Activation of cytosolic phospholipase A2 and fatty acid transacylase is essential but not sufficient for thrombin-induced smooth muscle cell proliferation

Nathan Gluck,* Ouri Schwob,* Miron Krimsky, and Saul Yedgar

Department of Biochemistry, Hebrew University Faculty of Medicine, Jerusalem, Israel

Submitted 21 May 2007; accepted in final form 25 March 2008

Gluck N, Schwob O, Krimsky M, Yedgar S. Activation of cytosolic phospholipase A2 and fatty acid transacylase is essential but not sufficient for thrombin-induced smooth muscle cell proliferation. Am J Physiol Cell Physiol 294: C1597–C1603, 2008. First published April 2, 2008; doi:10.1152/ajpcell.00206.2007.—Thrombin is a potent stimulant of smooth muscle cell (SMC) proliferation in inflammatory conditions, leading to pathological thickening of vascular walls in atherosclerosis and airway remodeling in asthma. Cell proliferation requires the formation and remodeling of cell membrane phospholipids (PLs), involving the activation of PL-metabolizing enzymes. Yet, the role of specific PL-metabolizing enzymes in SMC proliferation has hardly been studied. To bridge this gap, in the present study, we investigated the role of key enzymes involved in PL metabolism, the PL-hydrolyzing enzyme phospholipase A2 (PLA2) and the PL-synthesizing enzyme lysophosphaticidic acid-fatty acid transacylase (LPAAT), in thrombin-induced proliferation of bovine aortic SMCs (BASMCs). Concomitantly with the induction of BASMC proliferation, thrombin activated cytosolic PLA2 (cPLA2-α), expressed by selective release of arachidonic acid and mRNA expression, as well as LPAAT, expressed by nonselective incorporation of fatty acid and mRNA expression. Specific inhibitors of these enzymes, arachidonyl-trifluoromethyl-ketone for cPLA2 and thimerosal for LPAAT, suppressed their activities, concomitantly with suppression of BASMC proliferation, suggesting a mandatory requirement for cPLA2 and LPAAT activation in thrombin-induced SMC proliferation. Thrombin acts through the protease-activated receptor (PAR-1), and, accordingly, we found that thrombin-induced BASMC proliferation was suppressed by the PAR-1 inhibitor SCH-79797. However, the PAR-1 inhibitor did not prevent thrombin-induced mRNA expression of cPLA2 and LPAAT, implying that the activation of cPLA2 and LPAAT is essential but not sufficient for thrombin-induced proliferation of BASMCs.

MATERIALS AND METHODS

Materials. Culture medium, FCS, antibiotics, and trypsin (solution A) were purchased from Bet Haemek Biological Industries. [3H]thymidine, AA, and oleic acid (OA) were purchased from Amersham

* N. Gluck and O. Schwob contributed equally to this work.

Address for reprint requests and other correspondence: S. Yedgar, Dept. of Biochemistry, Hebrew Univ.-Hadassah Medical School, Jerusalem, Israel 91120 (e-mail: yedgar@md.huji.ac.il).
(Aylesbury, UK). Human thrombin was purchased from OMRIX Biopharmaceuticals (Brussels, Belgium). The protease-activated receptor (PAR)-1 inhibitor N⁶-cyclopropyl-1⁷-[4-(1-methyllethyl)phenyl]methy1-7H-pyrrolo[3, 2-f]quinazoline-1.3-diamine dihydrochro-
ride (SCH-79797) was purchased from Tocris Bioscience. BSA (es-
tentially FA free), trypsin blue solution, bromoeno lactone (BEL; 
iiPLA₂ inhibitor), arachidonil-trifluoromethyl-ketone (AAOCOF; 
cPLA₂ inhibitor), and all other buffers and chemicals were purchased 
from Sigma (St. Louis, MO).

Cell cultures. SMCs harvested from the bovine aorta (BASMCs) 
were generously provided by the laboratory of I. Vlodawsky (Hadas-
sah University Hospital, Jerusalem, Israel). Cells were grown at 5% 
CO₂ at 37°C in DMEM (4.5% glucose) with 10% FCS, glutamine, 
penicillin, and streptomycin. Medium was changed every 3–4 days. 

Thrombin-induced proliferation of BASMCs.

To assess the 
effect, we determined thymidine incorporation into confluent 
SMCs under increasing concentrations of thrombin and com-
pared it with SMC proliferation in serum-containing (10%) 
medium. As shown in Fig. 1, thrombin enhanced SMC prolif-
eration in a concentration-dependent manner, reaching FCS-
induced growth at 2 U/ml, which is conventionally used in 
other studies (8). However, the proliferation-inducing activity differed significantly between thrombin batches, and, there-
fore, every new batch was calibrated independently against 

For cPLA₂, PCR was performed using bovine-compatible human 
cPLA₂-α primers (sense: 5'-TGTTCCAACAGATTGTTGG-3' 
and antisense: 5'-AACAGAGCAACGAGATGG-3') to amplify a 900-bp 
fragment (15). cDNA (2.5 μl) was mixed with ReddyMix PCR 
MasterMix (1.5 mmol/l MgCl₂) (ABgene) for a total volume of 25 μl. 
PCRs were carried out in a PT-100 (MJ Research, Watertown, MA) 
programmable thermal controller with an initial 5-min denaturation at 
94°C followed by the cycled program of 30 s at 94°C (denaturation), 
30 s at 50°C (annealing), and 2 min at 72°C (extension). PCRs were 
carried out for 32 cycles, and a final extension of 10 min at 72°C 
ended the reaction. PCR products (20 μl) were analyzed by 1.5% 
agarose gel electrophoresis and visualized with ethidium bromide.

For LPAAT, PCR was performed using primers (sense: 5'-CTGT-
GTGGCCGTGAGGC-3' and antisense: 5'-CAGCATGGCGC-
CGTTGTGGTT-3') to amplify a 402-bp fragment (16). PCRs were 
carried out with an initial 5-min denaturation at 94°C followed by the 
cycled program of 30 s at 94°C (denaturation), 30 s at 62°C (anneal-
ing), and 30 s at 72°C (extension). PCRs were carried out for 26 
cycles, and a final extension of 10 min at 72°C ended the reaction. 
Results were normalized to 28S rRNA.

cPLA₂ phosphorylation by Western blot analysis. Cells were grown 
to confluence in six-well plates and synchronized in medium with 
0.2% serum) for 1, 4, or 12 h at 37°C. Cells were then washed in PBS, incubated in 0.2% FCS for 48 h.

Cell viability was determined by vital staining using 0.1% trypan 
blue.

Determination of BASMC proliferation. The cell growth capacity 
was determined by the conventional method of incorporation of 
radioactively labeled thymidine into DNA (14). Confluent cells were 
synchronized by "starvation" as described above and incubated under 
the different treatments for the desired times, and [³H]thymidine (2 
μCi/well) was added to the culture medium for 2 h. Cells were then washed with PBS, incubated in 10% trichloroacetic 
acid (TCA) at 4°C for 1 h, washed twice with 5% TCA, and incubated 
for 1 h in 0.2 N NaOH at 37°C. The fluid was collected, and its 
radioactivity was measured in a scintillation counter.

Determination of PLA₂ activity by the release of AA and OA. 
cPLA₂ is specific to AA-carrying PLs, whereas sPLA₂ and iPLA₂ 
have no preference for FAs and can release both AA and OA. 
Therefore, monitoring of the production of AA and OA by cells is 
conventionally used for the differentiation of cPLA₂, from sPLA₂ and 
iPLA₂ activities. To this end, confluent synchronized cells were 
metabolically labeled by an overnight incubation in culture medium 
supplemented with [³H]AA or [³H]OA (2.5 μCi/well in 24-well 
plates). Cells were then washed twice with PBS and incubated 
in 0.2% FCS for 48 h.

Insoluble cellular components were removed by centrifugation at 
10,000 rpm for 10 min at 4°C. The protein content of the supernatant 
was determined according to the Bradford method using BSA as the 
standard. Extracts (40 μg protein) were mixed with 4X loading buffer 
[125 mM Tris (pH 6.8), 20% glycerol, 10% mercaptoethanol, 4.6% 
SDS, and 0.02% bromophenol blue] and boiled for 5 min. Proteins 
were separated by 10% Tris-glycine SDS-PAGE and transferred onto 
nitrocellulose membranes (Protran BAS5, Schleicher & Schuell Bio-
science) at 150 mA for 2 h. For cPLA₂ (no. 2832, Cell Signaling) and 
phospho-cPLA₂ (no. 2831, Cell Signaling), membranes were blocked 
for 1 h in Tris-buffered saline-0.1% Tween 20 (TBST) containing 5% 
nonfat milk powder, washed three times for 5 min in TBST, and 
incubated with 1:1,000 diluted antibodies overnight at 4°C in TBST 
containing 5% BSA. Membranes were washed three times for 5 min in 
TBST, incubated with a 1:7,500 dilution of goat anti-rabbit sec-
ondary antibody (no. 111-035-144, Jackson ImmunoResearch) con-
jugated to horseradish peroxidase for 1 h, and washed three times for 
5 min in TBST, incubated with 1:1,000 diluted antibodies overnight at 
4°C in TBST containing 5% BSA. Membranes were washed three times for 5 min in 
TBST before detection using SuperSignal Chemiluminescent 
substrate (Pierce, Rockford, IL).

Measurement of FA acylation by incorporation of [³H]OA or 
[³H]AA into membrane PLs. Confluent synchronized cells were 
incubated under the different treatments, after which [³H]OA or [³H]AA 
was added for up to 2 h. Medium was subsequently removed, and cells 
were washed twice with medium containing 1% BSA. Cells were 
lysed with 1 ml of 1% triton, and the labeled AA or OA content was determined by scintillation 
counting. For total labeling, cells were washed to remove free [³H]FAs and 
then lysed with 1 ml of 1% triton, and the total AA or OA content was determined by scintillation counting.

mRNA expression of cPLA₂ and LPAAT by RT-PCR. Cells were 
grown to confluence in six-well plates and synchronized as described 
above. On the day of the experiment, cells were incubated with 
thrombin-supplemented or -deficient (control) fresh medium (with 
0.2% serum) for 1, 4, or 12 h at 37°C. Cells were then washed in PBS 
and lysed with 0.5 ml TriReagent (T-9424, Sigma). RNA was isolated 
according to the manufacturer’s instructions, and the RNA concentra-
tion and purity were determined by spectrophotometry. RNA was 
reverse transcribed into cDNA with Moloney murine leukemia virus 
reverse transcriptase (M1701, Promega). cDNA (5 μg) was mixed with 
RT buffer containing 50 mM Tris (pH 8.3), 75 mMol/l KCl, 3 mMol/l 
MgCl₂, and 10 mMol/l DTT, 200 units reverse transcriptase, 40 units 
RNAsin. 0.2 μg oligo DT, and 1 mMol/d NDT, for a total volume of 
20 μl. Samples were incubated for 2 h at 37°C followed by 5 min at 
95°C, supplemented with 80 μl of PCR water, and stored at −20°C 
until subjected to PCR amplification.

RESULTS

Thrombin-induced proliferation of BASMCs. To assess the 
effect, we determined thymidine incorporation into confluent 
SMCs under increasing concentrations of thrombin and com-
pared it with SMC proliferation in serum-containing (10%) 
medium. As shown in Fig. 1, thrombin enhanced SMC prolif-
eration in a concentration-dependent manner, reaching FCS-
induced growth at 2 U/ml, which is conventionally used in 
other studies (8). However, the proliferation-inducing activity differed significantly between thrombin batches, and, there-
fore, every new batch was calibrated independently against
and of iPLA2 (BEL) on BASMC proliferation under thrombin.

As noted in the Introduction, cPLA2 is specific to AA-carrying PLs, whereas sPLA2 and iPLA2 have no preference for a fatty acyl chain. Therefore, to test the possible activation of endogenous PLA2s by thrombin, cell membrane PLs were prelabeled with either radioactive AA ([3H]AA) or OA ([3H]OA), as described in MATERIALS AND METHODS, and cellular PLA2 activities were determined by the release of free AA or OA following treatment with thrombin for increasing time periods (up to 2 h). As shown in Fig. 2, it was found that thrombin enhanced the release of AA but had no effect on OA release. However, in the process of membrane formation/remodeling, the released FA might be rapidly reacylated and thus not accumulate in the extracellular medium and escape detection. To examine this possibility, the experiment was repeated in the presence of the reacylation inhibitor thimerosal (19, 28). The results shown in Fig. 2 demonstrate that the reacylation inhibitor indeed elevated the level of AA accumulation in the extracellular medium in a concentration-dependent manner but did not affect the OA level. It thus appears that thrombin activates cPLA2 but not the other two PLA2 types.

Effect of cPLA2- and iPLA2-specific inhibitors on SMC proliferation. As noted in the Introduction, a previous study (27) reported the requirement of iPLA2 activity in thrombin-induced proliferation of rat vascular SMCs, whereas sPLA2 and iPLA2 have no preference for a fatty acyl chain. Therefore, to test the possible activation of endogenous PLA2s by thrombin, cell membrane PLs were prelabeled with either radioactive AA ([3H]AA) or OA ([3H]OA), as described in MATERIALS AND METHODS, and cellular PLA2 activities were determined by the release of free AA or OA following treatment with thrombin for increasing time periods (up to 2 h). As shown in Fig. 2, it was found that thrombin enhanced the release of AA but had no effect on OA release. However, in the process of membrane formation/remodeling, the released FA might be rapidly reacylated and thus not accumulate in the extracellular medium and escape detection. To examine this possibility, the experiment was repeated in the presence of the reacylation inhibitor thimerosal (19, 28). The results shown in Fig. 2 demonstrate that the reacylation inhibitor indeed elevated the level of AA accumulation in the extracellular medium in a concentration-dependent manner but did not affect the OA level. It thus appears that thrombin activates cPLA2 but not the other two PLA2 types.

Conversely, no effect of the iPLA2 inhibitor was observed on AA release (not shown). To further test whether iPLA2 activation is required in this process, the BEL effect on cell proliferation was examined using two methods: cell counting and thymidine incorporation, as described in MATERIALS AND METHODS. In repeated experiments, no inhibition of thrombin-induced proliferation by BEL was observed, as shown in Fig. 3B. These results support the conclusion that iPLA2 does not play a significant role in this BASMC proliferation.

Thrombin-induced cPLA2 mRNA expression. After the above experiments, which suggested the requirement of cPLA2 activation for thrombin-induced BASMC proliferation, cPLA2 mRNA expression was determined after the induction of proliferation by thrombin. As shown in Fig. 4A, consistent with an increase in enzymatic activity (Figs. 2 and 3), thrombin elevated cPLA2 mRNA expression up to 2.5-fold.

Thrombin-induced cPLA2 activation was further demonstrated by the results shown in Fig. 4B, which show that (i) concomitant with the enhanced lipolytic activity, thrombin also induced cPLA2 phosphorylation, which is required for enzyme activation (12, 13).
transacylase activity in thrombin-induced BASMC growth, we determined the thrombin effect on AA and OA incorporation into cell membranes. As shown in Fig. 5A, thrombin enhanced OA incorporation into cell membrane PLs. However, whereas thrombin elevated the release of AA (Fig. 2), it did not increase the net AA incorporation into cell PLs (not shown). These findings suggest that during cell proliferation thrombin exerts membrane PL remodeling by increasing the level of PL-containing OA (and possibly other non-AA FAs) while releasing AA.

**Thrombin-induced LPAAT expression.** Subsequent to the thrombin-induced FA incorporation, we determined the thrombin effect on LPAAT mRNA expression. As shown in Fig. 5B, LPAAT mRNA expression was clearly enhanced by treatment of BASMCs with thrombin. The role of LPAAT is further documented by Fig. 6, showing that thimerosal, the LPAAT inhibitor, also suppressed BASMC proliferation, demonstrating the requirement for FA transacylation in thrombin-induced BASMC growth.

**Effect of PAR-1 inhibition on thrombin-induced BASMC proliferation and activation cPLA2 and LPAAT.** Thrombin action on SMCs has been reported to be mediated by the PAR family, which includes four isoforms: PAR-1, PAR-2, PAR-3, and PAR-4 (24). It is generally accepted that PAR-1 is present in SMCs and takes part in thrombin-induced proliferation, whereas the presence of other PARs in SMCs and their roles in proliferation are not unequivocally clear. It is likely that this differs between different aortic and venous SMCs (3, 6, 7). Accordingly, in the present study, we determined the effect of the PAR-1 inhibitor SCH-79797 (1) on thrombin-induced BASMC proliferation as well as its effect on the activation of cPLA2.

---

**Fig. 3.** Inhibition of thrombin-induced-AA release (A) and BASMC proliferation (B) by cytosolic phospholipase A2 (cPLA2) inhibitor. A: confluent/synchronized cells were labeled overnight with [3H]AA. Cells were washed and then incubated for 30 min with or without thimerosal in the presence of increasing concentrations of the cPLA2 inhibitor arachidonyl-trifluoromethylketone (AACOCF3). Cells were then stimulated with thrombin for 1 h, and the [3H]AA content in the culture medium was determined. Each data point is the mean ± SE for 5 replicates. *P = 0.02; **P = 0.2 × 10^{-4}; ▫P = 0.4 × 10^{-4}; ▫P = 0.4 × 10^{-6}. B: confluent/synchronized cells were incubated for 30 min with or without the cPLA2 inhibitor (AACOCF3) or the iPLA2 inhibitor bromoenolactone (BEL) and then incubated overnight in either control or thrombin-supplemented medium, and [3H]thymidine incorporation was measured during the following 2 h. Cells were washed and then lysed for the determination of the cellular label content. Each data point is the mean ± SE for 5 replicates. *P = 0.04; **P = 0.001.

**Fig. 4.** Induction of mRNA expression (A) and phosphorylation (B) of cPLA2 by thrombin. A: confluent/synchronized cells were incubated for the indicated times in either control or thrombin-supplemented medium, and enzyme mRNA expressions were determined by RT-PCR. Each data point is the mean ± SE for 3 replicates. *P = 0.003. B: cells were incubated for 1 h with or without thrombin, lysed, and then subjected to Western blot analysis using antibodies to cPLA2 or its phosphorylated form (cPLA2-P) as described in MATERIALS AND METHODS. The Shown are representative blots of 3 reproducible experiments in which treatment with thrombin increased cPLA2-P by 758%, 427%, and 149%, as determined by densitometry.
As shown in Fig. 7, the PAR-1 inhibitor clearly suppressed thrombin-induced BASMC proliferation. However, to our surprise, in three reproducible experiments, we found that the PAR-1 inhibitor did not affect either cPLA2 or LPAAT enzymatic activity. For example, [3H]AA release (a measure of cPLA2 activity) by cells treated with thrombin or thrombin + SCH-79797 was 4.17 ± 0.58% and 4.55 ± 0.25% of the total cell label, respectively. Similarly, [3H]OA incorporation (a measure of LPAAT activity) into cells treated with thrombin or thrombin + SCH-79797 was 6,937 ± 750 and 6,834 ± 562 disintegrations/min, respectively. To further support this surprising finding, we examined the SCH-79797 effect on mRNA expression of PL-metabolizing enzymes. As shown in Fig. 8, the PAR-1 inhibitor did not affect thrombin-induced cPLA2 or LPAAT mRNA expression, thereby suggesting a dual mechanism for thrombin action in the induction of BASMC proliferation, as discussed below.
SMC proliferation is a key process in pathological thickening of the vascular wall in the development of atherosclerosis and stenosis and in airway remodeling in asthma. Both processes are facilitated by inflammatory conditions. Cell growth involves the formation of new cell membrane components, PLs in particular. The formation of membrane PLs can be done by de novo synthesis and/or PL remodeling involving lipolysis and reconstitution. Both thrombin and products of PL hydrolysis are markedly enhanced in inflammatory conditions. Products of PL hydrolysis, commonly denoted inflammatory lipid mediators, such as lysophospholipid, lysophosphatidic acid in particular, as well as AA and its eicosanoid derivatives, induce SMC proliferation, leading to related pathology (2, 9, 10, 13, 18). PL lipolysis and the subsequent production of inflammatory lipid mediators can occur by multiple enzymatic pathways (20), but PL2 enzymes play a major role in these processes. In addition, extracellular sPLA2s, which are secreted by activated inflammatory cells, can act as receptor ligands, independent of their lipolytic activity, to activate cell signaling and subsequent cell proliferation (25). Yet, the possible involvement of PL-metabolizing enzymes in thrombin-induced SMC proliferation has not been well investigated, as discussed in the Introduction.

The present study shows that the thrombin-induced proliferation of BASMCs requires l) the activation of lipolytic enzyme cPLA2 (but not iPLA2 or sPLA2), as shown by its elevated mRNA expression and enzyme phosphorylation, selective AA release, and the concomitant inhibition of cell proliferation by the selective cPLA2 inhibitor AACOCF3; and 2) the activation of the FA-acylating enzyme LPAAT, as shown by its elevated mRNA expression, incorporation of FAs, and the concomitant inhibition of cell proliferation by the acylation inhibitor thimerosal.

Previous studies have demonstrated the involvement of iPLA2 (also called Ca2+-independent cPLA2-γ) in membrane remodeling (4) and FA transacylation (26). It was also reported that thrombin-stimulated activation of MAPKs in rabbit ven-

Fig. 8. PAR-1 inhibitor does not affect mRNA expression of cPLA2 and LPAAT. Confluent/synchronized cells were incubated with the PAR-1 inhibitor SCH-79797 for 30 min prior to treatment with thrombin for 12 h. mRNA levels of the enzymes were determined as described in MATERIALS AND METHODS. Each data point is the mean ± SE for 2 replicates. No significant differences were observed between the bars denoted with * or **.

DISCUSSION

SMC proliferation is a key process in pathological thickening of the vascular wall in the development of atherosclerosis and stenosis and in airway remodeling in asthma. Both processes are facilitated by inflammatory conditions. Cell growth involves the formation of new cell membrane components, PLs in particular. The formation of membrane PLs can be done by de novo synthesis and/or PL remodeling involving lipolysis and reconstitution. Both thrombin and products of PL hydrolysis are markedly enhanced in inflammatory conditions. Products of PL hydrolysis, commonly denoted inflammatory lipid mediators, such as lysophospholipid, lysophosphatidic acid in particular, as well as AA and its eicosanoid derivatives, induce SMC proliferation, leading to related pathology (2, 9, 10, 13, 18). PL lipolysis and the subsequent production of inflammatory lipid mediators can occur by multiple enzymatic pathways (20), but PL2 enzymes play a major role in these processes. In addition, extracellular sPLA2s, which are secreted by activated inflammatory cells, can act as receptor ligands, independent of their lipolytic activity, to activate cell signaling and subsequent cell proliferation (25). Yet, the possible involvement of PL-metabolizing enzymes in thrombin-induced SMC proliferation has not been well investigated, as discussed in the Introduction.

The present study shows that the thrombin-induced proliferation of BASMCs requires l) the activation of lipolytic enzyme cPLA2 (but not iPLA2 or sPLA2), as shown by its elevated mRNA expression and enzyme phosphorylation, selective AA release, and the concomitant inhibition of cell proliferation by the selective cPLA2 inhibitor AACOCF3; and 2) the activation of the FA-acylating enzyme LPAAT, as shown by its elevated mRNA expression, incorporation of FAs, and the concomitant inhibition of cell proliferation by the acylation inhibitor thimerosal.

Previous studies have demonstrated the involvement of iPLA2 (also called Ca2+-independent cPLA2-γ) in membrane remodeling (4) and FA transacylation (26). It was also reported that thrombin-stimulated activation of MAPKs in rabbit ven-

tricular myocytes is dependent on iPLA2 activity (5). Of particular relevance to the present study is the report of Yellaturu and Rao (27), which showed that thrombin-induced proliferation of rat thoracic aorta SMCs required increased activity of iPLA2, and this was inhibited by BEL (at 10 μM). In variance to that, in the present study, we could not verify the involvement of iPLA2 in thrombin-induced proliferation of BASMCs, since, except for AA, no release of other FA could be detected, even in the presence of the transacylation inhibitor (which further increased AA release). In addition, application of the same procedure of Yellaturu and Rao, namely, treatment with the iPLA2 inhibitor BEL (up to 50 μM in some experiments), did not affect the thrombin-induced AA release. Although we do not have a satisfactory explanation for the discrepancy between our findings and those of Yellaturu and Rao, this might be due to differences in species (rats vs. cows), or methodology, e.g., inhibition of FA reacylation, as applied in the present study. Also, a later study (21), using HEK-293 cells and Caki-1 cells, showed that iPLA2-β and iPLA2-γ may differ in their effect on cell growth and can be selectively inhibited by R- and S-enantiomers of BEL. This might suggest that the distinction between iPLA2 isoforms and their selective inhibition (by BEL enantiomers) should be considered when studying the role of iPLA2 in cell function.

On the other hand, we clearly found that thrombin-induced BASMC proliferation is associated with cPLA2 expression and selectively enhanced AA release. Both AA release and cell proliferation were suppressed by the cPLA2 inhibitor AACOCF3. These findings strongly suggest that cPLA2 activation is required for thrombin-induced BASMC proliferation. This conclusion is in accord with the report of Anderson and Marshall (2), which showed that the cPLA2-mediated (AACOCF3-inhibited) release of AA is critical for the proliferation of human artery vascular SMCs (although in growth medium, not thrombin-supplemented medium).

At the pathway of PL formation, the present study also showed that thrombin-induced BASMC proliferation requires the activation of FA transacylase, as shown by the enhanced FA incorporation and expression of LPAAT mRNA, and inhibition of all of the above by the acylation inhibitor. As noted above, both PL hydrolysis and reconstitution may occur by more than one enzymatic pathway. The present study shows that in thrombin-induced BASMC proliferation, membrane PL formation involves their hydrolysis by cPLA2 and FA incorporation by LPAAT.

Taken together, these findings suggest that by activation of AA-specific cPLA2 and non-FA-specific LPAAT, thrombin-induced BASMC proliferation is associated with the increased production of AA, a precursor for diverse proinflammatory eicosanoids. Since elevated thrombin levels and subsequent SMC proliferation are characteristic of inflammatory conditions, it is probable that the thrombin-induced AA and subsequent eicosanoid production are important mechanisms by which thrombin exerts its proinflammatory effect.

Of particular interest in this study is the finding that the PAR-1 inhibitor suppressed thrombin-induced cell proliferation without affecting the thrombin-induced activity of the above PL-metabolizing enzymes (Figs. 7 and 8). Yet, both cPLA2 and LPAAT inhibitors independently suppressed thrombin-induced BASMC proliferation (Figs. 3 and 6). Taken together, these findings appear to suggest that thrombin induces SMC proliferation by two inde-
pendent mechanisms: 1) via its proteolytic action on PAR-1 and 2) via PAR-1-independent activation of PL-metabolizing enzymes cPLA2 and LPAAT. It is possible, of course, that the thrombin-induced activation of PL-metabolizing enzymes is mediated by other PARs or another mechanism(s) altogether, which is yet to be explored. Nevertheless, the results shown in Figs. 3B, 6, and 7 demonstrate that each of the inhibitors tested, namely, the cPLA2 inhibitor (Fig. 3B), the LPAAT inhibitor (Fig. 6), and PAR-1 inhibitor (Fig. 7), abolished thrombin-induced BASMC proliferation, bringing it to that of control, thrombin-un-treated cells. This implies that the effects of the two mechanisms are not additive. Instead, both are essential since blockade of either of them eliminates the induction of BASMC proliferation by thrombin.

The PAR-mediated mechanism has been given considerable attention, whereas the other mechanism has hardly been studied, and its extensive investigation is clearly of interest. The findings of the present study thus open a new avenue in the study of thrombin function and might introduce new insights into the control of thrombin-associated pathological conditions by the regulation of cPLA2 and/or LPAAT.

REFERENCES


