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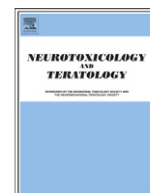

Phytanic acid induces Neuro2a cell death via histone deacetylase activation and mitochondrial dysfunction

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ABSTRACT

Phytanic acid is a branched fatty acid that is a metabolic intermediate of chlorophyll. In this study, the effects of phytanic acid on Histone deacetylase (Hdac) activity were examined in an *in vitro* enzyme assay and in neuronal Neuro2a cells. Several fatty acids have been shown to be Hdac inhibitors, but phytanic acid enhanced the enzyme activity *in vitro*. In Neuro2a cells, phytanic acid significantly reduced histone acetylation and induced cell death, which was inhibited by an Hdac inhibitor, sodium butyrate. Theophylline, a common Hdac activator, had a similar effect on Neuro2a cell viability, and this effect was also inhibited by sodium butyrate. Phytanic acid decreased the level of intracellular active mitochondria, while butyrate increased this level. The cytotoxic effect of phytanic acid was also abolished by a caspase-9 inhibitor. Apicidin, a Hdac2- and 3-specific inhibitor, reduced the cellular toxicity, which suggests that the toxicity of phytanic acid depends on activation of the Hdac2 and 3 subtypes. Overall, these results show that phytanic acid induces mitochondrial abnormality and cell death via activation of Hdac2, 3 in Neuro2a cells. This effect of Hdac activation by phytanic acid may produce neuronal damage in Refsum disease and other peroxisomal disorders, which is caused by accumulation of phytanic acid.

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1. Introduction

Phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) is a branched fatty acid that is a metabolic intermediate of chlorophyll. Serum levels of phytanic acid depend on intake from dietary sources including dairy products and red meat (Hellgren, 2010). Metabolism of phytanic acid occurs via α-oxidation and β-oxidation. In the α-oxidation pathway, mutation of the enzyme phytanoyl-CoA-2-hydroxylase causes Refsum disease, a rare genetic disease with neurological symptoms such as retinitis pigmentosa, polyneuropathy, and ataxia. Accumulation of phytanic acid may cause Refsum disease (Wierzbicki, 2007), but the mechanisms through which phytanic acid causes neuronal damage remain unclear.

Histone deacetylase (Hdac) is involved in epigenetic transcriptional regulation, with histone modifications including acetylation being associated with chromatin structure. Acetylation of lysine residues on histones neutralizes the positive charge of lysine and weakens binding to DNA, which induces an opened chromatin structure that is more accessible to transcriptional factors. Since Hdac deacetylates histones, Hdac acts as a transcriptional repressor, and consequently Hdac inhibitors up-regulate gene expression. These effects are of interest in cancer, with Hdac inhibitors shown to exert antitumor effects, such as induction of cell death and cell cycle arrest in cancer cells (Carew et al., 2008; New et al., 2012) via regulation of expression of tumor suppressor genes; and in neurodegenerative disease, in which Hdac inhibitors have neuroprotective functions (Chuang et al., 2009). For example, the fatty acid Hdac inhibitor sodium butyrate improves learning and memory in a neurodegenerative disease model mouse (Fischer et al., 2007).

In contrast to Hdac inhibitors, Hdac activators may accelerate neuronal damage. In this context, phytanic acid has been shown to cause mitochondrial dysfunction in neurons and glial cells (Busanello et al., 2013; R nicke et al., 2009), but through unknown mechanisms. Mitochondria are important organelles in eukaryotic cells and damage to mitochondria can cause cell death. Mitochondrial abnormality also occurs in neurodegenerative diseases such as Alzheimer, Parkinson, and Huntington diseases (Chaturvedi and Beal, 2013). Hdac inhibitors have protective functions against neuronal cell death in a neurodegeneration model (Zhu et al., 2014), which suggests that phytanic acid may affect mitochondrial function via modulation of Hdac activity. However, it is unknown if phytanic acid modulates Hdac activity or if enhancement of Hdac activity causes mitochondrial dysfunction.

Butyrate, a short-chain fatty acid, and valproic acid, a branched fatty acid, are neuroprotective Hdac inhibitors (Chuang et al., 2009), but the effects of long-chain branched fatty acids on Hdac function are unknown. In patients with Refsum disease, phytanic acid appears to be neurotoxic, rather than neuroprotective. The aim of this study was to examine the effects of phytanic acid on Hdac activity, and to determine how modulation of Hdac activity affects cell viability in

Abbreviations: FBS, fetal bovine serum; Hdac, histone deacetylase; MTT, 3-(4,5-dimethyl-2-thiazolyl) 2,5-diphenyl-2H-tetrazolium bromide; NaBu, sodium butyrate; PI, propidium iodide; SDS, sodium dodecyl sulfate

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neuronal Neuro2a cells, with the goal of establishment of strategies for neuroprotection in Refsum disease.

2. Materials and methods

2.1. Materials

Phytanic acid (Cayman Chemical, Ann Arbor, MI, USA); 3-(4,5-dimethyl-2-thiazolyl) 2,5-diphenyl-2H-tetrazolium bromide (MTT) (Dojindo, Kumamoto, Japan); a HDAC deacetylase fluorometric assay kit and a cellular histone acetylation assay kit (both Cyclex, Nagano, Japan); theophylline (Nacalai Tesque, Kyoto, Japan); the caspase-9 inhibitor Z-LEHD-FMK (R&D Systems, Minneapolis, MN, USA); and the Hdac inhibitors sodium butyrate (Alfa Aesar, Ward Hill, MA, USA), suberoylanilide hydroxamic acid (SAHA), MS-275 (both Cayman Chemical), apicidin, and MC1568 (both Sigma, St. Louis, MO, USA) were used in the study.

2.2. Cell culture

Mouse neuroblastoma Neuro2a cells were obtained from the Health Science Research Resources Bank, Japan, and maintained in Dulbecco's modified Eagle medium (D-MEM) (Sigma) supplemented with 10% fetal bovine serum (FBS), 10 U/ml penicillin, and 100 μg/ml streptomycin in a 5% CO_2 humidified atmosphere at 37 °C. During treatment, Neuro2a cells were cultured in medium without FBS.

2.3. Hdac enzyme activity assay

Hdac activity was measured using a Cyclex HDACs deacetylase fluorometric assay kit. Briefly, phytanic acid was added to the enzyme reaction mixture and the solution was incubated at 37 °C for 15 min. Fluorescence of the assay solution was measured at an excitation of 355 nm and an emission of 460 nm using a fluorescent microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.4. Quantification of histone acetylation

Cellular histone acetylation was quantified using a Cyclex cellular histone acetylation assay kit. Neuro2a cells were cultured in 96-well plates at a density of 2000 cells/well. After one day of culture, the cells were incubated with 10 μM phytanic acid dissolved in the medium for 48 h. Cells were then fixed and subjected to immunostaining. Acetylation levels were standardized based on cell viability evaluated using a MTT assay, as described below. Absorbance of the assay solution was measured at 450 nm using a microplate reader (Molecular Devices).

2.5. Cell viability assay

Cell viability was determined by MTT assay and double staining with calcein-AM and propidium iodide (PI) (Dojindo). Neuro2a cells were cultured in 96-well plates at a density of 2000 cells/well. After one day of culture, the cells were incubated with phytanic acid dissolved in the medium for 48 h. For the MTT assay (Nagai et al., 2006), 0.25 mg/ml MTT in D-MEM without FBS was added to the treated cells and incubated for 3 h, followed by termination by addition of 20% (*w*/*v*) SDS and 50% (*v*/*v*) dimethylformamide in water. Absorbance of the assay solution was measured at 570 nm using a microplate reader (Molecular Devices). MTT assay was performed quadruplicately and repeated independent 3 times for reproductivity ($n = 12$). For live/dead staining, Neuro2a cells were cultured in poly-lysine coated 24-well plates at a density of 12,000 cells/well. After treatment as described above, the cells were stained with 0.5 μg/ml calcein-AM and 0.5 μg/ml PI in medium without FBS at 37 °C for 20 min. Nuclei were simultaneously stained with 0.5 μg/ml Hoechst 33342 (Dojindo). After staining, the medium was replaced with a fresh medium without serum and the stained cells were observed by fluorescent microscopy. PI-positive cells were considered to be dead cells.

2.6. Mitochondrial staining

Mitochondrial staining was examined using a JC-1 mitochondrial staining kit (Sigma) and MitoPT TMRE assay kit (ImmunoChemistry Technology LLC, Bloomington, MN, USA). Briefly, for observation under fluorescent microscopy, Neuro2a cells in 24-well plates were treated as described above. Then the cells were stained with JC-1 at 37 °C for 15 min. After staining, the medium was replaced with a fresh medium without serum and the stained cells were observed by fluorescent microscopy. Intracellular mitochondria were quantified by imaging cytometry. Treated Neuro2a cells were harvested and stained with JC-1 or TMRE at 37 °C for 15 min. After staining, the medium was replaced with a fresh medium without serum and stained mitochondria were analyzed using a Tali Image-based cytometer (Life Technologies, Carlsbad, CA, USA).

2.7. Statistical analysis

Data are shown as mean ± SD. Differences were analyzed using one-way ANOVA followed by a Tukey–Kramer post-hoc test, with *p*-values <0.05 considered to be significant.

3. Results

3.1. Effects of phytanic acid on Hdac activity and cellular histone acetylation

The effects of phytanic acid on Hdac activity were evaluated in an *in vitro* enzyme assay using a fluorescently labeled substrate. As shown in Fig. 1A, phytanic acid at >30 μM significantly increased Hdac activity in this assay. To examine if phytanic acid increases intracellular histone acetylation, Neuro2a cells were treated with 10 μM phytanic acid for 48 h, after which the amount of histone acetylation was quantified. This treatment significantly decreased histone acetylation, similarly to the effect of 1 mM theophylline, a known Hdac activator (Fig. 1B). These data suggest that phytanic acid directly enhances Hdac enzyme activity and decrease intracellular histone acetylation in Neuro2a cells.

3.2. Effects of phytanic acid on Neuro2a cell viability and association with Hdac activation

Next, the effect of phytanic acid on Neuro2a cell viability via Hdac activation was analyzed. Neuro2a cells were treated with 5 or 10 μM phytanic acid for 48 h in the presence or absence of the Hdac inhibitor, sodium butyrate (2 mM). Cell viability was quantified by MTT assay. Phytanic acid significantly decreased the cell viability compared to untreated control cells. In the presence of sodium butyrate, the effect of phytanic acid on cell viability was significantly attenuated and viability increased (Fig. 2A). The Hdac activator theophylline (1 mM) also significantly decreased cell viability and butyrate attenuated this effect (Fig. 2B).

3.3. Induction of cell death by phytanic acid via Hdac activation

A decrease in cell viability may be due to induction of cell death or growth retardation. Thus, phytanic acid-treated cells were subjected to live/dead cell staining with calcein-AM and PI. Neuro2a cells were treated with 10 μM phytanic acid for 48 h in the presence or absence of 2 mM sodium butyrate. After treatment, the cells were stained with calcein-AM and PI, and nuclei were stained with Hoechst 33342. In untreated control cells, the percentage of PI-positive dead cells was 6.73 ± 3.23%. Phytanic acid treatment significantly increased this rate to 12.47 ± 2.42% (Fig. 3A, B), while butyrate alone decreased the rate to 4.58 ± 0.57%. In cotreatment with phytanic acid, butyrate attenuated the toxicity of phytanic acid, with a rate of dead cells of 4.71 ± 1.31% (Fig. 3A, B). Phytanic acid treatment also increased the number of brightly

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