Linoleic acid and TNF-α cross-amplify oxidative injury and dysfunction of endothelial cells

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Abstract Factors implicated in the development of atherosclerosis include metabolic alterations of the endothelium induced by certain lipids and inflammatory cytokines. To study the hypothesis that the combined presence of unsaturated fatty acids and inflammatory cytokines may cross-amplify their individual injurious effects, cultured endothelial cells were treated with 90 μM of linoleic acid (18:2 n-6) and/or 20 ng/ml (100 U/ml) of tumor necrosis factor-α (TNF) for up to 24 h. Disturbances in endothelial cell metabolism were determined by measuring cellular oxidative stress, oxidative stress-inducible nuclear factor-κB (NF-κB) and NF-κB-related transcription, intracellular calcium levels, and endothelial barrier function reflected by transendothelial albumin movement. Both 18:2 and TNF increased cellular oxidation, intracellular calcium, and endothelial barrier permeability. These changes were cross-amplified in cells treated both with 18:2 and TNF, compared with 18:2 or TNF alone. In contrast, a combined exposure to 18:2 and TNF did not potentiate effects mediated by 18:2 or TNF alone on NF-κB activation or NF-κB-related transcription. Pretreatment with 25 μM vitamin E attenuated 18:2 and/or TNF-mediated endothelial cell dysfunction. These results suggest that certain unsaturated fatty acids can potentiate TNF-mediated endothelial cell dysfunction and that oxidative stress may be partially responsible for these metabolic events. These findings have implications for understanding lipid-mediated inflammatory responses in atherosclerosis.—**Toborek, M., S. W. Barger, M. P. Mattson, S. Barve, C. J. McClain, and B. Hennig.** Linoleic acid and TNF-α cross-amplify oxidative injury and dysfunction of endothelial cells. *J. Lipid Res.* 1996. **37:** 123–135.

Supplementary key words atherosclerosis • fatty acids • inflammatory cytokines • oxidative stress • nuclear transcription factors • intracellular calcium • antioxidants • transfection • transcription

Among different factors that contribute to the development of atherosclerosis, lipids and inflammatory cytokines play crucial roles. Intra- and extracellular accumulation of lipids is an integral part of atherogenic processes. Moreover, there is strong evidence that lipids, including selective free fatty acids, may cause injury to the endothelium (reviewed in ref. 1). It was proposed that hydrolysis of triglyceride-rich lipoproteins mediated by lipoprotein lipase, a key enzyme in lipoprotein metabolism associated with the luminal site of endothelial cells, may be an important source of high concentrated fatty acid anions in the proximity to the endothelium (2, 3). In fact, activity of lipoprotein lipase is increased in atherosclerotic lesions (3, 4). Lipoprotein lipase-derived remnants of lipoproteins isolated from hypertriglyceridemic subjects as well as selective unsaturated fatty acids, such as linoleic acid, were demonstrated to disrupt endothelial integrity (5, 6).

Recent evidence suggests that linoleic acid (18:2 n-6) may play a critical role in the pathogenesis of atherosclerosis (1). Adipose tissue levels of 18:2, which reflect intake of this fatty acid over a period of time, were positively associated with the degree of coronary artery disease (7). In addition, concentrations of 18:2 were increased in the phospholipid fractions of human coronary arteries in cases of sudden cardiac death due to ischemic heart disease (8). LDL isolated from humans (9) or animals (10) fed linoleate- or corn oil-enriched diets were more susceptible to oxidative modification and more markedly disrupted endothelial barrier function as compared to LDL isolated from subjects fed diets enriched with more saturated lipids. Several mechanisms were proposed to explain injurious effects of 18:2 to endothelial cells. Due to very low basal activity of endothelial cell elongases and Δ5 and Δ9 desaturases, arachidonic acid is not produced from 18:2 significantly

Abbreviations: 18:2, linoleic acid; TNF, tumor necrosis factor; NF-κB, nuclear factor κ-B; DCF, 2,7-dichlorofluorescein; LDL, low density lipoprotein; GSH, reduced glutathione; EMSA, electrophoretic mobility shift assay; CAT, chloramphenicol acetyltransferase; $[Ca^{2+}]_i$, intracellular free calcium; ROS, reactive oxygen species.

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in this type of cell (11, 12). Consequently, 18:2 accumulates within endothelial cells (11, 13). Moreover, 18:2 decreases levels of intracellular ATP (14) and proteoglycans (15) and enhances Ca^{2+}-ATPase activity (16) and elastase-like activity (17). Linoleic acid-mediated disruption of endothelial barrier function also may be caused by its ability to inhibit gap-junctional intracellular communication (18) and to induce intracellular oxidative stress (19).

It is generally accepted that inflammatory mechanisms may contribute to the development of atherosclerosis. Recruitment of monocytes into the vessel wall during the early stages of atherosclerosis was observed in a number of animal and human studies (20). In addition, T cells accumulate in atherosclerotic lesions (21). Monocytes/macrophages and T cells are involved in several steps of atherogenesis. For example, monocyte-derived macrophages contribute to the formation of fatty streaks, produce several growth and cytotoxic factors for myocytes and endothelial cells, as well as increase oxidative stress within the vessel wall. Both macrophages and T cells, when activated, produce inflammatory cytokines, including tumor necrosis factor-α (TNF) (20). In fact, macrophages are the main source of TNF in vivo (22). Endothelial cells, which possess both the 55- and the 75-kD TNF receptors, are especially susceptible to injury induced by this cytokine (23). Although the detailed mechanisms of TNF-mediated endothelial cell injury are not fully understood, recent evidence suggests that TNF-stimulated oxidative stress, an increase in intracellular calcium (24), and activation of nuclear transcriptional factor kappa B (NF-κB) may be important factors in this process. A crucial role of TNF in the development of atherosclerosis is supported by the observation that production of this cytokine by mononuclear leukocytes is enhanced in patients with atherosclerosis (25). Moreover, increased levels of TNF are present in atherosclerotic vessels, mainly in intimal thickenings (26). Most interesting, lipoprotein lipase induces TNF gene expression in macrophages and TNF production by this type of cells (27, 28). This suggests that during lipoprotein lipase-mediated hydrolysis of triglycerides and subsequent endothelial cell exposure to free fatty acids, a concomitant exposure to elevated levels of TNF also may occur.

Consistent with this hypothesis, the main objective of the present study was to examine endothelial cell integrity in response to treatment with 18:2 and/or TNF. To mimic consecutive events which may occur in vivo, endothelial cells were first pre-treated with 18:2 and then exposed to TNF. Moreover, in some experiments, endothelial cultures were pretreated with vitamin E before exposure to 18:2 and/or TNF. Oxidative stress, antioxidant status, concentration of intracellular calcium, and endothelial barrier function were determined as markers of endothelial cell metabolism. Because NF-κB is thought to be implicated in many endothelial cell responses to injury and stress, activation of NF-κB and NF-κB-related transcriptional activity also were measured in endothelial cells exposed to 18:2 and/or TNF.

MATERIAL AND METHODS

Endothelial cell cultures

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 methods described by Hennig et al. (29). Cells were determined to be endothelial in origin by uniform cobblestone morphology and by quantitative determination of angiotensin-converting enzyme activity. Cells from passages 5–10 were used in the present study. Endothelial cell cultures were treated with 90 μM of stearic acid (18:0), oleic acid (18:1n–9), linoleic acid (18:2n–6), and linolenic acid (18:3n–3) (≥ 99% pure, Nu-Chek Prep, Elysian, MN) and/or TNF (20 ng/mL = 100 U/mL; Knoll Laboratories, Whippany, NJ).

Examples of typical experiments are as follows:

A. *Short-term exposure.* Endothelial cells were exposed to fatty acids for 6 h or to TNF for 1.5 h. When cells were exposed to 18:2 + TNF, 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.

B. *Long-term exposure.* Endothelial cells were exposed to 18:2 for 24 h or to TNF for 19.5 h. When cells were exposed to 18:2 + TNF, 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.

In some experiments, endothelial cell cultures were pretreated with 25 μM vitamin E for 12 or 24 h before exposure to 18:2 and/or TNF. Vitamin E was then maintained in experimental media during a fatty acid and/or cytokine treatment exposure.

Each experiment was carefully controlled. Endothelial cells were exposed to different factors at the same time and measurements were performed under the same experimental conditions.

Cellular oxidation studies

2,7-Dichlorofluorescein (DCF) fluorescence. This measurement of cell oxidation is based on reactive oxygen species (ROS)-mediated conversion of 2,7-dichlorofluorescin (loaded into cells as 2,7-dichlorofluorescin diacetate; Molecular Probes, Inc.) into fluorescent DCF, with increased fluorescence emission reflecting enhanced oxidative stress. The study was performed as

described by Mattson et al. (30). Briefly, treated endothelial cells were loaded with 50 μM 2,7-dichlorofluorescin diacetate (Molecular Probes, Inc.) by incubation for 50 min. Before analysis, cells were washed 3 times in Hank's and then imaged by confocal scanning laser microscopy using 488 nm excitation and 510 nm emission filters. Average pixel intensity was measured within each cell in the field and expressed in the relative units of DCF fluorescence. Values are mean ± SEM of individuals cells from two or three separate plates.

Glutathione determination

Total glutathione was determined using the method of Tietze (31) as modified by Bhat et al. (32). Specificity of this method for glutathione measurement is ensured by highly specific glutathione reductase. Briefly, treated confluent endothelial monolayers, cultured in 100-mm culture dishes (Corning, Corning, NY), were washed with phosphate-buffered saline, scraped into 5% trichloroacetic acid, and sonicated. Cell lysates were neutralized with triethanolamine. To determine the total glutathione level, 0.4-mL aliquots of neutralized cell lysates were mixed with 0.5 mL of 0.2 M sodium phosphate buffer, containing 0.01 M EDTA and 0.6 M NADPH, and with 100 μL 6 mM 5,5′-dithiobis-(2-nitrobenzoic acid). The reaction was initiated by the addition of 10 μL 50 U glutathione reductase/mL and recorded at 412 nm. The glutathione content was calculated on the basis of the standard curve obtained with known amounts of glutathione. Trichloroacetic acid precipitates were redissolved in aliquots of 0.1% sodium dodecyl sulfate in 0.2 M NaOH, brought to a total volume of 1 mL with buffer, and used for protein determination.

Vitamin E determination

Vitamin E was extracted from endothelial cells and cultured media as described by Hatam and Kayden (33) and measured by isocratic non-aqueous reversed-phase HPLC method according to Barua et al. (34). The samples were run in a Waters HPLC system (Millipore Corp., Waters Chromatography Division, Milford, MA) equipped with Waters Resolve C-18, (5 μm, 3.9 × 300 mm) column, 600E powerline multisolvent delivery system controller, Waters 717 plus autosampler, 486 IEEE tunable absorbance detector and 810 baseline computer workstation. Vitamin E was eluted using the mobile phase (filtered and degassed) acetonitrile–dichloromethane–methanol–1-octanol 90:15:10:0.1 (v/v/v/v) and detected spectophotometrically at 292 nm.

Electrophoretic mobility shift assay (EMSA)

Nuclear protein extracts were prepared from cells according to the method of Dignam, Lebovitz, and Roeder (35) and EMSA was performed using a commercially available kit (Gibco). The nuclear extracts were incubated with the end-labeled double-stranded oligonucleotide containing a tandem repeat of the sequence for NF-κB DNA binding site for 20–30 min at room temperature. After binding of the complexed and uncomplexed DNA, the DNA-protein complexes were resolved by electrophoresis in a 5% low ionic strength non-denaturing polyacrylamide gel.

Transfection and chloramphenicol acetyltransferase (CAT) assay

This assay reflects NF-κB-dependent transcription. Briefly, subconfluent endothelial cell cultures were exposed to 1 μg of pκB/TK5-CAT (generously provided by Dr. C. V. Jongeneel) (36), 5 μg carrier plasmid, and 8 μL of Lipofectin (Gibco) in serum-free, antibiotic-free minimal essential medium for 2 h. After the transfection medium was replaced, cells were incubated overnight with maintenance medium or in medium enriched with 25 μM vitamin E. Transfected cells were then exposed to 18:2 for 24 h and/or TNF for 19.5 h. After treatment exposure, cells were washed, scraped into 250 mM Tris-HCl (pH 7.5) and lysed by three freezing-thawing cycles. CAT activity of the transfected cells were determined following the method of Gorman, Moffat, and Howard (37). Cell extracts, normalized for protein levels, were incubated with 0.7 μCi/ml [^{14}C]chloramphenicol (50

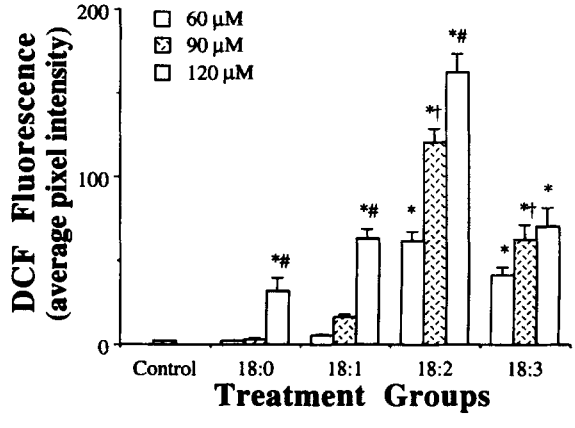


Fig. 1. Effect of different concentrations of 18-carbon fatty acids on endothelial cell oxidation as measured by DCF fluorescence. Endothelial cells were exposed to fatty acids for 6 h; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid. Values are mean ± SEM, n = 10. *Significantly different from controls. †Values in cells exposed to 90 μM fatty acids are significantly different from values in cells exposed to 60 μM fatty acids. #Values in cells exposed to 120 μM fatty acids are significantly different from values in cells exposed to 90 μM fatty acids.

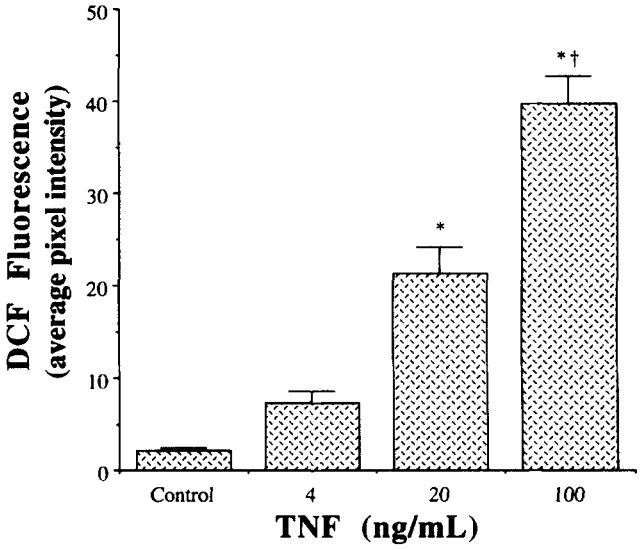


Fig. 2. Effect of different concentrations of TNF on endothelial cell oxidation as measured by DCF fluorescence. Endothelial cells were exposed to TNF for 1.5 h. Values are mean ± SEM, n = 10. *Significantly different from controls (0 ng TNF/mL) and 4 ng TNF/mL. †Values in cells exposed to 100 ng TNF/mL are significantly different from values in cells exposed to 20 ng TNF/mL.

mCi/mmol; New England Nuclear) and 0.44 mM acetyl coenzyme A (Sigma) in 250 mM Tris-HCl (pH 7.5) at 37°C for 2 h. Acetylated and non-acetylated forms of chloramphenicol were extracted with ethyl acetate and separated by thin-layer chromatography on polyester-backed silica gel plates (Whatman) using chloroform–methanol 95:5 (v/v). The non-acetylated, mono- and diacetylated chloramphenicol forms were localized on thin-layer chromatography plates by autoradiography. The degree of acetylation was determined by cutting the radioactive zones corresponding to acetylated or non-acetylated chloramphenicol from the plates and quantified by scintillation counting. CAT activity was expressed as percent of total chloramphenicol converted to acetylated forms per h per mg protein.

Measurement of intracellular free calcium levels

Intracellular free calcium ($[Ca^{2+}]_i$) was quantified according to the method of Mattson et al. (30). Briefly, treated endothelial cells cultured in polyethyleniminecoated glass-bottom 35-mm dishes 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 Hank's balanced salt solution (Gibco) containing 10 mM HEPES buffer. Cells were imaged on a Zeiss inverted microscope using a fluoro 40 ×, 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).

Determination of endothelial barrier function

Endothelial barrier function was measured as transendothelial albumin transfer using polystyrene chambers with a 0.8 μm pore size polycarbonate membrane (Millipore Corporation, Bedford, MA) according to the method of Hennig et al. (29). After reaching confluency, endothelial monolayers were treated with 18:2 for 24 h, TNF for 19.5 h or they were pretreated with 18:2 for 4.5 h and then exposed to 18:2 + TNF for additional 19.5 h. After treatments, chambers with endothelial cells attached to the membranes were washed with M-199 and exposed to 200 μM bovine serum albumin (fatty acid-free, Sigma Chemical Company, St. Louis, MO) in M-199 for 1 h. After incubation with albumin, the albumin

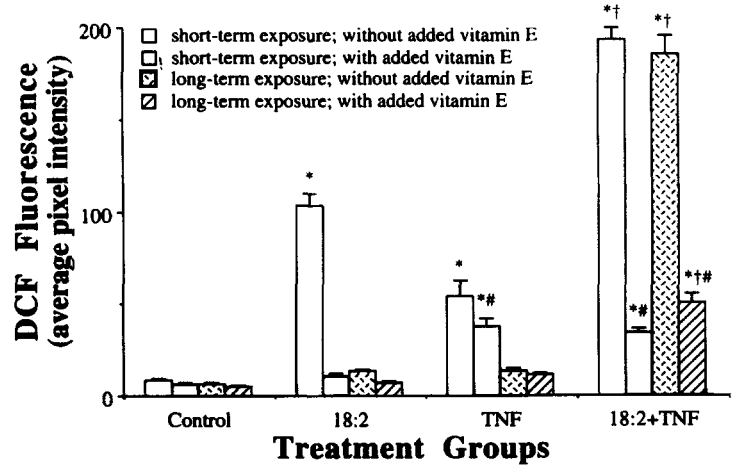


Fig. 3. Effect of 18:2 and/or TNF on endothelial cell oxidation as measured by DCF fluorescence. For a short-term exposure, endothelial cell cultures were treated with 18:2 for 6 h (18:2 group), TNF for 1.5 h (TNF group), or with 18:2 for 4.5 h before adding TNF for a 1.5 h combined exposure (18:2 + TNF group). For a long-term exposure, cells were treated with 18:2 for 24 h (18:2 group) or TNF for 19.5 h (TNF group) or with 18:2 for 4.5 h before adding TNF for a 19.5 h combined exposure (18:2 + TNF group). Some cultures were pretreated with 25 μM vitamin E for 24 h, before adding 18:2 and/or TNF. Values are mean ± SEM, n = 10. *Significantly different from controls at the corresponding time points. †Values in group 18:2 + TNF are significantly different from values in 18:2 or TNF groups at the corresponding time points. #Significantly different compared to values without added vitamin E at the corresponding time points.

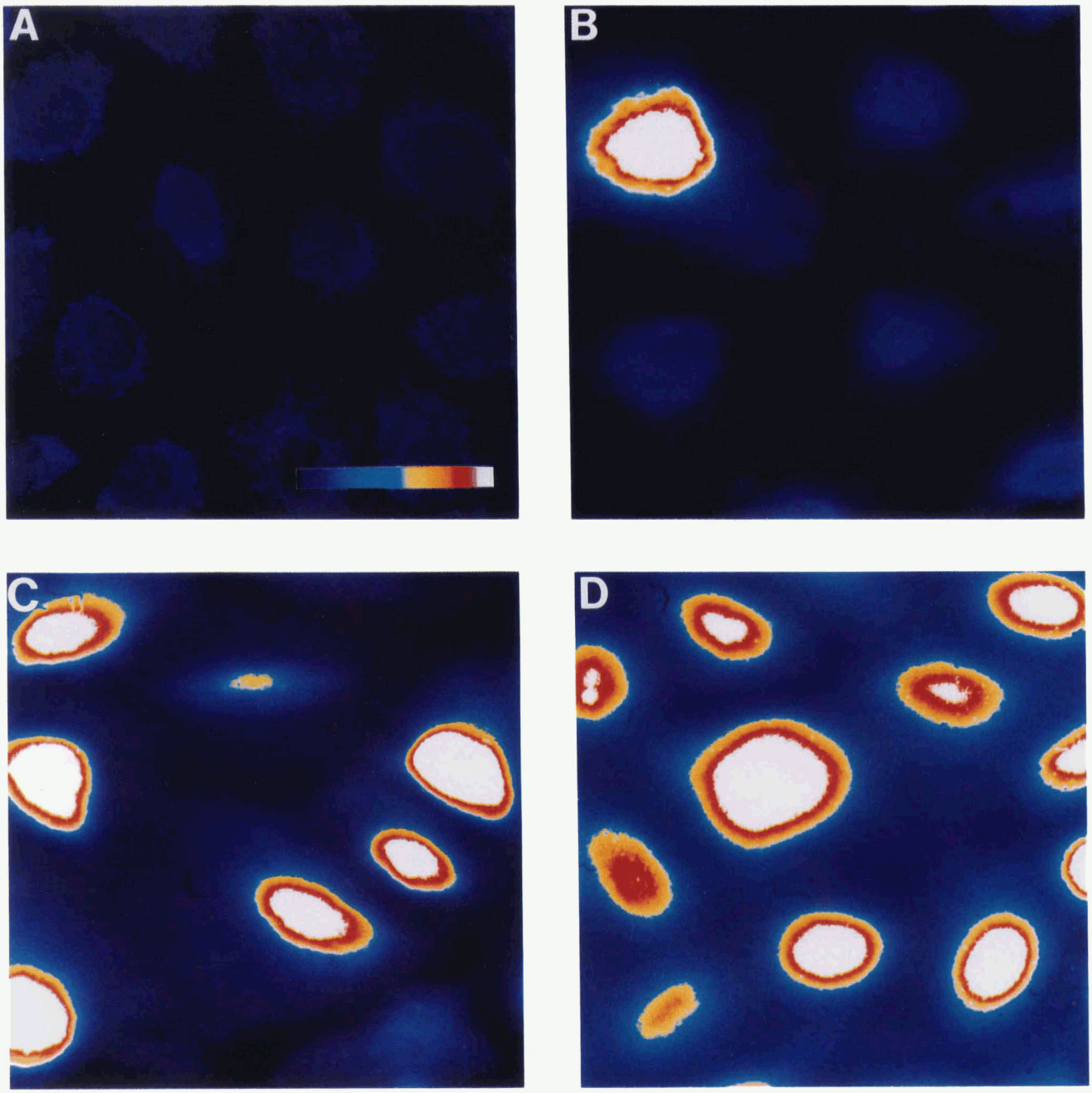


Fig. 4. Photomicrographs from confocal scanning microscopy visualizing oxidative stress as DCF fluorescence emission. A, control cells; B, cells exposed to TNF (20 ng/mL) for 1.5 h; C, cells exposed to 18:2 (90 μM) for 6 h; D, cells exposed to TNF + 18:2. In the 18:2 + TNF group, treatment with 18:2 began 4.5 h before adding TNF for a 1.5 h combined exposure. Blue color on the pseudocolor scale reflects low level of cellular oxidation, yellow intermediate, red high, and white the highest level of cellular oxidative stress.

transferred across endothelial monolayers was determined using bromcresol green (Sigma) and recorded spectophotometrically at 630 nm.

Protein determination

Protein was determined as described by Lees and Paxman (38).

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 (39). Statistical probability of $P \leq 0.05$ was considered significant.

RESULTS

Cellular oxidative stress in cells exposed to different concentrations of 18-carbon fatty acids or TNF

Concentration-dependent effects of 18-carbon fatty acids, differing in degree of unsaturation, as well as concentration-related effects of TNF on cellular oxidation were determined by DCF fluorescence (Figs. 1, 2, respectively). DCF fluorescence, expressed in relative units as an average of pixel intensity, reflects primarily hydrogen peroxide levels, an indicator of oxidative stress in intact cells. When endothelial cells were exposed to fatty acids at the concentrations of 60 and 90 μM for 6 h, only 18:2 and 18:3 induced oxidative stress. However, all tested fatty acids at the level of 120 μM enhanced cellular oxidation. Independent of concentrations, 18:2 induced endothelial cell oxidative stress most markedly as compared to all 18-carbon fatty acids used in the present study (**Fig. 1**).

Plasma free fatty acid concentrations can range from approximately 90 to 1200 μM, with values as high as 2500 μM under stressful conditions such as fasting, diabetes, or strenuous exercise. The majority of free fatty acids is bound to plasma components, mostly albumin. With plasma albumin concentration of approximately 600 μM, the molar ratio of free fatty acids can range from 0.15 to 4 under various physiologic conditions, with an average of approximately 1 (40, 41). In addition, the concentration of free fatty acids generated by the action of lipoprotein lipase near the endothelium may significantly exceed these levels. Therefore, to mimic physiologic conditions and to consider albumin concentrations in culture media, further experiments were performed at 18:2 concentration of 90 μM.

The effects of different concentrations of TNF on endothelial cell oxidation are reflected in **Fig. 2.** Exposure to TNF (20 and 100 ng/mL) for 1.5 h induced oxidative stress. Cellular oxidation was significantly higher in cells exposed to 100 ng TNF/mL as compared to the lower concentrations. On the basis of these results, TNF at the concentration of 20 ng/mL was used in further experiments in which endothelial cells were exposed to a combined treatment of 18:2 plus TNF. In addition, similar TNF levels were reported to be present in atherosclerotic vessels (26).

In experiments when endothelial cells were treated with both 18:2 and TNF, exposure times were established in our previous experiments. The maximal depletion of glutathione and the maximal activation of NF-κB occurred when cells were exposed to 18:2 for 6 h or TNF for 1.5 h (19, 24, 42). Moreover, the increase in endothelial cell permeability mediated by 18:2 or TNF is observed after a 24-h exposure (19, 24). Therefore, treatment exposures to 18:2 for 24 h and to TNF for 19.5 h also were used in some experiments.

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

The effect of 18:2 and/or TNF on cellular oxidation was determined by DCF fluorescence (**Fig. 3** and **Fig. 4**) as well as levels of glutathione and vitamin E (**Fig. 5** and **Fig. 6**, respectively), critical antioxidants for endothelial cells. A short-term exposure to TNF and 18:2 (18:2 for 6 h; TNF for 1.5 h) significantly increased endothelial cell oxidation. However, treatment with 18:2 exerted a more significant effect compared to treatment with TNF. A combined exposure to 18:2 and TNF resulted in a cross-amplified increase in DCF fluorescence as compared to effects of 18:2 and TNF alone. Pretreatment with vitamin E for 24 h before a short-term exposure to 18:2 and/or TNF effectively decreased cellular oxidation. In cells treated with 18:2, vitamin E normalized DCF fluorescence. In cells exposed to TNF or 18:2 + TNF, vitamin E attenuated cellular oxidation compared to cultures which were not pretreated with this antioxidant; however, DCF fluorescence remained elevated as compared to controls. The prolonged amplification of cellular oxidation observed in 18:2 + TNF-treated cells, compared to treatment with 18:2 or TNF alone, was most apparent after a long-term exposure (18:2 for 24 h; TNF for 19.5 h). Cellular oxidation returned to control values in 18:2- or TNF-treated cells; however, in cells exposed to 18:2 + TNF, oxidative stress remained greatly increased (Fig. 3).

Figure 4 shows photomicrographs visualizing DCF fluorescence in control cells and cells exposed to TNF, 18:2 or 18:2 + TNF (Figs. 4A, B, C, D, respectively).

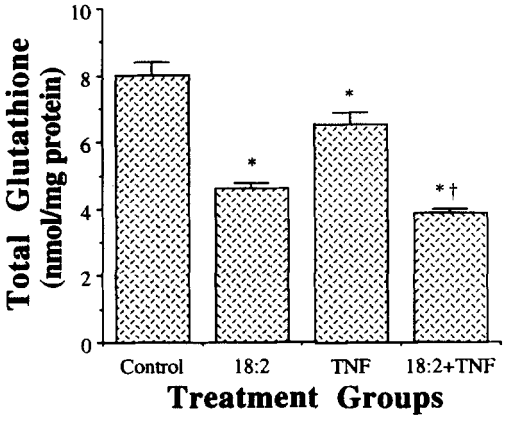


Fig. 5. Effect of 18:2 and/or TNF on intracellular total glutathione levels. Endothelial cell cultures were treated with 18:2 for 6 h (18:2 group), TNF for 1.5 h (TNF group), or with 18:2 for 4.5 h before adding TNF for a 1.5 h combined exposure (18:2 + TNF group). Values are mean ± SEM, n = 5. *Significantly different from controls. †Values in group 18:2 + TNF are significantly different from values in 18:2 or TNF groups.

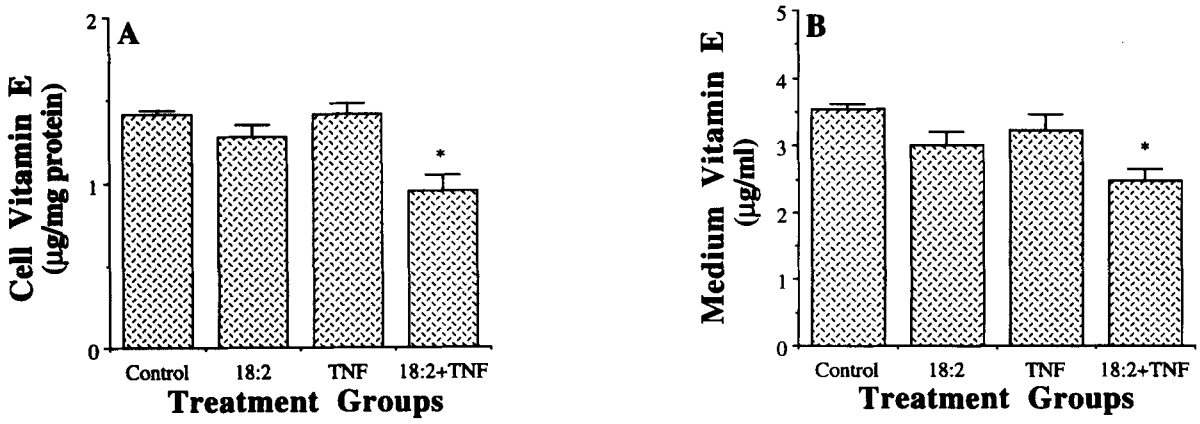


Fig. 6. Effect of 18:2 and/or TNF on cellular (A) or medium (B) vitamin E levels. Endothelial cell cultures were exposed to 18:2 (90 μM) for 24 h or TNF (20 ng/mL) for 19.5 h. In the 18:2 + TNF group, treatment with 18:2 began 4.5 h before adding TNF for a 19.5 h combined exposure. *Significantly different from controls.

Treatment exposures were 6 h for 18:2 and 1.5 h for TNF. The pseudocolor scale that reflects the levels of cellular oxidation is arranged in such a way that white color reflects the highest level of reactive oxygen species (ROS), red color high levels, yellow color intermediate, and blue color the lowest level of ROS.

Increased cellular oxidation results not only in enhanced levels of ROS but also in depletion of antioxidants. Therefore, we measured effects of 18:2 and/or TNF on glutathione (Fig. 5) as well as vitamin E levels in endothelial cells (Fig. 6A) and in surrounding media (Fig. 6B) of treated cultures. A short-time exposure to 18:2 or TNF resulted in the depletion of cellular glutathione. However, these effects were amplified when endothelial cells were exposed to 18:2 + TNF. A long-term exposure to 18:2 or TNF (18:2 for 24 h; TNF for 19.5 h) had no effect on cellular or medium vitamin E levels. Endothelial cell exposure to 18:2 + TNF decreased vitamin E content both in cells and medium.

Activation of NF-κB and induction of NF-κB-dependent transcription in 18:2 and/or TNF-treated endothelial cells

Recent evidence indicates that oxidative stress may affect cellular metabolism by induction of expression of genes regulated by nuclear transcription factor-κB (NF-κB). Therefore, we determined effects of 18:2 and/or TNF on activation of NF-κB in cultured endothelial cells, as measured by EMSA. These results are shown in **Fig. 7.** Both treatments with either TNF for 1.5 h or 18:2 for 6 h activated NF-κB. However, a combined exposure to 18:2 + TNF did not potentiate activation of NF-κB as compared to the cultures exposed to 18:2 or TNF alone.

To determine whether 18:2- and/or TNF-mediated activation of NF-κB may induce gene expression in endothelial cells, we transfected these cells with a plasmid (pκB/TK5-CAT) encoding bacterial protein, chloramphenicol acetyltransferase (CAT). Expression of this construct is controlled by a promoter responsive to NF-κB. Results of the CAT assay are shown in **Figs. 8A and B,** respectively. Figure 8A depicts an autoradiograph of a thin-layer chromatogram that indicates acetylated and non-actetylated forms of chloramphenicol after exposure to 18:2 for 6 h, as compared to control. Figure 8B shows quantified results of the CAT assay. CAT activity was elevated in cells exposed to 18:2 and/or TNF. However, no significant differences in 18:2, TNF or 18:2 + TNF groups were noted. A 24-h pretreatment with vitamin E normalized CAT activity in 18:2- and/or TNF-treated cells.

Injury to endothelial cells mediated by 18:2 and/or TNF

Effects of 18:2 and/or TNF on cell injury were measured by determination of intracellular calcium levels and transendothelial albumin movement across endothelial monolayers (**Fig. 9** and **Fig. 10**). Increased intracellular calcium ($[Ca^{2+}]_i$) is an important indicator of cell injury which eventually may lead to irreversible cell damage. Treatment with either 18:2 for 6 h, TNF for 1.5 h, or the combination caused an increase in $[Ca^{2+}]_i$, compared to controls. However, this short-term exposure did not cause any significant difference in $[Ca^{2+}]_i$ levels between 18:2, TNF, and 18:2 + TNF groups. A similar relationship was observed after a long-term exposure to 18:2 or TNF (18:2, 24 h; TNF, 19.5 h). In contrast, a long-term treatment of endothelial cells with 18:2 + TNF amplified the increase in $[Ca^{2+}]_i$. A 24-h pretreatment with vitamin E attenuated 18:2- and/or TNF-mediated elevation of $[Ca^{2+}]_i$ (Fig. 9).

Figure 10 shows effects of 18:2, TNF, and 18:2 + TNF treatments on endothelial barrier function as measured

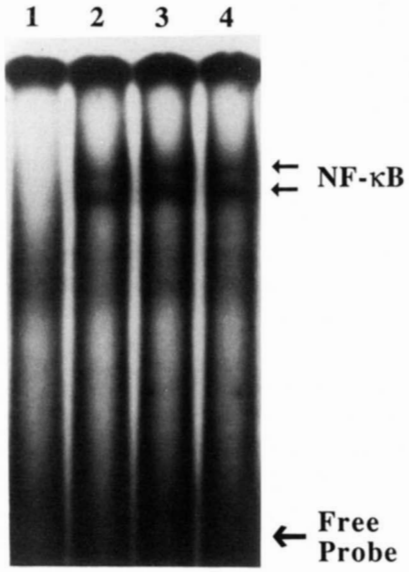


Fig. 7. Effect of 18:2 and/or TNF on activation of NF-κB in cultured endothelial cells. Lane 1, control cultures; lane 2, cultures exposed to 18:2 (90 μM) for 6 h; lane 3, cultures exposed to TNF (20 ng/mL) for 1.5 h; lane 4, cultures exposed to TNF + 18:2. In the 18:2 + TNF group, treatment with 18:2 began 4.5 h before adding TNF for a 1.5 h combined exposure.

by transendothelial albumin movement. Both a 24-h and a 19.5-h exposure to 18:2 or TNF, respectively, caused a significant disruption of endothelial barrier function. However, when cells were exposed to 18:2 + TNF, loss of endothelial integrity was more marked as compared to effects of 18:2 or TNF alone. Pretreatment with vitamin E completely prevented the 18:2, and partially the TNF or 18:2 + TNF-mediated disruption of endothelial barrier function.

DISCUSSION

Endothelial cells may be exposed in vivo to high concentrations of free fatty acids and inflammatory cytokines. Among the 18-carbon fatty acids used in the present study, 18:2 most markedly increased oxidative stress in cultured endothelial cells. This is consistent with our previous experiments where the most significant depletion of intracellular glutathione was observed in cells exposed to 18:2 as compared to other 18-carbon fatty acid treatments (19). One may suggest that a unique metabolism of 18:2 in endothelial cells is responsible for this effect. It was shown that 18:2 accumulates in this type of cells due to its insufficient conversion to arachidonic acid (11, 12). In addition, among several tested fatty acids, 18:2 disrupted endothelial barrier function most significantly. These observations confirmed that the biological effects of fatty acids are dependent not only on the degree of unsaturation and their oxidizability but also on other specific cellular mechanisms, which may indirectly influence oxidative stress. Due to its unique effects on endothelial cell metabolism, 18:2 was chosen to test metabolic effects of a combined exposure to fatty acids and cytokines, such as TNF.

Although 18:2 and TNF are injurious to the endothelium, our present study demonstrates that a combined exposure to 18:2 and TNF significantly cross-amplifies disturbances of endothelial integrity. Among the experimental groups, an increase in $[Ca^{2+}]_i$ and disruption of endothelial barrier function were the most markedly enhanced in 18:2 + TNF-treated cells. Several mechanisms may explain 18:2- or TNF-mediated disturbances in endothelial integrity; however, there is evidence that oxidative stress plays one of the crucial roles in this process (19, 24).

Endothelial cells are especially sensitive to disturbances mediated by oxidative processes (43). It is well known that oxidative stress may induce changes in cellular membrane structure, fluidity, transport, and antigenic characteristics (44) as well as in disturbances in fibrinolytic pathway (45) and prostacyclin synthesis (46). These abnormalities may ultimately contribute to endothelial cell injury, which is one of the earliest steps in the development of atherosclerosis (47).

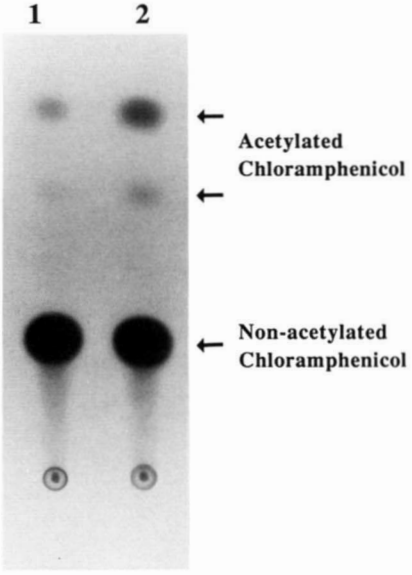
In the present study we provide evidence, using a new cell imaging technique, that both 18:2 and TNF can induce oxidative stress in viable cultured endothelial cells. Moreover, an exposure to 18:2 + TNF potentiated cellular oxidation when compared to treatment with 18:2 or TNF alone. Our data suggest that the maximal oxidative stress mediated by 18:2 or TNF occurs within the first hours of cell exposure. These findings correspond with our earlier observation where we showed that a short-term exposure to 18:2 or TNF was sufficient to decrease cellular glutathione levels significantly (19, 24). However, the overall cell dysfunction, as measured by increased $[Ca^{2+}]_i$ or transendothelial albumin flux, is maintained at 24 h.

Being an unsaturated fatty acid, 18:2 undergoes peroxidative pathways initiated by hydrogen abstraction followed by oxygen attack on the generated lipid alkyl radical (48). Several reports suggest that 18:2 can act as a potent prooxidant in endothelial cells in culture. For example, 18:2 enhances radical adduct formation in endothelial cells exposed to iron-induced oxidative stress (49), decreases glutathione levels (19), and increases peroxisomal β-peroxidation (50), a pathway that leads to the production of H_2O_2. Degradation of 18:2 via peroxidative pathways leads to formation of highly cytotoxic products, such as linoleic acid hydroperoxides or 4-hydroxy-2-(E)-nonenal (51).

In addition to unsaturated lipids, there are several lines of evidence suggesting that TNF also can contribute to the induction of cellular oxidative stress. It has been shown that the interaction between TNF and its cellular receptor stimulates formation of free radicals (52). Moreover, TNF causes damage to mitochondria and converts xanthine dehydrogenase to xanthine oxidase (53), an enzyme that produces superoxide anion radicals. Shultze-Osthoff et al. (54) showed that mitochondrial generation of free radicals at the ubiquinone site, detectable as early as 1 h after TNF treatment, may be the crucial event in TNF-mediated cellular injury/dysfunction. In addition, it was shown that TNF may participate in the generation of hydroxyl radicals (55).

Depletion of antioxidants is another marker of oxidative stress. Our earlier studies indicated that a short-time exposure to 18:2 or TNF decreased cellular glutathione levels in cultured endothelial cells (19, 24). In the present study we demonstrate that a combined exposure to 18:2 and TNF decreases glutathione more markedly than treatments with 18:2 or TNF alone. Decreased levels of this antioxidant may be a mechanism of NF-κB activation in cells treated with 18:2 or TNF. The critical role of decreased thiol groups in activation of NF-κB was previously demonstrated in human T and monocytic cell lines (56). In the present study we also provide evidence that vitamin E levels are diminished in cells treated with 18:2 + TNF for 24 h. Among experimental groups, the decrease in cellular vitamin E levels was statistically significant only in cells exposed to 18:2 + TNF. It is possible that interactions between reduced glutathione (GSH) and vitamin E are responsible for this effect. Vitamin E may serve as an antioxidant only in a reduced form. Therefore, compounds such as GSH are required to maintain the active form of vitamin E by reducing the oxidized form (57). The significance of interaction between GSH and vitamin E is supported by the observation that vitamin E-dependent inhibition of liver microsomal lipid peroxidation is approximately five times more active in the presence of GSH, than in its absence (58). Therefore, one may suggest that depletion of glutathione compromised the vitamin E redox cycle and ultimately led to decreased vitamin E levels observed in the present study. The most marked depletion of intracellular glutathione as well as cell and medium vitamin E indicates that the most significant oxidative stress occurred in cells exposed to 18:2 + TNF, compared to 18:2 or TNF treatments alone. This is consistent with our observation that cellular oxidation, measured by DCF fluorescence, was most markedly enhanced in cells treated with 18:2 + TNF.

Recent evidence suggests that ROS also may be involved in activation of NF-κB and thus induce expression of genes that are controlled by this transcription factor (42, 59, 60). NF-κB is activated by a number of stimuli including viruses, lipopolisaccharide, cytokines, and UV light (61). We reported that 18:2 is another



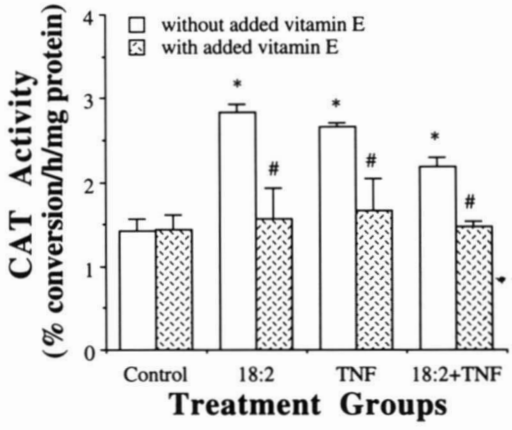


Fig. 8. A: Micrograph of an autoradiograph of a thin-layer chromatogram indicating non-acetylated and acetylated forms of chloramphenicol in endothelial cells transfected with a plasmid pκB/TK5-CAT. Lane 1, control endothelial cells; lane 2, endothelial cells treated with 18:2 for 24 h. B: Effect of 18:2 and/or TNF on NF-κB-related transcription, as measured by CAT activity in cultured endothelial cells. Endothelial cell cultures were exposed to 18:2 (90 μM) for 24 h or TNF (20 ng/mL) for 19.5 h. In the 18:2 + TNF group, treatment with 18:2 began 4.5 h before adding TNF for a 19.5 h combined exposure. Some cultures were pretreated with 25 μM vitamin E for 12 h, before adding 18:2 and/or TNF. Values are mean ± SEM, n = 4. *Significantly different from controls. #Significantly different compared to values without added vitamin E.

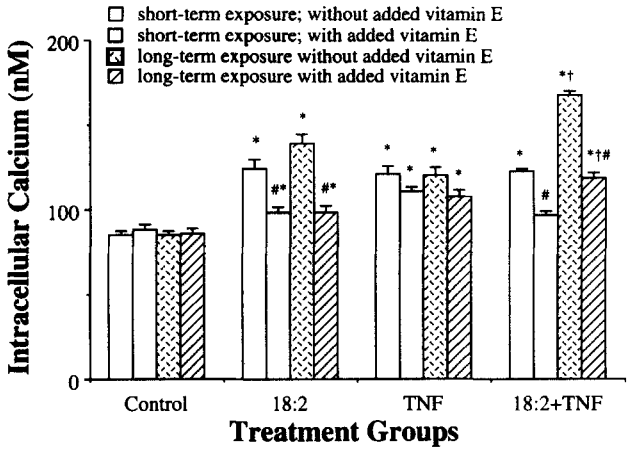


Fig. 9. Effect of 18:2 and/or TNF on intracellular calcium levels in cultured endothelial cells. For a short-term exposure, endothelial cell cultures were treated with 18:2 for 6 h (18:2 group), TNF for 1.5 h (TNF group), or with 18:2 for 4.5 h before adding TNF for a 1.5 h combined exposure (18:2 + TNF group). For a long-term exposure, cells were treated with 18:2 for 24 h (18:2 group) or TNF for 19.5 h (TNF group) or with 18:2 for 4.5 h before adding TNF for a 19.5 h combined exposure (18:2 + TNF group). Some cultures were pretreated with 25 μM vitamin E for 24 h, before adding 18:2 and/or TNF. Values are mean ± SEM, n = 10. *Significantly different from controls at the corresponding time points. †Values in group 18:2 + TNF are significantly different from values in 18:2 or TNF groups at the corresponding time points. #Significantly different compared to values without added vitamin E at the corresponding time points.

potent activator of NF-κB (42, 62). Although multiple transduction pathways are involved in activation of NF-κB by different factors, it appears that all of them lead to activation of protein kinase(s) and subsequent phosphorylation of NF-κB inhibitory subunits, called IκB proteins. Phosphorylated IκB are degraded, and the released NF-κB complex translocates into the nucleus and induces gene expression. There is strong evidence that phosphorylation of IκB and activation of NF-κB can be effectively blocked by antioxidants (63). In fact, we demonstrated that 18:2-mediated activation of NF-κB can be effectively blocked by pretreatment with N-acetylcysteine or vitamin E (42). It was proposed that induction of ROS is the common mechanism of NF-κB activation, regardless of the type of stimuli (61). Therefore, one may suggest that 18:2- and/or TNF-mediated activation of NF-κB, as demonstrated in the present paper, was also due to oxidative mechanisms. However, TNF may also activate NF-κB via the ceramide pathway (64).

Although both 18:2 and TNF may activate NF-κB, pretreatment with 18:2 with a subsequent exposure to TNF did not further affect activation of this transcriptional factor as compared to the effects of 18:2 or TNF alone. This phenomenon can be explained by the properties of NF-κB complex and IκB subunits. In general, it is known that activated NF-κB induces several forms of IκB which in turn inhibit further activation of NF-κB (63). Therefore, 18:2-activated NF-κB, observed in the present study, was most likely a subject of such feedback inhibition. One may suggest that this inhibition prevented further activation of NF-κB when 18:2-pretreated cells were additionally exposed to TNF.

As observed in the present study, changes in the activation of NF-κB by 18:2 and/or TNF were consistent with alterations in NF-κB-dependent transcription, as measured by CAT assay. CAT activity was elevated in cells exposed to 18:2 and/or TNF. However, compared to cultures exposed to 18:2 or TNF alone, no additive effect of the treatment with 18:2 + TNF on CAT activity was observed. Moreover, vitamin E, which completely blocks 18:2-induced activation of NF-κB (42), prevented 18:2- and/or TNF-mediated induction of NF-κB-related transcription.

In the present study, an increase in intracellular calcium ($[Ca^{2+}]_i$) was correlated with changes in endothelial barrier function, measured as transendothelial albumin movement. This finding is consistent with similar observations reported by Yamada et al. (65). Increased $[Ca^{2+}]_i$ could be one of the mechanisms that leads to 18:2- and/or TNF-mediated disruption of endothelial barrier function. Although ionized calcium is one of the most common cellular signaling factors, destabilization of calcium metabolism and increased levels of $[Ca^{2+}]_i$ may activate degradative processes, including oxidative damage (66). Increased $[Ca^{2+}]_i$ can promote ROS formation by activation of several mechanisms, such as activation of phospholipases, conversion of xanthine dehydrogenase to xanthine oxidase, or stimulation of nitric oxide and peroxynitrite production. In addition, enhanced $[Ca^{2+}]_i$ causes activation of cellular proteases, disruption of cytoskeleton, and membrane lysis. Increased $[Ca^{2+}]_i$ may eventually lead to cell death by initiation of cellular necrosis or apoptosis. On the other hand, elevated free radicals lead to increased $[Ca^{2+}]_i$ and thus, these two factors potentiate their injurious effects (67). In the present study, the relationship between increased $[Ca^{2+}]_i$, endothelial cell dysfunction, and induction of oxidative stress is supported by the observation that vitamin E effectively attenuated $[Ca^{2+}]_i$, transendothelial albumin movement, and cellular oxidation.

In conclusion, exposure of endothelial cells to 18:2 and/or TNF caused an increase in cellular oxidation, activation of NF-κB and NF-κB-related transcription, elevation of intracellular calcium, and disturbances in endothelial barrier function. These changes, except for NF-κB activation and NF-κB-related transcription, were potentiated when endothelial cells were treated with both 18:2 and TNF. Pretreatment with vitamin E attenuated 18:2- and/or TNF-mediated endothelial cell dysfunction. These results demonstrate that 18:2- and/or

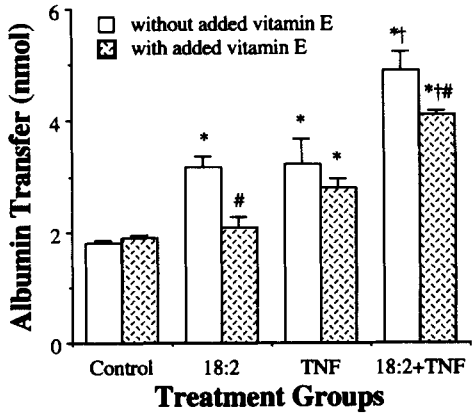


Fig. 10. Effect of 18:2 and/or TNF on endothelial cell barrier function as measured by transendothelial albumin transfer. Endothelial cell cultures were exposed to 18:2 (90 μM) for 24 h or TNF (20 ng/mL) for 19.5 h. In the 18:2 + TNF group, treatment with 18:2 began 4.5 h before adding TNF for a 19.5 h combined exposure. Some cultures were pretreated with 25 μM vitamin E for 24 h, before adding 18:2 and/or TNF. Values are mean ± SEM, n = 4. *Significantly different from controls. †Values in group 18:2 + TNF are significantly different from values in 18:2 or TNF groups at the corresponding time points. #Significantly different compared to values without added vitamin E.

TNF-mediated loss in endothelial barrier integrity is due, at least in part, to induction of cellular oxidative stress. These findings may have significant implications in understanding the role of lipid and/or inflammatory components in the development of atherosclerosis. These findings also suggest that diets high in unsaturated fats, e.g., linoleic acid-rich triglycerides, may contribute to atherogenesis by cross-amplifying inflammatory cytokine-mediated endothelial cell dysfunction.

This work was supported by grants HL36552 from the National Institutes of Health and the Kentucky Agricultural Experimental Station. We thank Dr. Swati Joshi-Barve for technical assistance with the electrophoretic mobility shift assay (EMSA).

Manuscript received 10 July 1995 and in revised form 6 October 1995.

REFERENCES

1. Hennig, B., M. Toborek, A. A. Cader, and E. A. Decker. 1994. Nutrition, endothelial cell metabolism, and atherosclerosis. *Crit. Rev. Food Sci. Nutr.* **34:** 253–282.
2. Zilversmit, D. B. 1973. A proposal linking atherogenesis to the interaction of endothelial lipoprotein lipase with triglyceride-rich lipoproteins. *Circ. Res.* **33:** 633–638.
3. Zilversmit, D. B. 1979. Atherogenesis: a postprandial phenomenon. *Circulation.* **60:** 473–485.
4. Ylä-Herttuala, S., B. A. Lipton, M. E. Rosenfeld, I. J. Goldberg, D. Steinberg, and J. C. Witztum. 1991. Macrophages and smooth muscle cells express lipoprotein lipase in human and rabbit atherosclerotic lesions. *Proc. Natl. Acad Sci. USA.* **88:** 10143–10147.
5. Hennig, B., B. H. Chung, B. A. Watkins, and A. Alvarado. 1992. Disruption of endothelial barrier function by lipolytic remnants of triglyceride-rich lipoproteins. *Atherosclerosis.* **95:** 235–247.
6. Hennig, B., A. Alvarado, S. Ramasamy, G. A. Boissonneault, E. A. Decker, and W. J. Means. 1990. Fatty acid-induced disruption of endothelial barrier function in culture. *Biochem. Arch.* **6:** 409–417.
7. Hodgson, J. M., M. L. Wahlqvist, J. A. Boxall, and N. D. Balazs. 1993. Can linoleic acid contribute to coronary artery disease? *Am. J. Clin. Nutr.* **58:** 228–234.
8. Luostarinen, R., M. Boberg, and T. Saldeen. 1993. Fatty acid composition in total phospholipids of human coronary arteries in sudden cardiac death. *Atherosclerosis.* **99:** 187–193.
9. Reaven, P., S. Parthasarathy, B. J. Grasse, E. Miller, D. Steinberg, and J. L. Witztum. 1993. Effects of oleate-rich and linoleate-rich diets on the susceptibility of low density lipoprotein to oxidative modification in mildly hypercholesterolemic subjects. *J. Clin. Invest.* **91:** 668–676.
10. Hennig, B., M. Toborek, G. A. Boissonneault, E. A. Decker, and P. R. Oeltgen. 1995. Animal and plant fats selectively modulate oxidizability of rabbit LDL and LDL-mediated disruption of endothelial barrier function. *J. Nutr.* **125:** 2045–2054.
11. Spector, A. A., T. L. Kaduce, J. C. Hoak, and G. L. Fry. 1981. Utilization of arachidonic and linoleic acids by cultured human endothelial cells. *J. Clin. Invest.* **68:** 1003–1011.
12. Debry, G., and X. Pelletier. 1991. Physiological importance of ϖ-3/ϖ-6 polyunsaturated fatty acids in man. An overview of still unresolved and controversial questions. *Experientia.* **47:** 172–178.
13. Hennig, B., and B. A. Watkins. 1989. Linoleic acid and linolenic acid: effect on permeability properties of cultured endothelial cell monolayers. *Am. J. Clin. Nutr.* **49:** 301–305.
14. Toborek, M., and B. Hennig. 1992. Effect of different fatty acids on ATP levels in cultured endothelial cells. *FASEB J.* **6:** A 1322.
15. 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.
16. 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.
17. 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.
18. de Haan, L. H., I. Bosselaers, W. M. Jongen, R. M. Zwijsen, and J. H. Koeman. 1994. Effect of lipids and aldehydes on gap-junctional intercellular communication between human smooth muscle cells. *Carcinogenesis.* **15:** 253–256.
19. 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.
20. Faruqi, R. M., and P. E. DiCorleto. 1993. Mechanisms of monocyte recruitment and accumulation. *Br. Heart J.* **69:(1 Suppl)** S19–29.

21. Hansson, G., J. Holm, and L. Jonasson. 1989. Detection of activated T lymphocytes in the human atherosclerotic plaque. *Am. J. Pathol.* **135:** 169–175.
22. 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.
23. Mantovani, A., F. Bussolino, and E. Dejana. 1992. Cytokine regulation of endothelial cell function. *FASEB J.* **6:** 2591–2599.
24. 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.
25. 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.
26. Rus, H. G., F. Niculescu, and R. Vlaicu. 1991. Tumor necrosis factor-alpha in human arterial wall with atherosclerosis. *Atherosclerosis.* **89:** 247–254.
27. Renier, G., M. Olivier, E. Skamene, and D. Radzioch. 1994. Induction of tumor necrosis factor α gene expression by lipoprotein lipase. *J. Lipid Res.* **35:** 271–278.
28. Renier, G., E. Skamene, J. B. DeSanctis, and D. Radzioch. 1994. Induction of tumor necrosis factor α gene expression by lipoprotein lipase requires protein kinase C activation. *J. Lipid Res.* **35:** 1413–1421.
29. 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.
30. 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.
31. Tietze, F. 1969. Enzymatic method for quantitative determination of nanogram amounts of total and oxidized glutathione. *Anal. Biochem.* **27:** 502–522.
32. Bhat, G., S. B. Tinsley, J. K. Tolson, J. M. Patel, and E. R. Block. 1992. Hypoxia increases the susceptibility of pulmonary artery endothelial cells to hydrogen peroxide injury. *J. Cell. Physiol.* **151:** 228–238.
33. Hatam, L. J., and H. J. Kayden. 1979. A high-performance liquid chromatographic method for the determination of tocopherol in plasma and cellular elements of the blood. *J. Lipid Res.* **20:** 639–645.
34. Barua, A. B., H. C. Furr, D. Janick-Buckner, and J. A. Olson. 1993. Simultaneous analysis of individual carotenoids, retinol, retinyl esters, and tocopherols in serum by isocratic non-aqueous reversed-phase HPLC. *Food Chem.* **46:** 419–424.
35. Dignam, J. D., R. M. Lebovitz, and R. G. Roeder. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* **11:** 1474–1489.
36. Shakhov, A. N., M. A. Collart, P. Vassalli, S. A. Nedospasov, and C. V. Jongeneel. 1990. κB-Type enhancers are involved in lipopolysaccharide-mediated transcriptional activation of the tumor necrosis factor α gene in primary macrophages. *J. Exp. Med.* **171:** 35–47.
37. Gorman, C. M., L. F. Moffat, and B. H. Howard. 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell. Biol.* **2:** 1044–1051.
38. Lees, M. B., and S. Paxman. 1972. SDS-Lowry protein estimation for lipid containing samples. *Anal. Biochem.* **47:** 184–192.
39. Snedecor, G. W., and W. G. Cochran. 1974. Statistical Methods. 7th ed., Iowa State University Press, Ames, IA.
40. Spector, A. A. 1975. Fatty acid binding to plasma albumin. *J. Lipid Res.* **16:** 165–179.
41. Potter, B. J., D. Sorentino, and P. D. Berk. 1989. Mechanisms of cellular uptake of free fatty acids. *Annu. Rev. Nutr.* **9:** 253–270.
42. Hennig, B., M. Toborek, S. Joshi-Barve, S. W. Barger, S. Barve, M. P. Mattson, and C. J. McClain. 1995. Linoleic acid activates NF-κB and induces NF-κB-dependent transcription in cultured endothelial cells. *Am. J. Clin. Nutr.* In press.
43. Hennig, B., and C. K. Chow. 1988. Lipid peroxidation and endothelial cell injury: implication in atherosclerosis. *Free Radical Biol. Med.* **4:** 99–106.
44. Grisham, M. B., and J. M. McCord. 1986. Chemistry and cytotoxicity of reactive oxygen metabolities. *In* Physiology of Oxygen Radicals. American Physiological Society. 1–18.
45. Holvoet, P., and D. Collen. 1994. Oxidized lipoproteins in atherosclerosis and thrombosis. *FASEB J.* **8:** 1279–1284.
46. Hempel, S. L., D. L. Haycraft, J. C. Hoak, and A. A. Spector. 1990. Reduced prostacyclin formation after reoxygenation of anoxic endothelium. *Am. J. Physiol.* **259:** C739–C745.
47. Ross, R. 1993. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature.* **362:** 801–809.
48. Cosgrove, J. P., D. F. Church, and W. A. Pryor. 1987. The kinetics of the autoxidation of polyunsaturated fatty acids. *Lipids.* **22:** 299–304.
49. Alexander-North, L. S., J. A. North, K. P. Kiminyo, G. R. Buettner, and A. A. Spector. 1994. Polyunsaturated fatty acids increase lipid radical formation induced by oxidant stress in endothelial cells. *J. Lipid Res.* **35:** 1773–1785.
50. Hennig, B., Y. Wang, G. A. Boissonneault, A. Alvarado, and H. P. Glauert. 1990. Effects of fatty acids enrichment on the induction of peroxisomal enzymes in cultured porcine endothelial cells. *Biochem. Arch.* **6:** 141–146.
51. Tamura, H., and T. Shibamoto. 1991. Gas chromatographic analysis of malonaldehyde and 4-hydroxy-2-(E)-nonenal produced from arachidonic acid and linoleic acid in a lipid peroxidation model system. *Lipids.* **26:** 170–173.
52. Collins, T. 1993. Endothelial nuclear factor-κB and the initiation of the atherosclerotic lesion. *Lab. Invest.* **68:** 499–508.
53. Friedl, H. P., G. O. Till, U. S. Ryan, and P. A. Ward. 1989. Mediator-induced activation of xanthine oxidase in endothelial cells. *FASEB J.* **3:** 2512–2518.
54. Schulze-Osthoff, K., A. C. Bakker, B. Vanhaesebroeck, R. Beyaert, W. A. Jacob, and W. Fiers. 1992. Cytotoxic activity of tumor necrosis factor is mediated by early damage of mitochondrial functions. Evidence for the involvement of mitochondrial radical generation. *J. Biol. Chem.* **267:** 5317–5323.
55. Yamauchi, N., N. Watanabe, H. Kuriyama, H. Neda, M. Maeda, T. Himeno, and Y. Tsuji. 1990. Suppressive effects of intracellular glutathione on hydroxyl radical production induced by tumor necrosis factor. *Int. J. Cancer.* **46:** 884–888.

56. Israël, N., M. A. Gougerot-Pocidalo, F. Aillet, and J. L. Virelizier. 1992. Redox status of cells influences constitutive or induced NF-κB translocation and HIV long terminal repeat activity in human T and monocytic cell lines. *J. Immunol.* **149:** 3386–3393.
57. Di Mascio, P., M. E. Murphy, and H. Sies. 1991. Antioxidant defense systems: the role of carotenoids, tocopherols, and thiols. *Am. J. Clin. Nutr.* **53:** 194S–200S.
58. Leedle, R. A., and S. D. Aust. 1990. The effect of glutathione on the vitamin E requirement for inhibition of liver microsomal lipid peroxidation. *Lipids.* **25:** 241–245.
59. Marui, N., M. K. Offermann, R. Swerlick, C. Kunsch, C. A. Rosen, M. Ahmad, R. W. Alexander, and R. M. Medford. 1993. Vascular cell adhesion molecule-1 (VCAM-1) gene transcription and expression are regulated through an antioxidant-sensitive mechanism in human vascular endothelial cells. *J. Clin. Invest.* **92:** 1866–1874.
60. Shu, H. B., A. B. Agranoff, E. G. Nabel, K. Leung, C. S. Duckett, A. S. Neish, T. Collins, and G. J. Nabel. 1993. Differential regulation of vascular cell adhesion molecule 1 gene expression by specific NF-κB subunits in endothelial and epithelial cells. *Mol. Cell. Biol.* **13:** 6283–6289.
61. Schreck, R., K. Albermann, and P. A. Baeuerle. 1992. Nuclear factor κB: an oxidative stress-responsive transcription factor of eukaryotic cells (a review). *Free Radical Res. Commun.* **17:** 221–237.
62. Toborek, M., S. Barve, C. J. McClain, V. Young, S. W. Barger, M. P. Mattson, and B. Hennig. 1995. Linoleic acid potentiates TNF-mediated endothelial cell dysfunction. *FASEB J.* **9:** A 322.
63. Thanos, D., and T. Maniatis. 1995. NF-κB: a lesson in family values. *Cell.* **80:** 529–532.
64. Kolesnick, R., and D. W. Golde. 1994. The sphingomyelin pathway in tumor necrosis factor and interleukin-1 signaling. *Cell.* **77:** 325–328.
65. Yamada, Y., T. Furumichi, H. Furui, T. Yokoi, T. Ito, K. Yamauchi, M. Yokota, H. Hayashi, and H. Saito. 1990. Roles of calcium, cyclic nucleotides, and protein kinase C in regulation of endothelial permeability. *Arteriosclerosis.* **10:** 410–420.
66. Clapham, D. E. 1995. Calcium signaling. *Cell.* **80:** 259–268.
67. 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.