Palmitate-Induced Apoptosis in Cultured Bovine Retinal Pericytes

Roles of NAD(P)H Oxidase, Oxidant Stress, and Ceramide

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Apoptosis of pericytes (PCs) is an early event in diabetic retinopathy. It is generally thought to be a consequence of sustained hyperglycemia. In keeping with this, long-term (>7 days) incubation of cultured PCs in a high-glucose media has been shown to increase apoptosis. We examine here whether the saturated free fatty acid palmitate, the concentration of which is often elevated in diabetes, has similar effects on cultured PCs. Incubation with 0.4 mmol/l palmitate for 24 h induced both oxidant stress and apoptosis, as evidenced by a sixfold increase in DCF fluorescence and a twofold increase in caspase-3 activation, respectively. NAD(P)H oxidase appeared to be involved in these responses, since overexpression of dominant-negative subunits of NAD(P)H oxidase, such as phox47(DN), diminished oxidant stress, and phox67(DN) and N-17 RAC1(DN) prevented the increase in caspase-3 activity. Likewise, overexpression of vRAC, a constitutively active RAC1, increased caspase-3 activity to the same extent as palmitate alone. The effects of vRAC and palmitate were not additive. In parallel with the increases in oxidative stress, the redox-sensitive transcription factor nuclear factor-κB (NF-κB) was activated in cells incubated with 0.4 mmol/l palmitate. Furthermore, inhibition of NF-κB activation by various means inhibited caspase-3 activation. Finally, incubation with palmitate increased the cellular content of ceramide, a molecule linked to apoptosis and increases in oxidative stress and NF-κB activation in other cells. In keeping with such a role, in PCs both coincubation with fumonisin B1 (a ceramide synthase inhibitor) and overexpression of ceramidase I reversed the proapoptotic effect of palmitate. On the other hand, they increased rather than decreased DCF fluorescence. In conclusion, the results suggest that palmitate-induced apoptosis in PCs is associated with activation of NAD(P)H oxidase and NF-κB and an increase in ceramide. The precise interactions between these molecules in causing apoptosis and the importance of oxidant stress as a contributory factor remain to be determined. *Diabetes* 54:1838–1845, 2005

Pericyte (PC) drop out due to apoptosis has long been considered an early event in diabetic retinopathy (1). Theories to explain why it occurs have predominantly focused on hyperglycemia. Thus, several groups (2–4) have reported that cultured retinal PCs exposed to high levels of glucose (25–30 mmol/l) for a period of 7 days or more show a higher rate of apoptosis than cells grown at 5.5 mmol/l glucose. Additionally, glucose-induced increases in sorbitol, hexosamines, advanced glycation end products (AGEs), and protein kinase C activity have all been linked to the death of endothelial and neural cells as well as PCs in the retina (5–9). High plasma free fatty acid (FFA) levels, like hyperglycemia, are a common feature of both poorly controlled type 1 and type 2 diabetes (10,11). In addition, elevated FFA levels are found in people with type 2 diabetes and are associated with obesity and the metabolic syndrome (12). A recent epidemiological study by Parvanova et al. (13) suggested that insulin resistance is an independent marker for proliferative diabetic retinopathy; this is a potentially important finding because insulin resistance has been demonstrated to have a positive correlation with increased plasma FFA levels (14,15). Despite this, the possibility that FFA excess contributes to the pathogenesis of diabetic retinopathy has received little attention.

In this study, we have examined the effects of incubation with the saturated fatty acid palmitate, which makes up 30–40% of plasma FFAs, on the behavior of PCs. More specifically, when initial studies demonstrated that palmitate promotes apoptosis, several factors that could have mediated this effect were evaluated. We first examined the pathogenetic role of oxidative stress and the enzymatic source of this stress. Next, the proapoptotic role of the redox-sensitive proinflammatory transcription factor nuclear factor-κB (NF-κB), which has been implicated in causing glucose-mediated apoptosis in PCs (3,16), was addressed. Finally, ceramide was studied since an accumulation of ceramide, synthesized de novo from saturated fatty acids and the amino acid serine, has been shown both to cause apoptosis in a variety of cells and to modulate oxidative stress (17–22).

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AGE, advanced glycation end product; DCF, dichlorohydrofluorescein diacetate; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; FFA, free fatty acid; GUS, β-glucuronidase; IκB, inhibitor of κB; IKK, inhibitor of κB kinase; NF-κB, nuclear factor κB; PC, pericyte; PEI, polyethylenimine; TUNEL, TdT-mediated dUTP nick-end labeling.

RESEARCH DESIGN AND METHODS

PC cell culture. Bovine eyes were purchased from a local slaughterhouse and processed the same day, as described by Gitlin and D'Amore (23). In brief, retinas were extracted from 18 to 20 eyes, minced, and digested in 0.2% type II collagenase (Worthington Biochemical, Lakewood, NJ), shaking for 1 h at 37°C. The digested retinas were then filtered through a 95-μm Nitex mesh (Sefar American, Depew, NY) to separate single cells and small microvessels from the nondigestible and larger materials. The collagenase was quenched by adding an equal volume of Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT). The mixture was centrifuged at 800*g* for 5 min and then resuspended in DMEM containing 5.5 mmol/l glucose, 10% FBS, 4 mmol/l glutamine, and 2% penstrep (final concentrations: 10 units/ml penicillin G, 10 μg/ml streptomycin sulfate, and 25 ng/ml amphotericin B; Gibco). The slurry was plated at three retinas per 21 cm (2) in 60-mm Falcon Primaria tissue culture dishes (Becton Dickinson, Franklin Lakes, NJ) and placed at 37°C in a 5% CO_2, 95% air incubator. The cells were passaged every 7–9 days once they reached their maximum confluence, which was typically 80–85%. PCs were characterized by morphology and by inability to uptake rhodamine-conjugated acetylated LDL. Cells in passages 3–6 with 80–85% confluence were used for experiments.

Incubation with fatty acids and assessment of apoptosis. PCs were incubated for the indicated period of time in 5.5 mmol/l glucose DMEM supplemented with 5% FBS, 10 mmol/l HEPES, 50 μmol/l carnitine, and the indicated concentration of palmitate or oleate (Sigma, St. Louis, MO) preconjugated with FFA-free BSA (Serologicals, Norcross, GA) at a 2:1 (FFA:BSA) molar ratio. Control cells were incubated with media containing FFA-free BSA (Serologicals) 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). Apoptosis was assessed by a TdT-mediated dUTP nick-end labeling (TUNEL) kit as per the manufacturer's instructions (Oncogene, Boston, MA) and caspase-3 activity with a commercially available kit (Molecular Probes, Eugene, OR) as described previously (24). The ceramide synthase inhibitor fumonisin B1 was purchased from Calbiochem (San Diego, CA), and the inhibitor of κ-B kinase (IKK) Bay 11-7085 was purchased from Biomol (Plymouth Meeting, PA).

Total ceramide measurement. Ceramide was quantified using sn-1, 2-diacylglycerol kinase (Calbiochem) to convert ceramide to (^{32}P) ceramide-1-phosphate in the presence of ^{32}P-ATP essentially as described in 25.

Recombinant adenovirus constructs and infection of PCs. Construction of recombinant replication-incompetent adenoviruses was performed essentially as previously described (26). The following genes were individually cloned into adenoviral constructs: β-glucuronidase (GUS) as an infection control (Invitrogen, Carlsbad, CA); ceramidase I (accession no. BC003204; ATCC, Manassas, VA); N17-RAC1 (accession no. BC003828; ATCC); p47DN, p67DN, and vRAC (kind gifts from Dr. David Pimentel, Boston University, Boston, MA); catalase (accession no. BC013447; ATCC); Cu, Zn superoxide dismutase (SOD1), and Mn superoxide dismutase (SOD2; kind gifts from Dr. Doug Sawyer, Boston University); and IκB-α unphosphorylatable mutant (BD Biosciences). Once the genes were cloned, the adenovirus plasmids were cut by *Pac*I and transfected to 293 human embryonic kidney cells (packaging cell line) grown in six-well plates. Recombinant adenoviruses appeared after 3–7 days of culture and were then reamplified by additional infection of 3–10 100-mm dishes of 293 cells. Purification of adenovirus was performed according to a procedure developed by one of the authors (Y.I.) that became the basis for Sartorius's Vivapure AdenoPACK purification and concentration system. Essentially, after reamplification, the 293 cells were scraped without removal of media. Both media and cells were aliquoted into 50-ml tubes and subjected to three freeze/thaw cycles in liquid N_2 to break up the cells and release the rest of the viral particles. This mixture was filtered through a Stericup 0.45-μm Durapore membrane filter to remove most of the cellular debris (Millipore, Billerica, MA). The viral particle containing eluate was diluted 1:1 (wt/wt) with dilution buffer (20 mmol/l bicine, 0.6 mol/l NaCl, pH 8.4) and then run through a strongly basic anion exchanger (Sartorius, Edgewood, NY), washed with 30–40 ml washing buffer (10 mmol/l bicine, 0.4 mol/l NaCl, pH 8.4), and eluted with 1 ml of elution buffer (10 mmol/l bicine, 0.72 mol/l NaCl, pH 8.2). The viral particle concentration was determined by multiplicity of infection. PCs were infected with adenoviruses at a titer of 100–200 × 10^6 pfu/ml mixed with polyethylenimine (PEI; Sigma [PEI molecules/virus particle = 10^6]) 2 days before a given experiment (27). PEI was used due to our observation that PCs are refractory toward infection unless the viral particles are electrostatically neutralized. Coincubating adenovirus with a cationic molecule such as PEI appears to take care of this problem, since it results in ~100% infection.

Oxidative stress measurements. 2′,7′-dichlorohydrofluorescein diacetate (DCF; Molecular Probes) is a cell-permeable nonfluorescent compound that is cleaved into fluorescent products in the presence of H_2O_2 and other radical oxygen species molecules and esterases. Thus, it allows for the measurement of oxidative stress within cells. Five hours before the end of an incubation with the indicated stimulus, DCF was added to each 100-mm plate at a final concentration of 10 μmmol/l. At the completion of the incubation, the cells were placed on ice, washed three times with ice-cold PBS, and then lysed in 1 ml of cold Cell Signaling Lysis Buffer (Cell Signaling Technology, Beverly, MA). The cells were scraped off the plate and sonicated on ice 10 times to rupture the cells. After this, the lysates were centrifuged at 14,000 rpm at 4°C for 5 min. The supernatants were transferred to cuvettes in which fluorescence was measured at an emission wavelength of 512 nm, using an excitation wavelength of 495 nm for the DCF fluorophore. All data are representative of at least three independent experiments and are expressed as means ± SE.

NF-κB reporter gene assay. Transactivation of NF-κB was assessed by transfection of the pNF-κB luciferase plasmid from the Mercury Profiling System (Clontech), which expresses firefly luciferase when activated. The constitutively active pCMV-renilla vector, which expresses renilla luciferase, was used to normalize for transfection efficiency. The two luciferase signals were assessed with a dual-luciferase assay kit (Promega, Madison, WI) and an LKB luminometer. Plasmids were transfected with the GenePorter 2 system (Gene Therapy Systems, San Diego, CA), following the manufacturer's protocol, 48 h before the experiment. All data are representative experiments of at least three independent repetitions and are shown as means ± SE.

Western blotting. After incubation of PCs with the indicated stimulus in six-well dishes, the cells were placed on ice and washed three times with ice-cold PBS. After this, 200 μl of Cell Signaling Lysis Buffer were added to each well and the cells were scraped. The cells were sonicated on ice 10 times to rupture the cells, then centrifuged at 14,000 rpm at 4°C for 5 min. The supernatants were transferred to new Eppendorf tubes and stored at −80°C for future analysis. After thawing, the lysates were assessed for protein by the bicinchoninic acid assay, and 25 μg of protein of each sample were loaded per lane onto a NuPAGE 4–20% gradient gel. The samples were run and then transferred to a polyvinylidine fluoride membrane as per manufacturer's instructions (Invitrogen). Western blots were carried out with anti-Bax antibody at a ratio of 1:1,000 (Santa Cruz Biotechnology, Santa Cruz, CA) and anti–Bcl-2 antibody (Santa Cruz Biotechnology) at a ratio of 1:1,000 at 4°C rocking overnight. A donkey anti-rabbit horseradish peroxidase–conjugated secondary antibody (Amersham, Piscataway, NJ) was used at a ratio of 1:10,000 for 1 h at room temperature. Enhanced chemiluminescence solution from Pierce (Rockford, IL) was used as the horseradish peroxidase substrate.

Statistics. All data were analyzed using the SAS program (SAS Institute, Cary, NC) with general linear mode procedure. P value <0.05 was taken as statistical significance. All the data were expressed as means ± SE.

RESULTS

Palmitate induces significant levels of apoptosis within 72 h. Apoptosis, as assessed by TUNEL staining, was increased by 10-fold in PCs incubated for 72 h with 0.4 mmol/l palmitate (Fig. 1*A* and *B*). Another index of apoptosis, caspase-3 activity, was increased twofold by palmitate in these cells at 24 h but was unchanged when the PCs were incubated with an equivalent concentration of oleate. The main difference between these indexes of apoptosis is that TUNEL staining measures nicked DNA, which is one of the final events of apoptosis, whereas caspase-3, an effector caspase, is activated before DNA fragmentation occurs. In fact, caspase-3 causes the activation of caspase-activated DNase, one of the enzymes responsible for DNA fragmentation during apoptosis (28,29). Due to the earlier detection of apoptosis with the caspase-3 assay, it was the index used in all subsequent studies.

NAD(P)H oxidase is responsible for the increase in oxidative stress. Research by Inoguchi et al. (30) demonstrated that in vascular endothelium and smooth muscle cells, large increases in NAD(P)H oxidase activity occur when they are incubated with 0.2 mmol/l palmitate. NAD(P)H oxidase is a membrane-associated multiprotein enzyme composed of many subunits, including gp91 (or one of its homologues), $p22^{phox}$, RAC, $p67^{phox}$, $p47^{phox}$, and $p40^{phox}$, that when activated generate superoxide and *NAD(P)+* (31–33). Superoxide production by NAD(P)H

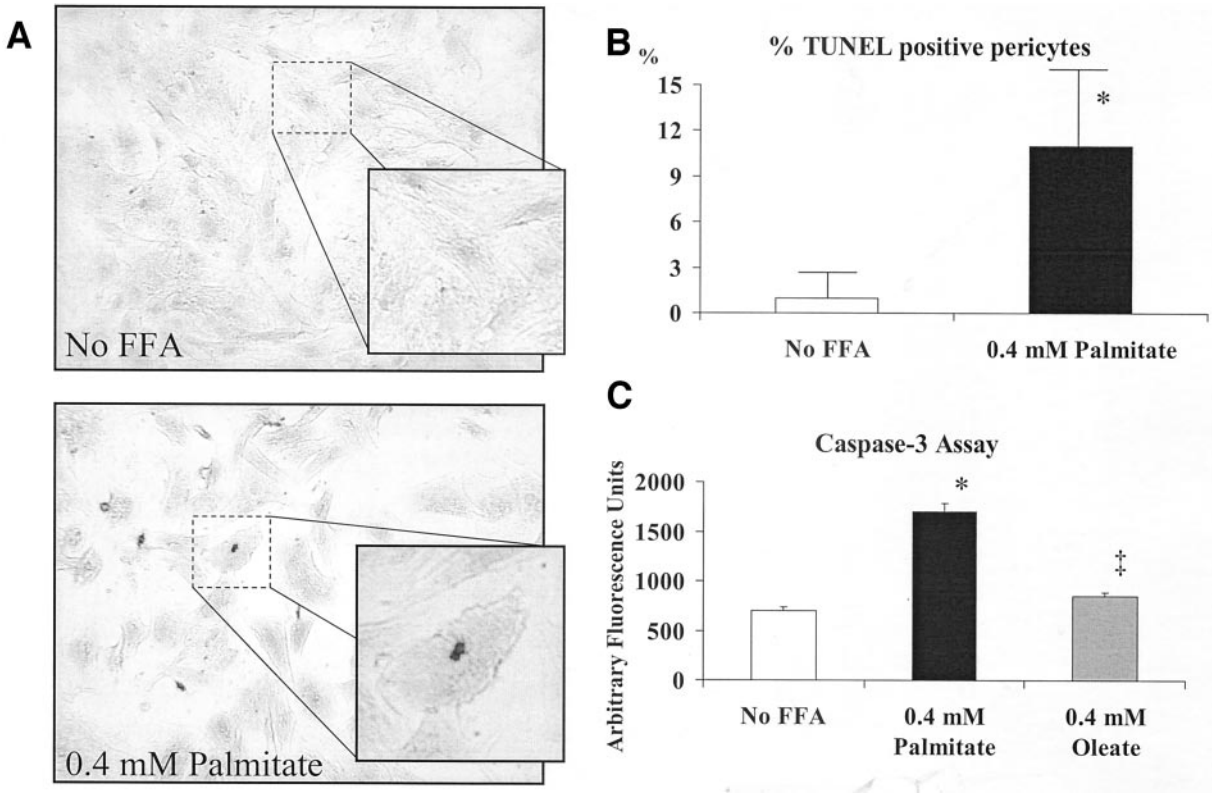

FIG. 1. Evaluation of PC apoptosis due to palmitate. *A*: Micrographs of TUNEL-stained apoptotic PCs. PCs were incubated for 3 days with DMEM media supplemented with 5% FBS, 50 μmol/l carnitine, and the indicated concentration of palmitate. TUNEL-positive cells, having dark brown color nuclei, were increased 10-fold due to palmitate incubation. Micrograph magnification is ×100 and the inset is ×200. *B*: Quantification of TUNEL staining from (*A*) ($n = 12$). *C*: Caspase-3 activity measured after 24 h of palmitate or oleate incubation. Palmitate increased caspase-3 activity twofold in this time frame. There were no significant statistical differences between the No FFA and oleate-treated groups. All data are from representative experiments ($n = 6$). *$P < 0.05$ vs. No FFA, ‡$P < 0.05$ vs. 0.4 mmol/l palmitate.

oxidase can be modulated by introducing dominant-negative or constitutively active forms of its subunits into a cell. To this end, PCs were infected with adenovirus to overexpress these mutant subunits, and the effect of doing so on PC viability was determined. Overexpression of a dominant-negative RAC1 (N17-RAC) or p67DN both decreased palmitate-induced caspase-3 activity to control levels or lower (Fig. 2*A*). p67DN even decreased basal caspase-3 activation to well below control levels. Conversely, overexpression of a constitutively active RAC (vRAC) led to an increase in caspase-3 activity in control cells equivalent to that caused by 0.4 mmol/l palmitate. An additive effect of palmitate and overexpression of vRAC on caspase activity was not observed (Fig. 2*A*).

Measurement of oxidant stress as reflected by DCF fluorescence revealed that it was increased by eightfold in PCs incubated with 0.4 mmol/l palmitate and that overexpression of a dominant-negative subunit such as p47DN substantially prevented this (Fig. 2*B*). These results indicate that NAD(P)H oxidase is involved in the increase in oxidative stress caused by palmitate; however, the relevance of this action to its cytotoxic effect is less clear. Thus, incubation with 0.1 mmol/l palmitate, a low-normal concentration of this fatty acid in plasma, increased oxidant stress nearly as much as 0.4 mmol/l palmitate, yet in our hands (J.M.C., unpublished observations) and in an earlier report (34), palmitate at this concentration was not toxic to cultured PCs. To determine independently if oxidant stress is necessary for the caspase-mediated response to palmitate in PCs, antioxidant genes including catalase, SOD1, and SOD2 were overexpressed before incubating PCs with 0.4 mmol/l palmitate (Fig. 2*C*). In both these cells, and cells incubated without added palmitate, overexpression of these genes dramatically diminished caspase-3 activation, suggesting that oxidant stress is a necessary component of this response.

NF-κB is activated by palmitate in the PCs. Oxidant stress is usually accompanied by changes in gene expression when it leads to alterations in a cell's fate. The redox-sensitive proinflammatory transcription factor NF-κB has received much attention in this context, since it is activated in the PCs of diabetic retina, atherosclerotic plaques, and in white blood cells in a diabetic milieu (3,35,36). Also, it is activated in response to increases in oxidative stress (37–39). NF-κB is normally found in the cytosol as a dimer bound to its inhibitor, inhibitor of κB (IκB). Stimuli that activate the NF-κB cascade usually begin by inducing kinases (such as IKK) that cause phosphorylation and subsequent ubiquitination and degradation of IκB proteins. This in turn exposes the nuclear localization signal of NF-κB and allows the NF-κB dimers to enter the nucleus and induce gene expression (40). Measurement of NF-κB reporter gene activity revealed a twofold increase in NF-κB transactivation within 24 h in PCs incubated with 0.4 mmol/l palmitate (Fig. 3*A*). Conversely, coincubation with an inhibitor of the upstream kinase of IκB, IKK, or overexpression of an unphosphorylatable and therefore undegradable IκB led to inhibition of

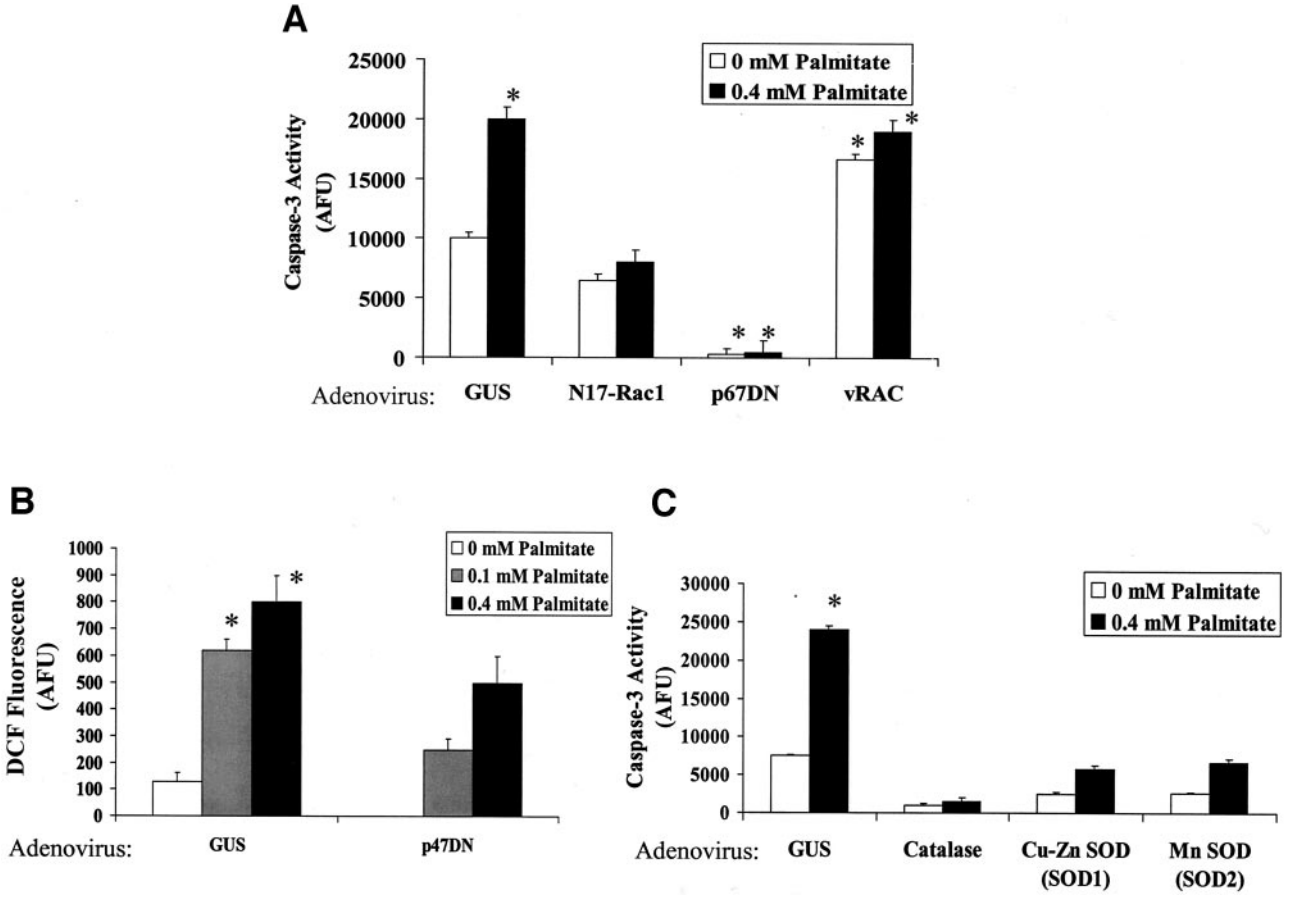

FIG. 2. Effects of modulating NAD(P)H oxidase subunits on caspase-3 activity and oxidative stress, and the effects of overexpression of antioxidant genes. *A*: Caspase-3 activity was measured after incubating the PCs for 24 h with high levels of palmitate. PCs were previously infected with a dominant-negative RAC1 (N17-RAC1), a dominant-negative p67^{phox} (p67DN), a constitutively active RAC1 (vRAC), or GUS as an infection control. *B*: Oxidative stress was measured via the DCF technique after 24 h of incubation, with the indicated concentration of palmitate and overexpression of either GUS as an infection control or a dominant-negative p47^{phox} (p47DN). *C*: Caspase-3 activity was measured after 24 h of incubation with palmitate and overexpression of the indicated genes (all wild type). Differences in caspase-3 activation in control cells in this and other studies (including the DCF experiments) are due to interassay variation possibly the result of differences in the amount of lysis buffer used. However, group differences were always very consistent within a given assay. All data are from representative experiments (n = 3). *P < 0.05 vs. 0 mmol/l palmitate.

caspase-3 activity in these cells (Fig. 3*B*). In keeping with these findings, the levels of the NF-κB–driven proapoptotic protein, Bax, were elevated in PCs that had been incubated with 0.4 mmol/l palmitate for 24 h, whereas the levels of Bcl-2, a prosurvival signal, were downregulated (Fig. 3*C*).

Ceramide accumulation occurs in PCs incubated with palmitate and is involved in apoptosis. Ceramide accumulation, induced by saturated fatty acids, has been linked to apoptosis and other manifestations of lipotoxicity in both pancreatic β-cells and cardiomyocytes (17–20). In these cells, ceramide accumulation has been shown to increase oxidative stress, probably by inhibiting electron transport at mitochondrial complex III and secondarily increasing superoxide generation (21,22). As shown in Fig. 4*A*, a dose-dependent increase in ceramide mass was observed in PCs incubated for 18 h with the indicated concentrations of palmitate. To determine whether ceramide is involved in the palmitate-induced apoptotic response, the cells were coincubated with a ceramide synthase inhibitor, fumonisin B1, for 24 h (Fig. 4*B*) or they were made to overexpress ceramidase I, an enzyme responsible for ceramide breakdown. As shown in Fig. 4*C*, both treatments markedly diminished the increase in caspase-3 activity caused by palmitate; indeed, in the cells overexpressing ceramidase I, caspase-3 activity was less than that observed in control cells (Fig. 4*C*). These data strongly suggest that ceramide plays a key role in palmitate-induced apoptosis in the PC.

As already noted, the higher rate of apoptosis in the PC incubated with 0.4 mmol/l palmitate was associated with a six- to eightfold increase in DCF fluorescence, indicating that it was associated with oxidative stress. Thus, an unexpected finding was that treatment with fumonisin B1 and overexpression of ceramidase, both of which rescued the PC from apoptosis, caused increases in DCF fluorescence (Fig. 4*D*).

DISCUSSION

Apoptosis of PCs is an early event in diabetic retinopathy. In the present study, we found that PCs undergo apoptosis when incubated in a medium containing the fatty acid palmitate (0.4 mmol/l) for 24 h. We also found that this effect of palmitate was linked to increases in NAD(P)H oxidase–generated oxidant stress, NF-κB activation, and ceramide accumulation.

Cytotoxicity induced by palmitate has been reported in

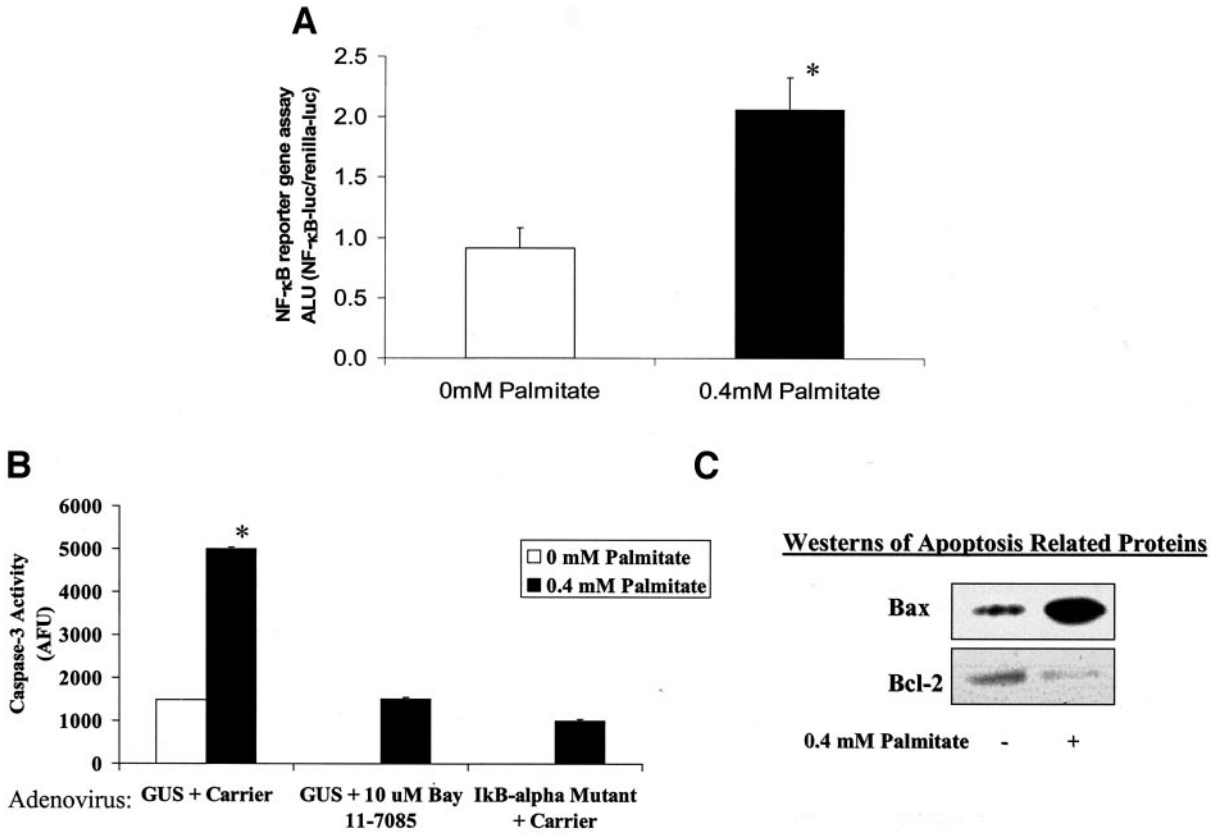

FIG. 3. Effects of palmitate on NF-κB transactivation and the role of NF-κB in palmitate-induced apoptosis. *A*: NF-κB reporter gene activity was assayed after 24 h of incubation with palmitate. Results are normalized to renilla luciferase (from a cytomegalovirus-driven vector) to account for transfection efficiency. Units are arbitrary light units (ALU). *B*: Caspase-3 activity was assayed after 24 h of incubation with palmitate and/or coincubation with the specific IKK inhibitor Bay 11-7085, carrier (DMSO), or overexpression of GUS (infection control) or the unphosphorylatable IκB-α mutant. *C*: Representative Western blots of PCs Bax and Bcl-2 protein levels after 24-h palmitate incubation with palmitate. All data are from representative experiments ($n = 3$). *$P < 0.05$ vs. 0 mmol/l palmitate.

multiple cells (endothelial cells, Chinese hamster ovary cells, astrocytes, pancreatic β-cells, and cardiomyocytes [17,41–44]); however, to our knowledge, this is the first report in which palmitate, by itself, has been demonstrated to produce apoptosis in retinal PCs. In an earlier study, Yamagishi et al. (34) failed to detect an increase in apoptosis in bovine retinal PCs incubated with 0.1 mmol/l palmitate for 48 h, although they did observe that palmitate at this concentration potentiated the apoptogenic effect of various AGEs. In agreement with their findings, we have observed that incubation of PCs with 0.1 mmol/l palmitate for 24 h has no effect on apoptosis (J.M.C., unpublished observations). Also, incubation with 0.4 mmol/l oleate, a monounsaturated FFA, did not have the cytotoxic effect of palmitate, suggesting that distinct FFAs may play different roles.

Ceramide, oxidant stress, and NF-κB activation have all been implicated in the pathogenesis of fatty acid–induced apoptosis in various settings; however, their relative role may be dependant on cell type (44). In the present study, ceramide accumulated in the PC in a dose-dependent manner when these cells were incubated with different concentrations of palmitate. We also found that blocking ceramide synthesis (Fig. 2*B*) or increasing its breakdown (Fig. 2*C*) rescued the PC from undergoing apoptosis. These findings are very similar to those obtained in pancreatic β-cells and cardiomyocytes, in which fumonisin B1 was demonstrated to inhibit fatty acid–induced apoptosis (45–47). It has been suggested that ceramide synthesized de novo from palmitate and serine causes apoptosis in β-cells and cardiomyocytes by inhibiting mitochondrial electron transport at complex III and secondarily increasing superoxide generation (21,22). We did not observe ceramide-induced increases in oxidative stress in this study; indeed, both treatment with fumonisin B1 and overexpression of ceramidase I increased oxidative stress (DCF fluorescence) at the same time they inhibited apoptosis (Fig. 2*D*). Thus, the prevention of apoptosis by fumonisin B1 and ceramidase must either be due to a decrease in oxidative stress not detected by measurements made here or it reflects an oxidative stress–independent action of ceramide.

Incubation with palmitate at concentrations of 0.1 and 0.4 mmol/l increased DCF fluorescence by six- and eightfold, respectively. These increments in palmitate-induced oxidant stress were most likely mediated by NAD(P)H oxidase (Fig. 3*B*), as described previously in vascular smooth muscle and endothelial cells (30). NAD(P)H oxidase also appeared to be in part responsible for the apoptosis induced by palmitate, since the death-promoting effect of the fatty acid was inhibited by the expression of DN-NAD(P)H oxidase subunits (Fig. 3*A*). The observation that overexpression of the antioxidant genes, catalase, Cu, Zn SOD, and MnSOD rescued the cells from palmitate-induced apoptosis suggests that oxidative stress is necessary for it to occur (Fig. 3*C*). On the other hand, the finding

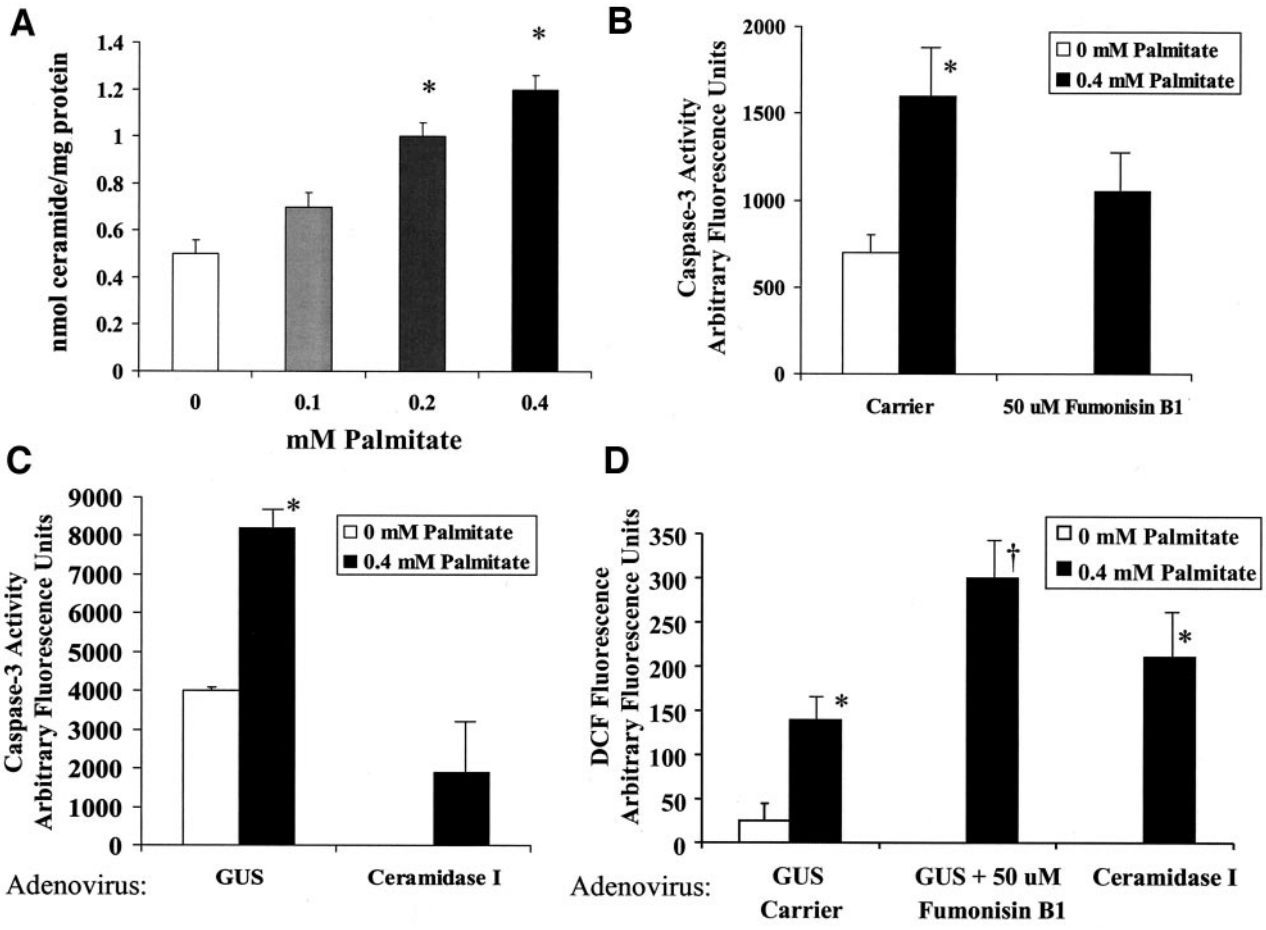

FIG. 4. Ceramide mass measurement, effect on apoptosis, and oxidative stress. *A*: Ceramide mass measurement in PCs after 18 h of incubation. *B*: Caspase-3 activity after 24 h of palmitate coincubated with the ceramide synthase inhibitor fumonisin B1 or carrier DMSO. *C*: Caspase-3 activity after 24 h of palmitate incubation with or without overexpression of ceramidase I, an enzyme that breaks down ceramide. GUS was used as infection control. *D*: Oxidative stress measured via DCF fluorescence. PCs were incubated with palmitate for 24 h and the indicated parameters as in *B* and *C*. All data are from representative experiments ($n = 3$). *$P < 0.05$ vs. 0 mmol/l palmitate, †$P < 0.001$ vs. 0 mmol/l palmitate.

that incubation with 0.1 mmol/l palmitate increased DCF fluorescence almost to the same extent as did 0.4 mmol/l palmitate is further evidence that by itself an increase in oxidant stress is not sufficient to cause apoptosis.

The redox-sensitive transcription factor NF-κB has been shown to be upregulated, as has the putatively NF-κB–driven proapoptotic factor Bax (Bcl-2–associated X protein) in retinal PCs of diabetic patients (3,16). Because of this and the observation that oxidative stress is an integral factor in palmitate-induced apoptosis (Fig. 2*C*), the contribution of NF-κB to apoptosis was examined in the present study. We found that palmitate induced a twofold increase in NF-κB transactivation (Fig. 4*A*) and a threefold increase in Bax protein in PCs. In addition, a concurrent decrease in the abundance of the prosurvival protein Bcl-2 was detected, seemingly tipping the balance between survival or death toward the latter (Fig. 3*C*). A similar effect was seen in Huh-7 cells (a human hepatic cell line), where there was a significant decrease in Bcl-2 gene expression upon 48 h of 0.15 mmol/l palmitate exposure (48). Interestingly, upregulation of at least five oxidative stress response genes involved in the formation of hydroperoxides and lipid peroxides were also observed in these cells. That NF-κB activation is involved in the enhancement of apoptosis by palmitate was further evidenced by the finding that inhibition of IKK with Bay 11-7085 or overexpression of an unphosphorylatable IκB-α mutant, both of which would attenuate NF-κB activation, inhibited palmitate-induced caspase-3 activity.

The abnormalities in cultured PCs incubated with palmitate reported here show many similarities to those observed in retinal PCs of humans with diabetic retinopathy (3,16). Common characteristics include increased oxidative stress, NF-κB activation, Bax accumulation, and, of course, programmed cell death itself. The precise interrelation of these changes to each other and to the pathogenesis of apoptosis is incompletely understood. As already noted, oxidative stress is necessary for palmitate-induced apoptosis but is not sufficient for it to occur. Thus, its interrelation to NF-κB activation and ceramide accumulation will require further study. A testable hypothesis is that the actions of both ceramide and NAD(P)H oxidase–derived oxidative stress converge to activate NF-κB, which in turn increases the expression of Bax. Bax then causes cytochrome C release from the mitochondria, which turns on the apoptosome and caspase-3 activity (49).

To establish the physiological significance of the findings reported here, experiments to determine whether similar events occur in PCs and perhaps other retinal cells in diabetic animals will be needed. Additionally, studies to examine how specific fatty acids interact with other factors to cause apoptosis could prove useful. In this context,

Yamagishi et al. (34) have observed that incubation of bovine retinal PCs with 0.1 mmol/l palmitate, which by itself increases oxidative stress but does not cause apoptosis, enhances the apoptogenic action of AGEs (possibly by increasing the expression of the AGE receptor RAGE). Also, in human umbilical vein endothelial cells, we have observed that a submaximal increase in IκB-α degradation, caused by a low concentration of palmitate (0.1–0.2 mmol/l), is enhanced when the concentration of glucose is elevated (50). Such findings suggest that moderately increased concentrations of glucose and palmitate (and possibly selected other FFAs) could complement each other in creating the metabolic stresses that activate NF-κB and lead to cellular dysfunction and apoptosis.

In conclusion, the results indicate that incubation with palmitate at a moderately elevated concentration increases apoptosis in cultured bovine retinal PCs. The apoptosis is associated with increases in NAD(P)H oxidase–mediated oxidative stress, ceramide accumulation, and NF-κB activation, and it is prevented when each of these changes is inhibited. Further studies are needed to determine the order in which these factors are altered and their interaction with each other.

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