Trans Fatty Acids Enhanced beta-Amyloid Induced Oxidative Stress in Nerve Growth Factor Differentiated PC12 Cells

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running title: trans fatty acids impair PC12 cells

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Abstract

The effects of trans fatty acids, elaidic acid (trans-9, C18:1) and linoelaidic acid (trans-9, trans-12 C18:2), at 20 or 40 μM in nerve growth factor differentiated PC12 cells with or without beta-amyloid peptide (Aβ) were examined. Elaidic acid treatment alone did not affect cell viability and oxidative injury associated markers (P>0.05). However, co-treatments of elaidic acid and Aβ led to more reduction in mitochondrial membrane potential (MMP) and Na^+-K^+-ATPase activity, and more increase in DNA fragmentation and 8-hydroxydeoxyguanosine (8-OHdG) production than Aβ treatment alone (P<0.05). Linoelaidic acid alone exhibited apoptotic and oxidative effects in cells via decreasing MMP and Na^+-K^+-ATPase activity, increasing reactive oxygen species (ROS) level, lowering glutathione content and glutathione peroxidase (GPX) activity (P<0.05). The co-treatments of linoelaidic acid with Aβ further enhanced oxidative damage via enhancing the generation of ROS, nitrite oxide and 8-OHdG, elevating caspase-3, caspase-8 and nitric oxide synthase activities, as well as declining GPX, catalase and superoxide dismutase activities (P<0.05). These results suggested that the interaction of linoelaidic acid and Aβ promoted oxidative stress and impaired mitochondrial functions in neuronal cells.

Keywords: Trans fatty acid; Amyloid; PC12 cells; Neurodegeneration; Mitochondrial dysfunction

Introduction

Alzheimer's disease (AD), a progressive neurodegenerative disorder, is the most common form of dementia in many countries. The 'amyloid cascade hypothesis' considers the beta-amyloid peptide (Aβ) a central role in the pathogenesis of AD [1, 2]. It has been documented that Aβ deposition accelerates the generation of oxidized glutathione (GSSG), reactive oxygen species (ROS) and nitrite, which in turn enhances oxidative stress and promotes neuronal cells apoptosis [3, 4]. Thus, any agent with the ability to favor Aβ induced oxidative damage in neuronal cells should be limited in order to avoid AD progression.

Dietary trans fat has been considered as a contributor for the development of chronic diseases such as coronal heart disease and diabetes because the intake of trans fatty acids (TFAs) is associated with enhanced oxidative stress in human [5, 6]. An epidemiological study indicated that intake of *trans*-unsaturated (hydrogenated) fats increased the risk of AD [7]. However, the mechanism remains unknown. Teixeira et al. [8] reported that a long-term intake of trans fat led to a significant brain incorporation of TFAs in rats, and highly linked to the development of movement disorders and memory acquisition. Phivilay et al. [9] also reported that dietary TFAs changed the brain fatty acid profile, but did not alter Aβ pathology in an AD animal model. Although those previous studies suggested that dietary TFAs could modify brain fatty acid composition and even affect brain functions, brain oxidative stress was not evaluated. Thus, the link between TFAs and brain health is still unclear. Further studies regarding the effects of TFAs and/or their interactions with Aβ on redox balance in neuronal cells could be helpful to understand the impact of TFA upon AD pathology. That is, it is hypothesized that TFAs elevate oxidative stress in neuronal cells. Both elaidic acid (trans-9, C18:1) and linoelaidic acid (trans-9, trans-12, C18:2) are trans 18 carbon fatty acids, and could be found in partially

hydrogenated vegetable oils. The studies of Zapolska-Downa et al. [10] and Bryk et al. [11] revealed that linoelaidic acid caused greater ROS production than elaidic acid in human endothelial cells. Thus, it is worthy to examine the effects of these two TFAs on oxidative injury and anti-oxidative defense in neuronal cells.

Nerve growth factor (NGF) differentiated PC12 cell line has been widely used as a sympathetic neurons model for investigating the survival of neuronal cells or antioxidant protection [12, 13]. In our present study, the effects of elaidic acid and linoelaidic acid upon the stability of NGF differentiated PC12 cells were evaluated. Furthermore, the interaction of these two TFAs and Aβ on oxidative stress in NGF differentiated PC12 cells was also examined.

Materials and Methods

Chemicals

Oleic acid (cis-9 C18:1, c18:1), elaidic acid (t18:1), linoelaidic acid (t,t18:2) and $A\beta_{1-42}$ were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Medium, plates, antibiotics and chemicals used for cell culture were purchased from Difco Laboratory (Detroit, MI, USA). NGF was purchased from Promega Co. (Madison, WI, USA). All chemicals used in these measurements were of the highest purity commercially available.

Cell Culture

PC12 cells were cultured in 35 mm dish containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated calf serum and 5% fetal bovine serum under 95% air/ 5% CO_2 at 37 °C. PC12 cells were treated with NGF (50 ng/ml) and allowed to differentiate for 5 days. The culture medium was changed every three days and cells were subcultured once a week. The medium was changed to serum-deprived medium and cells were washed with serum-free DMEM 24 h before experiments and

replanted in the 96 well plates.

Experimental Design

c18:1, t18:1 or t,t18:2 was dissolved in dimethyl sulfoxide (DMSO) and diluted with the medium. The final concentration of DMSO in the medium was 0.5%. At this concentration, DMSO did not affect any measurements (data not shown). Aβ was diluted in 10 mM sodium phosphate buffer (PBS, pH 7.2) to a final concentration for experiments. PC12 cells (10^5 cells/ml) were treated with c18:1, t18:1 or t,t18:2 at 20 or 40 μM for 36 h at 37 °C, which led to 98% incorporation of test fatty acid into cells. After the medium was discharged, these cells were followed by 24 h exposure of 10 μM Aβ at 37 °C. Control groups contained neither TFA nor Aβ.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay

MTT assay was performed to examine cell viability. Briefly, PC12 cells were incubated with 0.25 mg MTT/ml for 3 h at 37 °C. The amount of MTT formazan product was determined by measuring absorbance at 570 nm (630 nm as a reference) using a microplate reader (Bio-Rad, Hercules, CA, USA). Cell viability was expressed as a percent of control groups.

Lactate Dehydrogenase (LDH) Assay

The plasma membrane damage of PC12 cells was evaluated by measuring the amount of intracellular LDH in the medium. Fifty μl of culture supernatants were collected from each well. LDH activity (U/l) was determined by a colorimetric LDH assay kit (Sigma Chemical Co., St. Louis, MO, USA).

Measurement of Mitochondrial Membrane Potential (MMP)

MMP was monitored using a flow cytometry (Beckman-FC500, Beckman Coulter, Fullerton, CA, USA) and the fluorescent dye Rhodamine123 (Rh123) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). This assay is based on the depolarization of

MMP resulting in the loss of Rh123 from the mitochondria and a decrease in intracellular fluorescence. After incubation with Aβ and/or TFA, PC12 cells were centrifuged at 1200 xg for 5 min and resuspended in DMEM. Rh123 (100 μg/l) was added to PC12 cells (10^5 cells/ml) for 45 min at 37 °C. Cells were collected and washed twice with PBS. The mean fluorescence intensity (MFI) in PC12 cells was analyzed with flow cytometry.

Na^+-K^+-ATPase Activity Assay

Na^+-K^+-ATPase activity was determined by measuring the amount of inorganic phosphate (Pi) released from ATP [14]. The reaction mixture contained 100 mM NaCl, 20 mM KCl, 2 mM ATP, 30 mM Tris-HCl buffer (pH 7.4) and the freshly isolated cellular mitochondria. The assay was initiated by adding ATP, and terminated by adding 15% trichloroacetic acid after 15 min incubation at 37 °C. The released Pi was assayed by measuring the absorbance at 640 nm. A unit was defined as 1 μmol Pi released from ATP by 1 mg of protein during 1 h (μmol Pi/mg protein/h). The values of the treated cells were normalized against the values of control, and expressed as percentage of control.

Measurement of Caspase Activity

Activity of caspase-3 and -8 was detected by using fluorometric assay kits (Upstate, Lake Placid, NY, USA) according to the manufacturer's protocol. The intra-assay CV was 4.1-5.0 %, and the inter-assay CV was 4.8-6.7 %. In brief, control or treated cells were lysed in 50 ml of cold lysis buffer and incubated in ice for 10 min. Fifty μl cell lysates was mixed with 50 ml of reaction buffer and 5 ml of fluorogenic substrates specific for caspase-3 or -8 in a 96-well microplate. After incubation at 37 °C for 1 h, fluorescent activity was measured using a fluorophotometer with excitation at 400 nm and emission at 505 nm. Data were expressed as a percentage of control.

Determination of Malonyldialdehyde (MDA) and Reactive Oxygen Species (ROS)

Cells were washed and homogenized. The lysate was then centrifuged at 1000 xg for

10 min at 4 °C, and 200 μl supernatant was used to measure MDA level using a commercial assay kit (OxisResearch, Portland, OR, USA). The dye $DCFH_2$-DA was used to measure level of cellular ROS according to the method of Fu et al. [15]. PC12 cells were washed and suspended in RPMI 1640 medium. After incubating with 50 μmol/l dye for 30 min and washing with PBS, the cell suspensions were centrifuged at 412 xg for 10 min. Then, the medium were removed and cells were dissolved with 1% Triton X-100. Fluorescence changes were measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm using a fluorescence microplate reader.

Analyses for Glutathione (GSH), Oxidized Glutathione (GSSG) and Activity of Glutathione Peroxidase (GPX), Catalase and Superoxide Dismutase (SOD)

Cells were washed twice with PBS, then were scraped from the plates and followed by homogenizing in 20 mM PBS containing 0.5 mM butylated hydroxytoluene to prevent sample further oxidation. The homogenate was centrifuged at 3000 xg for 20 min at 4 °C, and the supernatant was used for these assays according to the manufacturer's instructions. GSH and GSSG concentrations (ng/mg protein) were determined by commercial colorimetric GSH and GSSG assay kits (OxisResearch, Portland, OR, USA). The activity (U/mg protein) of GPX, catalase and SOD in PC12 cells was determined by using assay kits (EMD Biosciences, San Diego, CA, USA). The amount of protein in cell extract was determined by the method of Lowry et al. [16].

Measurement of DNA Fragmentation

Cell death detection ELISA plus kit (Roche Molecular Biochemicals, Mannheim, Germany) was used to quantify DNA fragmentation. After incubation with Aβ and/or TFA, PC12 cells were lysed for 30 min at room temperature and followed by centrifugation at 200 xg for 10 min. Then, 20 μl supernatant was transferred onto the streptavidin-coated plate, and 80 μl freshly prepared immunoreagent was added to each well and incubated for 2 h at

room temperature. After washing with PBS, substrate solution was added and incubated for 15 min. The absorbance at 405 nm (reference wavelength 490 nm) was measured using a microplate reader. DNA fragmentation was expressed as the enrichment factor using the following equation:

enrichment factor = (absorbance of the sample) / (absorbance of the control)

DNA Oxidation Assay

8-hydroxydeoxyguanosine (8-OHdG) is an indicator of DNA oxidative stress. DNA fractions were obtained using a DNA Extractor WB kit (Wako Pure Chemical Industries Ltd., Tokyo, Japan). Extracted DNA was dissolved in water (1 mg/ml), and oxidative damage was determined using an ELISA kit for 8-OHdG (OXIS Health Products Inc, Portland, OR, USA).

Nitrite Assay and Nitric Oxide Synthase (NOS) Activity

The production of nitric oxide was determined by measuring the formation of nitrite. Briefly, 100 μl of supernatant was treated with nitrate reductase, NADPH and FAD, and followed by incubating for 1 h at 37 °C in the dark. After centrifuging at 6,000 xg, the supernatant was mixed with Griess reagent for color development. The absorbance at 540 nm was measured and compared with a sodium nitrite standard curve. The method described in Sutherland et al. [17] was used to measure total NOS activity by incubating 30 μl of homogenate with 10 mM NADP, 10 mM L-valine, 3000 U/ml calmodulin, 5 mM tetrahydrobiopterin, 10 mM $CaCl_2$, and a mixture of 100 μM L-arginine containing L-[^{3}H]arginine.

Statistical Analysis

The effect of each treatment was analyzed from eight different preparations (n=8). Data were reported as means ± standard deviation (SD), and subjected to analysis of variance. Differences among means were determined by the Least Significance Difference Test with

significance defined at $P<0.05$.

Results

Effects of TFAs on Aβ induced cytotoxicity in PC12 cells.

As shown in Figure 1, without Aβ treatment, the addition of c18:1 and t18:1 did not affect cell viability (1A) and membrane integration (1B) ($P>0.05$), but t,t18:2 alone reduced 10-20% cell viability and increased 20-30% plasma damage ($P<0.05$). Aβ treatment alone also significantly decreased cell survival ($P<0.05$). Furthermore, the co-treatments of Aβ plus t18:1 and Aβ plus t,t18:2 caused greater cell death and plasma damage when compared with Aβ treatment alone ($P<0.05$). t,t18:2 treatment alone lowered 20-30% MMP and Na^+-K^+-ATPase activity (Table 1, $P<0.05$). Aβ treatment alone decreased 50-60% MMP and Na^+-K^+-ATPase activity ($P<0.05$). The co-treatments of t18:1 or t,t18:2 plus Aβ led to greater reduction in both MMP and Na^+-K^+-ATPase activity than Aβ treatment alone ($P<0.05$). As shown in Figure 2, TFAs alone did not affect the activity of caspase-3 and caspase-8 ($P>0.05$); however, Aβ treatment significantly elevated the activity of these two enzymes ($P<0.05$). Co-treatments of t,t18:2 plus Aβ further raised both caspase-3 and caspase-8 activities than Aβ treatment alone ($P<0.05$).

Effects of TFAs on Aβ induced oxidative stress in PC12 cells.

The effects of fatty acid and/or Aβ upon levels of MDA, ROS, GSSG and GSH in NGF-differentiated PC12 cells are shown in Table 2. Without Aβ treatment, t,t18:2 significantly increased MDA, ROS and GSSG levels, and decreased GSH level ($P<0.05$). The co-treatments of Aβ with t18:1 at high dose or Aβ with t,t18:2 led to greater ROS and less GSH levels than Aβ treatment alone ($P<0.05$). The effects of fatty acid and/or Aβ upon activity of GPX, catalase and SOD are shown in Table 3. t,t18:2 treatment alone

significantly lowered GPX activity (P<0.05). Aβ plus t18:1 at high dose significantly decreased GPX and SOD activities than Aβ alone (P<0.05). Co-treatments of Aβ with t,t18:2 led to greater reduction in the activity of three test enzymes than Aβ treatment alone (P<0.05). As shown in Table 4, without Aβ treatment, TFA did not affect DNA fragmentation, determined as enrichment factor, and 8-OHdG level (P>0.05). Aβ alone significantly increased DNA fragmentation and 8-OHdG generation (P<0.05). The treatments of t18:1 at high dose or t,t18:2 plus Aβ led to greater elevation in both DNA fragmentation and 8-OHdG formation than Aβ treatment alone (P<0.05).

Effects of TFAs and Aβ on NO production and NOS activity in PC12 cells.

As shown in Figure 3, TFAs did not affect NO production (3A) and NOS activity (3B) (P>0.05). The treatments of Aβ plus t18:1 at high dose or t,t18:2 significantly enhanced NOS activity and NO production when compared with Aβ treatment alone (P<0.05).

Discussion

In our present study, elaidic acid (t18:1) alone did not cause apoptotic or pro-oxidant effects toward NGF-treated PC12 cells, but linoelaidic acid (t,t18:2) alone in NGF-treated PC12 cells markedly increased the production of ROS and GSSG, and decreased MMP and Na^+-K^+-ATPase activity, which consequently reduced cell viability. It is reported that linoelaidic acid caused more oxidative injury than elaidic acid in human endothelial cell lines [10, 11]. The results of our present study extended the pro-oxidant ability of linoelaidic acid to NGF-treated PC12 cells. On the other hand, the co-treatments of elaidic acid only at high dose with Aβ led to substantial apoptosis and oxidative damage in NGF-treated PC12 cells. However, the co-treatments of linoelaidic acid with Aβ caused more severe oxidative and apoptotic damage based on the increase in several biomarkers such as ROS, MDA, DNA fragmentation and caspases activity. These findings indicated

that these two TFAs were able to react with Aβ, which in turn enhanced oxidative stress and impaired neuronal cells. Furthermore, our results implied that linoelaidic acid had greater reactivity with Aβ. The study of Butterfield et al. [18] indicated that methionine at residue 35 of Aβ sequence was required for brain oxidative damage in mice with AD. Thus, it is possible that these two TFAs reacted with Met35 of Aβ residue, which exacerbated oxidative and apoptotic effects as we observed. Further study focused on the interaction of TFAs and Met35 of Aβ would be helpful to elucidate the relationship between these two molecules.

Na^+-K^+-ATPase is a transmembrane protein responsible for exchanging intracellular Na^+ for extracellular K^+. It has been indicated that the decreased Na^+-K^+-ATPase activity resulted from collapse of mitochondrial membrane potential could in turn cause apoptotic insult, cell damage and/or cell death [19]. On the other hand, it is reported that activation of caspase-3 and caspase-8 could act as an apoptotic executor for cell death in PC12 cells [20] because these caspases are directly responsible to the change in cell morphological events and to the cleavage of nuclear proteins in PC12 cells [21]. In our present study, linoelaidic acid with or without Aβ reduced Na^+-K^+-ATPase activity and mitochondrial membrane potential, as well as elevated capase-3 and caspase-8 activities in NGF-treated PC12 cells. These results revealed that this TFA at test doses was able to cause mitochondrial dysfunctions, which partially explained the observed cell death. 8-OHdG is a marker of DNA oxidative damage. The level of 8-OHdG in the cerebrospinal fluid (CSF) of AD patients was increased and positively correlated with the AD duration [22]. We found linoelaidic acid treatment alone failed to affect 8-OHdG level, but the co-treatments of this TFA and Aβ effectively facilitated DNA fragmentation and 8-OHdG production. These findings once again supported that linoelaidic acid highly reacted with Aβ, which in turn enhanced Aβ-induced oxidative injury and impaired nuclear

components in neuronal cells.

Increased levels of nitrites and nitrates in CSF of AD patients have been reported [23]. Aβ could evoke NO synthesis in NGF-differentiated PC12 cells [24]. Nitric oxide, via up-regulating redox-sensitive transcription factors such as nuclear factor kappa B, was another important factor responsible for oxidative and inflammatory reactions in AD progression [25, 26]. In our present study, linoelaidic acid treatment alone failed to affect NO level and NOS activity, but the combination of linoelaidic acid and Aβ effectively elevated NOS activity and stimulated NO over-production in NGF-differentiated PC12 cells. These findings further indicated that this TFA could enhance Aβ-induced oxidative stress via raising RNS, and implied that it might promote inflammatory progression in neuronal cells.

The protective effect of oleic acid against mitochondrial oxidative stress in human ECV-304 cells has been reported [27]. However, this cis 18 carbon fatty acid in our present study did not exhibit anti-oxidative or pro-oxidant effect in NGF-treated PC12 cells with or without Aβ. Elaidic acid is the 9-trans isomer of oleic acid, and is the predominant TFA found in human food supply [28]. Linoelaidic acid could be found in hydrogenated vegetable oil and frying oil [29]. So far, the association of elaidic acid with breast cancer and atherosclerosis has been reported [30, 31]. Our data suggested that the interaction of Aβ with elaidic acid or linoelaidic acid, not oleic acid, accelerated oxidative and apoptotic stress in NGF-treated PC12 cells. These findings implied that the trans double bond of these TFAs play a crucial role in determining their actions and functions. Furthermore, the adverse effects of these two TFAs upon AD progression could not be ignored. Since dietary TFAs could modulate brain fatty acid profile [7, 9], the intake of dietary TFAs, especially linoelaidic acid, should be limited for people with high risk of AD in order to avoid the enhanced oxidative injury from the interaction of TFAs and Aβ. In

addition, more studies are necessary to investigate the content of linoelaidic acid in food, and examine the role of this TFA in the progression of neurodegenerative diseases.

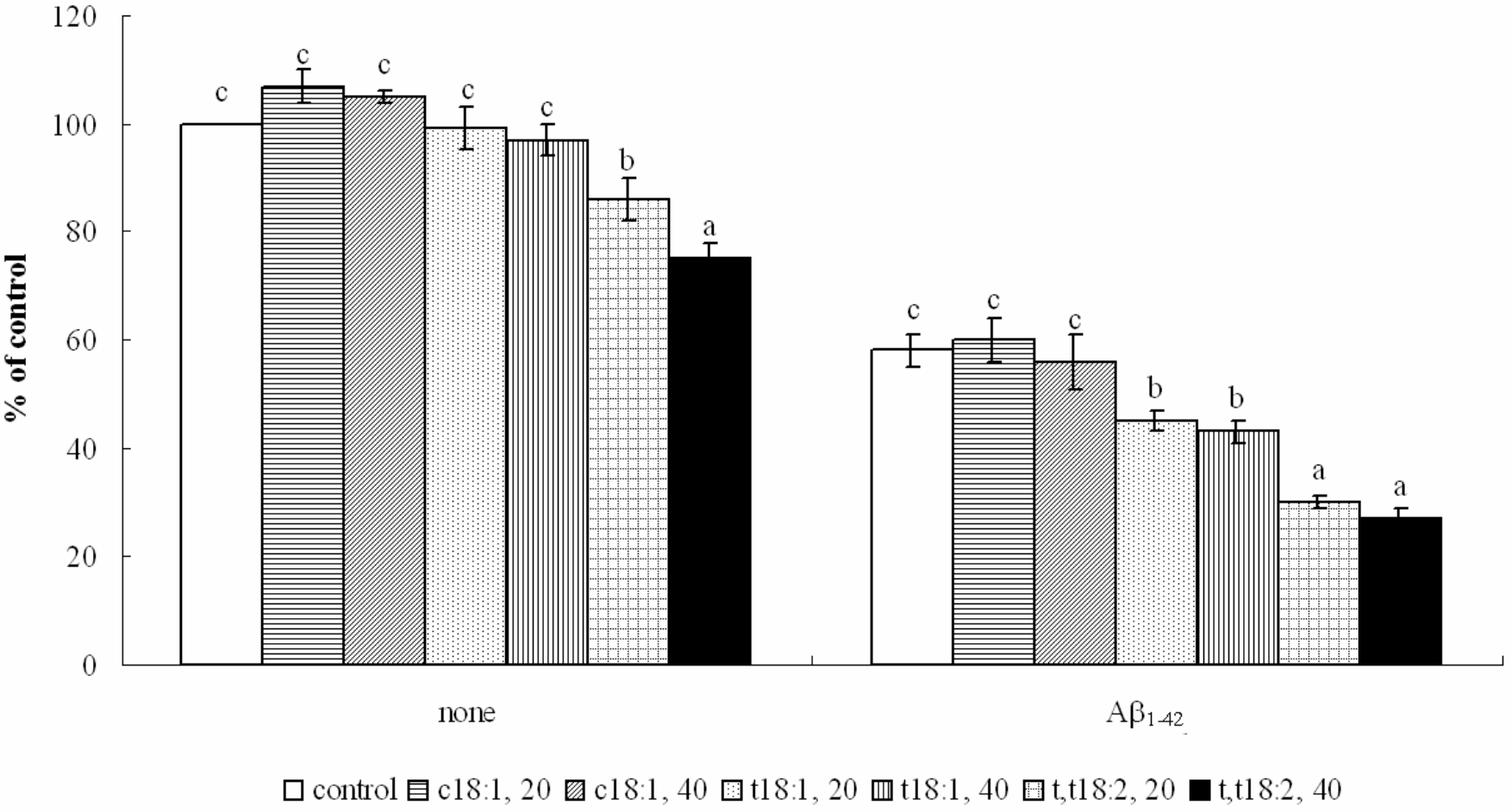
In conclusion, our present study provided evidence to link trans fatty acids and AD, and found the treatment of linoelaidic acid alone induced apoptotic and oxidative damage in NGF-differentiated PC12 cells. The co-treatments of Aβ with linoelaidic acid caused more severe oxidative and apoptotic stress via increasing production of ROS, NO and 8-OHdG, raising activity of caspase-3, caspase-8 and NOS, declining mitochondrial membrane potential, lowering the activity of GPX and Na^+-K^+-ATPase. The results implied that linoelaidic acid was a neuro-toxic agent.

Conflict of interest statement

None

Fig. 1 Effect of c18:1, t18:1 or t,t18:2 with or without Aβ upon cell viability determined by MTT assay (A) and plasma membrane damage determined by LDH assay (B). NGF differentiated-PC12 cells were pre-treated with c18:1, t18:1 or t,t18:2 at 20 or 40 μM and followed by adding 10 μM $A\beta_{1-42}$. Control contained no fatty acid. None means $A\beta_{1-42}$ was not added. Data are mean±SD (n=8). $^{a-d}$Means among bars without a common letter differ, $P<0.05$.

1A.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1B.

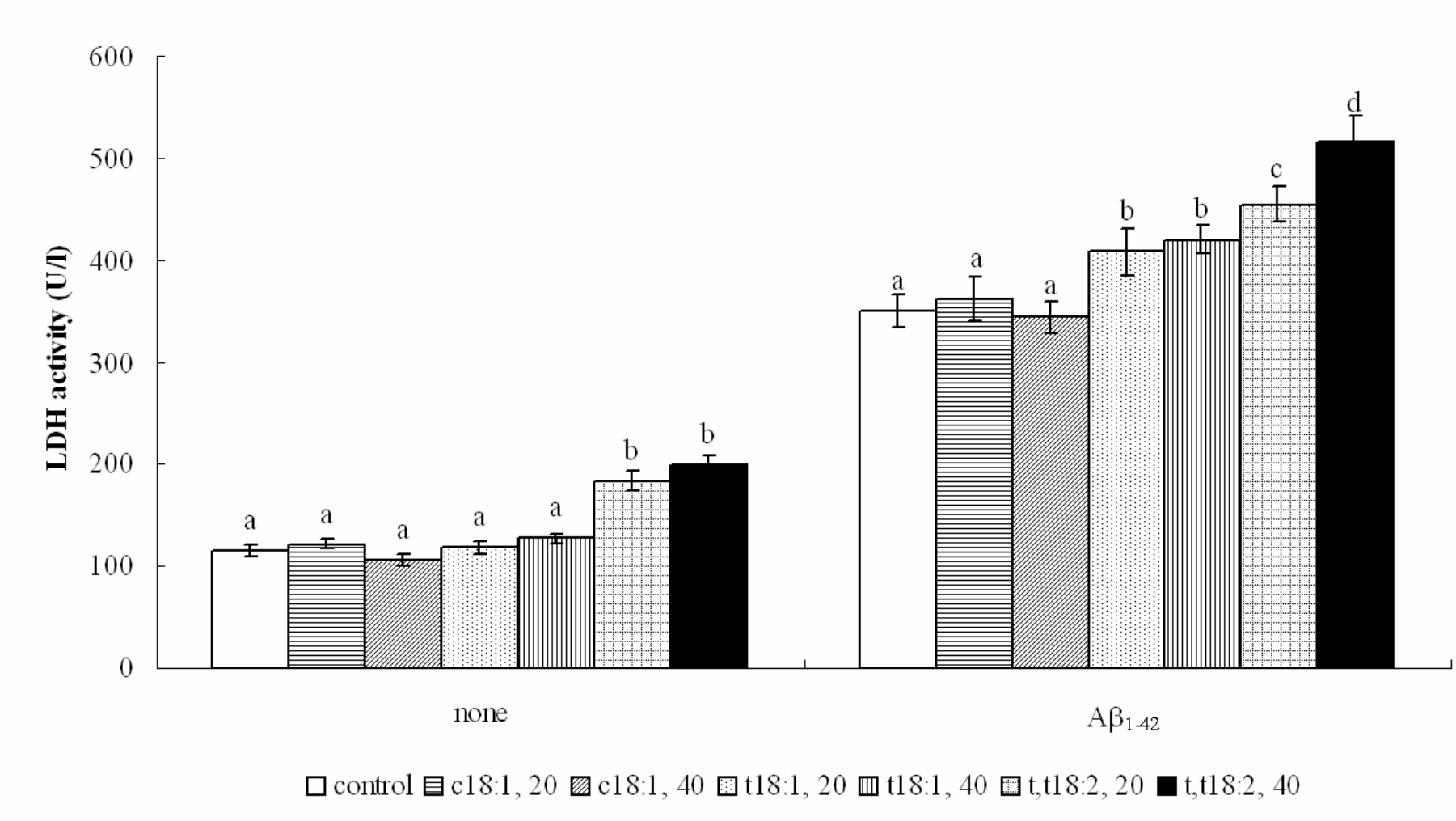

LDH activity (U/l)
600
500
400
300
200
100
0
a
a
a
a
a
b
b
a
a
a
b
b
c
d
none
Aβ1-42
control
c18:1, 20
c18:1, 40
t18:1, 20
t18:1, 40
t,t18:2, 20
t,t18:2, 40

Table 1 Effect of c18:1, t18:1 or t,t18:2 with or without Aβ upon MMP, determined as MFI, and Na^+-K^+-ATPase activity. NGF differentiated-PC12 cells were pre-treated with c18:1, t18:1 or t,t18:2 at 20 or 40 μM and followed by adding 10 μM $A\beta_{1-42}$. Data are mean±SD (n=8).

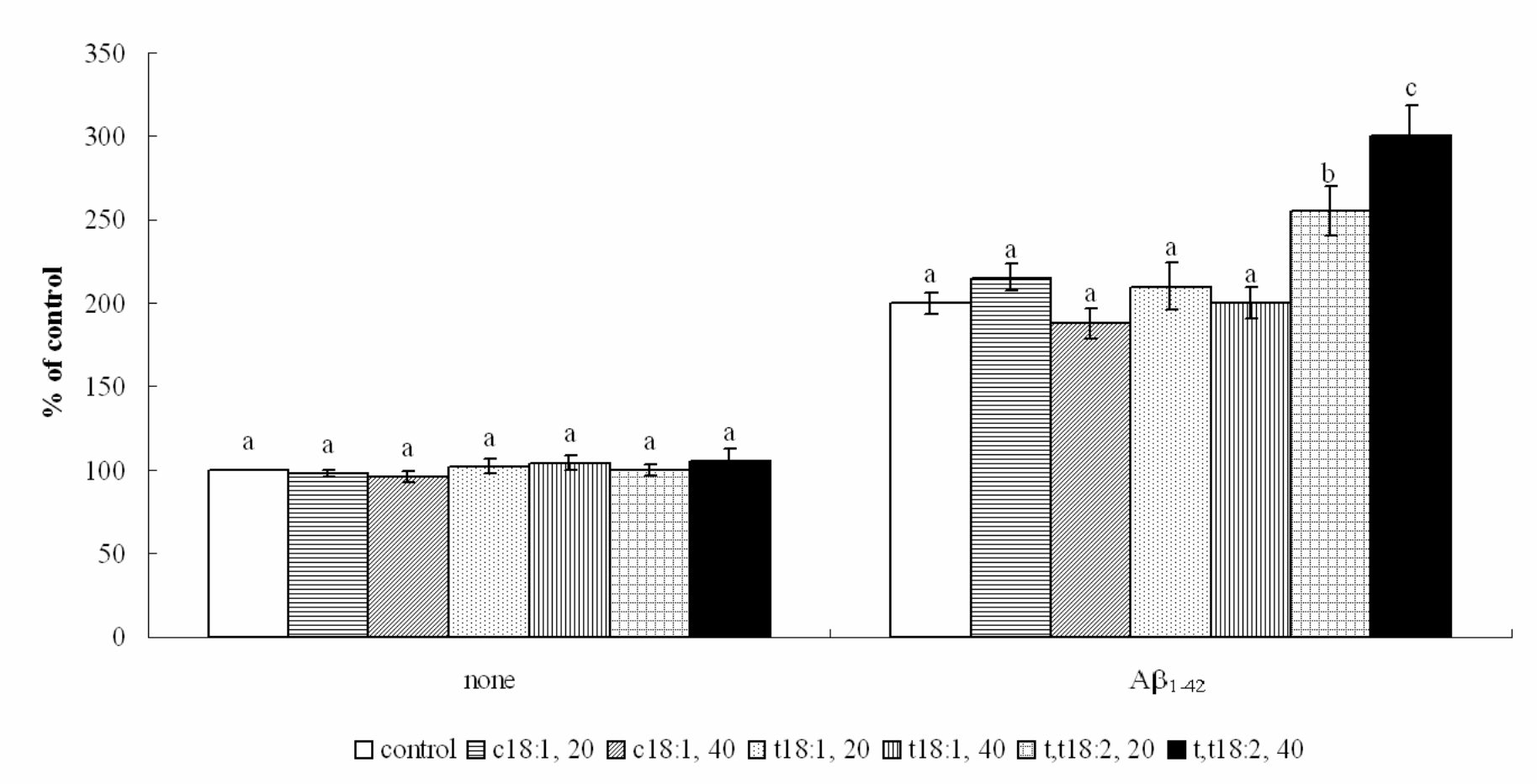
	MFI	Na^+-K^+-ATPase
Control[1]	100[f]	100[e]
c18:1, 20	102±3[f]	97±4[e]
c18:1, 40	96±2[f]	99±3[e]
t18:1, 20	104±5[f]	100±2[e]
t18:1, 40	99±3[f]	102±3[e]
t,t18:2, 20	80±2[e]	83±5[d]
t,t18:2, 40	69±4[d]	76±5[d]
$A\beta_{1-42}$	39±5[c]	53±2[c]
c18:1, 20+$A\beta_{1-42}$	41±2[c]	54±4[c]
c18:1, 40+$A\beta_{1-42}$	39±4[c]	51±3[c]
t18:1, 20+$A\beta_{1-42}$	34±1[b]	43±3[b]
t18:1, 40+$A\beta_{1-42}$	30±3[b]	40±4[b]
t,t18:2, 20+$A\beta_{1-42}$	22±2[a]	32±1[a]
t,t18:2, 40+$A\beta_{1-42}$	21±1[a]	28±3[a]

[1]Controls contained no fatty acid.

[a-f]Means in a column without a common letter differ, $P<0.05$.

Fig. 2 Effect of c18:1, t18:1 or t,t18:2 with or without Aβ upon activity of caspase-3 (A) and caspase-8 (B). NGF differentiated-PC12 cells were pre-treated with c18:1, t18:1 or t,t18:2 at 20 or 40 μM and followed by adding 10 μM $A\beta_{1-42}$. Control contained no fatty acid. None means $A\beta_{1-42}$ was not added. Data are mean±SD (n=8). $^{a-c}$Means among bars without a common letter differ, $P<0.05$.

2A.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a
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b
c
none
Aβ1-42
control
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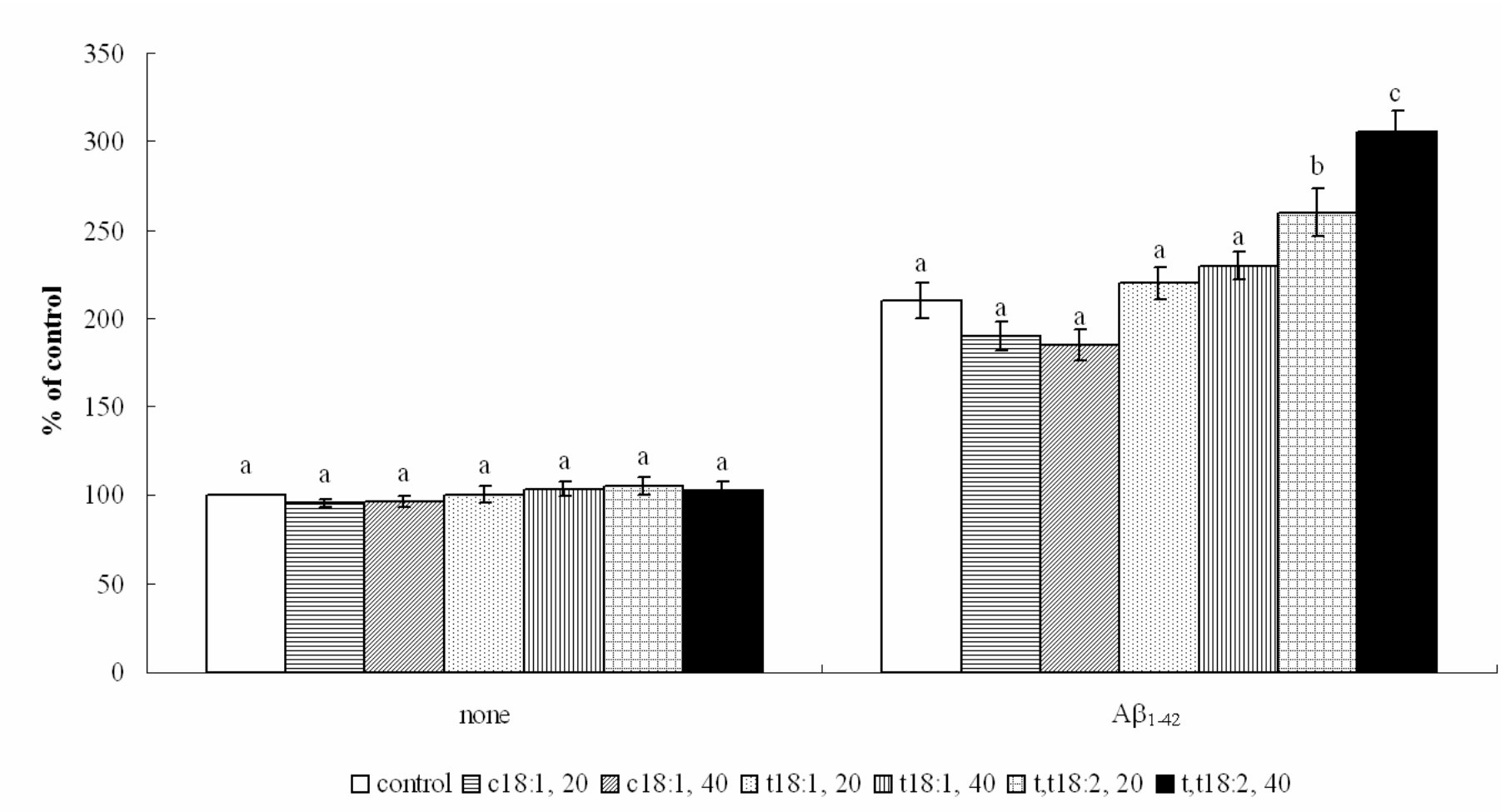
t,t18:2, 40

2B.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% of control
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b
c
none
Aβ1-42
control
c18:1, 20
c18:1, 40
t18:1, 20
t18:1, 40
t,t18:2, 20
t,t18:2, 40

Table 2 Effect of c18:1, t18:1 or t,t18:2 with or without Aβ upon level of MDA (μmol/mg protein), ROS (nmol/mg protein), GSH (ng/mg protein) and GSSG (ng/mg protein). NGF differentiated-PC12 cells were pre-treated with c18:1, t18:1 or t,t18:2 at 20 or 40 μM and followed by adding 10 μM $A\beta_{1-42}$. Data are mean±SD (n=8).

	MDA	ROS	GSH	GSSG
Control[1]	0.19±0.04[a]	0.23±0.03[a]	89±3[f]	1.3±0.2[a]
c18:1, 20	0.21±0.02[a]	0.20±0.05[a]	91±5[f]	1.4±0.1[a]
c18:1, 40	0.22±0.05[a]	0.22±0.04[a]	87±5[f]	1.2±0.3[a]
t18:1, 20	0.18±0.06[a]	0.25±0.04[a]	85±4[f]	1.3±0.3[a]
t18:1, 40	0.24±0.08[a]	0.29±0.03[a]	90±6[f]	1.2±0.4[a]
t,t18:2, 20	0.53±0.11[b]	0.65±0.08[b]	77±3[e]	1.7±0.6[b]
t,t18:2, 40	0.62±0.13[b]	0.93±0.12[c]	67±4[d]	1.9±0.7[b]
$A\beta_{1-42}$	1.16±0.15[c]	1.33±0.11[d]	52±5[c]	2.4±0.6[c]
c18:1, 20+$A\beta_{1-42}$	1.07±0.17[c]	1.24±0.21[d]	54±3[c]	2.5±0.8[c]
c18:1, 40+$A\beta_{1-42}$	1.12±0.20[c]	1.27±0.26[d]	56±5[c]	2.2±1.0[c]
t18:1, 20+$A\beta_{1-42}$	0.99±0.13[c]	1.46±0.20[d]	50±3[c]	2.6±1.2[c]
t18:1, 40+$A\beta_{1-42}$	1.14±0.19[c]	1.69±0.27[e]	43±5[b]	2.7±0.7[c]
t,t18:2, 20+$A\beta_{1-42}$	1.38±0.22[d]	1.85±0.19[e]	40±4[b]	3.3±0.9[d]
t,t18:2, 40+$A\beta_{1-42}$	1.60±0.25[e]	2.31±0.30[f]	29±6[a]	3.6±1.0[d]

[1]Controls contained no fatty acid.

[a-f]Means in a column without a common letter differ, $P<0.05$.

Table 3 Effect of c18:1, t18:1 or t,t18:2 with or without Aβ upon activity (U/mg protein) of GPX, catalase and SOD. NGF differentiated-PC12 cells were pre-treated with c18:1, t18:1 or t,t18:2 at 20 or 40 μM and followed by adding 10 μM $A\beta_{1-42}$. Data are mean±SD (n=8).

	GPX	Catalase	SOD
Control[1]	71.4±6.0[f]	2.5±0.4[c]	9.0±1.7[f]
c18:1, 20	72.2±5.1[f]	2.6±0.5[c]	9.1±1.2[f]
c18:1, 40	70.9±4.5[f]	2.5±0.3[c]	8.8±1.0[f]
t18:1, 20	71.3±3.8[f]	2.4±0.2[c]	9.2±0.8[f]
t18:1, 40	72.5±4.0[f]	2.3±0.3[c]	9.0±1.1[f]
t,t18:2, 20	66.3±2.7[e]	2.2±0.4[c]	8.7±0.9[f]
t,t18:2, 40	61.4±3.2[d]	2.1±0.3[c]	7.8±0.7[e]
$A\beta_{1-42}$	40.8±3.2[c]	1.0±0.1[b]	5.2±0.5[d]
c18:1, 20+$A\beta_{1-42}$	39.4±2.7[c]	1.1±0.2[b]	5.0±0.6[d]
c18:1, 40+$A\beta_{1-42}$	39.7±3.0[c]	1.2±0.2[b]	5.1±0.7[d]
t18:1, 20+$A\beta_{1-42}$	37.5±2.1[c]	1.0±0.3[b]	5.2±0.5[d]
t18:1, 40+$A\beta_{1-42}$	32.4±1.9[b]	1.1±0.1[b]	4.5±0.4[c]
t,t18:2, 20+$A\beta_{1-42}$	30.6±2.5[b]	0.6±0.1[a]	4.0±0.5[b]
t,t18:2, 40+$A\beta_{1-42}$	22.5±1.8[a]	0.5±0.2[a]	3.1±0.6[a]

[1]Controls contained no fatty acid.

[a-f]Means in a column without a common letter differ, $P<0.05$.

Table 4 Effect of c18:1, t18:1 or t,t18:2 with or without Aβ upon DNA fragmentation, determined as enrichment factor, and 8-OHdG level (ng/mg protein). NGF differentiated-PC12 cells were pre-treated with c18:1, t18:1 or t,t18:2 at 20 or 40 μM and followed by adding 10 μM $A\beta_{1-42}$. Data are mean±SD (n=8).

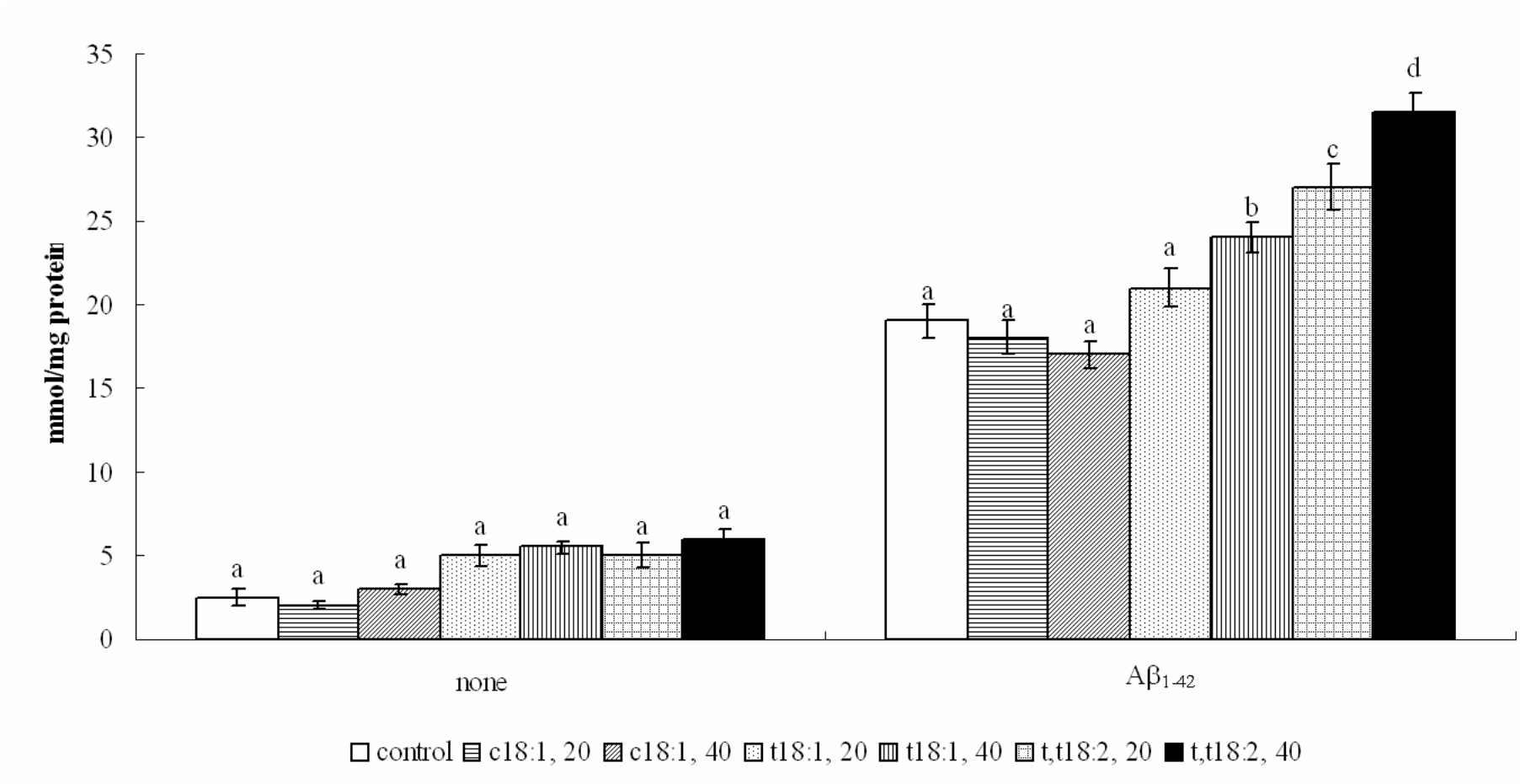
	enrichment factor	8-OHdG
Control[1]	1.0[a]	0.57±0.05[a]
c18:1, 20	0.95±0.08[a]	0.60±0.08[a]
c18:1, 40	1.05±0.09[a]	0.52±0.10[a]
t18:1, 20	0.92±0.07[a]	0.61±0.09[a]
t18:1, 40	1.03±0.10[a]	0.63±0.10[a]
t,t18:2, 20	1.11±0.12[a]	0.60±0.09[a]
t,t18:2, 40	1.04±0.11[a]	0.62±0.08[a]
$A\beta_{1-42}$	2.44±0.14[b]	1.93±0.21[b]
c18:1, 20+$A\beta_{1-42}$	2.31±0.16[b]	2.01±0.16[b]
c18:1, 40+$A\beta_{1-42}$	2.37±0.13[b]	1.96±0.19[b]
t18:1, 20+$A\beta_{1-42}$	2.42±0.18[b]	2.04±0.22[b]
t18:1, 40+$A\beta_{1-42}$	2.92±0.12[c]	2.65±0.18[c]
t,t18:2, 20+$A\beta_{1-42}$	3.33±0.17[d]	2.58±0.24[c]
t,t18:2, 40+$A\beta_{1-42}$	3.64±0.19[e]	3.02±0.26[d]

[1]Controls contained no fatty acid.

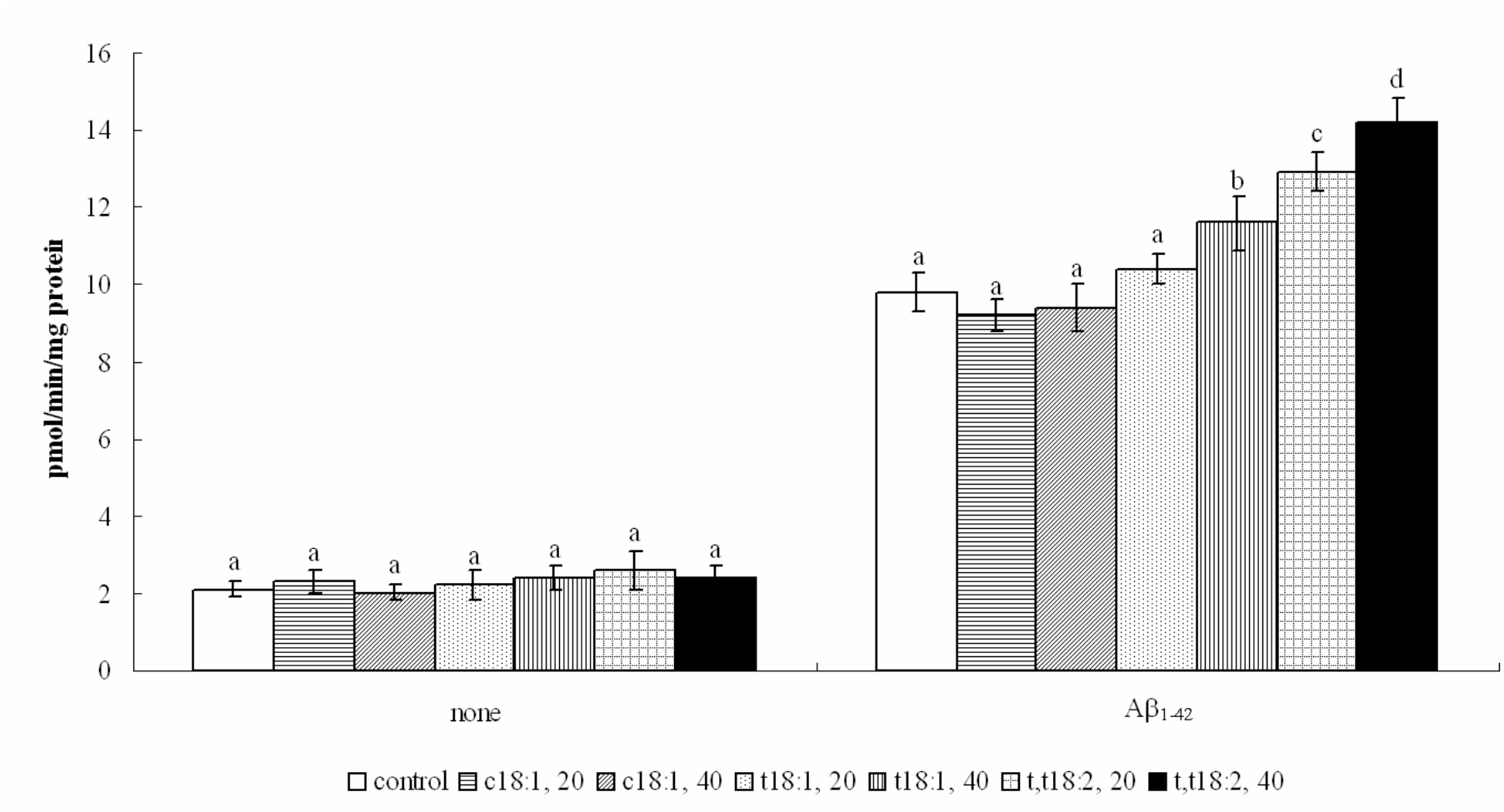
[a-e]Means in a column without a common letter differ, $P<0.05$.

Fig. 3 Effect of c18:1, t18:1 or t,t18:2 with or without Aβ upon nitric oxide level (A) and NOS activity (B). NGF differentiated-PC12 cells were pre-treated with c18:1, t18:1 or t,t18:2 at 20 or 40 μM and followed by adding 10 μM $A\beta_{1-42}$. Control contained no fatty acid. None means $A\beta_{1-42}$ was not added. Data are mean±SD (n=8). $^{a-d}$Means among bars without a common letter differ, $P<0.05$.

3A.


35
30
25
20
15
10
5
0
nmol/mg protein
a
a
a
a
a
a
a
a
a
a
a
b
c
d
none
Aβ1-42
control
c18:1, 20
c18:1, 40
t18:1, 20
t18:1, 40
t,t18:2, 20
t,t18:2, 40

3B.


pmol/min/mg protein
16
14
12
10
8
6
4
2
0
a
a
a
a
a
a
a
a
a
a
a
b
c
d
none
Aβ1-42
control
c18:1, 20
c18:1, 40
t18:1, 20
t18:1, 40
t,t18:2, 20
t,t18:2, 40

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