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Transforming growth factor-α stimulates prostaglandin generation through cytosolic phospholipase A_2 under the control of p11 in rat gastric epithelial cells

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1 The regulatory effects of transforming growth factor (TGF)-α on phospholipase A_2 (PLA_2) isozymes contributing to prostaglandin generation in rat gastric epithelial RGM1 cells were examined.

2 Stimulation with TGF-α for 24 h time-dependently induced prostaglandin E_2 generation with an increase in cyclo-oxygenase-2 protein. The TGF-α-induced prostaglandin E_2 generation was suppressed by NS-398, a cyclo-oxygenase-2 inhibitor.

3 TGF-α stimulated the activity and the protein synthesis of cytosolic PLA_2 ($cPLA_2$). A time-dependent increase in $cPLA_2$ protein occurred in parallel with PGE_2 generation, which was inhibited by methyl arachidonyl fluorophosphonate (MAFP), a $cPLA_2$ inhibitor. However, no change in activity of secretory PLA_2 or Ca^{+2}-independent PLA_2 was observed in the TGF-α-stimulated cells.

4 Stimulation with the Ca^{2+} ionophore A23187 for 10 min induced MAFP-sensitive arachidonic acid liberation. Interestingly, preincubation with TGF-α for 24 h diminished A23187-stimulated arachidonic acid liberation despite the increase in $cPLA_2$ protein.

5 Under the conditions, TGF-α was found to increase p11, an endogenous $cPLA_2$ suppressor, also known as annexin II light chain. The TGF-α-induced increase in p11 was suppressed by tyrphostin AG1478, an inhibitor of tyrosine kinase of epidermal growth factor receptor, which was also found to restore the inhibition by TGF-α of A23187-stimulated arachidonic acid liberation. However, TGF-α did not alter protein levels of annexin II heavy chain.

6 These results suggest that TGF-α stimulates prostaglandin generation through an increase in $cPLA_2$, the hydrolytic action of which may be under the control of p11.
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Keywords: p11 (annexin II light chain); phospholipase A_2; transforming growth factor-α; gastric epithelial cell

Abbreviations: AA, arachidonic acid; COX, cyclo-oxygenase; $cPLA_2$, cytosolic phospholipase A_2; EGF, epidermal growth factor; MAFP, methyl arachidonyl fluorophosphonate; $sPLA_2$, secretory phospholipase A_2; TGF, transforming growth factor

Introduction

The generation of prostaglandin depends on cyclo-oxygenase (COX)-catalyzed conversion of arachidonic acid (AA), and is an important event contributing to pathological conditions including inflammation. It is widely believed that the liberation of AA, which is a rate-limiting step in the generation of prostaglandin, is mainly mediated by the hydrolytic action of phospholipase A_2 (PLA_2) toward membrane glycerophospholipids (Waite 1985; Kudo *et al*., 1993). Among numerous types of mammalian PLA_2s, Ca^{2+}-dependent cytosolic PLA_2 ($cPLA_2$; type VI) and secretory PLA_2 ($sPLA_2$; types IIA and V) are responsible for AA liberation in a variety of cells (Dennis, 1997; Gijón & Leslie, 1999). Therefore, the acceleration of hydrolytic activity of $cPLA_2$ or $sPLA_2$ upon stimulation is critical to prostaglandin generation.

It has been generally accepted that prostaglandin generated in gastric cells, including epithelial cells and fibroblasts, is involved in the healing of gastric mucosal lesions and in the maintenance of gastric mucosal integrity (Robert *et al*., 1979; Kobayashi & Arakawa, 1995). Several growth factors including epidermal growth factor (EGF) (Nakano *et al*., 1995), transforming growth factor (TGF)-α (Sawaoka *et al*., 1999), basic fibroblast growth factor (Sasaki *et al*., 1998), and hepatocyte growth factor (Jones *et al*., 1999) have been shown to stimulate expression of an inducible COX isoform, termed COX-2, in guinea-pig gastric mucosal cells and rat gastric epithelial RGM1 cells, suggesting involvement of COX-2 in prostaglandin generation upon stimulation with these growth factors. Among these growth factors, TGF-α is produced by gastric epithelial cells (Beauchamp *et al*., 1989), and stimulates prostaglandin generation in the cells (Sawaoka *et al*., 1999). Furthermore, exogenous prostaglandin activates gastric fibroblasts to stimulate expression of hepatocyte growth factor (Takahashi *et al*., 1996), which induces proliferation of gastric epithelial cells (Takahashi *et al*., 1995). Thus, it is conceivable that these growth factors and prostaglandin cooperatively accelerate the ulcer healing process through autocrine and paracrine mechanisms. A recent report showed that amounts of TGF-α and EGF in gastric mucosa are increased in parallel with prostaglandin generation and COX-2 expression during the healing of stress-induced gastric lesions in an animal model (Konturek *et al*., 1998). Furthermore, a COX-2 inhibitor suppressed TGF-α-stimulated prostaglandin generation, proliferation and morphogenesis in RGM1 cells (Sawaoka *et al*., 1999). Collectively, TGF-α is implicated in

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the repair of gastric mucosal damage through, at least in part, COX-2-dependent prostaglandin generation. However, the mechanism underlying the activation of the PLA_2 isozyme(s) contributing to the TGF-α-induced prostaglandin generation remains to be elucidated.

TGF-α in the stomach exhibits numerous biological activities including inhibition of gastric acid secretion and stimulation of migration and proliferation of gastric epithelial cells (Barnard *et al*., 1995; Kobayashi *et al*., 1996), through binding to EGF receptor (Lenferink *et al*., 1998). It has been demonstrated that prostaglandin generation induced by EGF or TGF-α occurs continuously over several hours in gastric epithelial cells (Nakano *et al*., 1995; Sawaoka *et al*., 1999). Although TGF-α was shown to increase $cPLA_2$ activity and elicit translocation of the enzyme to membranes in mouse HEL-30 keratinocytes (Kast *et al*., 1993), it is unclear whether the mechanism underlying $cPLA_2$ activation is responsible for the continuous prostaglandin generation in gastric epithelial cells. It was reported that a similar continuous generation of prostaglandin induced by proinflammatory cytokines is mediated through $sPLA_2$ expression in rat mesangial cells (Pfeilschifter *et al*., 1993) and rat 3Y1 fibroblasts (Kuwata *et al*., 1998). However, the role of $sPLA_2$ in prostaglandin generation in gastric cells is poorly understood. In the present study, therefore, to clarify the mechanism underlying PLA_2 activation contributing to the sustained prostaglandin generation in TGF-α-stimulated gastric epithelial cells, we examined the role of the two PLA_2 isozymes, $cPLA_2$ and $sPLA_2$, in the generation of prostaglandin in RGM1 cells upon stimulation with TGF-α.

Methods

Cell culture

RGM1, a non-transformed epithelial cell line derived from normal rat gastric mucosa, was purchased from Riken Cell Bank (Tsukuba, Japan). RGM1 cells were maintained in Dulbecco's modified Eagle's medium/Ham's F-12 medium (DMEM/F-12; 1 : 1) supplemented with 20% heat-inactivated foetal calf serum, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin at 37°C under humidified air containing 5% CO_2. Cells were plated in 35-mm culture dishes at 1×10^6 cells in DMEM/F-12 containing 0.01% bovine serum albumin, and used for experiments the following day. The cells were washed and placed in DMEM/F-12.

Measurement of prostaglandin E_2 generation and AA liberation

RGM1 cells were plated in 35-mm culture dishes at 1×10^6 cells, and incubated with [^{3}H]-AA (100 Ci mmol^{-1}, 1 μCi ml^{-1}) for 24 h in DMEM/F-12 containing 0.01% bovine serum albumin. After being washed, the labelled cells were placed in DMEM/F-12, treated with 1 μM NS-398, a COX-2 inhibitor, or 1 μM methyl arachidonyl fluorophosphonate (MAFP), a $cPLA_2$ inhibitor, for 30 min, and then stimulated with TGF-α as described in the figure legends. In some experiments, the stimulated cells were washed, treated with 10 μM BW755C (a COX and lipoxygenase inhibitor) for 15 min, and further stimulated with 1 μM A23187 for 10 min. After lipids in the medium and the cells were extracted, the [^{3}H]-prostaglandin E_2 generated and [^{3}H]-AA liberated were analysed by t.l.c. as described previously (Akiba *et al*., 1998).

Immunoblot analysis for COX-2, $cPLA_2$, p11, and annexin II heavy chain

After stimulation with TGF-α, RGM1 cells were washed and scraped off in buffer A (100 mM NaCl, 2 mM EGTA, 100 μM *p*-(amidinophenyl)methanesulphonyl fluoride, 100 μM leupeptin, 20 mM β-glycerophosphate, 1 mM Na_3VO_4, and 10 mM Tris-HCl, pH 7.4) containing 0.05% Triton X-100. The cells (10 μg protein) were solubilized and subjected to SDS–PAGE, followed by immunoblot analysis using antibodies against COX-2, $cPLA_2$, p11, and annexin II heavy chain. In some experiments, RGM1 cells (8×10^6 cells) in 100-mm dishes were scraped off and sonicated in buffer A. After the lysate was centrifuged at $100{,}000 \times g$ for 30 min at 4°C, the resulting supernatant (cytosol fraction, 10 μg protein) and pellet (membrane fraction, 10 μg protein) resuspended in buffer A were subjected to immunoblot analysis.

Assay for $cPLA_2$, $sPLA_2$, and Ca^{2+}-independent PLA_2 activity

For the $cPLA_2$ assay, RGM1 cells were scraped off and sonicated in buffer A containing 0.05% Triton X-100. The lysate was diluted 10 times with buffer A and treated with 5 mM dithiothreitol at 37°C for 10 min to inhibit $sPLA_2$ activity. The sample (8 μg protein) was incubated with a mixture of 1-stearoyl-2-[^{3}H]-arachidonoyl-*sn*-glycero-3-phosphocholine and the unlabelled compound (125 Ci mol^{-1}, 2 μM) at 37°C for 1 h in the presence of 5 mM $CaCl_2$ and 50 mM Tris-HCl (pH 8.5). For the Ca^{2+}-independent PLA_2 assay, the cell lysate (40 μg protein) prepared as above was treated with 5 mM dithiothreitol at 37°C for 10 min, and incubated with a mixture of 1,2-dipalmitoyl-*sn*-glycero-3-[choline-*methyl*-^{14}C]-phosphocholine and the unlabelled compound (25 mCi mmol^{-1}, 20 μM) at 37°C for 1 h in the presence of 5 mM EDTA, 800 μM Triton X-100 and 50 mM HEPES (pH 7.5). For the $sPLA_2$ assay, RGM1 cells were stimulated in the presence of 0.2 mg ml^{-1} heparin. After the medium was centrifuged, the supernatant (50 μl), as an enzyme source, was incubated with 1-palmitoyl-2-[^{14}C]-inoleoyl-*sn*-glycero-3-phosphoethanolamine (55 mCi mmol^{-1}, 2 μM) at 37°C for 15 min in the presence of 5 mM $CaCl_2$ and 50 mM Tris-HCl (pH 8.5). The radioactivity of [^{3}H]-AA, [^{14}C]-lysophosphatidylcholine, and [^{14}C]-linoleic acid was determined as described previously (Akiba *et al*., 1999), and the enzyme activity was calculated.

Materials

Recombinant human TGF-α was obtained from Pepro Tech EC Ltd. (London). Interleukin-1β was from Collaborative Biomedical Products (Bedford, MA, U.S.A.). MAFP was from Cayman Chemical (Ann Arbor, MI, U.S.A.). A23187, NS-398 (*N*-(2-cyclohexyloxy-4-nitrophenyl)methanesulphonamide), and tyrphostin AG1478 (4-(3-chloroanilino)-6,7-dimethoxyquinazoline) were from Calbiochem (La Jolla, CA, U.S.A.). Antibodies against COX-2 and $cPLA_2$ were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). Antibodies against p11 and annexin II heavy chain were from Transduction Laboratories (Lexington, KY, U.S.A.). 1-Palmitoyl-2-[^{14}C]-linoleoyl-*sn*-glycero-3-phosphoethanolamine was from Amersham Pharmacia Biotech (Buckinghamshire). Other radio-labelled chemicals were from NEN Life Science Products, Inc. (Boston, MA, U.S.A.).

Statistical analysis

Values are expressed as the mean ± s.e.mean. Data were analysed by Student's *t*-test or by one-way analysis of variance and Dunnett's test. $P<0.05$ was considered statistically significant.

Results

TGF-α stimulates prostaglandin E_2 generation and cPLA$_2$ protein synthesis

As shown in Figure 1, stimulation of RGM1 cells with 50 ng ml^{-1} TGF-α (up to 24 h) induced a small but significant increase in the level of prostaglandin E_2 time- and dose-dependently. The TGF-α-induced prostaglandin E_2 generation was inhibited by 1 μM NS-398, a COX-2 inhibitor, and 1 μM MAFP, a cPLA$_2$ inhibitor (Figure 1b), suggesting involvement of COX-2 and cPLA$_2$ in the generation. TGF-α has been shown to stimulate COX-2 expression in RGM1 cells (Sawaoka *et al*., 1999). We confirmed that 50 ng ml^{-1} TGF-α time-dependently increased COX-2 proteins with a maximal increase observed 12 h after the stimulation (Figure 2a). In the present study, to examine the role of cPLA$_2$ in the prostaglandin E_2 generation, we further determined changes in PLA$_2$ isozymes in TGF-α-stimulated RGM1 cells (Figure 2). As shown in Figure 2a, immunoblot studies using anti-cPLA$_2$ antibodies revealed that 50 ng ml^{-1} TGF-α increased cPLA$_2$ protein in a time-dependent manner in RGM1 cells. The increase in cPLA$_2$ protein was relatively gradual as compared with the change in COX-2 protein. Amounts of cPLA$_2$ were also increased in the cytosol and membrane fractions (Figure 2b). We further confirmed that cPLA$_2$ activity in the cell lysate was increased upon stimulation with 50 ng ml^{-1} TGF-α (Figure 2c). Under the conditions, TGF-α did not alter sPLA$_2$ activity in the incubation medium (Figure 2d), while 5 ng ml^{-1} interleukin-1β, as a control, increased sPLA$_2$ activity in the incubation medium (Figure 2d) but not cPLA$_2$ activity in the cell lysate (Figure 2c). In addition to cPLA$_2$ and sPLA$_2$, Ca^{2+}-independent PLA$_2$ has been detected in a variety of cells and tissues (Ackermann & Dennis, 1995). Recently, we reported the possible involvement of Ca^{2+}-independent PLA$_2$ in stimulus-induced prostaglandin generation in macrophage-like P388D$_1$ cells (Akiba *et al*., 1999). We examined the effect of TGF-α on Ca^{2+}-independent PLA$_2$ activity in RGM1 cells. However, the activity of the PLA$_2$ isozyme in the cell lysate of unstimulated cells (16.6 ± 1.3 pmol min^{-1} mg protein^{-1}, $n=3$) was not affected by stimulation with 50 ng ml^{-1} TGF-α for 24 h (17.8 ± 1.9 pmol min^{-1} mg protein^{-1}). These results suggest that TGF-α-stimulated, COX-2-dependent prostaglandin E_2 generation may be mediated by cPLA$_2$ increased upon the stimulation.

Inhibitory effect of TGF-α on A23187-stimulated AA liberation

In human bronchial epithelial BEAS-2B cells, leukaemia inhibitory factor has been shown to enhance Ca^{2+} ionophore A23187-induced AA liberation through stimulation of cPLA$_2$ expression (Ikezono *et al*., 1997). In order to determine whether TGF-α exhibits a similar effect in RGM1 cells, after preincubation with TGF-α for 24 h, the cells were washed and stimulated with A23187 for 10 min. Unexpectedly, the result shown in Figure 3a indicates that

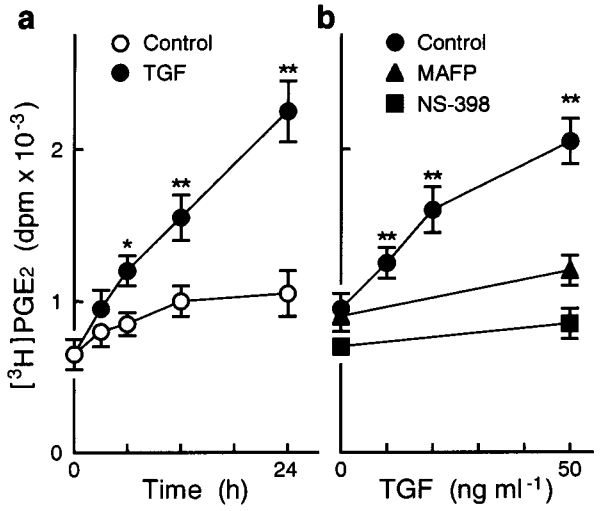

Figure 1 Prostaglandin E_2 generation upon stimulation with TGF-α. (a) [^{3}H]-AA-labelled RGM1 cells were stimulated with or without 50 ng ml^{-1} TGF-α (TGF) for the indicated periods. (b) The labelled cells were treated with 1 μM NS-398, a COX-2 inhibitor, 1 μM MAFP, a cPLA$_2$ inhibitor, or the vehicle for 30 min, and stimulated with various concentrations of TGF-α (TGF) for 24 h. The amounts of [^{3}H]-prostaglandin E_2 (PGE$_2$) generated was determined as described in the Methods. Each point represents the mean ± s.e.mean of three separate experiments. $^*P<0.05$, $^{**}P<0.01$; relative to the corresponding response of the unstimulated cells.

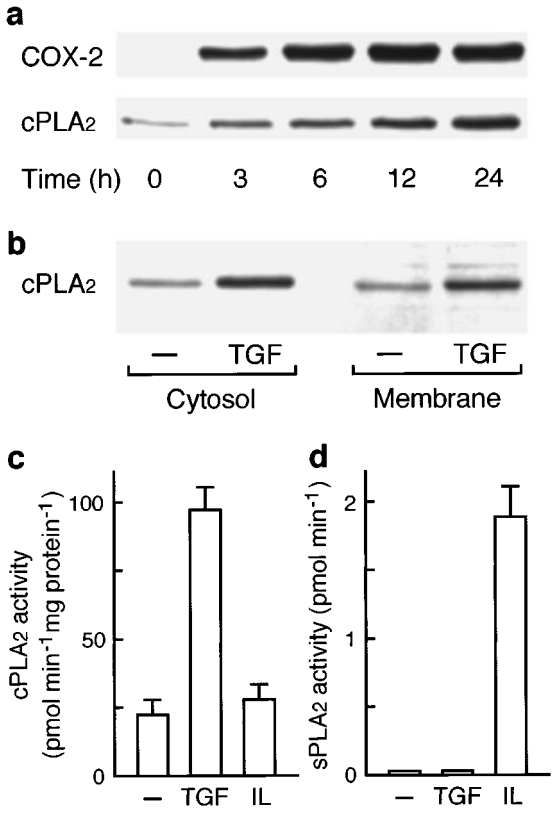

Figure 2 Changes in COX-2, cPLA$_2$ and sPLA$_2$ upon stimulation with TGF-α or interleukin-1β. (a) RGM1 cells were stimulated with 50 ng ml^{-1} TGF-α for the indicated periods. COX-2 and cPLA$_2$ in the cells were analyzed as described in the Methods. The results are representative of three separate experiments. (b) RGM1 cells were stimulated with (TGF) or without (−) 50 ng ml^{-1} TGF-α for 24 h. cPLA$_2$ in the cytosol and membrane fractions was analysed. (c, d) RGM1 cells were stimulated with 50 ng ml^{-1} TGF-α (TGF), 5 ng ml^{-1} interleukin-1β (IL), or the vehicle (−) for 24 h. cPLA$_2$ activity in the cell lysate (c) and sPLA$_2$ activity in the incubation medium (d) were determined as described in the Methods ($n=3$).

preincubation with 10 or 50 ng ml^{-1} TGF-α diminished 1 μM A23187-induced AA liberation. This inhibitory effect of TGF-α was apparently observed 3 h but not 0.5 h after the preincubation with TGF-α (Figure 3b). Under the conditions, amounts of the increased cPLA$_2$ proteins in response to 50 ng ml^{-1} TGF-α did not change even when TGF-α-preincubated cells were stimulated with 1 μM A23187, as shown in Figure 4a.

In order to confirm whether A23187-induced AA liberation is mediated by cPLA$_2$, the effect of MAFP was examined. We observed that A23187-induced AA liberation (15119±1251 d.p.m.; basal level, 4303±158 d.p.m.; mean±s.e.mean, $n=3$) was significantly inhibited by 10 μM MAFP (6228±479 d.p.m., $P<0.01$ vs A23187), which did not affect the basal level (4405±172 d.p.m.).

A recent report showed that preincubation of human colonic epithelial cells with TGF-α inhibits bradykinin-induced prostaglandin E$_2$ generation, this inhibitory effect being ascribable to termination of bradykinin-elicited signalling by prostaglandin E$_2$ generated during the preincubation with TGF-α (Beltinger *et al*., 1999). To examine whether such a mechanism is responsible for the effect of TGF-α in RGM1 cells, a COX-2 inhibitor was used to inhibit TGF-α-induced prostaglandin E$_2$ generation. However, pretreatment with 1 μM NS-398 did not overcome the inhibitory effect of 50 ng ml^{-1} TGF-α on 1 μM A23187-stimulated AA liberation (Figure 3a). Pretreatment with 1 μM indomethacin or 10 μM BW755C also had no influence (data not shown). TGF-α initiates intracellular signalling through binding to EGF receptor (Lenferink *et al*., 1998). To examine whether the inhibitory effect of TGF-α is mediated through activation of intracellular signalling, we tested the effect of tyrphostin AG1478, an inhibitor of intrinsic tyrosine kinase of EGF receptor. The result shown in Figure 3a indicates that the inhibitory effect of TGF-α was reversed by 0.1 μM tyrphostin AG1478, which had no effect on AA liberation induced by A23187 alone. Furthermore, as shown in Figure 4b, 0.1 μM tyrphostin AG1478 inhibited an increase in cPLA$_2$ protein induced by 50 ng ml^{-1} TGF-α. The quantitative results, which had been estimated by measuring the density of cPLA$_2$ band shown in Figure 4b, also revealed that TGF-α-increased amounts of cPLA$_2$ were significantly suppressed by tyrphostin AG1478 (Control, 10.1±1.5; AG1478 alone, 7.5±1.4; TGF-α alone, 21.2±1.2; AG1478+TGF-α, *14.4±1.3 arbitrary units; the mean±s.e.mean, $n=3$, *$P<0.05$ vs TGF-α alone).

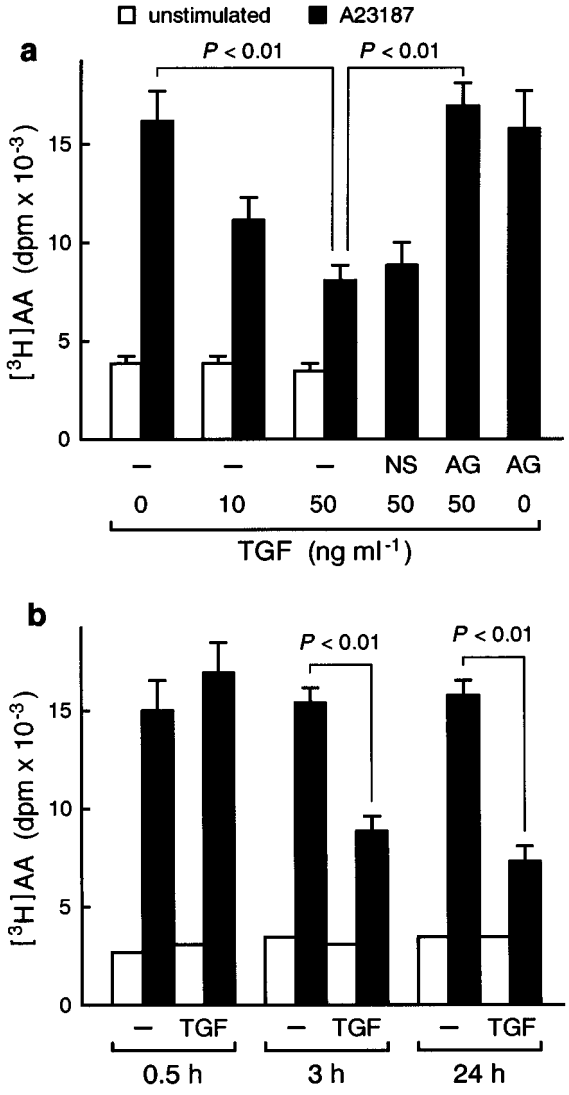

Figure 3 Effects of NS-398 and tyrphostin AG1478 on inhibition by TGF-α of A23187-induced AA liberation. (a) [^{3}H]-AA-labelled RGM1 cells were treated with 1 μM NS-398 (NS), 0.1 μM tyrphostin AG1478 (AG), a tyrosine kinase inhibitor, or the vehicle (−) for 30 min, and stimulated with or without 10 or 50 ng ml^{-1} TGF-α (TGF) for 24 h. The cells were washed, treated with 10 μM BW755C, and stimulated with or without 1 μM A23187 for 10 min. (b) The labelled cells were stimulated with (TGF) or without (−) 50 ng ml^{-1} TGF-α for 0.5, 3, or 24 h. After being washed, the cells were stimulated with A23187 as in (a). The amounts of [^{3}H]-AA liberated was determined as described in the Methods ($n=3$).

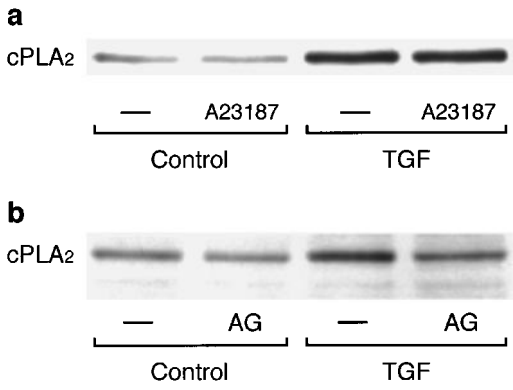

Figure 4 Effects of A23187 and tyrphostin AG1478 on TGF-α-induced increase in cPLA$_2$ protein. (a) RGM1 cells were stimulated with (TGF) or without (Control) 50 ng ml^{-1} TGF-α for 24 h. After being washed, the cells were stimulated with (A23187) or without (−) 1 μM A23187 for 10 min. (b) RGM1 cells were treated with (AG) or without (−) 0.1 μM tyrphostin AG1478 for 30 min, and stimulated with (TGF) or without (Control) 50 ng ml^{-1} TGF-α for 24 h. cPLA$_2$ in the cells was analysed as described in the Methods. The results are representative of three separate experiments.

TGF-α increases p11 protein

Recently, it was reported that dexamethasone inhibits A23187-induced AA liberation through expression of p11 (Yao *et al*., 1999), which is known to inhibit cPLA$_2$ activity directly (Wu *et al*., 1997). To examine the possible involvement of p11 in the inhibitory effect of TGF-α on A23187-induced AA liberation, we determined protein levels of p11 in TGF-α-stimulated RGM1 cells. As shown in Figure 5a, stimulation with 50 ng ml^{-1} TGF-α for 3 and 24 h induced an increase in p11 protein, although the augmentation was not observed 0.5 h after the stimulation. Amounts

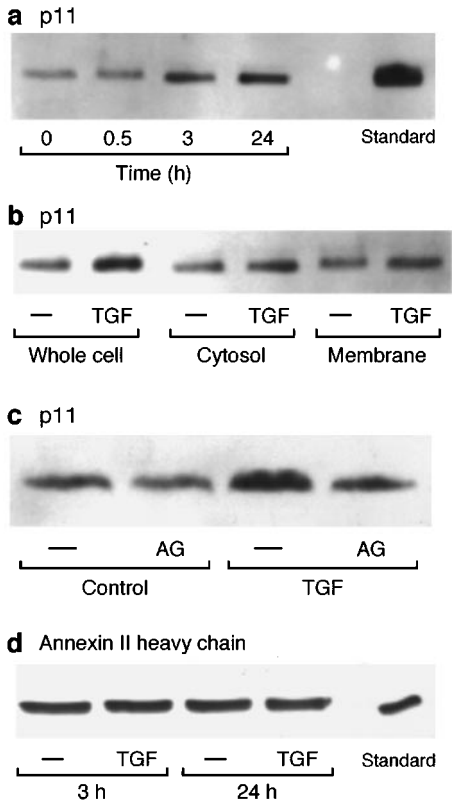

Figure 5 Changes in p11 and annexin II heavy chain proteins upon stimulation with TGF-α. (a) RGM1 cells were stimulated with 50 ng ml^{-1} TGF-α for the indicated periods. p11 in the cells was analysed as described in the Methods. (b) RGM1 cells were stimulated with (TGF) or without (−) 50 ng ml^{-1} TGF-α for 24 h. p11 in the cells and the cytosol and membrane fractions was analysed. (c) RGM1 cells were treated with (AG) or without (−) 0.1 μM tyrphostin AG1478 for 30 min, and stimulated with (TGF) or without (Control) 50 ng ml^{-1} TGF-α for 24 h. p11 in the membrane fraction was analysed. (d) Annexin II heavy chain in the cells stimulated with (TGF) or without (−) 50 ng ml^{-1} TGF-α for 3 or 24 h was analysed. The results are representative of three separate experiments.

of p11 were also increased in the cytosol and membrane fractions as well as whole cells upon stimulation with 50 ng ml^{-1} TGF-α for 24 h (Figure 5b). Furthermore, pretreatment with 0.1 μM tyrphostin AG1478 inhibited TGF-α-stimulated increase in p11 protein in whole cells (result not shown) and the membrane fraction (Figure 5c). The quantitative results of Figure 5c by densitometric analysis also demonstrated that the increased p11 in response to TGF-α was suppressed by tyrphostin AG1478 (Control, 9.9±1.3; AG1478 alone, 8.1±1.0; TGF-α alone, 19.3±2.1; AG1478+TGF-α, *9.1±1.3 arbitrary units; the mean± s.e.mean, $n=3$, $^*P<0.05$ vs TGF-α alone). It was shown that p11, known as annexin II light chain, binds to annexin II heavy chain, which also inhibits the hydrolytic activity of cPLA$_2$ through sequestration of substrates (Wu *et al*., 1997). We further determined changes in protein levels of annexin II heavy chain in TGF-α-stimulated RGM1 cells. However, as shown in Figure 5d, 50 ng ml^{-1} TGF-α did not alter amounts of annexin II heavy chain in the cells.

Discussion

Prostaglandin generated in gastric cells including epithelial cells plays an important role in the ulcer healing process and maintaining gastric mucosal integrity. In guinea-pig gastric mucosal cells and RGM1 cells, a contribution by COX-2, an inducible COX isoform, to prostaglandin generation has been suggested upon stimulation with EGF (Nakano *et al*., 1995), TGF-α (Sawaoka *et al*., 1999), basic fibroblast growth factor (Sasaki *et al*., 1998), and hepatocyte growth factor (Jones *et al*., 1999). We also confirmed that TGF-α increased COX-2 proteins with stimulation of prostaglandin E$_2$ generation, which was inhibited by NS-398, a COX-2 inhibitor, in RGM1 cells. Thus, it is likely that in gastric epithelial cells, prostaglandin generation induced by these growth factors depends on induction of COX-2 expression.

The PLA$_2$-catalyzed liberation of AA from membrane phospholipids is an essential step in the generation of prostaglandin. However, the role of TGF-α in activation of the PLA$_2$ isozyme(s) contributing to the prostaglandin generation in gastric epithelial cells remains uncertain. In this study, therefore, we examined the contribution of cPLA$_2$ and sPLA$_2$ to the prostaglandin generation in TGF-α-stimulated RGM1 cells. Our results showed that TGF-α-stimulated prostaglandin E$_2$ generation was suppressed by MAFP, a cPLA$_2$ inhibitor. Furthermore, we found that TGF-α increased the level of cPLA$_2$ protein, as well as activity, in parallel with prostaglandin E$_2$ generation, whereas it did not affect sPLA$_2$ or Ca^{2+}-independent PLA$_2$ activity. These results suggest that the increase in cPLA$_2$ in response to TGF-α is involved in the generation of prostaglandin E$_2$. In contrast to our findings, a previous report showed that EGF has no influence on PLA$_2$ activity in guinea-pig gastric mucosal cells (Nakano *et al*., 1995). Although TGF-α and EGF evoke their biological activities through binding to EGF receptor (Lenferink *et al*., 1998), the biological activities of TGF-α in the stomach are often more potent than those of EGF (Barnard *et al*., 1995). At present, however, we have no explanation for the differences between TGF-α-stimulated RGM1 cells and EGF-stimulated guinea-pig gastric mucosal cells.

We showed here that TGF-α continuously increased prostaglandin E$_2$ and cPLA$_2$ protein levels throughout the incubation periods (24 h) in RGM1 cells. It has been demonstrated that TGF-α induces a relatively rapid generation of prostaglandin within 30 min, through an increase in cPLA$_2$ activity and translocation of the enzyme to membranes without an increase in intracellular Ca^{2+} concentration in mouse HEL-30 keratinocytes (Kast *et al*., 1993). However, it is unclear whether such a mechanism is responsible for the relatively continuous prostaglandin E$_2$ generation observed in TGF-α-stimulated RGM1 cells. Considering our observation that TGF-α increased protein levels of cPLA$_2$ in the membrane fraction as well as whole cells, the sustained prostaglandin E$_2$ generation may be mediated by, in part, the membrane-associated cPLA$_2$ increased through its expression in response to TGF-α. Previously, it was shown that a similar sustained prostaglandin generation is mediated through induction of sPLA$_2$ expression in interleukin-1β-stimulated rat mesangial cells (Pfeilschifter *et al*., 1993). Furthermore, a recent report showed that cPLA$_2$ activation mediates sPLA$_2$ expression responsible for sustained prostaglandin generation in rat 3Y1 fibroblasts stimulated with interleukin-1β and tumor necrosis factor α (Kuwata *et al*., 1998). In RGM1 cells, however, no increase in sPLA$_2$ activity was observed in the incubation medium of TGF-α-stimulated cells. Therefore, we suggest that in RGM1 cells TGF-α stimulates prostaglandin E$_2$ generation through cPLA$_2$ expression.

A previous report showed that leukemia inhibitory factor stimulates cPLA$_2$ expression leading to enhancement of A23187-induced AA liberation in human bronchial epithelial

BEAS-2B cells (Ikezono *et al*., 1997). In contrast, our result showed that preincubation of RGM1 cells with TGF-α diminished A23187-induced AA liberation under the conditions where amounts of cPLA$_2$ were increased. Furthermore, the AA liberation in response to A23187 was suppressed by MAFP, a cPLA$_2$ inhibitor. Thus, TGF-α exhibited an inhibitory effect on cPLA$_2$-catalyzed AA liberation despite the increase in cPLA$_2$ protein. Similarly, preincubation of human colonic epithelial cells with TGF-α has been shown to inhibit bradykinin-induced prostaglandin E_2 generation (Beltinger *et al*., 1999). This inhibitory effect of TGF-α is suggested to be ascribable to impairment of bradykinin-elicited signaling by prostaglandin E_2 generated during the preincubation with TGF-α. However, in the present study, NS-398 suppressed TGF-α-stimulated prostaglandin E_2 generation, but did not overcome the inhibition by TGF-α of A23187-induced AA liberation. This result suggests that prostaglandin E_2 generation by TGF-α is not involved in the inhibitory effect of TGF-α. We further demonstrated that the inhibition by TGF-α of A23187-induced AA liberation was restored by tyrphostin AG1478, an inhibitor of intrinsic tyrosine kinase of EGF receptor. This inhibitor also suppressed TGF-α-stimulated increase in cPLA$_2$ protein. Our findings indicate that TGF-α-elicited signalling leads to inhibition of cPLA$_2$-catalyzed AA liberation in spite of an increase in cPLA$_2$ protein, suggesting the existence of a regulatory mechanism(s) for the hydrolytic activity of cPLA$_2$.

In regard to the regulation of cPLA$_2$ activity, it has been shown that p11, known as annexin II light chain, directly inhibits cPLA$_2$ activity through binding to the COOH-terminal region of the enzyme, thus probably acting as an endogenous regulator for the activity (Wu *et al*., 1997). In the present study, we found that stimulation of RGM1 cells with TGF-α induced an increase in p11 protein, which was consistent with the occurrence of the inhibitory effect of TGF-α on A23187-induced AA liberation (Figures 3b and 5a). Moreover, our results showed that tyrphostin AG1478 restored the inhibition by TGF-α of A23187-induced AA liberation with suppression of the increase in p11 in response to TGF-α. These results indicate that the inhibitory effect of TGF-α on A23187-induced AA liberation occurs in parallel with the increase in p11. Indeed, an increase in p11 due to its overexpression has been shown to result in suppression of A23187-induced AA liberation in BEAS-2B cells (Yao *et al*., 1999). It was shown that a monomer of annexin II heavy chain, which is often associated with p11, also inhibits indirectly cPLA$_2$ activity probably through sequestration of substrates (Wu *et al*., 1997). In our study, however, no change in annexin II heavy chain proteins was observed in TGF-α-stimulated RGM1 cells, suggesting that the inhibition by TGF-α of A23187-induced AA liberation is not attributable to an indirect inhibitory effect of annexin II heavy chain. Based on the results, we suggest that the increase in p11 protein is involved in the inhibitory effect of TGF-α on cPLA$_2$-catalyzed liberation of AA.

It has been shown that p11 inhibits cPLA$_2$ activity, which is measured using natural membranes of BEAS-2B cells as substrates, especially in the presence of a physiological concentration of Ca^{2+} (0.5 μM) (Wu *et al*., 1997). In the present study, however, cPLA$_2$ activity in lysate of TGF-α-stimulated cells did not decrease but rather increased in spite of an increase in p11, suggesting that the p11 in lysate is unable to inhibit the cPLA$_2$ activity. This difference may be due to our cPLA$_2$ assay conditions where liposomes as substrates and relatively higher concentration of Ca^{2+} (5 mM) were used. Considering the fact that TGF-α exhibited an inhibitory effect on AA liberation in A23187-stimulated cells, it is possible that p11 may efficiently regulate the catalytic action of cPLA$_2$ within the intracellular environment. It was shown that a decrease in constitutive p11 in HeLa cells expressing p11 antisense mRNA results in further increases in basal levels of free AA and A23187-induced AA liberation (Yao *et al*., 1999), suggesting that cPLA$_2$ activity is ordinarily regulated by p11 in cells. Collectively, we suggest that the cPLA$_2$-mediated generation of prostaglandin E_2 upon stimulation with TGF-α occurs under the control of p11, which might play a role in protection against excessive hydrolysis of phospholipid by the increased cPLA$_2$.

In summary, our findings indicate that TGF-α induces an increase in cPLA$_2$ protein in parallel with prostaglandin generation, while it increases p11 to regulate cPLA$_2$-catalyzed AA liberation in RGM1 cells. We suggest that TGF-α stimulates prostaglandin generation through the synthesis of cPLA$_2$, the hydrolytic action of which may be under the control of inducible p11.

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