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Acidic sphingomyelinase induced by electrophiles promotes proinflammatory cytokine production in human bladder carcinoma ECV-304 cells

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ABSTRACT

Electrophiles in environmental pollutants or cigarette smoke are high risk factors for various diseases caused by cell injuries such as apoptosis and inflammation. Here we show that electrophilic compounds such as diethyl malate (DEM), methyl mercury and cigarette smoke extracts significantly enhanced the expression of acidic sphingomyelinase (ASMase). ASMase activity and the amount of ceramide of DEM-treated cells were approximately 6 times and 4 times higher than these of non-treated cells, respectively. Moreover, we found that DEM pretreatment enhanced the production of IL-6 induced by TNF-α. Knockdown of ASMase attenuated the enhancement of TNF-α-dependent IL-6 production. On the other hand, enhancement of TNF-α-induced IL-6 production was observed in ASMase-overexpressing cells without DEM. Fractionation of the lipid raft revealed that the TNF receptor 1 (TNFR1) was migrated into the lipid raft in DEM-treated cells or ASMase-overexpressing cells. The TNF-α-induced IL-6 expression required the clustering of TNFR1 since IL-6 expression were decreased by the destruction of the lipid raft with filipin. These results demonstrated a new role for ASMase in the acceleration of the production of TNF-induced IL-6 as a pro-inflammatory cytokine and indicated that electrophiles could potentiate inflammation response by up-regulating of ASMase expression following formation of lipid rafts.

Introduction

Exposure to electrophiles in environmental pollutants derived from factories, agricultural chemicals and automobiles injures various organs and induces a variety of diseases such as respiratory and renal ailments, allergies, atherosclerosis and cancer. It is well known that cigarette smoke contains thousands of electrophiles and is a high risk factor for chronic obstructive pulmonary disease (COPD)[1], atherosclerosis, and carcinogenesis [1]. Although the particular molecular mechanisms of their toxicities still remain to be resolved, one possible mechanism would be the modification of thiol residues of proteins that are preferential targets of electrophiles for the alteration of cellular function. Conformational alteration of cellular signaling proteins causes alteration of their activities and gene expression in response to electrophiles such as acrylamide, hydrogen peroxide and arsenic [2–4]. These modifications can be a critical event resulting in injuries of target tissues. Previous studies suggest close links between electrophiles with inflammation and apoptosis, but the direct targets of electrophiles are poorly defined.

Sphingomyelinase (SMase) is an enzyme that catalyzes hydrolysis of sphingomyelin to ceramide and phosphocholine. Ceramide is a lipid mediator and is implicated in mediating and regulating diverse cellular processes, such as proliferation, differentiation, stress response, apoptosis and inflammation [5,6] and also the pathogenesis of numerous common diseases such as cancer, atherosclerosis, pulmonary edema in acute lung injury and cardiovascular disease through the activation of the cell death signal [7–9].

SMase is categorized into three types according to its optimal pH, namely neutral SMase (NSMase), alkaline SMase (AlkSMase), and acidic SMase (ASMase). ASMase expressed by almost all cell types is subclassified into two isoforms, a lysosomal ASMase and a secretory Zn^{2+}-dependent ASMase that is the only SMase responsible for the hydrolysis of SM in the extracellular leaflet of the plasma membrane. In addition to its housekeeping function in lysosomes, ceramide formed by ASMase participates in the formation of lipid rafts that serve as a cell surface signaling platform for various receptors such as Fas and TNF-α to mediate inflammation, apoptosis and differentiation [10–13]. More specifically, ASMase-dependent ceramide production participates in the induction of apoptosis caused by reactive nitrogen species and radiation [8,14].

ASMase is a key mediator of the pathogenesis of lung diseases such as pulmonary fibrosis, edema formation and asthma. Rapid

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[1] *Abbreviations used:* DEM, diethyl malate; SMase, sphingomyelinase; ASMase, acidic SMase; TNF-α, tumor necrosis factor-α; TNFR1, TNF receptor 1; IL-6, interleukin-6; SM, sphingomyelin; CSE, cigarette smoke extracts; CTX-B, cholera toxin B subunit; EpRE, electrophile-responsive element; ARE, antioxidant responsive element.

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activation of ASMase and induction of apoptosis were observed in lung inflammation treated in a mouse model of pulmonary fibrosis in mice and apoptosis was considerably attenuated in ASMase knockout mice [15]. Lung fibroblasts from ASMase knockout mice were resistant to elevation in ceramide levels and apoptosis induced by TNF-α [16]. Inhibition of ASMase also attenuates lipopolysaccharide-mediated release of inflammatory cytokines [17]. These data suggest that ASMase-dependent ceramide generation may exhibit powerful proapoptotic and proinflammatory properties benefits.

In this study, we investigated whether electrophiles are compounds responsible for inducing pro-inflammatory cytokines by ASMase-dependent ceramide generation. Our results showed that electrophiles powerfully enhanced the expression and activation of ASMase and led to the accumulation of ceramide. Increased expression of ASMase amplified the generation of IL-6 induced by TNF-α followed by the clustering of the TNF receptor to the lipid raft consisting of the newly generated ceramide.

Materials and methods

Cell culture

The human bladder carcinoma cell line ECV-304 cells were maintained in RPMI1640 medium (Nissui Co., Japan) supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 U/ml), streptomycin (100 mg/ml), L-glutamine (588 mg/ml), and 0.16% $NaHCO_3$ at 37 °C in an atmosphere of 95% air and 5% CO_2. Cells were seeded at a density of 1×10^5 cells per ml and total cell numbers were modified according to the experiment. Cells were allowed to attach to the dish for 24 h prior to experiments.

Preparation of cigarette smoke extracts (CSE)

Cigarette smoke extracts (CSE) were prepared as follows. Briefly, cigarette smoke derived from one cigarette (Mild Seven, JT, Japan) was drawn slowly into a 50 ml syringe from the filter side and bubbled through 10 ml of PBS repeatedly. The resulting solution was filtered through a 0.20 μm-pore filter and then diluted with RPMI1640 medium. After the pH was adjusted to neutral with NaOH, CSE containing medium was added to the dish.

Construction of ASMase expression vector

The ASMase expression vector (pcDNA/ASMaseV5-His) was generated by ligating the human ASMase ORF into a pcDNA3.1/V5-His vector (Invitrogen, USA), thus generating V5 and a His tag at the C terminus of the ASMase protein. The human ASMase ORF was generated by PCR using cDNA derived from ECV-304 cells as the template. PCR was performed for 30 cycles at a denaturing temperature of 94 °C for 15 s followed by annealing at 65 °C for 15 s and extension at 68 °C for 2 min. The forward primer was 5′-TAGGAATTCCCACCATGCCCCGCTACGGAG-3′ and the reverse primer was 5′-CCTGATATCATCGCAAAACAGTGGCCTTGG-3′. The forward primer contained the EcoRI site sequence, and the reverse primer contained the EcoRV site sequence. Amplified ASMase ORF was digested with restriction enzymes and electrophoresed on agarose gel. After purification from the gel, the products were ligated into the appropriately digested vector. The constructed vector was sequenced using an ABI3100 (Applied Bioscience, USA).

Establishment of stable ASMase overexpressing cells

ECV-304 cells (1×10^6) were seeded in 90 mm dishes and grown for 24 h in medium containing 10% FCS. Transfection of pcDNA/ASMaseV5-His was performed using Gene Juice Transfection Reagent (Novagen, USA) according to the manufacturer's protocols. Briefly, 15 μl of transfection reagent was added to 500 μl Opti-MEM medium (Invitrogen, USA) and then 5 μg of vector DNA was added and the solution was gently mixed. After a 20-min incubation period, the DNA–reagent complexes were added to the dish and incubated for 24 h and then medium was changed to fresh medium containing 10% FCS and maintained for another 24 h. For isolation of stable ASMase overexpressing cells, transfected cells were re-seeded at a density of 1×10^3 cells per 90 mm dish 48 h after the transfection and cultured with RPMI1640 medium containing 400 μg/ml G418 (Invitrogen, USA). Cells were cultured for 4 weeks by changing the medium every 4 days to fresh G418 containing medium. The resulting G418-resistant clones were isolated and the ASMase expression level was checked by RT-PCR and immunoblot analysis.

Knockdown of ASMase by siRNA

Cells (4.5×10^4) were seeded in 60 mm dishes and grown for 24 h in medium containing 10% FCS. Transfection of double strand siRNA was performed using Lipofectamine 2000 Reagent (Invitrogen, USA) according to the manufacturer's protocols. Briefly, 5 μl of transfection reagent was added to 500 μl Opti-MEM medium (Invitrogen, USA) and then 50 pmol of siRNA against human ASMase (Invitrogen, USA) or negative control siRNA (Ambion, USA) was added and gently mixed. After a 20 min incubation period, the DNA–reagent complexes were added and further incubated for 24 h. Then the medium was changed to fresh medium containing 10% FCS and maintained for another 24 h.

Measurement of intracellular ceramide content

Intracellular ceramide contents were measured by diacylglycerol kinase assay according to methods described previously [18]. Briefly, treated cells were rinsed with cold PBS twice and collected to a centrifugation tube. After centrifugation, cells were resuspended in sterile water and transferred to a glass tube. Lipids in the whole cell were extracted according to the method of Bligh and Dyer [19]. The resulting organic phase, which contains most of the lipids, was dried under N_2 gas and used as the sample. Lipids were resuspended in 10 μl of cardiolipin micelle buffer (1.25 mM cardiolipin/7.5% octyl-β-D-glucopyranoside) and combined with 70 μl of reaction buffer [50 mM imidazole–HCl (pH 6.6)/50 mM LiCl/12.5 mM $MgCl_2$/1 mM EDTA/2 mM DTT/5 μg diacylglycerol kinase (Wako, Japan)]. The reaction was started by adding 10 μl of ATP solution containing [γ-^{32}P] ATP (40 μCi/μmol) and incubated for 30 min at room temperature. After the reaction was stopped by adding 3 ml of chloroform:methanol (1:2), 0.7 ml of sterile water, 1 ml of chloroform, and 1 ml of sterile water were added and mixed by vortex in turn. After centrifugation, the organic phase was transferred to a new tube and dried using a centrifugation evaporator. Samples were resuspended to chloroform and spotted on TLC plate (Merck, USA) and developed with chloroform:acetone:methanol:acetic acid:water (10:4:3:2:1). The developed plate was dried and spots were detected using an imaging analyzer BAS-2000 II (Fuji film, Japan). Ceramide contents were correlated with protein concentration.

ASMase activity assay

ASMase activity was measured using NBD-C6 sphingomyelin (Molecular Probes, USA) as substrate. After treatment, cells were rinsed with cold PBS twice and activity buffer (50 mM NaOAc (pH 5.5)/2 mM EDTA/0.2% Triton X-100/protease inhibitor cocktail) was added and the cells were collected to a centrifuge tube by