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Penta-Acetyl Geniposide Induce Apoptosis in C6 Glioma Cells by Modulating the Activation of Neutral Sphingomyelinase-Induced p75 Nerve Growth Factor Receptor and Protein Kinase Cδ Pathway[S]

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ABSTRACT

In our previous studies, we demonstrated the apoptotic cascades protein kinase C (PKC) δ/c-Jun NH_2-terminal kinase (JNK)/Fas/caspases induced by penta-acetyl geniposide [$(Ac)_5$GP]. However, the upstream signals mediating PKCδ activation have not yet been clarified. Ceramide, mainly generated from the degradation of sphingomyelin, was hypothesized upstream above PKCδ in $(Ac)_5$GP-transduced apoptosis. Furthermore, nerve growth factor (NGF)/p75 is supposed to be involved because$(Ac)_5$GP-induced apoptosis was demonstrated previously in glioma cells. In the present study, $(Ac)_5$GP was shown to activate neutral sphingomyelinase (N-SMase) immediately, with its maximum at 15 min. The NGF and p75 enhanced by $(Ac)_5$GP was inhibited when added with GW4869, the N-SMase inhibitor, indicating NGF/p75 as the downstream signals of N-SMase/ceramide. To investigate whether N-SMase is involved in $(Ac)_5$GP-transduced apoptotic pathway, cells were treated with $(Ac)_5$GP added with or without GW4869. It showed that N-SMase inhibition blocked FasL expression and caspase 3 activation. Likewise, p75 antagonist peptide attenuated the FasL/caspase 3 expression. The PKCδ translocation induced by $(Ac)_5$GP was also eliminated by GW4869 and p75 antagonist peptide. To further confirm whether N-SMase activation plays an important role in $(Ac)_5$GP-induced apoptosis, cells were analyzed the apoptotic rate by 4′, 6-diamidino-2-phenylindole (DAPI) staining. $(Ac)_5$GP-induced apoptosis was reduced 40 and 80% by 10 and 20 μM GW4869, respectively. It indicated that N-SMase activation is pivotal in $(Ac)_5$GP-mediated apoptosis. In conclusion, SMase and NGF/p75 are suggested to mediate upstream above PKCδ, thus transducing FasL/caspase cascades in $(Ac)_5$GP-induced apoptosis.

Penta-acetyl geniposide [1-(β-D-2′,3′,4′,6′-tetraacetyl-glucopyranosyloxy)-1,4*a*,5,7*a*-tetrahydro-7-(acetomethyl)-cyclopentapyran-4-carboxylic acid methyl ester; $(Ac)_5$GP, Fig. 1] is an herbal derivative prepared from *Gardenia geniposide*. It has been suggested that $(Ac)_5$GP plays more potent roles than geniposide in chemoprevention (Peng et al., 2005a). In addition, $(Ac)_5$GP possesses the antitumor effect on apoptosis and growth arrest (Wang et al., 1993; Chang et al., 2002, 2004).

In our previous studies, we demonstrated that $(Ac)_5$GP may transduce the apoptotic signals through PKCδ activation and the downstream cascades of JNK/Jun phosphorylation, FasL/Fas elevation, and the subsequent activation of caspase 8 and caspase 3 (Peng et al., 2004, 2005b). PKCδ seems to play the pivotal role in $(Ac)_5$GP-mediated apoptosis of tumor cells. However, the upstream signals above PKCδ have not yet been clarified.

There exist different kinds of lipids in cell membrane, which could be released and transduce the secondary signals while cells were being stimulated. Among all of them, one derivative of sphingolipid, ceramide, showed to be highly

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ABBREVIATIONS: $(Ac)_5$GP, penta-acetyl geniposide; SMase, sphingomyelinase; N-SMase, neutral sphingomyelinase; A-SMase, acid sphingomyelinase; NGF, nerve growth factor; NGFR, nerve growth factor receptor; PKCδ, protein kinase C δ; MAP, mitogen-activated protein; JNK, c-Jun NH_2-terminal kinase; TNF-α, tumor necrosis factor-α; HPTLC, high-performance thin-layer chromatography; TEMED, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine; DAPI, 4′,6-diamidino-2-phenylindole; GSH, glutathione; GW4869, $C_{30}H_{28}N_6O_6{\cdot}2HCl$.

involved in apoptotic pathways (Obeid et al., 1993). The generation of ceramide mainly depends on the degradation of sphingomyelin, which is catalyzed by sphingomyelinase (SMase) (Jaffrezou et al., 1996). Ceramide regulates a variety of cell functions, including growth arrest, differentiation, aging, apoptosis, and immune response (Hannun, 1996). Obeid et al. (1993) showed that ceramide analog, such as TNF-α, induced DNA fragmentation in leukemia cells (Obeid et al., 1993). Defective sphingomyelin/ceramide signaling showed resistance to radiation therapy in Burkitt's lymphoma, breast carcinoma, and myeloid leukemia cells (Cai et al., 1997; Michael et al., 1997; Bruno et al., 1998).

It was suggested that ceramide could mediate apoptotic signals via PKC isoforms. Ceramide translocated PKCδ and -ε (from membrane) to the cytosol, thus induced the apoptosis of leukemia cells (Sawai et al., 1997). In prostate cancer cell lines, ceramide generation was found only in PKCδ (+) cells. Ceramide translocated PKCδ to mitochondria, inducing the subsequent release of cytochrome *c* and activation of caspase 9, thus further amplifying the ceramide-mediated mitochondrial amplification loop (Sumitomo et al., 2002). Ceramide has been shown to translocate PKCδ to Golgi apparatus, and then transduce the tyrosine phosphorylation, activation, and apoptotic signals (Kajimoto et al., 2004). Therefore, ceramide might be hypothesized as the possible upstream signal of PKCδ in $(Ac)_5$GP-induced apoptosis.

In the previous studies, apoptotic signals induced by $(Ac)_5$GP were demonstrated in C6 glioma cells, which is derived from the nervous system (Benda et al., 1968). It has been shown that nerve growth factor (NGF) regulates the growth, differentiation, and apoptosis of glioma cells (Rabin et al., 1998). The biological functions of NGF are mediated by its high-affinity receptor, trkA, and the low-affinity receptor $p75^{NTR}$ (Chao and Hempstead, 1995). trkA contains a cytosol tyrosine kinase domain, which is not found in the wide-expressed p75 receptor. The collaboration of trkA and p75 potentiate functional responses to neurotrophins (Hantzopoulos et al., 1994). It was also reported that p75 plays an important role in apoptotic pathway (Rabizadeh et al., 1993). The antibody to the extracellular domain of p75 NGF receptor (NGFR) mediated apoptosis in sympathetic neurons (Freidin, 2001), whereas antagonist to p75 neurotrophin receptor retarded apoptosis-driven hair follicle involution (Botchkarev et al., 2003). It was suggested that p75 NGFR has some functional similarities to the superfamily of TNF-α receptors, Fas and CD40 (Smith et al., 1994).

Ceramide and SMase have been reported as capable of inducing NGF synthesis in primary astrocyte cultures, implying that there may exist the cross-talks between ceramide and NGFR signals in nervous system (Galve-Roperh et al., 1997). Thus, in the present study, we investigated whether ceramide/SMase and NGF/NGFR are involved in $(Ac)_5$GP-mediated programmed death in glioma cells and clarified the upstream transduction pathway above PKCδ.

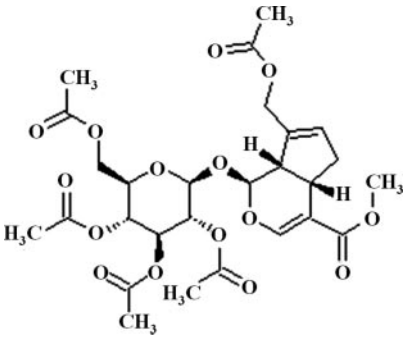

Fig. 1. Structure of $(Ac)_5$GP.

Materials and Methods

Chemicals. Minimal essential medium, fetal calf serum, penicillin, and streptomycin were purchased from Invitrogen (Carlsbad, CA). Amplex Red Sphingomyelinase kit is a product of Molecular Probes (Carlsbad, CA). SDS, bis-acrylamide, ammonium persulfate, TEMED, and nitrocellulose paper were from Bio-Rad (Hercules, CA). PKCδ antibody was purchased from BD Transduction Laboratories (Lexington, KY). Triton X-100, Tris base, β-actin antibody, nonhydroxy fatty acid ceramide, and DAPI were from Sigma (St. Louis, MO). GW4869, a reported specific inhibitor for N-SMase, was also purchased from Sigma (Luberto et al., 2002). Antibodies of NGF, NGFR p75, Fas-L, and caspase 3 were from Santa Cruz Biotechnology (Santa Cruz, CA). High-performance thin-layer chromatography (HPTLC) silica gel 60 plate was from Merck (Whitehouse Station, NJ). Lipofectamine was from Invitrogen (Carlsbad, CA). p75 antagonist peptide YCDIKGKECY (cysteine-cysteine bond resulting in cyclization is indicated by the underline), which has been reported as specific to NGF p75 receptor, was synthesized by Protech Technology (Reno, NV) with a minimum 90% purity (Li et al., 2005).

$(Ac)_5$GP Preparation. $(Ac)_5$GP was isolated and acetylated from *Gardenia fructus* with the same procedure used in our previous study (Wang et al., 1992). The stock solution of $(Ac)_5$GP was prepared in dimethyl sulfoxide, protected from light, and stored at −20°C. Before use, the $(Ac)_5$GP solution was freshly prepared in the medium at the desired concentration.

Cell Culture and Treatment. The rat C6 glioma cell line was originally derived from an *N*-nitrosomethylurea-induced rat brain tumor (Benda et al., 1968). C6 cells were maintained in minimal essential medium supplemented with 10% fetal calf serum and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin), at 37°C in a humidified atmosphere with 5% CO_2. Cells were seeded at a density of 5×10^5 onto each 10-mm Petri dish 24 h before drug treatment.

DAPI Staining. We used DAPI staining to assess morphological changes in the chromatin structure of C6 glioma cells undergoing apoptosis. Cells were trypsinized, mounted on glass slides, and fixed in 4% paraformaldehyde for 30 min followed by staining with 1 μg/ml DAPI for 30 min. Apoptosis was characterized by chromatin condensation and fragmentation when examined by fluorescence microscopy. The incidence of apoptosis in each preparation was analyzed by counting 300 cells and determining the percentage of apoptotic cells.

Cell Fraction and PKCδ Translocation. Subcellular fractionation was performed as follows. Cells were washed with phosphate-buffered saline and added with cold buffer consisting of 20 mM Tris, 0.03 mM Na_3VO_4, 2 mM $MgCl_2 \cdot 6H_2O$, 2 mM EDTA, 0.5 mM EGTA, 2 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 250 mM sucrose, and 10 μg/ml leupeptin, and then they were scraped into suspension and lysed by a homogenizer (Eyela Nazelax; Tokyo Rikakikai Co., Ltd., Tokyo, Japan) on ice. The lysate was centrifuged at 50,000 rpm at 4°C for 1 h. The supernatant was collected and used as the cytosol fraction. The pellet was solubilized with 20 mM Tris, 0.03 mM Na_3VO_4, 5 mM $MgCl_2 \cdot 6H_2O$, 2 mM EDTA, 0.5 mM EGTA, 2 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 5 mM NaF, 10 μg/ml leupeptin, and 0.1% Triton X-100 at 4°C and mixed by vortex every 10 min to extract soluble membrane proteins. One hour later, samples were recentrifuged at 50,000 rpm at 4°C for 1 h, and the supernatant was used as a membrane fraction. PKCδ expressed

in the two fractions were then analyzed with immunoblot and densitometer.

Electrophoresis and Immunoblotting. Cells were harvested into lysis buffer containing 50 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, and 5% β-mercaptoethanol and then lysed by sonication. Equal amounts of protein (10 μg/lane) were subjected to SDS-polyacrylamide gel electrophoresis and transfected onto nitrocellulose membranes. Membranes were blocked with 5% bovine serum albumin with 0.05% Tween 20 in phosphate-buffered saline and then incubated with the first antibody for 1 h. Membranes were then washed three times with 0.05% Tween 20 in phosphate-buffered saline and incubated with the secondary antibody conjugated to anti-mouse horseradish peroxidase (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Bands were visualized by using the enhanced chemiluminescence Western blotting detection system. Protein quantitation was determined by densitometry using AlphaImager Series 2200 software (Alpha Innotech, San Leandro, CA).

Antisense Knockout Assay. Sense (5′-CCTCTTACCTCAGTTACAATTTATA-3′) and antisense (5′-GCCGCAGAGAAAAGTTGTGCTTCAT-3′) oligonucleotides for N-SMase were synthesized by Mission Biotech (Taipei, Taiwan) (GenBank accession number BC091139). Cells were plated at 70% density 24 h before antisense oligonucleotide treatment. The cells were washed and then incubated with antisense oligonucleotide (5 μM) in serum-free minimal essential medium containing 10 μg/ml Lipofectamine at 37°C. The medium was changed to 5% fetal bovine serum minimal essential medium 6 h later before culturing at 37°C for 24 h.

SMase Activity Assay. SMase activity was analyzed with Amplex Red Sphingomyelinase Assay Kit. Cells were washed with phosphate-buffered saline and pelleted by centrifugation at 1500*g* for 10 min at 4°C. The cell pellet was resuspended and lysed in buffer containing 1% Triton X-100, 1 μg/ml aprotinin, 1 mM EDTA, and 100 μg/ml phenylmethylsulfonyl fluoride for 60 min on ice and then centrifuged at 17,000*g* for 10 min at 4°C to remove the nuclei. The protein concentration in the supernatant was measured with Bio-Rad Protein Assay. For analyzing the activity of N-SMase, the supernatant was diluted with 1× reaction buffer containing 0.1 M Tris-HCl and 10 mM $MgCl_2$, pH 7.4, and then inoculated into the 96-well plate as 100 μl/well. The total protein amount of each well was approximately 25 μg. The solution was added with 100 μl of working solution (containing 8 U/ml alkaline phosphatase, 0.2 U/ml choline oxidase, 2 U/ml horseradish peroxidase, and 0.5 mM sphingomyelin) and incubated for 45 min at 37°C. The H_2O_2 generated thus reacted with Amplex Red to elicit the fluorescent resorufin. The fluorescent intensity was measured with Fluorescence Multi-Well Reader (HTS 7000) immediately at excitation/emission levels of 571/585 nm. On the other hand, acid sphingomyelinase (A-SMase) activity was analyzed with two-step assay. First, we used 50 mM sodium acetate (not 1× reaction buffer) to dilute the supernatant and inoculate 100 μl to each well of the 96-well plate. To generate phosphorylcholine and ceramide, 10 μl of 5 mM sphingomyelin solution was added to each well and then incubated for 1 h at 37°C. Working solution (100 μl) containing 8 U/ml alkaline phosphatase, 0.2 U/ml choline oxidase, and 2 U/ml horseradish peroxidase was then added to each well to react with phosphorylcholine for 45 min at 37°C. H_2O_2 generated from the two-step procedure then reacted with Amplex Red to elicit resorufin, which was measured with the Fluorescence Multi-Well Reader as mentioned above.

Quantitative Measurement of Ceramide Content. Cells were harvested into lysis buffer containing 50 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, and 5% β-mercaptoethanol and then lysed by sonication. Lipids were extracted as the following procedures. In brief, 1 ml of cell lysate was suspended with 1.25 ml of chloroform and 2.5 ml of methanol, and then vortexed and incubated overnight at 4°C. After centrifugation for 5 min at 1700*g*, 1.25 ml of chloroform and 1.25 ml of 0.88% KCl were added to the supernatant. The tubes were vortexed and centrifuged for 5 min at 1700*g*. The organic lower phase was transferred into a new tube and evaporated under nitrogen. The lipids were dissolved into chloroform-methanol [2:1 (v/v)] and analyzed by HPTLC on an HPTLC silica gel 60 plate using dichloromethane-methanol-glacial acetic acid [100:2:5 (v/v)] for ceramides. Individual lipid classes were visualized by dipping each plate into $CuSO_4$ (3%)/H_3PO_4 (8%) and then charring the plate on a heat block at 180°C for 10 min. Densitometric scanning of the bands and evaluation of the data were done with LAS-3000 (Fuji Photo Film Co., Ltd., Tokyo, Japan). In addition to three replicates of samples, nonhydroxy fatty acid ceramide was added to each plate as the lipid standard.

Statistical Analysis. Statistical differences were evaluated using the unpaired *t* test and were considered significant at the $P < 0.05$ level.

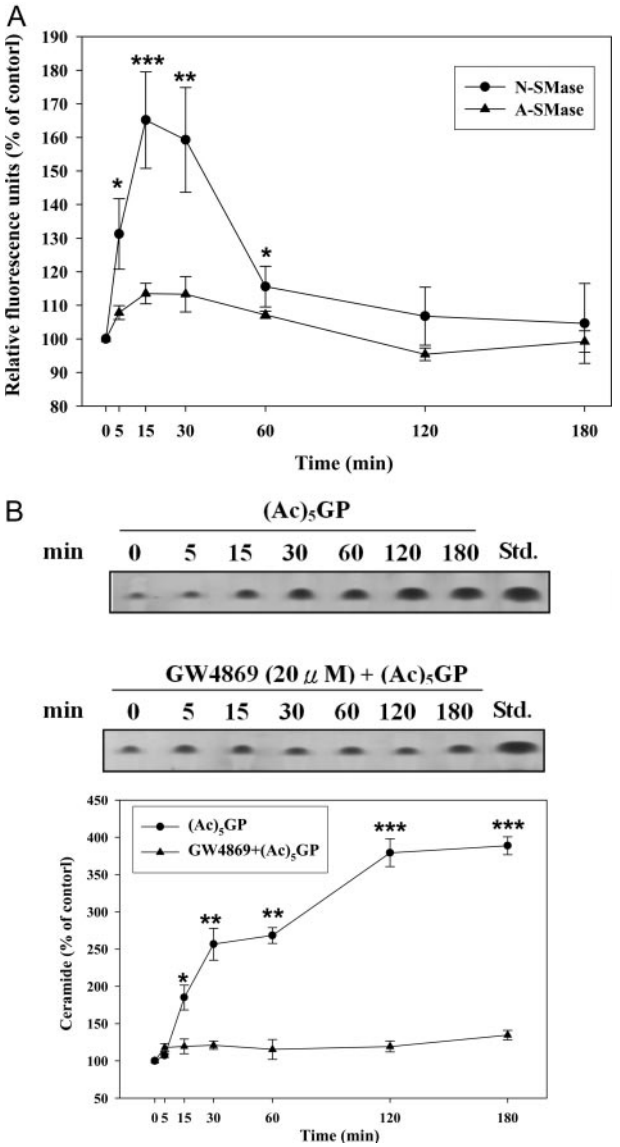

Fig. 2. SMase activated by $(Ac)_5$GP. A, cells were treated with 0.6 mM $(Ac)_5$GP for the indicated time. SMase activity was analyzed and was directly proportional to the fluorescence emitted. Data were presented as mean ± S.D., $n = 3$; *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$ compared with the control. B, cells were treated with 0.6 mM $(Ac)_5$GP with or without 20 μM GW4869 for the indicated time. Ceramide levels are shown as percentages of the control group at 0 h. Std., standard nonhydroxy fatty acid ceramide. Data are presented as mean ± S.D., $n = 3$; *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$ compared with the control.

Results

$(Ac)_5$GP Induces N-SMase Activation. C6 glioma cells were treated with 0.6 mM $(Ac)_5$GP at different time points until 3 h. SMase activity was presented as the percentage of fluorescence. Figure 2A showed that N-SMase reached the maximal activity at 15 min and then declined within 2 h, whereas A-SMase was not activated by $(Ac)_5$GP. The N-SMase activity showed to be up-regulated approximately 70% by $(Ac)_5$GP. Figure 2B showed the change of ceramide level. $(Ac)_5$GP increased approximately 4-fold of ceramide within 2 h. Addition of GW4869 blocked the increase of ceramide induced by $(Ac)_5$GP.

NGF/p75 Signals Downstream of N-SMase. C6 cells were treated with 0.6 mM $(Ac)_5$GP at different time points. The protein level of NGF increased 1.5-fold at 6 h and more than 2-fold at 24 h (Fig. 3A). p75 also showed to be elevated by $(Ac)_5$GP. At 9 h, p75 increased 2-fold compared with the control. Similar results were observed in ceramide-treated cells, implying that NGF and p75 could be downstream of ceramide generation (Fig. 3B). Figure 3C showed that the elevation of NGF and p75 by $(Ac)_5$GP was reduced by GW4869, the N-SMase inhibitor. These data indicate NGF/p75 as the downstream signals of $(Ac)_5$GP-induced N-SMase activation.

N-SMase Activation and NGF/p75 Interaction is Involved in $(Ac)_5$GP-Transduced FasL/Caspase 3 Cascades. To investigate whether N-SMase activation is involved in $(Ac)_5$GP-transduced FasL/caspase 3 pathway, cells were treated with 0.6 mM $(Ac)_5$GP added with or without GW4869 for 24 h. Figure 4A shows that $(Ac)_5$GP enhanced the amount of FasL. It also increased the protein level and activation of caspase 3. N-SMase inhibition dose-dependently blocked FasL expression and caspase 3 activation induced by $(Ac)_5$GP. In Fig. 4B, p75 antagonist peptide also is shown to reduce $(Ac)_5$GP-mediated FasL/caspase cascades. Thus $(Ac)_5$GP seems to transduce the death signals via N-SMase and NGF/p75.

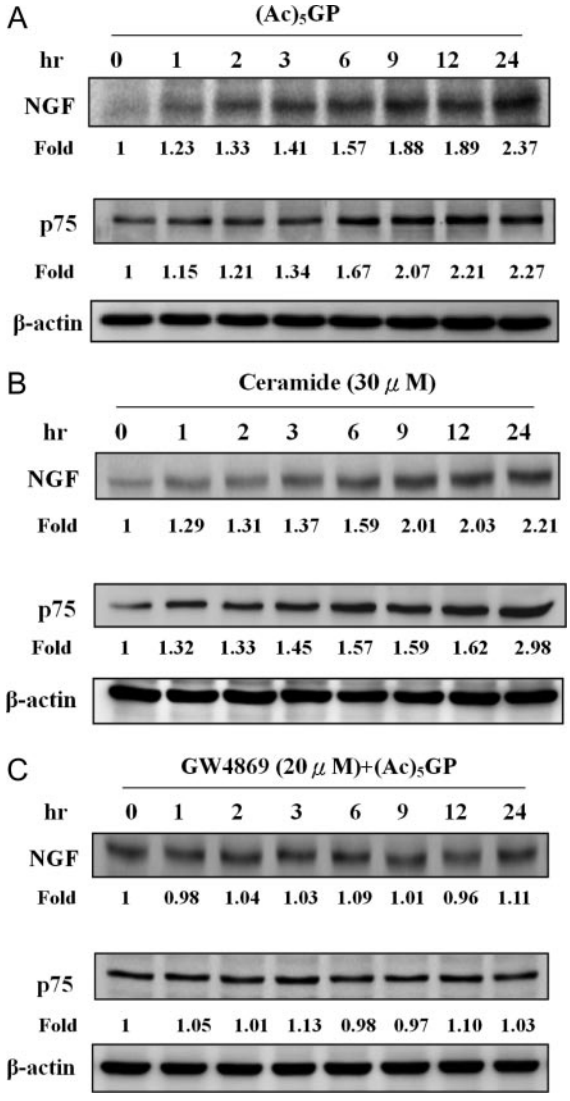

Fig. 3. NGF and p75 levels elevated by $(Ac)_5$GP and downstream of the SMase signal in C6 cells. A, cells were treated with 0.6 mM $(Ac)_5$GP at the indicated time. B, cells were treated with 30 μM ceramide at the indicated time. C, cells were added with 20 μM GW4869 for 1 h and then treated with 0.6 mM $(Ac)_5$GP at the indicated time. The expression of NGF and p75 was analyzed by Western blot and densitometer. Three independent experiments were conducted, which showed similar pattern of changes.

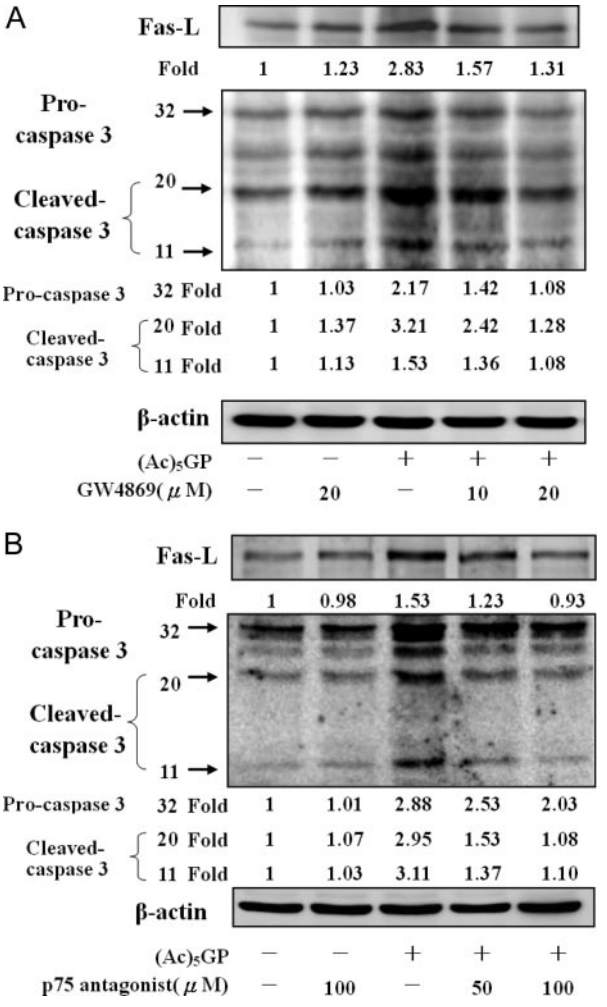

Fig. 4. SMase and p75 mediating FasL expression and caspase 3 activation induced by $(Ac)_5$GP in C6 cells. A, cells were treated with 0.6 mM $(Ac)_5$GP with or without pretreatment of 10 or 20 μM GW4869. B, cells were added with or without 50 or 100 μM p75 antagonist peptide for 1 h and then treated with 0.6 mM $(Ac)_5$GP for 24 h. Three independent experiments were conducted with the similar change.

N-SMase Activation and NGF/p75 Interaction Mediate PKCδ Translocation Induced by $(Ac)_5GP$. Cells were treated with 0.6 mM $(Ac)_5GP$ with or without GW4869 or p75 antagonist for 9 h. Figure 5A showed that N-SMase inhibition reduced the translocation of PKCδ. Likewise, blocking the NGF/p75 interaction by peptide antagonist inhibited PKCδ translocation dose-dependently (Fig. 5B). Figures 4 and 5 demonstrate that N-SMase and NGF/p75 are involved in $(Ac)_5GP$-transduced PKCδ activation and the downstream FasL/caspase cascades, which were reported in the previous study.

$(Ac)_5GP$ Induces Apoptosis through N-SMase Activation. To further confirm that N-SMase activation plays an important role in $(Ac)_5GP$-induced apoptosis, cells were treated with 0.6 mM $(Ac)_5GP$ with or without GW4869 for 24 h. The apoptotic rate was then analyzed by DAPI staining. Figure 6A, c and d, shows that $(Ac)_5GP$ induced apoptosis as ceramide-treated, whereas N-SMase inhibition dose-dependently eliminated the apoptosis induced by $(Ac)_5GP$ (Fig. 6A, e and f). As shown in the bar graph, $(Ac)_5GP$-induced apoptosis was reduced 40 and 80% by 10 and 20 μM GW4869, respectively, indicating that N-SMase activation is critical in $(Ac)_5GP$-mediated apoptosis. The pivotal role of N-SMase was further confirmed by antisense knockout assay (Fig. 6B). The antisense oligonucleotide of N-SMase eliminated more than 80% of apoptosis induced by $(Ac)_5GP$ (Fig. 6B, d and f).

Discussion

In the present study, we demonstrated that N-SMase plays a critical role as the upstream signal above PKCδ in $(Ac)_5GP$-

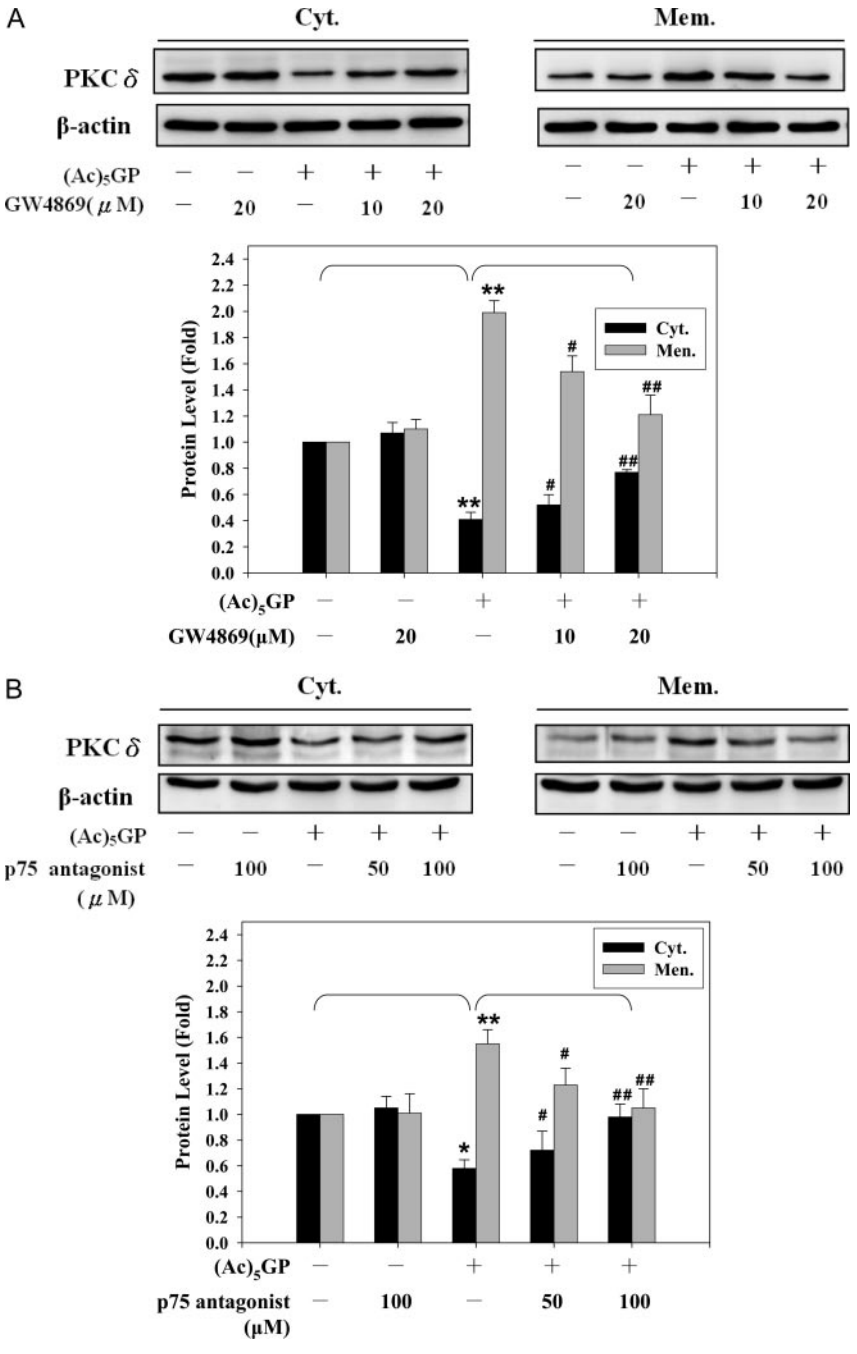

Fig. 5. SMase and p75 mediating the PKCδ translocation induced by $(Ac)_5GP$ in C6 cells. A, cells were treated with 0.6 mM $(Ac)_5GP$ with or without pretreatment of 10 or 20 μM GW4869. B, cells were treated with 0.6 mM $(Ac)_5GP$ with or without pretreatment of 50 or 100 μM p75 antagonist peptide. Translocation of PKCδ was analyzed as the protein level exist in different cell fractions. As shown in the bar graph, data are presented as mean ± S.D., $n = 3$; *, $P < 0.005$; **, $P < 0.001$ compared with the control. #, $P < 0.05$; ##, $P < 0.005$ compared with the cells treated with $(Ac)_5GP$ only.

induced apoptotic pathway. SMases are divided into the classes of 1) A-SMase, which is popular in lysosome; 2) zinc-dependent acid sphingomyelinase, which exists in the serum; 3) N-SMase, which was found in nervous system and mediates the TNF-α signaling; 4) Mg^{2+}-independent neutral sphingomyelinase, which was found in myelin sheath; and 5) alkaline sphingomyelinase, which exits in biliary and gastrointestinal tracts (Levade and Jaffrezou, 1999). Because C6 glioma cell is the cell line tested in the experiment, the SMase possibly involved could be N-SMase and/or A-SMase. Our data suggest that N-SMase, but not A-SMase, is activated and mediates the death signals in $(Ac)_5GP$-induced apoptosis.

Generation of oxidative stress has been reported as a critical event for death-inducing agent, including TNF-α, Fas, viral infections, and chemotherapeutic drugs. It has been found that depletion of GSH precedes the onset of apoptotic signals induced by various agents (Buttke and Sandstrom, 1994; Macho et al., 1997). Liu and Hannun observed that N-SMase from human leukemic cells was inhibited by GSH, suggesting that N-SMase may be a direct target transmitting the effect of GSH depletion (Liu et al., 1998). In the present study, we also found that GSH depletion is involved in $(Ac)_5GP$-induced N-SMase activation (see Supplementary Data S1). GSH depleted rapidly within 15 to 30 min, accompanying with the SMase activation shown in Fig. 2. The $(Ac)_5GP$-induced depletion of GSH was not altered by GW4869, the noncompetitive in-

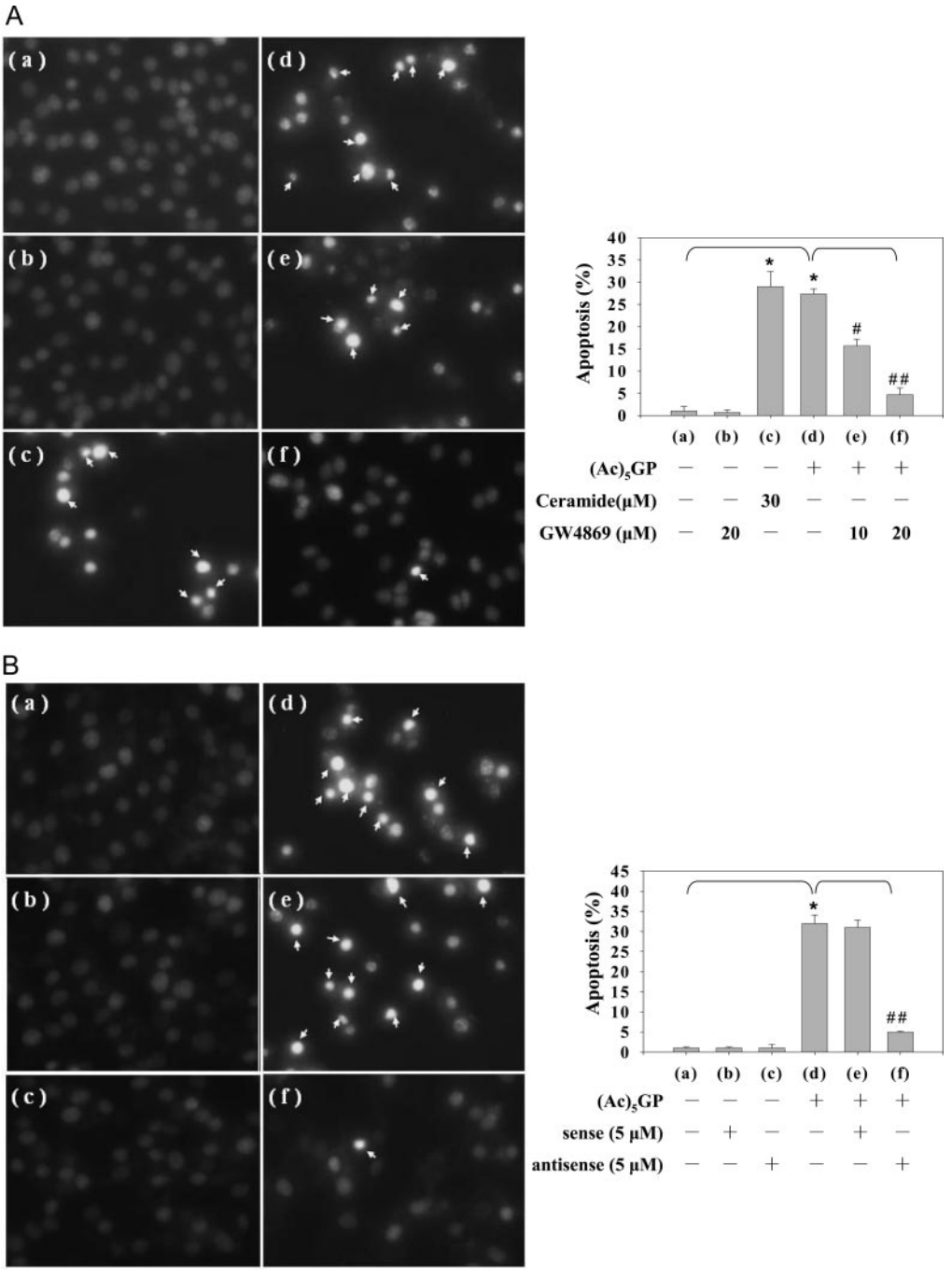

Fig. 6. SMase mediating $(Ac)_5GP$-induced apoptosis in C6 cells. Apoptosis rate was analyzed by DAPI staining. A, cells treated with vehicle only (a), 20 μM GW4869 (b), 30 μM ceramide (c), 0.6 mM $(Ac)_5GP$ (d), 0.6 mM $(Ac)_5GP$ added with 10 μM GW4869 (e), and 0.6 mM $(Ac)_5GP$ added with 20 μM GW4869 (f). As shown in the bar graph, data were presented as mean ± S.D., n = 3; *, $P < 0.001$ compared with the control. #, $P < 0.05$; ##, $P < 0.005$ compared with the cells treated with $(Ac)_5GP$ only. B, cells treated with Lipofectamine as the control group (a), 5 μM sense oligonucleotide (b), 5 μM antisense oligonucleotide (c), 0.6 mM $(Ac)_5GP$ (d), 0.6 mM $(Ac)_5GP$ added with 5 μM sense oligonucleotide (e), and 0.6 mM $(Ac)_5GP$ added with 5 μM antisense oligonucleotide (f). As shown in the bar graph, data are presented as mean ± S.D., n = 3; *, $P < 0.001$ compared with the control. #, $P < 0.05$; ##, $P < 0.005$ compared with the cells treated with $(Ac)_5GP$ only.

hibitor of N-SMase, implicating that GSH was upstream of N-SMase. The addition of GSH reduced the downstream apoptotic signals (PKCδ/Fas L/caspases) induced by (Ac)$_5$GP, just like the phenomenon observed in cells treated with GW4869. However, using thiobarbituric acid-reactive substances analysis, no oxidative stress was found after (Ac)$_5$GP treatment (see Supplementary Data S1). It might be supposed that in C6 glioma cells, (Ac)$_5$GP transduced N-SMase activation and the downstream apoptotic signals not via the oxidative stress. The impeding effect of GSH may be caused by the structural specificity or other indirect mechanism (Liu and Hannun, 1997).

(Ac)$_5$GP increased the expression of p75 within 9 h, whereas the protein amount was not altered after 12 h. The rapid elevation of p75 might be regulated in translational or post-translational level. However, it may also result from the attenuation of p75 degradation, which is supposed to occur in physiological condition. On the other hand, NGF level increased gradually even after 24 h of (Ac)$_5$GP treatment. It describes that NGF exerts different actions via binding with its receptors trkA or p75. trkA transduces NGF signals for survival and differentiation, whereas p75 induces the cell death (Rabizadeh et al., 1993). The consistent elevation of NGF shown in the present study could be supposed as amplification of the apoptotic signals, whereas the rebound effect for cell survival might be included. Thus, the stable expression of p75 should be viewed as the key point in NGF-mediated apoptosis induced by (Ac)$_5$GP.

The cyclic peptide YCDIKGKECY has been shown to be a specific antagonist to p75 receptor (Longo et al., 1997; Li et al., 2005). Early crystallographic studies revealed that three hydrophilic β-loops were contained in NGF surface, which constituted the likely domain for receptor binding. One of the hairpin loops was identified to be formed by NGF 29 to 35 region (McDonald et al., 1991). Ibanetz et al. (1992) found that NGF mutants modified in the 29 to 36 loop region inhibited the binding of p75. Subsequent study suggested that the cyclized peptide dimer exerted the survival-promoting and NGF-inhibitory activity, whereas the linear peptide was inactive (Longo et al., 1997). The short peptide interferes with the action of NGF because it lacks three-dimensional conformation, which may be necessary to activate NGF receptor.

Many previous studies reported that NGF regulated various cell responses via ceramide generation or SMase activation. NGF transduced the cell death of hippocampal neurons through the activation of N-SMase (Brann et al., 2002). NGF/p75 induced differentiation and ceramide-mediated apoptosis in Schwann cells cultured from degenerating nerves (Hirata et al., 2001). It also indicated that SMase is involved in NGF-mediated outgrowth of nerve cells (Brann et al., 1999). In the present study, we demonstrated NGF signaling downstream of SMase. Similar results were also reported by Golve-Roperch et al. (1997), who suggested that SMase/ceramide induces NGF synthesis via activation of MAP kinase. Taken together, some complicated cross-talks are predicted to exist between SMase and NGF, which may coordinate to promote the death signals.

The apoptotic pathway induced by (Ac)$_5$GP is shown in Fig. 7. (Ac)$_5$GP transduced SMase activation and NGF/p75 expression, thus mediating the PKCδ/JNK/Fas/caspase death signals. In fact, SMase/ceramide may modulate a variety of downstream targets, including kinase, phosphatase, and transcription factors. SMase and ceramide were reported to activate MAP kinase in HL-60 cells (Raines et al., 1993). It has been described that Raf phosphorylation is involved in SMase/ceramide-stimulated MAP kinase activation (Huwiler et al., 1996). SMase pathway has been suggested to control NGF synthesis via MAP kinase activation in astrocyte cultures (Galve-Roperh et al., 1997). In our undergoing investigation, we also found that MAP kinase is involved in NGF/p75 expression (data not shown). Whether MAP kinase is involved downstream of (Ac)$_5$GP-activated SMase, hence mediating the transcription of NGF/p75, will be further investigated in our future study.

In C6 glioma cells, (Ac)$_5$GP transduces apoptotic signals through the activation of SMase and downstream expression of NGF and p75, thus mediating PKCδ translocation and the

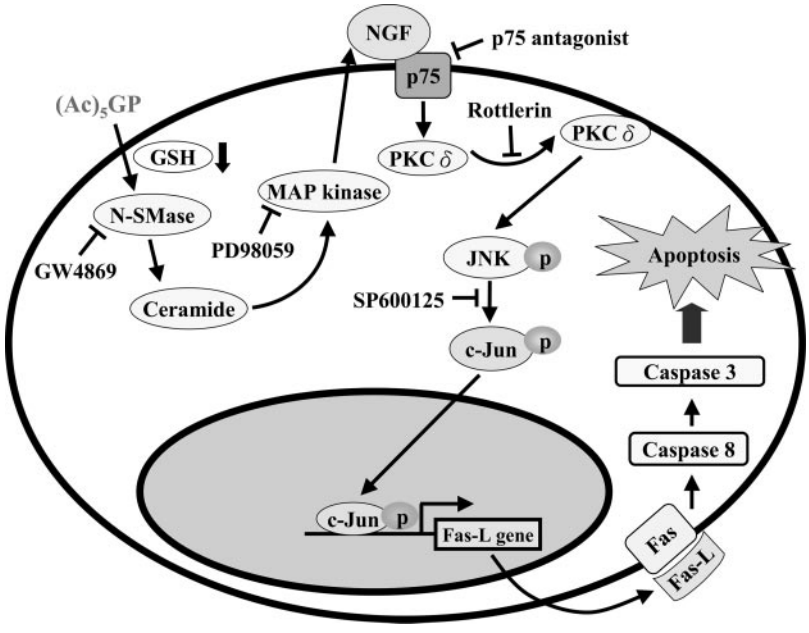

Fig. 7. The apoptotic pathway induced by (Ac)$_5$GP in C6 glioma cells. Signals downstream PKCδ were reported in our previous studies (Peng et al., 2004).

following JNK/Fas/caspase cascades. These results indicate that $(Ac)_5GP$ could potentially be developed as an antitumor drug.

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