2+}]_i$ response to exogenous H_2O_2 and ionopore A23187 was measured using the fluorescent probe, fura2-AM, in rat aortic smooth muscle cells(RASMCs). The differential expression levels of PLC isozymes were measured in cells exposed actually with H_2O_2 and ionopore. The subsequent signaling cascades related with PLC isozymes were also investigated in RASMCs. H_2O_2 (1 mM) inhibited the proliferation of RASMCs in DMEM containing 0.1% FBS and increased the $[Ca^{2+}]_i$ by a 50%. In H_2O_2-

stimulated RASMCs, the expression of PLC-δ1 was suppressed within 1 hour, but the other PLC isozymes, β-, and γ-form were not affected. In contrast, the expression of PLC-δ1 and PLC-γ1 were suppressed by ionophore, A23187 (10μM), for 3 hr. The activity of protein kinase C (PKC) was also suppressed in same trend of PLC-δ1, not PLC-γ1.

Conclusions The H_2O_2-induced Ca^{2+} overload may differentially suppress the expression of PLC isozymes in RASMCs and the decreased expression of PLC-δ1 subsequently inhibited the PKC activity. These results suggest that the PLC/PKC-regulated Ca^{2+} homeostasis is the crucial targets of exogenous ROS in H_2O_2-stimulated RASMC.

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I. Introduction

The reactive oxygen species may be involved in the regulation of vascular tone[1,2]. The primary trigger of smooth muscle cell contraction is a rise in cytoplasmic $[Ca^{2+}]_i$[3]. Changes in intracellular Ca^{2+} homeostasis are thought to play important roles in smooth muscle cell responses to oxidants[4]. Both neutrophils and reactive oxygen species (ROS) play important roles in ischemia/ reperfusion-induced cardiac abnormalities[5,6]. Low levels of ROS are regularly produced during a process of physiological metabolisms, and every cell contains several enzymes such as catalase, glutathione peroxidase, and superoxide dismutase, which scavenge ROS from cell[7]. High level of ROS are generated from a variety of sources such as xanthine oxidase system, the leakage of electrons from mitochondria, the cyclooxygenase pathway of arachidonic acid metabolism, and

the respiratory burst of phagocytic cells, and induce a variety of tissue damages[8].

H_2O_2 is often used as an experimental source of ROS. ROS has numerous intracellular targets, including second-messenger pathways, L-type Ca^{2+} channels, K^+ channels, ion transporters, and contractile proteins[9] (Fig. 1). In cardiac myocytes, H_2O_2 have been shown to inhibit Na^+ and Ca^{2+} pumps, accelerate rundown of L-type Ca^{2+} currents, activate Na^+-Ca^{2+} exchange, deplete internal caffeine-sensitive Ca^{2+} stores by inhibiting the sarcoplasmic reticulum Ca^{2+}-ATPase, and activate Na^+-H^+ exchange by inducing mitogen-activated protein kinases[10,11]. These effects are independent of metabolic inhibition, since the mitochondrial uncoupler, carbonylcyanide-p-trifluoro-methoxy- phenylhydrazone, or the metabolic inhibitor, 2-deoxyglucose, do not mimic the effects of ROS[12,13].

Although Ca^{2+}-overload in smooth muscle cells can be induced by ROS by directly affecting the Ca^{2+} handling proteins or indirectly by inducing membrane lipid peroxidation, this may not be the only mechanism for the occurrence of Ca^{2+}-overload and subsequent cell injury[14]. Enhanced adrenergic stimulation as that observed during reperfusion of the ischemic heart may also increase the uptake of extracellular Ca^{2+} into the myocardium and be another factor contributing to

Ca^{2+}-overload[15,16,17]. Proteolytic degradation may also contribute to Ca^{2+}-overload because several cellular proteins including sarcoplasmic recticular Ca^{2+}-pump ATPase are considered to be peroxidase, may also be targets for the Ca^{2+}-activated proteases; further studies are needed to confirm these possibilities. Thus due to the interactive nature of oxidative stress and Ca^{2+}-overload, it is rather difficult to decide whether these pathogenetic substrates for the Ca^{2+}-activating proteases (calpains)[18]. The endogenous antioxidant proteins such as superoxide dismutase, catalase and glutathione factors are causally related to complementary mechanisms of cellular injury.

Phospholipase C (PLC) hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) located in cellular membranes to generate diacylglycerol (DG) and inositol 1,4,5-triphosphate (IP_3), of which diacylglycerol and inositol 1,4,5-triphosphate (IP_3), serve as intracellular messengers for protein kinase C (PKC) activation and intracellular Ca^{2+} mobilization, respectively[19] (Fig.2). In cardiomyoctes, PLC isozymes can be activated by raised Ca^{2+} in the presence of added agonist, the possibility was addressed that Ca^{2+} overload was responsible for the observed

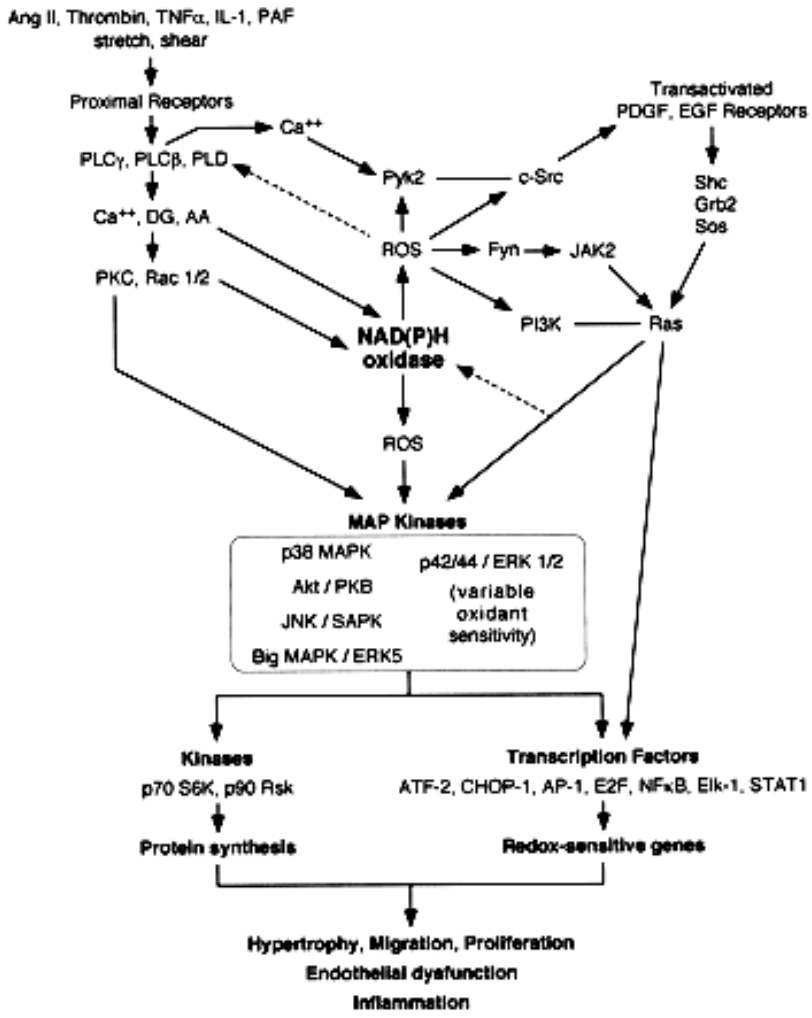

Fig.1. Redox-sensitive signaling pathways in vascular cells. Dotted lines depict pathways in which a relationship has been suggested but not proved. PAF indicates platelet-activating factor; PLC, phospholipase C; PLD, phsopholipase D; DG, diacylglycerol; AA, arachidonic acid; PKC, protein kinase C; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; and SAPK, stress-activating protein kinases.

release of IP_3[20]. The phosphorylation and activation of the plasma membrane Ca^{2+} pump was carried out by PKC activated with DG. The control of PLC activity is thus one of the earliest key events in the regulation of various cell functions. To date, three major types of phosphoinositide-specific phospholipase C species named β, γ and δ, have been characterized by a comparision of amino acid sequences and each type included multiple subtypes[21].

The PLCβ family is activated by G_q subfamily of heterotrimeric G proteins to induce cardiac hypertrophy and failure. Receptors that activate this G_q-PLCβ pathway include those bradykinin, angiotensin II, thromboxane A2, vasopressin and acetylcholine[22]. The PLCγ family is regulated by tyrosine phosphorylation in response to polypeptide growth factors, such as platelete-derived growth factor, epidermal growth factor, nerve growth factor, and hepatocyte growth factor. Tyrosine phosphorylation of PLCγ promotes its association with actin components of the cytoskeleton[19]. PLCγ family is also receptors coupled to PLD, cytosolic phospholipase A_2, or PI3-kinase in the absence of tyrosine phosphorylation. Although PLCδ family has four distinct activated directly by several lipid-derived second messengers and indirectly the

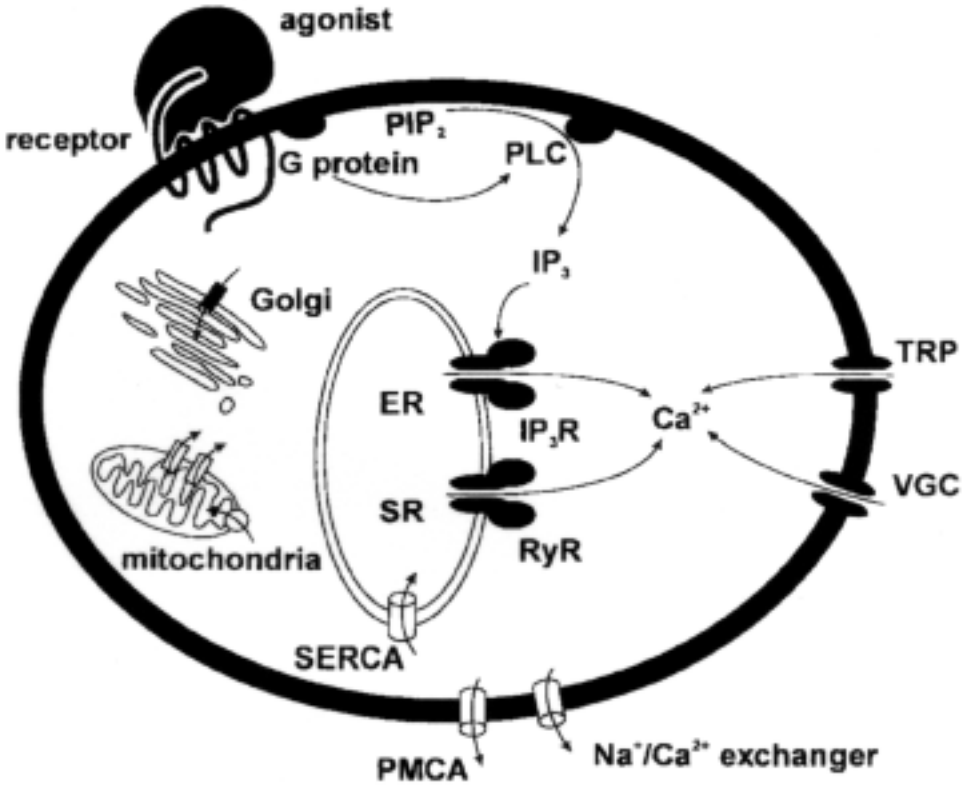

Fig.2. Generation of intracellular Ca^{2+} signals. The ER indicates endoplasmic reticulum; SR, sarcoplasmic reticulum; IP_3R, 1,4,5-triphosphate receptor; RyR, ryanodine receptor; VGC, voltage-gated Ca^{2+} channels.

isoforms, the mechanism by which these isozymes are coupled to membrane receptors remains unclear. It has been reported that PLCδ1was directly activated by a new class of GTP-binding protein (G_h, transglutaminase II) through coupling with α_1-adrenergic receptor[23]. This PLCδ1-G_h pathway thus may be an important player in the signaling pathway that regulates calcium homeostasis and modulates physiological processes, such as smooth muscle tone (i.e., blood pressure) and neurotransmitter release[24]. PLC isozymes are activated by intracellualr Ca^{2+}, but PLC-δ isozymes are more sensitive to Ca^{2+} compared to the other isozymes. An increase in the intracellular concentration of Ca^{2+} to a level sufficient to fix the C2 domain of PLC-δ might therefore trigger its activation[25]. Thus, activation of PLC-δ isozymes might occur secondarily to receptor-mediated activation of other PLC isozymes or Ca^{2+} channels.

In this study, we demonstrate that the acute exposure to high doses of H_2O_2 caused Ca^{2+}-overload in rat aortic smooth muscle cells and a possible link PLC isozymes activation and Ca^{2+} homeostasis at an increase cytosolic Ca^{2+}.

II. METHODS

1. Isolation and Culture of RASMCs

RASMCs were isolated by a modification of the method of Chamley-Campbell[26]. The thoracic aortas from 6-8 weeks-old Spraque-Dawley rats were removed and transferred on ice in serum-free DMEM containing 1% penicillin/ streptomycin. The aorta was free from connective tissue, transferred into a petri dish containing 5 ml of an enzyme dissociation mixture containing DMEM with 1 mg/ml of collagenase type I (Sigma, Deisenhofen, Germany) and 0.5 mg/ml elastase (Sigma, Deisenhofen, Germany) and was incubated for 30 min at 37℃. Then the arota was transferred into DMEM and the adventitia was stripped off forceps under a binocular microscope. The aorta was transferred into a plastic tube containing 5 ml of the enzyme dissociation mixture and was incubated for 2 h at 37℃. The suspension was centrifuged (1500 rpm for 10 min) and the pellet was resuspended in DMEM with 10% FBS. Cells were cultured over several passages (up to 10). RASMCs were cultured in DMEM supplemented with 10% FBS, 100 IU/ml penicillin, 100 μg/ml streptomysin in 75-cm^2 flasks at 37℃ in a

humified atmosphere of 95% air and 5% CO_2 (Forma Scientific, Inc., USA).

2. Cell Survival and Proliferation Assay

The proliferative response of RASMCs was determined using a tetrazolium-based colorimetric assay[27]. Before all the experiments, confluent RASMCs were rendered quiescent by culturing for 48 hours in 0.5% v/v FBS instead of 5%. The assay is dependent on the reduction of tetrazolium salt WST-1, which results in formation of a dark red formazan product, by various mitochondrial dehydrogenases of viable cells. Briefly, Premix WST-1 (10 μl/well) was added to RASMCs that were cultured in a 96-well culture dish ($3X10^4$/well) and pretreated with a control medium or media containing varying concentrations of H_2O_2 for 72 hr. The absorbance of the samples was measured at 450 nm using a microplate reader against a background control. Cell viability was determined with the trypan blue dye-exclusion method using a hemocytometer. RASMCs were seeded in 24-well culture plates ($2.5 X 10^4$ cells/well; well diameter 12 mm). After cells were further incubated in the DMEM medium containing 0.5% serum for 48 hours, cells were treated with H_2O_2 (0-2 mM) for 48 hours. Cells were

then harvested from dishes using a 0.1% w/v trypsin solution, and the viability was examined by the trypan blue dye exclusion test. The number of viable cells was estimated by microscopic cell counting using a hemacytometer.

3. Treatment of Cells with hydrogen peroxide

On day 3 following isolation, RASMCs were further incubated with DMEM containing 0.2% FBS for the complete serum-starvation. The cells were then rinsed twice with PBS. H_2O_2, at various concentrations, was added to medium and incubated with cells for various time intervals. For negative controls, cells were incubated with medium alone for equivalent amounts of time. All cells were then washed twice with PBS and either lysed in protein 1% Triton X-100 lysis buffer and stored at -20℃ for immunoblot analysis.

4. Ca^{2+} measurement

Intracellular Ca^{2+} concentration was measured in freshly isolated and cultured single rat aortic smooth muscle cells using fura2-AM photometry[28]. Briefly, cells were loaded with cell permeable ester (acetoxymethyl, AM) of fura-2 by

incubating the cells in DMEM containing 2μM fura-2/acetoxymethyl ester. After 60 min at 37℃, cells were centrifuged, washed twice, and resuspended in DMEM. Cells were then equilibrated for 10 min in the dark at room temperature, centrifuged, and resuspended in Ca^{2+}-free PBS. Cells were transferred into an experiment chamber and, depending on the experimental protocol used. Ca^{2+} concentration in each experiment is expressed as the 340:380 excitation ratio at 510-nm emission (F_{340}:F_{380};fura-2).

5. Imaging of Intracellular ROS generation

Intracellular oxidant stress was monitored by measuring changes in fluorescence resulting from intracellular probe oxidation[29]. H_2DCFDA (1-10 μM, Molecular Probes) enters the cells and can be oxidized by ROS including superoxide and/or hydroxyl radical to yield 2',7'-dichlorofluorescein (DCFH). Fluorescence intensity was detected on UV wavelength.

6. Immunoblot analysis

Immnoblotting was performed as described previously[23]. Proteins were

fractionated by sodium dodecyl sulfate (SDS) polyacrylamide gel (10-12%) electrophoresis and then electrotransferred to methanol-treated polyvinylidene difluoride membranes. The blotted membranes were washed twice with water and blocked by incubation with 5% nonfat dried milk in PBS buffer (8.0 g NaCl, 0.2 g KCl, 1.5 g NaH_2PO_4, 0.2 g K_2HPO_4 per liter). After a 1 hour incubation at room temperature, the membranes were probed overnight at 4℃ with monoclonal antibodies to PLCβ, PLCδ, and PLCγ followed by goat anti-rabbit IgG-peroxidase and detected by ECL. [e]

III. RESULTS

1. Effect of H_2O_2 concentration on the proliferation of RASMCs

   The acute treatment of H_2O_2 modulates so different kinds of intracellular signal transduction depending on concentration used. To investigate the concentration of H_2O_2 as second messengers, the proliferation of RASMCs was determined in a manner of dose-response. In Fig. 3, the proliferation of RASMCs was increased up to 500 μM H_2O_2, but high concentration of H_2O_2

inhibited the proliferation. The effective H_2O_2 concentration producing the cell death was 1 mM, based on the proliferation of RASMCS. To investigate the production of intracellular ROS at 1 mM H_2O_2, the cells were treated with a molecular probe (H_2DCFDA, 10 μM) in time course. In Fig.4, the fluorescence intensity of H_2O_2-treated cells was 30% higher compared to the untreated cells.

2. Increase of intracellular Ca^{2+} in H_2O_2-stimulated RASMCs

Generally, ROS can induce the Ca^{2+} overload by directly affecting the Ca^{2+} handling proteins. To examine which concentrations of extracellular H_2O_2 induce Ca^{2+} overload in RASMCs, cells were loaded with cell permeable ester (acetoxymethyl, AM) of fura 2 by incubating the cells in DMEM containing 2μM fura 2/acetoxymethyl ester. In Fig. 5, the intracellular Ca^{2+} concentration was increased by 1.5-fold after loading 500 μM H_2O_2, indicating that the addition of extracelluar H_2O_2 significantly changed intracellular Ca^{2+} concentration the rate at which the oxidation of DCF took place.

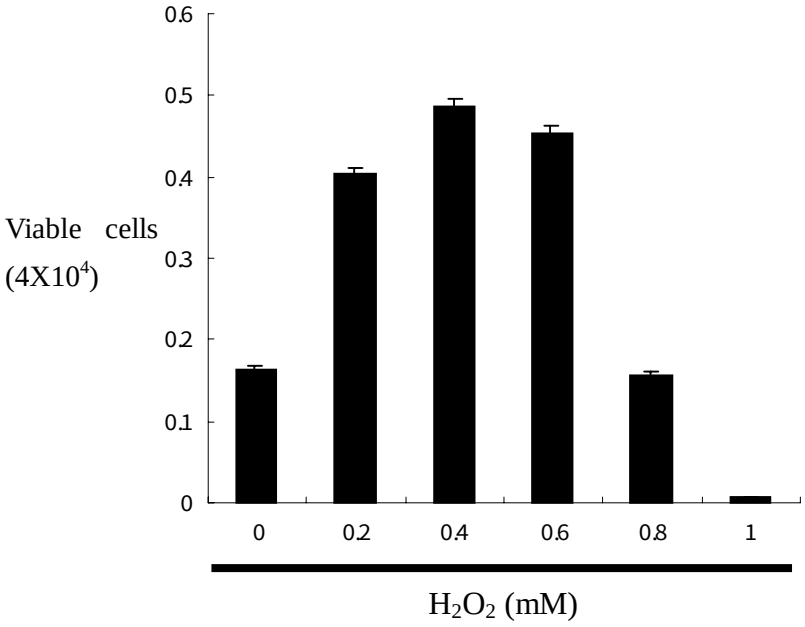

Fig. 3. Effect of H_2O_2 concentration on the proliferation of RASMCs. Quiescent RASMCs (2.7 X 10^4 cells per well) were stimulated with various concentrations of H_2O_2. After 72 h incubation, cells were harvested and the viability examined by trypan blue dye exclusion test. The number of viable cells was estimated using a hemocytometer.

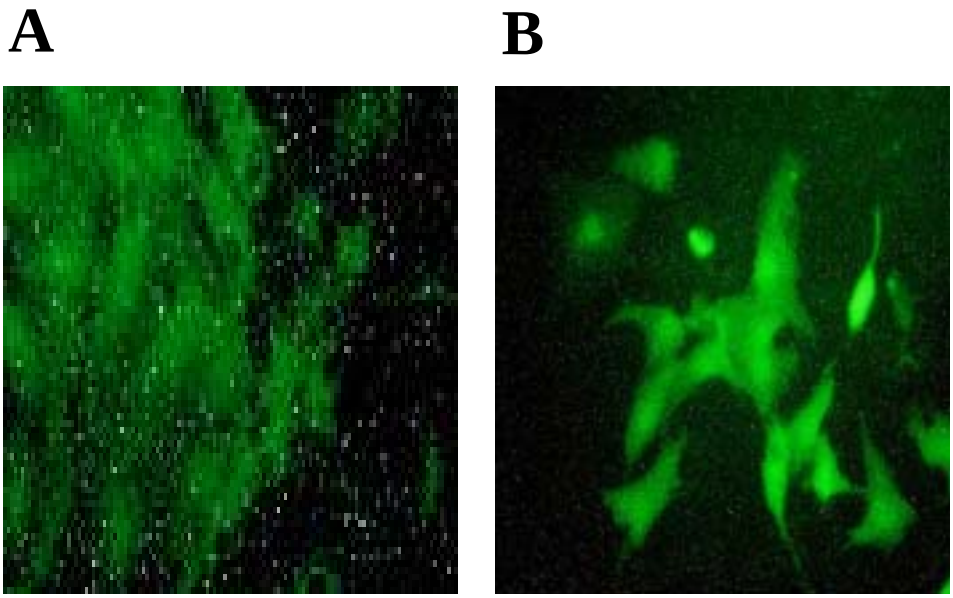

Fig. 4. Photograph of intracellular ROS induced with extracellular H_2O_2. H_2DCFDA (1-10 μM, Molecular Probes) enters the cells and can be oxidized into2',7'-dichlorofluorescein (DCFH). Fluorescence intensity was detected on UV wavelength.

3. Differential expression of PLC isozymes in H_2O_2-stimulated RASMCs

To assess the involvement of PLC isozymes in H_2O_2-induced Ca^{2+} overload of RASMCs, the expression levels of PLC isozymes were measured for variable times with 1 mM H_2O_2. In Fig. 6, the expression of PLC-δ1 was significantly decreased within 1 hr but that of PLC-γ1 and PLC-β1 was not affected, indicating that the response for decreased expression of PLC-δ1 was consistent with the production of intracellular ROS and Ca^{2+} overload. The expression level of PLC-δ1 was recovered after 1 hr in H_2O_2-induced RASMCs.

4. Inhibitory effect of PLC-δ1 on protein kinase C activation

To examine whether the decreased expression of PLC-δ1 is involved in PKC activity in H_2O_2-induced RASMCs, the activity of PKC was measured with anti-phpsho-PKC. In Fig. 7, the activity of PKC was decreased in the same tendency

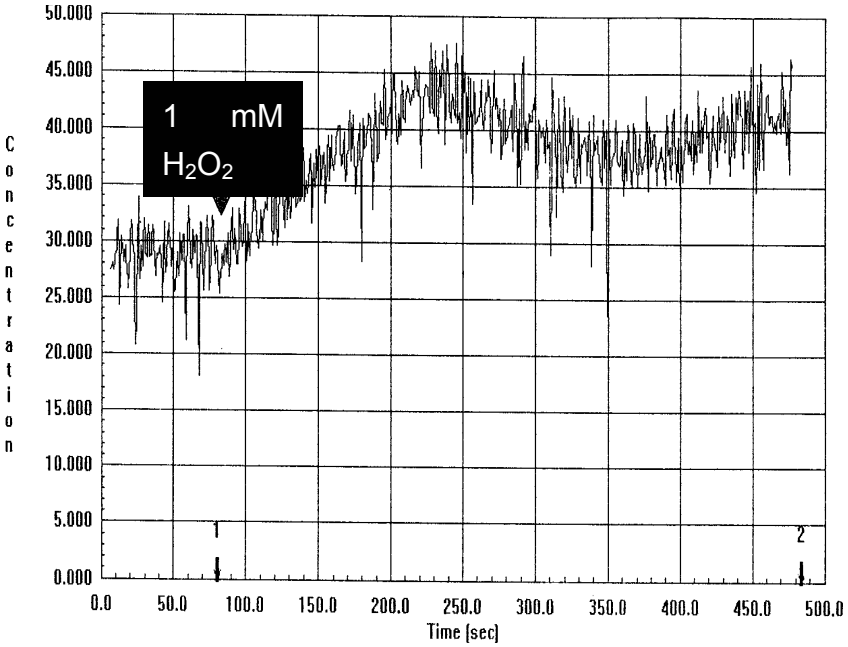

Fig. 5. Effect of H_2O_2 on intracellular Ca^{2+} concentration. Cells were loaded with cell permeable ester (acetoxymethyl, AM) of fura-2 by incubating the cells in DMEM containing 2μM fura-2/acetoxymethyl ester. After 60 min at 37℃, cells were equilibrated for 10 min in the dark at room temperature, centrifuged, and resuspended in Ca^{2+}-free PBS. Cells were transferred into an experiment chamber and, depending on the experimental protocol used. Ca^{2+} concentration in each experiment is expressed as the 340:380 excitation ratio at 510-nm emission (F_{340}:F_{380};fura-2).

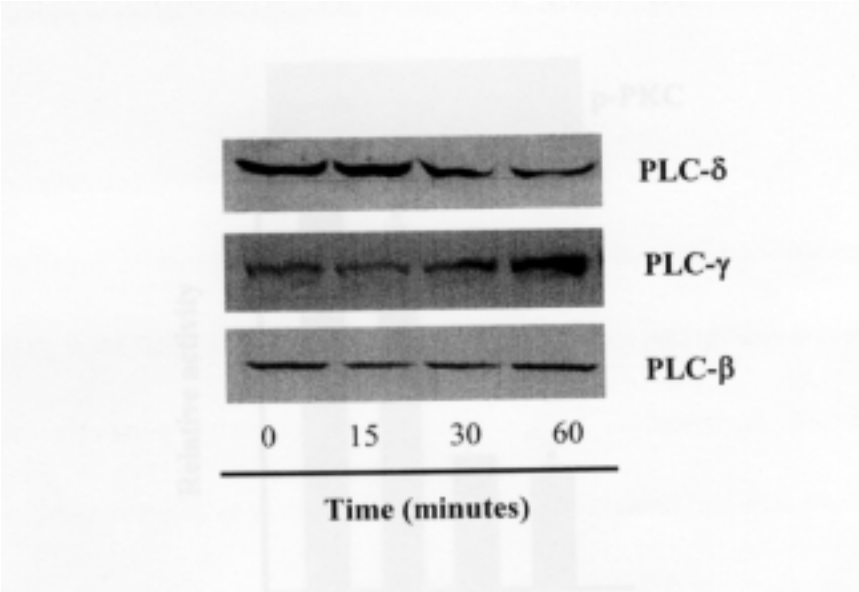

Fig. 6. The expression patterns of PLC isozymes in H_2O_2 –treated RASMCs. Confluent cells (approx. 7 X 10^6/10 cm diameter dish) was treated with 1 mM H_2O_2 for the times indicated. After H_2O_2 treatments, 100 μg of cell lysates was subjected to SDS-PAGE (10%) and analyzed with western blot.

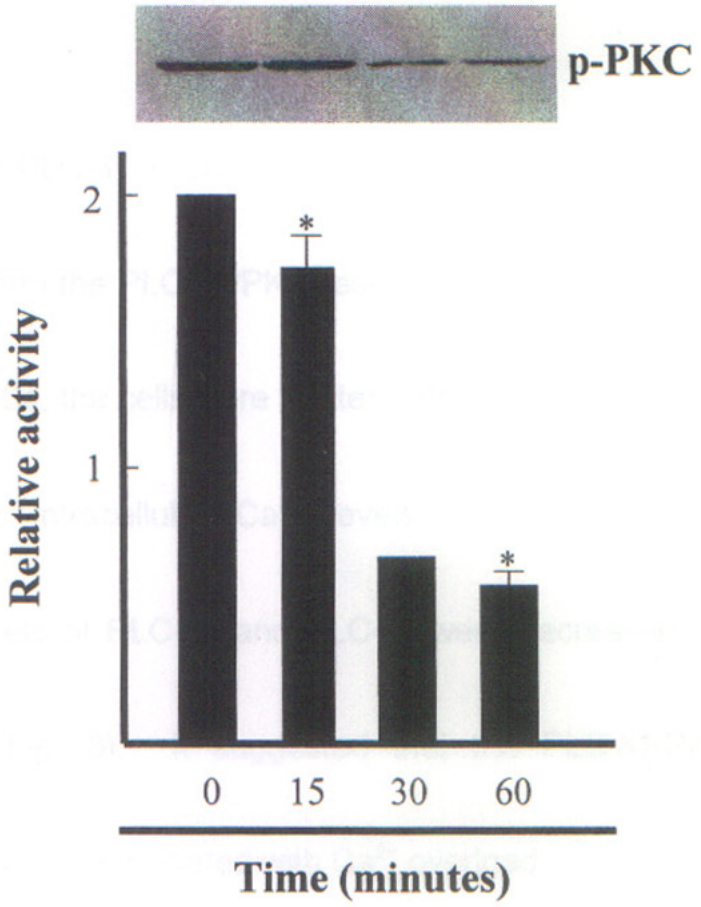

Fig. 7. Effect of extracelluar H_2O_2 on PKC activity in RASMCs. Confluent cells (approx. 7 X 10^6/10 cm diameter dish) was treated with 1 mM H_2O_2 for the times indicated. After H_2O_2 treatments, 100 μg of cell lysates was subjected to SDS-PAGE (10%) and analyzed with western blot.

of expression patterns of PLCδ1. It suggests that inositol 1,4,5-triphosphate and 1,2-diacylglycerol, produced by PLC, were decreased, leading to contribute the decreased activity of PKC.

5. Suppression of PLC-δ1 in ionopore-treated RASMCs

To further confirm the PLC-δ1/PKC response for Ca^{2+} overload by exogenous H_2O_2 in RASMCs, the cells were treated with ionophore, A23187 that is useful for increasing intracellular Ca^{2+} levels. After incubation of 3hr, the expression levels of PLC-δ1 and PLC-γ1 were decreased, but PLC-β1 was not affected (Fig. 8). It suggested that the PLC-δ1/PKC response by extracellualr H_2O_2 is associated with Ca^{2+} overload.

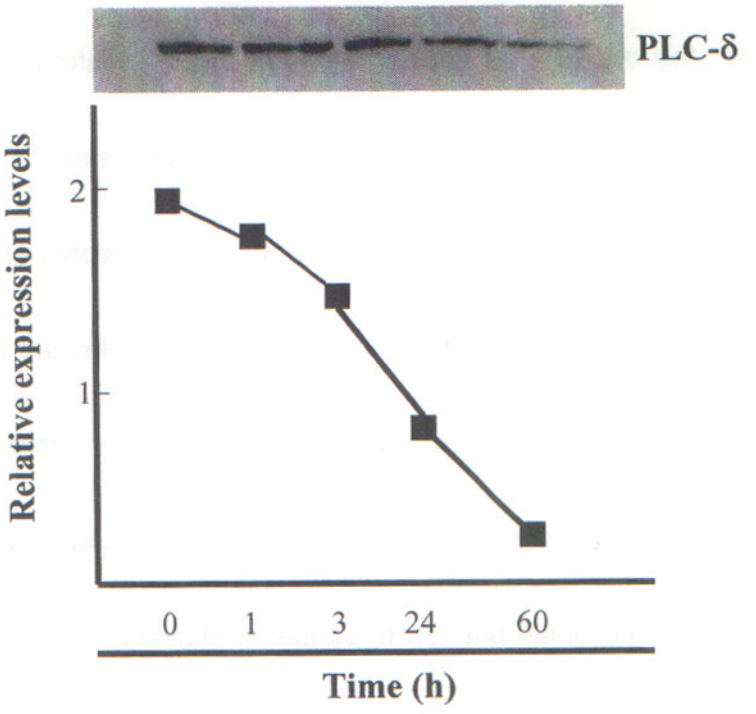

Fig. 8. Effect of ionophore on expression of PLC isozymes. Confluent cells (approx. 7 X 10^6/10 cm diameter dish) was treated with ionophore A23187 (10 μM) for 3 hour. After ionophore treatments, 100 μg of cell lysates was subjected to SDS-PAGE (10%) and analyzed with western blot.

IV. DISCUSSION

Oxidative stress resulting from ROS generation can lead to a decrease in calcium responsiveness of myofilaments either directly, by oxidative modification of contractile proteins (e.g., oxidation of critical thiol groups), or indirectly, by causing Ca^{2+} overload. In general, a whole range of cell functions is regulated by the free cytosolic Ca^{2+} concentration. The Ca^{2+} ions needed to control the activity of the cell can by supplied to the cytosol from intracellular Ca^{2+} stores or from the extracellular space. Ca^{2+} is released from the endoplasmic reticulum (ER) or the sarcoplasmic reticulum (SR) through two types of Ca^{2+} channels: inositol 1,4,5-triphosphate receptors (IP_3R) and ryanodine receptors (RyR). IP_3 is split off from its precursor PIP_2 when cell-surface receptors are activated by extracellular agonists. This reaction is catalyzed by phosphoinositide-specific phospholipase C (PLC) isozymes and results in the generation of two intracellular messengers, diacylglycerol (DAG) and IP_3. These messengers then promote the activation of protein kinase C and the release of Ca^{2+} from intracellular stores, respectively. IP_3 is further converted by the actions of several distinct kinases and

phosphatases to a variety of inositol phosphates, some of which are also implicated in intracellular signaling. In this study, we investigated whether the expressions of PLC isozymes were affected by acute exposure of extracellular H_2O_2 in RASMCs. As shown in Fig.6, the PLC-δ1 was affected by intracellular ROS induced with extracellular H_2O_2. Subsequently, decreased expression of PLC-δ1affected the activity of PKC activated by DAG. It shows that extracellular H_2O_2 can induce intracellular ROS, leading to the Ca^{2+} overload. This Ca^{2+} overload may be the result of changes in intracellular Ca^{2+} homeostasis that is regulated by PLC-δ1/PKC. In the case of ionophore, A23187, the direct increase of intracellular Ca^{2+} significantly suppressed the expression of PLC-γ1 as well as PLC-δ1 (Fig. 8). It suggests that the degree of increase in intracellular Ca^{2+} may be different between ROS and ionophore.

V. CONCLUSIONS

The H_2O_2-induced Ca^{2+} overload may differentially suppress the expression of PLC isozymes in RASMCs and the decreased expression of PLC-δ1 subsequently inhibited the PKC activity. These results suggest that the PLC/PKC-regulated Ca^{2+} homeostasis is the crucial targets of extracellular ROS in H_2O_2-stimulated RASMC.

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