Activation of AMP-Activated Protein Kinase Prevents Lipotoxicity in Retinal Pericytes

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Purpose. The recent FIELD study demonstrated that the lipid-lowering agent fenofibrate significantly reduces the development and progression of diabetic retinopathy (DR). These results suggest that lipids may play a causal role in DR. They also suggest that AMP-activated protein kinase (AMPK) activation could account for these findings given that fenofibrate is an AMPK activator. The authors previously demonstrated that free fatty acids, in addition to hyperglycemia, can induce apoptosis in retinal pericytes (PCs), the first cells lost in the diabetic retina. Incubation with the saturated fatty acid palmitate, but not the monounsaturated fatty acid oleate, elicited cytotoxicity in a manner dependent on oxidative stress, NF-κB activation, and ceramide accumulation. In this study, the authors explored whether AMPK can downregulate these pathways and, in doing so, protect PCs from apoptosis.

Methods. PCs were incubated with palmitate or oleate to determine whether the factors previously linked to lipotoxicity were uniquely increased by palmitate. The effects of AMPK activation on these parameters and on apoptosis were concurrently examined.

Results. Only palmitate increased NF-κB activation, ceramide and diacylglycerol mass, and apoptosis. Activation of AMPK with AICAR or, where used, expression of a constitutively active AMPK prevented all these effects. In contrast, both palmitate and oleate markedly increased oxidative stress, and the activation of AMPK did not prevent this.

Conclusions. AMPK activation prevents the metabolic abnormalities and apoptosis specifically caused by palmitate in cultured PCs. Pharmacologic agents that activate AMPK in the diabetic retina may warrant consideration as a therapeutic option to avert PC apoptosis and to maintain microvascular homeostasis. (*Invest Ophthalmol Vis Sci.* 2011;52:3630–3639) DOI:10.1167/iovs.10-5784

Diabetic retinopathy (DR) is a leading cause of blindness in adults worldwide.[1] During its course retinal cells of both vascular and neural origin undergo apoptosis that leads to interruptions in nutritive blood flow, neural dysfunction, and, ultimately, impaired vision. One of the earliest cells to undergo apoptosis in this setting is the microvascular pericyte (PC).[2] Various theories have attributed PC apoptosis to hyperglycemia-induced increases in sorbitol, hexosamines, advanced glycation-end products, and protein kinase C (PKC) activity, all of which have also been linked to the death of endothelial and neural cells in the retina.[3–7] In addition to hyperglycemia, dyslipidemia has recently been implicated in the pathogenesis of DR in humans. Thus, results from the Action to Control Cardiovascular Risk in Diabetes (ACCORD) eye study[8] and especially the Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) study[9] demonstrated that the lipid-lowering agent fenofibrate reduced progression to retinopathy, necessitating laser treatment by 31% in patients with type 2 diabetes and good glycemic control (HbA_{1C}, ~7.0%). Like hyperglycemia, dyslipidemia, including elevated serum free fatty acid (FFA) levels, is a common feature in patients with poorly controlled types 1 and 2 diabetes.[10,11] We have demonstrated that elevated levels of the FFA palmitate increase apoptosis in bovine retinal PCs by an effect dependent on increases in oxidative stress, ceramide synthesis, and NF-κB activation.[12] Evidence that elevated FFAs, and specifically saturated fatty acids such as palmitate, can cause dysfunction is strongly suggested by studies with other cultured cells. Thus, the incubation of pancreatic β cells, cardiomyocytes, skeletal muscle myotubes, and vascular endothelium with elevated levels of FFAs has all been shown to cause apoptosis, insulin resistance, or both.[13–17]

In addition to lowering plasma lipids, fenofibrate, the main drug tested in the FIELD study, is an activator of AMP-activated protein kinase (AMPK).[18] AMPK is a fuel and stress-sensing enzyme that is activated by such antidiabetic and lipid-lowering therapies as metformin, the thiazolidinediones and statins, and cellular energy deficits caused by hypoxia and exercise. Once activated, AMPK phosphorylates key metabolic enzymes resulting in an increase in processes that generate adenosine triphosphate (ATP), such as fatty acid oxidation (FAox), and a decrease in others that consume ATP but are not acutely necessary for survival, such as fatty acid and triglyceride synthesis.[17,19,20] In this regard, AMPK has the potential to protect cells against the adverse effects of high glucose and FFAs by preventing the accumulation of damaging or toxic secondary metabolites such as diacylglycerol (DAG) or ceramides and by effects on multiple transcriptional activators and coactivators.[21,22] In the present study, we tested whether AMPK activation protects PCs against palmitate-induced cytotoxic-

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Presented in part at the 2008 American Diabetes Association Scientific Sessions, San Francisco, California, June 6–10, 2008.

Supported by United States Public Health Service Grant PO1-HL68758-01-A1, a Diabetes Complications Center grant from the Juvenile Diabetes Research Foundation (NR), and the Kilo Foundation (YI).

Submitted for publication April 26, 2010; revised November 22, 2010, and January 14, 2011; accepted January 16, 2011.

Disclosure: **J.M. Cacicedo**, None; **S. Benjachareonwong**, None; **E. Chou**, None; **N. Yagihashi**, None; **N.B. Ruderman**, None; **Y. Ido**, None

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Investigative Ophthalmology & Visual Science, May 2011, Vol. 52, No. 6

ity. We also compared the effects on the PC of palmitate with that of oleate, a fatty acid that, at similar concentrations, is not toxic to PC.[12,23]

Materials and Methods

Pericyte Cell Culture

Bovine eyes were purchased from a local slaughterhouse and processed the same day, as previously described.[12] PCs were characterized by morphology, α-smooth muscle actin reactivity, and inability to take up rhodamine-conjugated acetylated low-density lipoprotein. Cells in passages 3 through 6, with 80% to 85% confluence, were used for all experiments.

Recombinant Adenovirus Constructs and Infection of Pericytes

Construction of recombinant replication-incompetent adenoviruses was performed as previously described.[24] The following genes were individually cloned into adenoviral constructs: β-glucuronidase (GUS) as an infection control (Invitrogen, Carlsbad, CA), CA-AMPK-α1 (in which Asp replaced Thr172, a generous gift from David Carling, London, UK), and ceramidase I (accession number BC003204; ATCC, Manassas, VA). Cloning, purification, and infection of PCs with adenovirus were performed as previously described.[12]

Incubation with Fatty Acids and Assessment of Apoptosis

PCs were incubated for the indicated period in DMEM containing 5.5 mM glucose supplemented with 5% FBS, 10 mM HEPES, and the indicated concentrations of palmitate or oleate (Sigma, St. Louis, MO) preconjugated with FFA-free BSA (Serologicals Co., Norcross, GA) at a 2:1 (FFA/BSA) molar ratio. This ratio was chosen because it is similar to that found in human blood, which at fasting ranges from 0.5:1 to 2:1 and averages 1.3:1.[25] The incubation media used in almost all the experiments (for exceptions, see Fig. 7C) contained 50 μM carnitine. Control cells (i.e., treated with No FFA or 0 mM palmitate) were incubated in media containing FFA-free BSA (Serologicals Co.) at the same concentration as the FFA-exposed cells. The BSA was very low in endotoxins as assessed by the supplier (3 EU/mg BSA vs. ~30–60 EU/mg BSA for standard albumin preparations). Cytotoxicity was measured by MTT assay in accordance with ATCC instructions. Apoptosis was assessed with a TdT-mediated dUTP nick end labeling (TUNEL) kit as per the manufacturer's instructions (Oncogene, Boston, MA) and by measurement of caspase-3 activity with a commercially available kit (Molecular Probes, Eugene, OR), as described previously.[26]

Studies of the Sphingolipid Cascade and Diacylglycerol

Measurements of total ceramide and DAG content, ^{3}H-serine incorporation into sphingolipids, and serine palmitoyltransferase (SPT) activity can be found in the Supplementary Methods (http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-5784/-/DCSupplemental).

Western Blot Analysis, Quantitative Real-Time PCR, and NF-κB Reporter Gene Assay

The methodologies for these measurements can be found in the Supplementary Methods (http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-5784/-/DCSupplemental).

Oxidative Stress Measurements

Methodologies for oxidative stress measurements, including dichlorofluorescein (DCF) fluorescence, 8-isoprostanes, and electron-spin resonance spectroscopy (ESRS), can be found in the Supplementary Methods (http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-5784/-/DCSupplemental).

Fatty Acid Oxidation Assay

Fatty acid oxidation (FAox) assay was assessed on the basis of ^3H_2O production from [9,10-^{3}H]-palmitate, as described by Moon and Rhead.[27] PCs grown to 85% confluence in Primaria six-well plates were preincubated for 24 hours in a medium consisting of Dulbecco's modified Eagle's medium (DMEM) with 5% FBS, 5.5 mM glucose, 1% penicillin/streptomycin, 50 μM carnitine, and 0.1 mM (nonradioactive) palmitate supplemented with a [9,10-^{3}H]-palmitate (18 μCi/mL) tracer. This preincubation was carried out to allow the ^{3}H-tracer concentration to reach equilibrium within the cells' internal fat pools, as described previously.[28] After prelabeling was completed, the media were changed to DMEM with 5% FBS, 5.5 mM glucose, 1% penicillin/streptomycin, 0.1 mM or 0.4 mM palmitate, supplemented with [9,10-^{3}H]-palmitate (with the same specific activity as the preincubation media), and the indicated FAox activator or inhibitor. Incubation media, containing the released ^3H_2O, were collected at the indicated times and transferred to glass test tubes. FFA-free BSA was added to the medium to a final concentration of 10% (wt/vol) followed by trichloroacetic (TCA) acid to precipitate the bound palmitate-BSA complexes. After this, the precipitated samples were centrifuged at 1000 rpm for 5 minutes at 4°C, and the supernatants were transferred to new test tubes and neutralized with NaOH. The neutralized samples were run through an AG1×8 (OH form) resin column (Bio-Rad, Hercules, CA) to remove the residual palmitate. Eluates were transferred to scintillation vials with 5 mL scintillation cocktail (Ecoscint; National Diagnostics, Irvine, CA). Samples were quantified by liquid scintillation counting and normalized by total cellular protein per well.

Statistical Analysis

All data were analyzed using either the SAS program (SAS Institute, Cary, NC) with the general linear mode procedure or with statistical graphing software (Prism, version 5.01 for Windows; GraphPad Software, San Diego, CA). Data were analyzed by *t*-test, or by one- or two-way ANOVA as appropriate. The Bonferroni method was used as the ANOVA posttest. $P < 0.05$ was taken as statistical significance. All the data are expressed as mean ± SE.

Results

AMPK Activation Prevents Palmitate-Induced Apoptosis in Pericytes

Time-course and dose-response experiments (Supplementary Fig. S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-5784/-/DCSupplemental) with concentrations of fatty acid that occur in the blood of persons with diabetes[29] demonstrated that palmitate, but not oleate, at concentrations of 0.4 mM or greater induced apoptosis-like cytotoxicity in PCs. To test more specifically for apoptosis, PCs were incubated with 0.4 mM palmitate for 72 hours and were stained for nicked DNA (TUNEL assay). As shown in Figures 1A and 1B, apoptosis, assessed by this means, was increased 10-fold. Coincubation with 1 mM AICAR, a cell-permeable AMPK activator, completely prevented this from occurring. Similarly, caspase-3 activity, another index of apoptosis, was increased by twofold when PCs were incubated with palmitate for 24 hours, and this was prevented both by AICAR (Fig. 1C) and adenoviral expression of a constitutively active AMPK.

Palmitate-Induced Ceramide and DAG Accumulation Is Prevented by the Activation of AMPK

We have previously shown that the sphingolipid ceramide, which can cause apoptosis by inhibiting mitochondrial respiration, accumulates in PCs incubated with palmitate for 24 hours in a dose-dependent manner and that this is necessary,

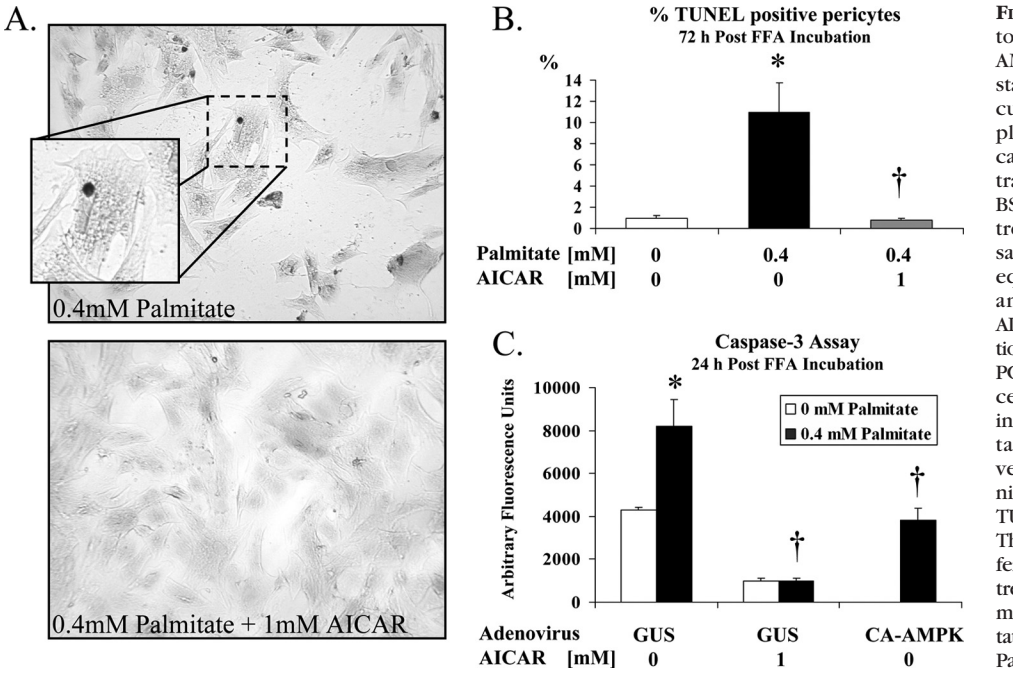

FIGURE 1. Palmitate-induced apoptosis is prevented by the activation of AMPK. (**A**) Micrographs of TUNEL-stained apoptotic PCs. PCs were incubated for 3 days with DMEM supplemented with 5% FBS, 50 μM carnitine, and the indicated concentration of palmitate conjugated to BSA at a 2:1 molar ratio. No FFA-treated PCs were incubated with the same medium, but it contained an equivalent concentration of BSA only and no FFA. The AMPK activator AICAR was coincubated at a concentration of 1 mM with the palmitate-treated PCs for the full 3 days. TUNEL-positive cells, with dark brown nuclei, were increased 10-fold because of palmitate incubation, which was prevented by AICAR coincubation. Magnification, 100×. (**B**) Quantification of TUNEL staining from **A** ($n = 12$). There were no significant statistical differences between *No FFA* and *AICAR*-treated groups. (**C**) Caspase-3 activity measured after 24 hours of BSA, palmitate, and coincubations with AICAR. Palmitate increased caspase-3 activity twofold in this time frame, which was prevented by AICAR or overexpression of a constitutively active AMPK (CA-AMPK). β-Glucuronidase (GUS) was used as an infection control. All data are from representative experiments ($n = 6$). *$P < 0.05$ vs. No FFA. †$P < 0.05$ vs. 0.4 mM palmitate.

though not sufficient, for palmitate-induced programmed cell death.[12] As demonstrated in Figure 2B, incubation of PCs with 0.4 mM palmitate for 24 hours caused a significant increase in ceramide accumulation that was prevented by coincubation with AICAR. In contrast, incubation with oleate had no effect on ceramide mass (Fig. 2B), in keeping with the observation

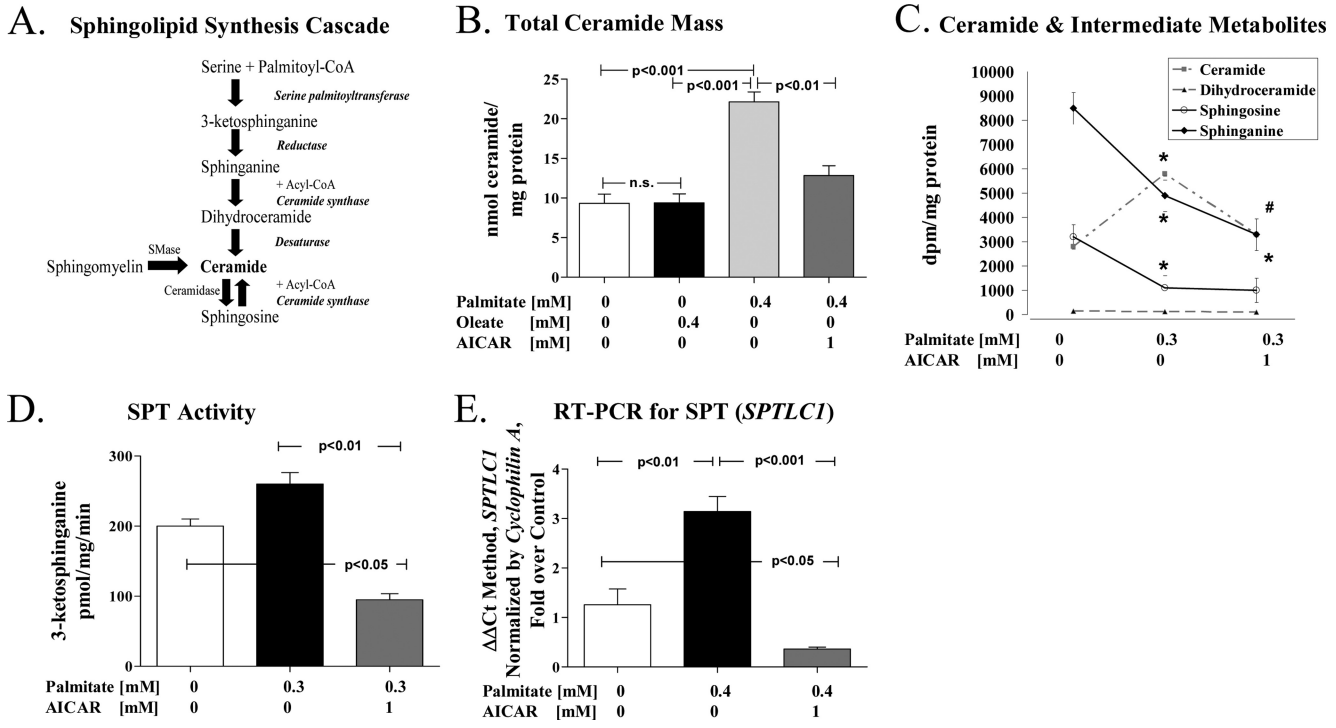

FIGURE 2. AICAR prevents ceramide accumulation by inhibiting the transcription of serine palmitoyl transferase. (**A**) De novo ceramide synthesis pathway. (**B**) Total ceramide mass was measured by the DAG kinase method from total lipid extracts after PCs were incubated with palmitate, oleate, or AICAR for 24 hours. (**C**) ^{3}H-L-serine incorporation into sphingolipids. PCs were incubated for 24 hours with the indicated concentration of palmitate or were coincubated with palmitate and AICAR. Then serine label incorporation into the various sphingolipid metabolites was measured, as described in Methods ($n = 4$). *$P < 0.05$ vs. 0 mM palmitate. #$P < 0.05$ vs. 0.4 mM palmitate. (**D**) PCs were incubated as in **C** but without the ^{3}H-L-serine tracer. SPT enzymatic activity was assessed from the microsomal fraction of the cellular lysates by the production of 3-ketosphinganine ($n = 4$). (**E**) PCs were incubated as in **D**. Their total RNA was then subjected to RT-PCR for SPT (*SPTLC1*) and *Cyclophilin A* gene transcripts ($n = 4$). All data are from representative experiments.

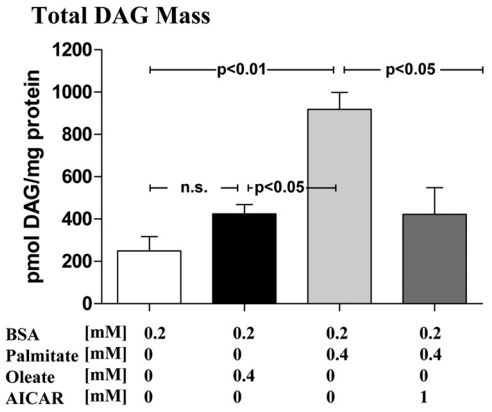

FIGURE 3. Effect of AICAR on palmitate-induced DAG mass accumulation after 24 hours of incubation. Because of the nature of the ceramide assay used in this study (i.e., DAG kinase method), total DAG levels could also be measured. DAG levels were four times higher in palmitate-treated PCs than in BSA-treated PCs. Coincubation with AICAR significantly prevented the rise in palmitate-induced DAG levels ($n = 4$).

that it does not increase PC apoptosis under these conditions.[12]

To determine at what step the activation of AMPK might inhibit sphingolipid synthesis (Fig. 2A), a tritiated serine tracer was given to PCs, and label incorporation into various sphingolipids was assessed by thin layer chromatography (Fig. 2C). Treatment with palmitate increased label incorporation into ceramide by twofold, compared with control BSA-treated cells, in which greater than 50% of the tracer was incorporated into sphinganine. Coincubation with AICAR prevented the incorporation of the tritiated serine into all pools of sphingolipids, suggesting that it downregulated the rate-limiting enzyme for de novo ceramide synthesis, SPT (Fig. 2A). Interestingly, SPT activity was increased by 31% in PCs incubated with palmitate, although the difference was not statistically significant. On the other hand, coincubation with AICAR fothreefold, and coincubationr 24 hours diminished activity by 60% below even control levels (Fig. 2D). In a subsequent experiment in which PCs were treated with AICAR for the last 2 hours of 24-hour palmitate incubation, AICAR had no effect on SPT activity (data not shown), suggesting that the effect of AMPK on SPT activity might have been indirect, perhaps by control of its transcription. To test this, PCs were incubated with palmitate in the presence or absence of AICAR for 24 hours, and their mRNA was subjected to RT-PCR for the SPT long chain 1 gene (*SPTLC1*). As shown in Figure 2E, palmitate increased the transcript levels of the *SPTLC1* gene by threefold, and coincubation with AICAR suppressed *SPTLC1* levels 50% below even control levels.

Another potentially toxic secondary lipid that has received much attention, especially in the DR literature, is DAG. Increased DAG levels have been reported in the retinas of diabetic animals, and it has been proposed that they alter retinal hemodynamics by effects on ECs and PCs. It has also been proposed that they contribute to retinopathy by activation of the PKCs.[30] Incubation with palmitate for 24 hours, but not oleate, increased DAG levels in the PCs by fourfold (Fig. 3), an effect completely prevented by coincubation with AICAR.

Palmitate-Induced NF-κB Activation Is Prevented by the Activation of AMPK

It has been demonstrated that palmitate induces NF-κB activation in PCs and that the prevention of such NF-κB activation by both genetic and pharmacologic means rescues the cells from palmitate-induced apoptosis.[12] In this context, it is noteworthy that NF-κB is upregulated, as is the NF-κB–driven mitochondrial pore-forming proapoptotic factor Bax (Bcl-2–associated X protein) in retinal PCs of persons with diabetes.[31,32] In the present study, incubating PCs with palmitate for 24 hours increased NF-κB reporter gene activity by approximately twofold (Fig. 4A), and coincubation with AICAR completely prevented this. In keeping with these findings, incubation with palmitate also increased the protein levels of the proapoptotic factor Bax threefold and diminished the levels of the prosurvival factor Bcl-2 (Fig. 4B). This palmitate-induced shift in the Bax/Bcl-2 ratio toward a proapoptotic phenotype (Fig. 1) was also completely prevented by AICAR. Incubation with oleate at concentrations equivalent to those of palmitate had no effect on NF-κB reporter gene activity (Fig. 4C).

To determine the specificity of AMPK in this pathway, we performed similar NF-κB reporter gene assays (as in Figure 4A) in human umbilical vein endothelial cells (HUVECs) transfected with plasmids encoding either CA- or dominant-negative AMPKs (Supplementary Fig. S2, http://www.iovs.org/lookup/

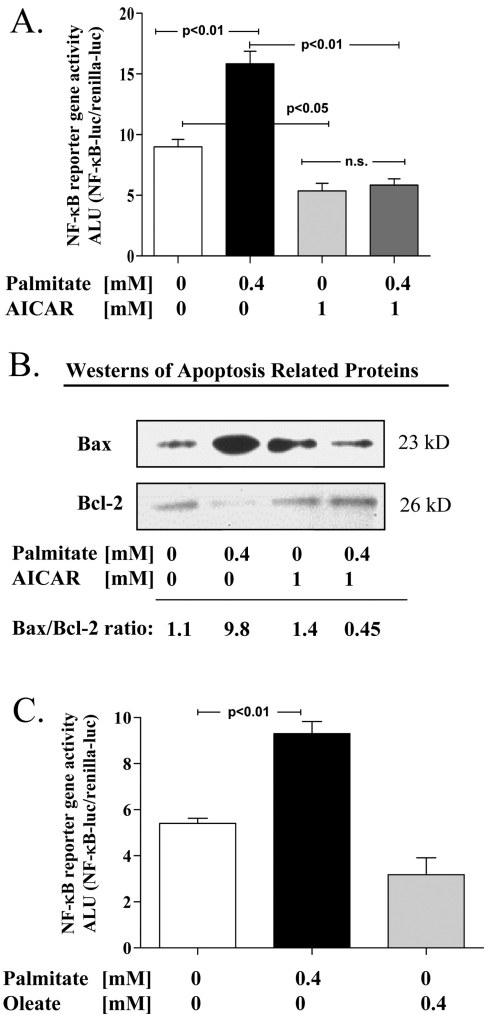

FIGURE 4. Effect of the AMPK activator AICAR on palmitate-induced NF-κB activation and apoptosis-related proteins. (**A**) NF-κB reporter gene activity was assayed after 24 hours of incubation with palmitate, AICAR, or both. Results are normalized to Renilla luciferase (from a cytomegalovirus-driven vector) to account for infection efficiency. Units are arbitrary light units (ALU). (**B**) Representative Western blots of the PCs Bax and Bcl-2 protein levels after 24 hours of incubation with palmitate, AICAR, or both. (**C**) NF-κB reporter gene activity was assayed after 24 hours of incubation with palmitate or oleate.

suppl/doi:10.1167/iovs.10-5784/-/DCSupplemental). HUVECs were used rather than PCs because they are more easily transfectable. We found that in the presence of CA-AMPK, NF-κB reporter gene activity was significantly suppressed under both basal and palmitate-stimulated conditions, whereas the DN-AMPK significantly upregulated both basal and palmitate-stimulated reporter gene activity.

Inhibition of De Novo Ceramide Synthesis Does Not Affect Palmitate-Induced NF-κB Activation

Activation of NF-κB has been reported when various cells are exposed to ceramides.[33,34] Thus, a decrease in ceramide accumulation hypothetically could account for the inhibition of palmitate-induced NF-κB activity caused by AMPK activation. To determine whether this occurred, PCs incubated with palmitate were coincubated with either myriocin, a specific SPT inhibitor, or fuminosin B1, a ceramide synthase inhibitor, or they were adenovirally infected with a construct to overexpress ceramidase I, an enzyme that increases ceramide breakdown. Although these treatments each produced a >80% decrease in palmitate-induced ceramide synthesis/accumulation (data not shown), none of them significantly diminished palmitate-induced NF-κB activation (Figs. 5A–C).

Palmitate and Oleate Cause Oxidative Stress in PCs That Activation of AMPK Does Not Prevent

Incubation with fatty acids causes oxidative stress that is thought to participate in generating apoptosis in various cultured cells, including cardiomyocytes, pancreatic β-cells, liver, vascular endothelium, and PCs.[12,16,35–37] In Figure 6A, we demonstrate that 24-hour incubation with 0.4 mM palmitate increased oxidative stress by eightfold, as measured by DCF fluorescence, and that this effect was not prevented by coincubation with AICAR. To confirm this result, electron spin resonance spectroscopy (ESRS) was used as a second and more specific method to measure NAD(P)H oxidase activity, the main oxidase activated by palmitate in PCs.[12] As demonstrated in Figure 6Bc, incubation with palmitate activated NAD(P)H oxidase, as evidenced by the presence of the paramagnetic spectra induced on ROS attack of the electron spin-trap (DMPO). BSA alone did not produce the same ESRS spectra (Fig. 6Ba), and coincubation with AICAR did not prevent it (Fig. 6Be). In contrast, coincubation of cell lysates with the specific NAD(P)H oxidase inhibitor DPI prevented the ROS-DMPO ES paramagnetic spectra (Figs. 6Bb, 6Bd, 6Bf), confirming that the oxidative stress measured was produced by NAD(P)H oxidase.

One of the consequences of prolonged oxidative stress is damage to various cellular components such as lipids. Thus, the lipid peroxidation product 8-isoprostane (or 8-iso-prostaglandin F2α) was measured as an index of long-term oxidative stress and to assess whether the stress was damaging cellular lipids. Incubation of PCs with either palmitate or oleate for 24 hours produced significant increases in 8-isoprostane release (Fig. 6C), suggesting that the generation of lipid peroxidation/oxidative stress is not a unique effect of palmitate.

Activation of AMPK by AICAR Does Not Lead to Increased Fatty Acid Oxidation

AMPK is a well-established activator of the β-oxidation of fatty acids in skeletal muscle and liver. In both tissues it acts, at least in part, by phosphorylating and deactivating mitochondrially associated ACC2, leading to a decrease in the concentration of malonyl CoA. Malonyl CoA is an inhibitor of carnitine palmitoyl transferase I (CPT1), which regulates the transport of fatty acyl CoA from the cytosol into the mitochondria (Fig. 7A).[20] Thus, by increasing the uptake of palmitate by mitochondria, AMPK would shunt it away from pathways leading to its esterification and, secondarily, the generation of potentially cytotoxic lipid metabolites, such as ceramide and DAG. To test whether activation of AMPK increases β-oxidation in the PCs, cells were incubated in media containing 0.1 or 0.4 mM palmitate, a ^{3}H-palmitate tracer, and carnitine with or without AICAR for up to 24 hours. Fatty acid oxidation assessed on the basis of

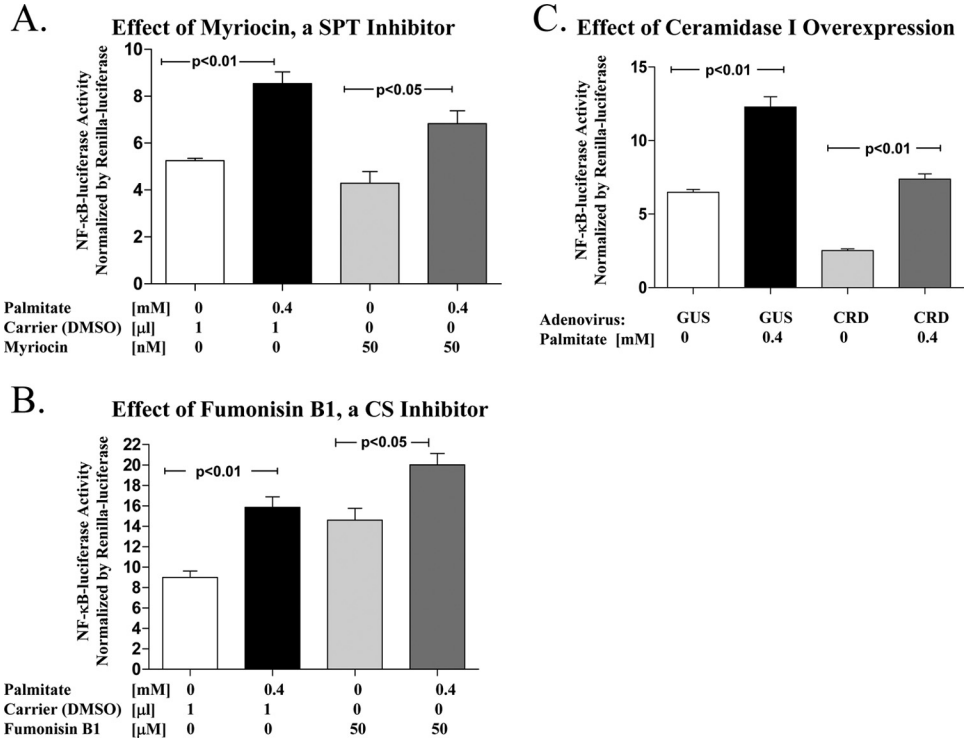

FIGURE 5. Effect of ceramide inhibition on palmitate-induced NF-κB activation. Seventy-two hours after infection with an NF-κB reporter gene adenovirus and CMV-Renilla-luciferase adenovirus, PCs were incubated with or without palmitate or coincubation with the SPT inhibitor myriocin (**A**) or the ceramide synthase inhibitor fumonisin B1 (**B**) for 24 hours. Cellular lysates were then measured for luciferase activity. NF-κB reporter gene activity (after 72 hours of infection) was also assessed in PCs that were coinfected with adenoviruses expressing GUS, as an infection control, or ceramidase I (CRD) after 24-hour incubation with or without palmitate (**C**). All data are from representative experiments ($n = 3$). Results are normalized to Renilla luciferase (from a cytomegalovirus-driven vector) to account for infection efficiency. Units are arbitrary light units (ALU). All experiments are $n = 3$–6.

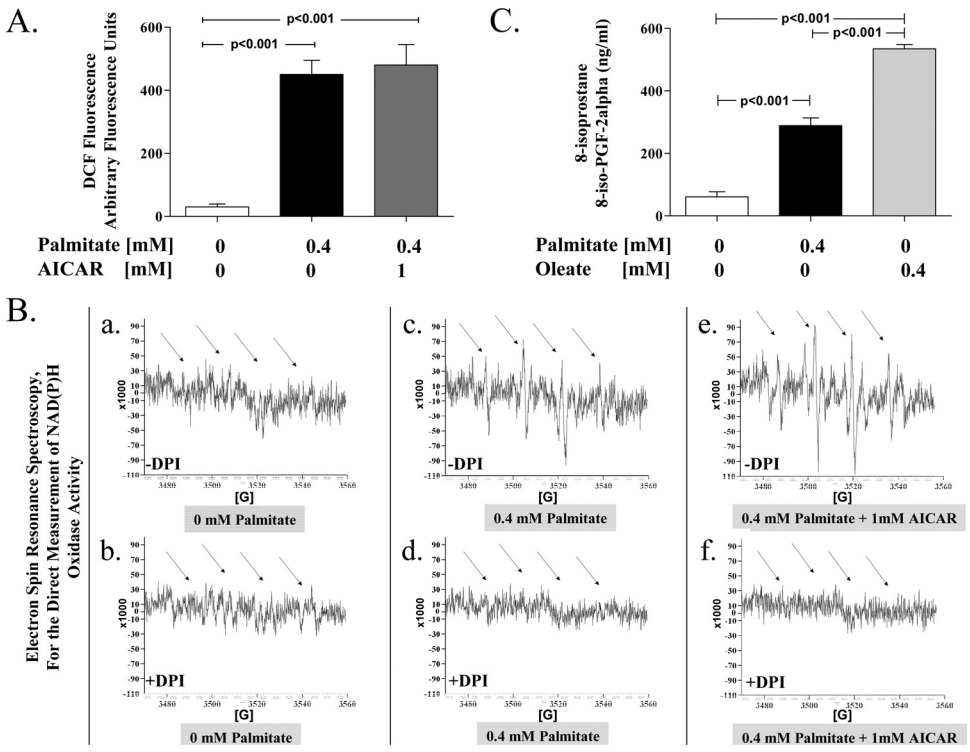

FIGURE 6. Activation of AMPK does not prevent palmitate-induced oxidative stress, and, surprisingly, oleate produces it. (**A**) Oxidative stress was measured with the fluorometric DCF technique on PCs that had been incubated with or without palmitate or coincubated with AICAR for 24 hours. (**B**) These are representative graphs of ROS-induced DMPO ESRS. PCs were treated with or without palmitate, AICAR, or both for 24 hours. The cells were homogenized, and their membrane proteins were extracted. Because activated NAD(P)H oxidase resides in the membrane, its ROS-producing activity is measurable by ESRS from a mixture of membrane proteins, an electron-trap (DMPO), and its substrate NAD(P)H (see Supplementary Methods, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-5784/-/DCSupplemental, for details). *Top*: ESRS spectra of membrane extracts from BSA-treated (**a**), palmitate-treated (**c**), and AICAR and palmitate-coincubated (**e**) PCs. *Arrows*: four spikes in the spectra that represent the paramagnetic signature of DMPO with ROS adducts. *Bottom*: paired samples from the top panels, but coincubated with the specific NAD(P)H oxidase inhibitor DPI (10 μM). Note that coincubation with DPI inhibited the DMPO-ROS signature, suggesting that NAD(P)H oxidase was indeed the oxidase generating the ROS. The unit on the *y*-axis is MHz ($\times$1000), and the unit on the *x*-axis is magnetic field strength (G). (**C**) The 8-isoprostanes, another index of oxidative stress (lipid peroxidation), were measured from the PC media after 24-hour incubation with BSA, palmitate, or oleate. All data are from representative experiments (n = 3).

^3H_2O production was linear through 24 hours at both palmitate concentrations, although the cells exposed to 0.4 mM palmitate had a statistically lower rate of oxidation, presumably because of palmitate-induced cytotoxicity (Fig. 7B). Of note, coincubation with AICAR failed to increase β-oxidation in either group. To confirm the functionality of the FAox assay, positive and negative controls and the effects of AICAR were tested again. For this purpose, PCs were incubated with 0.4 mM palmitate with or without carnitine (to increase long-chain fatty acyl-CoA β-oxidation), etomoxir (a specific inhibitor of CPT1; to decrease long-chain fatty acyl-CoA β-oxidation), or AICAR for 6 hours. The absence of carnitine led to an almost twofold decrease in β-oxidation, and etomoxir almost completely inhibited β-oxidation. Surprisingly, AICAR had no effect on any of the groups tested (Fig. 7C) despite the fact that within 15 minutes of its addition, AICAR activated AMPK, as evident from increases in its phosphorylation at Thr172 and that of its downstream target ACC at S79 (Figs. 7D, 7E). Furthermore, these changes persisted for 24 hours. Another possibility is that PCs lack ACC2. ACC has two isoforms, ACC1 and ACC2. ACC1 is cytosolic and generates the malonyl-CoA used for fatty acid synthesis, whereas mitochondrially associated ACC2 generates the malonyl-CoA that inhibits CPT1 (Fig. 7A). To assess whether ACC2 is present in PCs, protein lysates from bovine perirenal fat (ACC1 positive control), bovine heart (ACC2 positive control), and PCs were subjected to Western blot analysis for ACC isoform determination. As shown in Figure 7F, ACC1 was present, but ACC2 was not detectable in the PCs (Fig. 7F).

DISCUSSION

To our knowledge, this is the first report that the activation of AMPK prevents the apoptosis of retinal PCs caused by incubation with the saturated fatty acid palmitate. In doing so, AMPK concurrently prevented palmitate-induced increases in ceramide, DAG content, and NF-κB activity. In contrast, incubation with the monounsaturated fatty acid oleate, unlike palmitate, failed to increase these parameters and did not cause apoptosis.[12] Two surprising observations were that AMPK exerted its protective effects even though it did not alter the oxidation of palmitate or decrease oxidative stress.

In earlier studies, activation of AMPK by AICAR, metformin, or, where used, a constitutively active AMPK was found to diminish palmitate-induced apoptosis in astrocytes,[38] osteoblasts,[39] Chinese hamster ovary and H9c2 cardiomyoblasts,[40] and endothelial cells (ECs) (JMC, unpublished observations, 2004).[41] All these investigations began with the same initial hypothesis that the activation of AMPK would increase the β-oxidation of fatty acids and secondarily would decrease detrimental lipid accumulation (e.g., ceramides). However, this was not universally found. As we demonstrated in PCs (Figs. 1, 7), the activation of AMPK in astrocytes[38] and osteoblasts[39] prevented apoptosis but did not increase the β-oxidation of the fatty acid. Thus, the protective role of AMPK activation in many cells appears to extend beyond diverting fatty acids into the oxidative pathway and is more closely related to effects on ceramide synthesis (Fig. 2), DAG metabolism (Fig. 3), NF-κB activation (Fig. 4), and perhaps other factors.

We had previously demonstrated that the pharmacologic inhibition of ceramide synthesis with fumonisin B1 or the prevention of its accumulation by overexpression of ceramidase I prevents palmitate-induced apoptosis.[12] In the present study, we confirmed that palmitate increases ceramide accumulation in the PCs by approximately twofold after 24-hour incubation (Fig. 2B) and that this is associated with a twofold increase in de novo ceramide synthesis (Fig. 2C). This effect of

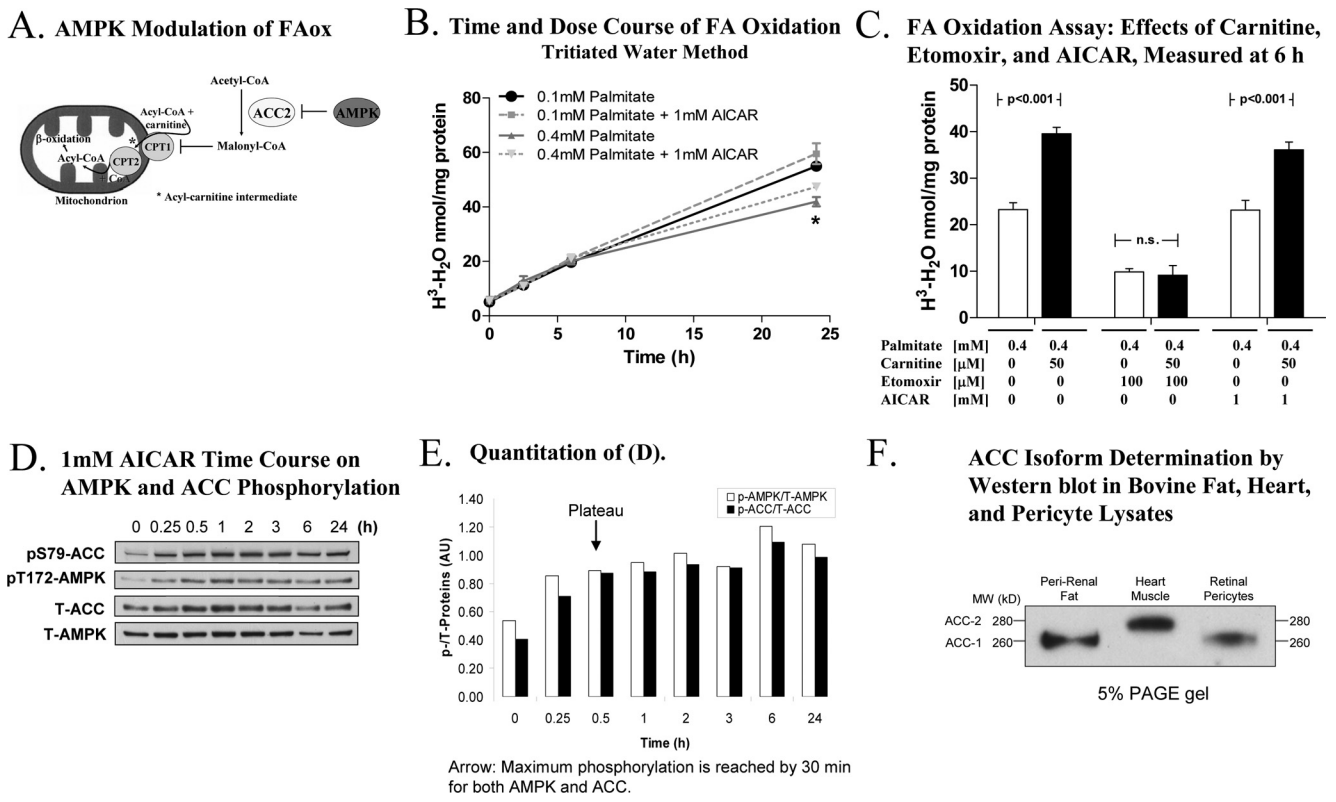

FIGURE 7. Effect of AMPK activation by AICAR on fatty acid oxidation (FAox). (**A**) Diagram of AMPK effect on FAox. Phosphorylation of ACC2 by AMPK inhibits ACC conversion of acetyl-CoA to malonyl-CoA at the mitochondrial surface.[20] Because malonyl-CoA is a natural inhibitor of CPT1, activation of AMPK relieves the inhibition of CPT1 by malonyl-CoA, and this increases the transport of long-chain fatty acyl-CoAs (such as palmitoyl-CoA) into the mitochondria. CPT1 takes acyl-CoAs from the cytosol, removes the CoA, and adds carnitine, forming an acyl-carnitine intermediate. CPT2, located on the matrix side of the mitochondria, receives the acyl-carnitine intermediate, removes the carnitine, and adds back the CoA, reforming the original acyl-CoA. Once inside the mitochondria, the acyl-CoA can then be oxidized by β-oxidation to generate ATP. (**B**) PCs were incubated in DMEM supplemented with 5% FBS, 50 μM carnitine, and 0.1 or 0.4 mM palmitate (with a ^{3}H-palmitate tracer at the same specific activity) with or without AICAR for the indicated amount of time. FAox was measured at each time point, by the ^3H_2O release method, and the results are plotted on an X-Y graph. The data demonstrate that the assay was linear all the way through 24 hours ($n = 3$ per condition). (**C**) Modulation of FAox by the CPT1 substrate and FAox activator carnitine, the CPT1 inhibitor etomoxir, and AICAR. After 6-hour treatment with the indicated compounds, FAox was measured ($n = 4$ per condition). This was the only experiment in which carnitine was omitted from certain groups, as indicated in the figure. (**D**) Time course of AMPK activation using 1 mM AICAR as the stimulus in PCs. (**E**) Quantitation of (**D**). (**F**) 15 μg protein lysates from PCs, bovine perirenal fat, and bovine heart muscle was run on a 5% PAGE gel for 2 hours to resolve the two ACC isoforms. White fat expresses only the 260-kDa ACC1 isoform, and heart muscle predominantly expresses the 280-kDa ACC2 isoform.

palmitate was associated with a 31% increase in SPT activity, which did not achieve statistical significance, suggesting an additional mass action effect (Fig. 2D). Such a conclusion is in keeping with our earlier observations that palmitate induces a dose-dependent increase in total ceramide levels.[12] In contrast, the decrease in ceramide synthesis caused by AICAR was clearly associated with decreases in SPT mRNA (Fig. 2E) and activity (Fig. 2D) and with total ceramide levels (Fig. 2B). We did not find these effects when the cells were incubated with AICAR for only the last 2 hours of palmitate treatment, suggesting that a rapid posttranslational modification of SPT by AMPK was not responsible for the change in its activity (data not shown). Collectively, these findings suggest that transcriptional regulation of the SPT gene is likely a key mechanism whereby AMPK exerts its protective effect. A similar conclusion was reached by Blásquez et al.,[38] who found that AMPK activation decreases SPT protein levels in astrocytes incubated with palmitate.

We also found that palmitate induced increases in DAG, which, by activating PKCs, could have cytotoxic effects in PCs. Yu and colleagues[42] demonstrated that palmitate increases de novo palmitate synthesis (Supplementary Fig. S3, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-5784/-/DCSupplemental), though why palmitate increased DAG synthesis more so than oleate remains to be determined. Possibly the enzymes involved in this pathway kinetically favor the addition of a saturated fatty acid over an unsaturated fatty acid to the growing DAG. AICAR blocked the production of both DAG and ceramide. As already noted, this is not related to an increase in fatty acid oxidation, which would steal cytosolic fatty acyl-CoA from the synthesis of these molecules. A likely explanation is that AMPK independently downregulated the transcription of key enzymes (e.g., SPT, GPAT) that regulate their synthesis.

We previously demonstrated that inhibition of the redox-sensitive transcription factor NF-κB by two distinct means (inhibition of the inhibitor of IκB kinase [IKK] or overexpression of an unphosphorylatable and thus undegradable IκB) rescued PC from palmitate-induced apoptosis.[12] In the present study, we found that NF-κB was probably not activated by FFA-induced oxidative stress because incubation with AICAR did not suppress palmitate-induced ROS generation (Figs. 6A, 6B) when it inhibited palmitate-induced NF-κB activation (Fig. 4A). Another possibility is that ceramide activated NF-κB, as it has been reported to do in HL60, Jurkat T, and PC12 cells.[33,34] We did not find this to be the case either because AMPK activation prevented both basal and palmitate-induced NF-κB activity (Fig. 4A), whereas AMPK-independent inhibition of ceramide accumulation only diminished basal NF-κB activity

(Fig. 5C). On the other hand, if a particular threshold of NF-κB activity was necessary to induce NF-κB–dependent gene transcription, ceramide levels could have been an influential factor. Nonetheless, the mechanism of AMPK-induced NF-κB inhibition remains elusive.

An unexpected result was that both palmitate and oleate generated oxidative stress (lipid peroxidation; Fig. 6C). We had previously demonstrated that incubation with palmitate, even at low noncytotoxic concentrations (0.1 mM), induces high levels of ROS.[12] These and other observations led us to conclude that palmitate-induced ROS is necessary, but not sufficient, for it to cause apoptosis in the PCs. A similar conclusion was drawn by Hickson-Bick et al.,[16] who found that at equivalent concentrations oleate caused a greater increase in DCF-measurable oxidative stress than did palmitate in cultured rat cardiomyocytes, even though only palmitate was toxic. This is not to say that excessive oxidative stress cannot induce apoptosis in PCs. As demonstrated by Shojaee et al.,[43] exposure of PCs to H_2O_2 or UV radiation causes significant apoptosis that is dependent on ROS and can be prevented by antioxidant treatment. Thus, there are likely distinct signaling events activated or deactivated by palmitate, oleate, H_2O_2, and UV that may explain the differences in their respective abilities to generate apoptosis in PCs At least with respect to palmitate and oleate, some of those signals have been discovered in this study.

Another unexpected observation was that AMPK activation did not inhibit palmitate-induced oxidative stress. Other groups have reported that AMPK can greatly diminish mitochondrial-mediated[44] and NAD(P)H oxidase-mediated[45] oxidative stress in HUVECs caused by hyperglycemia. Presumably, PCs lack a factor on which AMPK acts to prevent this. We had previously determined that palmitate-induced oxidative stress is generated from NAD(P)H oxidase and is not caused by ceramide accumulation,[12] as has also been reported for β-cells and cardiomyocytes.[46,47] Thus, the missing factor may be involved in the translocation of subunits, assembly, or function of the NAD(P)H oxidase holoenzyme. This could make the PC an interesting model for future studies of AMPK- and NAD(P)H oxidase-derived oxidative stress. Finally, it has been proposed that excessive β-oxidation of fatty acids can overwhelm the TCA cycle, leading to depletion of its intermediates, mitochondrial dysfunction, excessive ROS generation, and cellular damage.[48] It has been suggested that in such a setting, the activation of AMPK may be detrimental.[48] At least with PCs, this concern appears unwarranted because the activation of AMPK prevented cell death (Fig. 1).

AICAR was used to activate AMPK in most of the studies described here. It has been widely used as an AMPK activator; indeed, much of our knowledge of AMPK is based on its biological actions.[49] On the other hand, although a great majority of the actions of AICAR are AMPK mediated,[22,50] it may have some effects that are not, such as the inhibition of hepatic gluconeogenesis at fructose bisphosphatase.[51] That the effect of AICAR on apoptosis in PCs described here was AMPK mediated is strongly suggested by the fact that it was mimicked by overexpression of a CA-AMPK (Fig. 1C) and by the observation that AMPK activators diminish palmitate-induced caspase 3 and 9 activation in many cells.[38–41] Similarly, the effects of AICAR on NF-κB in the PCs have been demonstrated in other cell types, including ECs.[24,44] Where studied, they have been mimicked by other AMPK activators, including metformin[44] and adiponectin.[52] Similar studies on apoptosis, looking at the effects of AICAR on individual caspases, and experiments in which a DN-AMPK is expressed or in which inhibitors of AICAR (which have generally demonstrated AICAR specificity for AMPK activation[53,54]) conversion to AICAR-P are needed to establish beyond question whether AMPK prevents palmitate-induced abnormalities in retinal PCs.

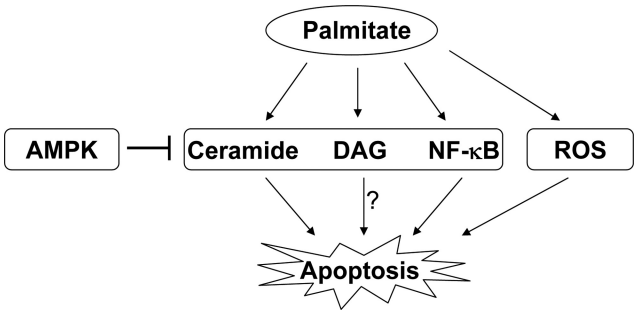

FIGURE 8. Palmitate-induced changes associated with apoptosis and their inhibition or lack of inhibition by AMPK. Previous studies have demonstrated that oxidative stress, ceramides, and NF-κB activation (and a growing number of pathways) all participate in palmitate-induced apoptosis in PCs and that blocking any of them can protect PC from apoptosis. Whether there is an epistatic relationship between those pathways is uncertain. Data presented here and in Cacicedo et al.[12] suggest that, at least with respect to the three mentioned pathways, a parallel relationship likely exists. What is better established is that when AMPK inhibits apoptosis, it prevents palmitate-induced increases in ceramides, NF-κB activation, and DAG content, but not oxidative stress in the PCs. Whether increased DAG levels contribute to palmitate-induced apoptosis has not been determined.

The abnormalities in cultured PCs incubated with palmitate reported here show many similarities to those observed in retinal PCs of humans with diabetic retinopathy. Common characteristics include increased oxidative stress, NF-κB activation, Bax accumulation, and programmed cell death itself.[31,32] All these abnormalities, as well as increased DAG levels, have also been found in the retinas of diabetic rodents.[6,55,56] To establish whether the activation of AMPK prevents these abnormalities in vivo, experiments in diabetic animals will be needed. For this purpose, systemic or perhaps local administration (e.g., by eyedrops) of AMPK activators such as AICAR, metformin, fenofibrate, and thiazolidinediones must be tested to determine whether they can affect the incidence of PC dropout and acellular capillary formation in the diabetic retina. We have demonstrated that apoptosis also occurs in macrovascular ECs incubated with moderate levels (0.4 mM) of palmitate[41] or (25 mM) glucose[26] for 72 hours and that the activation of AMPK by AICAR or CA-AMPK overexpression prevented it. This suggests that in addition to PCs, the activation of AMPK may be able to protect other cells (ECs and neural cells) in the retina that are susceptible to fuel-induced apoptosis and that AMPK activation could do so from more than one noxious stimulus (high glucose and high FFA).

In conclusion, these results indicate that the activation of AMPK, by AICAR or by overexpression of a CA-AMPK, can prevent PCs from undergoing palmitate-induced apoptosis. Activation of AMPK by AICAR prevented palmitate-induced increases in ceramide and DAG accumulation, NF-κB activation, increased Bax/Bcl-2 ratio, and, most important, apoptosis itself (Fig. 8). In contrast, oleate, a fatty acid that did not cause any of these changes, produced the same increases in oxidative stress as did palmitate. In addition, AICAR did not decrease the oxidative stress generated by palmitate. Further studies are needed to determine the precise mechanisms by which AMPK prevents lipotoxicity and whether AMPK activators can have a beneficial effect on the diabetic retina in vivo.

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