Palmitate-Induced Apoptosis of Microvascular Endothelial Cells and Pericytes

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Abstract

Background: Recent observations in the EURODIAB Complications Study demonstrated that markers of insulin resistance are strong risk factors for retinopathy incidence in patients with diabetes. However, the molecular mechanism underlying this remains to be elucidated. In this study, we investigated the influence of palmitate, a major saturated free fatty acid in plasma, on the apoptotic cell death of cultured microvascular endothelial cells (EC) and retinal pericytes.

Materials and Methods: The intracellular formation of reactive oxygen species (ROS) was detected using the fluorescent probe CM-H$_2$DCFDA. DNA synthesis was determined by measuring [^{3}H]-thymidine incorporation into cells. DNA fragmentations of EC were quantitatively analyzed in an enzyme-linked immunosorbent assay, and DNA laddering was evaluated on agarose gel electrophoresis.

Results: Palmitate increased ROS generation in microvascular EC. Furthermore, palmitate significantly inhibited DNA synthesis and induced apoptotic cell death in EC, which were completely prevented by an antioxidant, N-acetylcysteine. Palmitate up-regulated pericyte mRNA levels of a receptor for advanced glycation end products (AGE), and thereby potentiated the apoptotic effects of AGE on pericytes.

Conclusions: The results suggest that palmitate could induce apoptotic cell death in microvascular EC and pericytes through the overgeneration of intracellular ROS, and thus be involved in the development of diabetic retinopathy.

Introduction

The vessels of the microvasculature are composed of two types of cells, endothelial cells (EC) and pericytes. We have shown that retinal pericytes not only regulate the growth, but also preserve the prostacyclin-producing ability and protect against lipid-peroxide–induced injury of co-cultured EC, thus providing a basis for understanding how diabetic retinopathy develops consequent to pericyte loss, the earliest histopathologic hallmark of diabetic retinopathy (1–3). Accelerated apoptosis of retinal vascular cells, as demonstrated by the development of acellular capillaries, was recently found to precede the characteristic vascular changes in human diabetic retinopathy (4).

Several major studies have established the value of strict control of blood glucose in preventing the microvascular complications of diabetes (5,6). Other studies underline the importance of active metabolic intervention, including control of blood pressure and lipid as well as glucose, for the prevention of diabetic microvascular complications (7,8). An increase in plasma free fatty acids is frequently observed in diabetes mellitus and the insulin-resistant state (9,10). However, the influence of fatty acids on the apoptotic cell death of microvascular EC and pericytes remains to be elucidated.

In the present study, we investigated the effects of palmitate, which is a major saturated free fatty acid in plasma (11), on the apoptosis of cultured microvascular EC and retinal pericytes. Because we recently showed that advanced glycation end products (AGE) induced pericyte apoptosis through interaction with a receptor for AGE (RAGE) (12,13), we further investigated whether palmitate could potentiate the cytopathic effects of AGE on pericytes.

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Materials and Methods

BSA (fraction V), palmitate, N-acetylcysteine (NAC), and L-cycloserine were purchased from Sigma (St. Louis, MO, USA). D-glyceraldehyde, glycolaldehyde,

and protease inhibitor cocktails were obtained from Nakalai Tesque (Kyoto, Japan). D-glucose was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). [^{3}H]-Thymidine, [γ-^{32}P]ATP, and Hybond-N$^+$ nylon membrane were obtained from Amersham Pharmacia Biotech (Buckinghamshire, United Kingdom). Reverse transcriptase and T4 polynucleotide kinase were purchased from Takara (Kyoto, Japan).

Cells

EC from human skin microvessels were maintained in E-BM medium supplemented with 5% fetal bovine serum (FBS), 0.4% bovine brain extracts, 10 ng/ml human epidermal growth factor, and 1 μg/ml hydrocortisone according to the manufacturer's instructions (Clonetics Corp., San Diego, CA, USA) (14). Cells at 5–10 passages were used for the experiments. Palmitate treatment was carried out in medium lacking epidermal growth factor and hydrocortisone. Pericytes were isolated from bovine retinas and maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Rockville, MD, USA) supplemented with 20% FBS (ICN Biomedicals Inc., Aurora, OH, USA) (15). Palmitate and AGE treatments were carried out in medium containing 2% FBS.

Intracellular Reactive Oxygen Species

The intracellular formation of reactive oxygen species (ROS) was detected using the fluorescent probe CM-H$_2$DCFDA (Molecular Probes Inc., Eugene, OR, USA) as described previously (16). Briefly, cells (1 $\times$ 10^5/96 well-plate) were loaded with 10 μmol/L CM-H$_2$DCFDA, incubated for 45 min at 37°C, and analyzed in a EZS-FL fluorescent plate reader (Asahi Techno Glass, Tokyo, Japan) using the EZScan-FL for Windows program.

Measurement of [^{3}H]-Thymidine Incorporation into EC and Pericytes

EC were incubated with or without 100 μM palmitate in the presence or absence of NAC for 24 hr, and then [^{3}H]-thymidine incorporation into the EC was determined as described previously (17). Pericytes were treated with 100 μg/ml of various types of AGE-BSA or nonglycated BSA in the presence or absence of palmitate for 2 days, and then [^{3}H]-thymidine incorporation was measured (17).

Measurement of Apoptotic Cell Death in EC and Pericytes

EC were incubated with or without 100 μM of palmitate in the presence or absence of NAC for 24 hr. The cells were then lysed and the supernatant was analyzed in an enzyme-linked immunosorbent assay (ELISA) for DNA fragments (Cell Death Detection ELISA, Roche Molecular Biochemicals, Mannheim, Germany). Pericytes were treated with 100 μg/ml of various types of AGE-BSA or nonglycated BSA in the presence or absence of 100 μM of palmitate for 2 days, and then apoptotic cell death was measured.

Primers and Probes

Sequences of the upstream and downstream primers and the internal probe used in the quantitative RT-PCR for detecting bovine RAGE and β-actin mRNAs were the same as described previously (12).

Poly(A)$^+$RNA Isolation and Quantitative RT-PCR

Poly(A)$^+$RNAs were isolated (18) from pericytes treated with or without 100 μM of palmitate for 2 days, and then analyzed by RT-PCR as described previously (19). Ten-microliter aliquots of each RT-PCR reaction mixture were electrophoresed on a 1.2% agarose gel, transferred to a Hybond-N$^+$ nylon membrane, and the membrane was then hybridized with the respective ^{32}P-end labeled probes (20). The amounts of the poly(A)$^+$RNA templates (30 ng) and the cycle numbers (35 cycles) for amplification were chosen in quantitative ranges where the reactions proceeded linearly, which had been determined by plotting signal intensities as functions of the template amounts and cycle numbers (12,21). Signal intensities of hybridized bands were measured by microcomputer-assisted NIH Image (Version 1.56).

Preparation of AGE Proteins

AGE proteins were prepared as described previously (22). Briefly, BSA was incubated under sterile conditions with D-glucose for 8 weeks or with D-glyceraldehyde or glycolaldehyde for 7 days. Then unincorporated sugars were removed by dialysis against PBS. Control nonglycated BSA was incubated in the same conditions except for the absence of reducing sugars. Preparations were tested for endotoxin using Endospecy ES-20S system (Seikagaku Co., Tokyo, Japan); no endotoxin was detectable.

Statistical Analysis

All values are presented as means $\pm$ SEM. Statistical significance was evaluated using the Student's t-test for paired comparison; $p < 0.05$ was considered significant.

Results

Effects of Palmitate on ROS Generation in EC

As shown in Figure 1, palmitate significantly increased intracellular ROS generation in human microvascular EC. ROS generation in EC was increased by approximately 1.4-fold after treatment with 100 μM palmitate for 4 hr.

Effects of Palmitate on [^{3}H]-Thymidine Incorporation into EC

We investigated the effects of palmitate on [^{3}H]-thymidine incorporation into EC. As shown in
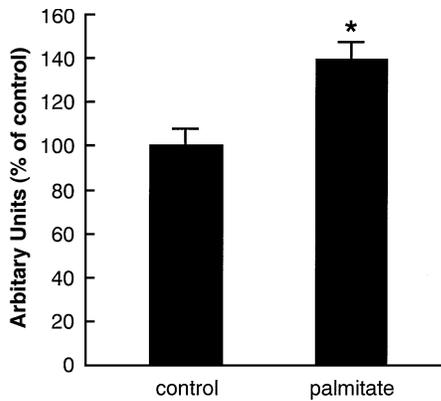
Fig. 1. Effects of palmitate on intracellular ROS generation in microvascular EC. Cells were treated with 100 μM palmitate for 24 hr, and then ROS were quantitatively analyzed. *$p < 0.01$ compared to the value of the control without palmitate.

Figure 2, palmitate significantly inhibited DNA synthesis in EC. EC displayed a 50% decrease in thymidine incorporation when exposed to 100 μM palmitate for 24 hr. To investigate whether intracellular ROS generation was involved in the inhibition of DNA synthesis induced by palmitate, the effects of NAC, an intracellular radical scavenger, on [³H]-thymidine incorporation into EC were examined. NAC was found to completely prevent the effects of palmitate (Fig. 2). L-cycloserine, an inhibitor of serine palmitoyltransferase, which ultimately blocks de novo ceramide synthesis, did not affect the palmitate-induced inhibition of DNA synthesis in EC (data not shown).
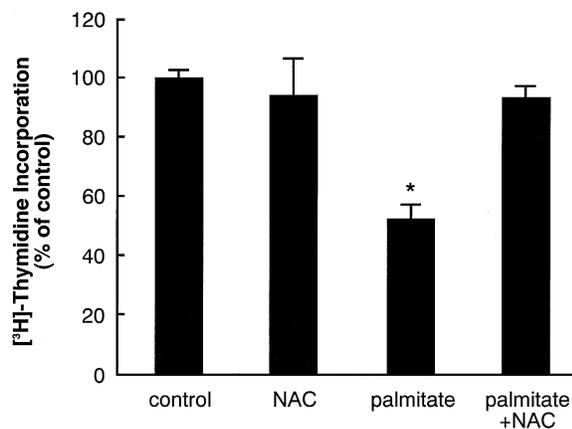

Fig. 2. Effects of palmitate on [³H]-thymidine incorporation into microvascular EC. Cells were treated with or without 100 μM of palmitate in the presence or absence of NAC for 24 hr. For the last 4 hr of culture, the cells were pulsed with 2 μCi of [³H]-thymidine. After cells were washed with ice-cold PBS, [³H]-thymidine incorporation into the cells was assayed. The percentage of [³H]-thymidine incorporation is indicated on the ordinate and related to the value without palmitate or NAC. *$p < 0.01$ compared to the value of the control without palmitate or NAC.
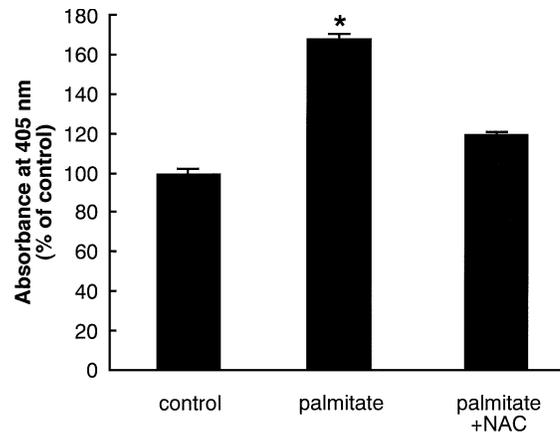

Fig. 3. Effects of palmitate on apoptotic cell death in microvascular EC. EC were treated with or without 100 μM palmitate in the presence or absence of NAC for 24 hr. Then cells were lysed and the supernatant analyzed by ELISA for DNA fragments. The percentage of absorbance at 405 nm is indicated on the ordinate and related to the value of the control without palmitate or NAC. *$p < 0.01$ compared to the value of the control of without palmitate or NAC.

Effects of Palmitate on Apoptotic Cell Death in EC

We next investigated whether palmitate could induce apoptotic cell death in microvascular EC. Apoptosis is characterized by DNA fragmentation due to endogenous endonuclease activation (23). Therefore, DNA fragments in the cytoplasm of EC exposed to palmitate were measured quantitatively. As shown in Figure 3, palmitate significantly induced apoptotic cell death in EC. As in the case of DNA synthesis, the effect of palmitate on apoptotic cell death in EC was significantly prevented by NAC treatment. Furthermore, it was also found that palmitate actually induced DNA ladder formation in EC (data not shown).

Effects of Palmitate on RAGE Gene Expression in Retinal Pericytes

We recently found that AGE induced apoptotic cell death in bovine retinal pericytes through interaction with RAGE (13). Therefore, poly(A)$^{+}$RNAs were isolated from pericytes treated with 100 μM of palmitate for 2 days, and analyzed by a quantitative RT-PCR technique to determine the effect of palmitate on the expression of RAGE genes. As shown in Figure 4, palmitate up-regulated RAGE mRNA levels in pericytes by approximately 2-fold compared with the basal level. An increase in the expression level of RAGE proteins was also confirmed in palmitate-treated pericytes (data not shown).

Effects of Palmitate on [³H]-Thymidine Incorporation into Pericytes

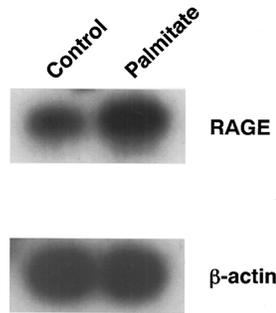To find any functional role of the palmitate-induced RAGE overexpression, pericytes were incubated

Fig. 4. Effects of palmitate on RAGE gene expression in retinal pericytes. Cells were treated with or without 100 μM of palmitate for 2 days, and then 30 ng poly(A)$^+$RNAs were transcribed and amplified by PCR. Each lower panel shows the expression of β-actin genes. PCR amplification for β-actin mRNA was performed for 20 cycles.

with 100 μg/ml of various types of AGE-BSA or nonglycated BSA in the presence or absence of palmitate. Although palmitate did not affect DNA synthesis in the pericytes exposed to nonglycated BSA, palmitate-treated pericytes were more sensitive to AGE because the growth inhibitory effects of AGE on pericytes were significantly enhanced in these pericytes (Fig. 5).

Effects of Palmitate on Apoptotic Cell Death in Pericytes

We next investigated whether palmitate-induced RAGE overexpression is actually related to pericyte apoptosis. As shown in Figure 6, although palmitate
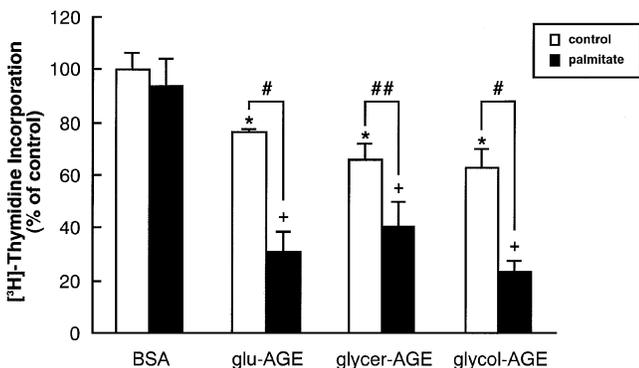

Fig. 5. Effects of palmitate on [^{3}H]-thymidine incorporation into retinal pericytes. Cells were treated with 100 μg/ml of various types of AGE-BSA or nonglycated BSA in the presence or absence of 100 μM of palmitate for 2 days. For the last 4 hr of culture, the cells were pulsed with 2 μCi of [^{3}H]-thymidine. After cells were washed with ice-cold PBS, [^{3}H]-thymidine incorporation into the cells was assayed. The percentage of [^{3}H]-thymidine incorporation is indicated on the ordinate and related to the value with nonglycated BSA alone. Glu-AGE, glucose-derived AGE; glycer-AGE, glyceraldehyde-derived AGE; glycol-AGE, glycolaldehyde-derived AGE. *$p < 0.01$ compared to the value of the control with nonglycated BSA alone. +$p < 0.01$ compared to the value of the control with nonglycated BSA and palmitate. ##$p < 0.05$. #$p < 0.01$ compared to the value of non–palmitate-treated cells.
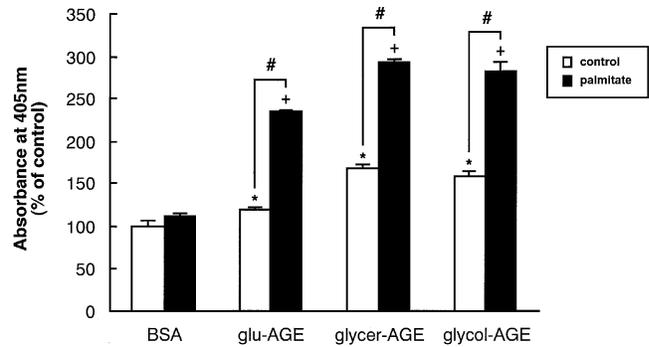

Fig. 6. Effects of palmitate on apoptotic cell death in pericytes. Pericytes were treated with 100 μg/ml of various types of AGE-BSA or nonglycated BSA in the presence or absence of 100 μM of palmitate for 2 days. Then cells were lysed and the supernatant analyzed by ELISA for DNA fragments. The percentage of absorbance at 405 nm is indicated on the ordinate and related to the value of the control with nonglycated BSA alone. Glu-AGE, glucose-derived AGE; glycer-AGE, glyceraldehyde-derived AGE; glycol-AGE, glycolaldehyde-derived AGE. *$p < 0.01$ compared to the value of the control with nonglycated BSA alone. +$p < 0.01$ compared to the value of the control with nonglycated BSA and palmitate. #$p < 0.01$ compared to the value of non–palmitate-treated cells.

itself did not affect apoptotic cell death in pericytes exposed to nonglycated BSA, AGE-induced pericyte apoptosis was significantly potentiated by treatment with 100 μM of palmitate.

Discussion

The present study demonstrates for the first time that palmitate, a major saturated free fatty acid in plasma, induces growth retardation and apoptotic cell death in microvascular EC. The evidence that it was palmitate-induced intracellular ROS generation, independent of ceramide synthesis, that caused the cytopathic effects of palmitate is as follows. First, palmitate increased intracellular ROS generation in microvascular EC (Fig. 1). Second, NAC, an intracellular radical scavenger, completely prevented the palmitate-induced DNA synthesis inhibition as well as apoptosis in EC (Figs. 2 and 3). Third, biochemical inhibition of de novo ceramide synthesis did not affect the growth inhibitory effect of palmitate on EC (data not shown). These observations are consistent with recent studies by Listenberger et al. (24), which showed that palmitate induced apoptosis of Chinese hamster ovary cells through the generation of ROS.

We previously showed that leptin induces ROS generation and monocyte chemoattractant protein-1 expression in aortic EC by increasing fatty acid oxidation (14). Because an increase of plasma free fatty acids, including palmitate, is frequently observed in diabetes and the insulin-resistant state (9,10), ROS overgeneration through mitochondrial fatty acid oxidation in EC might contribute to the development of diabetic micro- and macrovascular complications.

Recent observations in the EURODIAB Complications Study demonstrated that markers of insulin resistance are strong risk factors for retinopathy incidence in patients with diabetes, and this supports our hypothesis (25).

We recently found that AGE, glycated protein derivatives formed at an accelerated rate in diabetes, could induce the apoptotic cell death of retinal pericytes through interaction with RAGE. In the present study, palmitate supplementation was found to potentiate the cytopathic effects of AGE on pericytes through overexpression of RAGE proteins. Because we previously showed that RAGE gene expression is regulated by redox-sensitive transcriptional factor nuclear factor-κB (NF-κB), palmitate-induced ROS generation and the resultant activation of NF-κB may be involved in RAGE protein overexpression in pericytes (26).

Accelerated apoptosis of retinal microvascular wall cells such as EC and pericytes was recently found to precede the characteristic diabetic vascular changes and may therefore be a key event in the early phase of development of diabetic retinopathy (4). Bax protein is increased in the retina of diabetic subjects and is associated with pericyte apoptosis (4). Therefore, palmitate-induced potentiation of the AGE–RAGE interaction and the subsequent ROS generation could be implicated in Bax protein induction in diabetic retinopathy (27).

In conclusion, the present study indicates that palmitate, a major saturated free fatty acid in plasma, induces programmed cell death of microvascular EC through the overgeneration of intracellular ROS. Palmitate also potentiates the apoptotic effects of AGE on retinal pericytes. The results suggest that palmitate may play some role in the development of diabetic retinopathy.

Acknowledgments

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