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Oleate prevents palmitate-induced cytotoxic stress in cardiac myocytes

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Abstract

The cytotoxicity of saturated fatty acids has been implicated in the pathophysiology of cardiovascular disease, though their effects on cardiac myocytes are incompletely understood. We examined the effects of palmitate and the mono-unsaturated fatty acid oleate on neonatal rat ventricular myocyte cell biology. Palmitate (0.5 mM) increased oxidative stress, as well as activation of the stress-associated protein kinases (SAPK) p38, Erk1/2, and JNK, following 18 h and induced apoptosis in ~20% of cells after 24 h. Neither antioxidants nor SAPK inhibitors prevented palmitate-induced apoptosis. Low concentrations of oleate (0.1 mM) completely inhibited palmitate-induced oxidative stress, SAPK activation, and apoptosis. Increasing mitochondrial uptake of palmitate with L-carnitine decreased apoptosis, while decreasing uptake with the carnitine palmitoyl transferase-1 inhibitor perhexiline nearly doubled palmitate-induced apoptosis. These results support a model for palmitate-induced apoptosis, activation of SAPKs, and protein oxidative stress in myocytes that involves cytosolic accumulation of saturated fatty acids.
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Dyslipidemia has received increasing attention as a pathogenetic factor in the progression of diabetes as well as the high risk of end organ damage such as atherosclerosis, neuropathy, renal failure, and heart failure in the metabolic syndrome. While vascular disease leading to myocardial ischemia is one commonly accepted mechanism for cardiac dysfunction in diabetes, a direct effect of lipid accumulation in cardiac myocytes has also been demonstrated and may play an equal role in the cardiac dysfunction observed in obesity and diabetes [1,2].

The most well-characterized effect of saturated fatty acids on cell biology is induction of cell death. In vitro studies have demonstrated that saturated fatty acids induce apoptosis in isolated cardiac myocytes [3], suggesting one mechanism by which lipid accumulation may promote myocardial dysfunction. Cardiomyocyte apoptosis also occurs in vivo in both diabetic animal models [1] and in transgenic animals with targeted overexpression of the long-chain fatty acid transporter [4]. The mechanism by which saturated fatty acids induce apoptosis in myocytes remains incompletely understood.

Other forms of stress such as oxidative stress, either through the build-up of fatty acid intermediates or other mechanisms, has been implicated but remain controversial across cell types [5–8]. Finally, the effect of saturated fatty acids on a host of so-called 'stress-activated proteins kinases' (SAPK) in myocytes which are known to regulate myocyte structure, function, and survival [6,9–11] remains unknown. In this study, we demonstrate that palmitate has broad effects on myocyte biology, and that all of its effects can be prevented by low concentrations of the mono-unsaturated fatty acid oleate. These data support a

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model where the cardiotoxicity of excess saturated fatty acids is driven by cytosolic accumulation of saturated fatty acids in myocyte cytosol.

Methods

Primary culture of ventricular myocytes. Neonatal rat ventricular myocytes (NRVM) were isolated as previously described [12]. In brief, ventricles were excised from 1- to 2-day-old Sprague–Dawley rat pups. Hearts were digested with trypsin (Sigma) overnight followed by collagenase (Worthington Biochemical) the next morning. Following preplating to increase myocyte purity, cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco) with 7% fetal bovine serum (FCS) (Gibco) for 24 or 48 h before serum starvation or treatment.

Cell culture conditions and treatments. Palmitic and oleic acids (sodium salt) were added to DMEM containing 2% w/v fatty acid-free (unless otherwise noted) bovine serum albumin (BSA, Sigma Cat. #A0281) (for a final molar ratio of 0.6:1, BSA/FA when [FA] = 0.5 mM) and either 25 mM glucose ('high glucose') or 5 mM glucose + 20 mM mannitol ('low glucose'). The fatty acids were added to the media by first dissolving in 90 °C water. All reagents listed above as well as L-carnitine, fumonisin B1, *N*-acetyl cysteine, triacsin C, and perhexiline were purchased from Sigma. U0126, MnTMPyP, and SB202190 were purchased from Calbiochem. Adenovirally overexpressed dominant negative JNK and constitutively active ceramidase were used as previously described [6,13].

Flow cytometry (FACS). Apoptosis was quantified as previously described [12] using flow cytometry. In brief, following treatment, both nonadherent and trypsinized adherent cells were collected, resuspended in phosphate-buffered solution (PBS), and fixed with ethanol (1:2 PBS/EtOH) at −20 °C. In preparation for FACS, cells were resuspended in PBS containing 20 μg/ml propidium iodide (PI) (Sigma) plus 0.1 mg/ml (~5 kU/ml) of RNase A (USB) and incubated at room temperature for 2–4 h. Gating was performed on the cells to exclude small debris with >2 logs weaker staining for PI than G_0 cells. The hypodiploid population of cells remaining was considered apoptotic. Fluorescence of 10,000 cells was measured and apoptosis was calculated as the percent of cells in the sub-G_1/G_0 peak.

DNA ladder. Examination of apoptosis by DNA ladder was performed as previously described [12]. Following treatment, cells were rinsed 1× with phosphate buffer solution (PBS) and then lysed with 200 μl buffer containing 0.5% Triton X, 10 mM EDTA (pH 8.0), and 10 mM Tris–HCl (pH 7.4). Lysate was centrifuged for 20 min at 14,000*g*. RNase A (USB) was added to the supernatant at a final concentration of 0.4 mg/ml and incubated at 37 °C for 1 h, followed by proteinase K (Gibco-BRL) (0.4 mg/ml) for 1 additional hour at 37 °C. DNA was precipitated with 1 mM NaCl and 50% propanol overnight at −20 °C. DNA was resolved by agarose gel electrophoresis and examined for ethidium bromide fluorescence.

Western blot analysis. To characterize the activity of related kinases, Western blots were performed on primary culture lysates as previously described [9]. Cells were lysed in a modified RIPA buffer (1% NP-40 (Calbiochem), 0.25% deoxycholic acid, 50 mM Tris–HCl (pH 7.4), 1 mM EDTA, 150 mM NaCl, 1 mM NaF, 1 μg/ml leupeptin, 1 mM PMSF, and 1 mM sodium orthovanadate). Protein concentrations were quantified with the Bradford reagent (Bio-Rad) and normalized protein (50–100 μg) was run on a 10% Tris–HCl ready gel (Bio-Rad). Protein was transferred to a polyvinylidene difluoride (PVDF) membrane by semi-dry transfer (Bio-Rad) at a constant current of 200 mA/gel for 20 min. Western blotting for phospho-p38, phospho-JNK, phospho-Erk1/2 (cell signaling), and unphosphorylated Erk2 (Santa Cruz) was performed according to the recommendations of the manufacturer.

AMPK overexpression and detection. Following 20 h of plating, media were aspirated from NRVMs and replaced with DMEM + 7% FCS containing virus for a multiplicity of infection (MOI) between 10 and 50. AMPK activity was calculated in vitro following cell lysis. After washing cells 2× with PBS, cells were lysed in a buffer containing Tris–HCl (50 mM, pH 7.4), NaF (50 mM), NaPPi (5 mM), EDTA (1 mM), EGTA (1 mM), mannitol (250 mM), Triton X (1%), DTT (1 mM), and the protease inhibitors leupeptin and pepstatin. Protein concentration was calculated by the Bradford assay (Bio-Rad) and normalized protein was split into two tubes. Volume was also normalized prior to overnight immunoprecipitation with anti-AMPK-α 1 (vendor) at 4 °C. Protein A/G beads (Santa Cruz) were added in the morning to the IP mixture for 3 h at 4 °C. Beads were washed three times with a buffer similar to the lysis buffer, with the exception of mannitol and Triton X-100, and NaCl (1 M) was added. The beads were then washed two times with the reaction buffer which contained Hepes (40 mM, pH 7.4), NaCl (80 mM), $MgCl_2$ (5 mM), and DTT (1 mM). The in vitro reaction took place for 10 min at 37 °C, by incubating the beads with 1× reaction buffer, 0.467 mg/ml SAMS peptide, 110 ng/ml cold ATP, 0.2 μCi [γ-^{32}P]ATP, and either 0 or 70 μg/ml AMP. The reaction was stopped by placing the samples on ice and then a portion of the sample was spotted on p81 phosphocellulose paper (Whatman). Spotted paper was washed 5× 20 min with 1% phosphoric acid and then counted on a scintillation counter.

Statistical analysis. Quantified results were analyzed for statistical significance by either one-way or two-way ANOVA or Student's *t* test.

Results and discussion

Palmitate-induced apoptosis is inhibited by low concentrations of oleate

Treatment of NRVM with 0.5 mM palmitate, but not oleate, caused increased cell death (Fig. 1). The effect of palmitate was seen only when nominally fatty acid-free albumin (Sigma Cat. #A0281) was used as a fatty acid carrier. If standard, unpurified albumin was used (Sigma Cat. #A3059), the effect of palmitate on cell survival varied markedly depending on the lot (data not shown). The region of cells with hypodiploid DNA, considered to be apoptotic, is illustrated by bars on the representative histograms from $n > 10$ experiments (Fig. 1). The increase in hypodiploid myocytes was consistent with the observed phenotypical changes seen by phase contrast microscopy (Fig. 1). We examined the concentration dependence of palmitate-induced apoptosis and found that [palmitate] > 0.25 mM was sufficient to induce apoptosis while there was no significant difference between apoptotic rates at 0.4 mM vs. 0.5 mM palmitate (data not shown).

High glucose has been reported to induce apoptosis in various cell types [14–16], and this effect has also been implicated in the pathogenesis of diabetic cardiovascular complications. However, we found that 25 mM glucose had no effect on NRVM survival at 24 h either on its own, in the presence of palmitate (Fig. 2B) or in the presence of 0.5 mM oleate (data not shown). Co-treatment with low concentrations of the mono-unsaturated fatty acid oleate (0.1 mM) completely inhibited palmitate-induced apoptosis (Figs. 2A and B).

Palmitate-induced oxidative stress is reversed by low concentration oleate

Oxidative stress induces apoptosis in cardiac myocytes [6,9,17,18] and has been implicated in palmitate-induced apoptosis in other cell types. In CHO cells, the ROS scavengers pyrolidine dithiocarbamate and 4,5-dihydroxy-1,3-

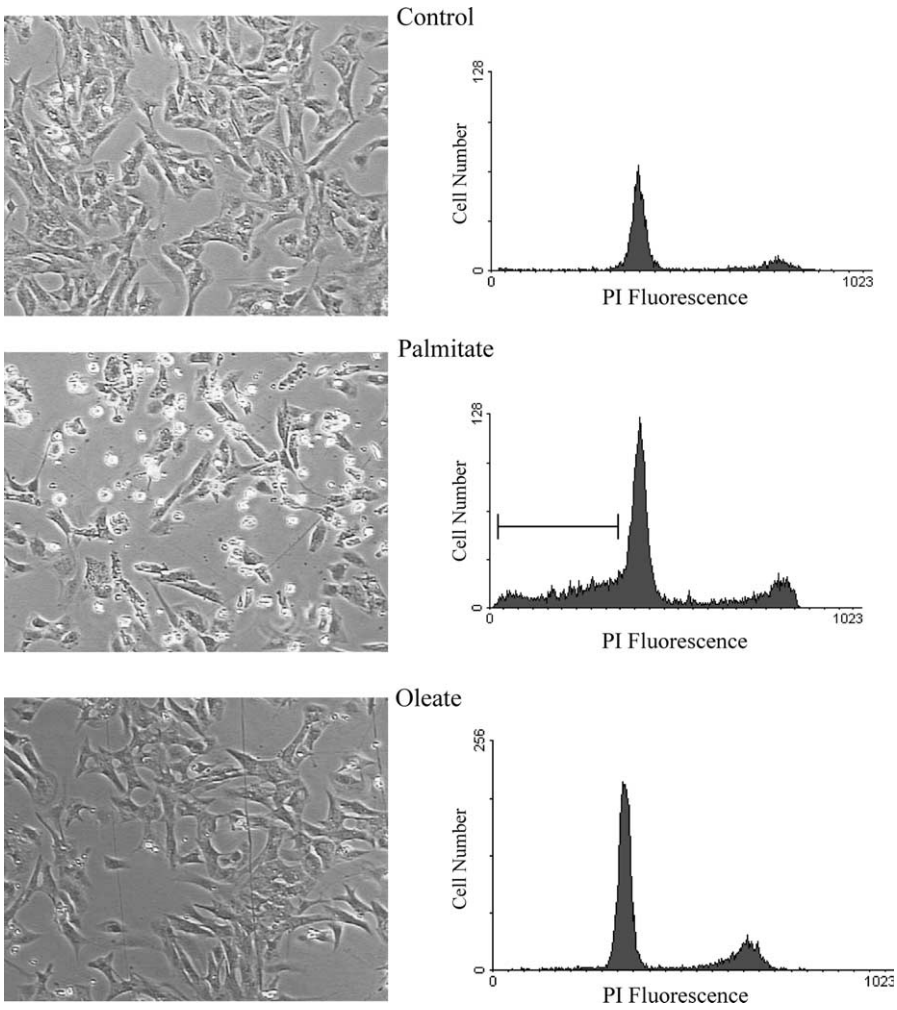

Fig. 1. The saturated fatty acid palmitate causes apoptosis in NRVM, whereas the mono-unsaturated fatty acid oleate has no effect. Phase contrast microscopic images following 24 h treatment with or without 0.5 mM palmitate or oleate. FACS histograms of PI fluorescence show an increase in the number of myocytes with hypodiploid DNA content in cells treated for 24 h with 0.5 mM palmitate (representative pictures from $n > 10$ experiments).

benzene-disulfonic acid (DBDA) both inhibited palmitate-induced caspase-3 activity and DNA laddering [5]. However, in NRVM the ROS scavengers DBDA, 5-aminosalicylic acid, and pyrolidine dithiocarbamate had no effect on palmitate-induced caspase-3 activity [8]. In this study, we examined palmitate- and oleate-induced oxidative stress in NRVM by measuring myocyte protein carbonyl production. We found that palmitate but not oleate caused an increase in protein carbonyls. Like palmitate-induced apoptosis, palmitate-induced carbonyl formation was completely prevented by addition of 0.1 mM oleate (Fig. 3A). The SOD/catalase mimetic MnTMPyP and the ROS scavenger *N*-acetyl-cysteine (NAC) are potent antioxidants that inhibit oxidative stress-induced apoptosis in myocytes [9]. However, neither MnTMPyP (50 μM) nor NAC (100 μM) had any effect on palmitate-induced apoptosis in NRVM (Fig. 3B). Thus, while palmitate does induce cellular oxidative stress, this appears to be qualitatively and/or quantitatively different from other forms of oxidative stress sufficient to induce apoptosis. The increase in permanent protein oxidative damage by palmitate, while not sufficient to induce myocyte death, may nevertheless play an important role in cardiac dysfunction in the setting of dyslipidemia. For example, whether palmitate-induced protein carbonyl formation is the mechanism for observed damage to cardiac sarcomeric proteins [19] warrants further investigation.

Palmitate-induced stress-associated protein kinase activation is inhibited by oleate

Stress-activated protein kinases have been implicated in the modulation of apoptosis [6,10]. We found that 0.5 mM palmitate treatment for 18 h increased phosphorylation of Erk1/2, p38, and JNK. Similar to apoptosis and carbonyl formation, co-treatment with 0.1 mM oleate prevented palmitate-induced activation of these kinases (Figs. 4A and B). We found that pretreatment of myocytes with either a MEK1/2 inhibitor (U0126, 10 μM) or a p38 kinase inhibitor (SB202190, 5 μM) had no effect on baseline or palmitate-induced apoptosis. Adenoviral overexpression of a dominant-negative JNK (dn-JNK) caused a slight

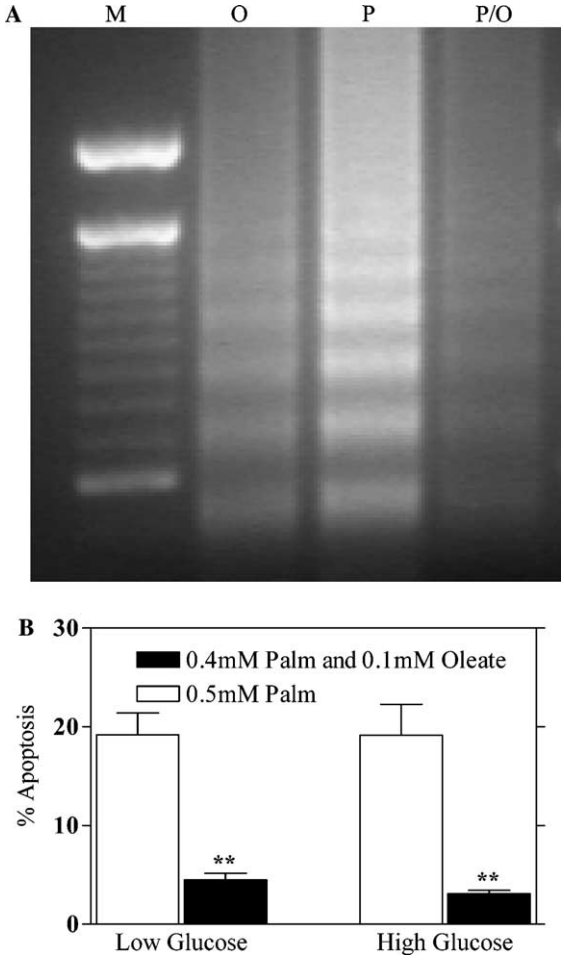

Fig. 2. Palmitate-induced apoptosis is prevented by low concentrations of oleate. Apoptosis following 24 h treatment with 0.5 mM oleate, palmitate or the two fatty acids combined was assessed (A) by DNA ladder on a 1.5% agarose gel (M, marker; O, 0.5 mM oleate; P, 0.4 mM palmitate; and P/O, 0.4 mM palmitate with 0.1 mM oleate) and (B) quantified by flow cytometry ($n = 4$, $^{**}p < 0.001$ vs. palm alone).

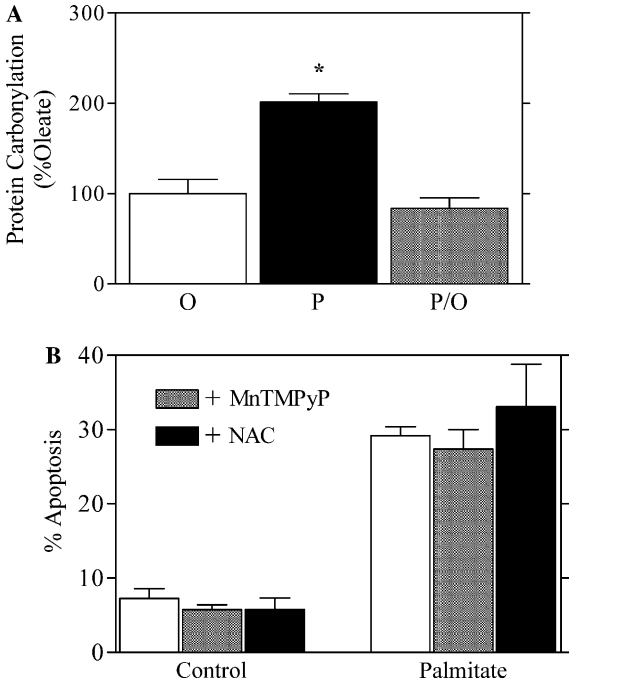

Fig. 3. Palmitate-induced oxidative stress is reversed by oleate. (A) Oxidative stress was determined by measurement of protein carbonylation. Following 18 h of treatment with 0.5 mM oleate, palmitate or a combination (0.4:0.1, palm/oleate), cells were lysed and analyzed for protein carbonylation ($n = 3$, $^{*}p < 0.05$ vs. both palm and palm/oleate). (B) Palmitate-induced apoptosis was unchanged following 24 h co-treatment with the SOD mimetic MnTMPyP or the ROS scavenger *N*-acetyl cysteine ($n = 3$, ns).

but statistically significant increase in myocyte apoptosis in control conditions. However, there was no significant interaction between palmitate and dn-JNK (Fig. 4C). Thus, these kinases do not appear to be mechanistic for palmitate-induced apoptosis in myocytes. However, given the general importance of these pathways in the regulation of myocyte gene expression, palmitate-induced SAPK activation may contribute to myocardial dysfunction through changes in expression of genes that regulate hypertrophy and protein turnover.

Alterations in FA metabolism and palmitate-induced apoptosis

Lipid signaling intermediates such as diacylglycerol (DAG) and ceramide have been implicated as potential mechanisms for saturated fatty acid-induced apoptosis in pancreatic β-cells [11], CHO cells [20], and adult rat ventricular myocytes in long-term culture [19]. Palmitate accumulation into DAG in skeletal muscle is suppressed by co-administration of oleate by favoring triacylglycerol (TAG) synthesis [21], a theory that has been implicated in oleate-mediated protection from palmitate-induced apoptosis [22], and may be extended to oleate rescue of other palmitate-induced stresses. For this model, the relative distribution of palmitate between mitochondrial oxidation, TAG synthesis, and accumulation as DAG and/or ceramide will determine the toxicity of palmitate within a given cell. We tested this model in myocytes by using pharmacologic and molecular strategies to alter the rate of palmitate metabolism and/or uptake into mitochondria.

Inhibition of palmitate mitochondrial uptake by the fatty acid transporter carnitine palmitoyl transferase-1 (CPT-1) with perhexiline (10 μM) caused a ~2-fold increase in palmitate-induced apoptosis (Fig. 5A). Triacsin C (10 μM), which prevents the movement of palmitate to TAG stores as well as mitochondrial uptake, through inhibition of fatty acyl CoA synthetase [23], also caused a ~2-fold increase in palmitate-induced apoptosis (Fig. 5C). In contrast, L-carnitine (30 mM), which increases mitochondrial long-chain fatty acid uptake via increasing CPT-1 activity, caused a significant decrease in apoptosis induced by palmitate (Fig. 5B).

Based upon these data, we hypothesized that activation of the enzyme 5′ adenosine monophosphate-activated protein kinase (AMPK) should also suppress palmitate-induced apoptosis via its activation of fatty acid

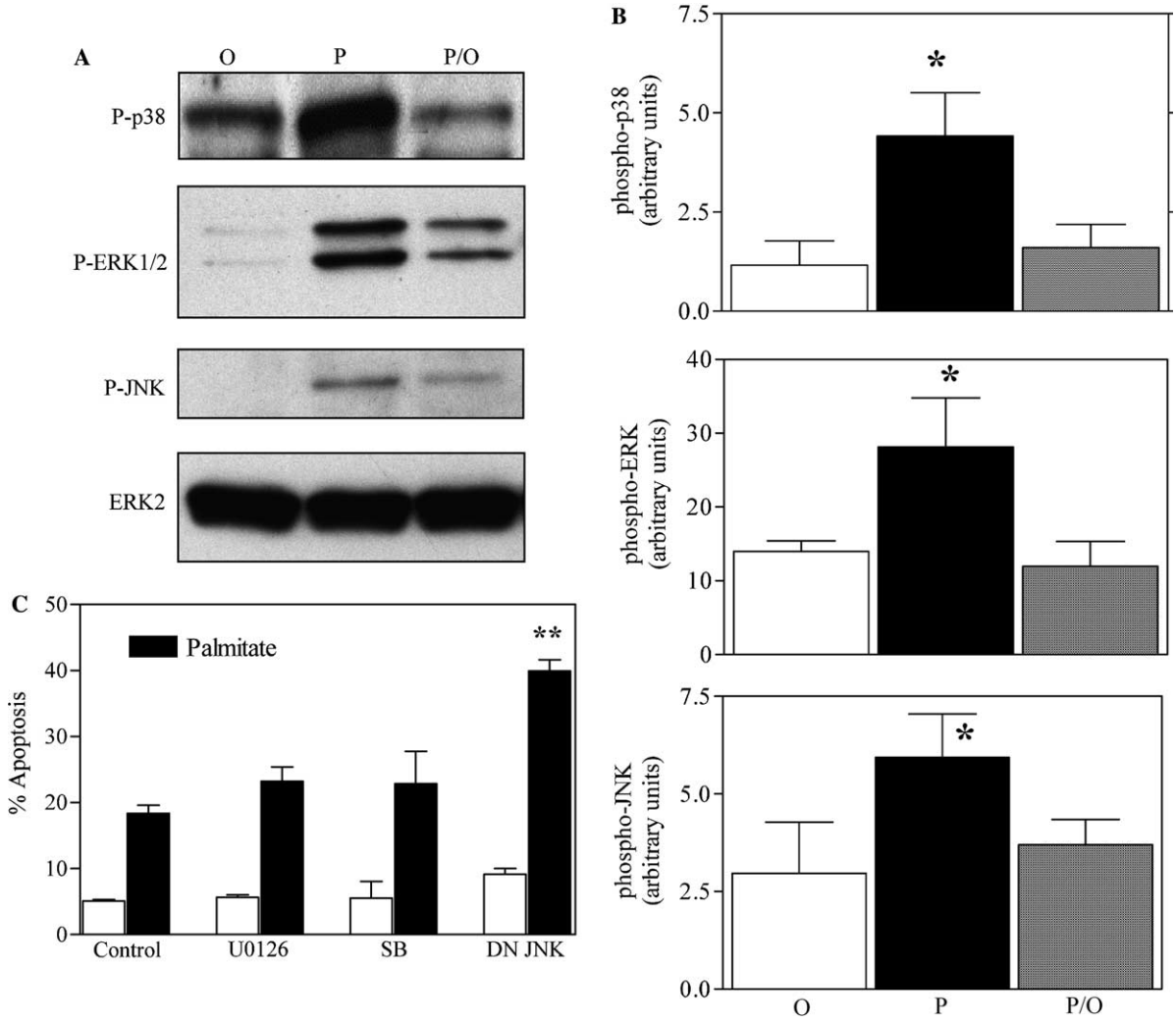

Fig. 4. Palmitate-induced activation of the p38, ERK1/2, and JNK SAPKs is reversed by oleate. (A) NRVMs were treated with 0.5 mM oleate, palmitate or a combination (0.4:0.1, palm/oleate) for 18 h prior to lysis and Western blot analysis (representative blots from four experiments). (B) Quantification of Western blot analysis, arbitrary units ($n = 4$, $^{*}p < 0.05$ vs. oleate and P/O). (C) Apoptosis was measured by flow cytometry following 24 h co-treatment with 0.5 mM palmitate and either U0126 (10 μM) or SB (5 μM). Cells were infected with dominant negative JNK-1 (dn-JNK) 24 h prior to palmitate treatment while control cells were infected with GFP. While dn-JNK caused a significant increase in baseline apoptosis ($^{**}p < 0.05$), no inhibitor had a significant interaction with palmitate-induced apoptosis ($n = 3$–5).

oxidation. We overexpressed α1-AMPK to levels that resulted in a >3-fold increase in specific activity, as measured by phosphorylation of SAMS peptide. We did not see any effect of AMPK overexpression on palmitate-induced apoptosis ($n = 3$, data not shown). This may, in part, be explained by the negative effect of AMPK on glycerol-3-phosphate acyltransferase (GPAT), the rate-limiting enzyme in TAG synthesis [24,25]. Thus, AMPK activation may activate palmitate utilization, but prevent palmitate shuttling to inert lipid stores. This is in contrast to agents that increase peroxisomal fatty acid oxidation in cardiac myocytes by activating PPARα (with no known effect on GPAT), which have been shown to limit palmitate-induced apoptosis [26].

One potential mechanism by which cytosolic palmitate might induce apoptosis is via induction of ceramide synthesis [7,13,19,27]. In NRVM, palmitate treatment lowers the rate of fatty acid oxidation and leads to the accumulation of ceramide in a time course similar to the induction of apoptosis [7,27,28]. However, we found that neither the ceramide synthase inhibitor fumonisin B1 nor adenovirally overexpressed constitutively active ceramidase caused a significant decrease in palmitate-induced apoptosis ($n = 3$, data not shown). This suggests that accumulation of palmitate alone, or metabolism to some other signaling intermediate such as diacylglycerol, may be responsible for palmitate-induced apoptosis in cardiac myocytes.

Collectively these data show that the saturated long-chain fatty acid palmitate induces multiple forms of cell stress in isolated cardiac myocytes, resulting in the activation of several stress-associated signaling pathways, protein oxidative stress, as well as apoptosis. The effects on apoptosis appear to be independent of palmitate's effects on oxidative stress and SAPKs. The induction of each

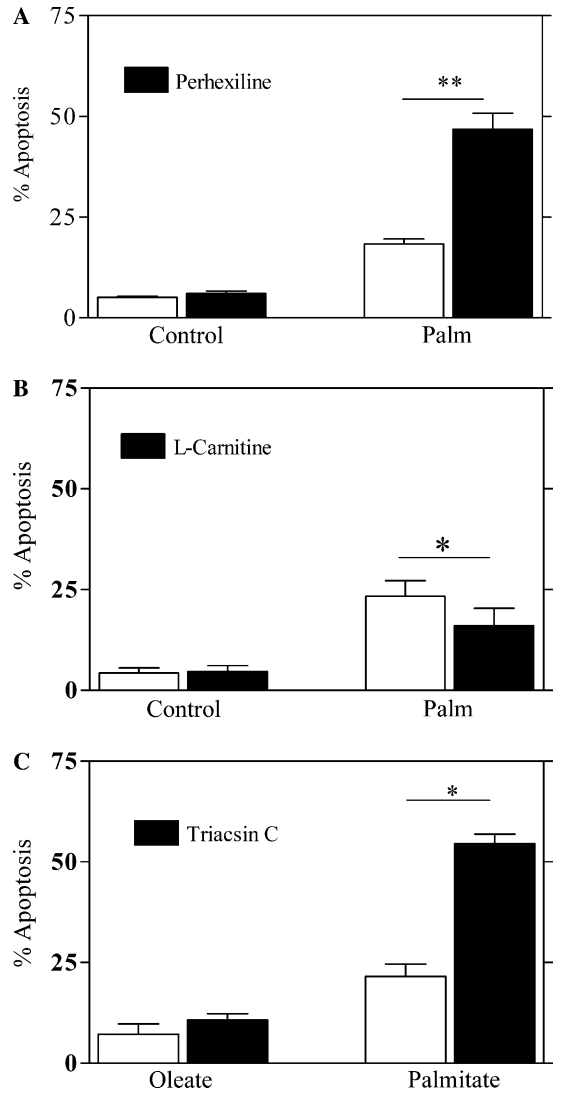

Fig. 5. Regulators of fatty acid metabolism alter palmitate-induced apoptosis. (A) Myocytes co-treated for 24 h with the carnitine palmitoyl transferase-1 inhibitor perhexiline (10 μM) caused a doubling in palmitate-induced apoptosis ($n = 3$, $^{**}p < 0.001$). (B) L-Carnitine (30 mM) slightly decreased palmitate-induced apoptosis ($n = 3$, $^{*}p < 0.01$). (C) Triacsin C (10 μM) caused a ~2-fold increase in palmitate-induced apoptosis ($n = 3$, $^{*}p < 0.01$).

of these events warrants further investigation as a potential contributor to the cardiac dysfunction that occurs in the setting of dyslipidemia. The prevention of these palmitate effects by low concentrations of oleate suggests that strategies that increase fatty acid oxidation and/or prevent cytosolic fatty acid accumulation will provide universal protection of the heart from saturated fatty acid induced toxicity.

Acknowledgments

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