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Ceramide kinase regulates TNFα-stimulated NADPH oxidase activity and eicosanoid biosynthesis in neuroblastoma cells

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ABSTRACT

A persistent inflammatory reaction is a hallmark of chronic and acute pathologies in the central nervous system (CNS) and greatly exacerbates neuronal degeneration. The proinflammatory cytokine tumor necrosis factor alpha (TNFα) plays a pivotal role in the initiation and progression of inflammatory processes provoking oxidative stress, eicosanoid biosynthesis, and the production of bioactive lipids. We established in neuronal cells that TNFα exposure dramatically increased Mg^{2+}-dependent neutral sphingomyelinase (nSMase) activity thus generating the bioactive lipid mediator ceramide essential for subsequent NADPH oxidase (NOX) activation and oxidative stress. Since many of the pleiotropic effects of ceramide are attributable to its metabolites, we examined whether ceramide kinase (CerK), converting ceramide to ceramide-1-phosphate, is implicated both in NOX activation and enhanced eicosanoid production in neuronal cells. In the present study, we demonstrated that TNFα exposure of human SH-SY5Y neuroblastoma caused a profound increase in CerK activity. Depleting CerK activity using either siRNA or pharmacology completely negated NOX activation and eicosanoid biosynthesis yet, more importantly, rescued neuronal viability in the presence of TNFα. These findings provided evidence for a critical function of ceramide-1-phospate and thus CerK activity in directly linking sphingolipid metabolism to oxidative stress. This vital role of CerK in CNS inflammation could provide a novel therapeutic approach to intervene with the adverse consequences of a progressive CNS inflammation.

1. Introduction

Many pathologies of the central nervous system (CNS) exhibit a strong inflammatory component that substantially contributes to neurodegeneration [1,2]. Microglia and astrocytes respond to acute injury, toxins, or invading pathogens by secreting inflammatory mediators such as eicosanoids, cytokines, and reactive oxygen species (ROS) [3,4]. Tumor necrosis factor alpha (TNFα), a pro-inflammatory cytokine, binds to two receptors, one coupled to the Mg^{2+}-dependent neutral sphingomyelinase (Mg^{2+}-nSMase) through the adaptor protein FAN, factor associated with nSMase [5,6]. SMases hydrolyze sphingomyelin to produce the bioactive lipid ceramide, which is implicated in mediating oxidative stress, and other apoptotic processes [7,8].

Ceramide, and its metabolites serve as key mediators of inflammatory pathways [9–11]. For instance, cytosolic phospholipase A_2 ($cPLA_2$), which liberates arachidonic acid (AA), translocates to distinct lipid microdomains in the endoplasmic reticulum, the Golgi apparatus, or the plasma membrane depending on the extrinsic stimulus [12,13]. Ceramide kinase (CerK), the enzyme responsible for the phosphorylation of ceramide, is localized both in the plasma membrane and the Golgi apparatus thus establishing a connection between the production of ceramide-1-phosphate (C1P) and $cPLA_2$ activation [14–16]. More recently, it was shown that $cPLA_2$ interacts with and is activated by C1P [17]. AA is the principal product of $cPLA_2$ activity and a pivotal precursor for they biosynthesis of eicosanoids [18]. Through conversion of AA, cyclooxygenase (COX) and lipoxygenase (LOX) generate prostaglandins and leukotrienes, pleiotropic inflammatory mediators, and also

Abbreviations: aSMase, acid sphingomyelinase; AA, arachidonic acid; BSA, bovine serum albumin; C1P, ceramide-1-phosphate; CerK, ceramide kinase; COX, cyclooxygenase; $cPLA_2$, cytosolic phospholipase A_2; DAPI, 4,6-diamidino-2-phynelindole; DCF, dichlorofluorescein; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DOTAP, 1,2-dioleoyl-2-trimehtylammonium-propane; DPI, diphenylene iodonium; FBS, fetal bovine serum; FITC, fluorescein; H_2DCFDA, 2′,7′-dihydrodichlorofluorescein diacetate; HBSS-CM, Hank's balanced salt solution with Ca^{2+} and Mg^{2+}; IDM, indomethacin; LOX, lipooxygenase; Mg^{2+}-nSMase, Mg^{2+}-dependent neutral sphingomyelinase; MK, MK-886; NAC, N-acetyl-L-cysteine; NBD, N-4-nitrobenzo-2-oxa-1,3-diazole; NOX, NADPH oxidase; PBS, phosphate buffered saline; PIC, protease inhibitor cocktail; PEG2000-DSPE, 1,2-distearoyl-*sn*-glucero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]; ROS, reactive oxygen species; SMase, sphingomyelinase; TNFα, tumor necrosis factor alpha.

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significant amounts of reactive oxygen species (ROS) as byproducts [19,20]. Moreover, AA is a critical component of membrane microdomains serving both as a membrane anchor for NADPH oxidase (NOX) as well as a cofactor for the NOX-proton channel [21–23]. In contrast to other oxidoreductases, ROS are the purposeful reaction products of NOX activity.

Utilizing a combination of pharmacological and molecular approaches, we demonstrated in human SH-SY5Y neuroblastoma cells exposed to TNFα that CerK activity was strictly required for the subsequent activation of several oxidoreductases (NOX, COX, and 5-LOX), and also enhanced eicosanoid biosynthesis as well as $cPLA_2$ activation. In addition, eliminating CerK activity rescued neuronal viability in the persistent presence of TNFα. This study demonstrated for the first time that CerK activation and thus C1P play an essential role in linking sphingolipid metabolism to increased oxidative stress thus attributing a therapeutic potential to CerK manipulation in CNS inflammation [24,25].

2. Materials and methods

2.1. Reagents

Recombinant human tumor necrosis factor alpha (TNFα) was purchased from Millipore (Temecula, CA). DMEM and Penicillin/Streptomycin solution were obtained from Mediatech (Herndon, VA). Fetal bovine serum was received from Atlanta Biologicals (Atlanta, GA). GlutaMAX-1 and trypsin/EDTA, as well as fluorescent siRNA, were from Invitrogen (Carlsbad, CA). The Mg^{2+}-nSMase inhibitor GW4869, the aSMase inhibitor desipramine, and the CerK inhibitor K1 were from EMD Biosciences (San Diego, CA). The XTT cell proliferation kit was from Trevigen (Gaithersburg, MD). ^{3}H-arachidonic acid was from American Radiolabeled Chemicals (St. Louis, MO). All other lipids were from Avanti Polar Lipids (Alabaster, AL). The siRNA directed against CerK, and non-targeting siRNA was from Dharmacon (Lafayette, CO). A polyclonal rabbit anti-human CerK antibody was from Exalpha (Shirley, MA). A polyclonal goat anti-human phospho-$cPLA_2$ antibody, a polyclonal goat anti-human phospho-$p40^{phox}$ antibody, a polyclonal rabbit anti-human GAPDH antibody, a polyclonal rabbit anti-human $p67^{phox}$ antibody, a monoclonal mouse anti-human β-actin antibody, and corresponding horseradish peroxidase-conjugated secondary antibodies were from Santa Cruz (Santa Cruz, CA). A polyclonal rabbit anti-human phospho-5-LOX antibody and all other reagents were purchased from Sigma (St. Louis, MO). VECTASHIELD hard set mounting medium with 4,6-diamidino-2-phenylindole (DAPI) was purchased from Vector Laboratories (Burlingame, CA). Streptavidin-agarose beads, sulfo-NHS-biotin, protease inhibitor cocktail (PIC), a Pico Super Signal chemoluminescent kit, and a BCA protein assay kit were obtained from Pierce (Rockland, IL).

2.2. Cell culture

Human SH-SY5Y neuroblastoma cells were grown in DMEM, 10% fetal bovine serum (FBS), 1% Glutamax, 100 U/ml Penicillin and 100 U/ml Streptomycin (humidified atmosphere, 5% CO_2, 37 °C, 100 mm dishes Falcon). For cell harvesting, cultures were incubated with trypsin (0.5 mg/ml)/EDTA (0.2 mg/ml) for 5 min, cells washed off with PBS, centrifuged (200 $\times g_{max}$, 2 min), and resuspended in growth medium.

2.3. Cationic nanoliposome formation and siRNA loading

Aliquots of 1,2-dioleoyl-2-trimethylammonium-propane (DOTAP), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (PEG2000-DSPE), and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), suspended in chloroform, were made in a 4.75:0.5:4.75 molar ratio, or alternatively, aliquots of DOTAP, PEG2000-DSPE, DOPE, and lissamine rhodamine B-labeled DOPE were made in a 4.75:0.5:4.7025:0.0475 molar ratio. Lipids were dried to a film under a stream of nitrogen, then hydrated by addition of 0.9% NaCl to a final lipid concentration of 25 mg/ml. Solutions were sealed, heated at 60 °C (60 min), mixed (Vortex), and sonicated until light no longer diffracted through the suspension. The lipid vesicle-containing solution was quickly extruded at 60 °C by passing the solution 10 times through 100 nM polycarbonate filters in an Avanti Mini-Extruder (Avanti Polar Lipids, Alabaster, AL). Nanoliposome solutions were stored at 4 °C until use, protected from light when necessary. To prepare siRNA-loaded cationic nanoliposomes, siRNA dissolved in 0.9% NaCl and nanoliposomes were mixed (10:1 w/w liposome to siRNA) and incubated overnight at room temperature prior to use. The RNA interference sequence was previously published by the Chalfant group (UGCCUGCUCUGUGCCUGUAdTdT and UACAGGCACAGAGCAGGCAdTdT) [17].

2.4. Confocal microscopy

Two-day old SH-SY5Y cultures (6-well plates, 5×10^5 cells per well) maintained in regular growth media were transfected with 200 nM FITC-labeled, non-targeted, siRNA loaded into rhodamine-labeled cationic nanoliposomes. After 24 h, SH-SY5Y cells were detached (0.5 mg/ml trypsin/ 0.2 mg/ml EDTA), and replated onto glass cover slips coated with poly-D-lysine (50 μg per cm^2) in growth medium for 24 h. After three washes with PBS, cultures were fixed 2% paraformaldehyde in PBS pH 7.0 (15 min RT). Cover slips were mounted onto glass slides with DAPI-containing mounting medium. Images were acquired using a Leica scanning confocal microscope (TCS SP2 AOBS) (Bannockburn, IL), and filter combinations for FITC, rhodamine, and DAPI.

2.5. Ceramide kinase assay

SH-SY5Y cells were grown in 6-well plates (5×10^5 cells per well) for 48 h. Following transfection with either CerK-targeted siRNA, or non-targeting siRNA (200 nM, 48 h), cultures were incubated with TNFα (100 ng/ml, 15 min), washed, scraped into PBS, and sonicated. A fluorescent CerK assay, adapted for a microplate reader, was performed using the method described by Don and Rosen [33]. Briefly, cell lysates (25 μg) were mixed with reaction buffer (100 μl, 20 mM Hepes (pH 7.4), 10 mM KCl, 15 mM $MgCl_2$, 15 mM $CaCl_2$, 10% glycerol, 1 mM DTT, 1 mM ATP) containing 10 μM NBD-labeled (green fluorescent probe) C6-ceramide conjugated to 0.2 mg/ml fatty acid-free BSA. Reactions were allowed to proceed for 30 min in the dark before the addition of 250 μl chloroform/methanol (2:1). Samples were vortex, centrifuged (20,000 $\times g_{max}$, 45 s), and 100 μl of the upper aqueous phase was transferred to a 96-well (black) microplate. Dimethylformamide (100 μl) was added to each sample well and NBD fluorescence was quantified with a fluorescent microplate reader with a 495 nM excitation filter and a 525 nM emission filter.

2.6. Arachidonic acid release assay

Two-day old SH-SY5Y cultures (6-well plates, 5×10^5 cells per well) were incubated over night with 5 nM ^{3}H-arachidonic acid (0.5 μCi/ml). Next, SH-SY5Y cells were rinsed (PBS) and unincorporated radioactivity removed (1 mg/ml BSA, 10 min). After a rinse with PBS, cultures were replenished with regular growth medium and incubated either with 10 μM GW4869 (1 h), 10 μM CerK inhibitor K1 (1 h), or 200 nM siRNA (2 d). Incubations and transfection spanned the ^{3}H-arachidonic acid labeling time. Next, SH-SY5Y cells were stimulated with TNFα (100 ng/ml) and media aliquots were removed at various time points. Aliquots were centrifuged (10,000 $\times g_{max}$, 10 min) and ^{3}H was measured in a scintillation counter (200 μl of media supernatant) to determine the relative amount of