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Dilinoleoyl-phosphatidylethanolamine from *Hericium erinaceum* protects against ER stress-dependent Neuro2a cell death via protein kinase C pathway

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Abstract

In many types of neurodegeneration, neuronal cell death is induced by endoplasmic reticulum (ER) stress. Hence, natural products able to reduce ER stress are candidates for use in the attenuation of neuronal cell death and, hence, in the reduction of the damage, which occurs in neurodegenerative disease. In this study, we investigated ER stress-reducing natural products from an edible mushroom, *Hericium erinaceum*. As a result of screening by cell viability assay on the protein glycosylation inhibitor tunicamycin-induced (i.e., ER stress-dependent) cell death, we found that dilinoleoyl-phosphatidylethanolamine (DLPE) was one of the molecules effective at reducing ER stress-dependent cell death in the mouse neuroblastoma cell line Neuro2a cells. A purified DLPE, commercially available, also exhibited a reducing effect on this ER stress-dependent cell death. Therefore, we concluded that DLPE has potential as a protective molecule in ER stress-induced cell death. From the structure of DLPE, it was hypothesized that it might activate protein kinase C (PKC). The activity of PKC-ε, a novel-type PKC, was increased by adding DLPE, and PKC-γ, a conventional-type PKC, was activated on the coaddition of diolein and DLPE, as shown by in vitro enzyme activity analysis. The protecting activity of DLPE was attenuated in the presence of a PKC inhibitor GF109203X but not completely diminished. Therefore, DLPE can protect neuronal cells from ER stress-induced cell death, at least in part by the PKC pathway.

Keywords: Neurodegeneration; Cell death; Stress; *Hericium erinaceum*; Phosphatidylethanolamine; Protein kinase C

1. Introduction

Neuronal cell death is an essential feature of neurodegenerative disease. Many types of neuronal cell death are induced by endoplasmic reticulum (ER) stress. Hence, we screened ER stress-attenuating molecules, which might reduce the risk of neurodegenerative disease, from an edible mushroom, *Hericium erinaceum*. *H. erinaceum* possesses certain compounds that induce the expression of neurotrophic factors (e.g., NGF) in astrocytes [1,2]. It has been reported that NGF has a protective or inducible activity on neuronal cell death through the Trk and p75 pathway [3]. Many types of neuronal cell death, for example, that associated with amyloid-β [4], glutamate [5] and NO [6], are thought to be caused by ER stress. In a large number of neurodegenerative diseases, including Alzheimer's, Parkinson's, Huntington's and the prion diseases, ER stress is reported to be the cause of neuronal cell death [7]. Thus, it is reasonable to suspect that molecules able to attenuate ER stress might reduce both the risk for and the extent of the damage in neurodegenerative disease. Recently, a few groups have reported molecules with potential for reducing the ER stress [8,9]. However, because neurodegenerative diseases have typically a long incubation period prior to diagnosis, reducing the risk before the identification of quite advanced disease is important. Therefore, reducing the risk of neurodegeneration by the consumption of food products might be a viable and important option for target populations [10,11].

The ER is an organelle in which newly synthesized proteins are folded and glycosylated. If proper folding in the ER fails and misfolded proteins accumulate, a stress

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response is induced, for example, an up-regulation of molecular chaperones [12,13]. Because many proteins synthesized through the ER are glycosylated, a protein glycosylation inhibitor, tunicamycin, induces misfolded protein accumulation in the ER and, ultimately, cell death. Thus, tunicamycin is generally used as a glycosylation inhibitor in ER stress models. We applied this model to this study.

The screened molecule dilinoleoyl-phosphatidylethanolamine (DLPE) is a phospholipid bearing two unsaturated fatty acids, namely, linoleic acids. Phospholipids, mainly phosphatidylserine, and unsaturated fatty acids are known to be activators of protein kinase C (PKC) [14]. Conventional-type PKCs, for example PKC-γ, are activated by Ca^{2+}, diacylglycerol and phosphatidylserine. Novel-type PKCs, for example, PKC-ε, are activated by unsaturated fatty acids or phosphatidylserine. As far as the role of PKC in cell death is concerned, there are reports that PKC activation attenuates neuronal cell death [15]. Therefore, it may be hypothesized that food products that can activate PKC may attenuate neuronal cell death. Dilinoleoyl-phosphatidylethanolamine is one such food product. In this article, we report the antiapoptotic activity of DLPE and the role PKC activation plays in this activity.

2. Methods and materials

2.1. Materials

Dilinoleoyl-phosphatidylethanolamine was purchased from Avanti Polar Lipids, USA. Tunicamycin was purchased from Nacalai Tesque, Japan.

2.2. Cell culture

Neuro2a cells were obtained from the Health Science Research Resources Bank, Japan, and maintained in the Dulbecco's modified Eagles medium (DMEM) (SIGMA, USA) supplemented with 10% fetal bovine serum (FBS), unless particularly noted.

2.3. Preparation of DLPE from H. erinaceum

Dried fruiting bodies of *H. erinaceum* were extracted with 85% ethanol and then acetone. The solvents were combined, concentrated under reduced pressure and partitioned between $CHCl_3$ and H_2O. The residue obtained after removing $CHCl_3$ was fractionated by silica gel flash column chromatography (90%, 70% $CHCl_3$/acetone, 90%, 50%, 30% $CHCl_3$/MeOH, MeOH) to obtain 14 fractions. Fraction 10, which exhibited the highest protective activity, was further separated by preparative reversed-phase TLC ($CHCl_3$/MeOH/H_2O=6/4/1) to afford a mixture of phosphatidyl ethanolamines having various fatty acids. Dilinoleoyl-phosphatidylethanolamine was purified from the mixture by reversed-phase HPLC ($CHCl_3$/MeOH/CH_3CN/H_2O=3/5/2/1).

2.4. Cell viability assay

Cell viability analysis was performed by 3-(4,5-dimethyl-2-thiazolyl)2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay and double staining method with Calcein-AM and propidium iodide (PI) (Dojindo, Japan).

Cell protection assay on ER stress confirmed by MTT assay was performed as follows. Neuro2a cells were cultured in a 96-well plate at a cell density of 5000 cells/well. After 1 day of culture, the cells were cultured in DMEM without FBS, and 0.5 μg/ml of tunicamycin and varying concentrations of DLPE or *H. erinaceum* extract were applied to the medium. The cells were incubated for 24 h, and then the viability was measured by MTT assay, as described previously [16]. Briefly, 0.25 mg/ml of MTT in DMEM without FBS were added onto the cells and incubated for 2 h. The incubation was terminated by the addition of 20% SDS (v/w) and 50% dimethylformamide (v/v) in water. The absorbance at 570 nm of reacted solution was measured by a microplate reader (Molecular Devices, USA).

Live/dead staining was performed as follows. Neuro2a cells were cultured in a polylysine-coated 24-well plate at a cell density of 50,000 cells/well. After 24 h of treatment with tunicamycin and DLPE, as described above, the cells were stained with Calcein-AM and PI (Dojindo). Stained cells were observed under fluorescent microscopy. Propidium iodide-positive cells were considered to be dead cells.

2.5. Caspase-12 activation assay

Caspase-12 activation, which is a marker of the ER stress signal, was investigated by western blotting, as described previously [17]. Briefly, 60 μg of cytosolic proteins were electrophoretically separated on a 12% polyacrylamide gel and transferred onto PVDF membrane (ATTO, Japan). The membrane was blocked with a blocking solution, phosphate-buffered saline containing 1% skimmed milk, and then incubated with anti-caspase-12 antibody (Chemicon, USA), followed by biotin-conjugated anti-rabbit IgG. The membrane was incubated with a streptavidin–biotin complex peroxidase kit (Nacalai Tesque). The specific immunoreactivity was visualized using a peroxidase staining kit for immunoblotting (Nacalai Tesque). Caspase-12 activation was estimated by decrease of pro-caspase-12.

2.6. Protein kinase C assay

Protein kinase C activity was measured as described before [18]. Briefly, PKC substrate peptide (Peptide Institute, Japan) was incubated with PKC-γ or PKC-ε (Calbiochem, USA) in a reaction buffer (50 μl) containing 20 mM Tris–HCl (pH 7.5), 5 mM Mg^{2+}-acetate (Wako, Japan), 0.1 mM $CaCl_2$ (Wako) and 10 μM ATP at 30°C for 10 min, and the reaction was terminated by incubation at 100°C for 5 min. By adding 10 μM DLPE (PKC-) or 10 μM DLPE and 2 μg/ml diolein (Wako) (PKC-γ) into the reaction solution, the PKC activation ratio was analyzed.

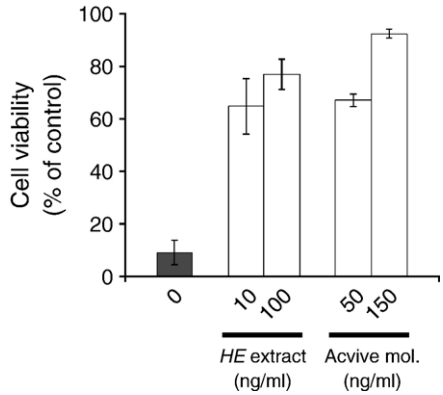

Fig. 1. Protective effect of *H. erinaceum* extract on ER stress-induced Neuro2a cell death. Neuro2a cells were incubated with 10 or 100 ng/ml of the extract, or 50 or 150 μg/ml of purified active molecule in the presence of 0.5 μg/ml of tunicamycin for 24 h. After treatment, cell viabilities were analyzed by MTT assay. The values are represented as the mean of the relative percentage of surviving cells±S.E.M. ($n=4$).

Nonphosphorylated substrate and phosphorylated product were separated and quantified by a reversed-phase HPLC system (Shimadzu, Japan) equipped with a TSK-GEL ODS-80$_{TM}$ column (Tosoh, Japan). The ratio of the product peak area to the product peak area plus substrate peak area was calculated as an index of PKC activity.

3. Results

3.1. H. erinaceum extract and DLPE reduces ER stress-induced cell death

We analyzed the reducing activity on ER stress-induced Neuro2a cell death from nonpolar extracts of *H. erinaceum*. By MTT assay, 0.5 μg/ml of tunicamycin treatment decreased the viability to 8.88±4.64%, whereas the addition of 10 and 100 ng/ml extracts increased the viability to 64.8±10.56 and 76.9±5.65%, respectively (Fig. 1). Thus, *H. erinaceum* contains certain molecules that protect the cells from ER stress-dependent cell death, and one such active molecule was purified from *H. erinaceum*. The purified molecule increased the cell viability to 67.0±2.32 and 92.4±1.63% upon the addition of 50 and 150 μg/ml, respectively (Fig. 1). By structural analysis, we investigated the structure of the active molecule in Neuro2a cell protection and found it to be a common phospholipid, that is, phosphatidylethanolamine bearing two linoleic acids (DLPE) (data not shown), and 50 μg/ml of purified molecule is equivalent to 67.9 μM of DLPE. Hence, the protective activity of commercially purchased purified DLPE on ER stress-dependent cell death was investigated. By MTT assay, 0.5 μg/ml of tunicamycin reduced the cell viability to 17.8±0.88%, whereas in the presence of 1, 10 and 100 μM of DLPE, the cell viabilities were increased to 22.2±2.32, 34.3±3.16, and 64.4±6.08%, respectively (Fig. 2A). Regarding the linoleic acid that is a metabolite of DLPE, the cell viabilities were only weakly changed, 11.43±0.85% in the absence of linoleic acid, and 10.7±2.74, 17.4±1.35, and 17.3±3.79% in 1, 10 and 100 μM of linoleic acid, respectively (Fig. 2B). Thus, DLPE itself rescued the cells from ER stress-induced cell death in a dose-dependent manner. By cell double staining assay, the ratios of PI-positive dead cells were determined as follows: control, 1.26%; DLPE only, 2.11%; tunicamycin only, 26.07%; and tunicamycin plus DLPE, 3.37% (Fig. 3 A, B). In caspase-12 activation assay, tunicamycin clearly reduced the amount of procaspase-12, whereas DLPE and *H. erinaceum* extract inhibited the decrease of procaspase-12 or caspase-12 activation. These data indicate that DLPE rescued the Neuro2a cells from ER stress-dependent cell death.

3.2. Protein kinase C activation by DLPE is involved in rescuing activity

Because DLPE is a phospholipid bearing unsaturated fatty acids, we speculated that DLPE might act as an activator of PKC. Hence, PKC activation activity of DLPE was analyzed by in vitro phosphorylation assay, using synthetic peptide, and the activity was quantitated by HPLC. In the absence of lipid, PKC-γ, a conventional-type PKC,

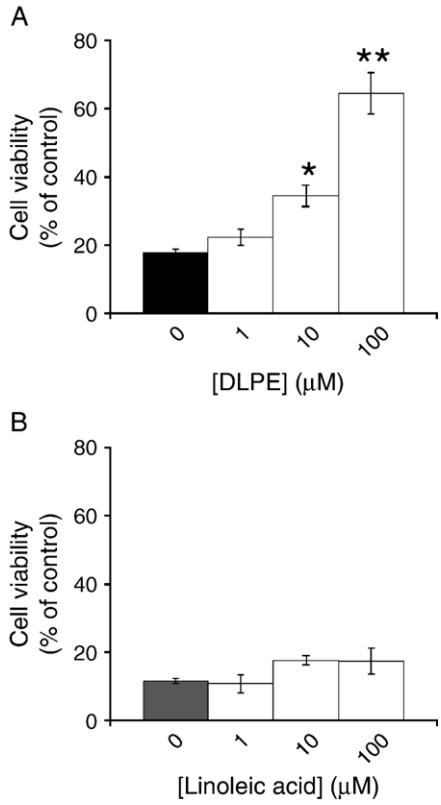

Fig. 2. Effects of DLPE and linoleic acid on ER stress-induced cell death. Neuro2a cells were incubated with various concentrations of DLPE (A) or linoleic acid (B) in the presence of 0.5 μg/ml of tunicamycin for 24 h. After treatment, cell viabilities were analyzed by MTT assay. The values are represented as the mean of the relative percentage of surviving cells±S.E.M. ($n=4$). *$P<.01$, **$P<.005$, nonpaired t test.

exhibited activity of 0.44±0.09 pmol/min. By the addition of 10 μM DLPE, the activity was observed to be 0.30±0.01 pmol/min. In the presence of diolein, a diacylglycerol, the activity was 1.09±0.35 and 2.39±0.13 pmol/min in the absence and presence of DLPE, respectively (Fig. 4). Thus, 10 μM DLPE did not stimulate the phosphorylation activity, whereas in the presence of diolein, DLPE activated PKC-γ. Therefore, it is suggested that DLPE binds to a phosphatidylserine binding site for activation of PKC-γ. On the other hand, PKC-ε, a novel-type PKC, activity was 0.52±0.09 pmol/min in the absence of DLPE, whereas by the addition of 10 μM DLPE, the phosphorylation activity was increased to 1.03±0.08 pmol/min (Fig. 4). Thus, DLPE can activate PKC-ε. From the data of both PKCs, it is

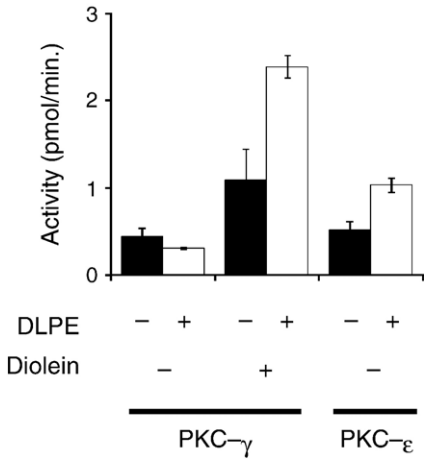

Fig. 4. Effects of DLPE on PKC activity. PKC-γ and PKC-ε (0.875 μg/ml) were incubated with 1 M DLPE in the reaction mixture. After incubation at 30°C for 10 min, the reaction was terminated and injected into the HPLC system. The activities are represented as the mean±S.E.M. (n=4) of phosphorylated product peptide synthesis rate (picomole per minute).

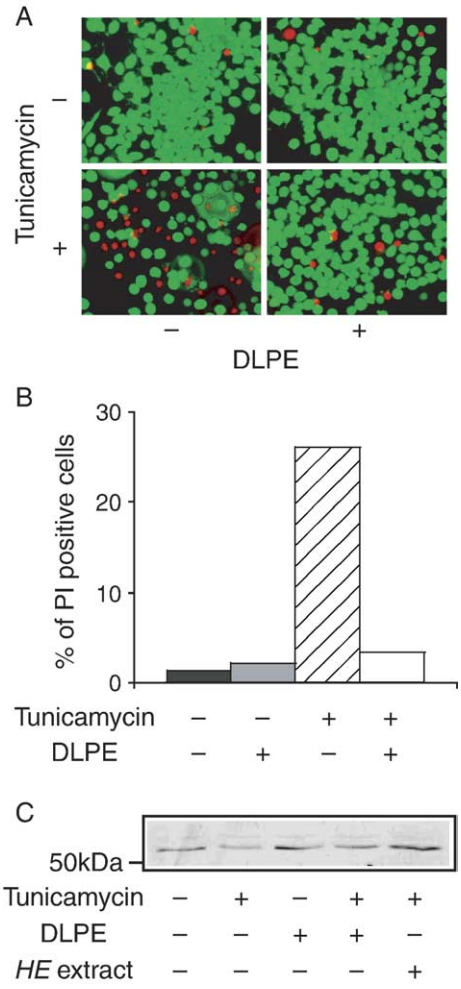

Fig. 3. Antiapoptotic effect of DLPE on ER stress-induced cell death. Neuro2a cells were incubated with 10 M of DLPE and 0.5 μg/ml of tunicamycin for 24 h. Then, the cells were stained with Calcein-AM and PI for live/dead analysis. (A) Typical staining pattern of live (Calcein-AM, green) and dead (PI, red) cells in each condition. (B) The value is represented as a relative percentage of dead (PI-positive) cells in total cells out of the total of all the cells. (C) Western blot analysis of procaspase-12. Neuro2a cells were treated with 100 μM of DLPE or 100 ng/ml of *H. erinaceum* extract in the presence or absence of 0.5 μg/ml of tunicamycin for 24 h. Sixty micrograms each of cytoplasmic proteins was subjected to western blotting.

evident that DLPE can bind to phosphatidylserine or unsaturated fatty acid binding sites and activate both conventional and novel-type PKCs. Hence, the role of PKC pathway on the cell-rescuing activity was investigated. We applied 1 μM of the PKC inhibitor GF109203X (GF) for cell protection analysis by MTT assay. In the absence of GF, the cell viability data were 19.9±0.77%, 51.3±2.29% and 89.2±6.10% in 0, 10 and 10 μM DLPE, respectively. In the presence of GF, the cell viability data were 13.0±3.58, 35.0±3.52 and 64.1±5.96% in 0, 10 and 10 μM DLPE, respectively (Fig. 5). With the addition of only GF, the cell viability was 94.2±16.4%. Thus, GF did not directly affect the cell viability under these conditions. From these data, 1 μM GF decreased the cell viability 34.7%, 31.8% and

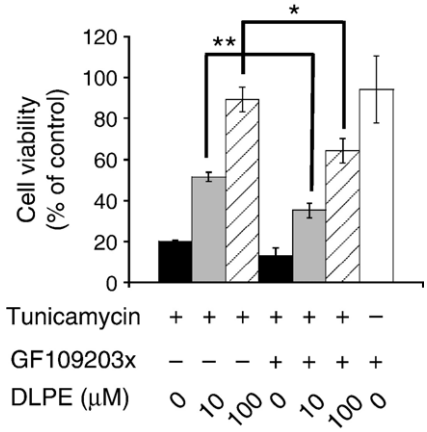

Fig. 5. Effects of PKC inhibitor on the protective effect of DLPE against ER stress-induced cell death. Neuro2a cells were incubated with 10 or 100 μM of DLPE and 0.5 μg/ml of tunicamycin for 24 h in the presence or absence of GF. After treatment, cell viabilities were analyzed by MTT assay. The values are represented as the mean of the relative percentage of surviving cells±S.E.M. (n=4). $^{**}P<.01$, $^{*}P<.015$, nonpaired t test.

28.2% in the presence of 0, 10 and 10 μM DLPE, respectively. Therefore, the PKC pathway appears to contribute to the cell protection mechanism against ER stress-dependent cell death.

4. Discussion

Treatments for reducing neuronal cell death are important for preventing as well treating neurodegenerative disease, including dementia and motor dysfunction. Because neuronal cell death is caused by ER stress in many neurodegenerative diseases, being able to attenuate ER stress would be expected to reduce the risk for and impact of neurodegenerative disease. However, neurodegenerative diseases have a long incubation period that are symptom-free; hence, there is a late diagnosis of the disease. This is a severe problem because once neurons are dead or neuronal circuits destroyed, brain function is almost impossible to restore. In other words, at the point symptoms have appeared, effective treatment options are highly constrained. Thus, reducing the risk contracting neurodegenerative disease might be an essential part of therapeutic intervention, and attenuating the risk of neurodegeneration via intake of a food product might be one of the optimal candidates in such an approach.

There are reports that nutritional molecules, including unsaturated fatty acids [10,19,20], exert a preventative effect on certain neurodegenerative diseases. However, in most of these, the detailed mechanisms by which protection is afforded have not been resolved. As shown in this report, DLPE protected neuronal cells from ER stress-dependent cell death. Therefore, the protective effect of unsaturated fatty acids on neurodegenerative diseases may in general depend on attenuating activity of ER stress. Because unsaturated fatty acids are mainly derived from fish and plants, intake of these foodstuffs might attenuate ER stress-dependent neuronal cell death and the diseases in which such death is the hallmark.

Regarding the molecular mechanisms that effect the protecting activity, we hypothesized a contribution of the PKC pathway. It is still controversial whether PKC reduces or contributes to cell death. For example, it is reported that PKC reduces cell death [15,21], including amyloid-β toxicity, whereas others report that PKC contributes to the induction of apoptosis [22]. In our study, the PKC inhibitor GF, partly reduced cell death and, thus, PKC had a role in the cell-rescuing pathway. However, the inhibitor could not completely inhibit cell death. These data suggest that there could be another intracellular signaling pathway for reducing ER stress-induced cell death. Recently, it was reported that the phosphatidylethanolamine binding protein (PEBP) reduced tumor necrosis factor α-induced apoptosis in human cancer cells [23]. Therefore, the PEBP-dependent signaling pathway might be a further candidate for the protective activity of DLPE.

In this study, the viability ratio obtained via MTT assay and the cell-staining method seem to be different. In the double staining assay, PI, which is a membrane-impermeable dye, can stain only the late phase of apoptotic cells [24]. Therefore, some of the cells, negative on MTT assay, might not be stained with PI. However, at least in both assays, the cell death induced by ER stress was consistently found to be significantly reduced by DLPE.

As shown in Fig. 1, the activity of the crude extract seemed to be higher than DLPE. In fact, we observed fractions that also appeared to have ER stress-reducing activity in the purification step. Thus, *H. erinaceum* would appear to contain additional molecules, which might reduce ER stress-induced cell death. However, the purification and structural analysis on these putative other factors remain to be carried out.

Our findings indicate that a common phospholipid, DLPE, a phosphatidylethanolamine bearing two linoleic acids, is able to protect neuronal cells from ER stress-induced cell death, and that the PKC pathway is involved in the protective mechanism. Further studies will elucidate the detailed molecular signaling pathway involved in the protective activity.

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