Activation of MAP kinase cascade induced by human pancreatic phospholipase A_2 in a human pancreatic cancer cell line

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Abstract We have found that the growth of human pancreatic cancer cells MIAPaCa-2, induced by human pancreatic phospholipase A_2 group I (hPLA$_2$-I), is mediated via its specific receptor but not via its catalytic property. The present study showed that the activation of mitogen-activated protein kinase (MAPK) cascade in MIAPaCa-2 cells is induced by hPLA$_2$-I: this digestive enzyme induced phosphorylation of MEK1/2, p44/42 MAPK and ATF-2, and the phosphorylation in the MAPK cascade was inhibited after the cells were pre-incubated with a selective inhibitor of MEK, PD98059. In addition, this inhibitor dose-dependently blocked the hPLA$_2$-I-induced MIAPaCa-2 proliferation, suggesting that activation of the MAPK cascade is essential for the hPLA$_2$-I-induced MIAPaCa-2 proliferation.
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Key words: Arachidonic acid metabolism; Human pancreatic phospholipase A_2; MAP kinase; MIAPaCa-2; Proliferation

1. Introduction

Phospholipase A_2 (PLA$_2$; EC 3.1.1.4) catalyzes the hydrolysis of the *sn*-2-acyl ester bond in glycerophospholipids to liberate free fatty acids and lysophospholipids [1]. The mammalian 14-kDa extracellular PLA$_2$s are classified into two types, designated group I (PLA$_2$-I) and group II (PLA$_2$-II), based on their primary structures [2]. PLA$_2$-II, found in the extracelluar spaces of some inflammatory regions [3–5], stimulates production of prostaglandin D_2 and E_2 in several cells and tissues [6,7], suggesting that PLA$_2$-II may play an important role in the pathogenesis of inflammatory diseases.

PLA$_2$-I, secreted from pancreatic acinar cells as an inactive zymogen, functions as an enzyme digesting glycerophospholipids after cleavage with trypsin [8]. Recent studies have shown that PLA$_2$-I exists not only in the pancreas, but also in non-digestive organs, such as lung or spleen [9,10], suggesting that PLA$_2$-I might have another function besides being a digestive enzyme. PLA$_2$-I binds to a specific receptor on mammalian cells and elicits a variety of biological responses in a receptor-mediated manner [11–16]. We have recently found that human PLA$_2$-I (hPLA$_2$-I) stimulates the growth of a human pancreatic cancer cell line, MIAPaCa-2, via hPLA$_2$-I binding to the specific receptor but not via hPLA$_2$-I catalytic property [17,18]. The intracellular signal transduction on the hPLA$_2$-I-induced cell response, however, remains unclear.

The activation of mitogen-activated protein kinase (MAPK) is catalyzed by MAPK kinase (MAPKK) or MEK, which phosphorylates MAPK on threonine and tyrosine residues [19,20]. MAPK is stimulated by a wide variety of hormones, cytokines and growth factors and in turn directs the phosphorylation of transcriptional factors [21,22]. This MAPK cascade has been suggested to regulate cell responses, such as cell growth [23] and differentiation [24].

The present study demonstrated that hPLA$_2$-I induces phosphorylation of MEK1/2, p44/42 MAPK and ATF-2 in MIAPaCa-2 cells when dosed to the culture medium. The hPLA$_2$-I-induced phosphorylation was inhibited by a selective MEK inhibitor, PD98059. Furthermore, PD98059 dose-dependently blocked the hPLA$_2$-I-induced MIAPaCa-2 proliferation, suggesting that activation of the MAPK cascade plays an important role in the hPLA$_2$-I-induced cell proliferation.

2. Materials and methods

2.1. Materials and cell culture

The mature form of hPLA$_2$-I was purified from human pancreatic juice by the method described previously [17,18]. The amino acid sequence of the purified material was confirmed by Edman degradation method using an autosequencer (PSQ-2; Shimadzu, Japan). Anti-phosphorylated MEK1/2, p44/42 MAPK, p38 MAPK, ATF-2, c-Jun and Elk1 antibodies were purchased from New England Biolabs, USA. A selective inhibitor of MAPK kinase or MEK, designated PD98059 (2′-amino-3′-methoxyflavone), was obtained from Calbiochem-Novabiochem, USA. Indomethacin and nordihydroguaiaretic acid (NDGA) were purchased from Wako Pure Chemical Industries, Japan, and Sigma Chemical, USA, respectively. PD98059, indomethacin or NDGA was dissolved in dimethyl sulfoxide. MIAPaCa-2 cell line was supplied by the Japanese Cancer Research Resources Bank and maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, USA) containing 10% fetal bovine serum (FBS; Gibco-BRL), 100 U/ml penicillin G (Sigma Chemical) and 100 μg/ml streptomycin (Sigma Chemical). The cells were grown in plastic flasks in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

2.2. Cell proliferation assays

The cell proliferation assay was performed by the method described previously [17,18]. MIAPaCa-2 (2.5×10^4) cells were grown for 24 h in 0.6 cm^2 in a 96-well plate using DMEM supplemented with 10% FBS. After the culture medium was replaced with the fresh same medium containing 0.1% FBS, the cells were grown in the same medium supplemented with or without hPLA$_2$-I. After incubation for 48 h, the cell proliferation was assayed using a Cell Counting Kit (Dojindo Laboratories, Japan). The absorbance at 450 nm of aliquots of supernatant from MIAPaCa-2 cells was measured using an automatic plate analyzer (ETY-3A; Toyo Sokki, Japan).

2.3. Arachidonic acid and its metabolites release assays

After MIAPaCa-2 (3.0×10^4) cells were grown for 24 h in DMEM containing 10% FBS using a 24-well plate, the culture medium was replaced with 0.5 ml of the fresh same medium containing 18.5 kBq

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Abbreviations: AA, arachidonic acid; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; h, human; MAPK, mitogen-activated protein kinase; MEK, MAPK or ERK kinase; NDGA, nordihydroguaiaretic acid; PAGE, polyacrylamide gel electrophoresis; PLA$_2$-I, group I of phospholipase A_2; PLA$_2$-II, group II of phospholipase A_2; SDS, sodium dodecyl sulfate

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(0.5 μCi) of [^{3}H]arachidonic acid (AA; 7.77 Tbq/mmol; 210 Ci/mmol; Moravek Biochemicals, USA) and followed by incubation for 20 h. The ^{3}H-labeled cells were washed twice with DMEM containing 0.1% FBS and incubated in the same medium supplemented with or without 50 nM hPLA$_2$-I. After incubation for the given times, the culture medium was aspirated and centrifuged to remove the debris. [^{3}H] radioactivity contained in the supernatant was measured in a liquid scintillation counter. The cell-associated radioactivity was also measured after lysing the cells with 0.5% Triton X-100. The amount of [^{3}H]AA released in the medium was normalized as the percentage of the total incorporated radioactivity (i.e. radioactivity associated with the cell plus that released in the medium).

2.4. Immunoblot analysis of phosphorylated kinases and transcritional factors

MIAPaCa-2 cells, treated with 50 nM hPLA$_2$-I for varying lengths of time, were lysed with a lysis buffer consisting of 100 mM Tris-HCl (pH 6.8), 4% sodium dodecyl sulfate (SDS), 12% glycerol, 4% 2-mercaptoethanol and 0.1% bromophenol blue. The lysates were boiled at 100°C for 5 min, and the 40-μg protein aliquots were subjected to 10% SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were electrophoretically transferred to a PVDF membrane (pore size 0.2 μm; Bio-Rad Laboratories, USA). Detections of phosphorylated kinases and transcritional factors were done using PhosphoPlus Antibody Kit series (New England Biolabs) and visualized by exposing to X-ray films (Hyperfilm-MP, Amersham International, UK).

2.5. Immunocytochemistry

MIAPaCa-2 cells, treated with or without 50 nM hPLA$_2$-I for 15 min, were fixed with 4% paraformaldehyde at 4°C for 10 min. The fixative was aspirated and the resulting cells were washed 3× for 5 min with 1 ml of TBST solution consisting of 50 mM Tris-HCl (pH 7.4), 150 mM NaCl and 0.1% Triton X-100. The fixed cells were incubated with 1 ml of TBST containing 1.5% normal goat serum. Localization of the phosphorylated p44/42 MAPK in the MIAPaCa-2 cells was detected using both a PhosphoPlus MAPK Antibody Kit (New England Biolabs) and a Vectastain ABC Kit (Vector Laboratories, USA).

3. Results and discussion

3.1. hPLA$_2$-I induces liberation of AA and its metabolites from MIAPaCa-2 cells

Previous studies have shown that the release of AA and its metabolites from mammalian cells is induced by porcine PLA$_2$-I, which is mediated via its specific receptor [14–16].

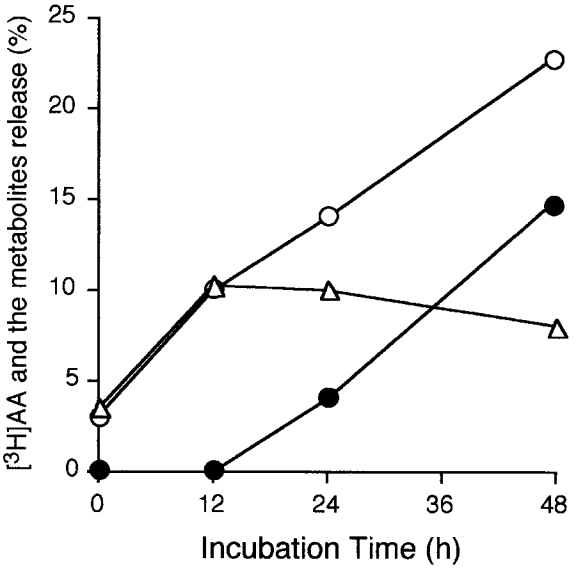

Fig. 1. [^{3}H]AA and its metabolites released from MIAPaCa-2 cells treated with or without hPLA$_2$-I. The amount of [^{3}H]AA and its metabolites released in the medium was normalized as the percentage of the total [^{3}H] radioactivity incorporated into the cells. The amounts of the specific release (●) was calculated by subtracting the basal amount (△) obtained without hPLA$_2$-I from the amount (○) with 50 nM hPLA$_2$-I. The data are expressed as the mean of triplicate measurements.

In the present study, we examined whether hPLA$_2$-I induces the release of AA and its metabolites from MIAPaCa-2 cells. Prolonged incubation (48 h) of MIAPaCa-2 cells with 50 nM hPLA$_2$-I significantly increased their basal release, whereas short-term incubation (< 12 h) did not (Fig. 1). We have previously shown that hPLA$_2$-I (50 nM) stimulates the growth of MIAPaCa-2 cells, which is mediated via its hPLA$_2$-I-specific receptor [17,18]. Since the metabolites derived from AA have been suggested to play an important role in cell proliferation [25,26], we tried to examine whether the release of AA and its metabolites from MIAPaCa-2 cells, induced by hPLA$_2$-I, gives rise to the cell proliferation. Cell responses,

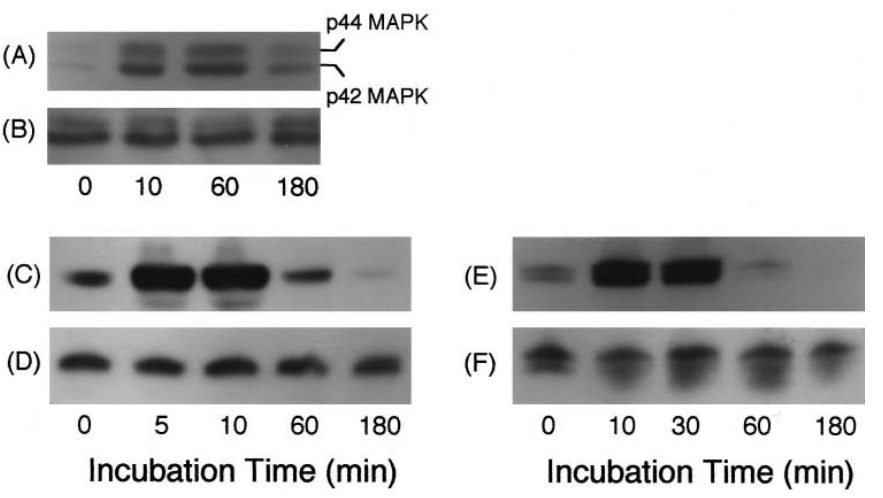

Fig. 2. hPLA$_2$-I-induced phosphorylation of p44/42 MAPK, MEK1/2 and ATF-2. MIAPaCa-2 cells were incubated with 50 nM hPLA$_2$-I for given times. The cell lysates were subjected to SDS-PAGE followed by immunoblotting with anti-phosphorylated p44/42 MAPK (A), with anti-non-phosphorylated p44/42 MAPK antibody (B); with anti-phosphorylated MEK1/2 antibody (C); with anti-non-phosphorylated MEK1/2 antibody (D); with anti-phosphorylated ATF-2 antibody (E) and with anti-non-phosphorylated ATF-2 antibody (F), respectively.

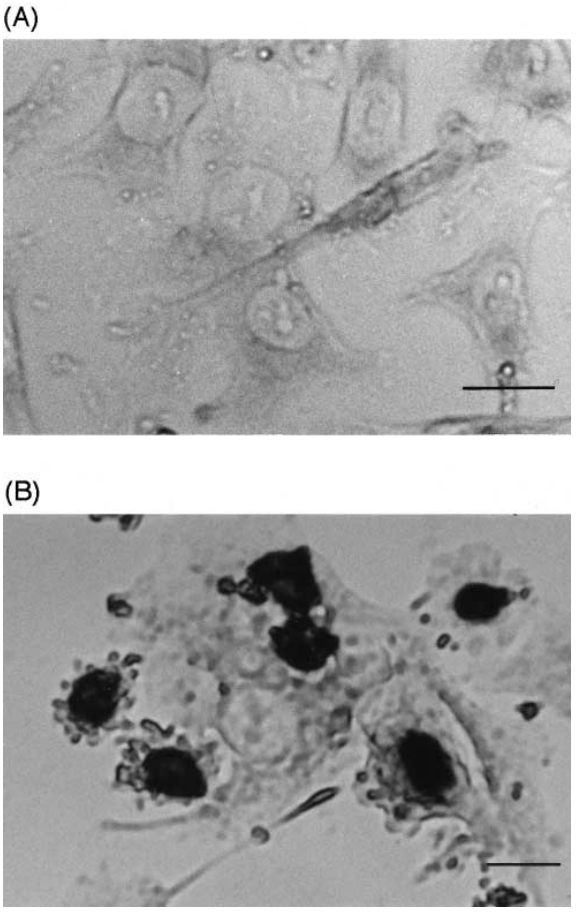

Fig. 3. Translocation of the phosphorylated p44/42 MAPK to the nuclei after incubation of MIAPaCa-2 cells with $hPLA_2$-I. A or B indicates light micrograph of the cells incubated without or with 50 nM $hPLA_2$-I, respectively. Bar, 10 μm.

stimulated by AA metabolites liberated from the cell membrane, are known to be blocked by indomethacin and nordihydroguaiaretic acid (NDGA) [14,26,27]. We found that the $hPLA_2$-I-induced proliferativity on MIAPaCa-2 cells is the same as that without indomethacin or NDGA, even when treated with each inhibitor (data not shown). These results suggest that the AA metabolic pathway may not contribute to the $hPLA_2$-I-induced cell proliferation.

3.2. *Phosphorylation of MEK1/2, p44/42 MAPK and ATF-2 induced by $hPLA_2$-I*

To evaluate activation of MAPK cascade, induced by $hPLA_2$-I, we examined whether MAPK in MIAPaCa-2 cells is phosphorylated when $hPLA_2$-I is added to the culture medium. MIAPaCa-2 cells were incubated in DMEM containing 0.1% FBS with or without 50 nM $hPLA_2$-I for the given times. The cells were lysed, and the resulting lysates were subjected to an immunoblot analysis using an anti-phosphorylated p44/42 MAPK antibody. Fig. 2A shows that the phosphorylated p44/42 MAPK was detectable within at least 10 min and disappeared within 180 min, after addition of $hPLA_2$-I. Total amounts of protein (Fig. 2B), detected by using anti-non-phosphorylated p44/42 MAPK antibody, did not change with different incubation periods. We observed that the phosphorylated p44/42 MAPK translocates to the nuclei in the MIAPaCa-2 cells treated with 50 nM $hPLA_2$-I for 15 min (Fig. 3B). On the other hand, phosphorylation of p38 MAPK, which may be activated under some stress, did not occur in MIAPaCa-2 cells treated with 50 nM $hPLA_2$-I (data not shown).

We investigated phosphorylation of MEK1/2 in the MIAPaCa-2 cells treated with 50 nM $hPLA_2$-I. MEK1/2 are dual-specific kinases and phosphorylate threonine and tyrosine residues in p44/42 MAPK [19,20]. Fig. 2C shows that the phosphorylated MEK1/2 increased clearly within at least 5 min and decreased to the basal level within 60 min. As described above, no detectable change in the total amounts of protein per each lane was noted (Fig. 2D). In addition, we investigated phosphorylation of transcriptional factors after treatment of MIAPaCa-2 cells with 50 nM $hPLA_2$-I. MAPK, activated by a wide variety of stimulants, directs the phosphorylation of transcriptional factors, such as c-Jun and TCF [21,22]. Fig. 2E shows that the phosphorylated ATF-2 was obviously detected at 10–30 min and decreased to the basal level within 60 min. However, transcriptional factors, such as c-Jun and Elk1, were not phosphorylated in MIAPaCa-2 cells by addition of 50 nM $hPLA_2$-I (data not shown).

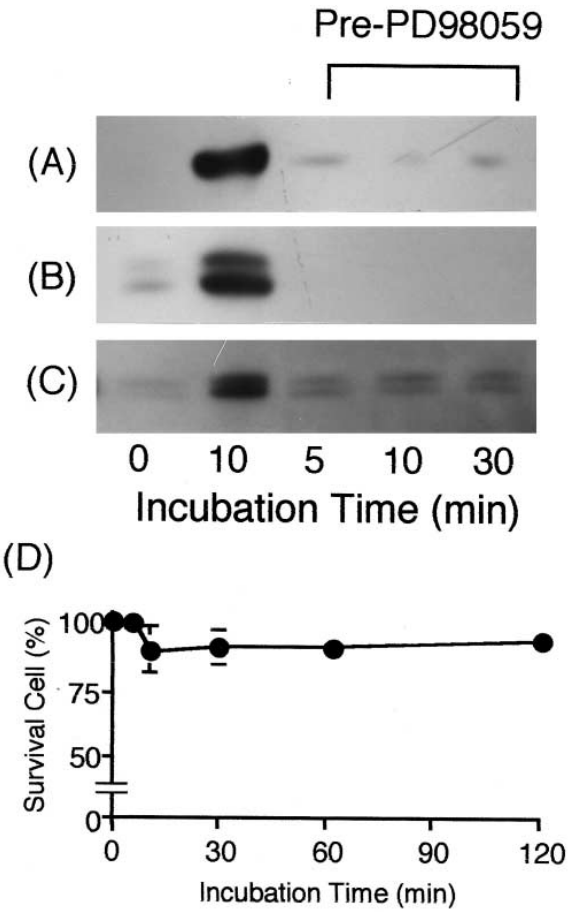

Fig. 4. Effects of PD98059 on the $hPLA_2$-I-induced phosphorylation of MEK1/2, p44/42 MAPK and ATF-2, and cell viability. The cells were treated with 50 μM PD98059 for 16 h before the addition of 50 nM $hPLA_2$-I for desired time. Immunoblot analyses with anti-phosphorylated MEK1/2 (A), anti-phosphorylated p44/42 MAPK (B) and anti-phosphorylated ATF-2 antibodies (C). (D) indicates viability of MIAPaCa-2 cells incubated with 50 nM $hPLA_2$-I for 2 h after pre-treatment with PD98059 for 16 h. The data are expressed as the mean and the standard deviation of triplicate measurements.

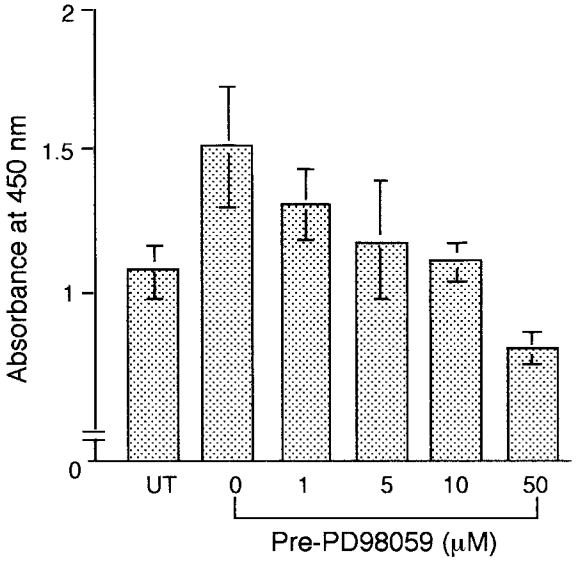

Fig. 5. Dose effects of PD98059 on the $hPLA_2$-I-induced MIAPaCa-2 proliferation. The cells were incubated with 50 nM $hPLA_2$-I for 48 h after pre-treatment with various concentrations of PD98059 for 16 h. The data are expressed as the mean and the standard deviation of triplicate measurements. UT; untreated with $hPLA_2$-I and PD98059.

3.3. *MAPK cascade required for $hPLA_2$-I-induced MIPaCa-2 proliferation*

PD98059 blocks phosphorylation of MAPK and several cell responses, which are mediated via the MAPK cascade [24,28]. We examined the effect of PD98059 on the phosphorylation of MEK1/2, p44/42 MAPK and ATF-2 in MIAPaCa-2 cells, which are induced by $hPLA_2$-I. After the cells were incubated with 50 mM PD98059 for 16 h prior to the treatment with 50 μM $hPLA_2$-I for the given times, the cell lysates were subjected to SDS-PAGE, and followed by immunoblotting with anti-phosphorylated antibodies. Fig. 4A–D shows that all phosphorylation of MEK1/2, p44/42 MAPK and ATF-2, which are induced by $hPLA_2$-I, were completely inhibited by pre-treatment with PD98059 without altering cell viability.

To evaluate the significant role of the MEK-MAPK pathway in the $hPLA_2$-I-induced cell proliferation, MIAPaCa-2 cells were treated with various concentrations of PD98059 for 16 h before addition of 50 nM $hPLA_2$-I. As shown in Fig. 5, PD98059 dose-dependently blocked the $hPLA_2$-I-induced MIAPaCa-2 proliferation. These data provide a strong support for the requirement of activation of the MAPK cascade in the $hPLA_2$-I-induced cell proliferation.

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