Effect of Vitamin E on Linoleic Acid–Mediated Induction of Peroxisomal Enzymes in Cultured Porcine Endothelial Cells

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ABSTRACT Linoleic acid decreases endothelial barrier function in culture. We hypothesize that the mechanism may involve induction of peroxisomes, with subsequent generation of hydrogen peroxide, and that vitamin E may protect against barrier function loss by preventing the induction of peroxisomal enzymes. To investigate this hypothesis, we exposed cultured endothelial cells to 0 or 90 μmol/L linoleic acid [18:2(n-6)], with or without 25 μmol/L supplemental vitamin E, for 5 d. The induction of peroxisomes by linoleic acid exposure was determined by measuring cellular peroxisomal β-oxidation and catalase activity. Vitamin E alone had no effect on β-oxidation or catalase activity, whereas linoleic acid exposure significantly increased both compared with control values. Vitamin E supplementation prevented induction of peroxisomal β-oxidation and catalase activity by 18:2. In contrast, cell enrichment with vitamin E had no effect on 18:2-induced accumulation of cytoplasmic lipid-like droplets. These results confirm our hypothesis that the protective effects of vitamin E against fatty acid–mediated endothelial cell injury may be due in part to the ability of vitamin E to prevent the induction of peroxisomal β-oxidation enzymes and thus the formation of excess hydrogen peroxide. J. Nutr. 120:331–337, 1990.

INDEXING KEY WORDS:
endothelial cells vitamin E fatty acids peroxisomes cell injury

The mechanism of atherosclerosis may involve damage to or dysfunction of the vascular endothelium, with subsequent reduced effectiveness of the endothelium as a barrier to plasma components [1, 2]. High levels of triglyceride-rich lipoproteins have been implicated in the endothelial injury process [3, 4]. Endothelial exposure to excessive amounts of free fatty acid anions, derived from the hydrolysis of triglyceride-rich lipoproteins, may result in the accumulation of these fatty acids within endothelial cells and may lead to cellular changes resulting in cell injury or dysfunction. This may in turn allow increased penetration of remnant lipoproteins into the arterial wall [3–5]. In support of this possibility, we showed that exposure to oleic [18:1(n-9)] [6] and especially to linoleic [18:2(n-6)], but not to linolenic [18:3(n-3)] [7], acid increased the transfer of albumin across cultured porcine endothelial cell monolayers. Prior cellular incubation with media containing 25 μmol/L vitamin E for 24 h protected endothelial cells from injury by pure linoleic acid and linoleic acid hydroperoxide, as indicated by a decreased rate of albumin transfer across the endothelium [8]. The mechanism of the linoleic acid–specific increase in albumin transfer is not clear.

One possible mechanism of linoleic acid–induced endothelial cell toxicity may involve peroxisomes, cytoplasmic organelles present in most if not all cells [9]. Several types of chemicals, such as hypolipidemic drugs, and nutritional alterations, such as feeding high-fat diets, have been shown to induce hepatic peroxisomal proliferation in rodents and other species [10–13]. The peroxisomal fatty acid β-oxidation pathway was specifically induced by peroxisome proliferators, whereas other enzymes, such as catalase, were slightly induced [10–13]. Because hydrogen peroxide is an end product of peroxisomal β-oxidation, it has been hypothesized that the adverse effects of peroxisome

1Supported in part by grants 1PO1 HL36552 and 1RO1 CA43719 from the National Institutes of Health, a grant from the American Heart Association, Kentucky Affiliate, and the Kentucky Agricultural Experiment Station.

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331
proliferators, such as hepatic tumors, are caused by the overproduction of hydrogen peroxide [10]. We recently demonstrated that peroxisomal β-oxidation and catalase activity are induced in cultured endothelial cells by the hypolipidemic drug ciprofibrate and by certain fatty acids [14, 15]. Compared to enrichment with stearic [18:0], oleic and linoleic acid, cellular enrichment with linoleic acid induced peroxisomal β-oxidation most markedly [15]. It therefore seems possible that a metabolic relationship may exist between 18:2-induced peroxisomal β-oxidation and the observed 18:2-induced increase in the transfer of albumin across cultured cell monolayers.

Vitamin E deficiency results in peroxisome proliferation in the liver [16]. More specifically, vitamin E deficiency in rats induced the activities of peroxisomal fatty acid CoA oxidase and carnitine acyltransferases, but not of catalase [16]. Furthermore, supplementation with vitamin E decreases the production of hydrogen peroxide from human polymorphonuclear leukocytes [17, 18]. Because vitamin E appears to be able to protect endothelial cells against 18:2-induced injury [8], the present study was designed to investigate whether this protective action of vitamin E may be due in part to its ability to prevent the induction of peroxisomal enzymes by fatty acids and thus to curtail the formation of cytotoxic hydrogen peroxide. Endothelial cells were exposed to linoleic acid in the presence or absence of vitamin E for 5 d. We then quantified the activities of the peroxisomal enzyme catalase and the peroxisomal fatty acid β-oxidation pathway. Ultrastructural changes also were assessed.

**MATERIALS AND METHODS**

**Cell culture.** Endothelial cells from porcine pulmonary arteries were obtained as described by Hennig et al. [6]. Briefly, blood vessels were resected under sterile conditions and rinsed with medium 199 (M-199, GIBCO Laboratories, Grand Island, NY) containing antibiotics; the lumen was then exposed to 0.1% collagenase (Worthington Biochemical, Freehold, NJ) for 5 to 10 min. After several rinses with M-199, the lumen was swabbed gently with a sterile, cotton-tipped applicator. The adherent endothelial cells were released into M-199 containing 10% fetal bovine serum (HyClone Laboratories, Logan, UT) and seeded into T-25 flasks. The flasks were capped loosely and placed in a CO₂ incubator (5%) at 37°C. The medium was changed after 24 h, and cultures were left in the incubator for approximately 5 to 8 d, when confluency was reached. The cells were then subcultured in M-199 containing 10% fetal bovine serum by using standard techniques and flasks obtained from Costar (Cambridge, MA). Cultures were determined to be endothelial by uniform morphology and by quantitative determination of angiotensin-converting enzyme activity. Cells from passages 10–15 were used in this study. Experiments were performed three times, and results were similar at cell passages 10–15. For the determination of fatty acid β-oxidation and catalase activity, cells were plated on T-75 flasks or P-100 dishes (Costar). For electron microscopy studies, cells were plated on gelatin-impregnated polycarbonate filters (Nucleopore, Pleasanton, CA; 13 mm diameter and 0.8 μm pore size) glued to polystyrene chemotactic chambers (ADAPS, Dedham, MA) [6].

The experimental media were composed of M-199 enriched with 5% fetal bovine serum, 90 μm/L (2.5 mg/100 mL) high-purity (≥99%) linoleic acid (Nu-Chek-Prep, Elysian, MN) and either 0 μm/L or 25 μm/L (1.1 mg/100 mL) vitamin E (α-tocopherol). Fatty acid- and vitamin E–enriched culture media were prepared as described earlier [6, 8].

**Experimental procedures.** Cells seeded for 4 h in medium without supplemental vitamin E or fatty acids were exposed to experimental media for 5 d to allow for maximal induction of peroxisomes. The experimental media were composed of M-199 enriched with 5% fetal bovine serum (control media) plus either 25 μm/L vitamin E, 90 μm/L 18:2, or 25 μm/L vitamin E and 90 μm/L 18:2. Cells were harvested by first washing them in ice-cold phosphate-buffered saline (PBS) and then scraping with silicone rubber blades. Harvested cells were centrifuged, suspended in PBS and sonicated, all in ice-cold buffer. Homogenates were then assayed for protein [19], catalase activity [20] or peroxisomal fatty acid β-oxidation [21]. To monitor vitamin E levels in the media and cells as they may relate to biological function, cell homogenates and media were assayed for vitamin E [22, 23] after incubation for 0, 2 and 5 d. For vitamin E determination, the method of Catignani [22] was modified by redissolving the lipid residue in methanol, by using 100% methanol as the mobile phase of the high performance liquid chromatography system and by implementing the fluorescence detection method of Hatam and Kayden [23].

Ultrastructural assessment involved the examination of 1) cells plated in control medium on gelatin-impregnated polycarbonate filters; 2) cells plated on filters in control medium supplemented with 18:2 (90 μm/L); and 3) cells cultured in control medium supplemented with vitamin E (25 μm/L) and 18:2 (90 μm/L). Cells grown in control medium supplemented with vitamin E alone also were examined. Exposure to 18:2, vitamin E, and vitamin E plus 18:2 was for 5 d. The cells on the filters were fixed in 3.5% cacodylate buffered glutaraldehyde fixative (pH 7.2–7.4) for 10 min at room temperature and then for an additional 80 min at 4°C. The remaining tissue processing procedures were similar to those detailed elsewhere [24]. All epoxy-embedded tissues were sectioned at 80 to 100 nm. From 30 to 50 cells from four to nine filters per treatment were imaged or examined in a Philips 400 electron microscope at 60 kV.
VITAMIN E, PEROXISOMES AND ENDOTHELIAL CELLS

**FIGURE 1** Effect of linoleic acid and vitamin E on peroxisomal \( \beta \)-oxidation in cultured endothelial cells. Peroxisomal \( \beta \)-oxidation is expressed as nmol palmitoyl CoA oxidized per min per mg protein. The experimental media consisted of M-199 enriched with 5% fetal bovine serum [Control] plus either 25 \( \mu \)mol/L vitamin E [VitE], 90 \( \mu \)mol/L linoleic acid (18:2) or 25 \( \mu \)mol/L vitamin E and 90 \( \mu \)mol/L linoleic acid. Values are means ± SEM \((n = 6)\). Data were analyzed by ANOVA, and when a significant difference occurred, a protected LSD test was performed. Value for linoleic acid was significantly greater than that for controls \((p \leq 0.05)\).

**Statistical analysis.** Data were analyzed statistically by analysis of variance, and when significant differences occurred, a protected LSD test was performed \((25)\). A statistical probability of \(p \leq 0.05\) was considered significant.

**RESULTS**

The effects of linoleic acid and vitamin E on peroxisomal \( \beta \)-oxidation in cultured endothelial cells are shown in Figure 1. The induction of peroxisomal \( \beta \)-oxidation was not affected by vitamin E alone. Exposure to 18:2, however, significantly increased \( \beta \)-oxidation compared with that in control cultures. This increase in peroxisomal \( \beta \)-oxidation was prevented by simultaneous enrichment with vitamin E. The chosen fatty acid concentration did not appear to adversely affect cell viability as established by trypan blue exclusion studies and morphological assessment by phase contrast microscopy, which showed maintenance of a typical cobblestone appearance.

The effects of 18:2 and vitamin E on peroxisomal catalase activity are shown in Figure 2. As with the \( \beta \)-oxidation data, 18:2 increased catalase activity compared with that in control cultures. Furthermore, simultaneous enrichment and vitamin E also prevented the fatty acid–induced increase in catalase activity.

**FIGURE 2** Effect of linoleic acid and vitamin E on peroxisomal catalase activity in cultured endothelial cells. Catalase activity is expressed as \( \mu \)mol hydrogen peroxide decomposed per min per mg protein. The experimental media consisted of M-199 enriched with 5% fetal bovine serum [Control] plus either 25 \( \mu \)mol/L vitamin E [VitE], 90 \( \mu \)mol/L linoleic acid (18:2) or 25 \( \mu \)mol/L vitamin E and 90 \( \mu \)mol/L linoleic acid. Values are means ± SEM \((n = 6)\). Data were analyzed by ANOVA, and when a significant difference occurred, a protected LSD test was performed. Value for linoleic acid was significantly greater than that for controls \((p \leq 0.05)\).

Figure 3 shows the levels of vitamin E in media and cells. Cell enrichment with vitamin E appeared to increase throughout the 5-d incubation period. As cells became enriched, vitamin E levels in the media fell. In contrast to media enrichment with vitamin E alone, enrichment with both vitamin E and 18:2 led to a complete depletion of vitamin E from the media after 5 d. Conversely, cells exposed to both 18:2 and vitamin E remained significantly enriched with vitamin E compared with control cultures, even after 5 d. The vitamin E level of endothelial cells immediately after plating, that is, before exposure to experimental media, was 0.037 ± 0.001 \( \mu \)g/mg protein.

The ultrastructure of cells grown on polycarbonate filters in control culture medium suggested viable, synthetically active and well-preserved cells [Fig. 4A]. The cells formed a monolayer, although occasional partial overlapping was observed. Infrequently, small amounts of cellular debris were present between the cells and the polycarbonate filters. The cell surface was uniform [Fig. 4A]. Vesicular invaginations were uncommon. The most prominent cytoplasmic components were heterogeneous lysosome-like bodies, rough endoplasmic reticulum, mitochondria, polyribosomes and, when in the plane of section, Golgi complex [Fig. 4A]. Lipid-like droplets were noted in only very few of the control
cultured cells. Nuclei were elongated and formed mostly of euchromatin, and they contained prominent nucleoli. The ultrastructure of cells grown in control medium supplemented with vitamin E was similar to that of cells grown in control medium alone. However, the vitamin E-supplemented cultures contained more cellular debris than unsupplemented cultures. The morphology of cells cultured in medium to which 18:2 (90 μmol/L) was added deviated from that of cells grown in control medium in two main respects. These cells appeared larger and contained markedly more cytoplasmic lipid-like droplets than those grown in control medium (Fig. 4B). With one exception, the structure of cell monolayers grown in medium supplemented with vitamin E and 18:2 was similar to that of cells treated with 18:2 alone: Monolayers exposed to vitamin E and 18:2 contained considerably more intercellular material than 18:2-treated cultures (Fig. 4C). The debris often elevated the viable cells from the polycarbonate filters (Fig. 4C).

**DISCUSSION**

Damage to or dysfunction of the vascular endothelium and the resulting disturbance in endothelial integrity may be involved in the process of atherosclerotic lesion formation (1, 2). High levels of triglyceride-rich lipoproteins and the free fatty acids derived from triglyceride hydrolysis have been implicated in the injury process of the endothelium and in a decrease in endothelial barrier function (3, 4). In support of this possibility, we have shown that exposure to oleic (6) and especially to linoleic, but not to linolenic (7), acid increased the transendothelial movement of albumin across cultured cell monolayers.

The mechanism of the linoleic acid–specific increase in albumin transfer is not known at present. In addition to a selective accumulation of this fatty acid in cellular phospholipid and triglyceride fractions (7), we speculate that metabolites of peroxisomal fatty acid β-oxidation, such as hydrogen peroxide, may be involved in the mechanism of endothelial cell injury (15) and the resulting decrease in the endothelial barrier function that was observed. There is evidence that high-fat diets induce peroxisomal β-oxidation in the liver (12, 13) and that polyunsaturated fatty acids appear to be used preferentially for total cell oxidation (26). In the present study, exposing cultured endothelial cells to linoleic acid markedly increased peroxisomal β-oxidation. Other 18-carbon fatty acids, such as stearic, oleic and linolenic acid, did not exhibit such a consistently marked influence on β-oxidation (15). Whether the twofold increase in catalase activity is sufficient to detoxify the hydrogen peroxide produced by peroxisomal β-oxidation is unclear.

**FIGURE 4** Transmission electron micrographs of endothelial cell monolayers cultured on polycarbonate micro pore filters. Cells were grown in control medium [A], or they were supplemented with 90 μmol/L 18:2 [linoleic acid] [B] or with 25 μmol/L vitamin E and 90 μmol/L 18:2 [C]. Note that fatty acid–treated cells contain considerably more lipid-like droplets [L] (lipid dissolved during processing). Cells treated with both vitamin E and fatty acid were characterized by the presence of intercellular debris [D]. Lysosome-like structures, L, Golgi complex, G, mitochondria, M, nucleus, N, nucleolus, Nu, polycarbonate filter, F, rough endoplasmic reticulum, arrow; polyribosomes, arrowhead. Calibration bar in the lower left corner is equal to 1 μm [×16,800].
VITAMIN E, PEROXISOMES AND ENDOTHELIAL CELLS
Vitamin E may be antiatherogenic by protecting endothelial cells from fatty acid- and from fatty acid hydroperoxide–induced cell injury [8]. Although the mode of cell protection by vitamin E is not clear, its beneficial action might be via its antioxidant activity [27], its ability to act as a biological membrane stabilizer [28, 29], or its regulatory function in cell turnover rate [30]. Furthermore, supplementation with vitamin E has been shown to decrease the production of hydrogen peroxide from human polymorphonuclear leukocytes [17, 18]. Previous work from our laboratory showed that supplemental vitamin E protected cultured endothelial cell monolayers from linoleic acid–induced loss of barrier function [8].

In this study we demonstrated that cells remained enriched with vitamin E as long as 5 d following a single vitamin E supplementation, allowing vitamin E to be available for its biological function as a protector against fatty acid–induced cell injury. Results from the present study suggest that one possible mechanism of vitamin E’s action is to reduce the induction of peroxisomal β-oxidation enzymes and thus the formation of excess hydrogen peroxide. Vitamin E could reduce 18:2-induced peroxisomal β-oxidation enzyme production by preventing excess fatty acid from entering the cell and preventing cellular accumulation of lipid-like droplets. However, our data do not support this mechanism because ultrastructural evaluation of vitamin E–enriched cells, which also were exposed to 18:2, revealed no reduction in lipid-like droplet accumulation in comparison to cells treated with 18:2 alone. It is possible that vitamin E reduces the 18:2-induced increase in peroxisomal β-oxidation by formation of vitamin E complexes with free fatty acids [29], possibly by formation of a hydrogen bond between the α-tocopherol chromanol nucleus hydroxyl and the carboxyl group of a fatty acid [31]. Vitamin E may thus prevent the induction of peroxisomal enzymes by blocking the carboxyl end of the fatty acid, an action that may prevent peroxisomal β-oxidation of fatty acids as well as excess formation of cytotoxic hydrogen peroxide, a by-product of peroxisomal β-oxidation. Since β-oxidation could be impeded by vitamin E, cellular lipid degradation might be reduced. In this event, a decrease in cytoplasmic lipid-like droplets should not be expected.

In summary, our data suggest that the protective effects of vitamin E against fatty acid–mediated endothelial cell injury may be due in part to the ability of vitamin E to reduce peroxisomal β-oxidation as a consequence of decreased fatty acid–mediated induction of peroxisomes. These protective properties of vitamin E against endothelial cell injury may have implications in regard to understanding the etiology of atherosclerosis. Further studies are needed to clearly define the relationship between peroxisome proliferation and atherosclerosis.

**LITERATURE CITED**

VITAMIN E, PEROXISOMES AND ENDOTHELIAL CELLS


