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Letter

Effects of estrogen on fatty-acid-induced cytotoxicity in mouse Neuro-2a neural cells

Masahiro Ogawa, Takahiro Kyoya, Takahiro Kimura and Megumi Terada

Life Science Research Institute, Kumiai Chemical Industry Co., Ltd.,
3360 Kamo, Kikugawa-shi, Shizuoka, 439-0031, Japan

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ABSTRACT — The accumulation of free fatty acids induces lipotoxicity in neural cells. Estrogen, 17β-estradiol (E2) protects against the damage of cells in various organs and tissues. However, the role of E2 on lipotoxicity in neural cells remains unclear. In this study, we investigated the effects of E2 on stearic acid (saturated fatty acid)- and oleic acid (unsaturated fatty acid)-induced cytotoxicity in retinoic acid-induced mouse neuroblastoma Neuro-2a differentiated into neural cells. Cell viability was evaluated by lactate dehydrogenase release from Neuro-2a neural cells. Stearic acid and oleic acids suppressed the cell viability in a dose-dependent manner. E2 prevented oleic acid-induced cytotoxicity but had no effect on stearic acid-induced cytotoxicity. ERα-selective agonist prevented cytotoxicity in Neuro-2a neural cells. In contrast, ERβ-selective agonist slightly significantly enhanced the cytotoxicity in the presence of oleic acid. Oleic acid, but not stearic acid, increased the mRNA level of *p62/Sqstm1*. E2 treatment statistically significantly, but slightly, enhanced the stearic acid-induced *Bax* expression. In contrast, E2 and ERα-selective agonist inhibited the oleic acid-induced the *p62/Sqstm1* expression. Our results suggested that fatty acids induced cytotoxicity in Neuro-2a neural cells, and estrogen prevented the oleic acid-induced cytotoxicity *via* ERα, but not ERβ. Further studies are needed to understand the role of ERβ in neuron injury under normal conditions.

Key words: Estrogen, Cytotoxicity, Neuro-2a, Apoptosis, Fatty acid, Lipotoxicity

INTRODUCTION

Excess accumulation of fatty acids in parenchymal cells of multiple tissues including skeletal and cardiac myocytes, hepatocytes, and pancreatic beta cells results in chronic cellular dysfunction and injury (lipotoxicity) (Weinberg, 2006). In addition, central nervous system injury from trauma and hypoxic-ischemia results in the degradation of membrane phospholipids with release of free fatty acids (Bazan and Rakowski, 1970; Homayoun *et al.*, 1997). The accumulation of free fatty acids in the brain may mediate much of the secondary damage (White *et al.*, 2000). There are three major types of morphologically distinct cell death: apoptosis, autophagic cell death, and necrosis. High-fat diet induces apoptosis and autophagy in rodent liver (Mei *et al.*, 2011; Masuda *et al.*, 2019). Fatty acids are chemically classified as saturated and unsaturated, and each has specific biological functions. Previous study indicated that saturated fatty acids induced apoptosis while unsaturated fatty acids induced autophagy in hepatocytes (Mei *et al.*, 2011).

Estrogen, one of main sex hormone, is responsible for female physical features and reproduction. Brain damage produced by cerebral ischemia is less in females than in males (Alkayed *et al.*, 1998; Jover *et al.*, 2002). Such protection in female rodents is abolished by ovariectomy and recovered by estrogen replacement (Rusa *et al.*, 1999;

Correspondence: Masahiro Ogawa (E-mail: m-ogawa@kumiai-chem.co.jp)

Dubal *et al.*, 1998; Rau *et al.*, 2003). Thus, it is assumed that estrogen has prevented from cell damage, but the mechanisms have not been elucidated.

Estrogen signaling is mediated by binding to estrogen receptor α (ERα) and/or ERβ, which are members of the nuclear receptor family. Estrogen prevents apoptosis *via* ERs-mediated mechanisms in many cell types (Lewis-Wambi and Jordan, 2009; Diakogiannaki *et al.*, 2007). Both of ERα and ERβ are expressed in brain (Broughton *et al.*, 2013) and peripheral neurons (Gu *et al.*, 2018), but the roles of estrogen and the two ER isoforms on neuron injury remain unclear.

In this study, we demonstrated that fatty acids induced cytotoxicity in mouse neuroblastoma Neuro-2a neural cells, and estrogen prevented the oleic acid-induced cytotoxicity *via* ERα, but not ERβ.

MATERIALS AND METHODS

Reagents

Stearic acids and oleic acid were obtained from Fujifilm Wako Pure. Chemical Co. (Osaka, Japan), 17β-estradiol; E2 was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), ICI 182,780 was obtained from Merck KGaA (Darmstadt, Germany), and propylpyrazole-triol (PPT; 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1-H-pyrazole) and diarylpropionitrile (DPN; 2,3-bis(4-hydroxyphenyl)propionitrile) were obtained from Tocris Bioscience (Bistrol, UK).

Cell culture and treatment

Mouse neuroblastoma Neuro-2a cells were obtained from JCRB cell bank (Osaka, Japan). Neuro-2a cells were grown to 50% confluence in phenol red-free Dulbecco's Modified Eagle Medium, high glucose (12100-046: Gibco, Waltham, MA, USA) supplemented with 10% charcoal-stripped fetal bovine serum, 1.5 g/L $NaHCO_3$, 100 units/mL penicillin, and 100 μg/mL streptomycin (growth medium) at 37°C in 5% CO_2 and 95% air atmosphere at 100% humidity. Cells were sub-cultured with the growth medium every 2-3 days.

To differentiate Neuro2a cells into neural cells, cells were grown to 50% confluence in the growth medium and then cultured in phenol red-free Dulbecco's Modified Eagle Medium, high glucose supplemented with 2% charcoal-stripped horse serum, 10 μM all-*trans* retinoic acid 1.5 g/L $NaHCO_3$, 100 units/mL penicillin, and 100 μg/mL streptomycin (differentiation medium) in 96-well plates at 37°C in 5% CO_2 and 95% air atmosphere at 100% humidity. The differentiation medium was replaced at 48 hr intervals, and cells were cultured for 4 days.

Cell viability

Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme in cells and is released into the cell culture supernatant during cytoplasmic membrane damage. In this study, the cytotoxic effects of fatty acids on Neuro-2a neural cells were investigated by measuring the activity of LDH in cell culture supernatant. In 96-well plates, Neuro-2a neural cells were incubated with the various concentrations of oleic acids or stearic acids in the absence or presence of 10 nM E2 for 24 or 48 hr, and four replicate wells were prepared for each treatment. LDH activity assay was measured using LDH Cytotoxicity Detection Kit (MK401; Takara Bio, Shiga, Japan).

Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted and reverse transcribed with PrimeScript RT reagent Kit (Takara Bio) from Neuro-2a neural cells in 24-wells plate. The resultant cDNA was subjected to quantitative real-time RT-PCR (qRT-PCR) using the following specific primers: *Esr1* (forward primer 5′-CCTCCCGCCTTCTACAGGT-3′ and reverse primer 5′-CACACGGCACAGTAGCGAG-3′), *Esr2* (forward primer 5′-TCCATCGCCAGTTATCACATCT-3′ and reverse primer 5′-CTGGACCAGTAACAGGGCTG-3′), *Bax* (forward primer 5′-TGAAGACAGGGGCCTTTTTG-3′ and reverse primer 5′-AATTCGCCGGAGACACTCG-3′), *p62/Sqstm1* (forward primer 5′- ATGTGGAACATGGAGGGAAGA-3′ and reverse primer 5′- GGAGTTCACCTGTAGATGGGT-3′), *Actb* (forward primer 5′-CAGCCTTCCTTCTTGGGTAT-3′ and reverse primer 5′-GCTCAGTAACAGTCCGCCTA-3′). The PCR profiles consisted of denaturation at 95°C for 1 min, primer-annealing at 55°C for 1 min, and primer extension at 72°C for 30 sec. The final primer extension was performed at 72°C for 10 min. The final primer extension was performed at 72°C for 10 min. The PCR in qRT-PCR was performed with SYBR Premix Ex TaqII (Takara Bio) on Thermal Cycler Dice, TP-900 (Takara Bio). Ct values were transformed into relative quantification data by $2^{-\Delta\Delta}$Ct method, and data were normalized to the *Actb*.

Statistical analyses

Data from Neuro-2a neural cells were statistically compared by Dunnett's multiple comparison tests or Tukey-Kramer test using the statistical analysis software Pharmaco Basic (version 15.0; Scientist Press Co., Ltd., Tokyo, Japan). Data are expressed as mean ± S.D., and differences were considered statistically significant at a p value of < 0.05 (n = 3 or 4).

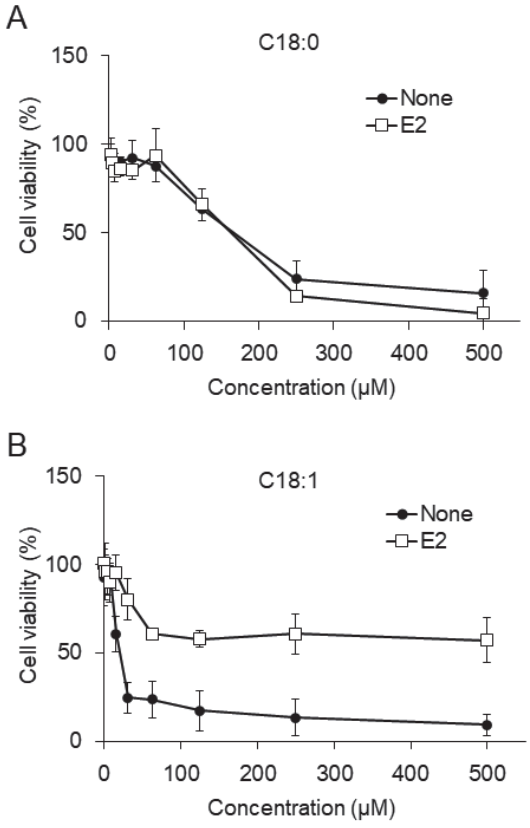

Fig. 1. Effects of estrogen on fatty-acids-induced cytotoxicity in Neuro-2a neural cells. Neuro-2a neural cells were treated with 10 nM E2 in the presence of various concentrations (2 μM to 500 μM) of stearic acid (A) or oleic acid (B) for 48 hr. Cytotoxicity was evaluated by LDH cytotoxicity assay. All experiments were performed in quadruplicate.

RESULTS

Effects of estrogen on fatty-acids-induced cytotoxicity in Neuro-2a neural cells

First, to determine whether fatty acids have cytotoxicity, Neuro-2a neural cells were incubated in various concentrations of fatty acids for 48 hr. Stearic and oleic acids decreased the cell viability in a dose-dependent manner. The half-maximal cytotoxic concentration for stearic acid and oleic acid was 176 μM and 38 μM, respectively.

To study the effects of estrogen on fatty acid-induced cytotoxicity, Neuro2a neural cells were treated with 500 μM stearic acid or 500 μM oleic acid in the presence or absence of E2. E2 prevented the oleic acid-induced cytotoxicity, but has no effects on the stearic acid-induced cytotoxicity (Fig. 1A, B).

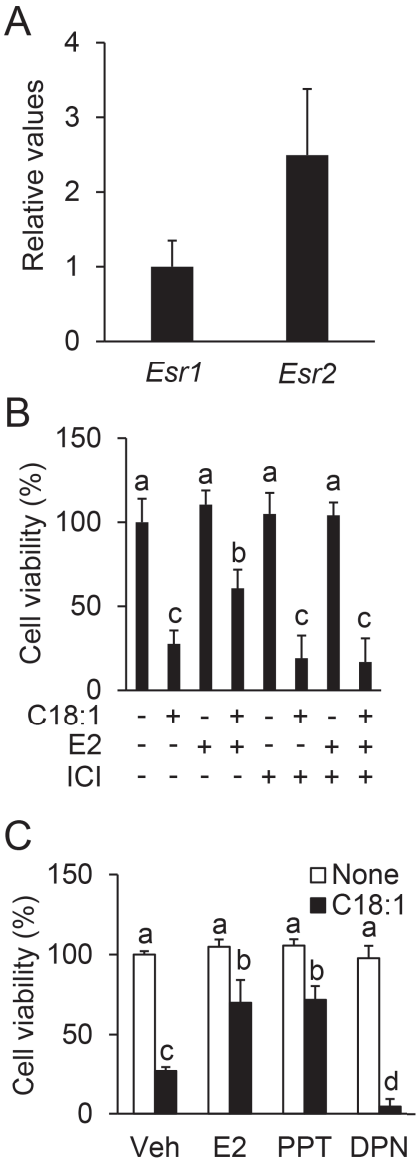

Fig. 2. The role of estrogen receptor isoforms on the inhibitory effects of estrogen on oleic acid-induced cytotoxicity. (A) In Neuro-2a neural cells, qRT-PCR was performed for *Esr1* and *Esr2* mRNA. Data were normalized to *Actb* mRNA as the internal control. (B) Neuro-2a neural cells were treated with 500 μM oleic acid in the presence or absence of E2 and/or ICI 182,780 (ICI) for 48 hr. (C) Neuro-2a neural cells were treated with 500 μM oleic acid in the presence or absence of E2, ERα selective agonist (PPT), or ERβ selective agonist (DPN) for 48 hr. Cytotoxicity was evaluated by LDH cytotoxicity assay. Different letters indicate statistically significantly different means ($p < 0.05$) in Tukey Kramer test. All experiments were performed in quadruplicate.

The role of estrogen receptor isoforms on the inhibitory effects of estrogen on oleic acid-induced cytotoxicity

Neuro-2a expressed the both of ERα and ERβ (Fig. 2A). To determine whether ERs are involved in fatty acid-induced cytotoxicity, Neuro-2a cells were treated with 500 μM oleic acid in the presence or absence of E2 and/or ER pure antagonist ICI 182,780. ICI 182,780 prevented the inhibitory effect of E2 on the oleic acid-induced cytotoxicity (Fig. 2B).

Furthermore, to determine the role of ER isoforms in inhibitory effects of E2 on oleic acid-induced cytotoxicity, Neuro-2a neural cells were incubated with oleic acid in the presence or absence of ER isoform selective agonists. ERα-selective agonist PPT prevented the cytotoxicity in Neuro-2a neural cells. Conversely, the ERβ-selective agonist DPN significantly enhanced the cytotoxicity in the presence of oleic acid (Fig. 2C).

Effects of estrogen on cell death-related gene expression

We determined whether fatty acids induced the cytotoxicity in Neuro-2a neural cells through apoptosis or autophagy. Stearic acid significantly increased the mRNA levels of *Bax* in a dose-dependent manner, and oleic acid statistically significantly, but only slightly ($p = 0.04$), increased the mRNA levels of *Bax* compared to the control (Fig. 3A). Oleic acid, but not stearic acid, increased the mRNA level of *p62/Sqstm1* at the concentrations of 50 μM and 500 μM (Fig. 3B). E2 treatment statistically significantly, but slightly, enhanced the stearic acid-induced *Bax* expression (Fig. 3C). In contrast, E2 inhibited the oleic acid-induced the *p62/Sqstm1* expression (Fig. 3D).

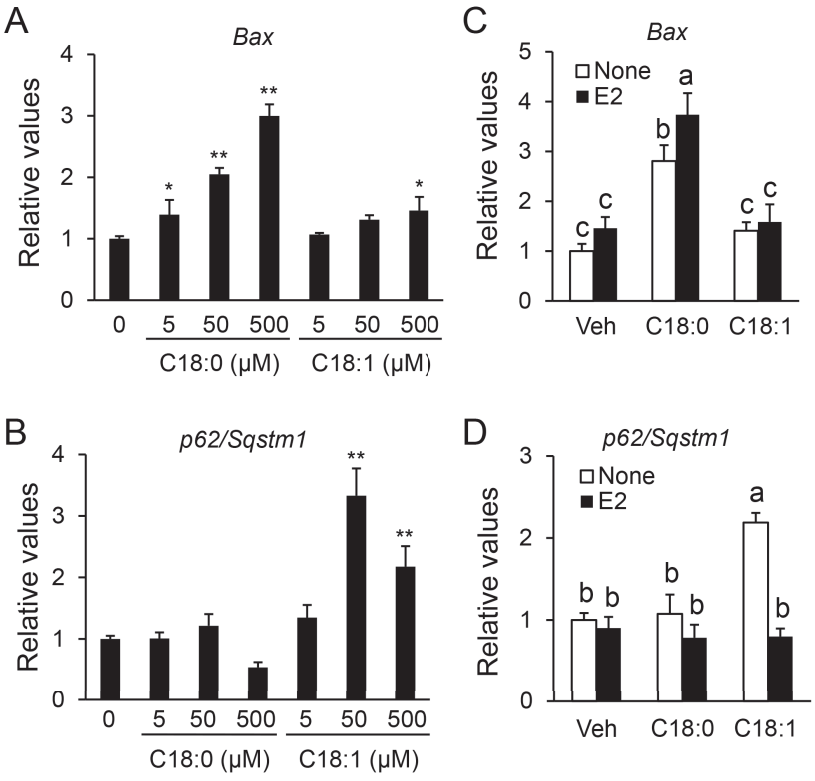

Fig. 3. Effects of estrogen on the levels of cell death-related genes in Neuro-2a neural cells treated fatty acids. Neuro-2a neural cells were treated with various concentrations (5 μM to 500 μM) of stearic acid or oleic acid for 48 hr. qRT-PCR was performed for *Bax* mRNA (A) and *p62*/*Sqstm1* mRNA (B). Neuro-2a neural cells were treated with 500 μM stearic acid or oleic acid in the presence or absence of E2. qRT-PCR was performed for *Bax* mRNA (C) and *p62*/*Sqstm1* mRNA (D). Data were normalized to *Actb* mRNA as the internal control. Values are indicated as mean ± S.D. *Left panel*; statistically significant differences compared with the control are indicated by *, $p < 0.05$ and **, $p < 0.01$ in Dunnett's test. *Right Panel*; different letters indicate statistically significantly different means ($p < 0.05$) in Tukey Kramer test. All experiments were performed in triplicate.

Effects of estrogen receptor isoforms on cell death-related gene expression

To determine whether ER is involved in the mechanisms by which E2 inhibits the oleic-acid-induced *p62/Sqstm1* expression, Neuro-2a neural cells were exposed to oleic acid and/or E2 in the presence of ICI 181,780. ICI 181,780 partially cancelled the inhibitory effect of E2 on the oleic acid-induced *p62/Sqstm1* expression (Fig. 4B). PPT inhibited the oleic acid-induced *p62/Sqstm1* expression similar to E2. In contrast, DPN enhanced the oleic acid-induced *p62/Sqstm1* expression (Fig. 4C).

DISCUSSION

E2 and PPT, but not DPN, prevented oleic acid-induced cytotoxicity in Neuro-2a neural cells. Fatty acids induce oxidative stress and inflammatory responses that cause cell death (Ryter *et al.*, 2007; Yang *et al.*, 2015). Estrogen mediates neuroprotection and anti-inflammatory effects through ERα signaling on astrocytes and neurons (Spence *et al.*, 2013). Estrogen also attenuates ischemic oxidative damage *via* ERα-mediated inhibition of NADPH oxidase activation (Zhang *et al.*, 2009). Taken together, estrogen has remarkable anti-inflammatory and antioxidant properties. Previous study indicated that cerebral ischemia was accompanied by an inflammatory reaction that contributes to the tissue damage, an effect mediated in part by toxic amounts of nitric oxide (NO) produced by the inducible isoform of NO synthase (iNOS). Other studies showed that the decrease of iNOS expression by estrogen is one of the factors mediating the resistance to cerebral ischemia in females (Park *et al.*, 2006). In female rats, a high fat diet led to increased iNOS expression, and decreased levels of E2 and ERα protein (Jovanovic *et al.*, 2017). Therefore indicating that E2 prevents the fatty acids-induced cell damage *via* ERα in Neuro-2a neural cells.

E2 inhibited oleic acid-induced cytotoxicity and tended to enhance stearic acid-induced cytotoxicity at high concentrations. Oleic acid induced autophagy but only had a minimal effect on apoptosis (Mei *et al.*, 2011). In contrast, palmitic acid suppressed autophagy, and significantly induced apoptosis in hepatocytes (Mei *et al.*, 2011). Indeed, saturated fatty acids and unsaturated fatty acids have differential effects on cell death in neuron cells. Therefore, these results suggest that E2 is likely to play different roles between saturated fatty acids and unsaturated fatty acids.

We demonstrated that stearic acid, but not oleic acid, decreased cell viability during the 24 hr incubation (unpublished data), but oleic acid had obvious cytotoxicity in Neuro-2a neural cells at 48 hr after treatment. Previous study has shown that saturated fatty acids induce apoptotic cell death in rat PC12 neural cells differentiated by nerve growth factor, but the exposure of unsaturated fatty acids for 24 hr did not have effects on cell viability (Ulloth *et al.*, 2003). Other study also showed that oleic acid did not induce the cytotoxicity in hepatocyte until 20 hr (Malhi *et al.*, 2007). In contrast, little has been

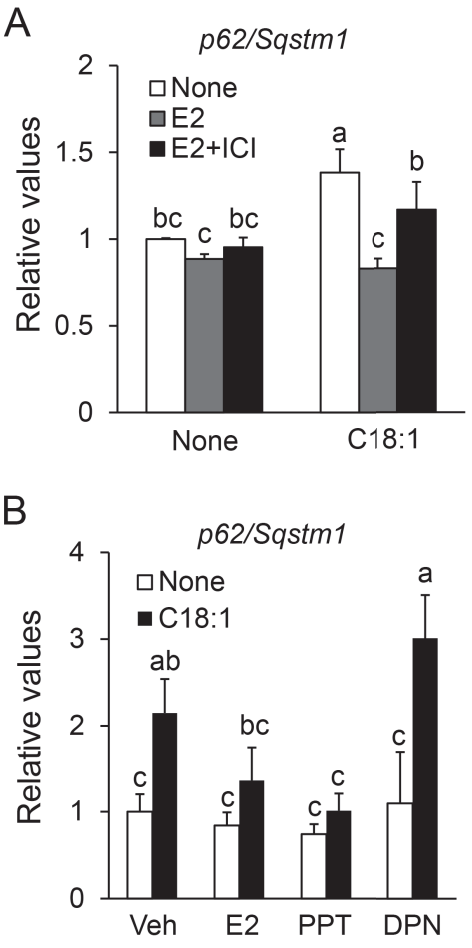

Fig. 4. Effects of agonists of estrogen receptor isoforms on the levels of cell death-related genes in Neuro-2a treated neural cells oleic acid. (A) Neuro-2a neural cells were treated with 500 μM oleic acid in the presence or absence of E2 and/or ICI 182,780 (ICI) for 24 hr. (B) Neuro-2a neural cells were treated with 500 μM oleic acid in the presence or absence of E2, ERα selective agonist (PPT), or ERβ selective agonist (DPN) for 24 hr. qRT-PCR was performed for *p62*/*Sqstm1* mRNA. Data were normalized to *Actb* mRNA as the internal control. Different letters indicate statistically significantly different means ($p < 0.05$) in Tukey Kramer test. All experiments were performed in triplicate.

reported on the cytotoxicity of oleic acid during the exposure of 48 hr. The cytotoxic effects of oleic acid may be later than that of stearic acid.

E2 and PPT inhibited oleic acid-induced increase of *p62/Sqstm1* mRNA. *p62/Sqstm1* gene is a well-known autophagy marker (Gómez-Sánchez *et al.*, 2016). Recent study showed that estrogen inhibits autophagy in endometrial cancer (Zhou *et al.*, 2019). Neuroprotective effects of E2 in peripheral neurons are partly related to the suppression of excessive autophagy (Lin *et al.*, 2016). Although the molecular mechanism remains to be elucidated, these results suggest that E2 has inhibitory effects of oleic acid-enhanced autophagy *via* ERα.

DPN slightly enhanced the oleic acid-induced cytotoxicity and induced the increase of *p62*/*Sqstm1* mRNA in Neuro-2a neural cells. ERα and ERβ are known to undertake different effects in various tissues (Hayashi *et al.*, 2003; Ogawa *et al.*, 2011). Recent study indicated that ERβ promotes autophagy in neural cells (Wei *et al.*, 2019). Therefore, ERβ may have the opposite effects of ERα in neural cells.

In conclusion, our results suggest that fatty acids induce cytotoxicity in Neuro-2a neural cells, and ERα prevents the oleic acids-induced cytotoxicity. Further studies are needed to understand the role of ERβ in neuron injury under normal conditions.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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