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Taiwan Cobra Phospholipase A_2 Elicits Posttranscriptional Up-regulation of ADAM17 in Human Neuroblastoma SK-N-SH Cells

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ABSTRACT

Taiwan cobra phospholipase A_2 (PLA_2) treatment promoted proADAM17 processing into mature ADAM17 in human neuroblastoma SK-N-SH cells. The abolishment of catalytic activity caused a drastic drop in the PLA_2 ability to induce ADAM17 maturation, and lysophosphatidylcholine treatment mimicked the effect of PLA_2. ADAM17 activity measurement, ADAM17 cell surface levels, TNFR2 ectodomain shedding, and ADAM17 mRNA transcription supported that posttranscriptional up-regulation of ADAM17 occurred in PLA_2-treated SK-N-SH cells. PLA_2 treatment induced p38 MAPK activation and ERK inactivation. p38 MAPK activation suppression by SB202190 (p38 MAPK inhibitor) abolished posttranscriptional up-regulation of ADAM17 in PLA_2-treated cells, while treatment with U0126 (MEK1 and MEK2 inhibitor) increased ADAM17 maturation in SK-N-SH cells. Constitutively active MEK1 expression abrogated PLA_2-induced ADAM17 maturation. Taken together, our data indicate that PLA_2-evoked p38 MAPK activation and ERK inactivation are involved in ADAM17 posttranscriptional up-regulation, and suggest that the action of PLA_2 is catalytic activity-dependent. J. Cell. Biochem. 111: 148–157, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: PLA_2; ADAM17; POSTTRANSCRIPTIONAL UP-REGULATION; ERK; p38 MAPK

A disintegrin and metalloprotease domain (ADAM) is a gene family of multidomain membrane-anchored proteins comprised of more than 30 members in various animal species and has been implicated in pathophysiological conditions [Mochizuki and Okada, 2007; Edwards et al., 2008]. The ADAM17/tumor necrosis factor α converting enzyme (TACE) is a member of the ADAM family and originally described as being responsible for the proteolytic cleavage of membrane-anchoring precursor form of tumor necrosis factor α (TNFα) to generate a soluble form of TNFα [Arribas and Ruiz-Paz, 2005]. Subsequent studies have shown that ADAM17 is also involved in the shedding of other biologically active proteins, including heparin-binding epidermal growth factor (HB-EGF), transforming growth factor α, tumor necrosis factor receptor 1 (TNFR1), TNFR2, EGF receptor (EGFR), vascular cell adhesion molecule-1, L-selectin, interleukin receptors, Notch and prion proteins [Seals and Courtneidge, 2003; Cisse et al., 2007]. ADAM17 has been shown to be synthesized as a zymogen, which is constitutively processed in the secretory pathway. An increase in the ADAM17 surface expression up-regulated ADAM17 sheddase activity [Santiago-Josefat et al., 2007]. Thus, in addition to protein expression, generation of mature ADAM17 from its proform is proved to be related to its sheddase activity and regulated by ERK-mediated phosphorylation of Thr735 of ADAM17 in HeLa and COS-7 [Soond et al., 2005]. Alternatively, ADAM17 sheddase activity in platelets and endothelial cells is up-regulated via the p38 MAPK-mediated pathway [Singh et al., 2005; Brill et al., 2009]. Recent studies show that Thr735 phosphorylation by p38 MAPK also regulates cell surface levels and ADAM17 processing [Xu and Derynck, 2010]. Noticeably, elevation in serum phospholipase A_2 (PLA_2) activity accompanied with an increase in soluble TNFR1 and TNFR2 levels is detected in the serum of schizophrenic patients [Gatta et al., 1990; Coelho et al., 2008]. Accordingly, PLA_2 treatment

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may elicit an increase in ADAM17 activity, which causes the release of soluble TNFRs (sTNFRs). Gatta et al. [1990] suggested that elevation in serum PLA_2 activity may reflect an increment of intraneuronal PLA_2 activity in the brain. Thus, the causal relationship between ADAM17 sheddase activity and PLA_2 treatment in neuronal cells merits further investigation. In this research, the effect of *Naja naja atra* (Taiwan cobra) PLA_2 on ADAM17 activity in human neuroblastoma SK-N-SH cells was examined. SK-N-SH cells have cellular characteristics representative of those in an immature nervous system and exhibit a neuronal phenotype with the expression of multiple neurochemical markers [Biedler et al., 1978]. Moreover, unlike primary cultures which are usually a mixture of different neurons, microglial cells, and fibroblasts, the model studied here with SK-N-SH cells allows us to directly investigate the effect of PLA_2 treatment in one single cell type. Our data indicated that PLA_2 treatment induced posttranscriptional up-regulation of ADAM17 through p38 MAPK activation and ERK inactivation pathways, suggesting that the PLA_2 effect is catalytic activity-dependent.

MATERIALS AND METHODS

PLA_2 from the venom of *Naja naja atra* (Taiwan cobra) was isolated as previously described [Chang et al., 1998]. Recombinant M-PLA_2, PLA_2(H48A), and PLA_2(D49K) were prepared according to our published procedure [Chiou et al., 2008; Liu et al., 2009]. PLA_2 activity was measured spectrophotometrically using the PLA_2 activity kit from Cayman Chemical (Ann Arbor, MI). The enzymatic activity of M-PLA_2 was 3.6% that of PLA_2, and PLA_2(H48A) and PLA_2(D49K) did not show significantly detectable enzymatic activity. Arachidonic acid, brefeldin, cycloheximide, lysophosphatidylcholine (LPC), MTT, MG-132, SB202190, U0126, and anti-β-actin antibody were obtained from Sigma–Aldrich, Inc. Anti-p38 MAPK, anti-phospho-p38 MAPK, anti-ERK, anti-phospho-ERK, anti-JNK, anti-phospho-JNK, anti-TNFR2, and anti-FasL antibodies were products of Cell Signaling Technology. Anti-ADAM17 (H-300) antibody (specifically recognized proADAM17) and anti-Fas (N-18) antibody were obtained from Santa Cruz Biotechnology, Inc., and anti-ADAM17-activation site (ab39163) antibody (specifically recognized mature ADAM17) was obtained from ABcam. Anti-TNFR1 antibody, monoclonal anti-human ADAM17-Fluorescein, monoclonal anti-human TNFR1-Fluorescein, and monoclonal anti-human TNFR2-Fluorescein were purchased from R&D System, Inc., and ADAM17 activity assay kit was the product of Calbiochem. Anti-ADAM10 antibody was purchased from AnaSpec, Inc., and horseradish peroxidase-conjugated secondary antibodies were obtained from Pierce. Cell culture supplies were purchased from GIBCO/Life Technologies, Inc. Unless otherwise specified, all other reagents were of analytical grade.

CELL CULTURE AND VIABILITY ASSAY

SK-N-SH cells were cultured with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, 2 mM glutamine, and penicillin (100 U/ml)/streptomycin (100 μg/ml) incubated at 37°C in an incubator humidified with 95% air and 5% CO_2. Exponentially growing cells (1×10^5) were plated in 96-well plates and treated with a series of concentrations of PLA_2 in serum-free medium. At suitable time intervals, MTT solution was added to each well at final concentration of 0.5 mg/ml and incubated for 4 h. Formazan crystals resulting from MTT reduction were dissolved by addition of 100 μl DMSO per well. The absorbance was detected at 595 nm using a plate reader.

RNA PREPARATION AND REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION (RT-PCR)

Total RNA was isolated from untreated control cells or PLA_2-treated cells using the RNeasy minikit (Qiagen, Inc., Valencia, CA) according to the instructions of the manufacturer. Reverse transcriptase reaction was performed with 2 μg of total RNA using M-MLV reverse transcriptase (Promega) according to the manufacturer's recommendations. A reaction without reverse transcriptase was performed in parallel to ensure the absence of genomic DNA contamination. After initial denaturation at 95°C for 10 min, PCR amplification was performed using GoTaq Flexi DNA polymerase (Promega) followed by 30 cycles at 94°C for 60 s, 55°C for 60 s, and 72°C for 60 s. After a final extension at 72°C for 5 min, PCR products were resolved on 2% agarose gels and visualized by ethidium bromide transillumination under UV light. Primer sequences were as follows: TNFR2 (forward): 5′-ACATCAGACGTGGTGTGCAA-3′; TNFR2 (reverse): 5′-CCAACTGGAAGAGCGAAGTC-3′; ADAM17 (forward): 5′-CAGCACAGCTGCCAAGTCATT-3′; ADAM17 (reverse): 5′-CCAGCATCTGCTAAGTCACTTCC-3′. The PCR reaction yielded PCR products of 323 and 235 bp for TNFR2 and ADAM17, respectively. Each reverse-transcribed mRNA product was internally controlled by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) PCR using primers 5′-GAGTCAACGGATTTGGTCGT-3′ (forward) and 5′-TGTGGTCATGAGTCCTTCCA-3′ (reverse), yielding a 512 bp PCR product. The TNFR2 and ADAM17 reverse transcriptase-PCR products were subsequently confirmed by direct sequencing.

DNA TRANSFECTION

The pCMV-MEK1 (expressed the constitutively active MEK1) vector was generous gift from Dr. W. C. Hung (National Sun Yat-Sen University, Taiwan). pCMV-MEK1 vector expressed the constitutively active MEK1 under control of the CMV promoter. Human ADAM17 expression plasmid (pME18S-ADAM17) and ADAM10 expression plasmid (pME18S-ADAM10) were kindly provided by Dr. E. Nishi (Kyoto University, Japan). The plasmids were transfected into SK-N-SH cells using Pipette-type Electroporator (MicroPorator-MP100, Digital Bio Tech. Co., Korea).

RNA INTERFERENCE

ADAM17 siRNA (catalog number sc-36604) and negative control siRNA (catalog number sc-37007) were purchased from Santa Cruz Biotechnology, Inc. For the transfection procedure, cells were grown to 60% confluence and ADAM17 or control siRNA were transfected using Lipofetamine™ 2000 (Invitrogen) according to the manufacturer's instructions. After 24 h posttransfection, the cells were exposed to PLA_2 for an additional 24 h, and then harvested for Western blot analyses.

DETERMINATION OF SOLUBLE TNFR2 BY ELISA

After treatment with PLA_2 for 24 h, the culture medium of SK-N-SH cells were collected and centrifuged at 12,000 rpm for 10 min, and the clarified supernatants were collected. ELISA for soluble TNFR2 (sTNFR2) was performed followed the manufacturer's protocol (Quantikine® sTNF-R II immunoassay kit, R&D system, Inc.). Developed assay plates were read at wavelength 450 nm using a plate reader, and the results were calculated using a standard curve generated each time an assay was performed.

MEASUREMENT OF ADAM17 ACTIVITY

After specific treatments, SK-N-SH cells were harvested and washed with PBS three times. The cells were resuspended in 20 mM Tris–HCl (pH 7.5) containing 1 mM $ZnCl_2$ and homogenized by sonication. Crude membranes were separated by precipitation at 12,500*g* for 20 min and dissolved in 20 mM Tris–HCl (pH 7.5) containing 1 mM $ZnCl_2$ and 2% Nonidet P-40 (Sigma). After incubation on ice for 1 h, followed by centrifugation at 12,500*g* for 5 min, the supernatant (referred to in the following as detergent extract) was used for enzyme analysis. ADAM17 activity was measured according to the manufacturer's protocol (Innozyme™ TACE activity kit, Calbiochem). The detergent extract of PLA_2-treated cells was added into ELISA plates precoated with anti-ADAM17 antibody, and the activity of captured ADAM17 is then measured. Its fluorescence-related enzymatic cleavage of substrate was monitored at 320 nm excitation and 405 nm emission wavelength using a microplate fluorescence reader.

FLOW CYTOMETRY ANALYSES OF CELL SURFACE EXPRESSION OF ADAM17, TNFR1, AND TNFR2

After specific treatment, nonspecific antibody binding sites were blocked by incubation with PBS containing 0.01% human IgG. One-fluorescent parameter flow cytometry was performed by staining cells with monoclonal anti-human ADAM17-Fluorescein, anti-human TNFR1-Fluorescein or anti-human TNFR2-Fluorescein according to the manufacturer's protocol (R&D System, Inc.). The stained cells were analyzed by a Beckman Coulter Epics XL flow cytometer.

WESTERN BLOT ANALYSIS

After specific treatments, cells were incubated in lysis buffer (20 mM Tris–HCl, pH 7.5, 1% Triton X-100, 1 mM EDTA, 150 mM NaCl, 10% glycerol, 1 mM Na_3VO_4, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixtures) for 20 min on ice. After insoluble debris was removed by centrifugation at 13,000*g* at 4°C for 15 min, the supernatants were collected and assayed for protein concentration using the Bradford method. An equal amount of protein per sample (15 μg) was resolved on SDS–PAGE and transferred onto a PVDF membrane. The transferred membranes were blocked using 5% nonfat milk in PBST (PBS containing 0.05% Tween-20) and incubated with appropriate primary antibodies and horseradish peroxidase-conjugated secondary antibodies. The immune complexes were detected by SuperSignal West Pico Chemiluminescent substrate kit (Pierce).

STATISTICAL ANALYSIS

All data are presented as mean ± SD. Significant differences among the groups were determined using the unpaired Student's *t*-test. A value of $P < 0.05$ was taken as an indication of statistical significance. All the figures shown in this article were obtained from at least three independent experiments with similar results.

RESULTS

As shown in Figure 1A, PLA_2 treatment induced an increase in ADAM17 protein expression accompanied with a decrease in proADAM17 protein expression in SK-N-SH cells in a concentration-dependent manner. Compared with that of PLA_2, higher concentrations of catalytically inactive PLA_2s including M-PLA_2, PLA_2(H48A), and PLA_2(D49K) were required to induce a notable ADAM17 up-regulation in SK-N-SH cells. Figure 1B shows that the transcriptional level of ADAM17 mRNA in PLA_2-treated cells did not significantly change as evidenced by RT-PCR assay. Consistent with Western blot analyses results, PLA_2 or catalytically inactive PLA_2 induced an increase in ADAM17 activity (Fig. 1C,D). To up-regulate ADAM17 activity to a similar extent, the used concentration of recombinant PLA_2s was at least 100-fold that of PLA_2. Apparently, abolishment of catalytic activity attenuated the ability of PLA_2 to up-regulate ADAM17 activity, suggesting that the PLA_2 effect was catalytic activity-dependent. Conversion of proADAM17 into mature ADAM17 is mediated through removing the prodomain by proprotein convertases in the late Golgi compartment. Mature ADAM17 is then trafficked to the membrane cell surface [Endres et al., 2003; Peiretti et al., 2003a]. Flow cytometry analyses showed that cell surface expression of ADAM17 increased after PLA_2 treatment (Fig. 1E), again supporting that PLA_2 treatment increasingly induced proADAM17 processing into ADAM17. Unlike arachidonic acid, LPC treatment up-regulated ADAM17 maturation (Fig. 1F). Consistently, an approximate 1.5-fold increase in ADAM17 activity was noted upon exposure to 50 μM LPC (data not shown). These results again supported that PLA_2 activity was involved in promoting the maturation of ADAM17.

Given that activated ERK- or p38 MAPK-mediated pathways were suggested to be involved in up-regulating ADAM17 activity [Singh et al., 2005; Soond et al., 2005; Brill et al., 2009; Xu and Derynck, 2010], phosphorylation of MAPKs was examined in PLA_2-treated cells. Figure 2A shows that the level of phospho-p38 MAPK was notably increased after 24 h of PLA_2 treatment, while that of phospho-ERK was reduced after PLA_2 treatment. In contrast, treatment with PLA_2 did not result in detectable levels of phospho-JNK. Catalytically inactive PLA_2s also induced p38 MAPK activation and ERK inactivation. SB202190 pretreatment abolished PLA_2-induced ADAM17 maturation (Fig. 2C), suggesting that p38 MAPK was involved in posttranscriptional up-regulation of ADAM17. The immunoblotting and flow cytometry analyses results showed that U0126 treatment elicited an increase in the conversion of proADAM17 into ADAM17 and the amount of detectable ADAM17 on the cell surface (Fig. 2D), reflecting that activated ERK suppressed the production of ADAM17. Overexpression of constitutively active MEK1 repressed PLA_2-elicited posttranscriptional up-regulation of

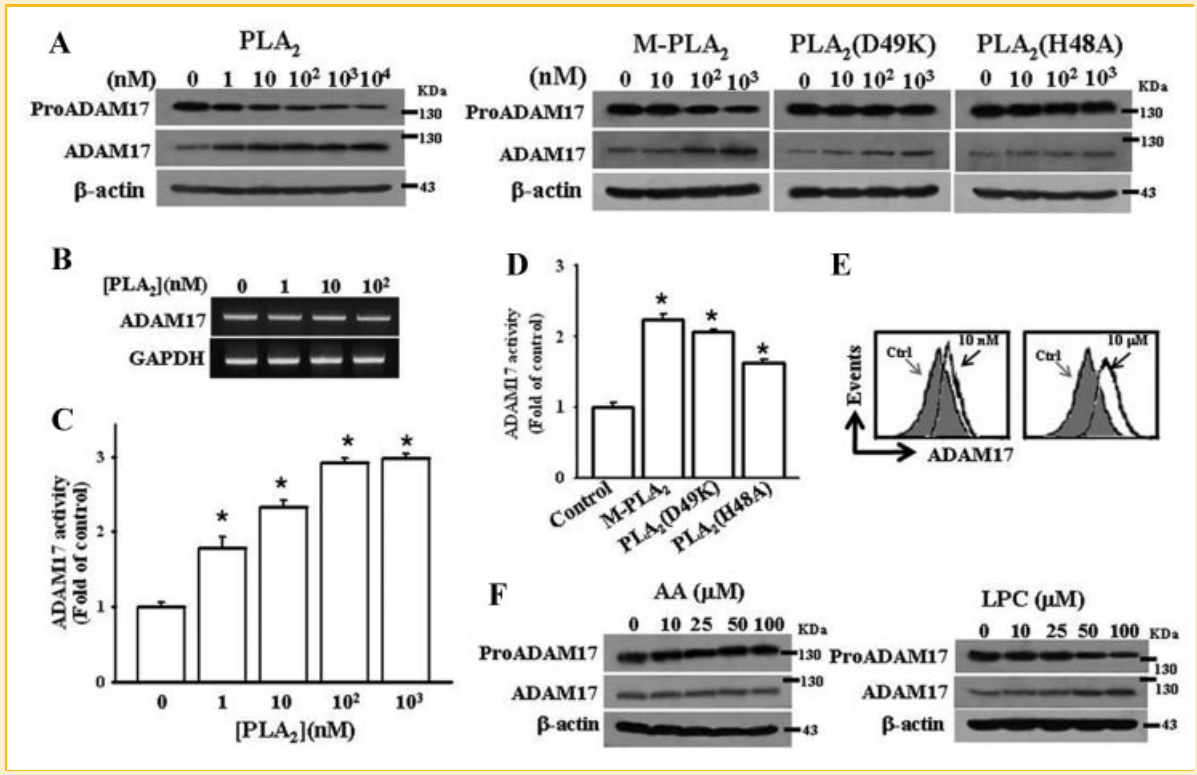

Fig. 1. PLA_2 treatment induced posttranscriptional up-regulation of ADAM17 in SK-N-SH cells. A: Effect of PLA_2, M-PLA_2, PLA_2(D49K), or PLA_2(H48A) on ADAM17 maturation. SK-N-SH cells were treated with indicated concentrations of PLA_2, M-PLA_2, PLA_2(D49K), or PLA_2(H48A) for 24 h. B: Detecting the transcription of ADAM17 mRNA using RT-PCR. SK-N-SH cells were treated with indicated concentrations of PLA_2 for 24 h. C: PLA_2 treatment induced an increase in ADAM17 activity. SK-N-SH cells were treated with indicated concentrations of PLA_2 for 24 h. The activity of ADAM 17 was detected according to the procedure described in the Materials and Methods Section (mean ± SD, $^*P < 0.05$, compared with untreated control cells). D: Effect of M-PLA_2, PLA_2(D49K), or PLA_2(H48A) on ADAM17 activity in SK-N-SH cells. SK-N-SH cells were treated with indicated concentrations of 1 μM M-PLA_2, 1 μM PLA_2(D49K), or 1 μM PLA_2(H48A) for 24 h (mean ± SD, $^*P < 0.05$, compared with untreated control cells). E: Surface expression levels of ADAM17 in PLA_2-treated cells. SK-N-SH cells were treated with 10 nM or 10 μM PLA_2 for 24 h, and then the cells were stained with anti-human ADAM17-Fluorescein to determine the relative expression levels of ADAM17 by flow cytometry. Results are representative of three independent experiments. F: Western blot analyses of ADAM17 protein expression in arachidonic acid (AA)-treated cells and LPC-treated cells. SK-N-SH cells were treated with indicated concentrations of AA (left panel) or LPC (right panel) for 24 h.

ADAM17 (Fig. 2D). ADAM17 activity measurement further supported the notion that ERK played a negative role in regulating ADAM17 maturation in SK-N-SH cells (Fig. 2E). Noticeably, U0126 treatment induced p38 MAPK activation in SK-N-SH cells, while transfection of constitutively active MEK1 suppressed PLA_2-elicited p38 MAPK activation (Fig. 2D). Taken together, the results inferred that ERK negatively regulated ADAM17 maturation through suppression of p38 MAPK phosphorylation. Treatment with 10 nM PLA_2 increased proADAM17 processing into ADAM17 in pME18S-ADAM17- or ADAM17 siRNA-transfected SK-N-SH cells as evidenced by Western blot analyses and ADAM17 activity assay (Fig. 2F). The detectable ADAM17 activity was in parallel with the ADAM17 surface expression, reflecting that ADAM17 activity closely responded to the maturation of ADAM17. Our recent studies showed that PLA_2 induced apoptosis in SK-N-SH cells [Chen et al., 2009]. Although treatment with 10 μM PLA_2 led to a notable reduction in approximate 40% viability of SK-N-SH cells (Fig. 2G), overexpression of ADAM17 or down-regulation of ADAM17 did not affect the cytotoxicity of PLA_2 on SK-N-SH cells. These results reflected that PLA_2-induced death of SK-N-SH cells was not associated with ADAM17 surface expression. Figure 2H shows that protein trafficking blockade by brefeldin abolished the PLA_2 effect on ADAM17 maturation but did not significantly change p38 MAPK phosphorylation. This implied that PLA_2-elicited p38 MAPK activation was an upstream event in promoting ADAM17 trafficking.

As shown in Figure 3A, PLA_2 treatment induced a decrease in TNFR2 protein expression in a concentration-dependent manner. Compared with that of TNFR2, Fas, and FasL did not significantly change after PLA_2 treatment (Fig. 3B), while protein expression of TNFR1 was marginally but significantly reduced. Flow cytometry analyses also revealed that PLA_2 treatment elicited a notable reduction in cell surface levels of TNFR2 compared with that of TNFR1 (Fig. 3C). Figure 3D shows that the ability of M-PLA_2, PLA_2(H48A), and PLA_2(D49K) to induce TNFR2 down-regulation was lower than that of native PLA_2. These reflected that catalytic activity-dependent pathway was involved in PLA_2-triggered TNFR2 down-regulation.

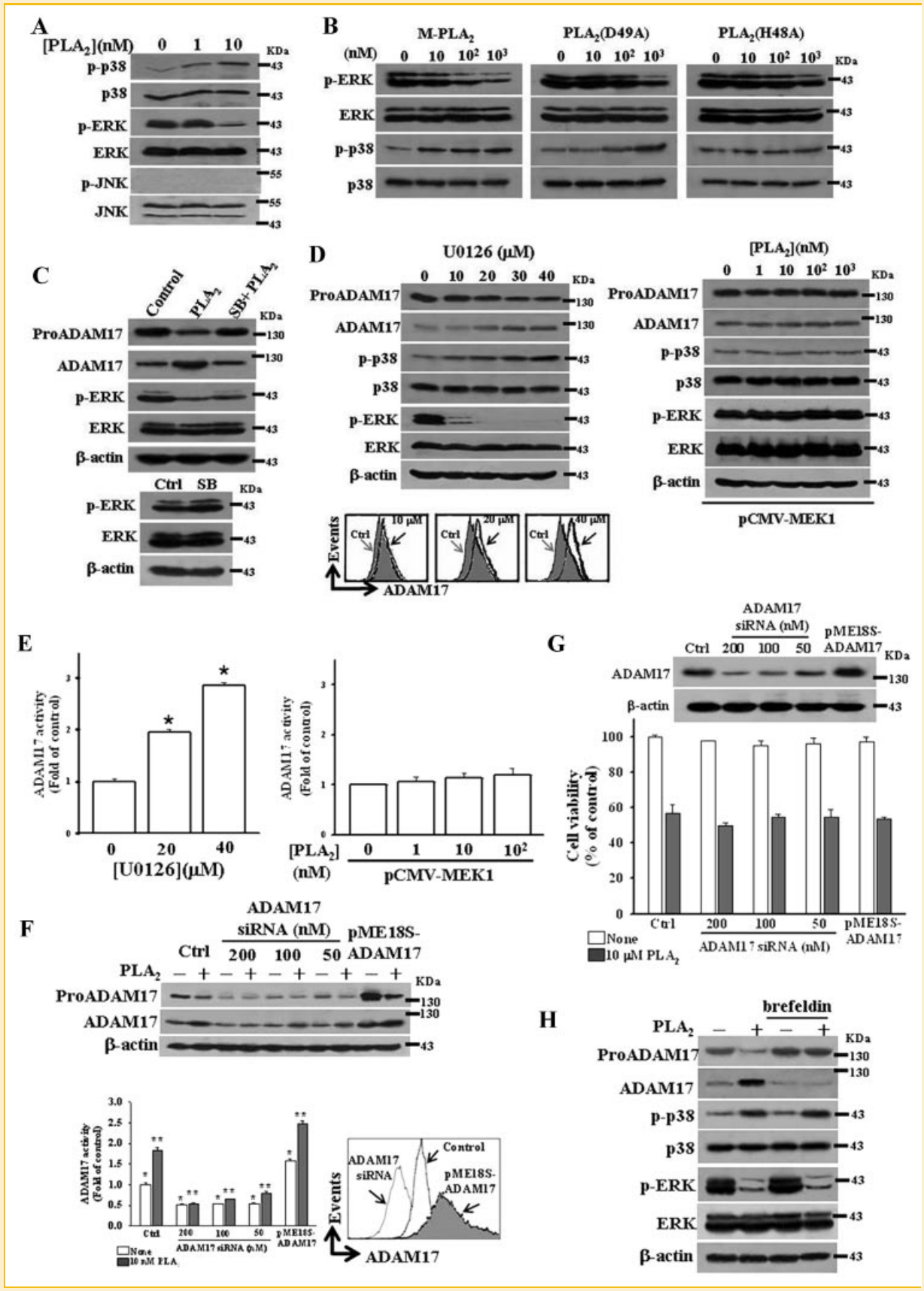
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Fig. 2.

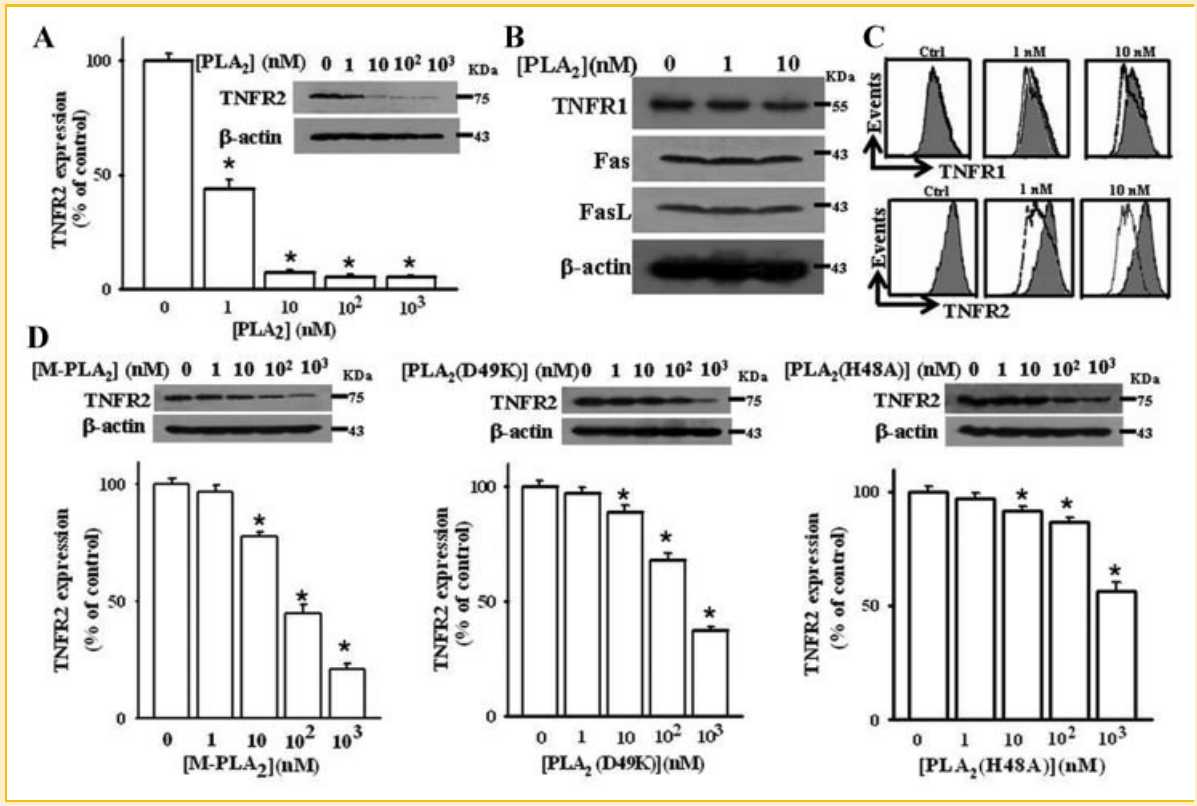

Fig. 3. PLA_2 down-regulated protein expression of TNFR2 in SK-N-SH cells. A: PLA_2-induced TNFR2 down-regulation. B: Effect of PLA_2 on TNFR1, Fas, or FasL protein expression. SK-N-SH cells were treated with 1 or 10 nM PLA_2 for 24 h. C: Surface expression levels of TNFR1 and TNFR2 in PLA_2-treated cells. SK-N-SH cells were treated with 1 or 10 nM PLA_2 for 24 h, and then the cells were stained with anti-human TNFR1-Fluorescein or anti-human TNFR2-Fluorescein to determine the relative expression levels of TNFR1 or TNFR2 by flow cytometry. Results are representative of three independent experiments. D: TNFR2 protein expression in M-PLA_2-, PLA_2(D49K)-, or PLA_2(H48A)-treated cells. SK-N-SH cells were treated with indicated concentrations of catalytically inactive PLA_2s for 24 h. Protein expression was quantified from Western blot analyses, and the results were shown under the results of Western blot analyses. Three independent experimental results were analyzed by densitometry (mean $\pm$ SD, $^*P < 0.05$, compared with untreated control cells).

Fig. 2. p38 MAPK activation and ERK inactivation promoted posttranscriptional up-regulation of ADAM17 in PLA_2-treated cells. A: Western blot analyses of phospho-p38 MAPK, phospho-ERK and phospho-JNK in PLA_2-treated cells. SK-N-SH cells were treated with indicated concentrations of PLA_2 for 24 h. B: The levels of phospho-p38 MAPK and phospho-ERK in M-PLA_2-, PLA_2(D49K)-, or PLA_2(H48A)-treated cells. SK-N-SH cells were treated with indicated concentrations of M-PLA_2, PLA_2(D49K), or PLA_2(H48A) for 24 h. C: Effect of SB202190 on PLA_2-induced ADAM17 maturation. Top panel: SK-N-SH cells were pretreated with 10 μM SB202190 for 1 h, and then incubated with 10 nM PLA_2 for 24 h. Bottom panel: SK-N-SH cells were treated with 10 μM SB202190 for 24 h. D: Effect of U0126 and constitutively active MEK1 on PLA_2-induced posttranscriptional up-regulation of ADAM17. Left panel: SK-N-SH cells were treated with indicated concentrations of U0126 for 24 h. Bottom of left panel: Surface expression levels of ADAM17 in U0126-treated cells. SK-N-SH cells were treated with 10, 20, or 40 μM U0126 for 24 h, and then the cells were stained with anti-human ADAM17-Fluorescein to determine the relative expression levels of ADAM17 by flow cytometry. Results are representative of three independent experiments. Right panel: SK-N-SH cells were transfected with pCMV-MEK1. After 24 h posttransfection, the cells were treated with indicated concentrations of PLA_2 for 24 h. E: Effect of U0126 and constitutively active MEK1 on ADAM17 activity. Left panel: SK-N-SH cells were treated with indicated concentrations of U0126 for 24 h. Right panel: SK-N-SH cells were transfected with pCMV-MEK1. After 24 h posttransfection, the cells were treated with indicated concentrations of PLA_2 for 24 h. The values represent averages of three independent experiments with triplicate measurements (mean $\pm$ SD, $^*P < 0.05$, compared with untreated control cells). F: PLA_2 treatment induced posttranscriptional up-regulation of ADAM17 in ADAM17 siRNA- or pME18S-ADAM17-transfected cells. SK-N-SK cells were transfected with ADAM17 siRNA (50, 100, or 200 nM) or pME18S-ADAM17, respectively. Control cells were co-transfected with control siRNA (100 nM) and control vector. After 24 h posttransfection, the cells were treated with 10 nM PLA_2 for 24 h. Top panel: Western blot analyses of proADAM17 and ADAM17. Left of bottom panel: ADAM17 activity assay. The values represent averages of three independent experiments with triplicate measurements (mean $\pm$ SD, *, $^{**}P < 0.05$, compared with control cells). Right of bottom panel: Surface expression of ADAM17 in ADAM17 siRNA (100 nM)- or pME18S-ADAM17-transfected cells. G: Effect of ADAM17 down-regulation and ADAM17 overexpression on viability of PLA_2-treated SK-N-SH cells. SK-N-SH cells were transfected with ADAM17 siRNA (50, 100, or 200 nM) or pME18S-ADAM17, respectively. Control cells were co-transfected with control siRNA (100 nM) and control vector. After 24 h posttransfection, the cells were treated with 10 μM PLA_2 for 24 h. Top panel: Western blot analyses of ADAM17 in ADAM17 siRNA- or pME18S-ADAM17-transfected cells. Bottom panel: The cell viability was determined by MTT assay. The values represent averages of three independent experiments with triplicate measurements (mean $\pm$ SD). H: Effect of brefeldin on PLA_2-induced posttranscriptional up-regulation of ADAM17. SK-N-SH cells were pretreated with 1 μg/ml brefeldin for 1 h, and then incubated with 10 nM PLA_2 for 24 h.

Given that TNFR2 protein expression was markedly reduced in PLA_2-treated cells compared with that of TNFR1, the causal relationship between ADAM17 and TNFR2 protein expression was further examined. Figure 4A shows that PLA_2 and catalytically inactive PLA_2s induced down-regulation of TNFR2 in a time-dependent manner. Figure 4B shows that the transcription of TNFR2 mRNA did not significant change in PLA_2-treated cells as revealed by reverse-transcription PCR amplification. Cycloheximide pretreatment further enhanced PLA_2-induced down-regulation of TNFR2 (Fig. 4C), suggesting that PLA_2 treatment posttranscriptionally regulated TNFR2 expression. Although TNFR2 protein expression is regulated by proteasome-mediated degradation [Chung et al., 2005], Figure 4D shows that pretreatment with MG132 (proteasome inhibitor) did not affect PLA_2-induced TNFR2 down-regulation. ELISA assay revealed that PLA_2 induced concentration-dependently an increase in sTNFR2 detected in culture medium of SK-N-SH cells (Fig. 4E). These results reflected that PLA_2 treatment increased TNFR2 shedding in SK-N-SH cells. SB202190 pretreatment abrogated PLA_2-induced TNFR2 down-regulation (Fig. 4F). Figure 4G and H show that U0126 elicited TNFR2 shedding in SK-N-SH cells, while expression of constitutively active MEK1 suppressed PLA_2-induced down-regulation of TNFR2 protein

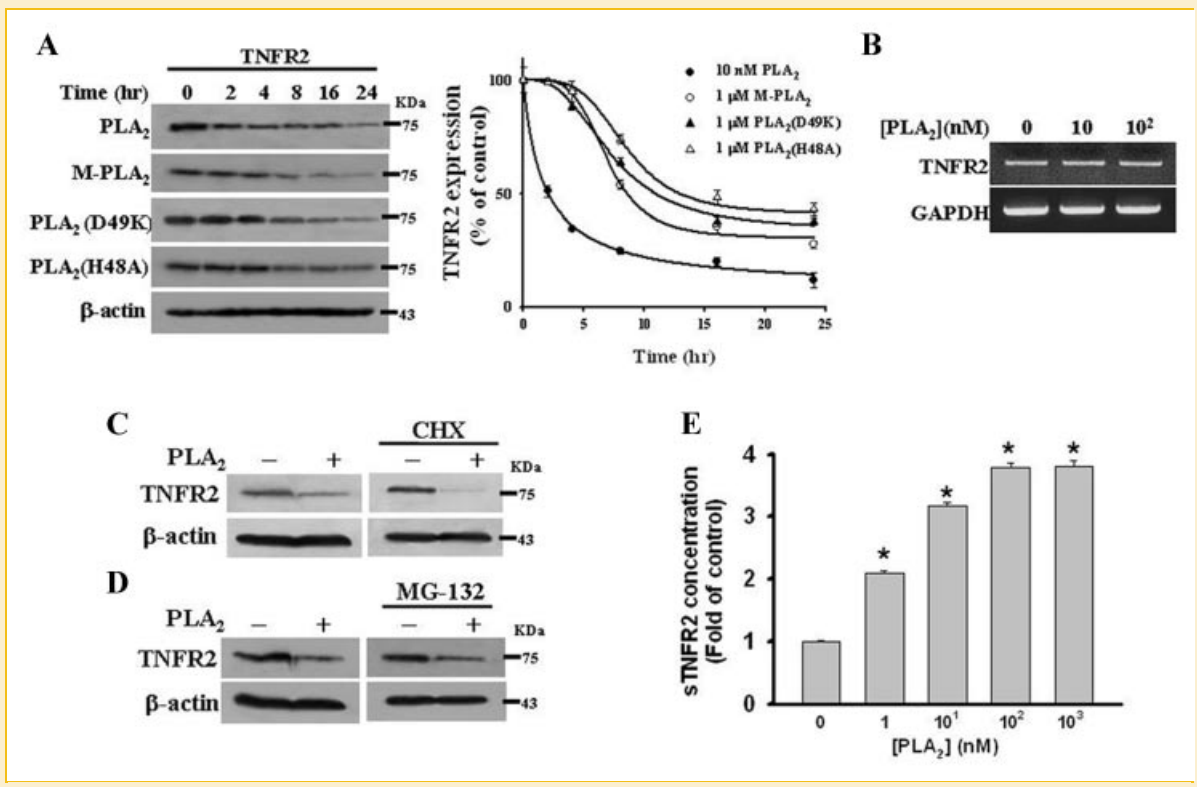

Fig. 4. PLA_2-induced down-regulation of TNFR2 as a result of increase in ectodomain shedding of TNFR2 in SK-N-SH cells. A: PLA_2 and catalytically inactive PLA_2s induced TNFR2 down-regulation in a time-dependent manner. Left panel: SK-N-SH cells were treated with 10 nM PLA_2, 1 μM M-PLA_2, 1 μM PLA_2(D49K), or 1 μM PLA_2(H48A) for indicated time periods. Right panel: Quantification of TNFR2 expression from Western blot analyses. Three independent experimental results were analyzed by densitometry. B: Detecting the transcription of TNFR2 mRNA using RT-PCR. SK-N-SH cells were treated with indicated concentrations of PLA_2 for 24 h. RT-PCR was conducted according to the procedure described in the Materials and Methods Section. C: Effect of cycloheximide on PLA_2-induced down-regulation of TNFR2. SK-N-SH cells were pretreated with 10 μM cycloheximide (CHX) for 1 h, and then treated with 10 nM PLA_2 for 24 h. D: Effect of MG-132 on PLA_2-induced down-regulation of TNFR2. SK-N-SH cells were pretreated with 10 μM MG-132 for 1 h, and then treated with 10 nM PLA_2 for 24 h. E: PLA_2 treatment increased soluble TNFR2 (sTNFR2) in culture medium of SK-N-SH cells. SK-N-SH cells were treated with indicated concentrations of PLA_2 for 24 h. The values represent averages of three independent experiments with triplicate measurements (mean ± SD, $^*P < 0.05$, compared with untreated control cells). F: Effect of SB202190 on PLA_2-induced TNFR2 shedding. SK-N-SH cells were pretreated with 10 μM SB202190 for 1 h, and then incubated with 10 nM PLA_2 for 24 h. G: Effect of U0126 and constitutively active MEK1 on PLA_2-induced TNFR2 shedding. Left panel: SK-N-SH cells were treated with indicated concentrations of U0126 for 24 h. Right panel: SK-N-SH cells were transfected with pCMV-MEK1. After 24 h posttransfection, the cells were treated with indicated concentrations of PLA_2 for 24 h. H: TNFR2 shedding in SK-N-SH cells after treatment with PLA_2, U0126 or a combination of SB202190 and PLA_2. SK-N-SH cells were treated with 10 nM PLA_2 or 10 μM U0126 for 24 h. Alternatively, SK-N-SH cells were pretreated with 10 μM SB202190 for 1 h, and then incubated with 10 nM PLA_2 for 24 h. The values represent averages of three independent experiments with triplicate measurements (mean ± SD, $^*P < 0.05$). I: Ectodomain shedding of TNFR2 in ADAM17 siRNA- or pME18S-ADAM17-transfected cells. SK-N-SH cells were transfected with ADAM17 siRNA (50, 100, or 200 nM) or pME18S-ADAM17, respectively. Control cells were co-transfected with control siRNA (100 nM) and control vector. After 24 h posttransfection, the cells were treated with 10 nM PLA_2 for 24 h. Top panel: Western blot analyses of TNFR2 protein expression in ADAM17 siRNA- or pME18S-ADAM17-transfected cells. Left of bottom panel: Effect of ADAM17 down-regulation or overexpression on the release of sTNFR2 in culture medium of SK-N-SH cells. The values represent averages of three independent experiments with triplicate measurements (mean ± SD, $^*, ^{**}P < 0.05$, compared with control cells). Right of bottom panel: Surface expression levels of TNFR2 in ADAM17 siRNA (100 nM)- or pME18S-ADAM17-transfected cells. The cells were stained with anti-human TNFR2-Fluorescein to determine the relative expression levels of TNFR2 by flow cytometry.

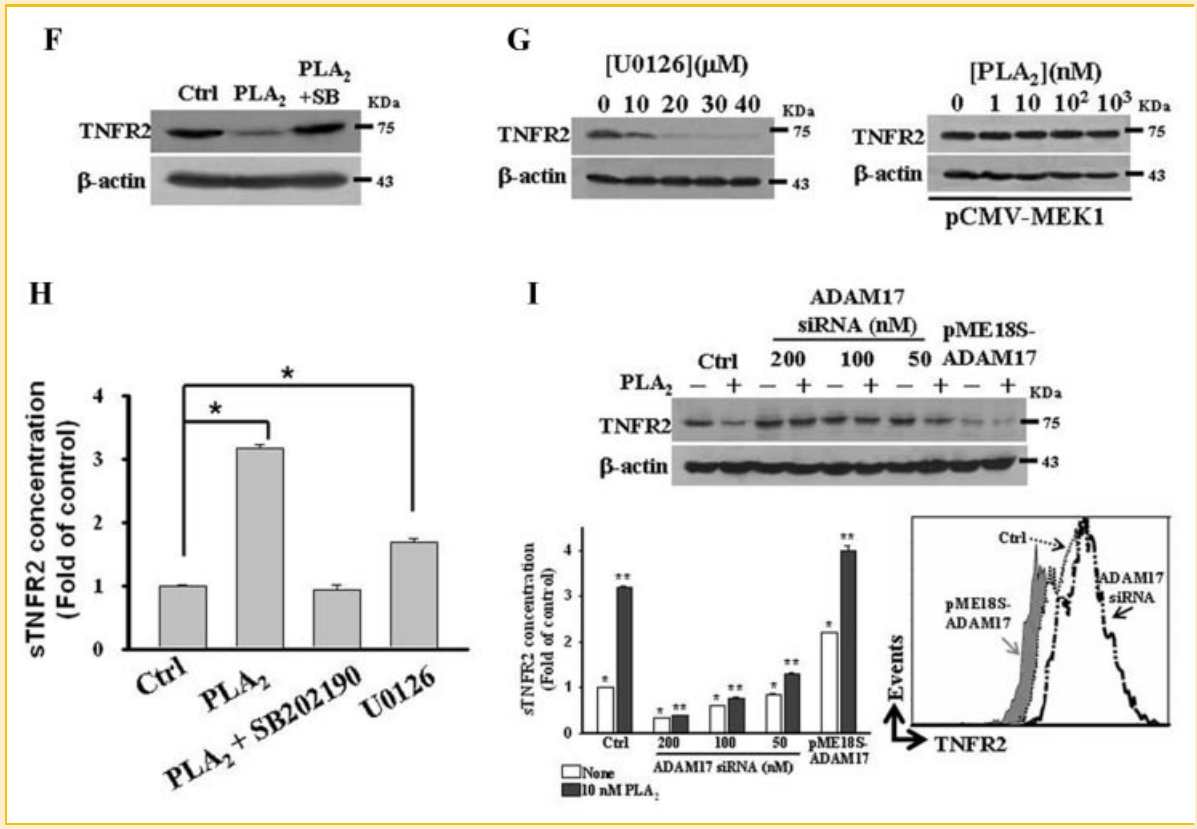

Fig. 4. (*Continued*)

expression. An increase in TNFR2 shedding was noted with pME18S-ADAM17-transfected SK-N-SH cells, while down-regulation of ADAM17 led to increase in TNFR2 protein expression (Fig. 4I). Treatment with PLA_2 further increased the release of sTNFR2 in a pME18S-ADAM17- or ADAM17 siRNA-transfected cell culture media. Collectively, the results suggested that posttranscriptional up-regulation of ADAM17 was involved in TNFR2 shedding in PLA_2-treated SK-N-SH cells, and that PLA_2-induced down-regulation of TNFR2 arose from increasing TNFR2 shedding. Likewise, the knock-down of ADAM17 by siRNA increased TNFR1 surface expression in SK-N-SH cells, while overexpression of ADAM17 down-regulated TNFR1 expression (Supplementary Fig. 1). Down-regulation of ADAM17 attenuated PLA_2-induced TNFR1 down-regulation. These results reflected that posttranscriptional up-regulation of ADAM17 was also contributed to TNFR1 down-regulation in PLA_2-treated cells.

DISCUSSION

ADAM17 is synthesized as an inactive zymogen in which its prodomain maintains the sheddase in an inactive and stable conformation [Milla et al., 1999; Schlondorff et al., 2000; Gonzales et al., 2004; Buckley et al., 2005]. Thus, proADAM17 conversion into mature ADAM17 appears to be a required step for catalytic activity activation. Nevertheless, it is not the only event involved because phosphorylation events [Soond et al., 2005; Xu and Derynck, 2010], reduction–oxidation modification [Wang et al., 2009] and association with other proteins [Zheng et al., 2002; Peiretti et al., 2003b; Canault et al., 2006] are described to be likely important. Although ADAM17 phosphorylation of by either ERK or p38 MAPK has been suggested [Soond et al., 2005; Xu and Derynck, 2010], the possibility that ERK or p38 MAPK activates ADAM17 via some other intermediate molecule cannot be completely ruled out. Noticeably, phosphorylation on Ser791 and Ser819 of ADAM17 is found to regulate p38 MAPK-mediated phosphorylation of Thr735 and ADAM17 activation [Xu and Derynck, 2010]. In view of our data showing that ERK inactivation acts in concert with p38 MAPK-mediated posttranscriptional ADAM17 up-regulation in SK-N-SH cells, the role of ERK in Ser791 and Ser819 phosphorylation may be considered. Compared with that of TNFR1, PLA_2 treatment notably increases TNFR2 ectodomain shedding. In agreement with our findings, sTNFR2 is greatly produced when ADAM17 activity in COS-7 cells and H9C2 cells is stimulated with high-density lipoproteins, while sTNFR1 is marginally increased [Tellier et al., 2008]. It appears that ADAM17 has higher substrate specificity toward TNFR2 than toward TNFR1. However, the molecular mechanism responsible for sheddase specificity in ADAM17 remains elusive [Arribas and Ruiz-Paz, 2005]. ADAM10 has been proven to be involved in FasL shedding [Schulte et al., 2007], while the

sheddase responsible for Fas shedding remains elusive. PLA_2 treatment does not significantly change ADAM10 protein expression (Supplementary Fig. 2A). Thus, it is no doubt that FasL expression is not significantly altered by PLA_2 treatment. ADAM10 shares most structure similarity and some substrate specificity with ADAM17. Previous studies revealed that ADAM10 may function as the TNFα sheddase in murine fibroblasts cells which lacked ADAM17 activity [Mezyk-Kopec et al., 2009]. However, there is no evidence supporting that ADAM10 is involved in TNFR1 and TNFR2 shedding. Overexpression of ADAM10 by transfection with pME18S-ADAM10 does not increase TNFR1 and TNFR2 shedding regardless of PLA_2 treatment (Supplementary Fig. 2B,C). Moreover, our data reveal that TNFR2 ectodomain shedding is correlated with ADAM17 expression. Taken together, PLA_2-induced the release of sTNFR2 reflects posttranscriptional up-regulation of ADAM17 in SK-N-SH cells.

Previous studies showed that elevation in serum PLA_2 activity accompanied with increase in sTNFR levels was detected in the serum of schizophrenic patients [Gatta et al., 1990; Coelho et al., 2008]. Our data indicate that PLA_2 treatment induces an increase in TNFR1 and TNFR2 shedding through posttranscriptional up-regulation of ADAM17. Catalytic activity inactivation causes a drastic drop in the ability of PLA_2 to regulate ADAM17 maturation, suggesting that catalytic activity is crucial for the PLA_2 effect. The finding that LPC mimics the action of PLA_2 in posttranscriptional up-regulation of ADAM17 activity again supports this proposition. These results indicate the casual relationship between an increase in PLA_2 activity and the release of sTNFRs. Noticeably, catalytically inactive PLA_2s weakly induce increased ADAM17 activity and TNFR2 shedding, reflecting that a catalytic activity-independent pathway is modestly involved in regulating ADAM17 sheddase activity. In summary, our data indicate that PLA_2-evoked p38 MAPK activation and ERK inactivation are involved in posttranscriptional up-regulation of ADAM17, and suggest a crucial role for catalytic activity in the PLA_2 action.

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