Linoleic acid potentiates TNF-mediated oxidative stress, disruption of calcium homeostasis, and apoptosis of cultured vascular endothelial cells

Michal Toborek,[1,*] Emmanuelle M. Blanc,[†] Simone Kaiser,[*,§] Mark P. Mattson,[†] and Bernhard Hennig[**]

Department of Surgery,* Sanders-Brown Research Center on Aging,† University of Potsdam and German Institute of Human Nutrition,§ and Department of Nutrition and Food Science,** University of Kentucky, Lexington, KY 40536

Abstract Diet-derived lipids may influence cytokine-mediated endothelial cell dysfunction, including TNF-induced apoptosis. To test this hypothesis, oxidative stress, intracellular calcium levels, endothelial barrier function, cell viability, and apoptosis were measured in vascular endothelial cells treated with 90 μM linoleic acid (18:2, n–6) and/or 20 ng/mL TNF (100 U/mL). For short-term exposure, endothelial cells were exposed to 18:2 for 6 h or to TNF for 1.5 h. For long-term exposure, endothelial cultures were treated with 18:2 for 24 h and with TNF for 19.5 h. In cells exposed to 18:2 + TNF, pretreatment with 18:2 began 4.5 h before additional exposure to TNF for either 1.5 h (short-term exposure) or 19.5 h (long-term exposure). After treatment, endothelial cultures were washed and incubated with maintenance medium for up to 4 days. Although initial treatment with TNF or 18:2 significantly increased oxidative stress and intracellular calcium levels, only exposure to TNF induced apoptosis in cultured endothelial cells. Furthermore, the combined exposure to 18:2 + TNF potentiated TNF-induced apoptosis. Additional treatments with BAPTA-AM, n-propyl gallate, vitamin E, and with aurintricarboxylic acid partially protected against TNF- or 18:2 + TNF-induced apoptosis. The present study suggests that changes in the cellular lipid environment may markedly influence local TNF-induced events in the vascular endothelium, including endothelial cell apoptosis. Such mechanisms may play a role in the damage and death of vascular endothelial cells in atherosclerosis.—**Toborek, M., E. M. Blanc, S. Kaiser, M. P. Mattson, and B. Hennig.** Linoleic acid potentiates TNF-mediated oxidative stress, disruption of calcium homeostasis, and apoptosis of cultured vascular endothelial cells. *J. Lipid Res.* 1997. **38:** 2155–2167.

Supplementary key words atherosclerosis • fatty acids • cytokines • oxidative stress • intracellular calcium • antioxidants • apoptosis

The vascular endothelium is a critical target for injuries mediated by free fatty acids or inflammatory cytokines, such as tumor necrosis factor-α (TNF). Due to hydrolysis of triglyceride-rich lipoproteins mediated by lipoprotein lipase, free fatty acids are released in the proximity of the endothelium. To support the initial hypothesis by Zilversmit (1) that free fatty acids may cause local injury to the endothelium, it was shown that selected fatty acids disrupted endothelial barrier function in an in vitro experimental setting (for review see ref. 2). Among different free fatty acids tested, linoleic acid (18:2, n–6) exerts the most harmful effects towards endothelial cells. For example, in cultured endothelial cells, 18:2 induced oxidative stress, increased intracellular calcium levels (3), diminished nitric oxide synthase activity (4), disturbed connective tissue metabolism (5, 6), and inhibited gap-junctional intercellular communication (7). In addition, oxidative derivatives of 18:2 also caused disturbances in endothelial cell metabolism. Linoleic acid hydroperoxide induced lipid peroxidation (8), evoked an increase in intracellular calcium levels, hydrolysis of phospholipids (9), transendothelial albumin transfer (10), and cytotoxicity (8) in cultured endothelial cells. Moreover, recent evidence indicates that hydroperoxides of unsaturated fatty acids, such as 15-hydroperoxyeicosatetraenoic acid (15-HPETE, a product of arachidonic acid peroxidation) or 13-hydroperoxydodecadienoic acid (13-HPODE, a product of 18:2 peroxidation) can induce apoptosis, decrease cellular viability, increase intracellular calcium, and induce DNA fragmentation in different T cell lines (11). It should be noted that HPODE is the major fatty acid oxidation product found in oxidized low den-

Abbreviations: 18:2, linoleic acid; TNF, tumor necrosis factor-α; 13-HODE, 13-(S)hydroxy-9(Z),11(E)-octadecadienoic; ATA, aurintricarboxylic acid; nPG, n-propyl gallate; DCF, 2′,7′-dichlorofluorescein; $[Ca^{2+}]_i$, intracellular calcium; MTT, 3-[4,5-dimethylthiazol-2]-2,5 diphenyl tetrazolium bromide.

[1]To whom correspondence should be addressed.

sity lipoprotein (LDL) (12), and highly oxidized LDL initiated apoptosis in arterial smooth muscle cells, macrophages, and fibroblasts (13). The role of oxidative stress in the induction of cellular apoptosis is further supported by the observation that low concentrations of hydrogen peroxide can induce apoptosis of cultured endothelial cells (14).

Elevated levels of inflammatory cytokines, especially TNF, may play a critical role in atherogenesis. The main sources of TNF within atherosclerotic vessels include monocyte/macrophages and T cells (15). Moreover, leukocyte production of TNF is increased in atherosclerosis patients (16) and elevated levels of this cytokine are observed in atherosclerotic vessels (17, 18). There is evidence that TNF can activate vascular endothelial cells (19, 20) as well as induce cytotoxic effects, including endothelial cell apoptosis (21).

Recent evidence indicates that fatty acid metabolism may be strongly associated with inflammatory cytokine- or TNF-mediated effects. Both oxidized LDL and 13-HPODE augment TNF-induced adhesion molecule expression in human endothelial cells (22). In addition, TNF enhanced monocyte or endothelial cell-mediated oxidation of LDL (23), and TNF-mediated cytolysis can be prevented by limiting the availability of arachidonic acid (24). Similarly, interleukin-1 stimulated oxidation of 18:2 into 13-(S)hydroxy-9(Z),11(E)-octadecadienoic (13-HODE) and 9-(R)hydroxy-10(E),12(Z)-octadecadienoic acids in cultured endothelial cells (25). Moreover, it is known that lipoprotein lipase can release free fatty acids from lipoproteins and also induce TNF gene expression and TNF production in macrophages by protein kinase C signaling pathway (26, 27). Thus endothelial cells may be simultaneously exposed to elevated levels of free fatty acids and TNF. We reported that such a combined exposure to 18:2 and TNF can cross-amplify oxidative injury and dysfunction of cultured endothelial cells (3).

Selected lipids and oxidative stress are implicated as mediators of apoptosis. In addition, the lipid environment and TNF may cross-influence their metabolic effects. Therefore, the hypothesis that 18:2 can modify TNF-induced oxidative stress and disruption of calcium homeostasis and thereby promote endothelial cell apoptosis was tested.

MATERIAL AND METHODS

Endothelial cell cultures and experimental media

Endothelial cells were isolated from porcine pulmonary arteries and cultured in medium M-199 (GIBCO Laboratories, Grand Island, NY) containing 10% calf bovine serum (HyClone Laboratories, Inc., Logan, UT) according to the method of Hennig et al. (28). Cultures were determined to be endothelial by uniform cobblestone morphology, determination of angiotensin-converting enzyme activity, and cellular uptake of fluorescent labeled acetylated LDL (1,1′-dioctadecyl-3,3,3′33-tetramethyl-indocarbocyanine perchlorate; Molecular Probes Inc., Eugene, OR). Cells from passages 6–10 were used in the present study. Subconfluent endothelial cell cultures were treated with 90 μM linoleic acid (18:2 n–6) (>99% pure, Nu-Chek Prep, Elysian, MN) and/or TNF (20 ng/mL = 100 U/mL; Knoll Laboratories, Whippany, NJ). Linoleic acid-enriched experimental media were prepared as described earlier (29).

Our previous studies indicated that the maximum oxidative stress in cells exposed to fatty acids or TNF occurs after 6 and 1.5 h incubation, respectively (29, 30). Therefore, for a typical experiment, endothelial cells were exposed to 18:2 and/or TNF as follows:

Short-term exposure. Endothelial cells were treated with 18:2 for 6 h (18:2 group) or with TNF to 1.5 h (TNF group). In 18:2 + TNF group, exposure to 18:2 began 4.5 h before adding TNF. A combined exposure to 18:2 + TNF was then maintained for 1.5 h.

Long-term exposure. Endothelial cells were treated with 18:2 for 24 h (18:2 group) or with TNF to 19.5 h (TNF group). In 18:2 + TNF group, exposure to 18:2 began 4.5 h before adding TNF. A combined exposure to 18:2 + TNF was then maintained for 19.5 h.

After treatment exposures, experimental media were removed, cells were washed, and oxidative stress, intracellular calcium, cell viability, DNA fragmentation, apoptosis, or albumin transfer across endothelial monolayers were measured. In addition, after the long-term treatment exposure, some cultures were washed and incubated with maintenance medium for up to 4 days. During consecutive days, several markers of cellular metabolism were measured.

In selected experiments, to determine possible mechanisms of TNF or 18:2 + TNF-mediated apoptosis, endothelial cells were additionally treated with aurintricarboxylic acid (ATA; endonuclease inhibitor; 10 μM), 1,2-bis(2-aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl)-ester (BAPTA-AM; cellular calcium chelator; 5 μM); n-propyl gallate (nPG; antioxidant; 5 μM) or vitamin E (antioxidant; 25 μM). ATA, BAPTA-AM or nPG were added to the culture media at the same time as 18:2 or TNF treatment. However, endothelial cell cultures were pretreated with vitamin E for 24 h before addition of fatty acid and/or cytokine.

Each experiment was conducted 3 times. The sample size per experimental group consisted of six individual cultures.

Cellular oxidative stress measurement

Imaging technique based on reactive oxygen species-mediated conversion of 2′,7′-dichlorofluorescin (DCF-H) into fluorescent 2′,7′-dichlorofluorescein (DCF) was used to determine cellular oxidation (31). The dye is applied to cells as 2′,7′-dichlorofluorescin diacetate (DCFH-DA). DCFH-DA freely crosses cell membranes, and it is converted by intracellular esterases into DCF-H localized in cells in hydrophobic lipid regions. Non-fluorescent DCF-H is then oxidized into fluorescent DCF) (32). These studies used a confocal laser scanning microscope (Molecular Dynamics, Sarastro 2000) coupled to an inverted microscope (Nikon). Treated endothelial cells cultured in polyethylenimine-coated glass-bottom 35-mm dishes (Mat-Tek Inc., Ashland, MA) were loaded with 100 μM of DCF-H (Molecular Probes, Inc.) by incubating for 50 min. The dye was excited at 488 nm and emission was filtered using a 510 nm barrier filter. The intensity of the laser beam and the sensitivity of the photodetector were held constant to allow quantitative comparisons of relative fluorescence intensity of cells between treatment groups. Values for average staining intensity per cell were obtained using the "Imagespace" software supplied by the manufacturer (Molecular Dynamics). The results, in relative units of DCF fluorescence, were expressed as average pixel intensity.

Measurement of intracellular free calcium levels

Intracellular free calcium ($[Ca^{2+}]_i$) was quantified according to the method of Mattson et al. (31). Briefly, treated endothelial cells cultured in polyethylenimine-coated glass-bottom 35-mm dishes (Mat-Tek Inc., Ashland, MA) were incubated with 6–8 μM acetoxymethylester form of the $[Ca^{2+}]_i$ indicator dye fura-2 (Molecular Probes) in 10% pluronic F 127 for 45 min. The excessive amounts of fura-2 were removed by washing the cells three times with fresh medium. Immediately before imaging, culture media were replaced with Hanks' balanced salt solution (GIBCO) containing 10 mM HEPES buffer. Cells were imaged on a Zeiss inverted microcsope using a fluoro 40X fluorescence objective and an Attofluor intensified CCD camera. Images were acquired using an Attofluor imaging system and software. The ratio of the fluorescence emission at two different excitation wavelengths (340 nm and 380 nm) was used to determine $[Ca^{2+}]_i$. The system was calibrated using solutions containing no Ca^{2+} (0 Ca^{2+} plus 1 mM EGTA) or a saturating level of Ca^{2+} (1 mM) according to Grynkiewicz, Poenie, and Tsien (33).

Mitochondrial function

MTT (3-[4,5-dimethylthiazol-2]-2,5 diphenyl tetrazolium bromide) conversion assay, performed according to Mattson et al. (31), was used to assess mitochondrial function, an indirect measure of cell viability. Briefly, MTT (thiazolyl blue) was added to the treated cells at the final concentration of 0.25 mg/mL and incubated 2 h to allow the conversion of MTT into purple formazon crystals. After washing in PBS, cells were lysed with 0.04 M HCl in isopropanol and absorbance was read at 595 nm.

Nuclear condensation and DNA fragmentation study

Nuclear condensation and DNA fragmentation were measured by staining endothelial cells with the fluroescent dye Hoechst 33342 as described earlier (34). Briefly, treated endothelial cells, cultured on glass-bottom dishes, were fixed in 4% paraformaldehyde. After washing, cells were stained with Hoechst 33342 at the concentration of μg/mL for 30 min. Cultures were washed again with PBS and water and the fluorescence was visualized using a 40× oil immersion lens. The dye was excited at 340 nm and emission was filtered with a 510 nm barrier filter. To quantify apoptotic process, endothelial cells with fragmented or condensed DNA and apparently normal DNA were counted.

Apoptosis studies

Cellular apoptosis was quantified using a flow cytometry assay (35). Briefly, treated endothelial cell cultures were gently trypsinized and washed twice with PBS. Cells were stained with Hoechst 33342 for 30 min, followed by staining with merocyanin 540 for 20 min, as described by Chiu et al. (36). Hoechst 33342 is a DNA-specific dye and merocyanin 540 detects membrane phospholipid domain changes and thus assesses alterations of membrane integrity associated with the early stages of apoptosis (35). Cells were analyzed and sorted using FACStar Plus cell sorter (Becton Dickinson). The primary laser was tuned to UV to excite Hoechst 33342-stained cells. The second laser was tuned to 488 nm to excite merocyanin 540-stained cells. Merocyanin 540 was detected using logarithmic amplification through a 575/25 band pass filter.

Endothelial barrier function in culture (albumin transfer studies)

Albumin transfer studies were performed as described earlier (28). Briefly, the chambers with attached polycarbonate filters with 0.8-μm pores were placed into wells having a diameter of 16 mm. Endothelial cells were seeded on the filters 2 days before treatment exposure. The chambers containing cells were washed three times with M-199 and incubated with experimental media. For albumin transfer measurements, cells first were washed three times with M-199, and fatty acid-free crystalline bovine serum albumin (Sigma Chemical Com-

pany) transfer was measured. The upper chamber, which contained 0.4 ml of M-199 enriched with 200 μM albumin, was placed into a well containing 0.6 ml of the same medium as above but without albumin. The albumin concentrations in the upper and lower chambers were determined by measuring the change in absorbance at 630 nm after addition of bromcresol green (Sigma Chemical Company).

Statistical analysis

Data were analyzed statistically using a one-way analysis of variance (ANOVA). For each endpoint, the treatment means were compared in pairs using Fisher's least significant difference procedure. Statistical probability of $P \leq 0.05$ was considered significant.

RESULTS

Oxidative stress in cells exposed to 18:2 and/or TNF

In the present study, cellular oxidation was measured by DCF fluorescence. This reaction is initiated primarily by peroxides, including hydrogen peroxide, as well as several products of hydrogen peroxide-mediated intracellular reactions (31, 32).

Figure 1 shows effects of a short-term or a long-term exposure to 18:2 and/or TNF, followed by a 1-day incubation with maintenance medium, on endothelial cell oxidation. A short-term exposure to 18:2 and/or TNF resulted in an increase in cellular oxidation in all experimental groups. However, after a long-term treatment, DCF fluorescence returned to control values in cells treated with 18:2 or TNF but it remained elevated in cells exposed to 18:2 + TNF. These results are in agreement with our previously published reports in which time- and dose-dependent effects of selected free fatty acids and/or TNF were measured (3, 29, 30, 37). However, when experimental media were replaced with maintenance medium for additional 1 day incubation, cellular oxidative stress remained markedly increased in cells previously exposed to TNF or 18:2 + TNF. These values exceeded control levels of cellular oxidation by 24 times in the TNF group and by 28 times in the 18:2 + TNF group.

Intracellular calcium ($[Ca^{2+}]_i$) levels in cells exposed to 18:2 and/or TNF

Increased levels of intracellular calcium are closely related to oxidative stress and cellular injury. For example, increased cellular oxidation may stimulate intracellular flux of $[Ca^{2+}]_i$. On the other hand, enhanced concentrations of $[Ca^{2+}]_i$ can stimulate cellular oxidative

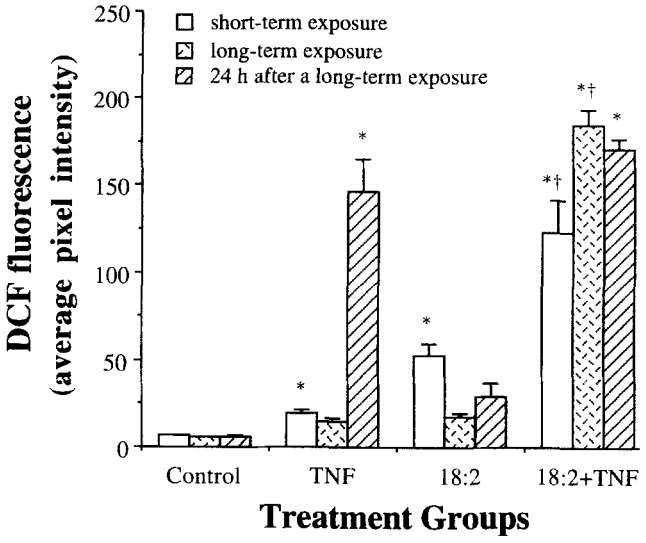


Fig. 1. Oxidative stress in cultured endothelial cells exposed to 18:2 and/or TNF as measured by DCF fluorescence. For short-term exposure, endothelial cell cultures were treated with 18:2 for 6 h or with TNF for 1.5 h. For long-term exposure cultures were treated with 18:2 for 24 h or with TNF for 1.5 h. In the 18:2 + TNF group, cells were exposed to 18:2 for 4.5 h before adding TNF for either 1.5 h (short-term exposure) or 19.5 h (long-term exposure). After the long-term treatment exposure, experimental media were removed, endothelial cultures were washed and maintained in normal medium for 24 h. Data are mean ± SEM; n = 6. *Significantly different from controls at the corresponding time points. †Values in group 18:2 + TNF are significantly different from values in TNF group at the corresponding time point.

stress. Several mechanisms, such as activation of phospholipases, xanthine oxidase, or generation of nitric oxide and peroxynitrite can be responsible for $[Ca^{2+}]_i$-mediated oxidative stress (for review see ref. 38).

Figure 2 shows effects of a short-term or a long-term exposure to 18:2 and/or TNF, followed by a 1-day incubation with maintenance medium, on endothelial cell concentration of $[Ca^{2+}]_i$. Exposure to 18:2 and/or TNF resulted in elevated levels of $[Ca^{2+}]_i$. The most marked increase in $[Ca^{2+}]_i$, (by more than 2-fold as compared to control values) was observed as a result of long-term exposure to 18:2 + TNF. When experimental media were replaced with maintenance medium for an additional 1 day, $[Ca^{2+}]_i$ remained elevated in TNF and 18:2 + TNF groups by approximately 20% and 40%, respectively, but returned to control values in cells previously exposed to 18:2 alone.

Mitochondrial function studies

The MTT conversion assay was used to indirectly measure viability of endothelial cells exposed to 18:2 and/or TNF. This assay takes advantage of the conversion of the yellow MTT to purple formazan crystals which occurs in mitochondria. This reaction occurs only in ac-

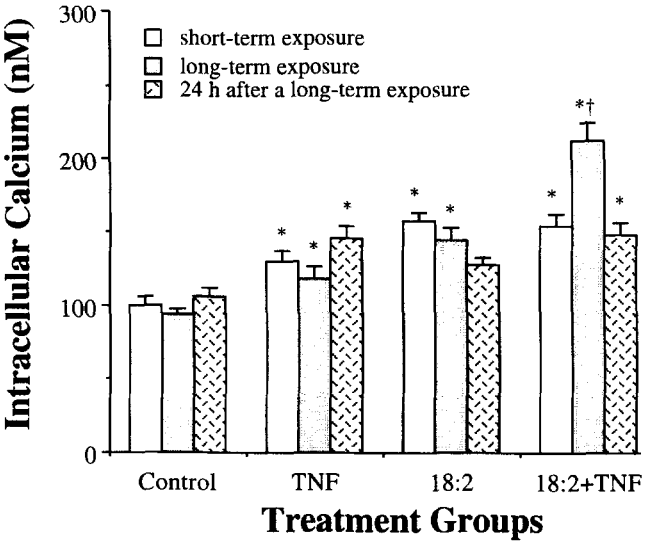


Fig. 2. Intracellular calcium levels in cultured endothelial cells exposed to 18:2 and/or TNF. For short-term exposure, endothelial cell cultures were treated with 18:2 for 6 h or with TNF for 1.5 h. For long-term exposure cultures were treated with 18:2 for 24 h or with TNF for 1.5 h. In the 18:2 + TNF group, cells were exposed to 18:2 for 4.5 h before adding TNF for either 1.5 h (short-term exposure) or 19.5 h (long-term exposure). After the long-term treatment exposure, experimental media were removed, endothelial cultures were washed and maintained in normal medium for 24 h. Data are mean ± SEM; n = 6. *Significantly different from controls at the corresponding time points. †Values in group 18:2 + TNF are significantly different from values in TNF group at the corresponding time point.

tive mitochondria of living cells. Results of the experiments involving MTT assay are shown on **Fig. 3A and B.**

Figure 3A shows effects of a long-term exposure to 18:2 and/or TNF, followed by the incubation in normal medium for up to 4 days, on mitochondrial function. The initial, long-term exposure to 18:2 and/or TNF did not affect conversion of MTT. When a long-term treatment with 18:2 and/or TNF was followed by a one-day incubation in normal medium, a decrease in MTT conversion, to approximately 65% of control values, was observed only in cells exposed to 18:2 + TNF. This decrease in mitochondrial function was prevented by additional treatment with ATA (data not shown). During consecutive days, after the long-term exposure, compromised MTT conversion was measured in cells treated with TNF and 18:2 + TNF. However, independent of the duration of cell maintenance in normal medium, the most marked decrease in MTT conversion, indicating the most diminished mitochondrial function and thus cellular viability, was observed in cultures treated with 18:2 + TNF. At the end of the incubation period, values of MTT conversion in the 18:2 + TNF group were only approximately 28% of those observed in control cultures.

Figure 3B reflects influence of ATA, BAPTA-AM, vitamin E, or nPG on MTT conversion in cells affected by a long-term exposure to 18:2 and/or TNF, followed by a 4-day incubation in normal medium. Partially protective effects of ATA, vitamin E, BAPTA-AM, and nPG against decreased mitochondrial function were observed in cells exposed to TNF or 18:2 + TNF.

DNA fragmentation

DNA fragmentation and condensation are considered characteristic features of cellular apoptosis. These morphological changes can be determined by staining cells with the DNA-binding fluorescent dye Hoechst 33342. **Figure 4A** shows a fluorescent image of initial stages of apoptosis of endothelial cells after a long-term exposure to TNF, followed by a 1-day incubation with maintenance medium and stained with Hoechst 33342. Selected cells with fragmented or condensed DNA are indicated by arrows.

Figure 4B shows the effect of a short-term or long-term exposure to 18:2 and/or TNF, followed by the incubation in normal medium for up to 4 days, on chromatin condensation and DNA fragmentation as determined by Hoechst 33342 staining. Long-term exposure to both TNF or 18:2 + TNF significantly increased the number of cells with fragmented or condensed DNA. In cultures exposed to TNF alone, the number of such cells with fragmented or condensed DNA remained approximately at the same level when the initial treatment was replaced by incubation in maintenance medium. However, during consecutive days of incubation with normal medium, the number of cells with fragmented or condensed DNA significantly increased in cultures previously exposed to 18:2 + TNF. At the fourth day of incubation in maintenance medium, the number of cells with fragmented or condensed DNA in 18:2 + TNF group exceeded those in the TNF group by approximately 3 times.

Endothelial cell apoptosis

Figure 5A shows effects of 18:2 and/or TNF on apoptosis of cultured endothelial cells as measured by flow cytometry. A long-term exposure to TNF and, in particular, to 18:2 + TNF, followed by a 1-day incubation with maintenance medium, resulted in a marked increase in number of apoptotic cells. The population of apoptotic cells in cultures exposed to 18:2 + TNF was significantly greater (by approximately 50%) than that in cells treated with TNF alone. ATA markedly protected against apoptosis in cells exposed to TNF or 18:2 + TNF. In cultures treated with 18:2 + TNF, partial protection against apoptosis also was observed in cells cotreated with BAPTA-AM, vitamin E, or nPG. In addition, a protective effect of nPG against TNF-induced en-

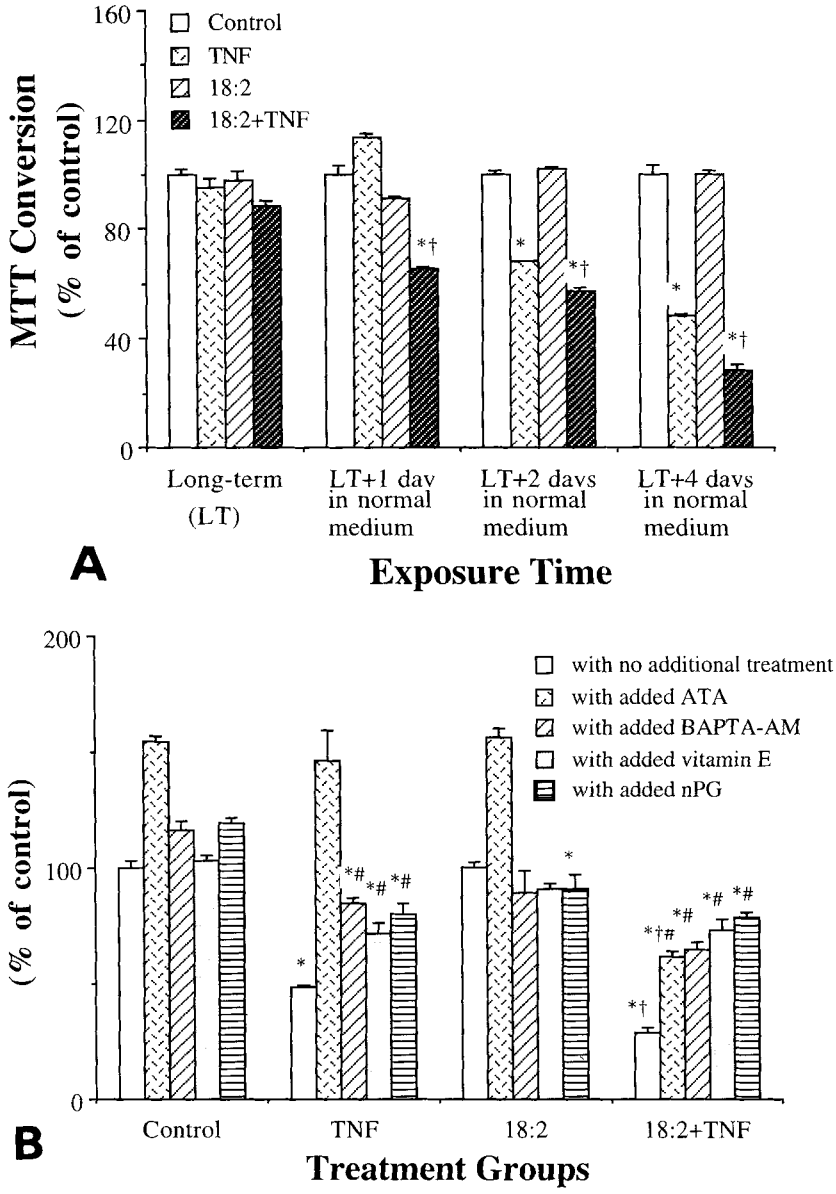
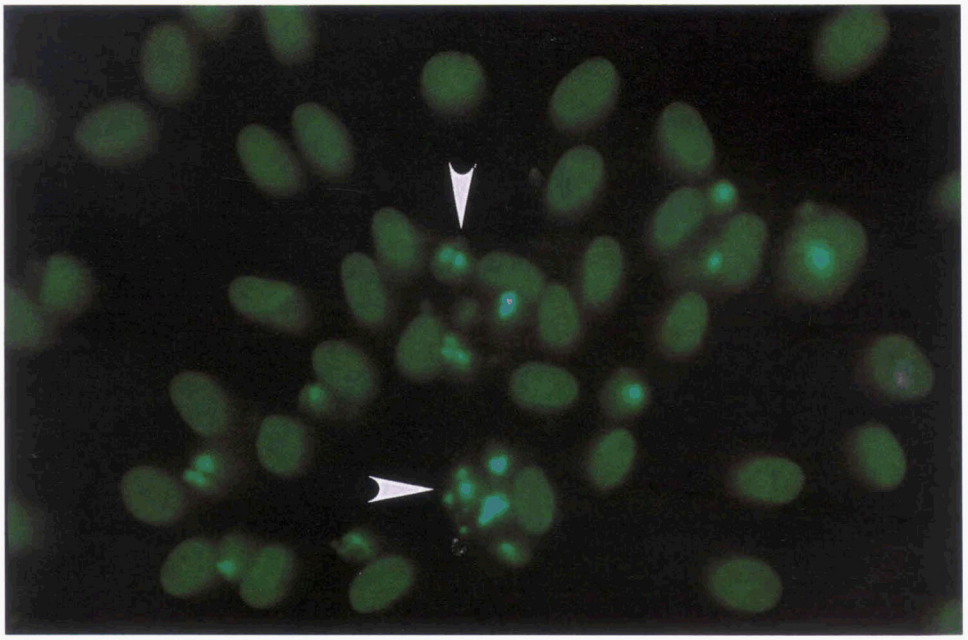


Fig. 3. A: Effect of long-term exposure and different incubation times in normal medium on endothelial cell viability, as measured by MTT conversion in cultured endothelial cells treated with 18:2 and/or TNF. For long-term exposure cultures were treated with 18:2 for 24 h or with TNF for 1.5 h. In the 18:2 + TNF group, cells were exposed to 18:2 for 4.5 h before adding TNF. After the long-term treatment exposure, experimental media were removed, endothelial cultures were washed and maintained in normal medium for up to 4 days. Data are mean ± SEM; n = 6. *Significantly different from controls at the corresponding time points. †Values in group 18:2 + TNF are significantly different from values in TNF group at the corresponding time point. B: Effect of aurintricarboxylic acid (ATA), BAPTA-AM, vitamin E, and n-propyl gallate (nPG) on MTT conversion in cultured endothelial cells treated with TNF and/or 18:2 + TNF. Cultures were exposed to 18:2 for 24 h or to TNF for 19.5 h. In the 18:2 + TNF group, cells were treated with 18:2 for 4.5 h before adding TNF. ATA, BAPTA-AM, and nPG were added to the selected endothelial cell cultures at the same time as 18:2 and/or TNF treatment. In addition, some cultures were pretreated with vitamin E 24 h before exposure to 18:2 and/or TNF. After the treatment exposures, experimental media were removed, endothelial cultures were washed and maintained in normal medium for 4 days. *Significantly different from controls at the corresponding time points. Data are mean ± SEM; n = 6. †Values in group 18:2 + TNF are significantly different from values in TNF group at the corresponding time point. #Significantly different compared to values with no additional treatment within the corresponding experimental group.



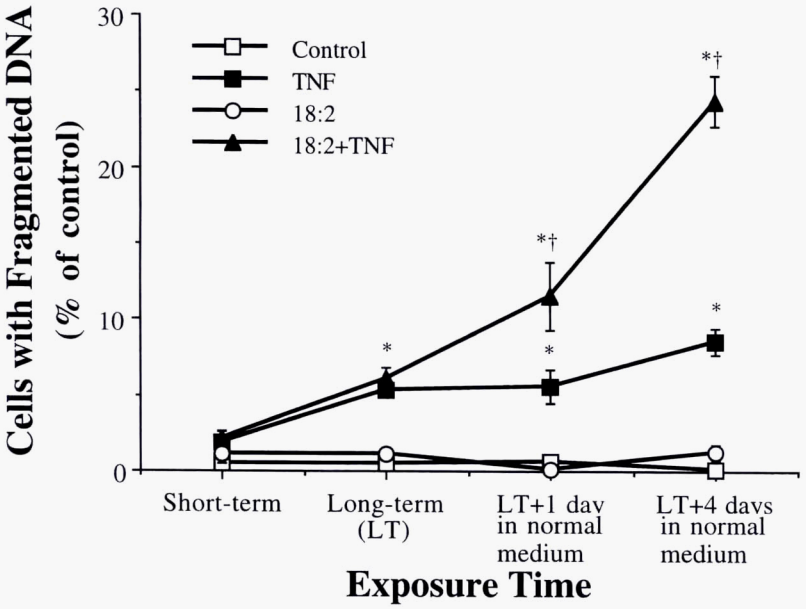


Fig. 4. Top: Fluorescent image of endothelial cells treated with TNF for 19.5 h, followed by a 1-day incubation with maintenance medium and stained with the DNA-binding fluorescent dye Hoechst 33342. Arrows show selected cells with fragmented or condensed DNA. Bottom: Effect of different exposure times and incubation in normal medium on number of endothelial cells with fragmented or condensed DNA, as identified by Hoechst staining, in cultures treated with 18:2 and/or TNF. For short-term exposure, endothelial cell cultures were treated with 18:2 for 6 h or with TNF for 1.5 h. For long-term exposure cultures were treated with 18:2 for 24 h or with TNF for 19.5 h. In the 18:2 + TNF group, cells were exposed to 18:2 for 4.5 h before adding TNF for either 1.5 h (short-term exposure) or 19.5 h (long-term exposure). After the treatment exposures, experimental media were removed, endothelial cultures were washed and maintained in normal medium for up to 4 days. Data are mean ± SEM; n = 6. *Significantly different from controls at the corresponding time points. †Values in group 18:2 + TNF are significantly different from values in TNF group at the corresponding time point.

dothelial cell apoptosis was observed. When a long-term exposure to TNF or 18:2 + TNF was followed by a 4-day incubation with the maintenance medium, numbers of apoptotic cells in those cultures were in the range of 90% of total cell population. In such advanced apoptotic stages, no differences between TNF or 18:2 + TNF groups were observed. In addition, partial protection against the development of apoptosis was detected in cells cotreated with ATA (data not shown).

Figure 5B shows examples of flow cytometry analyses of control cultures and changes in cell population as a result of a long-term exposure to 18:2 and/or TNF, followed by a 1-day incubation in normal medium. The pseudocolor scale is arranged to visualize different cell populations in such a way that gray color reflects necrotic cells, blue color reflects viable cells, and red color reflects apoptotic cells.

Endiothelial barrier function study

Figure 6 shows effects of 18:2, and/or TNF, and/or ATA on endothelial barrier function, measured as albumin transfer across endothelial monolayers. A long-

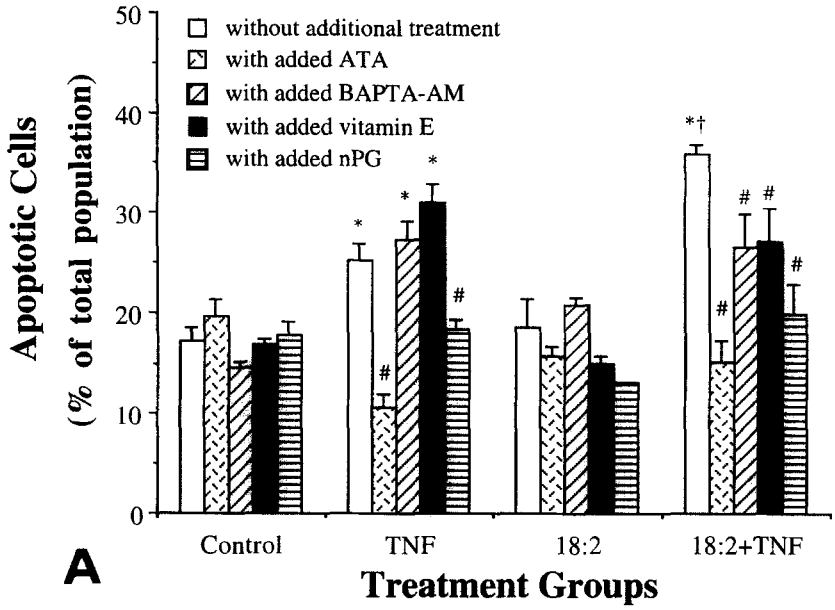


Fig. 5. A: Effect of aurintricarboxylic acid (ATA). BAPTA-AM, vitamin E, and n-propyl gallate (nPG) on number of apoptotic cells, measured by flow cytometry, in cultured endothelial cells treated with TNF and/or 18:2 plus TNF. Cultures were exposed to 18:2 for 24 h or to TNF for 19.5 h. In 18:2 + TNF group, cells were treated with 18:2 for 4.5 h before adding TNF. ATA, BAPTA-AM, and nPG were added to the selected endothelial cell cultures at the same time as 18:2 and/or TNF treatment. In addition, some cultures were pretreated with vitamin E 24 h before exposure to 18:2 and/or TNF. After the treatment exposures, experimental media were removed, endothelial cultures were washed and maintained in normal medium for additional 24 h. Data are mean ± SEM; n = 6. *Significantly different from controls at the corresponding time points. †Values in group 18:2 + TNF are significantly different from values in TNF group at the corresponding time point. #Significantly different compared to values with no additional treatment within the corresponding experimental group.

term exposure to both 18:2 or TNF markedly increased transendothelial flux of albumin; however, the most significant increase in albumin transfer was observed in cells treated with 18:2 + TNF. Additional treatment with ATA did not exert any effect on albumin transfer after the initial exposure to 18:2 and/or TNF. When the exposure to 18:2 and/or TNF was followed by a 1-day incubation with maintenance medium, albumin transfer in 18:2-treated cells returned to control values. In contrast, albumin flux in TNF- and, especially, in 18:2 + TNF-treated cultures was significantly higher than values obtained as a result of the initial treatment exposures. After a 1-day incubation in normal medium, albumin transfer exceeded control values by approximately 180% and 360% in TNF and 18:2 + TNF groups, respectively. Moreover, treatment with ATA partially protected against elevated albumin transfer across endothelial monolayers in cultures exposed to TNF and 18:2 + TNF and after an additional 1-day incubation in normal medium.

DISCUSSION

Recent evidence indicates that apoptotic processes may be involved in the development of atherosclerosis. In fact, a high incidence of apoptotic cells is detected in fibrous and fibro-fatty non-ulcerated atherosclerotic lesions (39, 40). Apoptotic material in atherosclerotic lesions consists mostly of smooth muscle cells and, to an even greater extent, of macrophages and T cells, i.e., cells involved in inflammatory reactions (39). In addition, apoptotic smooth muscle cells are detected in balloon-injured arteries in rats (41, 42) and in cholesterol-fed rabbits (43). Apoptosis of smooth muscle can be induced by TNF, interleukin-1β, or interferon-γ (44).

The involvement of endothelial cell apoptosis in the development or the progression of atherosclerosis is unknown. However, a number of apoptotic cells are detected in neointima of atherosclerotic vessels (41). On the basis of this observation, it was suggested that apoptosis might participate in remodeling of the vessel wall during atherogenesis (41). Moreover, endothelial cells are a major site of inflammatory reactions, and the intermediates of such reactions, such as reactive oxygen species (14), TNF (19), or elastase (45) trigger endothelial cell apoptotic processes. The present study demonstrates that the lipid environment may markedly influence a TNF-mediated increase in cellular oxidative stress and intracellular calcium and thus potentiate endothelial cell apoptosis mediated by this cytokine.

Selective diet-derived lipids, including 18:2, exert dif-

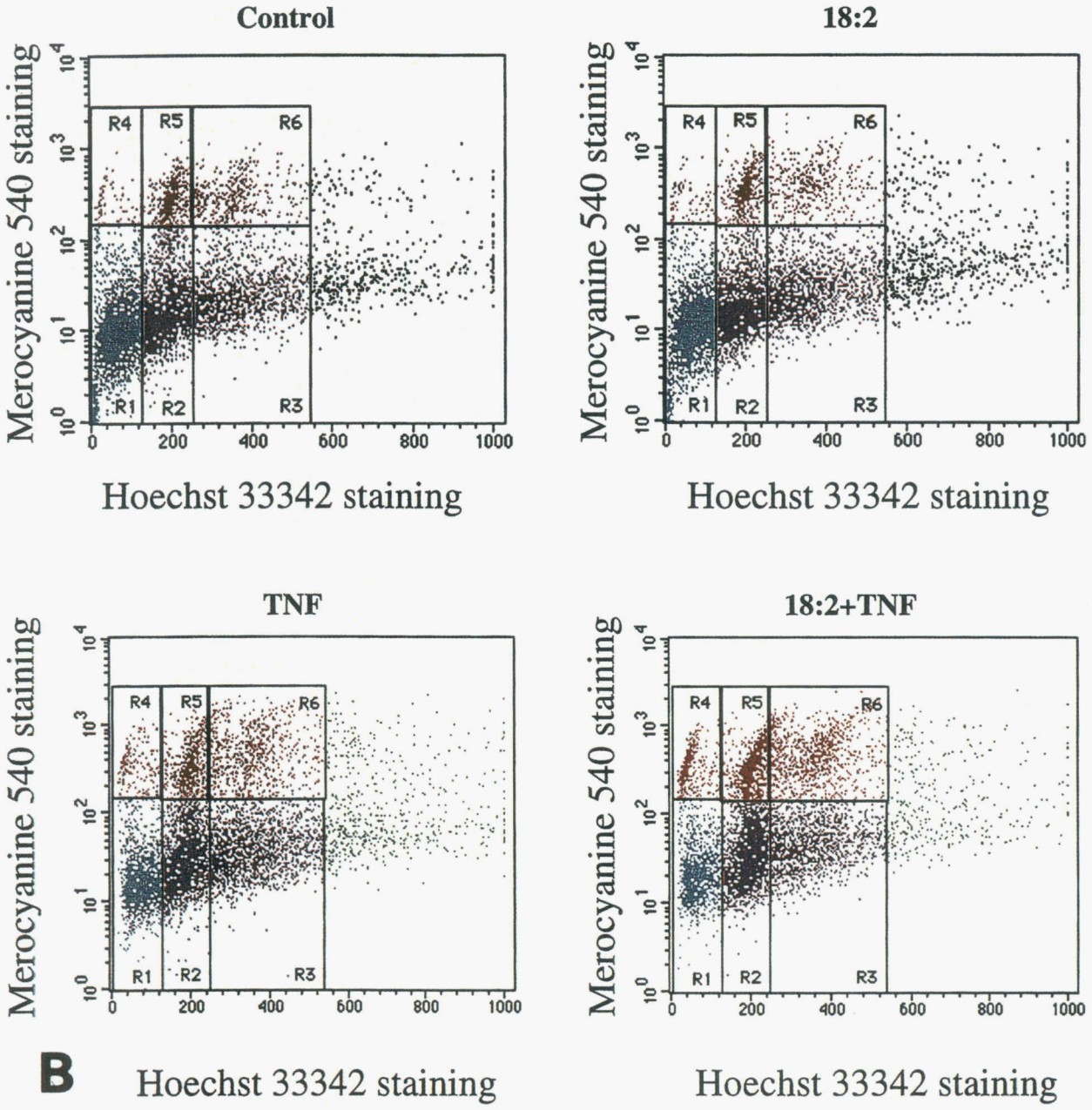


Fig. 5. B: Flow cytometry analysis of different populations of endothelial cells treated with 18:2 and/or TNF as described in Fig. 3A. Cells were dually stained with Hoechst 33342 and merocyanin 540. Six subpopulations of cells, differing in cell cycle and viability, were identified as follows: R1, necrotic cells and instrument noise (gray); R2, G_0/G_1, viable cells (blue); R3, S, G_2/M, viable cells (blue); R4, apoptotic cells with fragmented DNA (red); R5, G_0/G_1, apoptotic cells (red); R6, S, G_2/M, apoptotic cells (red).

ferent injury effects in cultured endothelial cells (for review see ref. 2). In addition, 18:2 is metabolized to oxidative derivatives, such as HPODE or 4-hydroxynonenal, which induce apoptosis in different cell types (11, 46). Interestingly, induction of apoptosis mediated by 4-hydroxynonenal in neuronal cells was linked to disruption of ion homeostasis, including intracellular calcium ($[Ca^{2+}]_i$ and disregulation of Na/K–ATPase (46). Although 18:2 was shown to induce similar changes in cultured endothelial cells (3, 47), no apoptosis was observed in endothelial cells exposed to this fatty acid in the current study (Figs. 4B and 5A). It is possible that potential 18:2-derived mediators of cellular apoptosis did not achieve threshold values needed for induction of endothelial cell death.

While it was previously reported that TNF can induce apoptosis in endothelial cells (21), mechanisms of TNF-mediated apoptosis of cultured endothelial cells are un-

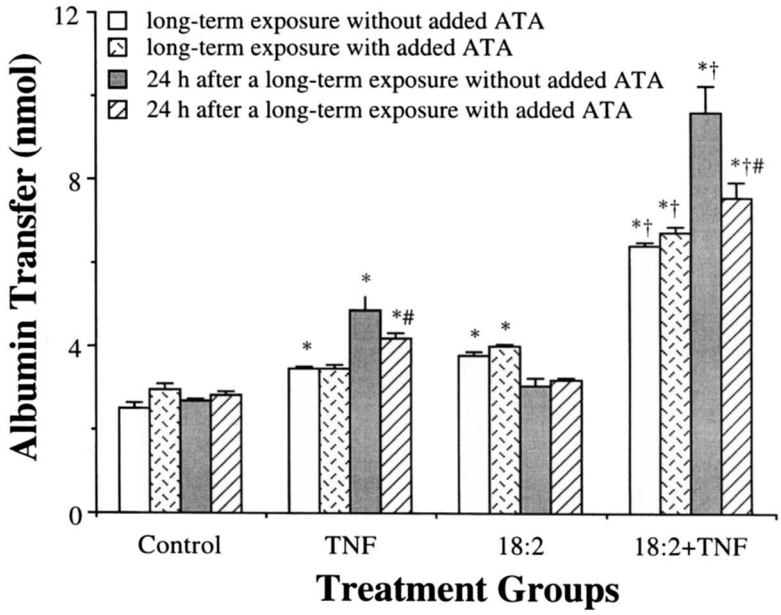


Fig. 6. Effect of aurintricarboxylic acid (ATA) on albumin transfer across endothelial monolayers exposed to 18:2 and/or TNF. Cultures were exposed to 18:2 for 24 h or to TNF for 19.5 h. In the 18:2 + TNF group, cells were treated with 18:2 for 4.5 h before adding TNF. ATA was added to the selected endothelial cell cultures at the same time as 18:2 and/or TNF. After the treatment exposures, experimental media were removed, endothelial cultures were washed and maintained in normal medium for additional 24 h. Data are mean ± SEM; n = 6. *Significantly different from controls at the corresponding time points. †Values in group 18:2 + TNF are significantly different from values in TNF group at the corresponding time point. #Significantly different compared to valuea without added ATA within the corresponding experimental group.

clear. For example, the sphingomyelin signaling pathway, which leads to generation of ceramide and cellular apoptosis, apparently is not activated (48) or only minimally induced (49) in endothelial cells exposed to TNF. However, there is evidence that c-Myc, a transcription factor of Myc family proteins might be involved in TNF-mediated apoptosis (50). Although endothelial cells are more resistant to TNF-mediated apoptosis, compared to other cell types, this process can be augmented by additional factors, such as cellular exposure to selected fatty acids as was demonstrated in the present study. In addition, inhibition of protein synthesis, either at the level of transcription by actinomycin D or translation by cycloheximide, has been shown to enhance TNF-mediated apoptosis of endothelial cells (21).

In the present study, an initial long-term exposure to TNF caused only minimal apoptosis as observed in Hoechst-stained cells and did not affect mitochondrial functions as measured by MTT assay (Figs. 4B and 3A, respectively). This observation is partially in agreement with a previously published report (48) where no apoptosis was noted in endothelial cells exposed to 100 U TNF/mL for 24 h. However, in the present study, endothelial cell apoptosis and compromised mitochondrial function increased during subsequent days after an initial treatment with TNF (Figs. 4B and 3A). Mechanisms of these delayed processes are difficult to explain. It is known that the dynamics of endothelial cell population is dependent on the balance among proliferation, necrosis, and apoptosis, modulated by exogenous stimuli (21). It is possible that such a balance is compromised only after several days following the initial TNF exposure. In addition, it is possible that additional treatment with 18:2 could affect this balance and shift the equilibrium towards accelerated apoptosis. To support this hypothesis, it should be noted that exposure to specific short-chain fatty acids increased cellular apoptosis in human colonic carcinoma cell lines (51).

Several mechanisms, including increased oxidative stress as well as disruption of ion homeostasis, can be responsible for potentiation of endothelial cell apoptosis in 18:2 + TNF-treated cells as compared to treatment with TNF alone. In the present study, we showed that endothelial cell treatment with 18:2 + TNF increased cellular oxidation more markedly as compared to exposure to TNF or 18:2 alone. In addition, DCF fluorescence was elevated 24 h after the termination of the initial, long-term treatment exposure to TNF or 18:2 + TNF, i.e., only in the experimental groups where apoptosis occurred (Fig. 1). The role of oxidative stress in induction of endothelial cell apoptosis by TNF and, in particular, by 18:2 + TNF, is further confirmed by the fact that antioxidants, such as vitamin E and nPG partially protected against apoptotic cell death (Fig. 5A).

It is generally accepted that oxidative stress may invoke apoptotic cell death (reviewed in ref. 52). Several oxidants, such as lipid hydroperoxides (12), oxidized LDL (13), or hydrogen peroxide (14) were shown to induce cellular apoptosis. Different mechanisms might be involved in oxidative stress-induced apoptosis (52). For example, an increase in oxidative stress may directly

lead to DNA damage and protein oxidation also may trigger apoptosis. Oxidative stress also can directly regulate gene expression by influence on specific transcription factors (52). Cellular redox status, regulated by cellular thiol and glutathione, may play a critical role in this process. It was shown that apoptosis is preceded by a decrease in cellular glutathione (53) or can be induced by oxidation of thiol groups (54). To support the role of thiols in cellular apoptosis, it was reported that endothelial cell exposure to 18:2 or TNF and, in particular, to 18:2 + TNF markedly decreased intracellular glutathione content (3).

Another mechanism by which 18:2 may increase endothelial cell vulnerability to TNF-mediated apoptosis may be related to the disruption of ion homeostasis. Exposure of endothelial cells to 18:2 or TNF and in particular to 18:2 + TNF, increased $[Ca^{2+}]_i$ (Fig. 2). Several mechanisms were proposed to explain the role of increased $[Ca^{2+}]_i$ in the induction of cellular apoptosis (for review see ref. 55). For example, elevated $[Ca^{2+}]_i$ might lead to activation of Ca^{2+}-dependent kinases and/or phosphatases with the subsequent induction of gene expression related to apoptosis. In addition, enhanced $[Ca^{2+}]_i$ can activate proteases and/or endonucleases(s) and thus induce DNA damage, a characteristic feature of cellular apoptosis (55). The role of elevated $[Ca^{2+}]_i$ levels in TNF and/or 18:2 + TNF-induced endothelial cell apoptosis is supported by the observation that BAPTA-AM, an intracellular calcium chelator, partially protected against apoptosis in these experimental groups (Figs. 5A). A similar protective effect of BAPTA-AM was observed previously in endothelial cell apoptosis induced by amyloid β-peptide (34).

Compromised endothelial integrity and barrier function may be due in part to endothelial cell apoptosis. In the present study, endothelial barrier function was measured by transendothelial albumin transfer. It is known that approximately 60% of albumin transfer across normal endothelial cell monolayers occurs via intercellular junctions (56). Because apoptosis of endothelial cells was only minimal after the initial long-term treatment with TNF or 18:2 + TNF (Fig. 4B), one may speculate that this process was not responsible for the increase in albumin flux observed at that time point. At least two facts support this hypothesis. *a*) Endothelial integrity was more markedly compromised as a result of the long-term treatment with 18:2 + TNF, as compared to cells exposed to TNF alone (Fig. 6) but the number of apoptotic cells was the same in both experimental groups (Fig. 4B). *b*) ATA, which protected against cellular apoptosis (Fig. 5A), did not influence the effect of a long-term exposure to TNF or to 18:2 + TNF on albumin transfer across endothelial monolayers (Fig. 6). However, when the initial, long-term exposure to TNF or 18:2 + TNF was followed by a 1-day incubation with normal medium, the number of apoptotic cells in 18:2 + TNF-treated cells was significantly higher as compared to cultures treated with TNF alone (Fig. 4B). Moreover, in that experimental setting, ATA partially protected against the TNF- and 18:2 + TNF-mediated increase in albumin transfer across endothelial monolayers (Fig. 6). The correlation between endothelial barrier function and MTT conversion assay (an indirect indicator of cell viability) was less clear. For example, compromised endothelial integrity was observed as a result of a long-term exposure to either TNF and 18:2 + TNF followed by a 1-day incubation in normal medium. However, at that time point, conversion of MTT was decreased only in the 18:2 + TNF group (Fig. 3A).

In conclusion, treatment of endothelial cells with 20 ng TNF/mL (100 U TNF/mL) for up to 19.5 h induced cellular apoptosis independent of the presence of TNF in experimental media at later time points. In addition, pre-exposure of endothelial cells with 18:2 potentiated TNF-mediated injury to endothelial cells, measured as changes in intracellular calcium, cellular oxidation, endothelial barrier function as well as mitochondrial function and apoptotic processes. Mediated by 18:2, potentiation of TNF-induced endothelial cell apoptosis and loss of mitochondrial function can be partially prevented by endonuclear inhibitor (aurintricarboxylic acid, ATA), cellular calcium chelator (BAPTA-AM), and antioxidants (n-propyl gallate, nPG; vitamin E). These results suggest that amplification of cellular oxidative stress and intracellular calcium observed in 18:2 + TNF-treated cells, compared to 18:2 or TNF alone, are mechanisms responsible for the potentiation of TNF-induced endothelial cell apoptosis and loss of viability. In addition, one may suggest that potentiation of endothelial cell apoptosis due to exposure to 18:2 + TNF may compromise endothelial barrier function and thus have significant implications in the pathogenesis of atherosclerosis.

This work was supported in part by grants from the National Institutes of Health (HL 36552, NS 29001, and NS 30583), American Heart Association, Kentucky Affiliate, USDA, University of Kentucky Research Funds, and Kentucky Agricultural Experimental Station.

Manuscript received 10 April 1997 and in revised form 23 July 1997.

REFERENCES

1. Zilversmit, D. B. 1973. A proposal linking atherogenesis to the interaction of endothelial lipoprotein lipase with triglyceride-rich lipoproteins. *Circ. Res.* **33:** 633–638.
2. Hennig, B., M. Toborek, C. J. McClain, and J. N. Diana.

1996. Nutritional implications in vascular endothelial cell metabolism. *J. Am. Coll. Nutr.* **15:** 345–358.
3. Toborek, M., S. W. Barger, M. P. Mattson, S. Barve, C. J. McClain, and B. Hennig. 1996. Linoleic acid and TNF-α cross-amplify oxidative injury and dysfunction of endothelial cells. *J. Lipid Res.* **37:** 123–135.
4. Davda, R. K., K. T. Stepniakowski, G. Lu, M. E. Ullian, T. L. Goodfriend, and B. M. Egan. 1995. Oleic acid inhibits endothelial nitric oxide synthase by a protein kinase C-independent mechanism. *Hypertension.* **26:** 764–770.
5. Toborek, M., and B. Hennig. 1993. Vitamin E attenuates induction of elastase-like activity by tumor necrosis factor-α, cholestan-3β,5α,6β-triol, and linoleic acid in cultured endothelial cells. *Clin. Chim. Acta.* **215:** 201–211.
6. Ramasamy, S., G. A. Boissonneault, D. W. Lipke, and B. Hennig. 1993. Proteoglycans and endothelial barrier function: effect of linoleic acid exposure to porcine pulmonary artery endothelial cells. *Atherosclerosis.* **103:** 279–290.
7. de Haan, L. H., I. Bosselaers, W. M. Jongen, R. M. Zwijsen, and J. Koeman. 1994. Effect of lipids and aldehydes on gap-junctional intercellular communication between human smooth muscle cells. *Carcinogenesis.* **15:** 253–256.
8. Reid, V. C., S. J. Hardwick, and M. J. Mitchinson. 1993. Fragmentation of DNA in P388D1 macrophages exposed to oxidised low-density lipoprotein. *FEBS Lett.* **332:** 218–220.
9. Sweetman, L. L., N. Y. Zhang, H. Peterson, R. Gopalakrishna, and A. Sevanian. 1995. Effect of linoleic acid hydroperoxide on endothelial cell calcium homeostasis and phospholipid hydrolysis. *Arch. Biochem. Biophys.* **323:** 97–107.
10. Hennig, B., C. Enoch, and C. K. Chow. 1987. Protection by vitamin E against endothelial cell injury by linoleic acid hydroperoxides. *Nutr. Res.* **7:** 1253–1260.
11. Sandstrom, P. A., D. Pardi, P. W. Tebbey, R. W. Dudek, D. M. Terrian. T. M. Folks, and T. M. Buttke. 1995. Lipid hydroperoxide-induced apoptosis: lack of inhibition by Bcl-2 over-expression. *FEBS Lett.* **365:** 66–70.
12. Folcik, V. A., and M. K. Cathcart. 1994. Predominance of esterified hydroperoxy-linoleic acid in human monocyte-oxidized LDL. *J. Lipid Res.* **35:** 1570–1582.
13. Bjorkerud, B., and S. Bjorkerud. 1996. Contrary effects of lightly and strongly oxidized LDL with potent promotion of growth versus apoptosis on arterial smooth muscle cells, macrophages, and fibroblasts. *Arterioscler. Thromb. Vasc. Biol.* **16:** 416–424.
14. de Bono, D. P., and W. D. Yang. 1995. Exposure to low concentrations of hydrogen peroxide causes delayed endothelial cell death and inhibits proliferation of surviving cells. *Atherosclerosis.* **114:** 235–245.
15. Tracey, K. J., and A. Cerami. 1992. Tumor necrosis factor and regulation of metabolism in infection: role of systemic versus tissue levels. *Proc. Soc. Exp. Biol. Med.* **200:** 233–239.
16. Vaddi, K., F. A. Nicolini, P. Mehta, and J. L. Mehta. 1994. Increased secretion of tumor necrosis factor-α and interferon-γ by mononuclear leukocytes in patients with ischemic heart disease. Relevance in superoxide anion generation. *Circulation.* **90:** 694–699.
17. Rus, H. G., F. Niculescu, and R. Vlaicu. 1991. Tumor necrosis factor-alpha in human arterial wall with atherosclerosis. *Atherosclerosis.* **89:** 247–254.
18. Barath, P., M. C. Fishbein, J. Cao, J. Berenson, R. H. Helfant, and J. S. Forrester. 1990. Detection and localization of tumor necrosis factor in human atheroma. *Am. J. Cardiol.* **65:** 297–302.
19. Read, M. A., A. S. Neish, F. W. Luscinskas, V. J. Palombella, T. Maniatis, and T. Collins. 1995. The proteasome pathway is required for cytokine-induced endothelial-leukocyte adhesion molecule expression. *Immunity.* **2:** 493–506.
20. Collins, T., M. A. Read, A. S. Neish, M. Z. Whitley, D. Thanos, and T. Maniatis. 1995. Transcriptional regulation of endothelial cell adhesion molecules: NF-κB and cytokine-inducible enhancers. *FASEB J.* **9:** 899–909.
21. Pulunovsky, V. A., C. H. Wendt, D. H. Ingbar, M. S. Peterson, and P. B. Bitterman. 1994. Induction of endothelial cell apoptosis by TNF alpha: modulation by inhibitors of protein synthesis. *Exp. Cell Res.* **214:** 584–594.
22. Khan, B. V., S. S. Parthasarathy, R. W. Alexander, and R. M. Medford. 1995. Modified low density lipoprotein and its constituents augment cytokine-activated vascular cell adhesion molecule-1 gene expression in human vascular endothelial cells. *J. Clin. Invest.* **95:** 1262–1270.
23. Maziere, C., M. Auclair, and J. C. Maziere. 1994. Tumor necrosis factor enhances low density lipoprotein oxidative modification by monocytes and endothelial cells. *FEBS Lett.* **338:** 43–46.
24. Hayakawa, M., N. Ishida, K. Takeuchi, S. Shibamoto, T. Hori, N. Oku, F. Ito, and M. J. Tsujimoto. 1993. Arachidonic acid-selective cytosolic phospholipase A2 is crucial in the cytotoxic action of tumor necrosis factor. *J. Biol. Chem.* **268:** 11290–11295.
25. Camacho, M., N. Godessart, R. Anton, M. Garcia, and L. Vila. 1995. Interleukin-1 enhances the ability of cultured human umbilical vein endothelial cells to oxidize linoleic acid. *J. Biol. Chem.* **270:** 17279–17286.
26. Renier, G., E. Skamene, J. B. DeSanctis, and D. Radzioch. 1994. Induction of tumor necrosis factor α gene expression by lipoprotein lipase. *J. Lipid Res.* **35:** 271–278.
27. Reiner, G., M. Olivier, E. Skamene, and D. Radzioch. 1994. Induction of tumor necrosis factor alpha gene expression by lipoprotein lipase requires protein kinase C activation. *J. Lipid Res.* **35:** 1413–1421.
28. Hennig, B., D. M. Shasby, A. B. Fulton, and A. A. Spector. 1984. Exposure to free fatty acid increases the transfer of albumin across cultured endothelial monolayers. *Arteriosclerosis.* **4:** 489–497.
29. Toborek, M., S. W. Barger, M. P. Mattson, C. J. McClain, and B. Hennig. 1995. Role of glutathione redox cycle in TNF-mediated endothelial cell dysfunction. *Atherosclerosis.* **117:** 179–188.
30. Toborek, M., and B. Hennig. 1994. Fatty acid-mediated effects on the glutathione redox cycle in cultured endothelial cells. *Am. J. Clin. Nutr.* **59:** 60–65.
31. Mattson, M. P., S. W. Barger, J. G. Begley, and R. J. Mark. 1995. Calcium, free radicals, and excitotoxic neuronal death in primary cell culture. *Methods Cell Biol.* **46:** 187–215.
32. Royall, J. A., and H. Ischiropoulos. 1993. Evaluation of 2′,7′-dichlorofluorescin and dihydrorhodamine 123 as fluorescent probes for intracellular H_2O_2 in cultured endothelial cells. *Arch. Biochem. Biophys.* **302:** 348–355.
33. Grynkiewicz, G., M. Poenie, and R. Y. Tsien. 1985. A new generation of calcium indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260:** 3440–3450.

34. Blanc, E. M., M. Toborek, R. J. Mark, B. Hennig, and M. P. Mattson. 1997. Amyloid β-peptide (25–35) induces cell monolayer albumin permeability, impairs glucose transport, and induces apoptosis in vascular endothelial cells. *J. Neurochem.* **68:** 1870–1881.
35. Reid, S., R. Cross, and E. C. Snow. 1996. Combined Hoechst 33342 and merocyanine 540 staining to examine murine B cell cycle stage, viability and apoptosis. *J. Immunol. Methods.* **192:** 43–54.
36. Chiu, L., H. Cherwinski, J. Ransom, and J. F. Dunne. 1996. Flow cytometric ratio analysis of the Hoechst 33342 emission spectrum: multiparametric characterization of apoptotic lymphocytes. *J. Immunol. Methods.* **189:** 157–171.
37. Hennig, B., M. Toborek, S. Joshi-Barve, S. W. Barger, S. Barve, M. P. Mattson, and C. J. McClain. 1996. Linoleic acid activates NF-κB and induces NF-κB-dependent transcription in cultured endothelial cells. *Am. J. Clin. Nutr.* **63:** 322–328.
38. Mattson, M. P., and S. W. Scheff. 1994. Endogenous neuroprotection factors and traumatic brain injury: mechanisms of action and implications for therapy. *J. Neurotrauma.* **11:** 3–33.
39. Bjorkerud, S., and B. Bjorkerud. 1996. Apoptosis is abundant in human atherosclerotic lesions, especially in inflammatory cells (macrophages and T cells), and may contribute to the accumulation of gruel and plaque instability. *Am. J. Pathol.* **149:** 367–380.
40. Geng, Y. J., and P. Libby. 1995. Evidence for apoptosis in advanced human atheroma. Colocalization with interleukin-1 beta-converting enzyme. *Am. J. Pathol.* **147:** 251–266.
41. Han, D. K., C. C. Haudenschild, M. K. Hong, B. T. Tinkle, M. B. Leon, and G. Liau. 1995. Evidence for apoptosis in human atherogenesis and in a rat vascular injury model. *Am. J. Pathol.* **147:** 267–277.
42. Bochaton-Piallat, M. L., F. Gabbiani, M. Redard, A. Desmouliere, and G. Gabbiani. 1995. Apoptosis participates in cellularity regulation during rat aortic intimal thickening. *Am. J. Pathol.* **146:** 1059–1064.
43. Kockx, M. M., G. R. De Meyer, J. Muhring, H. Bult. J. Bultinck, and A. G. Herman. 1996. Distribution of cell replication and apoptosis in atherosclerotic plaques of cholesterol-fed rabbits. *Atherosclerosis.* **120:** 115–124.
44. Geng, Y. J., Q. Wu, M. Muszynski, G. K. Hansson, and P. Libby. 1996. Apoptosis of vascular smooth muscle cells induced by in vitro stimulation with interferon-gamma, tumor necrosis factor-alpha, and interleukin-1 beta. *Arterioscler. Thromb. Vasc. Biol.* **16:** 19–27.
45. Yang, J. J., R. Kettritz, R. J. Falk, J. C. Jennette, and M. L. Gaido. 1996. Apoptosis of endothelial cells induced by the neutrophil serine proteases proteinase 3 and elastase. *Am. J. Pathol.* **149:** 1617–1626.
46. Mark, R. J., M. A. Lovell, W. R. Markesbery, K. Uchida, and M. P. Mattson. 1997. A role of 4-hydroxynonenal, an aldehydic product of lipid peroxidation, in disruption of ion homeostatis and neuronal death induced by amyloid β-peptide. *J. Neurochim.* **68:** 255–264.
47. Ramasamy, S., G. A. Boissonneault, E. A. Decker, and B. Hennig. 1991. Linoleic acid-induced endothelial cell injury: role of membrane-bound enzyme activities and lipid oxidation. *J. Biochem. Toxicol.* **6:** 29–35.
48. Slowik, M. R., L. G. De Luca, W. Min, and J. S. Pober. 1996. Ceramide is not a signal for tumor necrosis factor-induced gene expression but does cause programmed cell death in human vascular endothelial cells. *Circ. Res.* **79:** 736–747.
49. Modur, V., G. A. Zimmerman, S. M. Prescott, and T. M. McIntyre. 1996. Endothelial cell inflammatory responses to tumor necrosis factor α. Ceramide-dependent and -independent mitogen-activated protein kinase cascades. *J. Biol. Chem.* **271:** 13094–13102.
50. Packham, G., and J. L. Cleveland. 1995. c-Myc and apoptosis. *Biochim. Biophys. Acta.* **1242:** 11–28.
51. Heerdt, B. G., M. A. Houston, and L. H. Augenlicht. 1994. Potentiation by specific short-chain fatty acids of differentiation and apoptosis in human colonic carcinoma cell lines. *Cancer Res.* **54:** 3288–3293.
52. Slater, A. F., C. S. Nobel, and S. Orrenius. 1995. The role of intracellular oxidants in apoptosis. *Biochim. Biophys. Acta.* **1271:** 59–62.
53. Beaver, J. P., and P. Waring. 1995. A decrease in intracellular glutathione concentration precedes the onset of apoptosis in murine thymocytes. *Eur. J. Cell Biol.* **68:** 47–54.
54. Sato, N., S. Iwata, K. Nakamura, T. Hori, K. Mori, and J. Yodoi. 1995. Thiol-mediated redox regulation of apoptosis. Possible roles of cellular thiols other than glutathione in T cell apoptosis. *J. Immunol.* **154:** 3194–3203.
55. McConkey, D. J., and S. Orrenius. 1996. The role of calcium in the regulation of apoptosis. *J. Leukocyte Biol.* **59:** 775–783.
56. Siflinger-Birnboim, A., J. Schnitzer, H. Lum, F. A. Blumenstock, C-P. J. Shen, P. J. Del Vecchio, and A. E. Malik. 1991. Lectin binding to gp60 decreases specific albumin binding and transport in pulmonary artery endothelial monolayers. *J. Cell Physiol.* **149:** 575–584.