



# Thrombin Induced Apoptosis through Calcium-Mediated Activation of Cytosolic Phospholipase A<sub>2</sub> in Intestinal Myofibroblasts

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## Abstract

Thrombin is a serine protease that participates in a variety of biological signaling through protease-activated receptors. Intestinal myofibroblasts play central roles in maintaining intestinal homeostasis. In this study, we found that thrombin-induced apoptosis is mediated by the calcium-mediated activation of cytosolic phospholipase A<sub>2</sub> in the CCD-18Co cell. Thrombin reduced cell viability by inducing apoptosis and proteinase-activated receptor-1 antagonist attenuated thrombin-induced cell death. Endogenous ceramide did not affect the cell viability itself, but a ceramide-mediated pathway was involved in thrombin-induced cell death. Thrombin increased intracellular calcium levels and cytosolic phospholipase A<sub>2</sub> activity. The ceramide synthase inhibitor Fumonisin B<sub>1</sub>, intracellular calcium chelator BAPTA-AM, and cytosolic phospholipase A<sub>2</sub> inhibitor AACOCF<sub>3</sub> inhibited thrombin-induced cell death. Thrombin stimulated arachidonic acid release and reactive oxygen species generation, which was blocked by AACOCF<sub>3</sub>, BAPTA-AM, and the antioxidant reagent Trolox. Taken together, thrombin triggered apoptosis through calcium-mediated activation of cytosolic phospholipase A<sub>2</sub> in intestinal myofibroblasts.

**Key Words:** Thrombin, Arachidonic acid, ROS, CCD-18Co, Apoptosis

## INTRODUCTION

Thrombin is a serine protease produced during the coagulation process that plays a central role in hemostasis by promoting platelet aggregation. In addition to its role in the coagulation pathway, thrombin is also essential in numerous biological activities, which affect immune responses, tissue repair, and inflammation (Anrather *et al.*, 1997). Thrombin is found on the surface of mucosal organs such as the lungs (Meyer and Jaspers, 2015) and the intestine (Vergnolle, 2016). It has also been found in normal and inflamed intestinal mucosa (Denadai-Souza *et al.*, 2018); inflammation triggered the release of thrombin in intestinal epithelial cells (Motta *et al.*, 2011). It has been reported that thrombin shows a bimodal effect: enhanced growth at low concentrations, and impaired growth/apoptosis at higher concentrations (Ahmad *et al.*, 2000; Zain *et al.*, 2000). However, the mechanism of thrombin-induced apoptosis is not clearly understood.

The biological effects of thrombin are mediated through proteinase-activated receptor-1 (PAR-1) (Flynn and Buret,

2004). PAR-1 is a G protein-coupled receptor and is highly expressed in the various cell types, including enterocytes, endothelial cells, and myofibroblasts in the gastrointestinal tract (Corvera *et al.*, 1999; Seymour *et al.*, 2003). Intestinal myofibroblasts are located subjacent to the intestinal stem cells and have regulatory functions as intestinal stem cell niches by the release of paracrine niche factors (Powell *et al.*, 2011). Through the release of the niche factors, intestinal myofibroblasts are fundamental in growth and repair, and mucosal protection (Powell *et al.*, 1999).

Cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>), which is activated at the physiological concentration of calcium, is a key enzyme that generates multiple eicosanoid products (Pompeia *et al.*, 2002). cPLA<sub>2</sub> is implicated in the process of inflammation and apoptosis through the liberation of arachidonic acid (AA) and reactive oxygen species (ROS) generation (Kohjimoto *et al.*, 1999).

Here, we show the underlying mechanism of thrombin-induced apoptosis in CCD-18Co cells isolated from human normal colon tissue and known to have properties of intestinal

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myofibroblasts (Valentich *et al.*, 1997). The cells were treated with thrombin and assessed for cell viability and ceramide levels changes, AA, and intracellular ROS levels. Our study elucidated the mechanism of thrombin-induced apoptosis and the roles of calcium and cPLA<sub>2</sub> in the apoptosis in CCD-18Co cells.

## MATERIALS AND METHODS

### Materials

$\alpha$ -Thrombin (human plasma,  $\geq 2,800$  NIH units/mg protein) was purchased from Calbiochem (La Jolla, CA, USA) and Sigma-Aldrich (St. Louis, MO, USA). [9,10-<sup>3</sup>H(N)]palmitic acid, [5,6,7,8,9,11,12,14,15-<sup>3</sup>H(N)]arachidonic acid was purchased from Amersham Biosciences (Buckinghamshire, England, UK). MTT (Thiazolyl Blue Tetrazolium Bromide), C<sub>6</sub>-ceramide, bacterial sphingomyelinase, and 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from Sigma-Aldrich. BAPTA-AM (1,2-bis-(*o*-Aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid, tetraacetoxymethyl ester) was purchased from BIOMOL Research Labs., Inc (Ann Arbor, MI, USA). Silica gel 60 thin-layer chromatography (TLC) plates were purchased from Merck (Darmstadt, Germany). For the protein assay, the Bradford reagent was purchased from Bio-Rad (Hercules, CA, USA). Plasticware for tissue culture was purchased from NUNC (Waltham, MA, USA). The cleaved caspase-3 ELISA kit was purchased from Cell Signaling Technology (Danvers, MA, USA). AA and Fumonisin B<sub>1</sub> were purchased from Sigma-Aldrich. Trolox was obtained from Tocris (Minneapolis, MN, USA) and the Fluo-4 NW Calcium Assay Kit was purchased from Invitrogen (Carlsbad, CA, USA).

### Methods

**Cell cultures:** The intestinal subepithelial myofibroblast cell line CCD-18Co was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA; Cat. No. CRL-1459). Cells were grown in Eagle's minimum essential medium (MEM) (GIBCO, Grand Island, NY, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (GIBCO) and 1% penicillin/streptomycin (Sigma-Aldrich). Cells were grown in a humidified tissue culture incubator at 37°C in an atmosphere containing 5% (v/v) CO<sub>2</sub>. For experiments, cells were cultured in a media containing 2% FBS.

**MTT assay:** Cell viability was determined by a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay as described in a previous study (Li *et al.*, 2016). MTT was added to the culture medium at a final concentration of 1 mg/mL, and cells were incubated at 37°C for 3 h. After the medium had been carefully aspirated, the precipitated formazan was solubilized with DMSO overnight at 37°C and quantified spectrophotometrically at 540 nm using an EIA plate reader (Bio-Rad).

**Nuclear staining:** Apoptotic cells with condensed nuclei were visualized by DAPI staining (Jang *et al.*, 2015). After treatment, cells were washed once with ice-cold phosphate-buffered saline (PBS) before being fixed in a solution of methanol/acetic acid (3:1, v/v) for 30 min and stained with 1 mg/mL of DAPI for 15 min. The nuclear morphology of the cells was observed by fluorescence microscopy (excitation wavelength of 364 nm, an emission wavelength of 454 nm).

**Cell radiolabeling and lipid analysis:** The ceramide levels

were determined as described in a previous study (Won *et al.*, 2020). CCD-18Co cells were labeled with [<sup>3</sup>H]palmitic acid (1.0  $\mu$ Ci/mL) for 24 h. The labeled cells were washed twice with ice-cold PBS and incubated with MEM containing 2% FBS for 1 h. After thrombin treatment, the medium was aspirated, and the cells were rinsed twice with ice-cold PBS. The cells were lysed in 0.8 mL of ice-cold methanol containing 0.5 N-HCl, and lipids were extracted using chloroform. After centrifugation, the lower organic phase was dried under a speed vacuum dryer. Lipids were dispersed in 20  $\mu$ L of chloroform/methanol (1:1, v/v) and applied to TLC plates. [<sup>3</sup>H]ceramide was separated from other radiolabeled cellular lipids by Thin-Layer Chromatography (TLC) in a solvent system containing chloroform/methanol/acetic acid/water (85:4.5:5:0.5, v/v/v/v). Commercial lipid standards (Sigma-Aldrich) were chromatographed on the sample plate. After drying, lipids were visualized by iodine vapor staining. The radioactive spots corresponding to ceramide were scraped, mixed with 2 mL of scintillation solution (Instagel-XF, Packard Instrument Co., Meriden, CT, USA), and counted for radioactivity using a  $\beta$ -scintillation counter (Tri-carb 1600 TR; Packard Instrument Co.).

**AA release assay:** AA release was measured as described in a previous study, with modifications (Choi *et al.*, 2013). Sub-confluent cells grown in 6-well plates were labeled for 24 h with [<sup>3</sup>H]AA (0.5  $\mu$ Ci/mL) in MEM containing 10% FBS. After labeling, the medium was aspirated and cells were washed twice with ice-cold PBS and incubated with MEM containing 2% FBS for 1 h. The cells were then treated with thrombin for the indicated time, and the aliquots (150  $\mu$ L) of medium were mixed with 1 mL of scintillation solution. The radioactivity was measured using a liquid  $\beta$ -scintillation counter. Radioactivity of released [<sup>3</sup>H]AA was normalized to the total radioactivity of the monolayer extracted with 1% Triton X-100.

**Detection of intracellular ROS:** Intracellular production of ROS was measured using a 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) assay (Walsh *et al.*, 2014). CCD-18Co cells were preincubated in the culture medium with 25  $\mu$ M DCFH-DA for 30 min. Then they were treated with thrombin for the indicated time. After treatment, the cells were harvested and washed with a 1 mL HEPES buffer [137 mM NaCl, 2.7 mM KCl, 0.4 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.5 mM MgCl<sub>2</sub>, 10 mM HEPES (pH 7.4), 1.8 mM CaCl<sub>2</sub>, 5 mM glucose]. The cells were lysed by sonication on ice. The formation of 2',7'-dichlorofluorescein was determined fluorometrically with an FL600 fluorescence spectrophotometer (Bio Tek, VT, USA) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Results were calculated as fluorescence intensity/mg of protein and represented as percentage of control.

**Cleaved caspase-3 ELISA:** The manufacturer's protocol was followed for the analysis of cleaved caspase-3. Briefly, 40  $\mu$ g of cell lysates were added to the antibody-coated plate and incubated overnight at 4°C. The plate was washed four times and 100  $\mu$ L of detection antibody was added before incubation for 1 h at 37°C. After washing the plate, 100  $\mu$ L of HRP-linked secondary antibody was added and the plate was incubated for 30 min at 37°C. After washing the plate, 100  $\mu$ L of TMB substrate was added and the plate was incubated for 10 min at 37°C. STOP solution was then added to each well and absorbance read at 450 nm.

**cPLA<sub>2</sub> activity assay:** cPLA<sub>2</sub> activity was analyzed with the cPLA<sub>2</sub> Assay Kit (Cayman Chemicals, Ann Arbor, MI, USA) according to the manufacturer's instructions. In brief, the cells

were harvested and washed three times with ice-cold PBS. After centrifugation (10 min at 1,000×g at 4°C), the cell pellet was lysed by sonication in the ice-cold buffer (50 mM HEPES, pH 7.4 containing 1 mM EDTA). The lysed sample was centrifuged at 10,000×g for 15 min at 4°C. The supernatant was stored on ice. The protein concentration of each sample was determined using the Pierce BCA method (Smith *et al.*, 1985). To avoid the measurement of secretory PLA<sub>2</sub> and Ca<sup>2+</sup>-independent PLA<sub>2</sub>, the secretory PLA<sub>2</sub>-specific inhibitor thioetheramide-PC and the Ca<sup>2+</sup>-independent PLA<sub>2</sub>-specific inhibitor bromoenol lactone were added to the samples before the assay. The results were represented as a ratio of (absorbance rate)/(μg of protein).

**Calcium assay:** Intracellular calcium levels were measured with the Fluo-4 NW Calcium Assay Kit (Karakhanova *et al.*, 2014). The cells were plated at 4.0×10<sup>4</sup> cells per well in 96 well plates and grown overnight. The medium was removed and 100 μL of the dye loading solution was added to each well of a 96-well plate. Fluorescence was measured using spectramax (Molecular Devices, CA, USA) for excitation at 494 nm and emission at 516 nm. The relative fluorescence was used for the estimation of changes in intracellular calcium levels.

### Statistical analysis

All experiments were conducted with at least three biological replicates. Data shown in column graphs represent the mean ± SEM, as indicated in the figure legends. When normality could be assumed, Student's t-tests were used to compare the difference between the two groups. Otherwise, the Mann-Whitney test was chosen. \**p*<0.05, \*\**p*<0.01, \*\*\**p*<0.001 were considered significant. Statistical analysis was performed with GraphPad Prism9 software. Each of these experiments was independently repeated at least three times.

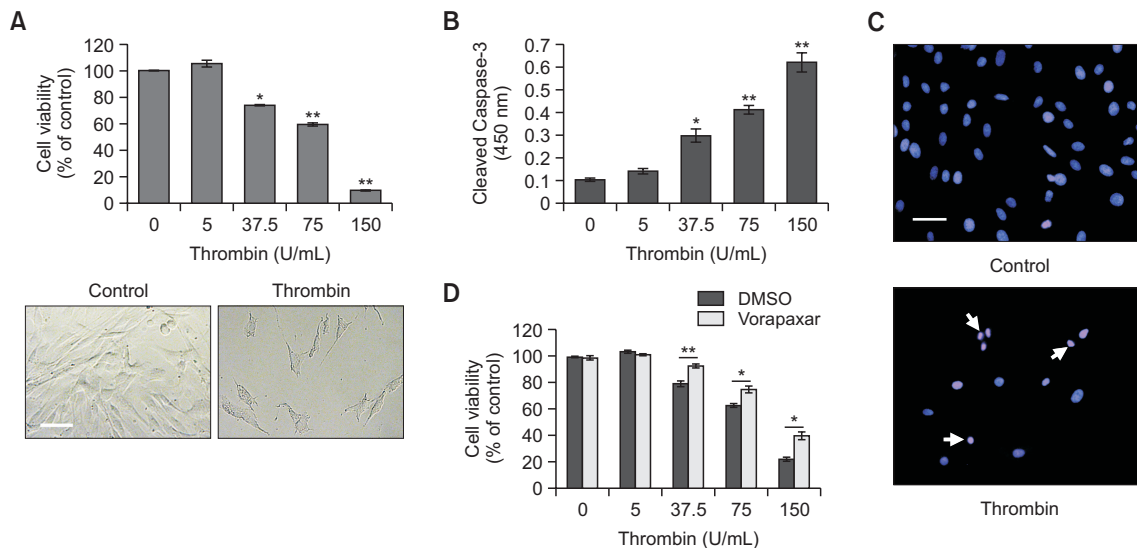
## RESULTS

### Thrombin induced apoptosis through PAR-1-mediated pathway in CCD-18Co cells

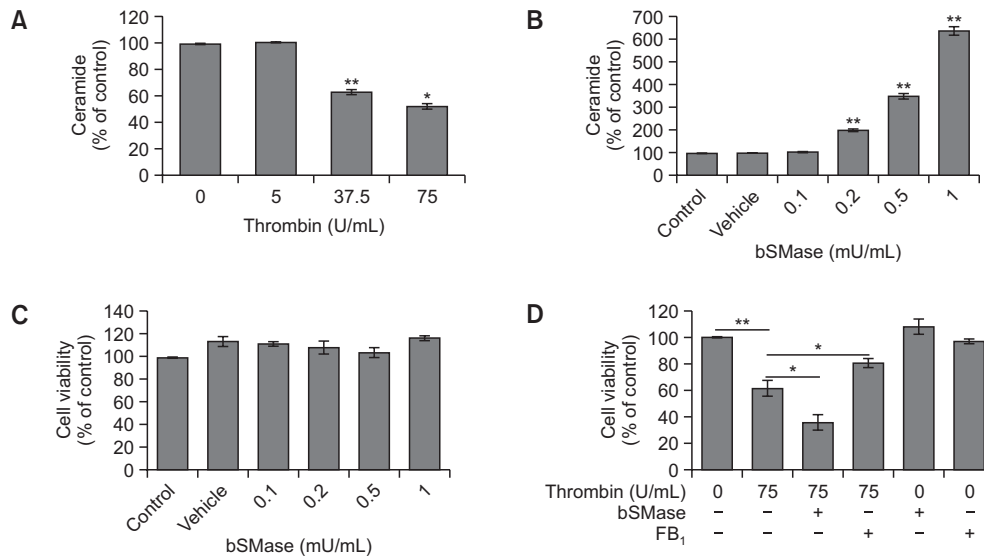
CCD-18Co cells were treated with thrombin (0, 5, 37.5, 75, or 150 U/mL) for 18 h, and cell viability was assessed by MTT assay. We found that thrombin decreased cell viability in a dose-dependent manner (Fig. 1A). Thrombin showed obvious toxicity on the cells under microscope (Fig. 1A). Treatment of thrombin (37.5 or 75 U/mL) reduced cell viability by 74.03 or 59.73%, respectively. At a dose of 150 U/mL, thrombin reduced cell viability by 10.25% in CCD-18Co cells (Fig. 1A). To determine whether thrombin induces apoptotic cell death, we examined the level of apoptosis marker cleaved caspase-3 and condensed fragmented nuclei using a fluorescent DNA-binding agent, DAPI. Thrombin increased cleaved caspase-3 in a dose-dependent manner (Fig. 1B). At a dose of 150 U/mL, thrombin increased cleaved caspase-3 level by 5.8-fold compared to the control. As shown in Fig. 1C, thrombin-treated cells exhibited condensed fragmented nuclei, the indication of apoptotic cell death. Next, we investigated the cytotoxicity of thrombin through the PAR-1-mediated pathway. Treatment of PAR-1 antagonist vorapaxar attenuated thrombin-induced cell death compared to control (Fig. 1D). These results demonstrated that thrombin reduced cell viability by inducing apoptosis through the PAR-1 mediated pathway in CCD-18Co cells.

### Thrombin induced cell death through the ceramide-mediated pathway

Ceramide is a bioactive lipid proposed to be an endogenous mediator of apoptosis (Pettus *et al.*, 2002). We examined the effect of thrombin on ceramide levels using [<sup>3</sup>H]palmitic acid, which was used as a lipid precursor to trace the newly



**Fig. 1.** Thrombin reduced cell viability and triggered apoptosis via PAR1 in CCD-18Co cells. (A) CCD-18Co cells were treated with thrombin (0, 5, 37.5, 75, or 150 U/mL) for 18 h and the cell viability was determined by MTT assay. Microscopic pictures showed the thrombin-treated and untreated CCD-18Co cells. The thrombin dose was 150 U/mL. Scale bar: 50 μm. (B) Cleaved caspase-3 levels in thrombin-treated CCD-18Co cells. Cell lysates were prepared from 0, 5, 37.5, 75, or 150 U/mL of thrombin-treated cells after 6 h, and cleaved caspase-3 levels were evaluated from the same amounts of cell lysates by ELISA. Relative expression levels were indicated as absorbance values at 450 nm. (C) CCD-18Co cells were treated with 150 U/mL of thrombin. After treatment, the cells were fixed and stained with DAPI. Scale bar: 50 μm. (D) CCD-18Co cells were treated with thrombin in the absence or presence of vorapaxar (10 μM) for 18 h and the cell viability was determined by MTT assay. \**p*<0.05, \*\**p*<0.01.



**Fig. 2.** Thrombin reduced the ceramide levels and thrombin-induced cell death was inhibited by Fumonisin B<sub>1</sub>. (A) The change of ceramide levels by thrombin treatment was analyzed by labeling with [<sup>3</sup>H]palmitic acid (1 μCi/mL). The cells were treated with the indicated concentration of thrombin for 18 h. For analysis of ceramide, the lipids were extracted and radioactivity was detected using β-liquid scintillation counting. (B) To evaluate the effect of endogenous ceramide on cell viability, CCD-18Co cells were treated with bSMase (0.1, 0.2, 0.5, or 1 mU/mL; the vehicle is 50% glycerol PBS) and the ceramide levels were analyzed. (C) Cell viability was determined by MTT assay in bSMase-treated cells. (D) After pretreatment of bSMase (0.2 mU/mL) or FB<sub>1</sub> (10 μM) for 12 h, thrombin was treated to the cells and then cell viability was determined by MTT assay. \**p*<0.05, \*\**p*<0.01.

synthesized ceramide, verified by detecting [<sup>3</sup>H]palmitate incorporation in CCD-18Co cells. As shown in Fig. 2A, thrombin decreased ceramide generation in a dose-dependent manner. Treatment of thrombin (37.5 or 75 U/mL) reduced newly synthesized ceramide by 37.22 or 47.94%, respectively. Next, we used bacterial sphingomyelinase (bSMase) to investigate the generation of endogenous ceramide that affects cell death. Ceramide production was increased by bSMase treatment in a dose-dependent manner and 1 mU/mL of bSMase increased ceramide level by 6.3-fold (Fig. 2B). In contrast, the influence of bSMase on the cell viability elicited scarcely any response (Fig. 2C). We further examined whether the alteration of ceramide generation affects cell viability in thrombin-treated cells. While bSMase-pretreated cells showed increased susceptibility to thrombin-induced cell death, pretreatment of ceramide synthase inhibitor Fumonisin B<sub>1</sub> (FB<sub>1</sub>) recovered cell viability in thrombin-treated cells (Fig. 2D). These results showed that ceramide itself did not affect cell viability, but might be used as a mediator for thrombin-induced apoptosis in CCD-18Co cells.

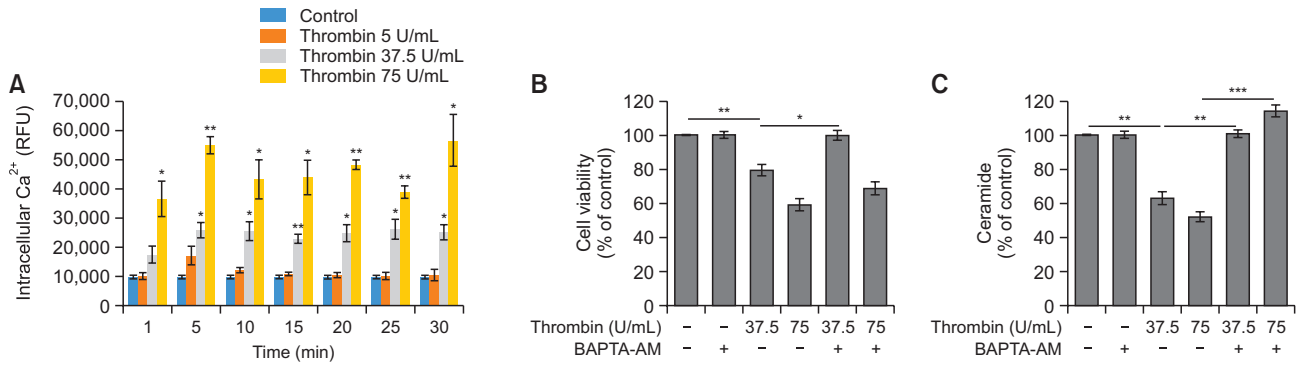
### Thrombin induced cell death through calcium-mediated activation of cPLA<sub>2</sub> in CCD-18Co cells

Elevations of intracellular calcium led to exaggerated impaired energy production and resulted in apoptosis (Pinton *et al.*, 2008). Increased intracellular calcium levels in many cell types, including CCD-18Co cells (Sacks *et al.*, 2008), were attributed to thrombin. In Fig. 3A, thrombin increased intracellular calcium levels in a dose-dependent manner; the levels spiked during the first minutes of thrombin treatment. After spiking, the average calcium level remained up to about 30 min in the 37.5 and 75 U/mL of thrombin-treated cells (Fig. 3A). Next, we examined the effects of intracellular calcium chelator BAPTA-AM on thrombin-induced cell death to investigate whether in-

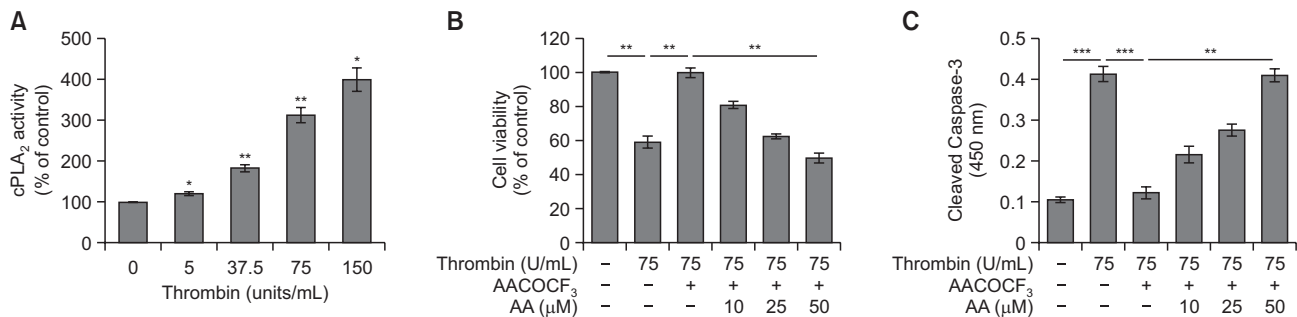
tracellular calcium levels were involved in thrombin-induced cell death. CCD-18Co cells were preincubated with BAPTA-AM (10 μM) for 1 h and the cells were treated with 37.5 or 75 U/mL of thrombin. The BAPTA-AM only treated cells showed no change in cell viability (Fig. 3B). BAPTA-AM pretreatment completely blocked the 37.5 U/mL of thrombin-induced cell death. In the presence of BAPTA-AM, 75 U/mL of thrombin-treated cells showed a 28.8% increased cell viability compared with 75 U/mL of thrombin-treated cells (Fig. 3B). Interestingly, the BAPTA-AM recovered the ceramide levels in thrombin-treated cells (Fig. 3C). These results demonstrated that the intracellular calcium levels involved in thrombin-induced cell death, and possibly ceramide, was used for the pathway involved in cell death. Ceramide-1-phosphate is a ceramide metabolite and is required for the cPLA<sub>2</sub> activation (Kriem *et al.*, 2005). cPLA<sub>2</sub> activity is also calcium-dependent and is attributed to initiating apoptosis through AA release (Kohjimoto *et al.*, 1999). Indeed, thrombin treatment increased cPLA<sub>2</sub> activity in a dose-dependent manner in CCD-18Co cells. At a dose of 150 U/mL, thrombin increased cPLA<sub>2</sub> activity by 4.0-fold compared to control (Fig. 4A). Next, we examined the effect of cPLA<sub>2</sub> inhibitor AACOCF<sub>3</sub> on thrombin-induced cell death. Treatment of AACOCF<sub>3</sub> recovered thrombin-induced cell death (Fig. 4B) and reduced cleaved caspase-3 level increased by thrombin (Fig. 4C). However, co-treatment of AA attenuated the effect of AACOCF<sub>3</sub> on cell death and cleaved caspase-3 in a dose-dependent manner (Fig. 4B, 4C). Taken together, thrombin-induced cell death through calcium-mediated activation of cPLA<sub>2</sub> and, AA triggered apoptosis in CCD-18Co cells.

### Thrombin stimulated AA release through calcium-mediated cPLA<sub>2</sub> activation

It is well known that thrombin stimulates AA release in many



**Fig. 3.** Thrombin reduced cell viability through the calcium-mediated pathway. (A) Intracellular calcium levels were analyzed using fluorescent calcium indicator Fluo-4 NW. The cells were treated with 0, 5, 37.5, or 75 U/mL of thrombin for the indicated time. Fluorescence was measured (excitation at 494 nm and emission at 516 nm) and the relative fluorescence reflected the estimation of changes in intracellular calcium levels. (B) CCD-18Co cells were preincubated with 10  $\mu$ M of BAPTA-AM for 1 h. The medium was replaced with the medium containing 37.5 or 75 U/mL of thrombin and the cells were incubated for 18 h. Cell viability was determined by MTT assay. (C) CCD-18Co cells were labeled with [<sup>3</sup>H]palmitic acid (1  $\mu$ Ci/mL) and treated with the indicated condition. The ceramide level was analyzed through radioactivity detection by  $\beta$ -liquid scintillation counting. \* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001.



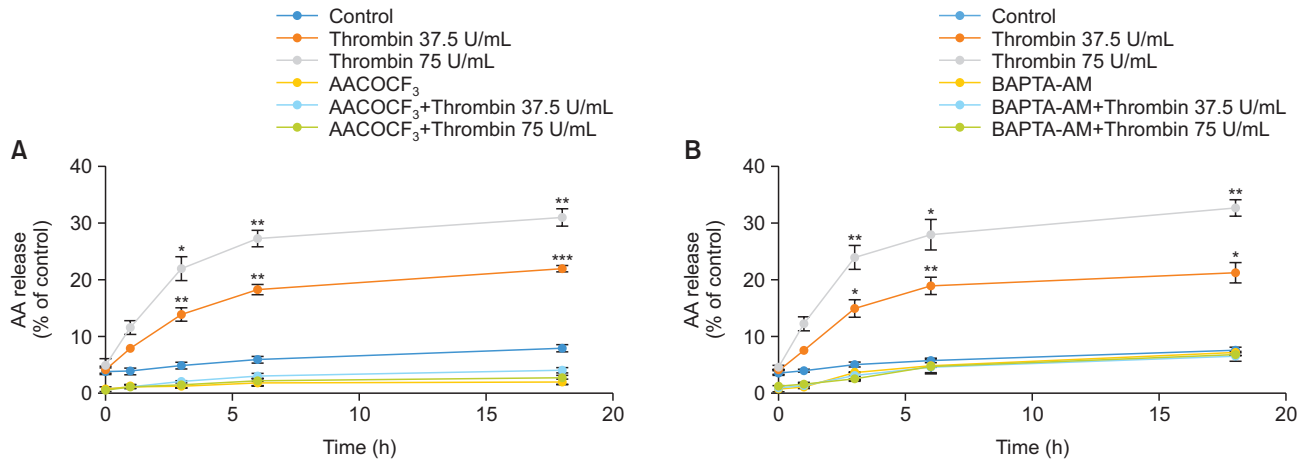
**Fig. 4.** Thrombin induced cell death through the cPLA<sub>2</sub>-mediated pathway. (A) CCD-18Co cells were treated with thrombin (0, 5, 37.5, 75, or 150 U/mL) for 6 h, and the cPLA<sub>2</sub> activity in the cell lysates was measured. (B) CCD-18Co cells were preincubated with 10  $\mu$ M of AACOCF<sub>3</sub> for 1 h. The medium was replaced with the medium containing 75 U/mL of thrombin and the cells were incubated for 18 h in the absence or presence of AA (10, 25, and 50  $\mu$ M). Cell viability was determined by MTT assay. (C) Cleaved caspase-3 levels were evaluated from the same amounts of cell lysates by ELISA. Relative expression levels were indicated as absorbance values at 450 nm. \* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001.

cell types such as platelets, smooth muscle cells, astrocytes, and osteoblasts (Suzuki *et al.*, 1997). We investigated whether thrombin induces AA release in the CCD-18Co cells. The CCD-18Co cells were pre-labeled with [<sup>3</sup>H]AA and the cells were treated with 37.5 or 75 U/mL of thrombin for the indicated time. Thrombin-induced [<sup>3</sup>H]AA release began within 1 h and increased steadily until 6 h when it reached a plateau. This [<sup>3</sup>H]AA release was blocked by AACOCF<sub>3</sub> (Fig. 5A) and BAPTA-AM (Fig. 5B). These results showed that thrombin stimulated AA release through the calcium-mediated cPLA<sub>2</sub> signaling pathway in CCD-18Co cells.

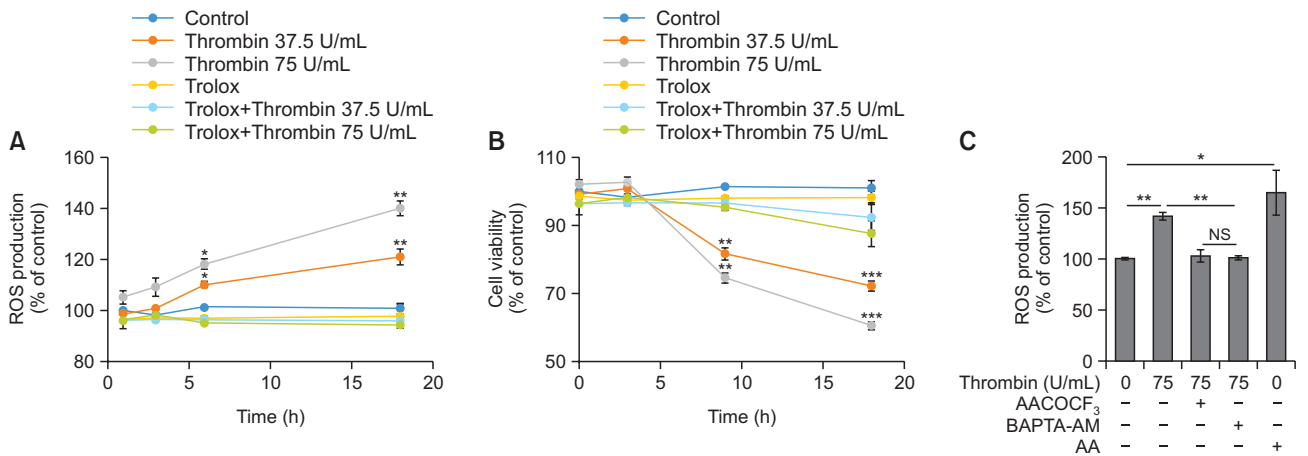
### Thrombin reduced cell viability via ROS generation in CCD-18Co cells

AA stimulated ROS generation, which played a significant role in the initiation of apoptosis (Simon *et al.*, 2000; Rukoyatkin *et al.*, 2013). We examined thrombin-induced ROS generation and its effect on cell viability. DCFH-DA was used for the assessment of intracellular ROS formation in CCD-18Co cells. CCD-18Co cells were incubated with 37.5 or 75 U/mL of thrombin for the time indicated. Thrombin stimulated ROS

generation in a time-dependent manner. Thrombin treatment (37.5 or 75 U/mL) enhanced ROS production by 120 and 140%, respectively at 18 h treatment (Fig. 6A). At the dose of 37.5 and 75 U/mL, thrombin decreased cell viability by 28 and 40%, respectively at 18 h treatment (Fig. 6B). To determine whether thrombin-induced cell death occurred through ROS generation, we treated an antioxidant reagent, the water-soluble derivative of vitamin E, Trolox (Messier *et al.*, 2013). The thrombin-induced ROS generation and cell death were inhibited by Trolox treatment. (Fig. 6A, 6B). These results demonstrated that thrombin-induced cell death was mediated by ROS generation. Next, we examined whether calcium release by thrombin itself contributes to thrombin-induced ROS generation. While AA treatment showed an increased ROS generation, AACOCF<sub>3</sub> and BAPTA-AM inhibited thrombin-induced ROS generation (Fig. 6C). There was no significant difference in ROS levels between AACOCF<sub>3</sub>- or BAPTA-AM-treated cells (Fig. 6C).



**Fig. 5.** Thrombin induced AA release via calcium- and cPLA<sub>2</sub>-mediated pathway. Thrombin induced-AA release was analyzed by labeling with [<sup>3</sup>H]AA. CCD-18Co cells were labeled with [<sup>3</sup>H]AA (0.5μ Ci/mL) in MEM medium containing 10% FBS for 24 h, and the cells were washed twice with PBS. The washed cells were incubated with MEM containing 2% FBS for 1 h, and treated with 37.5 or 75 U/mL of thrombin for indicated times in the absence or presence of 10 μM of AACOCF<sub>3</sub> (A) or 10 μM of BAPTA-AM (B). The AA radioactivity was analyzed with a liquid β-scintillation counter. \**p*<0.05, \*\**p*<0.01, \*\*\**p*<0.001.



**Fig. 6.** Thrombin stimulated ROS generation and reduced cell viability in CCD-18Co cells. (A) CCD-18Co cells were preincubated with 25 μM of DCFH-DA for 30 min. Then, 37.5 or 75 U/mL of thrombin was treated for the indicated time in the absence or presence of Trolox (25 μM). The formation of 2',7'-dichlorofluorescein was determined at an excitation wavelength of 485 nm, and an emission wavelength of 530 nm. (B) CCD-18Co cells were treated with 37.5 or 75 U/mL of thrombin for the indicated time in the absence or presence of Trolox (25 μM). The cell viability was determined by MTT assay. (C) ROS generation was measured from the cells treated with the indicated condition for 18 h. The 10 μM of AACOCF<sub>3</sub> and BAPTA-AM, and 25 μM of AA were treated, respectively. \**p*<0.05, \*\**p*<0.01, NS, not significant.

**DISCUSSION**

Previous studies have suggested that thrombin could be a therapeutic target for the treatment of intestinal bowel disease (IBD) (Chande *et al.*, 2008; Scaldaferri *et al.*, 2011). Thrombin release and its activity increased with the severity of inflammation in IBD patients (Denadai-Souza *et al.*, 2018; Motta *et al.*, 2021b). A recent study supports that thrombin release is from the intestinal epithelium (Motta *et al.*, 2019). These findings showed that thrombin itself might contribute to the development of IBD, independent of its role in coagulation.

PAR-1 activation is implicated in the inflammatory response of the gastrointestinal tract through the upregulation of inflammatory mediators such as tumor necrosis factor-α (TNF-α)

and interleukin-6 (Vergnolle, 2004; Rezaie, 2014). PAR-1 activation is vital in the execution of apoptosis through the initiation of the caspase cascade (Flynn and Buret, 2004). Previous reports identified that PAR-1-activation induced apoptosis in intestinal epithelial cells (Chin *et al.*, 2003), and inhibition of PAR-1 attenuated pathological severity in the colitis model (Motta *et al.*, 2021a).

In this study, we used CCD-18Co cells, which exhibited similar properties as intestinal myofibroblasts in situ (Valentich *et al.*, 1997). CCD-18Co cells are a useful tool for defining the biological functions of intestinal myofibroblasts and elucidating their role in intestinal physiology (Zhu *et al.*, 2003). Intestinal myofibroblasts release niche factors, including Wnts, BMPs, and EGF, which support epithelial proliferation and the

maintenance of epithelial barrier integrity via secretion of cytokines and metabolites of AA (Beltinger *et al.*, 1999). In a recent study, the crucial roles of the cells in intestinal regeneration were demonstrated. Dysfunction of intestinal myofibroblast caused the failure of epithelial regeneration in the DSS-induced colitis model (Horiguchi *et al.*, 2017).

In Fig. 1, thrombin induced apoptosis through PAR-1 activation in CCD-18Co cells. This result demonstrated that thrombin is able to exert cytotoxicity in myofibroblasts. It shows that increased thrombin release could be a cause of exacerbation of IBD by inducing apoptosis of myofibroblasts that play a significant role in epithelial regeneration (Horiguchi *et al.*, 2017).

Ceramide is a sphingolipid that has gained attention as an important signaling molecule in various vital cell processes. The production of ceramide contributes to inducing apoptosis or inhibiting cell proliferation (Kolesnick and Hannun, 1999). Thrombin treatment reduced ceramide levels (Fig. 2A), and the endogenous ceramide generation did not affect cell viability in CCD-18Co cells (Fig. 2B, 2C). In Fig. 2D, pretreatment of bSMase increased susceptibility to thrombin-induced cell death but FB<sub>1</sub> pretreatment inhibited the cytotoxic effect of thrombin. Thus, we hypothesized that ceramide was not directly involved in the apoptosis process, but a ceramide-mediated pathway was involved in the thrombin-induced apoptosis in CCD-18Co cells. In Fig. 3A, thrombin increased intracellular calcium levels; it is known that elevations of intracellular calcium led to the initiation of apoptosis (Pinton *et al.*, 2008). Indeed, BAPTA-AM treatment blocked thrombin-induced cell death (Fig. 3B), and the ceramide reduced by thrombin treatment was recovered by BAPTA-AM (Fig. 3C). Taken together, thrombin-induced cell death was mediated by intracellular calcium. The ceramide might play a role as a mediator rather than directly triggering cell death by itself.

Ceramide kinase (CERK) stimulated the conversion of ceramide to ceramide-1-phosphate (C-1-P), and CERK activity was observed to be calcium-dependent (Kolesnick and Hemmer, 1990). It was demonstrated that C-1-P activates cPLA<sub>2</sub> directly (Pettus *et al.*, 2004). cPLA<sub>2</sub> catalyzes the hydrolysis of the *sn*-2 position of glycerophospholipids to release AA, and AA serves as the precursor to produce eicosanoids, including prostaglandins and leukotrienes (Smith, 1992). It also potentiates apoptosis via oxidative stress generation (Korbecki *et al.*, 2013).

In Fig. 4A, thrombin treatment increased cPLA<sub>2</sub> activity, and thrombin-induced apoptosis was blocked by AACOCF<sub>3</sub>. This inhibition of apoptosis by AACOCF<sub>3</sub> was attenuated in the presence of AA (Fig. 4B, 4C). Thrombin stimulated AA release, which was blocked by AACOCF<sub>3</sub> and BAPTA-AM (Fig. 5). These results demonstrated that thrombin-induced cell death through calcium-mediated activation of cPLA<sub>2</sub>. AA is an effector molecule to thrombin-induced apoptosis in CCD-18Co cells.

Vitamin E has been evaluated to improve oxidative stress-associated diseases (Bhalla *et al.*, 2009). Thrombin stimulated ROS production and reduced cell viability. Thrombin-induced ROS generation and cell death were inhibited by the Trolox (Fig. 6A, 6B). These results showed that thrombin-induced apoptosis was mediated by ROS production. We compared the ROS levels of AACOCF<sub>3</sub>- and BAPTA-AM-treated cells to examine whether calcium release itself contributes to ROS generation. There was no significant difference in ROS levels between AACOCF<sub>3</sub>- or BAPTA-AM-treated cells (Fig. 6C).

This result showed there was no additional ROS generation by calcium release itself.

It was reported that putative signaling pathways initiate a pro-apoptotic response to PAR-1 activation. This pathway includes JAK/STAT, RhoA, myosin light chain kinase, ERK1/2, and caspase activation (Turgeon *et al.*, 1998; Flynn and Buret, 2004). Thrombin induced apoptosis in neurons and astrocytes via a pathway requiring tyrosine kinase and RhoA activities (Donovan *et al.*, 1997). Other pathways inducing apoptosis mediated by thrombin should be investigated.

In summary, thrombin induces apoptosis through calcium-mediated cPLA<sub>2</sub> activation and stimulation of AA-induced ROS generation in human colonic myofibroblasts. This study supports the strategy that targeting the thrombin-cPLA<sub>2</sub>-AA axis may be applied as a therapeutic approach for the management or prevention of inflammation-related disorders in the intestine.

## CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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