Platelet protease-activated receptor (PAR)4, but not PAR1, associated with neutral sphingomyelinase responsible for thrombin-stimulated ceramide-NF-κB signaling in human platelets

Wei-Fan Chen,[1,2] Jie-Jen Lee,[3] Chao-Chien Chang,[2,4] Kuan-Hong Lin,[5] Shwu-Huey Wang,[6] and Joen-Rong Sheu[1,2]

[1]Graduate Institute of Medical Sciences and [2]Department of Pharmacology, School of Medicine, Taipei Medical University, Taipei,; [3]Department of Surgery, Mackay Memorial Hospital, Taipei; [4]Department of Cardiology, Cathay General Hospital, Taipei; [5]Central Laboratory, Shin-Kong Wu Ho-Su Memorial Hospital, Taipei; and [6]Core Facility Center, Office of Research and Development, Taipei Medical University, Taipei, Taiwan

ABSTRACT

Thrombin activates platelets mainly through protease-activated receptor (PAR)1 and PAR4. However, downstream platelet signaling between PAR1 and PAR4 is not yet well understood. This study investigated the relationship between nSMase/ceramide and the NF-κB signaling pathway in PARs-mediated human platelet activation. The LC-MS/MS, aggregometry, flow cytometry, immunoprecipitation, and mesenteric microvessels of mice were used in this study. Human platelets stimulated by thrombin, 3-OMS (a neutral sphingomyelinase [nSMase] inhibitor) and Bay11-7082 (an NF-κB inhibitor) significantly inhibited platelet activation such as P-selectin expression. Thrombin also activated IκB kinase (IKK)β and IκBα phosphorylation; such phosphorylation was inhibited by 3-OMS and SB203580 (a p38 MAPK inhibitor). Moreover, 3-OMS abolished platelet aggregation, IKKβ, and p38 MAPK phosphorylation stimulated by PAR4-AP (a PAR4 agonist) but not by PAR1-AP (a PAR1 agonist). Immunoprecipitation revealed that nSMase was directly associated with PAR4 but not PAR1 in resting platelets. In human platelets, C24:0-ceramide is the predominant form of ceramides in the LC/MS-MS assay; C24:0-ceramide increases after stimulation by thrombin or PAR4-AP, but not after stimulation by PAR1-AP. We also found that C2-ceramide (a cell-permeable ceramide analog) activated p38 MAPK and IKKβ phosphorylation in platelets and markedly shortened the occlusion time of platelet plug formation *in vivo*. This study demonstrated that thrombin activated nSMase by binding to PAR4, but not to PAR1, to increase the C24:0-ceramide level, followed by the activation of p38 MAPK-NF-κB signaling. Our results showed a novel physiological significance of PAR4-nSMase/ceramide-p38 MAPK-NF-κB cascade in platelet activation.

Introduction

Platelets play a crucial role in normal hemostasis, and platelet-dependent arterial thrombosis underlies most myocardial infarctions. Several agonists, such as collagen, ADP, and thrombin, bind to their specific platelet receptors and promote platelet activation. Thrombin is the most potent platelet activator; it activates platelets by interacting with protease-activated receptors (PARs). Of the 4 known PAR isoforms, PAR1, PAR3, and PAR4 constitute the active thrombin receptors on human platelets.[1] PAR2 is activated by trypsin, tryptase, and coagulation factor Xa, but not by thrombin.[1] PAR1 and PAR4 are essential for thrombin-induced human platelet activation, whereas PAR3 and PAR4 mediate normal responsiveness to thrombin in mouse platelets.[2] The downstream signaling pathways of PAR1 and PAR4 in human platelets are not yet fully understood. Initially, these receptors were thought to couple the same set of heterotrimeric G-proteins.[3] However, a recent study showed that PAR1-mediated platelet activation required ADP to be secreted for a complete platelet response, whereas PAR4 was activated without ADP.[4] Platelets are anucleate cells derived from megakaryocytes in the bone marrow. Despite being anucleated, platelets express some transcription factors, including estrogen receptors,[5] peroxisome proliferator-activated receptors,[6] and retinoid X receptors.[7] Nuclear factor (NF)-κB, a transcription factor, regulates diverse cell functions ranging from inflammation to cell death. As the term 'nuclear factor' implies, the actions of NF-κB require its translocation from the cytosol to the nucleus, where it binds cognate nuclear DNA sequences. Although NF-κB is known to be activated after platelet activation,[8] the functional significance of NF-κB in platelets remains unclear. The question of whether this transcription factor is functionally present in platelets in a novel way, unrelated to transcriptional regulation, has yet to be answered. Platelets thus provide a good model for studying non-genomic functions of transcription factors. Sphingolipid signaling pathways are known to play a crucial role in regulating the pathogenesis of atherosclerosis.[9] Sphingomyelin (SM) is one of the major sphingolipids present on the plasma membrane. SM is enriched in lipid rafts which are considered microdomains of the plasma membrane and critical for signal transduction.[10] The neutral sphingomyelinase (nSMase)-mediated hydrolysis of SM is emerging as a major pathway of the ceramide production that occurs in response to various stimuli and stressors.[11] Platelets may use ceramide as a second messenger to induce platelet activation.[12] Although previous studies

 doi:10.3324/haematol.2012.072553
The online version of this article has a Supplementary Appendix.
Manuscript received on June 21, 2012. Manuscript accepted on October 2, 2012.
Correspondence: shwu@tmu.edu.tw/sheujr@tmu.edu.tw

have suggested that ceramide is a potent activator of NF-κB,[13] few details have been documented on the specific role of ceramide in platelet pathophysiology. This study investigated the relationship between nSMase/ceramide and the NF-κB signaling pathway in PARs-mediated human platelet activation.

Design and Methods

Human platelet aggregation

Human platelet suspensions were prepared as previously described.[14] The study protocol was approved by the Institutional Review Board of Taipei Medical University, and conformed to the principles outlined in the Declaration of Helsinki. All human volunteers provided informed consent. In brief, blood was collected from healthy volunteers who had not taken any medicine during the preceding two weeks and this blood was mixed with an acid-citrate-dextrose (ACD) solution (9:1, v/v). After centrifugation, the supernatant (platelet-rich plasma; PRP) was supplemented with EDTA (2 mM) and heparin (6.4 U/mL). The washed platelets were suspended in a Tyrode solution containing bovine serum albumin (BSA) (3.5 mg/mL). The final concentration of Ca^{2+} in a Tyrode solution was 1 mM.

A turbidimetric method was applied to measure platelet aggregation[14] using a Lumi-Aggregometer (Payton, Scarborough, Ontario, Canada). Platelet suspensions (3.6×10^8 cells/mL) were pre-incubated with reagents for 3 min before the addition of agonists, and the reaction was allowed to proceed for 6 min. The extent of aggregation was expressed as a percentage of the control (in the absence of reagents) in light-transmission units.

Measurement of platelet relative $[Ca^{2+}]i$ mobilization by Fura 2-AM fluorescence

Citrated whole blood was centrifuged at 120 g for 10 min. The supernatant was incubated with Fura 2-AM (5 μM) for 1 h. Human platelets were then prepared as described. Finally, the external Ca^{2+} concentration of the platelet suspensions was adjusted to 1 mM. The rise in $[Ca^{2+}]i$ was measured using a fluorescence spectrophotometer (CAF 110, Jasco, Tokyo, Japan).[14]

Expression of P-selectin in platelets

The method by Shcherbina and Remold-O'Donnell was modified to measure the expression of P-selectin in washed platelets.[15] In brief, washed platelets (3.6×10^8 cells/mL) were pre-incubated with reagents, followed by the addition of anti-human P-selectin-fluorescein mAb (1 μg/mL) for 20 min. Data acquisition and analysis were performed by flow cytometry (Beckman Coulter, Miami, FLA, USA).

Immunoprecipitation

In brief, washed platelets (1×10^9/mL) were stimulated with thrombin (0.05 U/mL) for 10-60 s, and were then lysed in an immunoprecipitation (IP) buffer as described previously.[16] An equal amount of protein from each supernatant was pre-cleared with protein A/G-agarose-conjugated beads for 2 h. Samples were then rotated overnight with primary antibodies against PAR1 and PAR4 (1 mg/mL). The following day, 20 μL of beads were added and rotated overnight. Immunoprecipitates were washed 3 times and were then analyzed by immunoblotting assay.

Quantification of ceramides by liquid chromatography coupled with tandem mass spectrometry

The quantity of ceramide was determined as previously described, with a slight modification.[17] For analysis, washed platelets (1×10^6/mL) were stimulated with thrombin (0.05 U/mL), PAR1-AP (1 μM), and PAR4-AP (25 μM) for 10-180 s to trigger various types of C16:0, C18:0, C24:0, and C24:1-ceramide formation. The platelet suspensions were then extracted with 600 μL of methanol containing the internal standards (C17:0-ceramide). The supernatants were collected and the extraction step was repeated; thereafter, combined organic phases were used to quantify ceramide levels. The amounts of C16:0, C18:0, C24:0, and C24:1-ceramide were determined by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Chromatographic separation was accomplished under gradient conditions using an XDB C18 column (150 mm × 2.1 mm ID; 5 μm particle size; Agilent Technologies, Santa Clara, CA, USA). The HPLC mobile phases consisted of acetonitrile-water-formic acid (5:95:0.1; v/v/v) (A) and acetonitrile-tetrahydrofuran-formic acid (50:50:0.1; v/v/v) (B). The initial buffer composition was 60% (A) to 40% (B) held for 0.5 min, then linearly changed to 0% (A) to 100% (B) at 4.5 min and held for 5 min, and then linearly changed to 60% (A) to 40% (B) and held for 4 min. We injected 25 μl of each sample, and the total run time was 15 min. The MS/MS analyses were performed on an API 4000 triple quadruple mass spectrometer with a Turbo V source (Applied Biosystems; Darmstadt, Germany). Precursor-to-product ion transitions of *m/z* 538.5→264.2 for C16:0-ceramide, of *m/z* 566.7→264.2 for C18:0-ceramide, of *m/z* 648.6→264.2 for C24:1-ceramide, of *m/z* 650.6→264.2 for C24:0-ceramide, and of *m/z* 552.5→264.6 for C17:0-ceramide were used for multiple reaction monitoring. Concentrations of the calibration standards, quality controls, and unknowns were evaluated by Analyst software 1.4.2 (Applied Biosystems). The level of relative abundance of each ceramide species was expressed as the percentage of C24:0-ceramide in resting platelets.

Fluorescein sodium-induced platelet thrombi in mesenteric microvessels of mice

As described,[18] mice were anesthetized, and an external jugular vein was cannulated with PE-10 for administration of the dye and drug (by an intravenous bolus). A segment of the small intestine was placed onto a transparent culture dish for microscopic observation. Venules (30-40 μm) were selected for irradiation to produce a microthrombus. In the epi-illumination system, light from a 100 W mercury lamp was passed through a B-2A filter (Nikon, Tokyo, Japan) with a DM 510 dichromic mirror (Nikon). Filtered light, from which wavelengths below 520 nm had been eliminated, was used to irradiate a microvessel; the area of irradiation was approximately 100 μm in diameter on the focal plane. We administered C2-ceramide, dihydroceramide (DHC), or an isovolumetric solvent control (0.5% DMSO) 1 min after fluorescein sodium (15 μg/kg) treatment. Five minutes after administration of fluorescein sodium, we began irradiation by filtered light, simultaneously with the video timer, and platelet plug formation was observed on a television monitor. The time lapse for inducing thrombus formation leading to the cessation of blood flow was measured.

Information concerning reagents and antibodies, immunoblotting study and statistical analysis is available in the *Online Supplementary Appendix*.

Results

Functional activities of nSMase/ceramide and NF-κB on platelet activation in human platelets stimulated by thrombin

Ceramide plays an important role as mediator of several cellular pathways; however, its role in platelet activation

remains obscure. To explore the relationship between the actions of nSMase/ceramide and NF-κB in human platelets, we examined the functional activities of 3-OMS (an nSMase inhibitor)19 and Bay11-7082 (an IκBα inhibitor) in thrombin-induced platelet activation. As shown in *Online Supplementary Figure S1A*, Bay11-7082 (10 and 20 μM) concentration-dependently inhibited platelet aggregation stimulated by thrombin (0.05 U/mL) was approximately 50% and 80%, respectively; however, 3-OMS (10 and 30 μM) was approximately 32% and 35% under the same conditions. Therefore, Bay11-7082 exhibited greater potency than did 3-OMS in inhibiting platelet aggregation stimulated by thrombin. Furthermore, relative $[Ca^{2+}]i$ mobilization was markedly increased on stimulation with thrombin but was significantly inhibited by 3-OMS (30 μM) and Bay11-7082 (20 μM), by approximately 42% and 74%, respectively (*Online Supplementary Figure S1B*). After platelet activation, P-selectin is rapidly translocated from cytosol to the cell membrane; thus, P-selectin expression is an important biomarker of platelet activation. As shown in *Online Supplementary Figure S1C*, in resting platelets (green line) P-selectin expression was minimal, but was markedly enhanced after thrombin (0.05 U/mL) stimulation (blue line). Pre-treatment with 3-OMS (30 μM) (red line) or

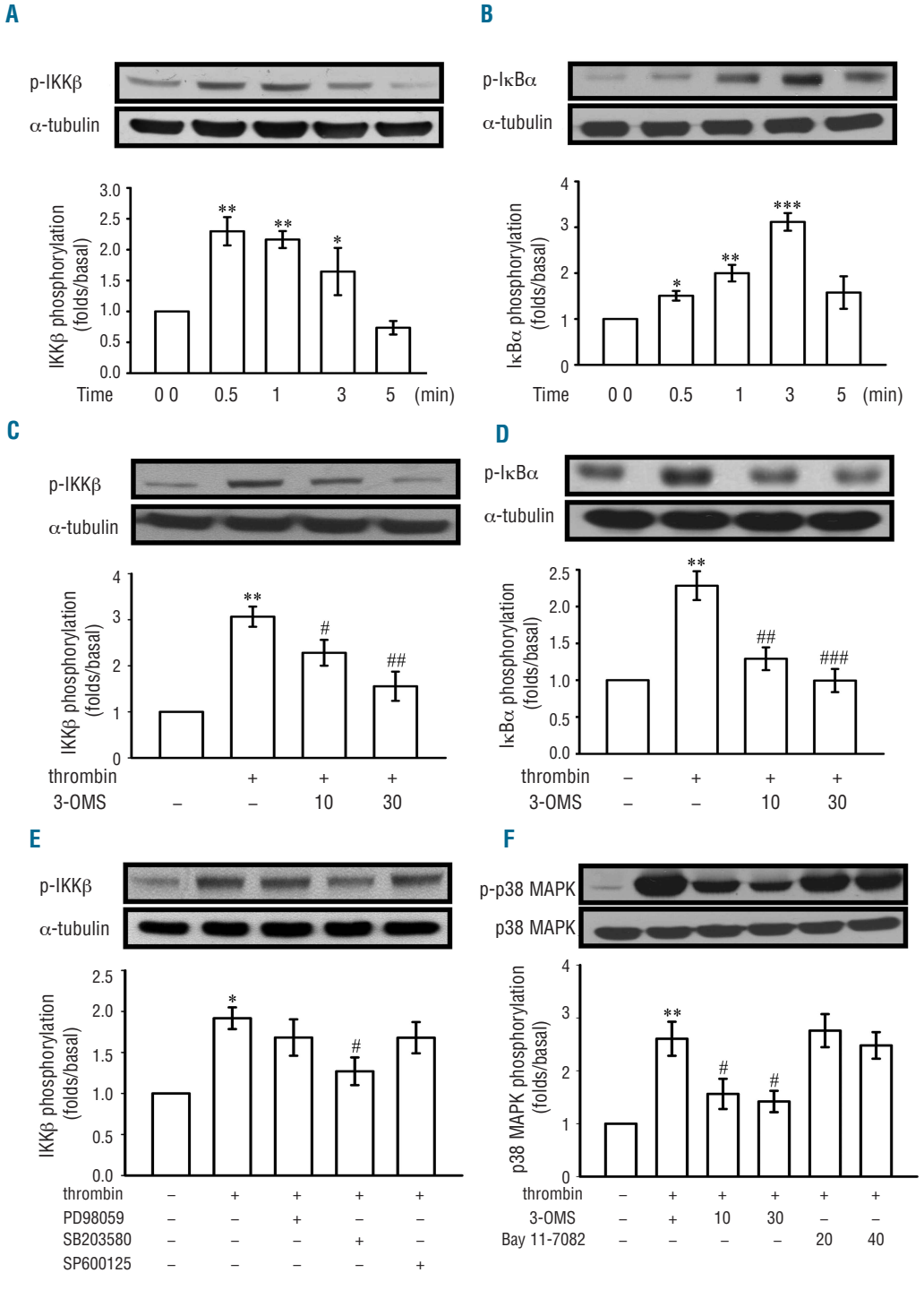

Figure 1. Effects of 3-OMS on NF-κB activation and p38 MAPK phosphorylation in thrombin-activated platelets. Washed platelets (1x10⁹/mL) were pre-incubated with 3-OMS (10 and 30 μM), Bay11-7082 (10 and 20 μM), PD98059 (20 μM), SB203580 (10 μM), or SP600125 (10 μM). Thereafter, thrombin (0.05 U/mL) was added to trigger the phosphorylation of (A, C, and E) IKKβ, (B, D) IκBα, and (F) p38 MAPK for the indicated times (0.5 to 5 min). Data are presented as the means ± S.E.M. (n=4). **P*<0.05, ***P*<0.01, and ****P*<0.001, compared with the control (resting) group; #*P*<0.05, ##*P*<0.01, and ###*P*<0.001, compared with the thrombin-treated group.

Bay11-7082 (20 μM) (brown line) significantly diminished P-selectin expression, by approximately 54% and 88%, respectively.

Role of nSMase/ceramide in NF-κB and p38 MAPK activation in platelets

To demonstrate the occurrence of NF-κB activation in anucleated human platelets, the platelets were treated by thrombin. The results indicated a rapid and time-dependent increase in the activation of NF-κB signals. As shown in Figure 1A and B, stimulation by thrombin (0.05 U/mL) resulted in a significant increase of IκB kinase (IKK) β and IκBα phosphorylation at 0.5 and 1.0 min, respectively. To investigate whether NF-κB-mediated platelet activation was regulated by nSMase/ceramide, we examined the effect of 3-OMS on thrombin-induced NF-κB activation. We observed that 3-OMS (10 and 30 μM) concentration-dependently reduced both IKKβ and IκBα phosphorylation at 0.5 and 1.0 min after thrombin stimulation, respectively (Figure 1C and D). Our previous study had indicated that NF-κB activation can be regulated by mitogen-activated protein kinases (MAPKs) in smooth muscle cells.[20] Three

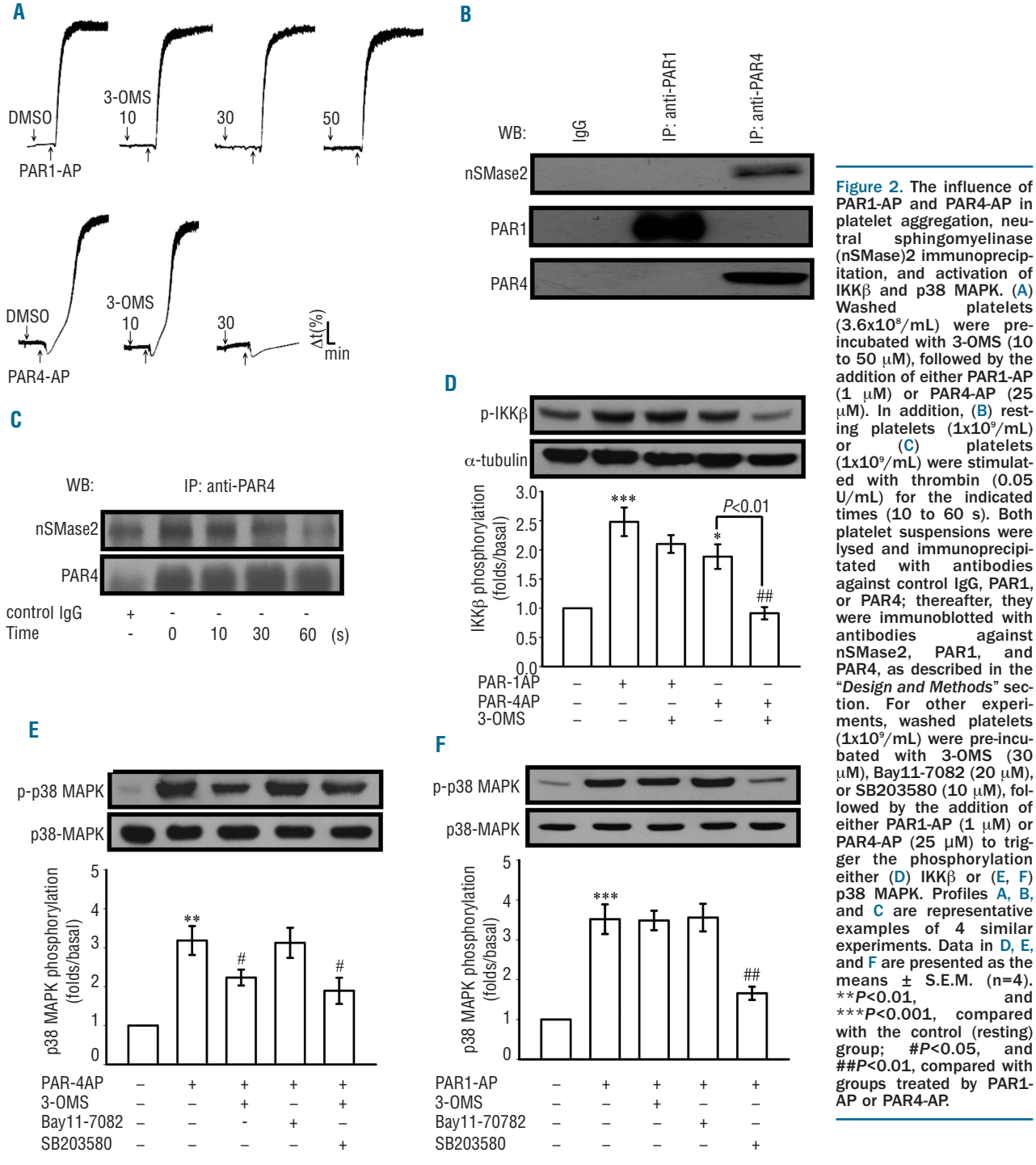

Figure 2. The influence of PAR1-AP and PAR4-AP in platelet aggregation, neutral sphingomyelinase (nSMase)2 immunoprecipitation, and activation of IKKβ and p38 MAPK. (A) Washed platelets ($3.6x10^8$/mL) were pre-incubated with 3-OMS (10 to 50 μM), followed by the addition of either PAR1-AP (1 μM) or PAR4-AP (25 μM). In addition, (B) resting platelets ($1x10^9$/mL) or (C) platelets ($1x10^9$/mL) were stimulated with thrombin (0.05 U/mL) for the indicated times (10 to 60 s). Both platelet suspensions were lysed and immunoprecipitated with antibodies against control IgG, PAR1, or PAR4; thereafter, they were immunoblotted with antibodies against nSMase2, PAR1, and PAR4, as described in the "*Design and Methods*" section. For other experiments, washed platelets ($1x10^9$/mL) were pre-incubated with 3-OMS (30 μM), Bay11-7082 (20 μM), or SB203580 (10 μM), followed by the addition of either PAR1-AP (1 μM) or PAR4-AP (25 μM) to trigger the phosphorylation either (D) IKKβ or (E, F) p38 MAPK. Profiles A, B, and C are representative examples of 4 similar experiments. Data in D, E, and F are presented as the means ± S.E.M. (n=4). $^{**}P<0.01$, and $^{***}P<0.001$, compared with the control (resting) group; $\#P<0.05$, and $\#\#P<0.01$, compared with groups treated by PAR1-AP or PAR4-AP.

MAPKs have been identified in platelets, namely ERK2, p38 MAPK, and JNK1.[21] We used their respective inhibitors PD98059, SB203580, and SP600125 to determine the relationship between these MAPKs and NF-κB activation in platelets. As shown in Figure 1E, administration of SB203580 (10 μM), but not that of PD98059 (20 μM) or SP600125 (10 μM), significantly inhibited IKKβ phosphorylation stimulated by thrombin. Moreover, 3-OMS (10 and 30 μM) but not Bay11-7082 (20 μM) attenuated thrombin-induced p38 MAPK phosphorylation; a higher concentration (40 μM) of Bay11-7082 remained ineffective (Figure 1F). These results suggested that the nSMase/ceramide-p38 MAPK-NF-κB cascade might play a crucial role in thrombin-induced platelet activation.

PAR4 but not PAR1 associated with nSMase responsible for ceramide formation

We employed PAR1-AP (SFLLRN) and PAR4-AP (AYPGFK) to determine thrombin receptors that would mediate ceramide formation; PAR1-AP is a PAR1 agonist and PAR4-AP is a PAR4 agonist.[22] As shown in Figure 2A, 3-OMS (30 μM) abolished platelet aggregation induced by PAR4-AP (25 μM) but not by PAR1-AP (1 μM). A higher concentration of 3-OMS (up to 50 μM) remained ineffective against PAR1-AP-induced aggregation. We observed that 3-OMS (30 μM) also reduced relative $[Ca^{2+}]i$ mobilization and P-selectin expression induced by PAR4-AP (25 μM) but not by PAR1-AP (1 μM) (*Online Supplementary Figure S2A and B*). Furthermore, Bay11-7082 (20 μM) significantly inhibited

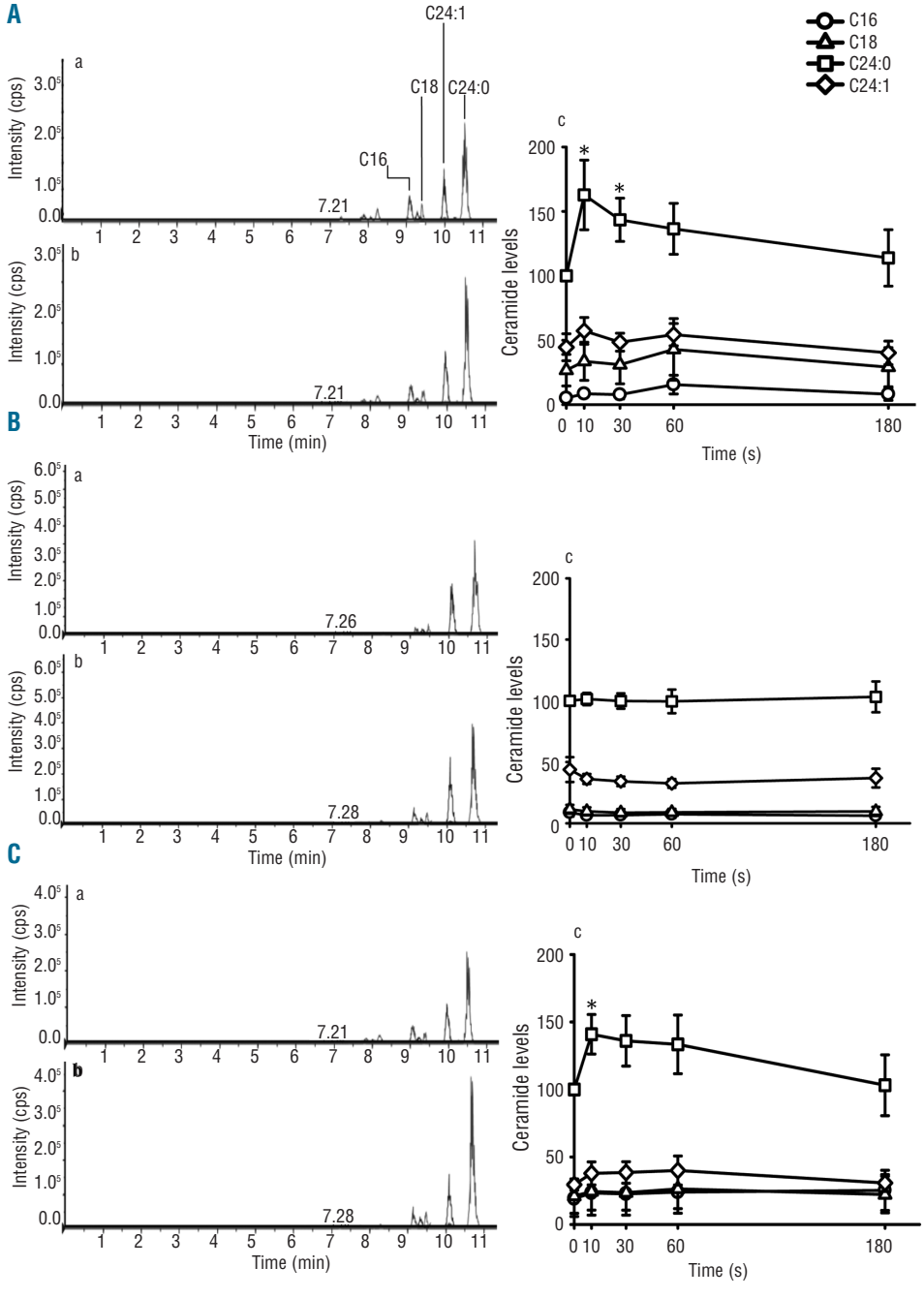

Figure 3. Various quantitative types of ceramide levels in platelets stimulated by thrombin, PAR1-AP or PAR4-AP by LC-MS/MS. Resting (A-C, a) or activated platelets (A-C, b) were stimulated by (A) thrombin (0.05 U/mL), (B) PAR1-AP (1 μM), or (C) PAR4-AP (25 μM) to trigger C16:0, C18:0, C24:0, and C24:1-ceramide formation. The full procedure for LC-MS/MS analysis is described in the *Design and Methods* section. Data in panels A to C (c) indicate the time-dependent ceramide formation (circle, C16:0; triangle, C18:0; square, C24:0; diamond, C24:1), shown as the means ± S.E.M. (n=6); **P*<0.05, compared with the resting group.

platelet aggregation, relative [Ca^{2+}]i mobilization, and P-selectin expression induced by both PAR4-AP (25 μM) and PAR1-AP (1 μM) (*Online Supplementary Figure S2*). In addition, platelets were immunoprecipitated with anti-PAR1 or PAR4 pAb, followed by immunoblotting with anti-nSMase2 mAb. As shown in Figure 2B, in resting platelets the nSMase was directly associated with PAR4 but not with PAR1. When platelets were stimulated with thrombin (for 10-60 s), associated nSMase was gradually dissociated from PAR4 in a time-dependent manner (Figure 2C). To establish the functional role of PAR4-nSMase in NF-κB activation, we tested the effect of 3-OMS on IKKβ phosphorylation induced by PAR1-AP or PAR4-AP. We observed that 3-OMS (30 μM) almost completely abolished IKKβ phosphorylation that occurred in response to PAR4-AP (25 μM) but did not affect phosphorylation caused by PAR1-AP (1 μM) (Figure 2D). Moreover, 3-OMS (30 μM) markedly diminished p38 MAPK phosphorylation stimulated by PAR4-AP (Figure 2E) but not by PAR1-AP (Figure 2F). By contrast, BAY11-7082 (20 μM) exerted no effect on p38 MAPK phosphorylation induced by either PAR4 or PAR1 (Figure 2E and F). Overall, the results clearly indicated that thrombin-induced nSMase activation was mediated by PAR4 but not by PAR1.

Quantification of ceramide types in platelets by LC/MS-MS

This study is the first to report accurate quantification of various types of ceramides in human platelets using a liquid chromatography mass spectrometer. The spectrometer was equipped with electrospray ionization and a double-quadruple mass analyzer with high sensitivity and resolution. Linearity of the calibration curve was proven for C16:0, C18:0, C24:0, and C24:1-ceramides from 10 to 1000 ng/mL. The coefficient of correlation (γ) for all measured sequences was at least 0.99 (*data not shown*). As shown in Figure 3A, C24:0-ceramide was the predominant form of ceramide present in human platelets; it increased 1.6-fold compared with the resting platelets 10 s after thrombin stimulation. By contrast, thrombin stimulation did not change the levels of C16:0, C18:0, and C24:1-ceramide (Figure 4A, c). We found no significant change for any type of ceramide in platelets stimulated by PAR1-AP (1 μM) (Figure 3B and C). By contrast, PAR4-AP (25 μM) treatment markedly increased C24:0-ceramide to approximately 1.4-fold compared with that of resting platelets (Figure 3C). In addition, pre-treatment with 3-OMS (30 μM) significantly inhibited C24:0-ceramide formation after PAR4-AP (25 μM) stimulation (*Online Supplementary Figure S3*). These results indicated that PAR4, but not PAR1, mediated thrombin-induced C24:0-ceramide formation in human platelets.

Exogenous C2-ceramide triggered platelet activation ex vivo and in vivo

We observed that C2-ceramide (50 μM), which is a cell-permeable ceramide analog, markedly activated p38 MAPK and IKKβ phosphorylation in a time-dependent manner. The maximal reaction was attained within 20-30 min after treatment (Figure 4A and B). Neither SCH79797 (1 μM), which is a PAR1 antagonist, nor tcY-NH2 (300 μM), which is a PAR4 antagonist, significantly inhibited p38 MAPK phosphorylation at 30 min after C2-ceramide stimulation. In addition, we found no effect of either 3-OMS (30 μM) or BAY11-7082 (20 μM) in this reaction (Figure 4C). In addition, the lactate dehydrogenase (LDH) study revealed that C2-ceramide (50 and 75 μM) incubated with platelets for 30 min did not significantly increase LDH activity (*Online Supplementary Figure S4A*), indicating that C2-ceramide did not affect platelet permeability or induce platelet cytolysis.

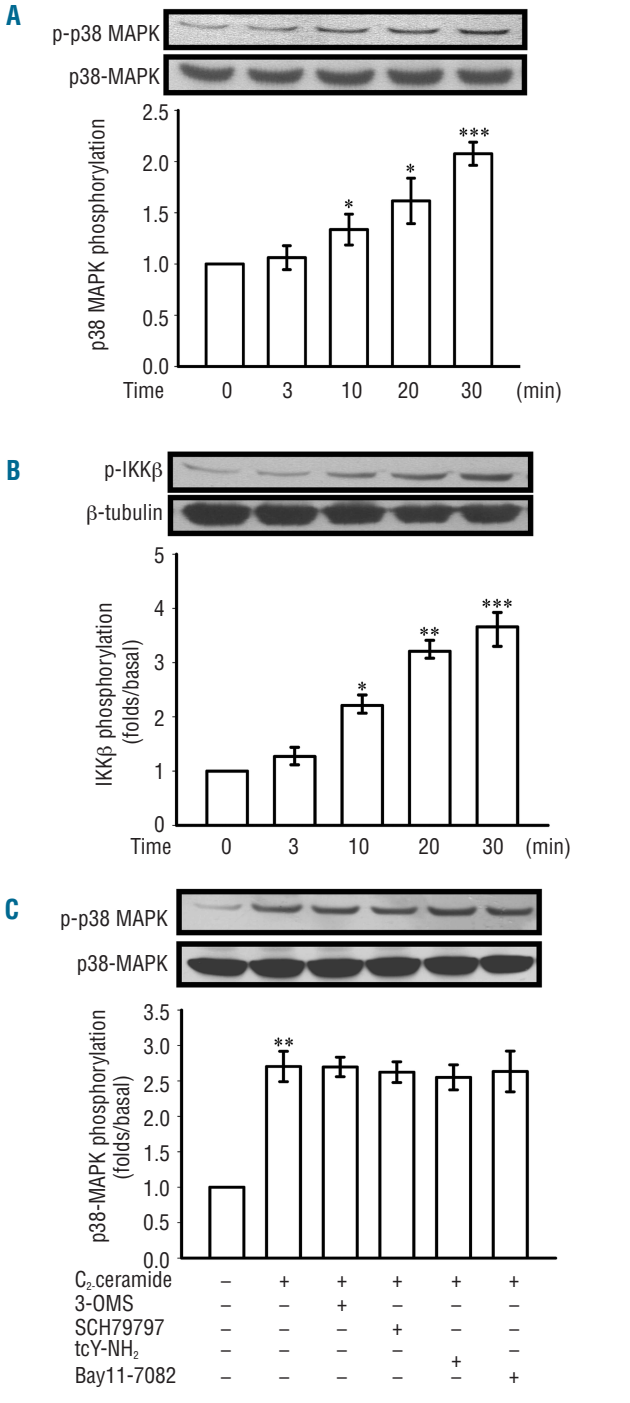

Figure 4. Exogenous c2-ceramide regulation of IKKβ and p38 MAPK phosphorylation. Washed platelets (1.0x10⁹/mL) were pre-incubated with 3-OMS (30 μM), SCH79797 (1 μM), trans-cinnamoyl-YPGKF-NH2 (tcY-NH2; 300 μM) or Bay11-7082 (20 μM). Thereafter, C2-ceramide (50 μM) was added for 3 to 30 min to trigger (A, C) p38 MAPK and (B) IKKβ phosphorylation. Data are presented as the means ± S.E.M. (n=4); *P<0.05, **P<0.01, and ***P<0.001, compared with the control (resting) group.

It clearly shows no cytotoxic effects of C2-ceramide on platelets at these concentrations. C2-ceramide (50 μM) did not directly trigger platelet aggregation (*Online Supplementary Figure S4B*). However, C2-ceramide (50 μM) significantly potentiated platelet activation, such as platelet aggregation (*Online Supplementary Figure S4B*), relative $[Ca^{2+}]i$ mobilization (*Online Supplementary Figure S4C*), and P-selectin expression (*Online Supplementary Figure S4D*) induced by a sub-threshold concentration of thrombin (0.03 U/mL) in washed human platelets.

As shown in Figure 5A, during thrombus formation in the microvessels of mice pre-treated with fluorescein sodium (15 μg/kg), the occlusion time required was approximately 100 s. When C2-ceramide (10 mg/kg) was administered after pre-treatment with fluorescein sodium, the occlusion time was significantly shorter than in the control group (0.5% DMSO, 101.5±14.6 s; C2-ceramide, 67.2±5.1 s; n=5, $P<0.05$) (Figure 5A). By contrast, no significant change was noted in the time required for thrombus formation in mice pre-treated with dihydroceramide (DHC; 10 mg/kg), which is an inactive form of C2-ceramide (0.5% DMSO, 97.4±4.4 s *vs.* DHC, 91.0±2.4 s; n=5, $P>0.05$) (Figure 5A). Figure 5B shows typical microscopic images of a microthrombus formed during treatment with fluorescein sodium. The mesenteric microvessels exposed to irradiation for either 5 or 60 s after fluorescein sodium treatment revealed no thrombotic platelet plug formation either in the control group (0.5% DMSO treatment) (Figure 5B) or in the DHC-treated group (Figure 5B). In microvessels treated with C2-ceramide, platelet plug formation was evident at 60 s but not at 5 s after irradiation (Figure 5B). The blood flow rate of the C2-ceramide-treated group was slower than that of the solvent- or DHC-treated group because of the thrombotic platelet plug development (Figure 5B).

Discussion

This study demonstrated, for the first time, that throm-

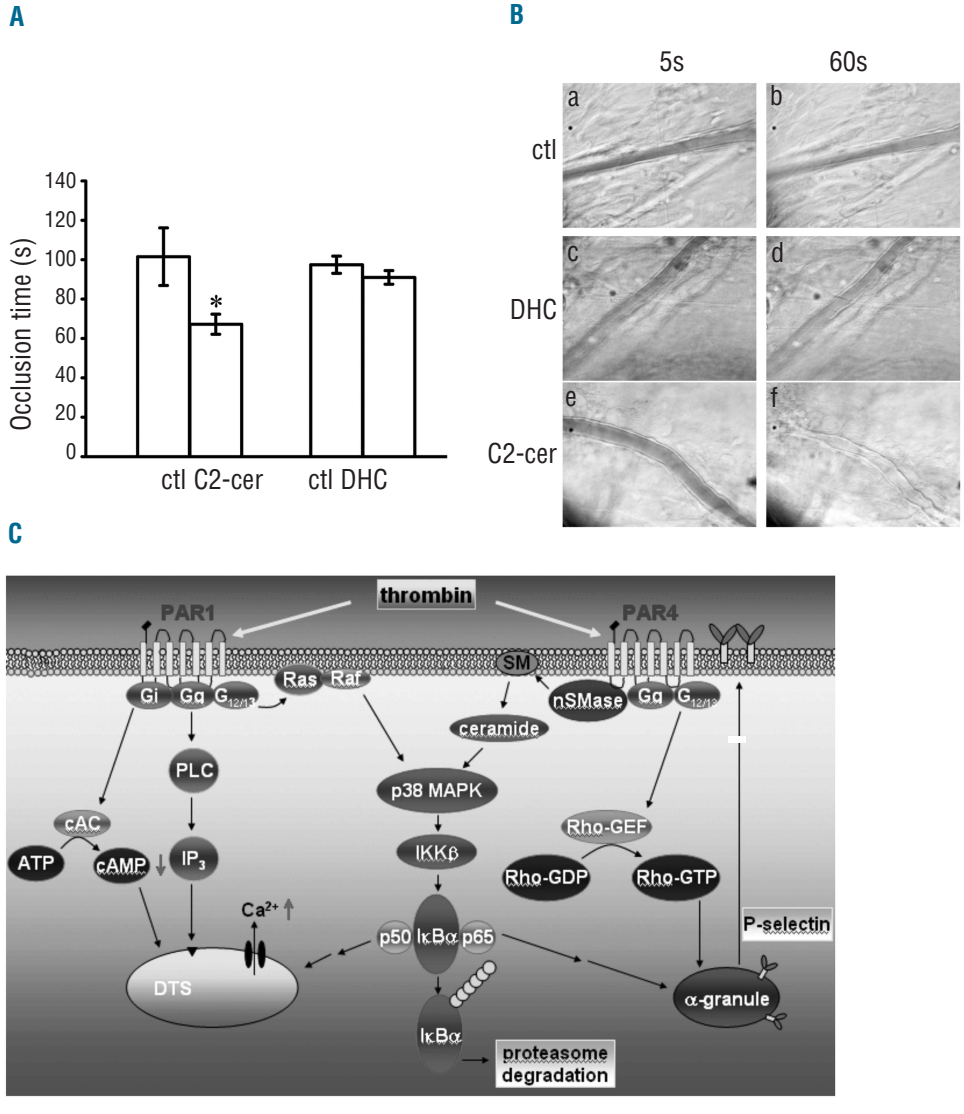

Figure 5. C2-ceramide shortened the occlusion time for inducing thrombus formation in mesenteric venules of mice and the hypothetical scheme of signal pathways in thrombin-induced platelet activation. Mice were administered isovolumetric 0.5% DMSO (solvent control; ctl), dihydroceramide (DHC; 10 mg/kg), or C2-ceramide (C2-cer; 10 mg/kg). Thereafter, mesenteric venules were selected for irradiation to induce microthrombus formation. The bar graphs (A) present the means ± S.E.M. of the occlusion time (s) for inducing platelet plug formation (n=6). *$P<0.05$ compared with the solvent control. Microscopic images (B) were obtained at 5 s (a, c, e) and 60 s (b, d, f) after administration of the fluorescent dye (15 μg/kg) and transillumination in mice treated with either solvent (a and b), DHC (10 mg/kg) (c and d), or C2-cer (10 mg/kg) (e and f). Photographs are representative examples of 6 similar experiments (400×). (C) Thrombin bound to G protein-coupled PAR1, and activated the PAR1-mediated signal events (such as PLC-IP3-$[Ca^{2+}]i$ pathway or inhibition of cAC), resulting in decreased cAMP formation. Thrombin also bound to PAR4, which was associated with nSMase, and subsequently activated the ceramide-p38 MAPK-NF-κB cascade, ultimately stimulating $[Ca^{2+}]I$ mobilization and platelet activation (P-selectin expression). cAC: cytosolic adenylate cyclase; cAMP: cyclic AMP; DTS: dense tubular system; IP3: inositol 1,4,5-trisphosphate; nSMase: neutral sphingomyelinase; PAR: proteinase-activated receptor; PLC: phospholipase C.

bin receptor PAR4 but not PAR1 was associated with nSMase in human platelets. PAR4-nSMase can be activated by thrombin to increase the intracellular ceramide levels, and this is followed by activation of downstream p38 MAPK-NF-κB signaling (Figure 5C). The sphingolipids are a family of lipids found ubiquitously in eukaryotic plasma membranes; they have emerged as active signaling molecules involved in the cell regulation process.[23] The putative second messenger ceramide is generated by the SM cycle, in which membrane SM is hydrolyzed in response to acid SMase or nSMase. Several biological responses are associated with nSMase activation, including inflammation, atherogenesis, and apoptosis.[24,25]

Two forms of mammalian nSMase have been identified, namely nSMase1 and nSMase2.[26] nSMase1 is localized in the endoplasmic reticulum and Golgi, and most likely functions as lysophospholipase C but not as an SMase.[26] nSMase2 is localized in Golgi and the inner leaflet of the plasma membrane, and can function as an SMase in cells.[27] Ceramide plays an important role in regulating cell proliferation and differentiation, the cell cycle, and apoptosis.[28] The most common ceramides display acyl chain lengths of 16 to 24 carbon atoms (C16-C24) and are produced in response to various stress stimuli, including TNF-α and oxidative stress.[28] One study demonstrated that downregulation of nSMase2 by siRNA prevented a confluence-induced increase in the C24:0-ceramide level of MCF7 cells.[29] Our findings confirmed that the C24:0-ceramide level was increased after thrombin or PAR4-AP stimulation in human platelets.

The role of ceramide in the regulation and development of cardiovascular diseases has been extensively studied recently. This has revealed that ceramide-mediated signaling involves a novel pathophysiological mechanism, which underlies the endothelial dysfunction associated with the overproduction of cytokines in ischemic heart diseases.[30] We also demonstrated for the first time that ceramide time-dependently triggered platelet activation (i.e. p38 MAPK and NK-κB) *ex vivo,* and significantly potentiated platelet plug formation *in vivo*.

Thrombin activates human platelets by cleaving and activating its receptors, which include PAR1 and PAR4. In turn, these receptors activate Gq, G12/13, and possibly the Gi family, which leads to the activation of PLC, PI3-kinase, and the monomeric G proteins (i.e. Rho). The activation also causes an increase in cytosolic Ca^{2+} concentration and inhibits cyclic AMP formation (Figure 5C).[31] PAR1 has been shown to induce a rapid transient spike in Ca^{2+} concentration, whereas PAR4 induces a robust prolonged response.[4] These differences in the timing and magnitude of the 2 PAR signals suggest distinct roles. This study played a pioneering role in determining that the pathway of PAR4, but not that of PAR1, is associated with nSMase in human platelets (Figure 5C).

The function of NF-κB has been extensively studied in nucleated cells. Genes regulated by NF-κB include those involved in the processes of inflammation, cell survival and differentiation, and proliferation responses.[32] Therefore, NF-κB is an attractive target for therapeutic interventions against various diseases. The pleiotropic NF-κB normally exists as an inactive cytoplasmic complex, the predominant form of which is a heterodimer composed of p50 and p65 subunits. These subunits are tightly bound to inhibitory proteins of the IκB family (Figure 5C).[32] After IκBα is phosphorylated by the IKK complex, it starts dissociating from NF-κB subunits, and is ubiquitinated and rapidly degraded by the proteasome.[32] Three IKK family members (α, β, and γ) are expressed in platelets, with the β form being the most strongly expressed. IKK phosphorylation has been proposed as a major upstream regulator for IκBα phosphorylation, leading to NF-κB activation (Figure 5C).[32] Although prior studies have shown that NF-κB is activated after platelet activation,[32] the functional significance of NF-κB remains unclear. Pre-treatment with an NF-κB inhibitor has been found to prevent multiple platelet activities, including the following: platelet adhesion to fibrinogen, integrin αIIbβ3 activation, P-selectin expression, and thromboxane A2 (TxA2) formation.[8] In the present study, time-dependent phosphorylation of both IKKβ and IκBα was observed in platelets activated by thrombin, PAR1-AP, PAR4-AP, and C2-ceramide. In addition, Bay11-7082 (an inhibitor of NF-κB) attenuated platelet aggregation, relative $[Ca^{2+}]i$ mobilization, and P-selectin expression stimulated by thrombin. By contrast, Bay11-7082 did not attenuate p38 MAPK phosphorylation stimulated by thrombin, PAR1-AP, PAR4-AP or C2-ceramide. This finding suggests that NF-κB might participate in physiopathological functions in anucleated platelets, and might be regulated by upstream regulators such as nSMase/ceramide and p38 MAPK (Figure 5C).

The physiopathological roles of JNKs and ERKs in platelets remain unclear, but might entail the suppression of αIIbβ3 integrin activation or the negative regulation of platelet activation.[33] Nevertheless, p38 MAPK provides a crucial signal for platelet activation.[34] Among the numerous downstream targets of p38 MAPK, the most physiologically relevant in platelets is cytosolic phospholipase A2, which catalyzes arachidonic acid release to produce TxA2.[34] This study demonstrated that p38 MAPK, but not ERKs or JNKs, is an upstream regulator for NF-κB activation in human platelets.

Previous studies have reported that nSMase plays an important role in regulating p38 MAPK and NF-κB activation in macrophages.[20] Our findings also revealed that 3-OMS, an inhibitor of nSMase, attenuated platelet activation (i.e. NF-κB activation and p38 MAPK phosphorylation) stimulated by thrombin and PAR4-AP, but not by PAR1-AP or C2-ceramide, indicating that PAR4-nSMase/ceramide acted as an upstream regulator for platelet activation.

In conclusion, the most important finding of this study was that thrombin activates nSMase by binding to PAR4, but not to PAR1, to increase the C24:0-ceramide level. This is followed by activation of p38 MAPK, which in turn initiates NF-κB activation, and ultimately induces platelet activation. Our results revealed a novel physiological significance of PAR4-nSMase activation, and identified a new role for the ceramide-p38 MAPK-NF-κB cascade in platelet activation.

Funding

This work was supported by grants from the National Science Council of Taiwan (NSC97-2320-B-038-016-MY3 and NSC100-2320-B-038-021-MY3) and Cathay General Hospital-Taipei Medical University (100-CGH-TMU-12 and 101-CGH-TMU-08).

Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org

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