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Oleate prevents palmitate-induced mitochondrial dysfunction, insulin resistance and inflammatory signaling in neuronal cells

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ABSTRACT

Elevated circulating levels of saturated free fatty acids (sFFAs; e.g. palmitate) are known to provoke inflammatory responses and cause insulin resistance in peripheral tissue. By contrast, mono- or poly-unsaturated FFAs are protective against sFFAs. An excess of sFFAs in the brain circulation may also trigger neuroinflammation and insulin resistance, however the underlying signaling changes have not been clarified in neuronal cells. In the present study, we examined the effects of palmitate on mitochondrial function and viability as well as on intracellular insulin and nuclear factor-κB (NF-κB) signaling pathways in Neuro-2a and primary rat cortical neurons. We next tested whether oleate preconditioning has a protective effect against palmitate-induced toxicity. Palmitate induced both mitochondrial dysfunction and insulin resistance while promoting the phosphorylation of mitogen-activated protein kinases and nuclear translocation of NF-κB p65. Oleate pre-exposure and then removal was sufficient to completely block subsequent palmitate-induced intracellular signaling and metabolic derangements. Oleate also prevented ceramide-induced insulin resistance. Moreover, oleate stimulated ATP while decreasing mitochondrial superoxide productions. The latter were associated with increased levels of peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α). Inhibition of protein kinase A (PKA) attenuated the protective effect of oleate against palmitate, implicating PKA in the mechanism of oleate action. Oleate increased triglyceride and blocked palmitate-induced diacylglycerol accumulations. Oleate preconditioning was superior to docosahexaenoic acid (DHA) or linoleate in the protection of neuronal cells against palmitate- or ceramide-induced cytotoxicity. We conclude that oleate has beneficial properties against sFFA and ceramide models of insulin resistance-associated damage to neuronal cells.

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1. Introduction

A high concentration of saturated free fatty acids (sFFAs) in the circulation provokes inflammation in various tissues [1,2], including liver, muscle, adipocytes [3,4] and brain [5,6]. In vitro studies show that one sFFA (palmitate) activates the intracellular nuclear factor-κB (NF-κB) signaling pathway in myotubes [7–9], adipocytes [10] and endothelial cells [11]. Adipose tissue in particular is prone to sFFA mediated inflammation, triggering the release of cytokines tumor necrosis factor-α (TNF-α) and interleukin (IL)-1 and -6 [1,3,12]. These proinflammatory cytokines may cooperate with elevated sFFAs to sustain or aggravate inflammation in obese animals.

The nuclear factor-κB (NF-κB) family of transcription factors includes p65/RelA, RelB, c-Rel, p100/p52 and p105/p50 [13,14]. The p65 and p50 heterodimer is most ubiquitous and is well known to regulate gene expression in response to harmful cellular stimuli such as reactive oxygen species (ROS), inflammatory cytokines, lipopolysaccharides (LPS) and palmitate [12,13,15]. In its inactive state, NF-κB is complexed with inhibitory κBα (IκBα) proteins thereby masking the nuclear localization signals. With degradation of IκBα by IκB kinase β (IKKβ), NF-κB dissociates from the IκBα complex and translocates into the nucleus to induce expression of target genes [14,15]. For instance, translocation and promoter binding of NF-κB in myotubes exposed to palmitate result in the expression of TNF-α [8]. It is also known that in reverse, TNF-α can activate NF-κB [16]. Conversely, inhibition of NF-κB signaling reduced palmitate-induced cytotoxicity [17]. Thus, NF-κB signaling plays an important role in palmitate-induced intracellular inflammatory responses.

Abbreviations: AD, Alzheimer's disease; DAG, diacylglycerol; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; HSP, heat shock protein; IFN, interferon; IκBα, inhibitory κBα; IKKβ, IκB kinase β; IL, interleukin; IRS, insulin receptor substrate; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharides; MAPK, mitogen-activated protein kinase; MCI, mild cognitive impairment; NF-κB, nuclear factor-κB; N2a, Neuro-2a; PCN, primary rat cortical neuron; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator-1α; PKA, protein kinase A; PKC, protein kinase C; PPAR, peroxisome proliferator-activated receptor; PUFAs, polyunsaturated n-3 fatty acids; ROS, reactive oxygen species; sFFAs, saturated free fatty acids; TG, triglyceride; TLC, thin layer chromatography; TNF-α, tumor necrosis factor-α; WST-1, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate

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Palmitate activates the NF-κB machinery via stimulation of mitogen-activated protein kinase (MAPK) signaling pathway. For example, palmitate increases phosphorylation of extracellular signal-regulated kinase (ERK) 1/2, c-Jun N-terminal kinase (JNK) or p38 MAPK in muscle [9,18], pancreatic cells [19] and human hepatocytes [20]. The activation of the MAPK signaling pathway has been implicated in both the generation of proinflammatory cytokines [21] and in the pathogenesis of insulin resistance [1,22]. Palmitate-induced insulin resistance has been identified in several animal and cell lines. However, palmitate either may or may not provoke insulin resistance in hypothalamic neuroendocrine cells in recent reports [23,24]. Elevated levels of plasma sFFAs resulted in insulin resistance in animals fed high-fat diets [6,25]. Recent in vitro studies demonstrate that palmitate treatment also induces both mitochondrial dysfunction [26,27] and insulin resistance [8,28] in cultured muscle cells. Since palmitate-induced insulin resistance is closely associated with mitochondrial dysfunction [27,29], it is postulated that mitochondrial dysfunction is what triggers insulin resistance [30]. On the other hand, insulin resistance can induce mitochondrial dysfunction in type 2 diabetes [31].

From the above, sFFAs induce intracellular MAPK signaling, NF-κB activation, mitochondrial dysfunction and insulin resistance in cells. By contrast, polyunsaturated n-3 fatty acids (PUFAs) such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are known to prevent or alleviate inflammation and insulin resistance [32, 33]. Interestingly, several recent in vitro studies in myotubes reported that coincubation of palmitate with oleate, a monounsaturated n-9 fatty acid, prevents palmitate-induced cytotoxicity, insulin resistance and mitochondrial dysfunction [27,28,34,35]. While excessive circulating sFFAs may also trigger inflammation and insulin resistance in brain as in peripheral tissue, the signaling cascade has not been clarified in neuronal cells. This possibility has obvious relevance to the possible connection between obesity-induced insulin resistance in the brain and neurodegeneration, for instance in mild cognitive impairment (MCI) and Alzheimer's disease (AD) [36,37]. In the present study, we examined the effects of palmitate on mitochondrial function and cell viability as well as on insulin and NF-κB signaling in neuronal cells. Next, we tested whether oleate has a protective effect against palmitate-induced toxicity and if preconditioning alone was sufficient. We found that palmitate induced mitochondrial dysfunction and insulin resistance associated with phosphorylation of MAPK and nuclear translocation of NF-κB p65. Pre-exposure to oleate completely blocked these palmitate-induced intracellular signaling and metabolic derangements, an effect outlasting its removal. Oleate also prevented ceramide-induced insulin resistance. Mechanistically, inhibition of protein kinase A (PKA) attenuated the protective effect of oleate against palmitate. Oleate increased triglyceride (TG) levels while palmitate increased diacylglycerol (DAG) synthesis and we show that oleate blocked palmitate-induced DAG as another explanation for its preconditioning protection effect. Finally, oleate preconditioning was superior to docosahexaenoic acid or linoleate in the protection of neuronal cells from such insults. These findings recommend that oleate has beneficial properties against sFFAs and ceramide models of neuronal cell injury.

2. Materials and methods

2.1. Cell culture

Mouse neuroblastoma Neuro-2a (N2a) cells were purchased from ATCC (Manassas, VA). Cells were cultured in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS; Invitrogen), 25 mM D-glucose, 2 mM L-glutamine, 1 mM sodium pyruvate and 1% penicillin/streptomycin and maintained at 37 °C in 5% CO_2. N2a cells were used under 15 passages after purchase and maintained at below 80% confluence. Neuronal phenotypes were confirmed in >99% cells with neuron-specific makers γ-enolase and β-tubulin (not shown). Primary rat cortical neurons (PCNs) were cultured from E18 Sprague–Dawley rat fetal cortex (Charles River, Wilmington, MA) as described [38]. Briefly, isolated fetal cerebral cortex was dissociated into single cells and then seeded to 12-well plates coated with poly-D-lysin at 4×10^5 cells per well. PCNs were cultured in neurobasal medium (Invitrogen, Carlsbad, CA) containing 2% B27 without insulin, 25 mM D-glucose, 0.5 mM L-glutamine and 1% penicillin/streptomycin for 7 days before experiments.

2.2. Reagents

Sodium salts of palmitate, oleate, cis-4,7,10,13,16,19-docosahexaenoic acids (DHA) and linoleate and fatty acid-free bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). For western blots, anti-phospho-ERK1/2 (pERK1/2, Thr202/Tyr204), ERK1/2, phospho-JNK (pJNK, Thr183/Tyr185), JNK, phospho-NF-κB p65 (pNF-κB p65, Ser536), NF-κB p65, IκBα, lamin A/C, caspase-3, caspase-9, phospho-Akt (pAkt, Ser473) and Akt antibodies were purchased from Cell Signaling Technology. Anti-PGC-1α, actin (Sigma), tubulin (Cymbus Biotechnology), heat shock protein (HSP) 90 (Stressgen, San Diego, CA) and γ-enolase (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies were purchased from the cited manufacturers. Recombinant human tumor necrosis factor-α (TNF-α; Invitrogen), interferon-γ (IFN-γ; Invitrogen) and insulin (Sigma) were dissolved in dH_2O and stored at −20 °C. PKI, an inhibitor of protein kinase A (PKA), was purchased from Invitrogen and dissolved in dH_2O. U126, a highly selective inhibitor of MAPK/ERK kinase1/2, was purchased from Santa Cruz. Rotenone, an inhibitor of mitochondrial complex I, and antimycin A, an inhibitor of mitochondrial complex III, were purchased from Sigma. Myriocin, an inhibitor of serine palmitoyltransferase, was purchased from Sigma. N-Acetyl-D-sphingosine (C2 ceramide), N-acetyl-D-erythro-sphinganine (C2-dihydroceramide), retinoic acid and Oil Red O were purchased from Sigma. The chemicals were dissolved in DMSO at a concentration of 10 mM and stored at −20 °C.

2.3. Fatty acid preparation

Fatty acid stock solutions (20 mM) of palmitate, oleate, DHA and linoleate were complexed to fatty acid-free BSA in cell culture medium. Briefly, sodium palmitate, oleate, DHA and linoleate were dissolved each in 0.1 M NaOH at 70 °C for 30 min and then added to cell culture medium containing 3.3 mM of fatty acid-free BSA. The fatty acids and BSA stock mixtures were incubated at 37 °C for 1 h to complex FFAs with BSA. The physiological ratio of fatty acids to BSA is 1–1.5:1 in normal condition. However, the ratio can reach up to 6:1 in human diabetic patients [39]. Thus, we employed this ratio (6:1) for the present study. Control BSA was prepared by mixing 1 ml of 0.1 M NaOH with 9 ml of cell culture medium containing 3.3 mM of fatty acid-free BSA.

2.4. Nuclear, cytosolic and total protein extraction

To extract total proteins, N2a cells or PCNs were washed in cold PBS and lysed in lysis buffer (20 mM Tris–HCl, pH 8, 150 mM NaCl, 10% glycerol, 2 mM EDTA, 1% Nonidet P-40 (NP-40)) containing protease inhibitor cocktail (Roche, Mannheim, Germany) at 4 °C for 30 min. The whole cell lysate was centrifuged at 14,000 rpm at 4 °C for 10 min and the supernatant saved at −20 °C for western blot analysis. To fractionate nuclear and cytosolic proteins, N2a cells were scraped from culture dishes and washed with cold PBS. Cells were gently suspended in hypotonic buffer (20 mM Tris–HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$) and then incubated at 4 °C for 15 min. NP-40 detergent was added at a final concentration of 0.5%. The cells were immediately vortexed for 10 s and then centrifuged at 3000 rpm at 4 °C for 5 min. The supernatant was saved for cytosolic fraction. The pellet was washed with PBS and then lysed in lysis buffer at 4 °C for 30 min. The lysate was centrifuged at 14,000 rpm at 4 °C for 30 min, after which the supernatant was saved as the nuclear fraction. The concentration of each nuclear,

cytosolic and total protein was determined using a Bio-Rad Protein Assay kit (Bio-Rad, Richmond, CA).

2.5. Western blot

Each protein sample (20 μg) was heated at 95 °C for 10 min in Laemmli sample buffer, separated on 10% SDS polyacrylamide gels or NuPAGE 4–12% Bis–Tris gels (Invitrogen), and then electrotransferred onto polyvinylidene-difluoride (PVDF) membranes. The membranes were blocked in 5% nonfat dry milk in Tris-buffered saline (TBS; 20 mM Tris, pH 7.6, 0.8% NaCl) containing 0.1% Tween 20 (TBST) for 1 h and then hybridized with primary antibodies (1:500–2000 dilution) in blocking buffer at 4 °C overnight. After incubation with primary antibodies, the membranes were washed with TBST, incubated in HRP-conjugated secondary antibodies (1:4000 dilution; Cell Signaling Technology) in blocking buffer at room temperature for 1 h, and then washed with TBST again. The signal was detected using enhanced chemiluminescence reagents and film (GE Healthcare, Piscataway, NJ). Band density was measured from the film using ImageJ software (NIH).

2.6. Cell viability

For 24 h coincubation or preconditioning experiments, N2a cells were cultured to 60–70% confluence in 96-well plates. Prolonged incubation with palmitate or ceramide up to 96 h was performed in 24-well plates. 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) solution (Roche, Mannheim, Germany) was added per well for the last 4 h of the experimental period. WST-1 reduction was detected using absorbance at 490 nm by a Vmax microplate reader (Molecular Device, Sunnyvale, CA).

2.7. Intracellular ATP and mitochondrial superoxide levels

The level of intracellular ATP was measured using ATP Bioluminescence Assay Kit HS II (Roche, Mannheim, Germany). Briefly, N2a cells

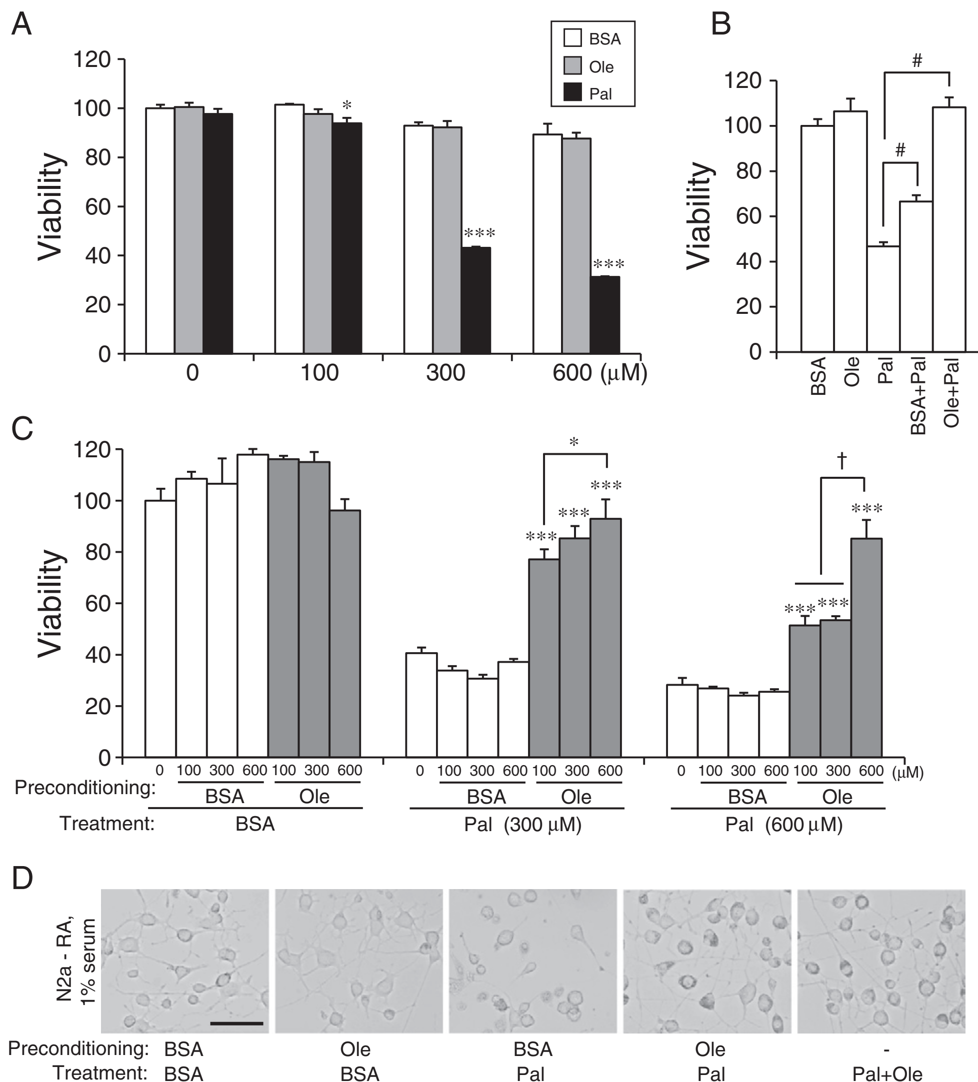

Fig. 1. Effect of palmitate and oleate on N2a cell viability. (A) Palmitate (Pal), but not oleate (Ole), decreased viability. N2a cells were incubated with palmitate, oleate or BSA at concentrations shown for 24 h and WST-1 reduction (490 nm Abs units) was measured for the last 4 h. Viability units were normalized to the BSA (0 μM) group. $^*p < 0.05$ vs. BSA, $^{***}p < 0.001$ vs. BSA, n = 4 per group. (B) Coincubation of oleate with palmitate completely reverses palmitate-induced cell loss. N2a cells were incubated with palmitate (300 μM) and/or oleate (300 μM). Culture duration, WST-1 assay and normalization as above. $\#p < 0.001$, n = 6 per group. (C) Oleate preconditioning prevents palmitate-induced decrease in viability. N2a cells were incubated with oleate or BSA for 24 h, washed, and then incubated with palmitate or BSA for the next 24 h in the absence of oleate. $^*p < 0.05$, $\dagger p < 0.001$, $^{***}p < 0.001$ vs. BSA (0 μM)/Pal (300 or 600 μM), n = 4 per group. (D) Oleate preconditioning, as well as coincubation, prevents palmitate-induced cytotoxicity in differentiated N2a cells. N2a cells differentiate into neuron morphology in culture medium containing 1% fetal bovine serum and 5 μM retinoic acid (RA) for 2 days. The N2a neurons were incubated with oleate (300 μM) or BSA in differentiation media for 24 h and then incubated with palmitate (300 μM) or BSA for another 24 h in the absence of oleate. Cells were photographed under phase contrast conditions. Scale bar: 100 μm.

were collected in lysis buffer (100 mM Tris–HCl, 4 mM EDTA, pH 7.8, 1% Triton X-100). After centrifugation, cell supernatant was mixed with the luciferase reagent at 1:1 ratio. Bioluminescence was measured using a CytoFluor 4000 microplate reader (Perseptive Biosystems, Framingham, MA). The level of mitochondrial superoxide production was measured using the MitoSOX Red reagent (Molecular Probes, Eugene, OR). The reagent is a cell-permeant, fluorogenic dye that is highly selective for the detection of superoxide in mitochondria in living cells. N2a cells were incubated in Hank's balanced salt solution containing 5 μM of the MitoSOX Red at 37 °C in 5% CO_2 for the last 10 min of the experimental period. Cells were washed with PBS and then collected in the lysis solution. Fluorescence was measured at 530 nm (excitation) and 580 nm (emission) by a CytoFluor 4000 microplate reader.

2.8. Thin layer chromatography (TLC)

Lipids were extracted from N2a cells in chloroform/methanol (2:1, vol/vol) as described [40]. The amounts of DAG and TG were measured by TLC as described [41]. Briefly, the lipid extract from N2a cells was separated using hexane/ethyl acetate (3:2, vol/vol) on a silica gel TLC plate. The plates were then soaked in 10% phosphomolybdic acid solution for 10 s, dried under hot air, and immediately heated at 200 °C for 2 min. The spot density was measured from the plates using ImageJ software.

2.9. Oil Red O staining

PCNs were washed with PBS and then fixed with 4% paraformaldehyde for 10 min. PCNs were rinsed again with PBS followed by a wash in 60% isopropyl ethanol. PCNs were stained with 0.3% Oil Red O solution for 15 min and then washed with 60% isopropyl ethanol. PCNs were finally rinsed in dH_2O and photographed under light microscopy.

2.10. Statistical analysis

Statistical significance across treatment groups was detected by two-tailed Student's t-test and/or one-way ANOVA with Newman–Keuls post-hoc tests (Prism, GraphPad Software). All data are presented as the mean ± standard error of the mean.

3. Results

3.1. Oleate preconditioning prevents palmitate toxicity

We first tested the hypothesis that palmitate should also decrease viability of neuronal cells. N2a cells were treated with palmitate, oleate or BSA for 24 h and WST-1 viability was measured for the last 4 h of the 24 h period. Palmitate, but not oleate, decreased viability in a concentration-dependent manner (Fig. 1A). Previous work has shown in fact that oleate blocks the cytotoxic action of palmitate on myotubes

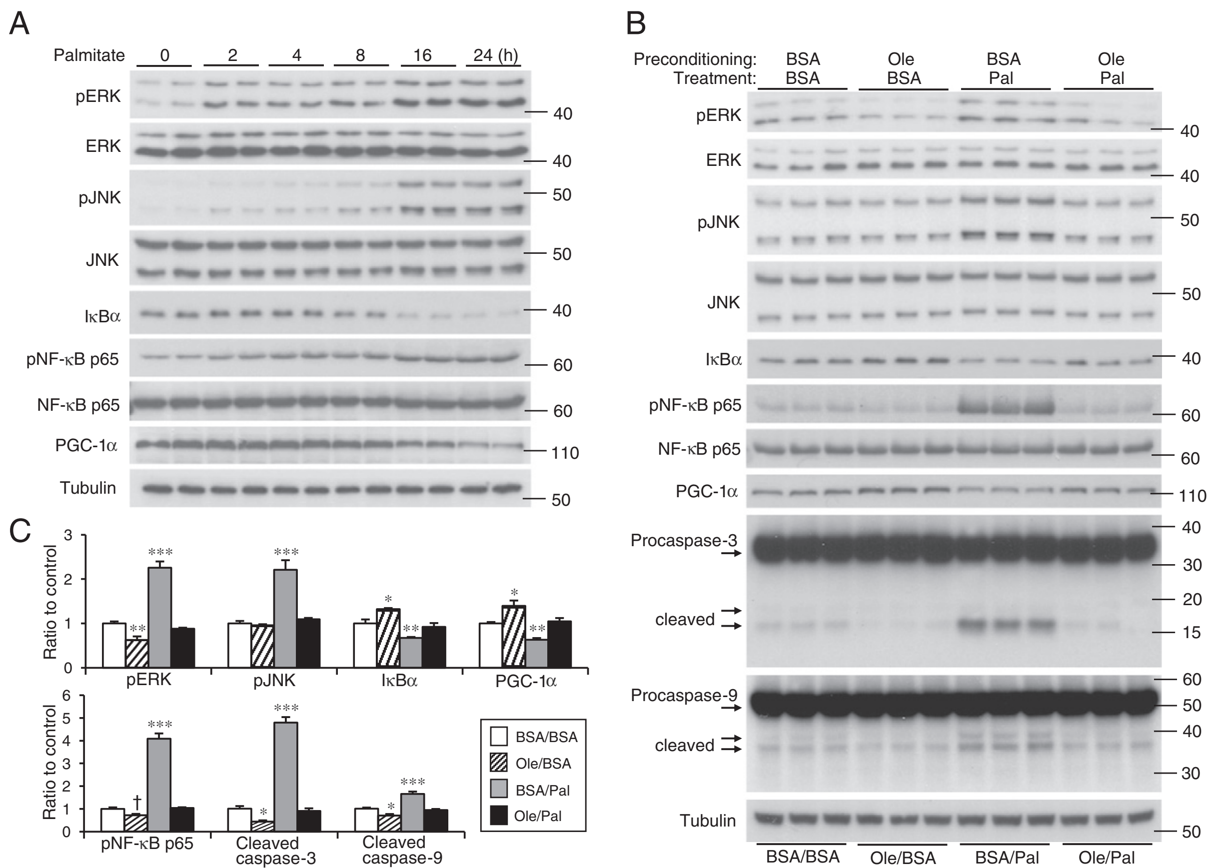

Fig. 2. Effect of palmitate and oleate on intracellular signaling. (A) Palmitate increased the phosphorylation of ERK1/2, JNK and NF-κB p65 and decreased the IκBα and PGC-1α levels in N2a cells. N2a cells were collected at indicated hours after palmitate (300 μM) treatment. Total proteins were analyzed by western blot. Bands are quantified in Supplemental Fig. S2A. (B) Oleate preconditioning prevents palmitate-induced ERK, JNK and NF-κB p65 signaling as well as cleavages of caspase-3 and caspase-9. N2a cells were incubated with oleate (Ole, 300 μM) or BSA for 24 h and then incubated with palmitate (Pal, 300 μM) or BSA for 24 h in the absence of oleate. (C) Densitometric quantification. Oleate preconditioning (dark bars) blocked palmitate-induced phosphorylation of ERK, JNK and NF-κB p65, degradation of IκBα and PGC-1α levels and cleavages of caspase-3 and -9 (gray bars). Note, oleate preconditioning alone decreased the basal levels of cleaved caspase-3 and -9, pERK1/2 and pNF-κB p65 and increased the total IκBα and PGC-1α. Relative levels of pERK, pJNK and pNF-κB p65 were normalized to each respective total protein level. Relative levels of cleaved caspase-3 and -9 and total levels of IκBα and PGC-1α were normalized to tubulin. *$p < 0.05$ vs. BSA/BSA, **$p < 0.01$ vs. BSA/BSA, ***$p < 0.001$ vs. BSA/BSA, †$p < 0.05$ vs. BSA/BSA (t-test), n = 6 per group, representative duplicates (A) and triplicates (B) shown by western blot.

[27,35]. Therefore, we first examined if oleate alleviates palmitate-induced cytotoxicity when it is coincubated in cultures of N2a cells. N2a cells were concurrently exposed to palmitate (300 μM) and/or oleate (300 μM) or control BSA for 24 h and WST-1 viability was measured as above. Oleate completely reversed palmitate-induced cell death (Fig. 1B). Coincubation of palmitate with BSA alone had a much smaller but still significant effect (Fig. 1B). Other studies have shown a similar effect in myotubes exposed to palmitate with/without BSA [35] and that even BSA alone has some anti-oxidative [42] and anti-apoptotic [43] actions. However, as shown in the results of Figs. 1C and 8, oleate, not BSA, is the outstanding protectant. We next examined if oleate *pretreatment* alone was sufficient to protect N2a cells from palmitate-induced cytotoxicity. Importantly, oleate is removed before exposure to palmitate. N2a cells were preconditioned with oleate or BSA for 24 h. Media containing oleate or BSA were replaced by new media containing palmitate or BSA and then incubated for an additional 24 h. Preconditioning with 3 different concentrations (100, 300 and 600 μM) of oleate, but not BSA, greatly attenuated the effect of palmitate (300 and 600 μM) on viability as measured by WST reduction (Fig. 1C). Preconditioning with oleate was also concentration-dependent (Fig. 1C). These results indicate that oleate preconditioning, but no longer BSA alone, protects N2a cells from subsequent palmitate-induced cytotoxicity.

To test this effect in N2a's committed to neuron phenotype, cells at 10–20% confluence were differentiated in culture medium containing 1% FBS and 5 μM retinoic acid for 2 days. N2a neurons were then preconditioned with oleate (300 μM) or BSA for 24 h as above. Oleate or BSA containing media was replaced by fresh differentiation media containing palmitate (300 μM) or BSA and incubated for another 24 h (in the absence of oleate). Palmitate treatment induced cytotoxicity including neurite degeneration in N2a neurons (Fig. 1D, middle panel). Oleate preconditioning, as well as coincubation, also prevented palmitate-induced cytotoxicity in N2a neurons (Fig. 1D). The protective effect of oleate against palmitate was also confirmed in N2a cells using trypan blue exclusion by manual cell count (not shown). We also tested the protective effect of oleate on palmitate-induced cytotoxicity in primary rat cortical neurons (PCNs). Similar to N2a cells, palmitate induced neurite degeneration in PCNs (Supplemental Fig. S1A). Oleate preconditioning, as well as coincubation, protected PCNs from palmitate, measured by trypan blue exclusion assay (Supplemental Fig. S1B).

3.2. Palmitate increases phosphorylation of ERK1/2, JNK and NF-κB p65 and decreases IκBα and PGC-1α

To examine the changes in previously reported intracellular signaling pathways in our N2a cells, 300 μM of palmitate was applied to the culture medium. N2a cells were collected at 0, 2, 4, 8, 16 and 24 h after treatment and total proteins were extracted. The levels of phosphorylated ERK1/2, JNK and NF-κB p65 and total IκBα and peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α were measured by western blot. Palmitate increased the levels of phosphorylated ERK1/2, JNK and NF-κB p65 in a time-dependent manner (Fig. 2A). Maximal increases were observed at 16 and 24 h after palmitate treatment. Palmitate decreased the levels of total IκBα and PGC-1α in a similar time-dependent manner (Fig. 2A). Band quantifications are given in Supplemental Fig. S2A. BSA treatment (control) produced no changes in any of these signaling proteins over time (not shown).

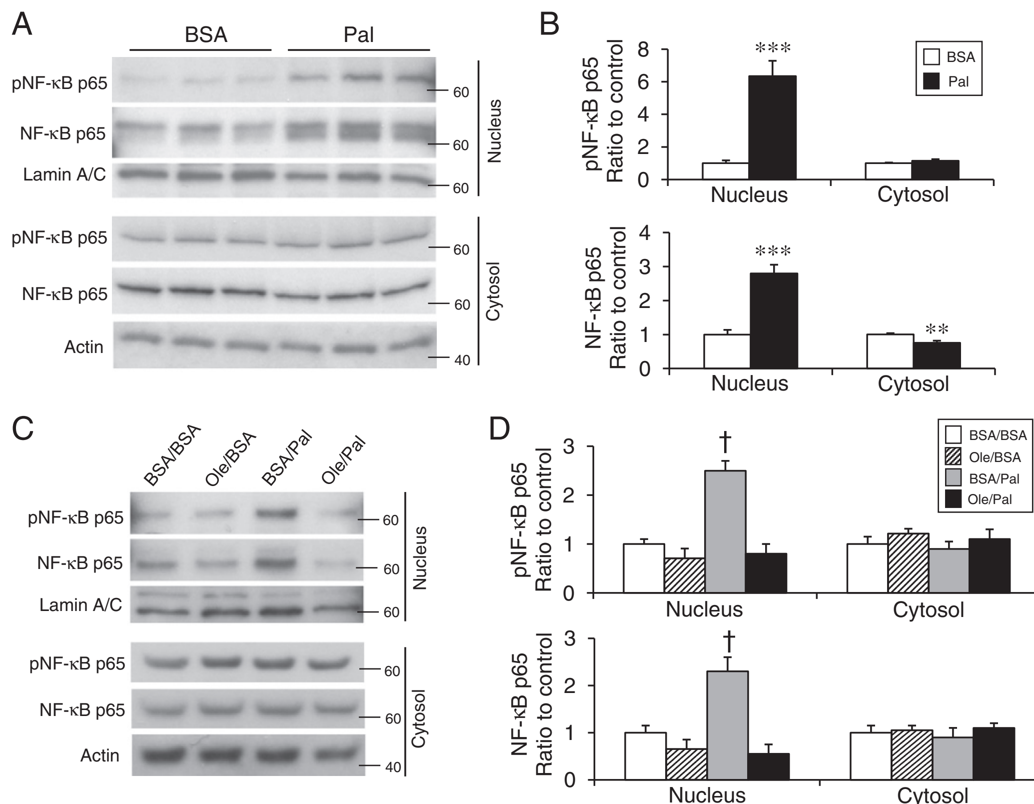

Fig. 3. Effect of palmitate and oleate on nuclear translocation of NF-κB p65. (A) Palmitate induced nuclear translocation of NF-κB p65. N2a cells were collected at 24 h after palmitate (300 μM) or BSA treatment. Nuclear and cytosolic fractions were analyzed by western blot. (A) Representative western blots and (B) densitometric quantification. Palmitate increased the nuclear translocation of pNF-κB p65 and total NF-κB p65. Relative levels of pNF-κB p65 and total NF-κB p65 were normalized to lamin A/C or actin. $^{**}p < 0.01$ vs. BSA, $^{***}p < 0.001$ vs. BSA, n = 6 per group. (C) Oleate preconditioning (lane 4) blocked palmitate-induced nuclear translocation of pNF-κB p65 and total NF-κB p65 (lane 3). N2a cells were incubated with oleate (Ole, 300 μM) or BSA for 24 h and then incubated with palmitate (Pal, 300 μM) or BSA for 24 h in the absence of oleate. (C) Representative western blots and (D) densitometric quantification. Bands are normalized as above. $^{\dagger}p < 0.01$ vs. BSA/BSA, n = 3 per group.

3.3. Oleate preconditioning prevents palmitate-induced signaling changes and caspase activation

To examine if oleate preconditioning prevents palmitate-induced intracellular signaling abnormalities, N2a cells were incubated with oleate (300 μM) or control BSA for 24 h. Media containing oleate or BSA were washed once with PBS and then replaced by new media containing palmitate (300 μM) or BSA and incubated for another 24 h (in the absence of oleate). Total proteins were extracted for western blot. Oleate preconditioning was sufficient to completely inhibit palmitate-induced phosphorylation of ERK1/2, JNK and NF-κB p65 (Ole/Pal vs. BSA/Pal; Fig. 2B and C). Oleate preconditioning also inhibited palmitate-induced decreases in total IκBα and PGC-1α (Fig. 2B and C). Interestingly, the oleate alone preconditioned group (Ole/BSA) modestly decreased the phosphorylation of ERK1/2 and NF-κB p65 and increased the total IκBα and PGC-1α compared with the BSA/BSA control without any palmitate (Fig. 2B and C). We observed similar effects of oleate against palmitate-induced signaling pathway changes in PCNs (not shown). It was reported that palmitate increased cleaved caspase-3 and -9 in cultured human mesangial cells, implicating apoptosis mechanisms and that caspase activation was blocked by oleate coincubation [44]. Therefore, we also tested if oleate preconditioning has a protective effect on palmitate-induced caspase cleavages in N2a cells. Palmitate treatment (BSA/Pal) greatly increased the levels of cleaved (activated) caspase-3 and -9 (Fig. 2B and C). Palmitate-induced cleavages of caspase-3 and -9 were also blocked by oleate preconditioning (Ole/Pal) (Fig. 2B and C). Here again, the oleate preconditioned group (Ole/BSA, no added palmitate) decreased the basal levels of cleaved caspase-3 and -9 compared with the BSA/BSA control group (Fig. 2B and C).

3.4. Oleate preconditioning prevents palmitate-induced increases in nuclear translocation of NF-κB p65

To examine if palmitate induces nuclear translocation of NF-κB p65, N2a cells were incubated with palmitate (Pal, 300 μM) or BSA for 24 h. Nuclear and cytosolic fractions were analyzed by western blot. Palmitate greatly increased the levels of pNF-κB p65 and total NF-κB p65 in the nucleus (Fig. 3A and B). In the cytosol, the level of total NF-κB p65 was correspondingly decreased (Fig. 3A and B). This result suggests that newly phosphorylated NF-κB p65 is translocated and presumably activated for transcription following palmitate treatment. We next examined if oleate preconditioning prevents palmitate-induced nuclear translocation of NF-κB p65. N2a cells were incubated with oleate (300 μM) or BSA for 24 h. Media containing oleate or BSA were replaced by new media containing palmitate (300 μM) or BSA and then incubated for another 24 h (in the absence of oleate). Palmitate treatment (BSA/Pal) increased the levels of pNF-κB p65 and total NF-κB p65 in the nucleus as before (Fig. 3C and D). Oleate preconditioning (Ole/Pal) completely blocked these palmitate-induced pNF-κB p65 responses (Fig. 3C and D). The quality of nuclear and cytosolic subcellular fractions was confirmed by western blot using anti-HSP90, lamin and tubulin antibodies. Western blots showed no contamination between nuclear and cytosolic fractions (Supplemental Fig. S2B).

3.5. ERK1/2 is not directly upstream of TNF-α- or palmitate-induced increases in NF-κB p65 signaling

As shown above, oleate preconditioning has cellular protective effects against palmitate in N2a cells and prevents NF-κB p65 activation by palmitate. It is accepted that inflammatory cytokines (e.g. TNF-α) can reciprocally induce activation of intracellular signaling pathways including NF-κB signaling [8,16,21]. Therefore, we examined if oleate preconditioning can also prevent TNF-α-induced intracellular signaling pathways in N2a cells. Cells were incubated with oleate (300 μM) or BSA for 24 h. Media containing oleate or BSA were replaced by new media containing TNF-α (10 ng/ml). N2a cells were collected at 0, 0.5, 1, 3, 8 and 24 h after TNF-α treatment and total proteins were extracted for western blot. TNF-α increased the levels of pERK1/2, pJNK and pNF-κB p65 in the BSA (control) preconditioned group as expected (Fig. 4A). Oleate preconditioning completely blocked TNF-α-induced phosphorylation of ERK1/2, but not JNK or NF-κB p65 at the 0.5 and 1 h time points (Fig. 4A). Unlike pERK1/2 and pJNK, TNF-α-induced pNF-κB p65 was maintained at high levels until 24 h in both BSA and oleate preconditioned groups. Of note, the level of IκBα decreased at 0.5 h after TNF-α treatment in both BSA (control) and oleate preconditioned groups concurrent with the increase in pNF-κB p65 as expected, but then quickly achieved basal or slightly increased levels thereafter (Fig. 4A). Thus, oleate blockade of TNF-α-induced ERK1/2 activation did not extend to any effects on pJNK, pNF-κB p65 or IκBα. We further examined if ERK1/2 signaling plays any role in the regulation of the NF-κB p65 signaling pathway and total level of PGC-1α following palmitate exposure. Since ERK1/2 is highly activated following 8 h of palmitate exposure (Fig. 2A), N2a cells were incubated with 10 μM of U0126, a highly selective MEK1/2 inhibitor, for the last 12 h of the 20 h palmitate (300 μM) exposure period. The control group was treated with vehicle (DMSO). U0126 completely inhibited palmitate-induced phosphorylation of ERK1/2 (Fig. 4B). However, U0126 had no effect on

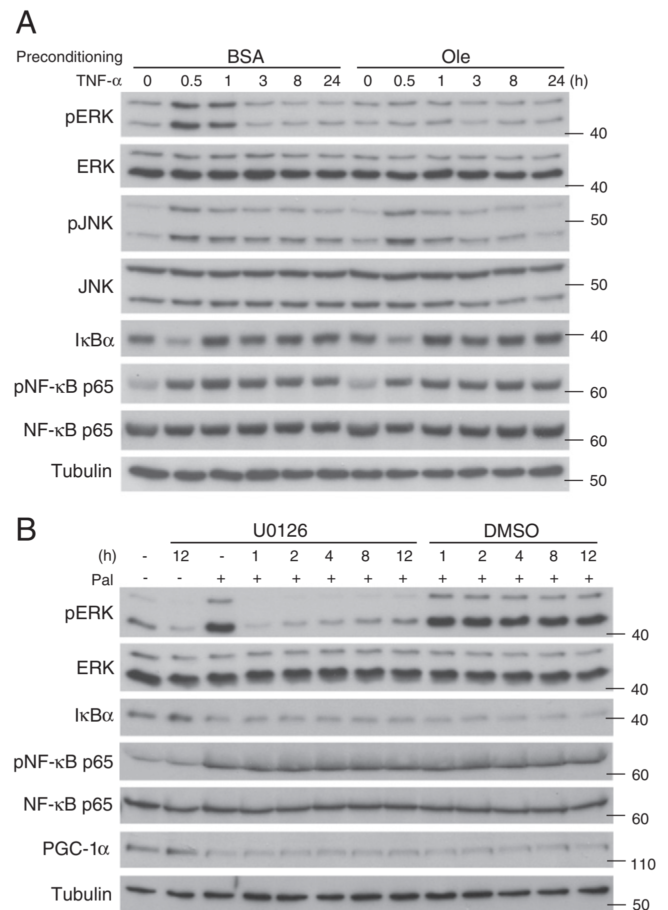

Fig. 4. Oleate effects on TNF-α-induced signaling. ERK1/2 activation is not linked to NF-κB p65 signaling. (A) Effect of oleate preconditioning on TNF-α-induced intracellular signaling. N2a cells were incubated with oleate (Ole, 300 μM) or BSA for 24 h and then incubated with TNF-α (10 ng/ml) in the absence of oleate. Cells were collected at indicated hours after TNF-α treatment and total protein extracts were analyzed by western blot. Oleate preconditioning completely blocked TNF-α-induced pERK1/2, but had no effect on the levels of pJNK, pNF-κB p65 and total IκBα. (B) Effect of U0126 on palmitate-induced NF-κB p65 signaling. N2a cells were incubated with palmitate (300 μM) for 20 h in the presence of U0126 (10 μM) or DMSO for indicated hours. Blockade of ERK1/2 activation by U0126 did not affect palmitate-induced NF-κB p65 signaling or levels of PGC-1α. Experiment was repeated in triplicate with similar results.

the phosphorylation of NF-κB p65 nor any effect on the levels of palmitate-induced degradation of IκBα and PGC-1α (Fig. 4B). In another experiment, N2a cells were concurrently coincubated with palmitate (300 μM) and U0126 (10 μM) for 0, 2, 4, 8, 16 and 24 h. Again, we observed that U0126 completely inhibited palmitate-induced ERK1/2 activation up to 8 h and partially inhibited at 16 and 24 h, but without effect on pNF-κB p65 or the levels of palmitate-induced degradation of IκBα and PGC-1α (Supplemental Fig. S3). In addition, U0126 blockade of ERK1/2 activation had no effect on the TNF-α-induced phosphorylation of NF-κB p65 and degradation of IκBα (not shown). In summary, while the action of palmitate to induce NF-κB p65 phosphorylation is not via its effect to also activate ERK, it is reversed by oleate. On the other hand, the action of TNF-α to do the same is not oleate sensitive, indicating a downstream phenomenon to fatty acid signaling.

3.6. Oleate preconditioning prevents palmitate-induced mitochondrial dysfunction

Previous non-neuronal cell culture studies have demonstrated that palmitate decreases intracellular ATP generation and increases intracellular ROS production [27,29]. We examined if oleate preconditioning prevents palmitate-induced mitochondrial dysfunction by measuring ATP loss and superoxide production. N2a cells were incubated with oleate (300 μM) or BSA for 24 h and then media were replaced by new media containing palmitate (300 μM) or BSA. Cells were incubated for another 24 h (intracellular ATP) or 8 h (mitochondrial superoxide) in the absence of oleate. Palmitate significantly decreased the intracellular ATP production and increased the levels of mitochondrial superoxide (Fig. 5A and B). Oleate preconditioning prevented both changes (Fig. 5A and B). Interestingly, oleate preconditioning itself also decreased the level of mitochondrial superoxide compared to the control group (Fig. 5B). We also examined if oleate has a protective effect against mitochondrial complex inhibitors. N2a cells were incubated with oleate (300 μM) or BSA for 24 h and the media were replaced by fresh media containing rotenone or antimycin A. Cells were incubated for another 24 h in the absence of oleate. WST-1-based viability measurements were made in the last 4 h. Vehicle control groups (0 μM inhibitor) were treated with the same amount of DMSO. Rotenone and antimycin A decreased viability in a concentration-dependent manner equally in both oleate and BSA preconditioned groups (Fig. 5C and D). From this, palmitate does not apparently act like an electron transport poison.

3.7. Oleate preconditioning prevents palmitate- or ceramide-induced insulin resistance

To examine if oleate preconditioning prevents another mechanism of palmitate toxicity, mainly the induction of insulin resistance, N2a cells were incubated with oleate (300 μM) or BSA for 24 h and the media were replaced by new media containing palmitate (300 μM) or BSA. N2a cells were incubated for another 24 h and then stimulated with insulin (20 nM) for 15 min before lysis. Total proteins were extracted and analyzed by western blot for changes to Akt. Palmitate exposure (BSA/Pal) decreased the levels of insulin-stimulated and basal (unstimulated) pAkt (Fig. 6A). Oleate preconditioning (Ole/Pal) rescued palmitate-induced decreases in the levels of both insulin-stimulated and basal pAkt (Fig. 6A). Next, we examined if oleate preconditioning also prevents ceramide-induced insulin resistance. Preliminary data had showed that treatment of 50 μM ceramide for 6 h significantly decreased the level of insulin-stimulated pAkt in N2a cells (not shown). N2a cells were incubated with oleate (300 μM) or BSA for 24 h and the media were replaced with new media containing ceramide (50 μM) or dihydroceramide (50 μM), a metabolically inactive control, for 6 h. Cells were stimulated with insulin (20 nM) for 15 min before lysis. Ceramide treatment (BSA/Cer) decreased the levels of insulin-stimulated and unstimulated pAkt similar to palmitate (Fig. 6B). Oleate preconditioning (Ole/Cer) also restored basal and insulin-stimulated signaling under ceramide conditions (Fig. 6B). We also observed similar

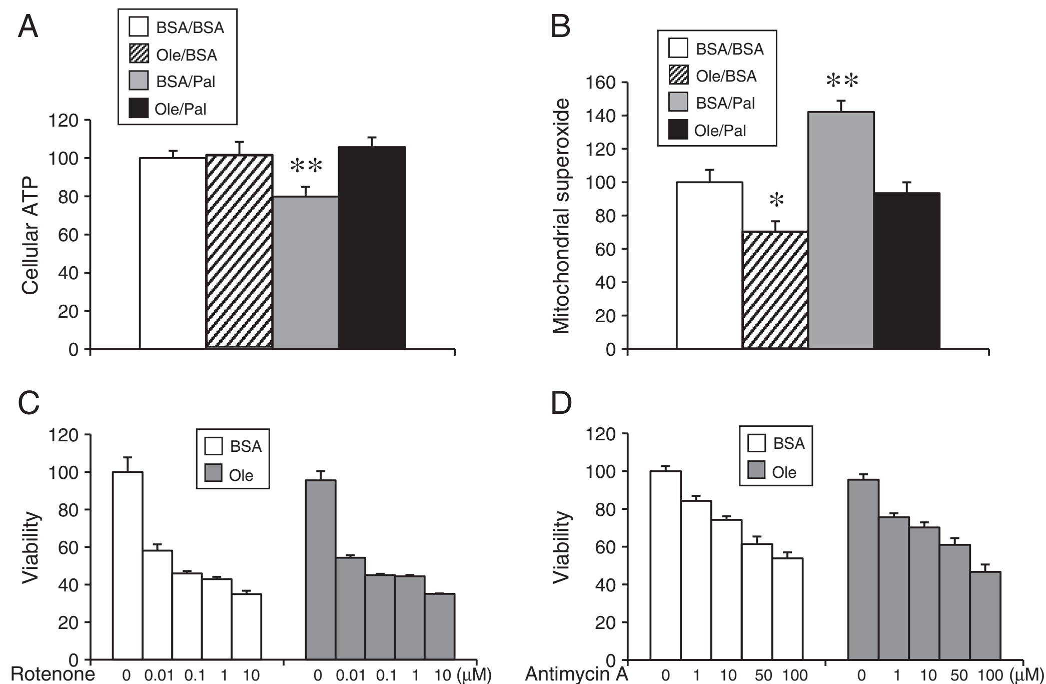

Fig. 5. Effect of oleate preconditioning on mitochondrial outcomes. Oleate preconditioning prevented the palmitate-induced cellular ATP decrease (A) and mitochondrial superoxide production (B). N2a cells were incubated with oleate (Ole, 300 μM) or BSA for 24 h and then incubated with palmitate (Pal, 300 μM) or BSA for 24 h (ATP) or 8 h (superoxide) in the absence of oleate. Relative levels of cellular ATP and mitochondrial superoxide were normalized to the BSA/BSA control group. *$p < 0.05$ vs. BSA/BSA, **$p < 0.01$ vs. BSA/BSA, n = 6 per group. Oleate preconditioning had no effect on rotenone- (C) or antimycin A- (D) induced decrease in viability. N2a cells were incubated with oleate (300 μM) or BSA for 24 h and then with various concentrations of rotenone or antimycin A for another 24 h after washout of oleate. WST-1 viability was measured for the last 4 h of the 24 h rotenone or antimycin A incubation period. Relative levels of WST-1 viability were normalized to the BSA group treated with a 0 μM inhibitor, n = 4 per group.

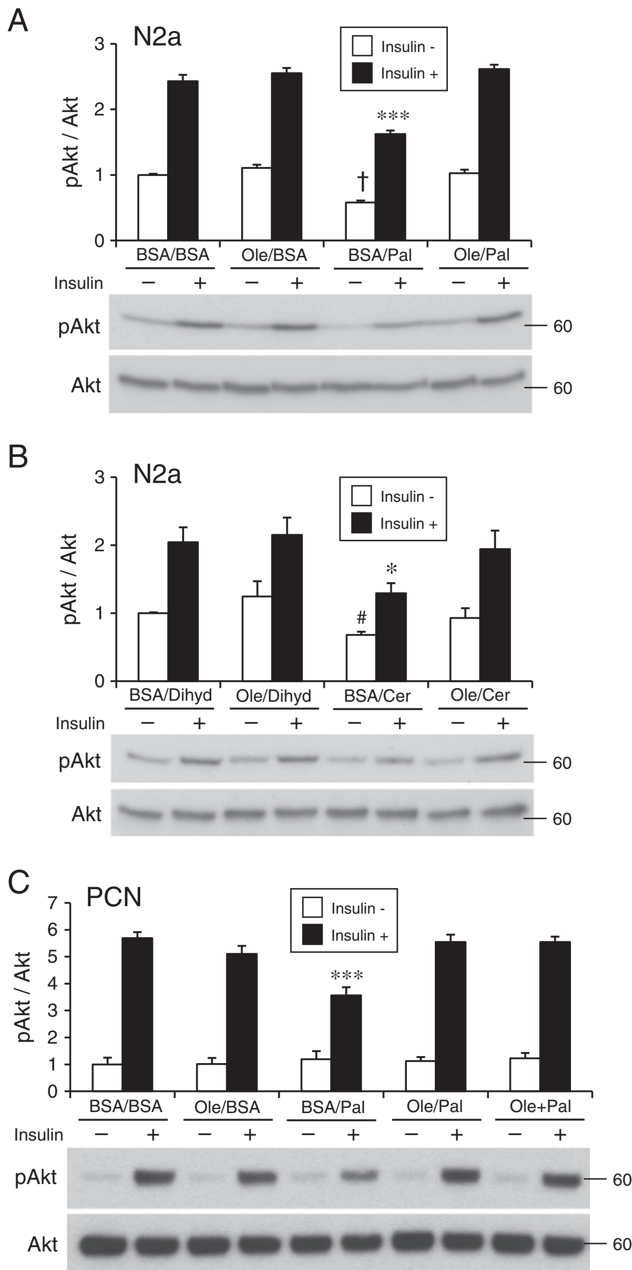

Fig. 6. Effect of oleate preconditioning on insulin resistance. (A) Oleate preconditioning prevented palmitate-induced insulin resistance in N2a cells as determined by Akt activation. †$p < 0.001$ vs. BSA/BSA without insulin, ***$p < 0.001$ vs. BSA/BSA with insulin, n = 6 per group. (B) Oleate preconditioning prevented ceramide-induced insulin resistance in N2a cells. #$p < 0.001$ vs. BSA/Dihyd without insulin (t-test), *$p < 0.05$ vs. BSA/Dihyd with insulin, n = 4 per group. Dihydroceramide (Dihyd, 50 μM) was used as control. N2a cells were incubated with oleate (Ole, 300 μM) or BSA for 24 h and then with palmitate (Pal, 300 μM) for 24 h or with ceramide (Cer, 50 μM) for 6 h in the absence of oleate. (+) = N2a cells were stimulated with insulin (20 nM) for 15 min before lysis. (C) Oleate preconditioning prevented palmitate-induced insulin resistance in PCNs. PCNs were incubated with oleate (Ole, 300 μM) or BSA for 24 h and then with palmitate (Pal, 300 μM) for another 24 h in the absence of oleate. (+) = PCNs stimulated with insulin (20 nM) for 15 min before lysis. ***$p < 0.001$ vs. BSA/BSA with insulin, n = 3 per group. Relative levels of pAkt were normalized to total Akt. Representative western blots (bottoms), densitometric quantifications given at top.

protective action of oleate against palmitate-induced insulin resistance in PCNs (Fig. 6C). PCNs were incubated with oleate (300 μM) or BSA for 24 h and then with palmitate (300 μM) for another 24 h in the absence of oleate. PCNs were also coincubated with oleate and palmitate for 24 h without preconditioning. PCNs were incubated in fresh neurobasal medium without B27 for 3 h before stimulation with insulin (20 nM, 15 min). Similar to N2a cells, oleate preconditioning, as well as coincubation, prevented palmitate-induced insulin resistance in PCNs (Fig. 6C). Moreover, oleate coincubation, but not preconditioning, completely blocked ceramide-induced insulin resistance in PCNs (Supplemental Fig. S4). In addition, we checked if inhibition of de novo synthesis of ceramide blocks palmitate-induced insulin resistance. N2a cells were incubated with palmitate (Pal, 300 μM) in the presence of myriocin (0.1, 1 or 10 μM), an inhibitor of ceramide synthesis, for 24 h and then stimulated with insulin (20 nM) for 15 min before lysis. Levels of pAkt were measured by western blot. Myriocin did not reverse palmitate-induced insulin resistance by this measure in N2a cells (Supplemental Fig. S5).

3.8. Effect of oleate preconditioning on other cytotoxic conditions

We examined if oleate preconditioning protects N2a cells from a comparative range of several cytotoxic conditions. N2a cells were incubated with oleate (300 μM) or BSA for 24 h. Media containing oleate or BSA were replaced by new media containing ceramide (50 μM), H_2O_2 (500 μM), and TNF-α/IFN-γ (300 ng/ml each) or by serum free media for another 24 h in the absence of oleate. Among these, oleate preconditioning only attenuated ceramide-induced cytotoxicity to any significant extent (Fig. 7A). Oleate preconditioning showed no effect in either H_2O_2 or TNF-α/IFN-γ conditions or in the absence of tropic support although all of these were shown to be cytotoxic (Fig. 7B). Next, to test if oleate protects N2a cell from palmitate in the absence of tropic support, N2a cells were cultured in serum-free culture medium for 2 days and then preconditioned with oleate (300 μM) or BSA for 24 h. Serum-free media containing oleate or BSA were replaced by fresh serum-free media containing palmitate (300 μM) or BSA and then incubated for another 24 h (in the absence of oleate). Palmitate treatment induced severe cell death (>95%) in serum-deprived N2a cells, as expected (Fig. 7C, middle panel). Oleate preconditioning, as well as coincubation, prevented palmitate-induced cell death even in serum-deprived N2a cells (Fig. 7C).

3.9. PKA in the protective effect of oleate against palmitate

A previous study suggested that the protective effect of oleate against palmitate is dependent on PKA activation in skeletal muscle cells [28]. We tested if the PKA pathway plays such a role in the protective effect of oleate against palmitate in neuronal cells. N2a cells were incubated with oleate (300 μM) and PKI (PKA inhibitor) for 24 h and then with palmitate (300 μM) and PKI for another 24 h in the absence of oleate. The control group was treated with BSA and PKI. WST-1 viability was measured for the last 4 h of the 24 h palmitate incubation period. PKI (50 μM) significantly attenuated the protective effect of oleate against palmitate (Fig. 8A). PKI did not however change the basal levels of N2a cell viability, either in the presence of 300 μM oleate, palmitate or BSA alone for 24 h (not shown).

3.10. Oleate increases TG while blocking palmitate-induced DAG

To examine lipid induction in the presence of oleate or palmitate, N2a cells were incubated with oleate (300 μM) and/or palmitate (300 μM) for 24 h. N2a cells were also pre-incubated with oleate for 24 h and then switched to palmitate for another 24 h. Oleate increased cellular triglyceride (TG) levels while palmitate increased diacylglycerol (DAG) levels (Fig. 8B and C). Both oleate preconditioning (Ole/Pal) and coincubation (Ole + Pal) blocked palmitate-induced DAG synthesis (Fig. 8B and C). Using Oil Red O staining, we confirmed that oleate, but not palmitate, increased the cellular TG levels in PCNs (Supplemental Fig. S6). In coincubations of oleate and palmitate, the TG levels also rose (Supplemental Fig. S6).

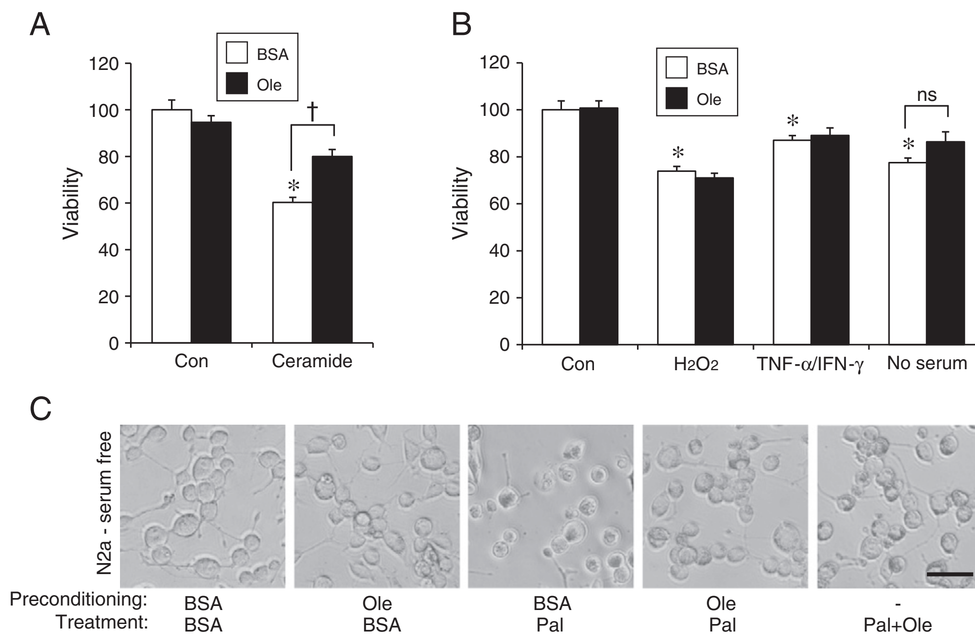

Fig. 7. Effect of oleate preconditioning on several cytotoxic conditions. (A) Oleate preconditioning attenuated ceramide-induced cytotoxicity. N2a cells were incubated with oleate (300 μM) or BSA for 24 h. Media containing oleate or BSA were replaced by new media containing ceramide (50 μM) or dihydroceramide (Con, 50 μM) and then incubated for another 24 h in the absence of oleate. WST-1 viability was measured for the last 4 h of the 24 h ceramide incubation period. Relative levels of viability were normalized to the BSA/Con group. *$p < 0.01$ vs. BSA/Con, †$p < 0.001$, n = 4 per group. (B) Oleate preconditioning had no effect on H_2O_2-, TNF-α/IFN-γ-induced cytotoxicity or serum deprivation-induced decreases in viability. N2a cells were preconditioned with oleate as described above and then treated with H_2O_2 (500 μM), TNF-α/IFN-γ (300 ng/ml each) or serum deprived for another 24 h in the absence of oleate. WST-1 viability was measured as described above. Control (Con) groups were treated with dH_2O as vehicle. Relative levels of viability were normalized to the BSA/Con group. *$p < 0.05$ vs. BSA/Con, n = 4 per group. (C) Oleate preconditioning, as well as coincubation, prevents palmitate-induced cell death in serum-deprived N2a cells. N2a cells were cultured in serum-free medium for 2 days, then incubated with oleate (300 μM) or BSA for the next 24 h and then with palmitate (300 μM) or BSA for the final 24 h in the absence of oleate. Cells were photographed under phase contrast conditions. Scale bar: 50 μm.

3.11. Protective effect of oleate compared to DHA and linoleate against palmitate-induced cytotoxicity

Since some PUFAs are known to be cytoprotective, we compared oleate with DHA and linoleate in N2a cells. First, we examined if either DHA or linoleate had intrinsic effect on cell viability. In Fig. 1, oleate showed no such effect compared to BSA up to 600 μM. Similar to previous reports [45,46], high concentrations (≥200 μM) of DHA or linoleate alone decreased cell viability somewhat (Fig. 9A). N2a cells were incubated with various concentrations of DHA, linoleate or oleate with palmitate (300 μM) for 24 h and WST-1 viability was measured in the last 4 h of the 24 h period. All three unsaturated FFAs completely or partially blocked palmitate-induced decreases in viability (Fig. 9B). Oleate however appeared to have the same advantage over DHA, especially at high concentrations. We next compared the preconditioning effects of middle concentrations of the three unsaturated FFAs on palmitate- or ceramide-induced cytotoxicity in N2a cells. Cells were preconditioned in 100 μM of DHA, linoleate, oleate or BSA for 24 h. Media containing DHA, linoleate, oleate or BSA were replaced by new media containing palmitate (300 μM) or ceramide (70 μM) and then further incubated for 24–96 h in the absence of any of the three unsaturated FFAs. Oleate preconditioning showed the strongest protection against palmitate-induced cytotoxicity (LD50~300 μM) compared with DHA or linoleate at 48, 72 and 96 h (Fig. 9C). In Fig. 9D, preconditioning with either oleate or linoleate showed partial protection against the much more toxic ceramide (LD50~70 μM) compared with DHA.

4. Discussion

Elevated plasma sFFAs are a major pathogenic factor in diabetes and cardiovascular and liver diseases [47,48]. They induce cellular degeneration through inflammation and insulin resistance. Conversely, PUFAs may prevent or even reverse these impairments [32,33]. Most research on FFAs and biologic effects has focused on peripheral tissue (e.g. muscle, liver and adipocytes). Growing evidence also suggests a possible link between sFFA-induced metabolic impairments and neurodegeneration [49,50].

To address the possible link between FFAs and neuronal degeneration, we examined the effects of a major sFFA on intracellular signaling, metabolism and survival in neuronal cells. We found that palmitate induced mitochondrial dysfunction and insulin resistance and it promoted the phosphorylations of ERK1/2 and JNK and nuclear translocation of NF-κB p65. Importantly, exposure to oleate completely blocked these palmitate-induced intracellular signaling activation and metabolic derangements. The lasting effect following oleate removal on preventing subsequent palmitate-induced cell damage is novel and striking. Oleate also prevented ceramide-induced insulin resistance. Even more dramatic, was its ability to reverse the increases in caspase and NF-κB p65 activations attributable to palmitate. Furthermore, oleate restored the basal production of mitochondrial superoxide and PGC-1α levels to control values. Oleate preconditioning did not block TNF-α-induced NF-κB p65 signaling and did not affect TNF-α/IFN-γ-induced cytotoxicity, as could be expected once these cytokines are formed. Mitochondrial complex inhibitor-induced cytotoxicity was also unaffected, all which indicate that oleate action is proximal to these steps. Inhibition of PKA attenuated the protective effect of oleate against palmitate and oleate increased TG accumulation while blocking palmitate-induced DAG synthesis. Oleate preconditioning was superior to DHA or linoleate in the protection of N2a cells against palmitate- or ceramide-induced cytotoxicity.

sFFAs induce intracellular inflammatory signaling pathways that trigger expression of inflammatory cytokines [1,51]. Activation of NF-κB p65 is a major link in the cells' response to inflammatory stimuli. We showed that palmitate increased phosphorylation of NF-κB p65 at serine 536 and induced its translocation into the nucleus. One possible mechanism is via a decrease in IκBα. Oleate preconditioning increased IκBα and restored

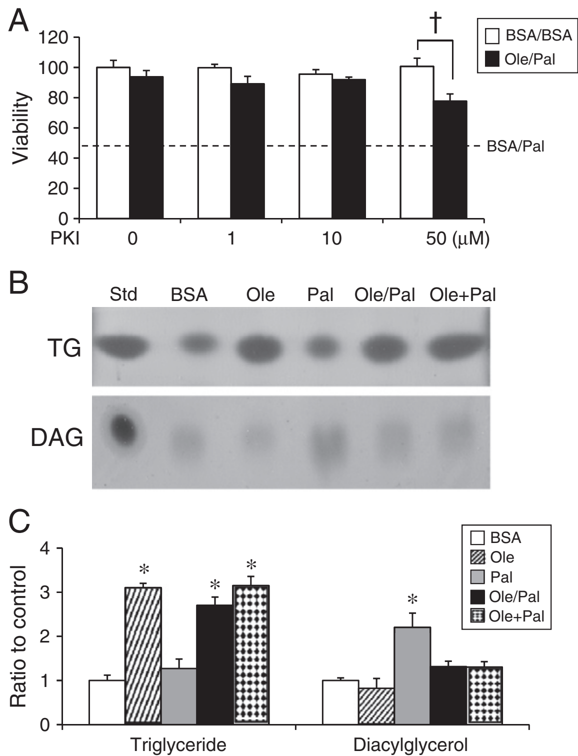

Fig. 8. Involvement of protein kinase A and triglyceride formation in the protective effect of oleate. (A) Inhibition of protein kinase A attenuated the protective effect of oleate. N2a cells were incubated with oleate (Ole, 300 μM) and PKI for 24 h and then with palmitate (Pal, 300 μM) and PKI for another 24 h in the absence of oleate. The control group was treated with BSA and PKI. WST-1 viability was measured for the last 4 h of the 24 h palmitate incubation period. Relative levels of viability were normalized to the BSA/BSA group treated with a 0 μM PKI. †$p < 0.01$, n = 5–6 per group. (B) Oleate increased triglycerides (TG) and blocked palmitate-induced diacylglycerols (DAG). N2a cells were incubated with oleate (300 μM) and/or palmitate (300 μM) for 24 h. N2a cells were also pre-incubated with oleate for 24 h and then incubated with palmitate for another 24 h (Ole/Pal). Lipids were extracted and TG and DAG were measured by thin layer chromatography (TLC). (B) Representative TLC plates and (C) densitometric quantification. Each group was normalized to the BSA group. Due to saturation of TG spots, 1/10 of each lipid sample was spotted for this panel compared with DAG. Std: lipid standard marker. *$p < 0.01$ vs. BSA, n = 4 per group.

basal NF-κB signaling. Our study in neuronal cells and PUFAs demonstrates opposite cellular protective and anti-inflammatory effects [32,33].

Consistent with previous non-neuronal studies [7,9,18], palmitate induced the activation of the ERK1/2 and JNK signaling pathways. In addition to NF-κB p65, these kinases also mediate the inflammatory response. In our hands, oleate preconditioning completely prevented palmitate-induced phosphorylation of ERK1/2 and JNK. Previous studies in myotubes [18,27] employed concurrent incubation with oleate to prevent palmitate-induced phosphorylation of ERK1/2 and JNK. Our finding that pre-exposure to oleate in neuronal cells is sufficiently preventative is novel and has mechanistic implications. Contrary to other reports [7,9] implicating ERK1/2 activation by palmitate as directly upstream of NF-κB in muscle cells, our results showed that inhibition of ERK1/2 had no effect on palmitate-induced NF-κB signaling in neuronal cells. Differences in cell-type may account for the dissociation since another study [10] in adipocytes similarly reported no direct regulation by ERK1/2 on palmitate-induced NF-κB activation. We also showed that oleate preconditioning completely blocked TNFα-induced phosphorylation of ERK1/2, but had no effect on TNFα-induced JNK and NF-κB p65 phosphorylations. Thus, while it appears that oleate reverses palmitate- and TNFα-induced ERK1/2 signaling, the latter is not responsible for the observed effects on NF-κB p65.

In skeletal muscle cells, palmitate causes mitochondrial dysfunction [26,27]. Consistent with these reports, we found that palmitate decreased ATP generation and increased mitochondrial superoxide production in N2a cells. Palmitate-induced mitochondrial dysfunction was also prevented herein by oleate preconditioning. As noted in myotubes [27], coincubation with oleate blocked palmitate-induced mitochondrial dysfunction. Our study newly points to another possible mechanism behind the oleate preconditioning phenomenon, that is to increase PGC-1α levels that outlast the presence of oleate. PGC-1α is a transcriptional coactivator for gene expression involved in energy metabolism and supports mitochondrial biogenesis [52]. Increased PGC-1α expression and decreased NF-κB activation following oleate treatment were attributed to enhanced peroxisome proliferator-activated receptor (PPAR) and protein kinase A activation in skeletal muscle [28]. Since the NF-κB pathway is involved in the regulation of PGC-1α [7] and in mitochondrial gene expression [53], our finding that oleate reduced NF-κB signaling may be linked to a sustained rise in PGC-1α. Increased PGC-1α may therefore play an important role in the protective effect of oleate against palmitate-induced mitochondrial dysfunction in neurons as in muscle [27]. PGC-1α plays an important role to induce several ROS scavenging enzymes such as superoxide dismutase 2 [54]. Indeed we show in Fig. 5B that oleate reverses oxidative stress (mitochondrial superoxide) induced by palmitate. This effect may also be due to an increase in PGC-1α, however further experimentation is needed to prove this causation. Since a direct oleate effect on electron transport function is lacking, the mitochondrial trophic and scavenging actions appear sufficient to account for the improvement in ATP generation (Fig. 5A).

Palmitate induces insulin resistance in muscle cells [8,28] and other non-neuronal cell types [19,20,29,55,56]. The few existing data on neuronal cells is discordant. One study demonstrated that palmitate induced insulin resistance in hypothalamic neurons [23], while another did not [24]. Our results clearly support the former view. Palmitate-induced insulin resistance appears to be mediated in part by DAG, ROS, and/or ceramide. Subsequently, protein kinase C (PKC), JNK and IKK signaling pathways are activated, leading to the phosphorylation of inhibitory sites on Akt and insulin receptor substrate 1 (IRS-1). Decreased insulin-stimulated glucose-transport type 4 activity and glucose uptake ensue [2,51]. Similar to but also extending the myotube coincubation studies [27,28,34,35], we found that oleate preconditioning completely blocked palmitate-induced insulin resistance in neuronal cells. Another recent study in muscle cells [57] demonstrated that oleate prevented palmitate-induced insulin resistance by activating AMP-activated protein kinase. Yet another mechanism for this observation is the promotion of triglyceride synthesis and mitochondrial β-oxidation by oleate, thereby preventing abnormal DAG synthesis and PKC/NF-κB activations [28]. Clearly, the protective mechanism of oleate against palmitate-induced insulin resistance will require more definition. Preliminary results in serum-free conditions (Fig. 7C) suggest that oleate action is independent of tropic factors such as insulin.

Ceramide is a bioactive lipid implicated in insulin resistance, type 2 diabetes and metabolic derangements associated with obesity [58]. Excess ceramide synthesized by peripheral tissue (e.g. liver, adipocytes) circulates in the blood and may trigger neurodegeneration in AD [59]. Palmitate-induced insulin resistance in muscle cells is in part mediated by de novo synthesis of ceramide [27] and oleate may block ceramide formation [60]. However, de novo synthesis of ceramide was not necessary for palmitate-induced insulin resistance in our neuronal cells, as was also reported in hepatocytes [61]. We observed that oleate blocked pre-formed ceramide-induced insulin resistance and toxicity. This result suggests that oleate can directly antagonize ceramide action as an added mechanism, since palmitate toxicity was independent of ceramide synthesis (Supplemental S5). Of interest then, it remains to test in neurons if oleate diverts palmitate from incorporation into ceramide (or into DAG) as in muscle [27,28,60] or inhibits biosynthetic enzymes, serine palmitoyltransferase and/or ceramide synthase.

Palmitate-induced cell death involves caspase activation. The increased cleavage of caspase-3 and -9 following palmitate treatment appears to support an apoptotic mechanism in non-neuronal cells [23,26,

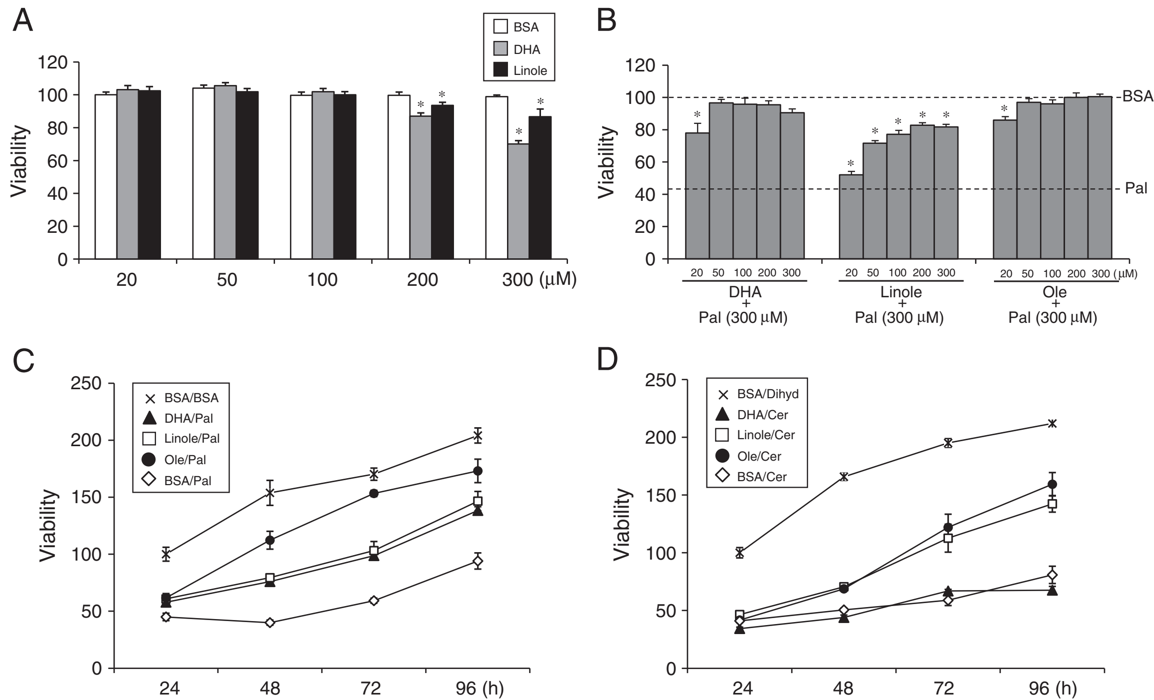

Fig. 9. Comparison of DHA, linoleate and oleate. (A) High concentrations (≥200 μM) of DHA or linoleate (Linole) alone decreased cell viability (compare to Fig. 1A, oleate). (B) DHA, linoleate and oleate (Ole) completely or partially blocked palmitate-induced decrease in viability. Various concentrations of DHA, linoleate or oleate were incubated with or without palmitate (Pal, 300 μM) for 24 h in N2a cells and WST-1 viability was measured for the last 4 h of the 24 h period. *$p < 0.01$ vs. BSA, n = 4 per group. Results are normalized to BSA control. Palmitate alone treated cultures were 42% viable (see Fig. 1A). (C) Oleate preconditioning yielded stronger protection against palmitate compared to DHA or linoleate at the longer 48, 72 and 96 h time points ($p < 0.01$). (D) Oleate or linoleate preconditioning showed stronger protection against ceramide compared to DHA at 48, 72 and 96 h ($p < 0.01$). N2a cells were incubated with each 100 μM of DHA, linoleate, oleate or BSA for 24 h. Media containing DHA, linoleate, oleate or BSA were replaced by new media containing palmitate (300 μM) or ceramide (Cer, 70 μM) and then further incubated for 24–96 h. n = 3 per group. Dihydroceramide (Dihyd, 70 μM) was used as control.

44,62]. Palmitate also increased cleaved caspase-3 and -9 in N2a cells and we extend the result to show that oleate preconditioning completely reversed this and prevented cell death. Oleate cytoprotection is demonstrated in various non-neuronal cell types [27,44,63].

We showed involvement of PKA in the protective effect of oleate against palmitate. PKA activation induces downstream proteins such as PGC-1α and PPARα [28]. Perhaps resulting from PGC-1α action, oleate via the PKA signaling pathway contributes, at least in part, to protect neuronal cells from palmitate-induced cytotoxicity. We also demonstrated that oleate, either preconditioning with or in coincubations with palmitate, increased cellular TG in neuronal cells. Moreover, oleate reversed palmitate-induced DAG synthesis. These two changes are consistent with previous reports showing that oleate may further protect palmitate-induced toxicity by increasing the sequestering TG droplets in non-neuronal cells [28,64].

PUFAs are promoted in therapeutic supplements against degenerative or inflammatory diseases [32,65]. The consumption of mono- or poly-unsaturated FFAs and the reduced risk of MCI and AD have been recently reviewed [50]. In this study, we give multiple levels of evidence that oleate has superior protective effects against palmitate damage to neuronal cells, even compared to other PUFAs such as DHA (n-3) and linoleate (n-6) that have received wider clinical study [66,67]. The oleate effect extends to ceramide toxicity in neuronal cells. It is also specific, in the sense of not mitigating several other damaging paradigms. Our findings recommend in vivo studies to determine effects of systemic oleate supplementation on neuronal protection against circulating sFFAs and ceramide in models of neurodegeneration.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2014.04.004.

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