Group II phospholipase A_2 activates mitogen-activated protein kinase in cultured rat mesangial cells

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Abstract Group II phospholipase A_2 (PLA_2) is a mediator of inflammation in various disease including glomerulonephritis. We recently found that urinary excretion of PLA_2 was increased in patients with mesangial proliferative glomerulonephritis and that interleukin-1 (IL-1) enhanced platelet derived growth factor-stimulated mesangial cell proliferation through the action of group II PLA_2 secreted in response to IL-1 stimuli. Here we report signal transducing mechanism through group II PLA_2 in mesangial cells. Group II PLA_2 (1–15 U/ml) rapidly activated mitogen-activated protein (MAP) kinase. IL-1β activated MAP kinase in two phases and the slow activation in the late phase, proceeding in parallel with increased group II PLA_2 secretion elicited by IL-1 treatment, was inhibited by the specific antibody raised against group II PLA_2. This suggests that the late phase activation of IL-1-induced MAP kinase was mediated, at least in part, by secreted group II PLA_2.

Key words: Group II phospholipase A_2; MAP kinase; Mesangial cell; Glomerulonephritis

1. Introduction

Recently an increasing number of patients have been introduced into maintenance dialysis due to chronic glomerulonephritis. Thus the mechanism involved in the progression of glomerulonephritis must be explored and clarified. Proliferation of glomerular mesangial cells is the predominant finding of glomerulonephritis at its progressive stage. Recent studies have shown that many cytokines, growth factors and vasoactive substances stimulate the proliferation of mesangial cells [1]. Interleukin-1 (IL-1) has been known to act as an autocrine factor to promote the progression of some types of glomerulonephritis in human and in experimental models [2–4]. Mesangial cells express IL-1 receptors and respond to IL-1 stimuli to promote cell proliferation, expression of cytokines and cell-adhesion molecules, and production of proinflammatory substances such as prostaglandins. We have reported that IL-1 enhanced the platelet derived growth factor (PDGF)-stimu-

lated proliferation of mesangial cells through the increased secretion of group II phospholipase A_2 (PLA_2) from these cells [5]. Group II PLA_2, believed to be a rate limiting enzyme of arachidonic acid cascade, participates in mediating inflammatory processes in various diseases such as rheumatoid arthritis, systemic lupus erythematosus, ulcerative colitis and Crohn's disease [6,7]. Concerning renal disease, we found that urinary excretion of group II PLA_2 was significantly increased in patients with mesangial proliferative glomerulonephritis including IgA nephropathy, suggesting that group II PLA_2 plays an important role in the inflammatory process of glomerulonephritis (manuscripts prepared).

Despite these previous studies, the signaling pathway of IL-1 and group II PLA_2 in mesangial cells remains to be elucidated. Recently Huwiler et al. reported that IL-1 stimulated biphasic activation of mitogen-activated protein (MAP) kinase in mesangial cells [8]. MAP kinase, activated quickly in response to various extracellular signals, plays a key role in integrating diverse signaling pathways [9,10]. We report here that group II PLA_2 activates MAP kinase rapidly in mesangial cells and group II PLA_2-induced MAP kinase activation is involved in the prolonged activation of MAP kinase by IL-1. These results are consistent with the action of group II PLA_2 as an autocrine mitogenic factor and suggest the involvement of group II PLA_2-induced MAP kinase activation in the progression of mesangial proliferative glomerulonephritis.

2. Materials and methods

2.1. Materials

Recombinant human IL-1β (5×10^8 U/mg) was obtained from Genzyme (Cambridge, MA). RPMI 1640 medium was obtained from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan). Bovine serum albumin (BSA; fatty acid free), bovine myelin basic protein (MBP) and lysophosphatidic acid were purchased from Sigma. Group II PLA_2 was purified from rat spleen [11,12] and group I PLA_2 was purified from rat pancreas [13] according to the method described previously. Immunoglobulin against group II PLA_2 was prepared as described previously [14]. This immunoglobulin at the concentration of 570 μg/ml almost completely inhibited PLA_2 activity in culture media of IL-1-stimulated mesangial cells (activity of PLA_2 was inhibited from 1.84 to 0.16 U/mg cell protein). Monoclonal mouse anti-MAP kinase antibody was obtained from Zymed.

2.2. Mesangial cell culture and stimulation

Rat mesangial cells were isolated and cultured as described previously [15,16]. Mesangial cells in passage 6–12 were grown to 80% confluent state and then cultured in RPMI 1640 containing 0.5% fetal calf serum for 48 h. Quiescent cells in 100 mm dishes were incubated in RPMI 1640 containing 1 mg/ml BSA and 20 mM HEPES (pH 7.4) for 1 h before stimulation. Then various stimulants were added in the medium and cells were incubated for indicated period.

2.3. Preparation of cytosol fraction

After the treatment of cells with stimulants, cytosol fractions were prepared as described previously [17]. Briefly, cells were scraped off with ice-cold freshly prepared homogenizing buffer (20 mM Tris-HCl, pH 7.4, containing 2 mM EGTA, 10 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1 μM p-amidinophenyl-methanesulfonyl fluoride and 100 kallikrein inactivator U/ml aprotinin) and homogenized by brief sonication. The homogenate was ultracentrifuged and the supernatant was collected as the cytosol fraction.

2.4. Measurements of PLA₂ activity

PLA$_2$ activity in culture medium was assayed after the treatment with IL-1β as described previously using 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphoglycerol as a substrate [7,12]. One unit of PLA$_2$ activity was represented as 1 nmol of oleic acid released from 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphoglycerol per minute.

2.5. Immunoblotting and assay of MAP kinase activity

Immunoblotting of MAP kinase was performed as described previously [17]. To detect signals of immunoblotting, biotin-labeled goat anti-mouse IgG and avidin/biotin system were used (Vector Labs Inc., Burlingame, CA). Activity of MAP kinase was measured by the in-gel kinase assay as described previously, using bovine MBP as the substrate [18,19]. In brief, each aliquots (40 μg protein) of cytosol fraction of mesangial cells were applied on SDS-10% polyacrylamide gel containing 0.5 mg/ml MBP. After electrophoresis, the gels were immersed in 6 M guanidine hydrochloride containing 50 mM Tris-HCl and 5 mM 2-mercaptoethanol, and then transferred to renaturing buffer (50 mM Tris-HCl containing 0.08% polyoxyethylene sorbitan monolaurate (Tween 20) and 5 mM 2-mercaptoethanol). The gels were incubated with [γ-^{32}P]ATP after renaturation, dried on filter paper and subjected to autoradiography. The radioactivity was measured by a bioimaging analyzer and the amount of incorporated phosphate was calculated using specific activity of [γ-^{32}P]ATP. (BAS2000; Fuji Photo Film Co. Ltd., Tokyo, Japan).

3. Results

3.1. MAP kinase activation by IL-1

Quiescent mesangial cells were incubated with 200 U/ml IL-1β and then MAP kinase activity of the cell lysate was measured. In-gel kinase assay showed two bands at 44- and 42-kDa positions (Fig. 1A). Immunoblotting using anti-MAP kinase monoclonal antibody confirmed the bands corresponded to MAP kinases (Fig. 1C). We confirmed IL-1-induced biphasic activation of MAP kinase as reported previously [8]. The activity of 44-kDa MAP kinase (p44 MAP kinase) reached its maximum 20 min after the stimulation and then returned to basal level in 1 h, followed by a steady increase lasting over 24 h (Fig. 1B). Similar results were obtained for the p42 MAP kinase. Huwiler et al. observed that the late phase-increase in MAP kinase activity was accompanied by de novo synthesis of MAP kinase protein. However, little increase in MAP kinase proteins was detectable during the incubation in our experiments, suggesting that MAP kinase activation is mainly caused by enhancing the kinase activity but not by increasing the kinase protein. During this time period, cell number was not changed.

3.2. MAP kinase activation by group II PLA₂

We therefore investigated the mechanism involved in the IL-1-induced late phase activation of MAP kinase. It is reported that IL-1β stimulates mesangial cells to secrete group II PLA$_2$ [20] and we have already obtained evidence that the secreted group II PLA$_2$ mediates the IL-1β-induced proliferation of mesangial cells [5]. Fig. 1D shows the activity of group II PLA$_2$ appeared in the culture medium. Since the time course of the IL-1-induced late phase activation resembled to that of
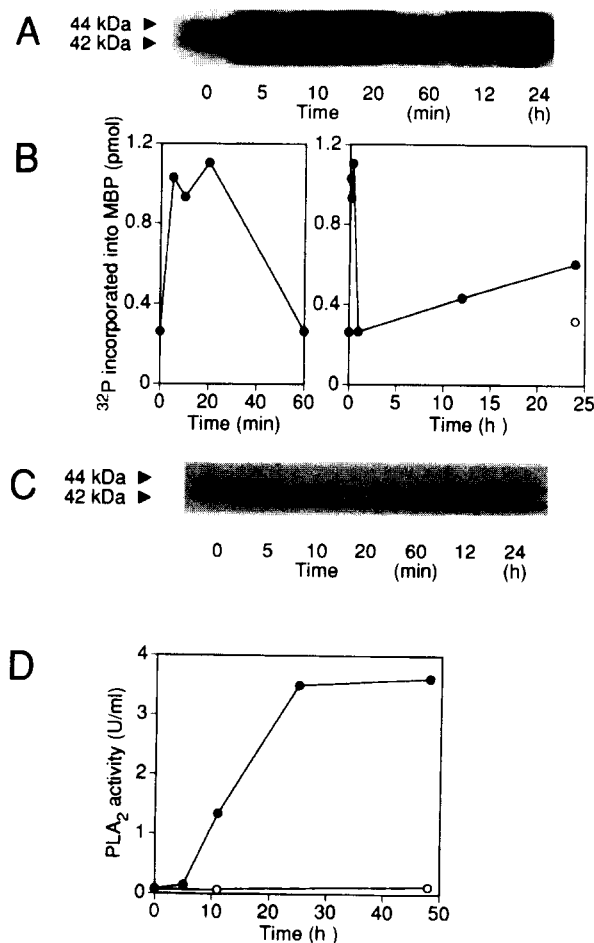

Fig. 1. Time courses of MAP kinase activation and group II PLA$_2$ secretion by IL-1β. Rat mesangial cells in passages 6–12 were stimulated by 200 U/ml IL-1β. Cytosol fractions of mesangial cells exposed to 200 U/ml IL-1β for indicated times were processed as described in section 2. (A) Autoradiographic detection of ^{32}P-labeled MBP by in-gel kinase assay. (B) IL-1β induced changes in p44 MAP kinase activity measured by bioimaging analyzer. ● = MAP kinase activity of mesangial cells stimulated by 200 U/ml IL-1β; ○ = the basal activity of MAP kinase measured under the same condition without IL-1β. (C) Immunoblotting of the cell lysate with anti-MAP kinase antibody. (D) The secretion of PLA$_2$ from mesangial cells. The PLA$_2$ activities in the culture media from 200 U/ml IL-1β-stimulated (●) and non-stimulated (○) mesangial cells were measured as described in section 2.

the secretion of PLA$_2$, we studied the effects of exogenous group II PLA$_2$ on MAP kinase activity to elucidate the signal transducing mechanism of group II PLA$_2$ in mesangial cell proliferation. Addition of 14 U/ml group II PLA$_2$ rapidly activated 44-kDa and 42-kDa MAP kinase (p44 and p42 MAP kinase) (Fig. 2A,B). As p44 MAP kinase and p42 MAP kinase were activated similarly, the figures show the activity of p44 MAP kinase as the representative. The activity reached its maximum in 10 min and increased almost linearly up to 10 U/ml to get to the plateau (Fig. 2C,D). These data suggest that the IL-1-induced late phase activation of MAP kinase may be mediated by secreted group II PLA$_2$. On the contrary, group I PLA$_2$ (10 U/ml), the other isoform of secretory PLA$_2$, activated MAP kinase weakly by at most 25% of the increments in response to group II PLA$_2$ stimulation at the same concentration (Fig. 3).

3.3. Inhibition of MAP kinase activation by anti-group II PLA₂ antibody

Next we studied the effect of anti-group II PLA₂ immunoglobulin on IL-1-induced MAP kinase activation to examine whether group II PLA₂ was involved in the IL-1β-evoked signaling pathway. Anti-group II PLA₂ immunoglobulin used in this study blunted the stimulatory effect of IL-1 on mesangial cell proliferation (data not shown). As shown in Fig. 4, the activation of MAP kinases was inhibited 10 h after IL-1β-stimulation when cells were treated with anti-group II PLA₂ immunoglobulin. Immunoglobulin prepared from non-immunized rabbit did not affect MAP kinase activity (data not shown).

3.4. Effects of lysophosphatidic acid

Lysophospholipids are putative signaling molecules produced by PLA₂-catalyzed reaction. We have already demonstrated that lysophosphatidic acid (LPA) was the most potent mitogen among several lysophospholipids for mesangial cells. LPA has been reported to activate MAP kinase in some types of cell [21–23]. We observed here that LPA activated MAP kinase also in mesangial cells. The activity reached maximum
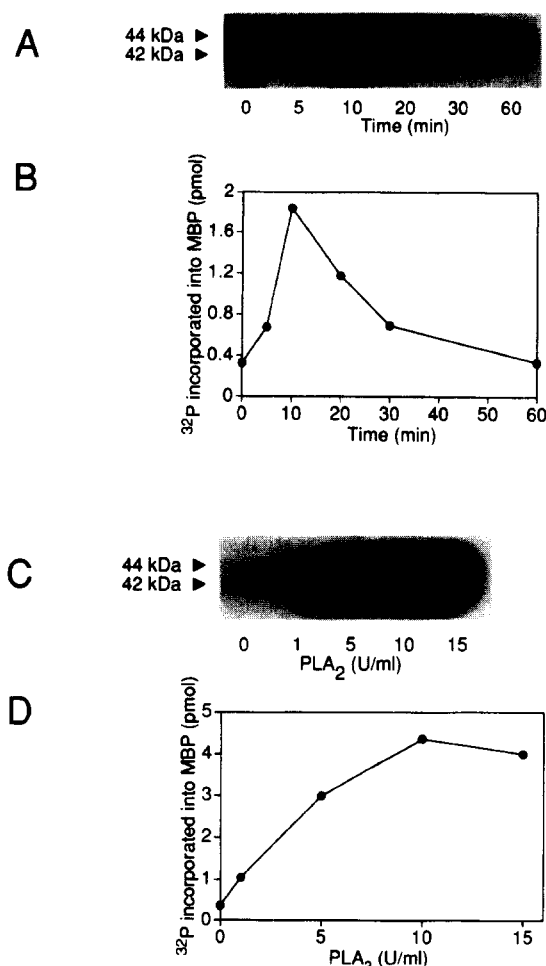
Fig. 2. MAP kinase activation by group II PLA₂. Experimental conditions were the same as in Fig. 1 except that rat mesangial cells were incubated with group II PLA₂ in place of IL-1β. (A and B) Time course of MAP kinase activation induced by the addition of 14 U/ml group II PLA₂. (C and D) Dose-dependence of MAP kinase activation by group II PLA₂.
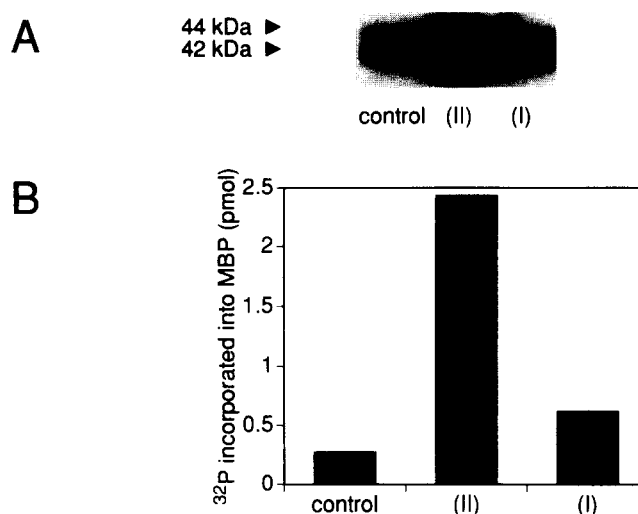
Fig. 3. Effects of group I PLA₂ on MAP kinase activation. Rat mesangial cells were incubated in the culture media containing no ligand (control), 10 U/ml group I PLA₂ (I) or 10 U/ml group II PLA₂ (II) for 10 min. The activity of p44 MAP kinase was measured as described under legend of Fig. 1.

in 10 min and the maximal activity was eight times higher than the basal activity (activity of p44 MAP kinase: 1.37 pmol and 0.17 pmol).

4. Discussion

The present study demonstrates the activation of MAP kinase by exogenous group II PLA₂ in cultured mesangial cells. IL-1β activates MAP kinase in two phases in agreement with previous results: a rapid and prominent activation, followed by a continuous stimulation persisting for more than 24 h. The time course of the late phase activation was paralleled to that of the secretion of group II PLA₂ from mesangial cells stimulated by IL-1β and the late phase activation was notably inhibited by the specific antibody against group II PLA₂. Taken together with the observation that exogenous group II PLA₂ rapidly activated MAP kinase, these data suggest that the prolonged MAP kinase-activation might be mediated through the secretion of group II PLA₂ from IL-1-stimulated mesangial cells. In addition, the deprivation of IL-1 from culture medium 6 h after the stimulation did not attenuate the late phase MAP kinase-activation (data not shown), suggesting that the IL-1 existing in media did not affect the prolonged MAP kinase-activation. Concerning the rapid and prominent activation of MAP kinase by IL-1, it may be possible that the rapid activation contributes as the initial signaling event to the secretion of group II PLA2, though the function is still to be elucidated.

Recent studies demonstrated that the activation of MAP kinases was involved in a variety of cell function. Some growth factors besides IL-1 was also shown to activate MAP kinase biphasically, and a steady increase in MAP kinase activity in the late phase is supposed to be important for DNA synthesis and cell cycle progression [24]. Considering our previous data that group II PLA₂ amplified PDGF-stimulated proliferation of mesangial cells, the late phase activation of MAP kinase by IL-1 through the secretion of group II PLA₂ may also contribute to the cell cycle progression.
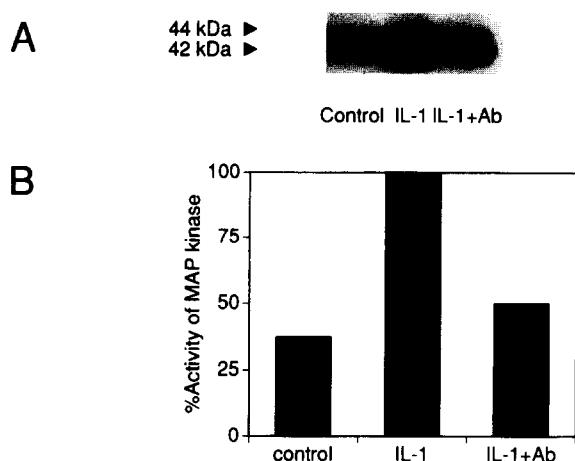
A

44 kDa ▶
42 kDa ▶

Control IL-1 IL-1+Ab

B

Fig. 4. Inhibition of MAP kinase activation by anti-group II PLA$_2$ antibody. Rat mesangial cells were stimulated by IL-1β as in Fig. 1 with or without anti-group II PLA$_2$ immunoglobulin (140 μg/ml) in culture media. Activity of MAP kinase was measured 10 h after stimulation using in-gel kinase assay. (A) Autoradiography. (B) MAP kinase activity expressed as percentage of p44 MAP kinase activity with IL-1β minus immunoglobulin. The basal activity (control) was measured without IL-1 and immunoglobulin.

Concerning the signal transduction pathway through which group II PLA$_2$ activated MAP kinase, the following two possible mechanism can be considered. First, group II PLA$_2$ may activate MAP kinase through yielding metabolites of PLA$_2$-catalyzed reaction. Lysophosphatidic acid (LPA) is one of these metabolites. We previously observed that LPA as well as group II PLA$_2$ enhanced the PDGF-stimulated proliferation of mesangial cells. Very recently, Fourcade et al. reported that group II PLA$_2$ generates LPA in membrane microvesicles shed from activated cells [25]. LPA has been reported to activate MAP kinase in some types of cells [21–23]. We confirmed that LPA stimulated the activation of MAP kinase also in mesangial cells. It is likely that LPA generated by the action of group II PLA$_2$ on mesangial cell membrane induced the action of MAP kinase. Another metabolite of PLA$_2$-catalyzed reaction, fatty acids, have been also reported to play some physiological role in cell function. Among them, arachidonic acid is a well-known precursor of potent pro-inflammatory substances such as prostaglandins and leukotriens. However, arachidonic acid had no significant effect on mesangial cell proliferation (manuscripts submitted). Thus we turned our attention to LPA as a signaling molecule. Second, group II PLA$_2$ may activate the MAP kinase via its specific receptor. Recently, the receptors for secretory PLA$_2$ has been cloned [26,27], and they were involved in various physiological responses such as DNA synthesis [28] and eicosanoid production [29]. Lambeau et al. cloned and characterized the receptor for snake venom PLA$_2$ from rabbit skeletal muscle. The receptor had binding capacity to mammalian secretory PLA$_2$ with high affinity, and the affinity for group II PLA$_2$ was about ten times higher than that for group I PLA$_2$ [27] This is consistent with the fact that group I PLA$_2$ did not activate MAP kinase so strongly as group II PLA$_2$ using the equivalent units of activity. However, more recently Ancian et al. also cloned the human receptor for PLA$_2$ and showed that the expressed receptor had little binding affinity toward both group I and group II PLA$_2$ [30]. The role of the receptor for PLA$_2$ in

the MAP kinase activation induced by PLA$_2$ still remains to be elucidated.

In conclusion, we first demonstrated that group II PLA$_2$ activated MAP kinase in rat mesangial cells and that the IL-1-induced late phase activation of MAP kinase was mediated, at least in part, by secreted group II PLA$_2$, suggesting that group II PLA2-induced MAP kinase activation is involved in the progression of mesangial proliferative glomerulonephritis. These results will throw light on the mechanism of the progression of mesangial proliferative glomerulonephritis.

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