Fenretinide inhibited de novo ceramide synthesis and proinflammatory cytokines induced by Aggregatibacter actinomycetemcomitans

Hong Yu, Michael Valerio, and Jacek Bielawski

Abstract - Ceramides play an essential role in modulating immune signaling pathways and proinflammatory cytokine production in response to infectious pathogens, stress stimuli, or chemotherapeutic drugs. In this study, we demonstrated that Aggregatibacter actinomycetemcomitans, the pathogen for aggressive periodontitis, induced de novo synthesis of ceramide in Raw 264.7 cells. In addition, we identified that fenretinide, a synthetic retinoid, suppressed the de novo synthesis of ceramide induced by A. actinomycetemcomitans. Moreover, fenretinide attenuated interleukin (IL)-1β, IL-6, and cyclooxygenase-2 mRNA expression induced by A. actinomycetemcomitans. Fenretinide also decreased IL-1β, IL-6, and prostaglandin E2 proinflammatory cytokine levels in Raw 264.7 cells induced by A. actinomycetemcomitans. However, fenretinide had no significant effects on tumor necrosis factor alpha mRNA or protein levels. Furthermore, we showed that fenretinide inhibited the janus kinase-signal transducer and activator of transcription, phosphatidylinositol 3-kinase-Akt, protein kinase C, and nuclear factor-kappaB signaling pathways, whereas fenretinide up-regulated the mitogen-activated protein kinase signaling pathways after bacterial stimulation. This study emphasizes the de novo ceramide synthesis pathway in response to bacterial stimulation and demonstrates the anti-inflammatory role of fenretinide in the bacteria-induced immune response.

Supplementary key words - dihydroceramide • cytokine • inflammation • macrophage • protein kinase

Sphingolipids play important roles in regulating cell growth, death, senescence, adhesion, migration, inflammation, angiogenesis, and intracellular trafficking. Among the sphingolipids, ceramides have been recognized as "second messengers" in modulating immune signaling transduction (1). Ceramides can be generated via three pathways (2) (Fig. 1). The first pathway is a de novo pathway, which occurs in the endoplasmic reticulum (ER) and possibly at ER-associated membranes, such as the perinuclear membrane and mitochondria-associated membranes. De novo synthesis of ceramides begins with the condensation of palmitate and serine to form 3-keto-dihydrosphingosine byserine-palmitoyltransferase (SPT). 3-Keto-dihydrosphingosine is reduced to dihydrosphingosine (dhSph) and followed by acylation to produce dihydroceramide (dhCer). Finally, dhCer is catalyzed by dihydroceramide desaturase (DEGS) to generate ceramides. The second pathway is the sphingomyelinase (SMase) pathway, which occurs in the plasma membrane and the endosomal/lysosomal compartments. Ceramides are generated by hydrolysis of sphingomyelin (SM) by SMase. The third pathway is the salvage pathway, which occurs in the acidic subcellular compartments, such as the late endosomes and the lysosomes. In the acidic subcellular compartments, complex sphingolipids and glycosphingolipids are degraded to form sphingosine (Sph). Sph can be converted to ceramides by ceramide synthase. Conversely, ceramides can...
also serve as substrates for synthesis of SM by SM synthase (SMS) or be hydrolyzed by ceramidase to form Sph. Sph can be metabolized by Sph kinase to generate sphingosine 1-phosphate (S1P). Ceramides can be generated at high concentration within the cell membrane in response to various infectious pathogens, stress stimuli, or chemotherapeutic drugs (3, 4). As “second messengers,” ceramides can form large, ceramide-rich membrane platforms (also called lipid raft platforms). These lipid raft platforms have been shown to initiate and greatly amplify immune signaling transduction by recruiting transmembrane and intracellular signaling molecules (1, 3, 4).

Fenretinide, also called n-4-hydroxyphenyl retinamide, is a synthetic retinoid. Fenretinide has been widely used in clinical trials for breast cancer chemoprevention (5) and is a synthetic retinoid. Fenretinide has been widely used in clinical trials for breast cancer chemoprevention (5) and is a synthetic retinoid. Fenretinide has been widely used in clinical trials for breast cancer chemoprevention (5) and is a synthetic retinoid. Fenretinide has been widely used in clinical trials for breast cancer chemoprevention (5) and is a synthetic retinoid.

Fig. 1. Ceramide synthesis pathways and the role of fenretinide in the de novo pathway. Ceramides can be generated by de novo, SMase, and salvage pathways. Fenretinide is an agonist for SPT, whereas it is an inhibitor for DEGS.

MATERIALS AND METHODS

Cells and reagents

Raw 264.7 (mouse monocyte/macrophage) cells were obtained from American Type Cell Collection (ATCC, Manassas, VA) and cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin in a 37°C incubator with 5% CO2. Raw 264.7 cells were treated with DMEM with 1% FBS for 16 h before treatment with fenretinide or stimulation by A.a. Fenretinide was obtained from Sigma Aldrich (St. Louis, MO) and diluted in ethanol.

Culture of A.a

A.a (serotype b, strain Y4) was purchased from ATCC. A.a frozen stock solution was streaked on anaerobe 5% sheep blood agar plates (BD Biosciences, Sparks, MD) and incubated in a 37°C incubator with 10% CO2 for 24 h. A.a colonies were cultured in NIH thioglycollate broth (BD Biosciences) overnight at 37°C with 10% CO2 for 24 h. Bacteria were centrifuged and fixed with 4% paraformaldehyde for 1 h. Finally, the bacteria were washed with PBS five times, centrifuged, and resuspended in PBS. The bacterial solution was adjusted to an absorbance A600 of 1 (4 × 10^9 colony-forming units [CFUs]/ml) and stored at −20°C.

Cell viability assay

Raw 264.7 cells (1 × 10^5) in a 96-well plate were incubated with fenretinide (2.5–5 µM) or control vehicle ethanol for 4–16 h. The cell viability in various treatment groups was analyzed by CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI).

Mass spectrometry analyses for sphingolipids

Sphingolipids were extracted from the samples by the Lipidomics Core Facility using the Bligh Dyer technique. Sphingolipid analysis was performed using ESI-MS/MS on a Thermo Finnigan TSQ 7000 triple quadrupole mass spectrometer operating in a multiple reaction monitoring positive ionization mode. This technique has been previously described by Bielawski et al. (22).

Quantitative real-time PCR

Total RNA was isolated from cells using TRIZOL (Invitrogen) according to the manufacturer’s instructions and quantified by a spectrophotometer. CDNA was synthesized by a TaqMan reverse transcription kit (Applied Biosystems, Foster City, CA) using 1,000 ng of total RNA. Real-time PCR was performed using a StepOnePlus Real-Time PCR System (Applied Biosystems) using conditions as follows: 50°C for 2 min, 95°C for 10 min, and 50 cycles of 95°C for 15 s, 60°C for 1 min. Amplicon primers of mouse IL-1ß, mouse IL-6, mouse tumor necrosis factor alpha (TNF-α), mouse cyclooxygenase 2 (Cox-2), and mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Applied Biosystems. Amplicon concentration was determined using threshold cycle values compared with standard curves for each primer. IL-1ß, IL-6, TNF-α, and COX-2 mRNA levels were normalized to an endogenous control GAPDH expression and expressed as fold change compared with control groups.
ELISA
IL-1β, IL-6, and TNF-α ELISA kits were purchased from R&D systems (Minneapolis, MN). The prostaglandin (PG)E2 ELISA kit was purchased from Cayman Chemical Co. (Ann Arbor, MI). Protein concentration in cell lysate was determined by a DC Protein Assay Kit (Bio-Rad Laboratories). The concentration of cytokines was normalized by total protein concentration in cell lysate.

Western blot assays
Total proteins were extracted by RIPA buffer (Cell Signaling Technology, Beverly, MA) supplemented with a proteinase inhibitor cocktail and a phosphatase inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Proteins were loaded on 10% Tris-HCl Ready Gel (Bio-Rad Laboratories, Hercules, CA) and electrotransferred to nitrocellulose membranes, blocked in 5% milk, and incubated overnight at 4°C with primary antibodies. P-STAT1, p-phosphatidylinositol 3-kinases (PI3K), 5% milk, and incubated overnight at 4°C with primary antibodies. P-STAT1, p-phosphatidylinositol 3-kinases (PI3K), p-Akt, p-protein kinase C (PKC), p-inhibitor of nuclear factor kappa-B kinase subunit alpha IκBα, p-nuclear factor-kappaB (NF-kB) p65, p-Jun N-terminal kinase (JNK), p-ERK, p-p38, and GAPDH were purchased from Cell Signaling Technology. The presence of the primary antibodies was detected on radiographic film by using HRP-conjugated secondary antibodies (Cell Signaling Technology) and SuperSignal West Pico Chemiluminescent film by using HRP-conjugated secondary antibodies (Cell Signaling Technology). Values are expressed as mean ± SD. A P value of ≤ 0.05 was considered significant.

Statistical analysis
Data were analyzed by unpaired two-tailed Student’s t test with Welch’s correction for unequal variances. All statistical tests were performed using GraphPad Prism software (GraphPad Software Inc., La Jolla CA). Values are expressed as mean ± SD. A P value of ≤ 0.05 was considered significant.

RESULTS
A.a stimulated the de novo synthesis of ceramide
Previous studies have shown that bacteria induced ceramide generation by the SMase pathway (23–25), but the effect of A.a on sphingolipid metabolism has not been determined. Because ceramides serve as “second messengers” modulating immune signaling transduction, it is important to determine the effect of A.a on sphingolipid metabolisms, especially on ceramide synthesis. To address this, we treated Raw 264.7 cells with A.a (100 CFUs) for various times (30–120 min) and analyzed various sphingolipid levels (including dhSph, dhCer, ceramide, Sph, S1P, and SM) in cell extracts by mass spectrometry analysis. The dhSph and total dhCer levels significantly increased after A.a stimulation (Fig. 2A, B). The total ceramide level also significantly increased 120 min after A.a stimulation (Fig. 2C). These data support the hypothesis that A.a induced the generation of ceramide by the de novo pathway. In addition, the Sph and S1P levels significantly increased 60 min and 120 min after A.a stimulation (Fig. 2D, E). Furthermore, we observed mild increases of total SM 30 min to 120 min after A.a stimulation (Fig. 2F). However, no significant difference of total SM levels in Raw 264.7 cells was observed before or after A.a stimulation. This result indicates that the SMase pathway might not play a significant role in ceramide accumulation in Raw 264.7 cells after A.a stimulation. Because SM is synthesized from ceramide by SM synthase (Fig. 1), the mild increases in total SM might be caused by the increased levels of ceramide in Raw 264.7 cells after bacterial stimulation. A.a mainly increased long-chain (C16) and very-long-chain (C22, C24, and C24:1) dhCer species (Fig. 2G–J) and ceramide species (Fig. 2K–N).

Fenretinide inhibited the de novo synthesis of ceramide after A.a stimulation
In previous studies, fenretinide has been confirmed as an agonist for SPT (13–15) but also as a potent inhibitor for DEGS (16, 17). Because SPT and DEGS are critical in the de novo pathway for ceramide biosynthesis, we hypothesized that fenretinide would interfere with ceramide synthesis induced by A.a. To test our hypothesis, Raw 264.7 cells were treated with fenretinide (5 μM) or control vehicle ethanol for 4 h. Cells were unstimulated or stimulated with A.a (100 CFUs) for an additional 4 h. Various kinds of sphingolipid levels, including dhSph, dhCer, ceramide, Sph, S1P, and SM, were evaluated in cell extracts by mass spectrometry analysis. In cells without bacterial stimulation, there were significant increases of dhSph (Fig. 3A; 2.5-fold) and total dhCer (Fig. 3B; 19.5-fold) in fenretinide-treated cells compared with sphingolipid levels in vehicle-treated cells. In contrast, there was a significant 36% decrease of the total ceramide in fenretinide-treated cells compared with vehicle-treated cells (Fig. 3C). There was no significant difference of Sph, S1P, and total SM levels between the two groups of cells (Fig. 3D–F). Fenretinide mainly increased long-chain (C16) and very-long-chain (C22, C24, and C24:1) dhCer species (Fig. 3G), whereas it significantly decreased very-long-chain (C22, C24, and C24:1) ceramide species (Fig. 3H). These data support the results from previous studies, which showed that fenretinide is a stimulator of SPT (13–15) but an inhibitor of DEGS (16, 17).

Previously we have shown that dhSph, dhCer, ceramide, Sph, and S1P levels significantly increased in Raw 264.7 cells after short-term (30–120 min) stimulation by A.a (Fig. 2). In vehicle-treated cells, we observed significant increases of dhSph (Fig. 4A; 2.2-fold), total dhCer (Fig. 4B; 1.34-fold), total ceramide (Fig. 4C; 1.5-fold), and Sph (Fig. 4D; 2.1-fold) 4 h after A.a stimulation, supporting the hypothesis that A.a induced ceramide generation via the de novo pathway. In contrast, there were no significant differences of S1P and total SM levels in vehicle-treated cells before or after bacterial stimulation (Fig. 4E, F). A.a significantly induced the production of very-long-chain (C24 and C24:1) dhCer species (Fig. 4G). In addition, A.a significantly increased the production of long-chain (C16) and very-long-chain (C22, C24, and C24:1) ceramide species (Fig. 4H).

In cells stimulated with A.a, we observed significant increases in dhSph (Fig. 5A; 2.2-fold) and total dhCer (Fig. 5B; 14.4-fold) in fenretinide-treated cells compared
Fig. 2. A.a induced de novo synthesis of ceramide in Raw 264.7 cells. Raw 264.7 cells were untreated or stimulated with A.a (100 CFUs) for various times. Purified dhSph (A), total (dhCer (B), total ceramide (C), Sph (D), S1P (E), total SM (F), dihydroC16-ceramide (dhC16-Cer) (G), dihydroC22-ceramide (dhC22-Cer) (H), dihydroC24-ceramide (dhC24-Cer) (I), dihydroC24:1-ceramide (dhC24:1-Cer) (J), C16-ceramide (K), C22-ceramide (L), C24-ceramide (M), and C24:1 ceramide (N) were analyzed and normalized by total protein. The data are representative of two separate experiments. Data are plotted as mean ± SD (n = 3; *P < 0.05, **P < 0.01).

with vehicle-treated cells (Fig. 5A, B). There was a 63% decrease of the total ceramide in fenretinide-treated cells compared with the total ceramide level in vehicle-treated cells (Fig. 5C) and a 21% decrease in the Sph level in fenretinide-treated cells compared with the Sph level in vehicle-treated cells (Fig. 5D). There was no significant difference of S1P between the two groups of cells after bacterial stimulation. However, we observed a 21% decrease of total
Fenretinide attenuated bacterial-induced immune responses

SM in fenretinide-treated cells compared with vehicle-treated cells (Fig. 5F). Because SM is generated from ceramide by SM synthase, the decrease of total ceramide level in fenretinide-treated cells might further inhibit the synthesis of SM, resulting in the lower total SM levels seen in fenretinide-treated cells compared with controls. Fenretinide mainly enhanced long-chain (C16) and very-long-chain (C22, C24, and C24:1) dhCer species (Fig. 5G). In contrast, fenretinide significantly inhibited long-chain (C16) and very-long-chain (C22, C24, and C24:1) ceramide species induced by A.a (Fig. 5H). In conclusion, these data support our hypothesis that fenretinide promoted the biosynthesis of dhSph and dhCer but it inhibited the de novo synthesis of ceramide induced by A.a. In addition, fenretinide might play a role in suppression of SM synthesis.

Fenretinide inhibited IL-1β, IL-6, and PGE2 cytokine expression induced by A.a

Previous study has shown that fenretinide inhibited chemokine (18) and chemokine receptor expression (19) and suppressed inflammatory response in animal models (20, 21). We hypothesized that fenretinide had an anti-inflammatory role against A.a stimulation. Although fenretinide has inhibited the proliferation of many tumor cell lines (7–11), it has been shown to have minimal cytotoxicity to nonmalignant cells (7, 12). In our study, fenretinide (2.5–5 μM) did not induce toxicity in Raw 264.7 cells after up to 16 h treatment (n = 4; P > 0.05; data not shown), which confirmed that fenretinide had minimal cytotoxicity. To study the effect of fenretinide on proinflammatory cytokine expression induced by A.a, Raw 264.7 cells were treated with fenretinide (2.5–5 μM) or control vehicle ethanol for 4 h. Then the cells were unstimulated or stimulated with A.a (100 CFUs) for 4 h. Fenretinide did not induce the mRNA production of IL-1β, IL-6, COX-2, and TNF-α in Raw 264.7 cells without bacterial stimulation (Fig. 6A–D). In contrast, compared with control cells stimulated with A.a, the mRNA levels of IL-1β, IL-6, and COX-2 in fenretinide-treated cells were significantly reduced in a dose-dependent manner after bacterial stimulation (Fig. 6A–C). However, there was no significant difference of TNF-α mRNA levels among treatment groups after bacterial stimulation (Fig. 6D). Accordingly, fenretinide did not induce the cytokine levels of IL-1β, IL-6, PGE2, and TNF-α in Raw 264.7 cells without bacterial stimulation.
the phosphatidylinositol 3-kinase (PI3K)-protein kinase B (PKB) (also called Akt) pathway (28, 29), the PI3K-PKC pathway (30, 31), and the mitogen-activated protein kinase (MAPK) signaling pathways (32, 33). The MAPKs consist of three major families, including extracellular-signal-regulated kinase (ERK), cJNK, and p38. Activation of PI3K-Akt and PI3K-PKC also influences the phosphorylation of inhibitor of nuclear factor kappa-B kinase subunit alpha (IkB), nuclear factor kappaB (NF-kB) (30, 34) and MAPK activities (35, 36). To determine which immune signaling pathways are influenced by fenretinide, Raw 264.7 cells were treated with fenretinide (5/10/20/100 M) or control vehicle ethanol for 4 h. Then the cells were unstimulated or stimulated with A.a for various times (30–120 min). The phosphorylation (p) status of various signaling proteins, including p-STAT1, p-PI3K, p-Akt, p-PKC, p-IkBa, p-NF-kB, p-ERK, p-JNK, and p-p38, was evaluated by Western blot. Cells treated with fenretinide exhibited lower levels of p-STAT1 (Fig. 8A, B), p-PI3K (Fig. 8A, C), p-Akt (Fig. 8A, D), p-PKC (Fig. 8A, E), p-IkBa (Fig. 8A, F), and p-NF-kB p65 (Fig. 8A, G) before and after bacterial stimulation compared with protein levels in cells treated with ethanol. In contrast, fenretinide-treated cells only showed decreased levels of p-JNK (Fig. 8A, H) and p-ERK (Fig. 8A, I) before bacterial stimulation (Fig. 7A–D). In contrast, compared with control cells stimulated with A.a, the cytokine levels of IL-1β, IL-6, and PGE2 in fenretinide-treated cells were significantly attenuated in a dose-dependent manner after bacterial stimulation (Fig. 7A–C). However, there was no significant difference of TNF-α protein expression among all groups of cells (Fig. 7D). In summary, this study demonstrated that fenretinide had an anti-inflammatory effect on IL-1β, IL-6, and PGE2 cytokine production but had no significant effect on TNF-α generation after bacterial stimulation in Raw 264.7 cells.

Fenretinide inhibited janus kinase-signal transducer and activator of transcription, PI3K-Akt, PKC, and NF-kB signaling pathways in Raw 264.7 cells induced by A.a

Because ceramides serve as “second messengers,” which regulate immune signaling transduction (1, 3, 4), we hypothesized that inhibition of the de novo synthesis of ceramide by fenretinide would affect immune signaling pathways, thus influencing downstream proinflammatory cytokine production. Many immune signaling pathways regulate proinflammatory cytokine production associated with bacterial stimulation, including the janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway (26, 27),

![Fig. 4.](image-url) A.a induced the production of dhSph, dhCer, ceramide, and Sph in vehicle-treated Raw 264.7 cells. Raw 264.7 cells were treated with vehicle (Veh) ethanol for 4 h. Then the cells were unstimulated or stimulated with A.a (100 CFUs) for 4 h. dhSph (A), total dhCer (B), total ceramide (C), Sph (D), SIP (E), total SM (F), dhCer species (G), and ceramide species (H) were analyzed and normalized by total protein. The data are representative of two separate experiments. Data are plotted as mean ± SD (n = 3; *P < 0.05, **P < 0.01, ***P < 0.001).
Fenretinide attenuated bacterial-induced immune responses

In addition, Sims et al. (43) reported that Kdo₂-lipid A, a TLR4-specific agonist, induced de novo sphingolipid biosynthesis in Raw 264.7 cells, which is essential for the induction of autophagy. In animal studies, inhibition of de novo ceramide synthesis prevented alveolar cell apoptosis, oxidative stress, and emphysema induced by SU5416, a specific inhibitor of vascular endothelial growth factor receptor (44). In addition, inhibition of de novo ceramide synthesis alleviated inflammatory cytokines in the spinal cord induced by administration of morphine (45). Furthermore, inhibition of SPT with myriocin reduced radiation-induced pulmonary inflammation and delayed the onset of radiation-induced pulmonary fibrosis in animals (46). In this study, we demonstrated that the de novo pathway contributed to the immune response to bacterial stimulus. As shown in Fig. 2 and Fig. 4, A.a induced the de novo synthesis of dhSph, dhCer, and ceramide. Previous studies have shown that Pseudomonas aeruginosa (23, 24) and Neisseria gonorrhoeae (25) activated acid SMase at early time points (5–45 min). Our previous study also demonstrated that P. aeruginosa activated acid SMase up to 300 min after bacterial stimulation (24). In the current study, we did not observe a significant reduction of total SM at an

**DISCUSSION**

De novo sphingolipid synthesis plays an important role in regulating immune response. Previous studies have shown that de novo synthesis of sphingolipids is critical for regulating ceramide and other sphingolipid levels in response to chemotherapeutic and proapoptotic agents (37–40), heat stress (41), and inflammatory cytokines (42). In addition, Sims et al. (43) reported that Kdo₂-lipid A, a TLR4-specific agonist, induced de novo sphingolipid biosynthesis in Raw 264.7 cells, which is essential for the induction of autophagy. In animal studies, inhibition of de novo ceramide synthesis prevented alveolar cell apoptosis, oxidative stress, and emphysema induced by SU5416, a specific inhibitor of vascular endothelial growth factor receptor (44). In addition, inhibition of de novo ceramide synthesis alleviated inflammatory cytokines in the spinal cord induced by administration of morphine (45). Furthermore, inhibition of SPT with myriocin reduced radiation-induced pulmonary inflammation and delayed the onset of radiation-induced pulmonary fibrosis in animals (46). In this study, we demonstrated that the de novo pathway contributed to the immune response to bacterial stimulus. As shown in Fig. 2 and Fig. 4, A.a induced the de novo synthesis of dhSph, dhCer, and ceramide. Previous studies have shown that Pseudomonas aeruginosa (23, 24) and Neisseria gonorrhoeae (25) activated acid SMase at early time points (5–45 min). Our previous study also demonstrated that P. aeruginosa activated acid SMase up to 300 min after bacterial stimulation (24). In the current study, we did not observe a significant reduction of total SM at an
difference among groups of cells (Fig. 5E). One of the reasons is that S1P is a single-chain lipid that has sufficient aqueous solubility to pass across cell membrane (2). It has been shown that S1P has low nanomolar concentrations in the cells but higher nanomolar concentrations in serum, where it is associated with lipoproteins and albumin (2, 50). In addition, S1P can be further irreversibly degraded by S1P lyase to release ethanolamine phosphate and hexadecenal (51). The lack of significant difference of S1P 4 h after bacterial stimulation among all groups of cells might be caused by the quick extracellular release and degradation by S1P lyase.

In this study, although there were high levels of dhSph and dhCer in Raw 264.7 treated with fenretinide before bacterial stimulation (Fig. 3A, B), those sphingolipids did not induce proinflammatory cytokine mRNA or protein expression before bacterial stimulation (Fig. 6 and 7); nor did they activate immune signaling pathways before bacterial stimulation (Fig. 8). This phenomenon might be caused by those sphingolipids’ hydrophobic property and their restricted subcellular localization (2, 51). It is known that the de novo synthesis of sphingolipids occurs in the ER and possibly at ER-associated membranes, such as the perinuclear and mitochondria-associated membranes (2, 51). The dhSph and dhCer might be restricted in the ER and possibly the ER-associated membrane compartments and therefore might not interact with receptors on the plasma membrane, which would prevent them from initiating

earlier time point (15 min, data not shown) and 30 to 120 min after A.a stimulation (Fig. 2F). One of the causes of discrepancy might be the different strain of bacteria used. Some bacteria, including pseudomonas, possess bacterial SMase (47, 48), which can hydrolyze SM, resulting in the generation of ceramide. A.a might lack bacterial SMase. Another possible reason for the discrepancy is that we used paraformaldehyde-fixed dead bacteria. The paraformaldehyde fixation process might inactivate bacterial SMase. Further study is needed to determine if live A.a possess bacterial SMase and could hydrolyze SM.

Although the total ceramide level in fenretinide-treated cells with bacterial stimulation (Fig. 5C) was significantly reduced to levels similar to those without bacterial stimulation (Fig. 3C), the Sph level was significantly enhanced in fenretinide-treated cells with bacterial stimulation (Fig. 5D; P < 0.01) compared with fenretinide-treated cells without bacterial stimulation (Fig. 3D). These results suggest that bacterial stimulation might activate the salvage pathway, which would explain the accumulation of Sph after bacterial stimulation. It is estimated that the salvage pathway contributes to about 50% to 90% of sphingolipid biosynthesis and turnover (49); therefore, it is not surprising that the salvage pathway could contribute to ceramide generation induced by bacterial stimulation.

Although we observed a significant reduction of ceramide level in fenretinide-treated cells 4 h after bacterial stimulation (Fig. 5C), the S1P did not manifest a significant difference among groups of cells (Fig. 5E). One of the reasons is that S1P is a single-chain lipid that has sufficient aqueous solubility to pass across cell membrane (2). It has been shown that S1P has low nanomolar lipid that has sufficient aqueous solubility to pass across cell membrane (2). It has been shown that S1P has low nanomolar concentrations in the cells but higher nanomolar concentrations in serum, where it is associated with lipoproteins and albumin (2, 50). In addition, S1P can be further irreversibly degraded by S1P lyase to release ethanolamine phosphate and hexadecenal (51). The lack of significant difference of S1P 4 h after bacterial stimulation among all groups of cells might be caused by the quick extracellular release and degradation by S1P lyase.

In this study, although there were high levels of dhSph and dhCer in Raw 264.7 treated with fenretinide before bacterial stimulation (Fig. 3A, B), those sphingolipids did not induce proinflammatory cytokine mRNA or protein expression before bacterial stimulation (Fig. 6 and 7); nor did they activate immune signaling pathways before bacterial stimulation (Fig. 8). This phenomenon might be caused by those sphingolipids’ hydrophobic property and their restricted subcellular localization (2, 51). It is known that the de novo synthesis of sphingolipids occurs in the ER and possibly at ER-associated membranes, such as the perinuclear and mitochondria-associated membranes (2, 51). The dhSph and dhCer might be restricted in the ER and possibly the ER-associated membrane compartments and therefore might not interact with receptors on the plasma membrane, which would prevent them from initiating
Fenretinide attenuated bacterial-induced immune responses

Fenretinide attenuated bacterial-induced immune responses 197

fenretinide. Previous work has shown that dhCers are involved in cell cycle arrest, apoptosis, autophagy, and oxidative stress (54), which could affect immune signaling transduction. There was an up-regulation of MAPK in fenretinide-treated cells after bacterial stimulation; especially the p-p38 and p-JNK levels were higher at 60 min in fenretinide-treated cells after bacterial stimulation compared with controls (Fig. 8H, J). Previous studies have demonstrated that fenretinide plays a role in up-regulation of MAPK signals (55, 56), which is dependent on the reactive oxygen species induced by fenretinide (55). We speculate that the up-regulation of protein kinase signals after bacterial stimulation in fenretinide-treated cells might be associated with the high level of dhCer, which further enhanced the oxidative stress induced by bacterial stimulation. The TNF-α production might be regulated by MAPK and NF-κB signals. The up-regulation of MAPK signals after bacterial stimulation offset the down-regulation of NF-κB signals, which resulted in no significant difference of TNF-α levels among all treatment groups after bacterial stimulation. On the other hand, IL-1β, IL-6, and PGE2 might be mainly regulated by the NF-κB signaling pathway and less affected by the oxidative stress; therefore, we observed significant reductions of those cytokine levels in fenretinide-treated cells after bacterial stimulation.

A previous study (21) demonstrated that long-term (18 h) treatment with fenretinide inhibited TNF-α in microglial cell culture induced by LPS stimulation. In our study, Raw 264.7 cells pretreated with fenretinide for 16 h and stimulated with

Fenretinide attenuated IL-1β, IL-6, and PGE2 cytokine expression in Raw 264.7 cells induced by A.a. Raw 264.7 cells were treated with fenretinide (2.5–5 μM) or control vehicle ethanol for 4 h. Then the cells were unstimulated or stimulated with A.a (100 CFUs) for 4 h. IL-1β (A), IL-6 (B), PGE2 (C), and TNF-α (D) were analyzed by ELISA in cell lysate. The concentration of cytokines was normalized by total protein concentration in cell lysate. The data are representative of three separate experiments. Data are plotted as mean ± SD (n = 3; **P < 0.01, ***P < 0.001).
Fig. 8. Fenretinide attenuated p-signal transducer and activator of transcription 1 (p-STAT1), p-PI3K, p-Akt, p-PKC, p-IκBα, and p-NF-kBp65 in Raw 264.7 cells induced by _A.a_. Raw 264.7 cells were treated with fenretinide (5 μM) or control vehicle ethanol for 4 h. Then the cells were unstimulated or stimulated with _A.a_ (100 CFUs) for various times (30–120 min). Phosphorylated (p) proteins were evaluated by Western blot assay. GAPDH served as a protein loading control. A: p-STAT1, p-PI3K, p-Akt, p-PKC, p-IκBα, p-NF-kBp65, p-JNK, p-ERK, p-p38, and GAPDH expression in Raw 264.7 cells. Protein density/mm² of p-STAT1 (B), p-PI3K (C), p-Akt (D), p-PKC (E), p-IκBα (F), p-NF-kBp65 (G), p-JNK (H), p-ERK (I), and p-p38 (J) was normalized by GAPDH. The data are representative of three separate experiments. Data are plotted as mean ± SD (n = 3; *P < 0.05, **P < 0.01, ***P < 0.001).
A. a for an additional 4 h significantly decreased TNF-α expression induced by A. a compared with controls (data not shown). However, pretreatment with fenretinide for 4 h had no significant effect on alleviating TNF-α expression induced by A. a. Moreover, we observed even higher TNF-α mRNA and protein levels in Raw 264.7 cells treated with a high dose (10 μM) of fenretinide with bacterial stimulation compared with controls (data not shown). This discrepancy might be caused by the activities of dhCer, and possibly dhSph, involved at various times. The impact of various activities associated with dhCer (54), such as cell cycle arrest, apoptosis, autophagy, and oxidative stress, might be variable during different time points. In the long-term (18 h) treatment study of fenretinide, the antiproliferative and autophagy roles associated with dhCer might have a significant impact on cells, leading to cell growth arrest or cell death and thus less TNF-α production. In contrast, in this short-term (4 h) treatment study, the antiproliferative and autophagy roles associated with dhCer might only have a minor effect on cells, whereas the oxidative stress induced by both bacterial stimulus and dhCer might have a major impact on cells, which could cause an up-regulation of MAPK after bacterial stimulation.

In this study, inhibition ceramide de novo synthesis by fenretinide significantly attenuated p-P3K, p-Akt, and p-PKC expression after bacterial stimulation compared with control. Our data show that ceramide played an essential role in regulating the PI3K-Akt and P3K-PKC signaling pathways. Previous studies have shown that Akt was recruited to the ceramide-rich microdomains after PI3K activation (57, 58). However, these studies also found conflicting results regarding how ceramide regulated the PI3K-Akt and PKC signaling pathways. Monick et al. (59) demonstrated that LPS induced the generation of ceramide and the activation of PI3K-Akt in human alveolar macrophages. They also showed that blocking ceramide synthesis by D609 blocked LPS-induced PI3K-Akt activation. D609 is a potent inhibitor of the phosphatidylinositol-specific phospholipase C, an enzyme known to be involved in acid SMase activation (25, 60). In addition, Barsacchi et al. (60) showed that activation of neutral SMase by TNF-α induced the generation of ceramide and the activation of the PI3K-Akt signaling pathway. Our results are in line with the findings by Monick et al. (59) and Barsacchi et al. (60) that ceramide plays a role in activation of the PI3K-Akt signaling pathway. However, there was also evidence that synthetic exogenous ceramide inhibited the PI3K-Akt and PKC signaling pathways. Exogenous C6-ceramide inhibited PKCa activity (61) and suppressed Akt activation (62, 63). In addition, exogenous C2-ceramide led to dephosphorylation of Akt (64–68). The inhibition of Akt activity by C2-ceramide or C6-ceramide had no effect on PI3K activity (63, 64) or was associated with down-regulation of PI3K activity (66, 68). It is important that natural, long-chain ceramides have a significant difference from the synthetic short chain ceramides. The long-chain ceramides are not water soluble and are therefore unable to dissipate the mitochondrial inner membrane potential (1). In contrast, short-chain ceramides, particularly C2-ceramide and C6-ceramide, are water soluble and therefore membrane permeable. Therefore, using exogenous short-chain ceramides as mimics of endogenous long-chain ceramides is, in general, not justified (1). In addition, previous studies have shown that the PI3K pathway plays a dual role in modulating the immune response (36, 69). PI3K serves as an essential proinflammatory signaling molecule activating the NF-κB signals (34). On the other hand, PI3K also has an anti-inflammatory role, which negatively regulates the p38 MAPK signal pathways (69). That might explain why we observed an up-regulation of p-p38 in fenretinide-treated cells without bacterial stimulation compared with control (Fig. 8A, J).

In conclusion, our results support that de novo synthesis of ceramide plays an important role in host immune response to A. a. Fenretinide inhibited the de novo synthesis of ceramide and suppressed JAK-STAT, PI3K-Akt, PKC, and downstream NF-κB signaling pathways. Furthermore, it attenuated IL-1β, IL-6, and PGE2 proinflammatory cytokine expression induced by A. a.

REFERENCES

1. van Blitterswijk, W. J., A. H. van der Luit, R. J. Veldman, M. Verheij, and J. Borst. 2003. Ceramide: second messenger or modulator of membrane structure and dynamics? Biochem. J. 369:199–211.
2. Hannun, Y. A., and L. M. Obeid. 2008. Principles of bioactive lipid signalling: lessons from sphingolipids. Nat. Rev. Mol. Cell Biol. 9:139–150.
3. Grasse, H., J. Riethmüller, and E. Gubins. 2007. Biological aspects of ceramide-enriched membrane domains. Prog. Lipid Res. 46:161–170.
4. Schenck, M., A. Carpineiro, H. Grasse, F. Lang, and E. Gubins. 2007. Ceramide: physiological and pathophysiological aspects. Arch. Biochem. Biophys. 462:171–175.
5. Zanardi, S., D. Serrano, A. Argusti, M. Barile, M. Puntoni, and A. Decensi. 2006. Clinical trials with retinoids for breast cancer chemoprevention. Endocr. Relat. Cancer 13:51–68.
6. Villablana, J. G., M. D. Krailo, M. M. Ames, J. M. Reid, G. H. Reiman, and C. P. Reynolds. 2006. Phase I trial of oral fenretinide in children with high-risk solid tumors: a report from the Children’s Oncology Group (CCG 09709). J. Clin. Oncol. 24:3423–3430.
7. O’Donnell, P. H., W. X. Guo, C. P. Reynolds, and B. J. Maurer. 2002. N-(4-hydroxyphenyl)retinamide increases ceramide and is cytotoxic to acute lymphoblastic leukemia cell lines, but not to non-malignant lymphocytes. Leukemia. 16:902–910.
8. Padovalli, V. K., Y. Saito, R. Xu, G. P. Kourakis, V. A. Levin, and A. P. Krivits. 1999. Fenretinide activates caspases and induces apoptosis in gliomas. Clin. Cancer Res. 5:2230–2235.
9. Lovat, P. E., S. Oliverio, M. Ranalli, M. Corazza, C. Rodolfo, F. Bernassola, K. Aughton, M. Maccarrone, Q. D. Hewson, A. D. Pearson, et al. 2002. GADD153 and 12-lipoxygenase mediate fenretinide-induced apoptosis of neuroblastoma. Cancer Res. 62:5158–5167.
10. Kalemkerian, G. P., R. Slusher, S. Ramalingam, S. Gadgeel, and M. Mabry. 1995. Growth inhibition and induction of apoptosis by fenretinide in small-cell lung cancer cell lines. J. Natl. Cancer Inst. 87:1674–1680.
11. Kabbout, M., A. Hatoum, G. Abou-Lteif, I. Chakroun, F. R. Homaidan, and N. Darwiche. 2004. Stage-specific effect of N-(4-hydroxyphenyl)retinamide on cell growth in squamous cell carcinoma. Mol. Carcinog. 40:12–23.
12. Li, X., W. Ling, A. Pennisi, S. Khan, and S. Yacoby. 2009. Fenretinide inhibits myeloma cell growth, osteoclastogenesis and osteoclast viability. Cancer Lett. 284:175–181.
13. Maurer, B. J., L. S. Metelitsa, R. C. Seeger, M. G. Cabot, and C. P. Reynolds. 1999. Increase of ceramide and induction of mixed
apo-physiology and activation of the NF-kappaB $\delta$ and/or subunit.

Mol. Cell. Biol. 19: 4798–4805.

Aksamitiene, E., A. Kiyatkin, and B. N. Kholodenko. 2012. Cross-talk between mitogenic Ras/MAPK and survival PI3K/Akt pathways: a fine balance. Biochem. Soc. Trans. 40: 139–146.

Gund, F., and G. Schahbauser. 2008. Recent advances in the genetic analysis of PTEN and PI3K innate immune properties. Immunobiology 213: 759–765.

Bose, R., M. Verheij, A. Haimovitz-Friedman, K. Scotto, Z. Fux, and R. Kolesnick. 1995. Ceramide synthesize mediates tauonurocin-induced apoptosis: an alternative mechanism for generating death signals. Cell 82: 405–414.

Chalfant, C. E., R. Rathbun, R. L. Pinkerman, R. E. Wood, L. M. Obeid, B. Ogretmen, and Y. A. Hannun. 2002. De novo ceramide regulates the alternative splicing of caspase 9 and Bcl-x in A549 lung adencarcinoma cells. Dependence on protein phasease-1. J. Biol. Chem. 277: 12587–12595.

Kroesen, B. J., S. Jacobs, B. J. Petrus, H. Sietama, J. W. Kok, Y. A. Hannun, and L. F. de Leij. 2003. Boz-induced apoptosis involves differential regulation of C16 and C24-ceramide formation and sphingolipid pool congratulation of the pro tease. J. Biol. Chem. 278: 14723–14731.

Perry, D. K., J. Carton, A. K. Shah, F. Meredith, D. J. Uhlinger, and Y. A. Hannun. 2000. Serine palmitoyltransferase regulates de novo ceramide generation during etoposide-induced apoptosis. J. Biol. Chem. 275: 9078–9084.

Cowart, L. A., and Y. A. Hannun. 2007. Selective substrate supply in the regulation of yeast de novo sphingolipid synthesis. J. Biol. Chem. 282: 12330–12340.

Hanada, K. 2003. Serine palmitoyltransferase, a key enzyme of sphingolipid metabolism. Biochim. Biophys. Acta. 1632: 16–30.

Sambrook, J., C. A. Haynes, S. L. Berridge, K. A. West, A. Momin, M. Leipelt, D. Reichert, C. K. Glass, M. C. Sullards, et al. 2010. Kdo2-lipid A, a TLR-specific agonist, induces de novo sphingo lipid biosynthesis in RAW264.7 macrophages, which is essential for induction of apoptosis. J. Biol. Chem. 285: 38568–38579.

Petracek, L., V. Natarajan, L. Zhen, T. R. Medler, A. T. Richter, C. Cho, W. C. Hubbard, E. V. Berdichev, and R. M. Tuder. 2005. Ceramide upregulation causes pulmonary cell apoptosis and emphysema-like disease in mice. Nat. Med. 11: 491–498.

Ndengele, M. M., S. Cuzzocrea, E. Masini, M. C. Vinci, E. Esposito, C. Muscoli, D. N. Petrusca, V. Mollo, E. Mazzon, D. Li, et al. 2009. Spinal ceramide modulates the development of morphine antinociceptive tolerance via peroxinirite-mediated nitroxidative stress and neuroimmune activation. J. Pharmacol. Exp. Ther. 329: 64–75.

Gorschhova, I., T. Zhou, B. Mathew, J. R. Jacobson, D. Takekoshi, P. Bhattacharya, B. Smith, B. Aydogen, R. R. Weichselbaum, V. Natarajan, et al. 2012. Inhibition of serine palmitoyltransferase delays the onset of radiation-induced pulmonary fibrosis through the negative regulation of sphingosine kinase-1 expression. J. Lipid Res. 53: 1553–1568.

Ibarguren, M., P. H. Bomans, P. M. Frederik, M. Stonehouse, A. I. Vasil, M. L. Vasil, A. Alonso, and F. M. Goni. 2010. End-products di glycerolceramide and ceramide modulate membrane fusion induced by a phospholipase C/sphingomyelinase from Pseudomonas aeruginosa. Biochim. Biophys. Acta. 1798: 59–68.

Millhas, D., C. J. Clarke, and Y. A. Hannun. 2010. Sphingomyelin metabolism at the plasma membrane: implications for bioactive sphingolipids. FEBS Lett. 584: 1887–1894.

Kitatani, K., J. Idkowiak-Baldys, and Y. A. Hannun. 2008. The sphingolipid salvage pathway in ceramide metabolism and signaling. Cell Signal 20: 1010–1018.

Ozawa, F. 2002. Plasma lipoproteins behave as carriers of extracellular sphingosine 1-phosphate: is this an atherogenic mediator or an anti-atherogenic mediator? Biochim. Biophys. Acta. 1582: 132–137.

Bartke, N., and Y. A. Hannun. 2009. Bioactive sphingolipids: metabolism and function. J. Lipid Res. 50(Suppl): S91–S96.

Lopez-Montero, I. N., Rodriguez, S. Cribier, A. Pohl, M. Velez, and P. F. Devaux. 2005. Rapid transilaurant movement of ceramides in phospholipid vesicles and in human erythrocytes. J. Biol. Chem. 280: 25811–25819.

Cuzzocrea, S., H. P. Deigner, T. Genovese, E. Mazzon, E. Esposito, G. Crisafulli, R. Di Paola, P. Bramanti, G. Matuschak, and D. Salvemini. 2009. Inhibition of ceramide biosynthesis ameliorates pathophysiological consequences of spinal cord injury. Shock 31: 634–641.

Cubillas, G., J. Munoz-Olava, F. Gregolini, P. Signorelli, J. Casas, V. Gagliostro, and R. Ghidoni. 2012. Dihydroceramide desaturase and...
55. Osone, S., H. Hosoi, Y. Kuwahara, Y. Matsumoto, T. Iehara, and T. Sugimoto. 2004. Fenretinide induces sustained-activation of JNK/p38 MAPK and apoptosis in a reactive oxygen species-dependent manner in neuroblastoma cells. *Int. J. Cancer.* **112:**219–224.

56. Samuel, W., R. K. Katty, S. Sekhar, C. Vijayasarithy, B. Wiggert, and T. M. Redmond. 2008. Mitogen-activated protein kinase pathway mediates N-((4-hydroxyphenyl)retinamide-induced neuronal differentiation in the ARPE-19 human retinal pigment epithelial cell line. *J. Neurochem.* **106:**591–602.

57. Lasserre, R., X. J. Guo, F. Conchouaoud, Y. Hamon, O. Hawchar, A. M. Bernard, S. M. Soudja, P. F. Lenne, H. Rigneault, D. Olive, G. Bismuth, J. A. Nunes, B. Pavastre, D. Marguet, and H. T. He. 2008. Raft nanodomains contribute to Akt/PKB plasma membrane recruitment and activation. *Nat. Chem. Biol.* **4:**538–547.

58. Gao, X., P. R. Lowry, X. Zhou, C. Depry, Z. Wei, G. W. Wong, and J. Zhang. 2011. PI3K/Akt signaling requires spatial compartmentalization in plasma membrane microdomains. *Proc. Natl. Acad. Sci. USA.* **108:**14509–14514.

59. Koide, N., T. Sugiyama, I. Mori, M. M. Mu, T. Yoshida, and T. Yokochi. 2003. C2-ceramide inhibits LPS-induced nitric oxide production in RAW 264.7 macrophage cells through down-regulating the activation of Akt. *J. Endotoxin Res.* **9:**85–90.

60. Fukao, T., and S. Koyasu. 2003. PI3K and negative regulation of TLR signaling. *Trends Immunol.* **24:**358–363.