Differential Regulation of Cyclooxygenase-2 Expression by Phytosphingosine Derivatives, NAPS and TAPS, and its Role in the NAPS or TAPS-Mediated Apoptosis

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We investigated the effect of novel phytosphingosine derivatives, N-acetyl phytosphingosine (NAPS) and tetra-acetyl phytosphingosine (TAPS), on induction of apoptosis in HaCaT cells in comparison with C2-ceramide. NAPS/TAPS effectively decreased cell viability in a dose dependent manner mainly due to apoptosis. An apoptosis expression array analysis showed that in the TAPS treated cells 13 genes including COX-2 encoding cyclooxygenase-2, the most induced by TAPS, were up-regulated while 23 others down-regulated. Therefore, we examined the mechanism underlying the altered expression of COX-2. Assays with inhibitors and antibodies against proteins involved in signal transduction demonstrated that NAPS and TAPS elevated COX-2 expression via tyrosine kinase, src, PI-3 kinase and PKC, followed by ERK activation. However, P38 was not involved in the NAPS-mediated COX-2 expression but in the TAPS-mediated. We further demonstrated by FACS analyses that NAPS- or TAPS-mediated apoptosis was greatly increased in cells treated with celecoxib, a selective COX-2 inhibitor. Inhibition of the ERK pathway apparently involved in the NAPS/TAPS-mediated COX-2 expression enhanced the NAPS/TAPS-mediated apoptosis, whereas inhibition of the P38 pathway did not. These results suggest that expression of COX-2 in the TAPS- or NAPS-treated cells may be increased to counteract the effect of those compounds on apoptosis.


Sphingolipids are found in all eukaryotic cells and enriched in the plasma membranes, Golgi membranes, and lysosomes (Merrill et al, 1997; Huwiler et al, 2000). Sphingolipid metabolites such as ceramides and sphingosines play important parts in regulating cellular activities, and have been implicated as putative second messengers in the signaling pathways (Merrill et al, 1997). Ceramides regulate cell differentiation, cell cycle arrest, proliferation, and apoptosis (Mathias et al, 1998; Perry and Hannun, 1998). Sphingosine also induces apoptosis and growth inhibition in various cell types as an intracellular signal mediator (Spiegel and Merrill, 1996).

Phytosphingosine (PS) is abundant in fungi and plants (Dickson, 1998) and is present in animals, including humans (Schurer et al, 1991). PS is structurally similar to sphingosine and ceramide with one major difference: PS possesses a hydroxyl group at C-4 of the sphingoid long-chain base, whereas sphingosine and ceramide both contain a trans double bond between C-4 and C-5 (Lee et al, 2001). It has been reported that both PS and N-acetyl phytosphingosine (NAPS) were involved in the heat stress response of Saccharomyces cerevisiae (Jenkins et al, 1997; Wells et al, 1998), and induced cell death in Chinese hamster cells (Lee et al, 2001). Little is known, however, about the cellular functions of the PS derivatives.

Cyclooxygenase (COX)-2 overexpression in cancer cells may promote tumor growth by imparting resistance to apoptosis. Consistent with this, a null COX-2 mutation in APC(C716 knock-out mice) is a murine model of familial adenomatous polyposis, restored the apoptosis and reduced the size and the number of colorectal adenomas (Oshima et al, 1996). The role of COX-2, however, in the induction of apoptosis and in skin tumor development is still controversial. Overexpression of the COX-2 protein has been reported to induce cell cycle arrest independently of the prostaglandins, which leads to the induction of apoptosis (Trifán et al, 1999). In addition, a specific COX-2 inhibitor, celecoxib induced apoptosis but its proapoptotic effect was independent of COX-2 inhibition in mouse embryo fibroblasts, which were derived from COX-2(+/-) and COX-2(-/-) mice (Williams et al, 2000). Moreover, observations relating to the proapoptotic effect of nonsteroidal anti-inflammatory drugs has resulted in contradictory conclusions in that the proapoptotic activity of the COX inhibitors occurs via either COX-dependent or COX-independent mechanisms (Rigas and Shiff, 2000).

It was reported that ceramides induce apoptosis and enhance COX-2 expression. C2-ceramide was shown to increase COX-2 expression through a signal transduction pathway mediated by the extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase, and p38 mitogen-activated protein kinase (MAPK), which belong to the MAPK family in human mammary

Abbreviations: COX-2, cyclooxygenase-2; FAK, focal adhesion kinase; NAPS, N-acetyl phytosphingosine; PKC, protein kinase C; SSPE, sodium chloride sodium phosphate EDTA; TAPS, tetra-acetyl phytosphingosine; TBST, tris-buffered saline with Tween 20; TUNEL, terminal deoxyribonucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling.

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epithelial cells (Subbaramaiah et al., 1998). The MAPK family consists of a set of serine threonine protein kinases, which leads to proto-oncogene expression (Cobb, 1999; Thomson et al., 1999; Pages et al., 2000). The activated MAPK may translocate to the nucleus, where they phosphorylate their target proteins (Coso et al., 1995). Ceramides mediate their effects by activating phospho-protein phosphatases (Dobrowsky and Hannun, 1992) and serine/threonine kinases (Liu et al., 1994) that may increase Raf activity (Zhang et al., 1997), and by inhibiting phospholipase D (Gomez-Munoz et al., 1994).

In this study, we examined the effects of the novel PS derivatives, NAPS and tetraerythro-tetraphosphine (TAPS), on the induction of programmed cell death of an immortalized human keratinocytes cell line HaCaT, and identified the genes of altered expression in the TAPS-treated cells. TAPS remarkably increased COX-2 expression at the mRNA level. The focus of this study was therefore to examine (i) signaling pathways, by which novel PS derivatives, NAPS and TAPS, turn on the expression of COX-2, and (ii) the role of COX-2 in NAPS- or TAPS-mediated apoptosis.

MATERIALS AND METHODS

Materials

The PS derivatives, NAPS and TAPS, were provided from Doosan Biotech (Yongin, Korea), and N-acetyl-D-erythrophosphine (Cayman Chemicals, USA). Synthesis of these PS derivatives was confirmed by nuclear magnetic resonance spectroscopy and matrix-assisted laser desorption ionization-time of flight mass spectrometry. The purity of the NAPS or TAPS was greater than 98% by high performance liquid chromatography (Lee et al., 2001) (Fig. 1). NAPS and TAPS in dimethyl sulfoxide were aliquoted in small amounts and stored at -20°C until used. An In Situ Cell Death Detection Kit was obtained from Boehringer Mannheim (Mannheim, Germany). The MTX (3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide) and all other chemicals were purchased from Sigma. Celecoxib was kindly provided by Pharmacia Biotech Inc. (San Francisco, California). [32P] deoxycytidine triphosphate (3000 Ci/mmol) was purchased from Amersham (Piscataway, New Jersey). Dulbecco’s modified Eagle medium and fetal bovine serum were obtained from Gibco BRL (Rockville, Maryland). Anti-COX-2 and anti-β-tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, California), and anti-p-MEK1/2 and p-p38 antibodies were obtained from Cell Signaling Technology (Beverly, Massachusetts). PIP, U73122, and GF109203X were obtained from Biomol (Plymouth Meeting, Pennsylvania). Genistein was purchased from Alexis (Plymouth Meeting, Pennsylvania). NAPS or TAPS was kindly provided by Professor N. Fuseng (German Cancer Research, German Cancer Research Center, Germany). Cells were maintained as monolayer cultures in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum, 100 units per mL penicillin, and 100 μg streptomycin per mL at 37°C in a humidified atmosphere containing 5% CO2. The cells were then seeded in a 6 well plate and kept on ice for 60 min. Cells were then permeabilized in a reagent consisting of 0.5% Triton X-100, 230 mM NaCl, 50 mM Tris-Cl (pH 7.5), and then kept on ice for 60 min. Cells were then washed with serum-free medium, replaced with media without fetal bovine serum for 12 h prior to treatment with PS derivatives.

Assessment of cell viability (MTT assay)

The immortalized human keratinocyte cell line, HaCaT, was kindly provided by Professor N. Fuseng (German Cancer Research, Germany). Cells were maintained as monolayer cultures in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum, 100 units per mL penicillin, and 100 μg streptomycin per mL at 37°C in a humidified atmosphere containing 5% CO2. The cells were then seeded in a 6 well plate and kept on ice for 60 min. Cells were then permeabilized in a reagent consisting of 0.5% Triton X-100, 230 mM NaCl, 50 mM Tris-Cl (pH 7.5), and then kept on ice for 60 min. Cells were then washed with serum-free medium, replaced with media without fetal bovine serum for 12 h prior to treatment with PS derivatives.

Assessment of cell viability (MTT assay)

The cell viability was determined colorimetrically using the MTT reagent. The MTT assay was performed as previously described (Wilson and Spier, 1987).

TUNEL (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick ending-labeling) assay

TUNEL assay of the nucleosomal DNA fragments using a commercially available “In Situ Cell Death Detection Kit” (Boehringer Mannheim) was employed according to the manufacturer’s protocol. This method was used to ensure the safe discrimination between apoptosis and necrosis.

Treatment of cells with inhibitors

Cells were pretreated with 20 μM LY294002 (an inhibitor of phosphatidylinositol (PI3)-kinase), 40 μM PD98059 (a selective ERK inhibitor), 5 μM SB203580 (a p38 inhibitor), 10 μM U0126 (a MEK inhibitor), 10 μM PPI (a src family specific inhibitor; Klint et al., 1999), 100 μM genistein (a tyrosine kinase inhibitor), or 5 μM GF109203X (a protein kinase C (PKC) inhibitor; Wolfe et al., 1996) for 1 h in serum-free medium, and then exposed to 30 μM NAPS or TAPS for the indicated time points. For fluorescence-activated cell sorter (FACS) analysis, cells were also pretreated with 10 μM celecoxib (a specific COX-2 inhibitor) for 1 h, before treatment with 30 μM of either NAPS or TAPS.

Immunohistochemical analysis

Cells were harvested and lyzed with a RIPA buffer containing 0.5% sodium dodecyl sulfate, 1% nonidet P40, 1% sodium deoxylcholate, 150 mM NaCl, 50 mM Tris–Cl (pH 7.5), and protease inhibitors. The protein concentration of each sample was determined using a BCA protein assay kit (Pierce, Rockford, Illinois). Twenty micrograms of the total protein for COX-2 detection were electrophoresed on 8% polyacrylamide gels and 80 μg for phospho-MAPK detection on 12% polyacrylamide gels. The proteins were transferred on to the nitrocellulose membranes by a semidry electrophoretic transfer process on a Bio-Rad TransBlot SD (Bio-Rad Laboratories, Hercules, CA) apparatus at 15 V for 60–75 min. The nitrocellulose membranes were blocked overnight at 4°C in 5% bovine serum albumin. The membranes were incubated with the primary antibodies in TBST containing 5% bovine serum albumin, for 2 h. This was followed by incubation with the secondary antibodies at room temperature for 1 h. Anti-goat IgG for COX-2 or anti-rabbit IgG for p-MEK1/2 or p-p38 was used as a secondary antibody (1:5000 dilution in TBST containing 1% bovine serum albumin). The signals were detected using ECL (Amersham).

Flow cytometric analysis

FACS analysis was performed using a slight modification of the method described by Telford et al. (1994). Briefly, the cells were pelleted at 350 g and washed once with 10 mL of ice-cold phosphate-buffered saline. The pellets were resuspended in 70% ethanol, and then kept on ice for 60 min. Cells were then permeabilized in a reagent consisting of 0.5% Triton X-100, 230 μg/mL RNaseA and propidium iodide to 50 μg/mL in phosphate-buffered saline. The samples were kept at 37°C for 30 min followed by flow cytometry analysis (Becton Dickinson FACSCan Becton Dickinson, Franklin Lakes, NJ), and the apoptotic populations (sub-G1 phase) were analyzed by the CellQuest program (BD Bioscience Franklin Lakes, NJ).

Apoptosis microarray assay

An aliquot of 2 μg of total RNA was used in the microarray analyses, which were performed according to the Human Apoptosis Expression Array kit manual (R&D, Minneapolis, Minnesota). Total RNA was reverse-transcribed with avian myeloblastosis virus reverse transcriptase using apoptosis-specific primers and labeled with [32P]-cytidine triphosphate. Labeled cDNA were purified using Sephadex G-25 spin column and hybridized with the array overnight at 65°C according to the manufacturer (R&D). Reagents included in the array kit were used for all hybridization. Wash solution I (0.5 × SSPE, 1% (w/v) sodium dodecyl sulfate) and wash solution II (0.1 × SSPE, 1% (w/v) sodium dodecyl sulfate) were used for washing steps. Membranes were exposed on Fuji RX X-ray film.

Analysis of the array results

Gene expression data were normalized against the housekeeping gene such as β2M, β-actin, cyclophilin A, HLA-A (0201) heavy chain, hypoxanthine–guanine phosphoribosyltransferase, L19 transferrin R, and α-tubulin which were shown at a constant level in all arrays. The Genesight 3.2 program from Biodiscovery (Biodiscovery Inc. Micororay Systems, Marina del Ray, CA) was used to analyze the data, and all gene values were normalized against the housekeeping gene.

Data analysis

Scanning densitometry was performed using an Image Master VDS (Pharmacia Biotech Inc.). The one-way ANOVA procedure was used to test the significant differences among the treatment groups. For each significant effect of treatment, the Newman–Keuls test was used for significance set at p < 0.05 or p < 0.01.
RESULTS

Effects of PS derivatives on the cell viability of the human keratinocytes HaCaT in a concentration-dependent manner

The effects of the PS derivatives, NAPS and TAPS, and C2-ceramide on the viability of HaCaT cells were examined using the MTT assay. After incubating the HaCaT cells in the presence of 1 to 30 μM of each compound for 24 h, the cell viability was reduced as the concentration increased (Fig 2). Considering the ED50 value, NAPS and TAPS induced the death of keratinocyte cells as effective as C2-ceramide in cytotoxicity, but they exerted strong cytotoxicity at 30 μM (viability 20%), compared with C2-ceramide (viability 40%). It was reported that the hydroxyl group at C4- of the sphingosine long-chain base is critical for its anti-proliferative activity and the N-acetyl group is also important for cytotoxicity (Lee et al., 2001). These results suggest that PS as well as sphingosine and C2-ceramide trigger HaCaT cell death.

Altered expression of genes encoding apoptosis-related proteins in TAPS-treated cells

To figure out factors responsible for the apoptosis mediated by a PS derivative, TAPS, we performed apoptosis expression array comprising 198 genes involved in apoptosis. Total mRNA was isolated from the TAPS-treated cells and the control cells, respectively. We identified genes, whose expression altered by at least 2-fold in the TAPS-treated cells compared with the control cells. The genes with altered expression in the TAPS-treated HaCaT cells were classified based on their cellular function and listed in Tables I and II. Among 198 genes on the array membrane, 13 genes were increased in TAPS-treated cells compared with control cells, whereas 23 genes were repressed (Tables I and II). Five genes were cell cycle regulator genes: p21, p53, cyclin A2, CDK2, and RdAp48. It is interesting to note that p21 and p53 were differentially expressed and that p21 was induced, whereas p53 was reduced. Apoptosis suppressor survivin and IAP-1 were downregulated by TAPS. Interestingly, apoptosis array analysis showed that expression of COX-2, which is known to be involved in the proliferation blocking apoptosis in cancer cells, dramatically increased (approximately 16.6-fold) with TAPS (30 μM) treatment.

PS derivatives induce apoptosis in a concentration-dependent manner

A TUNEL assay was performed in order to determine if the cytotoxicity of the PS derivatives was related to apoptosis (Fig 3). The results show that the TUNEL-positive cells were detected in the NAPS-, TAPS-, or C2-ceramide-treated cells at 10 μM and 30 μM, respectively. The percentage of TUNEL-positive cells for TAPS, NAPS, or C2-ceramide was 29%, 35%, or 17% at 30 μM, respectively, suggesting that TAPS or NAPS induced apoptosis more effectively than C2-ceramide.

NAPS and TAPS induce COX-2 protein more than C2-ceramide

In this study, our focus was to examine the induction mechanism of COX-2 protein by the PS derivatives, NAPS and TAPS, and compared them with PS and C2-ceramide. The human keratinocyte cell line, HaCaT, was treated with 30 μM of PS, NAPS, TAPS, or C2-ceramide for 24 h.
Western blot analysis showed that the COX-2 protein level was significantly higher in the NAPS- or TAPS-treated cells (approximately 15- and 22-fold, respectively) than in the control cells [Fig 4A]. In contrast, the C2-ceramide- and PS-treated cells showed a relatively small increase in the COX-2 protein level compared with the control cells. The induction of COX-2 protein was not apparent at a concentration of between 1 and 10 μM, but 30 μM of NAPS, TAPS, or C2-ceramide dramatically increased COX-2 induction [Fig 4B]. In addition, this concentration induced apoptosis efficaciously in the HaCaT cells [Fig 3]. Samples were taken at the indicated time points in order to monitor COX-2 production with respect to time after treatment. Production of COX-2 at 30 μM of TAPS became apparent 4 h after treatment, its level reached a maximum at 8 h and decreased thereafter [Fig 4C]. Unlike TAPS, NAPS-induced COX-2 expression increased steadily to a maximum 16 h after treatment with SB203580, rather it increased slightly.

ERK and p38 contribute to the induction of COX-2 by TAPS, but p38 is not involved in the NAPS-induced COX-2 induction It has been reported that the C2-ceramide-induced COX-2 promoter activity was inhibited by the dominant negative mutant of ERK 2 and p38 (Chen et al, 2001). In order to determine if ERK or p38 is also involved in the signaling pathways for the COX-2 induction by the novel PS derivatives NAPS or TAPS in HaCaT cells, the NAPS- or TAPS-induced COX-2 protein level was examined after suppressing either p38 or ERK using their inhibitors. As shown in Fig 5(A,B), blocking ERK with a selective ERK inhibitor, PD98059 (40 μM), almost completely abrogated the TAPS-induced COX-2 increase at 8 h, whereas it inhibited the NAPS-induced COX-2 increase to a lesser degree. The induction of the COX-2 protein by TAPS was also significantly inhibited by pretreatment with the p38 inhibitor, SB203580 (5 μM), but no alteration was observed in the COX-2 production induced by NAPS despite the treatment with SB203580, rather it increased slightly.

Furthermore, 10 μM U0126, a MEK inhibitor, effectively inhibited the TAPS-induced COX-2 protein, but only inhibited the NAPS-induced COX-2 protein level to a lesser degree, the extent of which was comparable with that inhibited by PD98059, an ERK inhibitor. These results suggest that TAPS increases COX-2 expression via the ERK and p38 pathway. ERK, however, is a greater contributor than p38, and NAPS may not act via p38 pathway.

NAPS and TAPS also increase the COX-2 expression through PI3-kinase It has been reported that ceramide regulates gene expression is through activating the PI3-kinase signal transduction pathway (Subbaramaiah et al, 1998). Therefore, this study examined whether or not the induction of PI3-kinase with LY294002 blocks the COX-2 induction by both NAPS and TAPS. Immunoblot analysis showed that a pretreatment with 20 μM LY294002 for 1 h almost completely prevented the increase in COX-2 protein levels by NAPS or TAPS. These results show that both NAPS and TAPS increase COX-2 expression through PI3-kinase [Fig 6A,B].

Tyrosine kinase, src, and PKC are differentially involved in the induction of COX-2 by NAPS or TAPS Figure 6 shows that PI3-kinase is involved in the NAPS- or TAPS-mediated COX-2 induction. One of the mechanisms by which ceramide regulates gene expression is through activating the PKC signal transduction pathway (Subbaramaiah et al, 1998). COX-2 is a target of activated PKC (Subbaramaiah et al, 1998). Tyrosine kinase phosphorylates src and PI3-kinase, which is followed by activation of phospholipase C or PKC. Therefore, genistein (100 μM), which is a tyrosine kinase inhibitor, was
used to examine if tyrosine kinase is involved upstream of PI3-kinase in NAPS- or TAPS-induced COX-2 induction. Immunoblot analysis showed that the genistein pretreatment reduced the TAPS-induced COX-2 protein levels indicating involvement of a tyrosine kinase (Fig 7B). We also examined if src is involved in the TAPS-induced COX-2 protein induction. The COX-2 protein induction by TAPS was completely prevented by pretreatment with 10 μM PP1, a src inhibitor (Fig 7B). In addition, GF109203X, a PKC inhibitor, inhibited the induction of the COX-2 protein by TAPS (Fig 7B) at 5 μM. The induction of the COX-2 protein by NAPS, however, was not blocked by pretreatment with genistein, PP1, or GF109203X (Fig 7B). These results suggest that NAPS and TAPS differentially regulate COX-2 expression. TAPS might regulate COX-2 expression through autophosphorylation of tyrosine kinase, which is followed by the activation of src and PI3-kinase, and finally by the activation of the PKC and/or ERK pathway. The COX-2 expression by NAPS might be also mediated through the PI3-kinase and ERK pathway. Upstream signal molecules that regulate the COX-2 expression induced by NAPS, however, needs to be further studied.

Figure 4. NAPS and TAPS induce the production of COX-2 protein more than C2-ceramide. (A) Immunoblot analysis of the COX-2 protein and the relative COX-2 protein level. Confluent HaCaT cells were treated with PS, NAPS, TAPS, or C2-ceramide (30 μM) and the total cell lysate was extracted from the cells after 24 h incubation. Each lane was loaded with 20 μg of the cell lysates. The detailed procedure is described in Materials and Methods. The relative COX-2 protein levels were normalized by scanning densitometry. The data are shown as a mean ± SEM of three independent experiments. One-way analysis of variance was used for the comparisons of the multiple group means followed by Newman–Keuls test (significance compared with control, *p<0.05 or **p<0.01) (control level = 1). (B) The representative immunoblot shows that HaCaT cells treated with NAPS, TAPS, or C2-ceramide for 24 h showed a marked increase at a concentration of 30 μM (C) Immunoblot analysis for COX-2 protein was performed in NAPS-, TAPS-, or C2-ceramide-treated HaCaT cells. The total cell lysate was extracted from the cells at the indicated times. The data were confirmed by two repeated experiments.

Figure 5. ERK and p38 contribute to the expression of COX-2 by TAPS, but p38 is not involved in the NAPS-induced COX-2 expression. Induction of the COX-2 protein by NAPS (A) and TAPS (B). The level of the COX-2 protein was assessed by immunoblot, and the relative COX-2 protein levels were plotted after scanning densitometry. Expression of the COX-2 protein was measured from NAPS-treated cells for 16 h or TAPS-treated cells (30 μM) for 8 h after 1 h pretreatment with ERK inhibitor PD98059 (40 μM), p38 inhibitor SB203580 (5 μM), and MEK inhibitor U0126 (10 μM). Data represent the mean ± SEM of three separate experiments. One-way analysis of variance was used for the comparisons of the multiple group means followed by Newman–Keuls test (significance compared with the control, **p<0.01; significant compared with NAPS or TAPS, ††p<0.01) (control level = 1).
Treatment with various kinase inhibitors indirectly showed that upregulation of COX-2 by TAPS or NAPS occurs through the ERK pathway or PI3^PKC pathway. We further examined whether TAPS indeed facilitates phosphorylation of MEK or p38, moreover, if these inhibitors are blocking their target pathways as predicted. Results showed that phosphorylation of MEK was detected at early time (≈5 min) point after TAPS treatment and sustained until 12 h (Fig 8). Phospho-p38 was also increased in TAPS-treated cells. TAPS-induced phosphorylation of MEK or p38, however, was inhibited by the preincubation of cells with PD98059 or SB203580. These results suggest that the ERK pathway is mainly involved in COX-2 induction by TAPS. Regarding the signaling pathway related to the COX-2 induction of C2-ceramide compared with that of NAPS or TAPS appeared to adopt a signal pathway similar to that of TAPS (Fig 9).

Inhibition of COX-2 function enhances significantly NAPS- or TAPS-mediated apoptosis NAPS- and TAPS-induced apoptosis was previously shown by TUNEL assay. The effects of the COX-2 inhibition by a selective COX-2 inhibitor, celecoxib, on NAPS- or TAPS-induced apoptosis were examined in order to test if induction of COX-2 is part of a protective mechanism to compensate for NAPS- or TAPS-induced apoptosis. We used 30 μM of NAPS or TAPS, at which concentration COX-2 protein production as well as the sub-G1 population was remarkably elevated. Twenty-four hours after HaCaT cells were exposed to either NAPS or TAPS with or without celecoxib (10 μM) cells were collected and treated with propidium iodide for the FACSScan to determine the sub-G1 DNA content. FACS analysis showed that the cells exposed to either NAPS or TAPS and celecoxib resulted in a remarkable increase in the cell death rate (Fig 10B). Co-treatment with NAPS or TAPS and celecoxib increased the sub-G1 percentage from 26.1 to 56.57% or from 33.4 to 60.47% when compared with the treatment with NAPS or TAPS alone. These results demonstrate that COX-2 protects the cells from NAPS- or TAPS-induced apoptosis, and that an impairment of COX-2 function leads to a pronounced increase in apoptosis.

The sub-G1 population was monitored by FACS analysis after adding 40 μM PD98059 to the cells treated with NAPS or TAPS (Fig 10B) in order to address whether or not the ERK signaling pathway involved in the COX-2 expression by NAPS or TAPS plays an important part in protecting cells from NAPS- or TAPS- induced apoptosis. It should noted that the cells cotreated with PD98059 increased the sub-G1 population by up to 44.60% and 53.05%, when it was combined with both NAPS or TAPS. In contrast, p38 inhibition using SB203580 failed to increase the sub-G1 population by TAPS, and even blocked the sub-G1 population induced by NAPS from 26.1 to 20.19%. This result corresponds to the fact that p38 inhibition slightly increased the COX-2 expression level induced by NAPS. These results show that the ERK signaling pathway protects cells from the

Figure 6. NAPS or TAPS also increase the expression of COX-2 through PI3-kinase. HaCaT cells were treated with NAPS (A) and TAPS (B) after preincubation for 1 h with PI3-kinase inhibitor 20 μM LY294002 16 h or 8 h later, cell lysate was extracted from the cells and immunoblot analysis was performed as described in Fig 5. The data were confirmed by three independent experiments.

Figure 7. Tyrosine kinase, src, and PKC are involved in the NAPS- or TAPS-mediated COX-2 induction. The cell lysate was extracted from the cells treated with NAPS (A) for 16 h or TAPS (B) for 8 h after preincubation for 1 h with the tyrosine inhibitor, genistein (GT) 100 μM, the src inhibitor, PP1 (10 μM), and the PKC inhibitor, GF109203X (5 μM), and immunoblot analysis was performed, as described in Fig 5. The data were confirmed by two repeated experiments.

Figure 8. TAPS phosphorylates MAPK. The cell lysate was extracted from the cells treated with TAPS at indicative times and then immunoblot analysis was performed using anti-p-MEK1/2, anti-p-p38, anti-MEK1/2, and anti-p38 antibodies. To determine the specificity of the MAPK inhibitors, HaCaT cells were harvested from the cells treated with TAPS for 8 h after preincubation for 1 h with ERK inhibitor PD98059 and p38 inhibitor SB203580. Each lane was loaded with 80 μg of the cell lysates. The data were confirmed by three times repeated experiments.
apoptosis caused by NAPS or TAPS by being involved in the COX-2 expression. Furthermore, these results provide a notion that p38 might negatively regulate the COX-2 expression by NAPS.

DISCUSSION

This study showed that the PS derivatives, NAPS and TAPS, induced apoptosis more effectively than C2-ceramide. It was found through expression array analysis that COX-2 was the most strongly induced gene among the genes upregulated by TAPS at the mRNA level.

It has been reported that a variety of stimuli, including tumor necrosis factor-α, interleukin-1β, phorbol esters, γ-interferon, and ultraviolet light stimulate the hydrolysis of sphingomyelin by sphingomyelinase (Smase), which results in the formation of ceramide, and induces COX-2 (Okazaki et al., 1990; Herschman, 1996; Spiegel et al., 1996; Huang et al., 1997; Sbrissa et al., 1997; Buckman et al., 1998). Therefore, it is possible that these agonists induce COX-2 at least in part, via ceramide signaling. It is known that ceramide induces apoptosis, whereas COX-2 overexpression inhibits apoptosis (Tsujii and DuBois, 1995; Verheij et al., 1996). Subbaramaiah et al (1998) proposed that the proapoptotic effects of ceramide may be counteracted, in part, by its ability to upregulate COX-2. A detailed mechanism demonstrating the relation between the induction of apoptosis and COX-2 expression by ceramide, however, has not been studied. The mechanism by which novel PS derivatives, NAPS and TAPS, induce COX-2 expression, and its role in the NAPS- or TAPS-mediated apoptosis in human keratinocytes are unclear. Therefore, this study examined the induction mechanism of the COX-2 gene by NAPS and TAPS, and the role of COX-2 in inducing apoptosis.

Ceramide signaling can be mediated by PKC (Sbrissa et al., 1997). PKC can activate Raf-1, which in turn, regulates the MAPK activity (Reunanen et al., 1998). Ceramide was previously

Figure 9. C2-ceramide induces COX-2 protein through tyrosine kinase, PI3-kinase, and ERK pathway. The cells were treated with 30 μM C2-ceramide after preincubation for 1 h with the PI3-kinase inhibitor, LY294002, the ERK inhibitor, PD98059, the p38 inhibitor, SB203580, the MEK inhibitor, U0126, the tyrosine inhibitor, genistein (GT), the src inhibitor, PP1 and the PKC inhibitor, GF109203X. Eight hours later, the cell lysate was extracted from the cells and immunoblot analysis was performed as described in Fig 5. The data were confirmed by two independent experiments.

Figure 10. Inhibition of COX-2 induction enhanced NAPS- or TAPS-mediated apoptosis. (A) The cells were exposed to celecoxib, NAPS, or TAPS at a concentration of 10 or 30 μM for 24 h. Cell were then harvested for FACS analysis. (B) The cells were pretreated with 40 μM PD98059 and 5 μM SB203580 for 1 h. After 24 h coinubation with either NAPS or TAPS at a concentration of 30 μM, the cells were collected for FACS analysis. The M cell population represents the apoptotic cells from each sample.
shown to induce the p38, c-Jun N-terminal kinase, and ERK/MAPK activities (Reununen et al., 1998). COX-2 expression can be affected by changes in the MAPK activity (Xie and Herschman, 1995, 1996; Guan et al., 1998). Several lines of evidence suggest that the COX-2 induction by ceramide occurs via ERK, c-Jun N-terminal kinase, and p38 MAPK activation (Xie and Herschman, 1995, 1996; Guan et al., 1998; Subbaramiah et al., 1998). This study showed that the induction of COX-2 by TAPS was blocked by GF109203X, a potent and selective PKC inhibitor. In addition, it was found that MEK, ERK, and p38 are important for mediating the induction of COX-2 by TAPS. Moreover, PI3-kinase was involved in the COX-2 induction by either NAPS or TAPS; however, we cannot rule out the possibility that the hydrocarbon chains of PS may facilitate a hydrophobic interaction with their cellular targets such as fatty acids, thereby indirectly exerting these effects.

To elucidate further the mechanism by which TAPS signaling induces COX-2 expression, tyrosine kinase and src were also examined to determine if they are involved in COX-2 expression. The results showed that TAPS increased the COX-2 protein level by activating tyrosine kinase and src. This is consistent with previous reports showing that ceramide formation in fibroblasts stimulates the tyrosine kinase activity (Hanna et al., 1995, 1996; Guan et al., 1996). However, we cannot rule out the possibility that the hydrocarbon chains of PS may facilitate a hydrophobic interaction with their cellular targets such as fatty acids, thereby indirectly exerting these effects.

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