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The effect of 5,8,11,14-eicosatetraynoic acid on endothelial cell gene expression

Karl M. Stuhlmeier *, Chi Tarn, Fritz H. Bach

Sandoz Center for Immunobiology, Beth Israel Deaconess Medical Center, Harvard Medical School, 99 Brookline Avenue, Boston, MA 02115, USA

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Abstract

The endothelium plays a key role in inflammation, hemostasis and organ rejection. We report here that a synthetic polyunsaturated fatty acid, 5,8,11,14-eicosatetraynoic acid (ETYA), selectively inhibits the up-regulation of several genes on endothelial cells. ETYA suppresses endothelial cell activation by inhibiting the up-regulation of adhesion molecules like E-selectin. A runoff assay for E-selectin demonstrated that the suppression is at the level of transcription. The fact that ETYA inhibits E-selectin upon stimulation with a diverse group of stimuli like lipopolysaccharide, tumor necrosis factor-α or phorbol 12-myristate 13-acetate, suggests that ETYA does not exert its effect by modifying membrane-bound receptors. The messenger RNA for interleukin-8 and glyceraldehyde phosphate dehydrogenase are not affected. Pre-treatment of endothelial cells with ETYA also prevents the adherence of monocytes to tumor necrosis factor-α-stimulated cells.

Keywords: ETYA (5,8,11,14-eicosatetraynoic acid); Endothelial cell activation; Polyunsaturated fatty acid; Gene regulation; Inhibition; Adhesion molecule

1. Introduction

The polyunsaturated fatty acid 5,8,11,14-eicosatetraynoic acid (ETYA), a synthetic arachidonic acid analog, was described for the first time nearly 40 years ago (Osbond et al., 1961; Osbond and Wickens, 1959). Subsequently, it has been used in a variety of in vitro and in vivo experiments, including clinical trials to lower cholesterol levels in humans. However, more recently, ETYA has been mainly used as an inhibitor of arachidonic acid metabolism, as this reagent has been shown to be a potent inhibitor of cyclooxygenase, lipoxygenase and cytochrome P-450 (Abramson and Weissmann, 1989; Gerrard, 1985). Despite this, ETYA has been known for a long time to possess anti-inflammatory and antipyretic properties (Willis, 1974) which are thought to be due to the inhibition of prostaglandins, leukotrienes and thromboxanes (Smith and FordHutchinson, 1974).

It has been shown that diets rich in polyunsaturated fatty acids can modulate the immune response in a number of ways, e.g., by modifying cell membrane fluidity and by influencing the precursor pool for eicosanoids (Foegh et al., 1983; Jordon, 1991). We were interested in whether we could make use of the reported anti-inflammatory properties of polyunsaturated fatty acids (Kort et al., 1991; Mertin, 1976; Mertin and Hunt, 1976; Ring et al., 1974) in an inflammation model using porcine endothelial cells to test several polyunsaturated fatty acids, including arachidonic acid, linoleic acid and gamma linoleic acid, for their potency in inhibiting gene up-regulation in response to stimulation with tumor necrosis factor-α (TNF), lipopolysaccharide, interleukin-1 or phorbol 12-myristate 13-acetate (PMA). In the course of these experiments it was found that ETYA may possess certain beneficial properties like other polyunsaturated fatty acids, unrelated to the inhibition of prostaglandin biosyntheses which might be helpful in controlling inflammation. The results of these studies, investigating the effect of ETYA on endothelial cell activation, are reported here.

2. Materials and methods

2.1. Preparation of porcine aortic endothelial cells

Porcine endothelial cells were isolated and passaged in our laboratory as previously described (Stuhlmeier et al.,

* Corresponding author. Tel.: (1-617) 632-0862; Fax: (1-617) 632-0880; e-mail: kstuhlm@bidmc.harvard.edu

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1994). In brief, endothelial cells were cultured in Dulbecco's modified Eagle's medium with 4.5 g/l glucose and supplemented with 10% heat-inactivated fetal bovine serum (Hyclone), L-glutamine and 50 U/ml penicillin/streptomycin. Experiments were performed in Dulbecco's modified Eagle's medium under serum-free conditions. Before incubating endothelial cells with ETYA, cells were washed three times with pre-warmed serum-free Dulbecco's modified Eagle's medium. TNF and PMA were diluted in serum-free medium and added to the endothelial cells; in cases where lipopolysaccharide was used as a stimulus, fetal calf serum was added simultaneously with lipopolysaccharide to a final concentration of 5%. Human umbilical vein endothelial cells were a gift from Dr. B. Ewenstein (Brigham Women's Hospital, Boston, MA, USA). Pig endothelial cells from passage 3–7 were used in these experiments. Human umbilical vein endothelial cells were used after the first passage to 96-well plates.

2.2. Stimulants and reagents

The following reagents were purchased from Sigma (St. Louis, MO, USA): lipopolysaccharide; serotype 055:B5, PMA, (3-4,5-dimethylthizol-2-yl)-2,5-diphenyltetrazolium bromide and citrated porcine plasma. 5,8,11,14-Eicosatetraynoic acid (ETYA); ≥ 98% pure and peroxide-free arachidonic acid were purchased from Cayman (Ann Arbor, MI, USA). TNF was a gift from Sandoz (Basel, Switzerland). Interleukin-1α was purchased from Genzyme. The 2′,7′-bis-(2-carboxyethyl)-5(and-6)-carboxyfluorescein, acetoxymethyl was purchased from Molecular Probes (Eugene, OR, USA). RNAzol B was purchased from Tel-Test (Friendswood, TX, USA). Lactate-dehydrogenase release was measured with a kit (CytoTox-96) purchased from Promega (Madison, WI, USA). The antinuclear factor-κB (NF-κB), p65 subunit antibody was purchased from Boehringer Mannheim and the consensus oligonucleotide for NF-κB was purchased from Promega. The NF-κB elements from the interleukin-8 promotor were synthesised by Midland Certified Reagent (Midland, TX, USA).

2.3. Enzyme-linked immunosorbent assay for detection of E-selectin

Enzyme-linked immunosorbent assays were performed as described (Stuhlmeier et al., 1994). In brief, cells in 96-well tissue culture plates, 1–3 days post-confluence, were stimulated as indicated, washed and fixed in ice-cold 0.05% glutaraldehyde at 4°C for 10 min. After three washes, cells were incubated with an anti-human E-selectin antibody (BBA1, British Biotechnology), diluted 1:10 000 in phosphate-buffered saline plus 0.05% polyoxyethylene-20-orbitan monolaurate. Despite the fact that this antibody has been previously shown to cross-react with human P-selectin on platelets by flow cytometry analysis, using an enzyme-linked immunosorbent assay for detection of E-selectin up-regulation, we were not able to detect P-selectin expression on human or pig endothelial cells at early time points (5–20 min) post-stimulation, where P-selectin expression peaks. These control experiments and the time-course of the protein recognized by BBA1 (Stuhlmeier et al., 1994) make us confident that our enzyme-linked immunosorbent assay system was specific for E-selectin expression. As a detection system, a horseradish peroxidase-coupled goat anti-mouse antibody (Pierce) was used.

2.4. Viability assay

Viability of endothelial cells exposed to ETYA or solvent controls was evaluated using a (3-4,5-dimethylthizol-2-yl)-2,5-diphenyltetrazolium bromide conversion assay, or a lactate-dehydrogenase release assay. The (3-4,5-dimethylthizol-2-yl)-2,5-diphenyltetrazolium bromide conversion assay was carried out as described elsewhere (Denizot and Lang, 1986). In brief, the assay was carried out in 96-well plates where Dulbecco's modified Eagle's medium had been replaced with serum- and phenol red-free medium at 80 μl/well. Two hours before termination of the assay, 20 μl of the (3-4,5-dimethylthizol-2-yl)-2,5-diphenyltetrazolium bromide stock solution (5 mg/ml in phosphate-buffered saline) was added. At the end of the incubation period, the plates were washed twice with phosphate-buffered saline and the formazan was solubilized by adding ethanol (50 μl/well). The optical density of each well was measured using an automatic plate reader with a 560 nm test wavelength and a 690 nm reference wavelength. Lactate-dehydrogenase release was measured using the CytoTox-96 cytotoxicity assay according to the manufacturer's instructions.

2.5. Measurement of tissue factor activity

Endothelial cells were pre-treated with 10, 25, 45 and 60 μM ETYA for 30 min and stimulated with 5 ng/ml TNF for an additional 8 h. After that time, the medium was removed and the cells were washed 3 times with barbital-buffered saline (clotting buffer). The cells were removed from the culture plate with a rubber policeman and pipetted into polystyrene tubes. The cells were pelleted by centrifugation and resuspended in 100 μl clotting buffer after which 100 μl of citrated porcine plasma and 100 μl of 30 mM $CaCl_2$ were added. The time from $CaCl_2$ addition to formation of the first definite fibrin strands was noted.

2.6. Adhesion assay

Pig endothelial cells were pre-incubated with 5, 20, 40 and 65 μM ETYA for 30 min and stimulated for 4 h with

TNF or medium. After this, in some experiments, the endothelial cells were fixed with 0.05% glutaraldehyde for 10 min at 4°C followed by three washes with Hanks' balanced salt solution, supplemented with 3% bovine serum albumin and, in some otherwise identical experiments, non-fixed cells were used. For the preparation of monocyte-like cells, U937 cells were labeled with 1 ng/ml of 2′,7′-bis-(2-carboxyethyl)-5(and-6)-carboxyfluorescein, acetoxymethyl, 50 μg were dissolved in 50 μl dimethyl sulfoxide. The cells were incubated with 2′,7′-bis-(2-carboxyethyl)-5(and-6)-carboxyfluorescein, acetoxymethyl for 30 min at 37°C and then washed twice with Hanks' balanced salt solution supplemented with 3% bovine serum albumin and finally resuspended in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. The extent of U937 adhesion to TNF-activated, ± ETYA-pretreated endothelial cells was determined and compared to untreated endothelial cells by adding U937 to the wells and allowing adhesion at 37°C for 30 min. Nonadherent cells were washed away with Hanks' balanced salt solution and fluorescence determined with a 7620 Microplate Fluorometer (Cambridge Technology, Cambridge, MA, USA) with the 485 nm excitation filter and the 530 nm emission filter. A standard curve relating fluorescence to cell number was prepared for each plate and used to calculate the number of adherent cells. The ETYA-induced inhibition of U937 binding to stimulated endothelial cells is shown. The adhesion of TNF-stimulated endothelial cells represented 100% adhesion and cells bound to unstimulated endothelial cells represented zero percent.

2.7. Northern blot analysis

Total cellular RNA was isolated from cultured pig endothelial cells using RNAzol B according to the manufacturer's suggestions (Chomczynski and Sacchi, 1987). Equal amounts of RNA (20 μg/lane) were loaded and electrophoresed in a 1% agarose/formaldehyde gel. The RNA was then transferred to a nylon membrane (Micron Separations) and hybridized to the complementary DNA fragments for pig E-selectin (derived by R. De Martin and H. Winkler in our laboratories), human intracellular adhesion molecule-1 (ICAM-1) (Staunton et al., 1988) (a gift from T. Springer, Boston, MA, USA), human vascular cell adhesion molecule-1 (VCAM-1) (a gift from Dr. T. Collins, Boston, MA, USA), mink plasminogen activator inhibitor-1 (PAI-1) (a gift from Dr. B. Kallin, Karolinska Institute, Stockholm, Sweden), pig interleukin-8 (derived by Drs. E. Hofer and R. De Martin in our laboratories), pig endothelial cell inducible gene-6 (ECI-6) (derived by Dr. R. De Martin, VIRCC, Vienna, Austria) (De Martin et al., 1995), human monocyte chemoattractant protein-1 (MCP-1) (a gift from Dr. T. Yashimura, NCL, Frederick, MD, USA) and pig glyceraldehyde phosphate dehydrogenase (GAPDH). All probes were labeled with a random primer labeling system from Stratagene (La Jolla, CA, USA).

2.8. Nuclear runoff analysis

The runoff assay was carried out essentially as previously described (Stuhlmeier et al., 1994). In short, after changing the medium to serum-free Dulbecco's modified Eagle's medium, pig endothelial cells were incubated with ETYA for 50 min, then exposed to 5 ng/ml TNF for 2 h. Cells were then placed on ice, harvested and lysed in a solution containing 10 mM Tris-HCl (pH 7.4), 10 mM NaHCl, 3 mM $MgCl_2$ and 0.4% Nonidet P-40. The lysates were incubated on ice for 5 min and centrifuged at $300 \times g$ for 5 min at 4°C. The nucleus pellets were resuspended in the above buffer containing 0.025% NP-40. The nuclei were then again collected by centrifugation for 5 min at 4°C and the pellet resuspended in pre-chilled glycerol storage buffer (50% glycerol, 75 mM NaCl, 0.5 mM ethylenediaminetetraacetic acid and 1 mM dithiothreitol). Labeling of transcripts and recovery of transcripts were carried out as described previously (Farrell, 1993). The complementary DNA inserts (2 μg per dot) were bound to Hybond-N membrane. Before hybridization the membrane was pre-hybridized for 1 h at 42°C in a solution containing $5 \times$ SSC, 50% formamide and $4 \times$ Denhardt's solution. Hybridization was carried out for 4 days at 40°C with shaking. After several washing steps the membrane was exposed for one month at −80°C and analyzed on a densitometer.

2.9. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts from porcine and human endothelial cells were prepared as described (Johnson et al., 1995). The double-stranded NF-κB consensus oligonucleotides were end-labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP. The DNA fragment representing the interleukin-8 NF-κB element (Kunsch and Rosen, 1993) (5′-AAT TCA AAT CGT GGA ATT TCC TCT GAC-3′) as well as a mutated form (5′-AAT TCA GAG TGG AAT TTC CTA GAG GAG G-3′) were labeled using Klenow and [α-^{32}P]ATP. After labeling, 5 μg of nuclear extract were incubated with 100 000 cpm of labeled probe in the presence of 3 μg poly(dI-dC) at room temperature for 30 min followed by separation of this mixture on a 6% polyacrylamide gel in Tris/glycine/EDTA buffer at pH 8,5. For supershift assays, 1 μl of the monoclonal anti-NF-κB p56 subunit antibody (Boehringer-Mannheim, Indianapolis, IN, USA) was added to the nuclear extract simultaneously with the labeled probe. Running conditions and additional controls for specificity of binding (data not shown) were performed as published (Stuhlmeier et al., 1994).

2.10. Statistics

Beside the runoff experiment, all experiments were repeated at least three times; representative experiments

are shown. Optical density values for the enzyme-linked immunosorbent assays and cell numbers in the adhesions assay are derived from quadruplicate wells and the resulting standard deviations are shown in the graphs. Asterisks indicate that the upregulation of genes by a given stimuli is significantly inhibited by ETYA and arachidonic acid (*t*-test) (P values ≤ 0.05 are considered to be significant).

3. Results

3.1. ETYA inhibits adhesion molecule up-regulation on endothelial cells

ETYA inhibits the up-regulation of several adhesion molecules on the protein level in a dose-dependent manner. We tested the effect of ETYA as an inhibitor for E-selectin, VCAM-1 and ICAM-1 up-regulation. These molecules play an important role in inflammation and are up-regulated in response to stimulation by TNF, interleukin-1 or lipopolysaccharide (Cotran and Pober, 1987; Gerritsen and Bloor, 1993). We used an enzyme-linked immunosorbent assay to measure the up-regulation of these adhesion molecules following stimulation with 5 ng/ml TNF, 100 ng/ml lipopolysaccharide, 5×10^{-7} M PMA or 5 ng/ml interleukin-1α. These doses have been found to lead to optimal expression of the adhesion molecules tested. Fig. 1A shows that a 30 min pre-incubation with ETYA inhibits the up-regulation of E-selectin in a dose-dependent manner. Similar inhibition is seen when arachidonic acid is used instead of ETYA under otherwise identical conditions (Fig. 1D). Adding 65 μM of ETYA inhibits the TNF-induced E-selectin up-regulation by 95%. Similar results are obtained when lipopolysaccharide (Fig. 1B) or PMA (Fig. 1C) is used as stimulus for E-selectin up-regulation. The inhibition of VCAM-1 and ICAM-1 expression by ETYA was tested using human umbilical

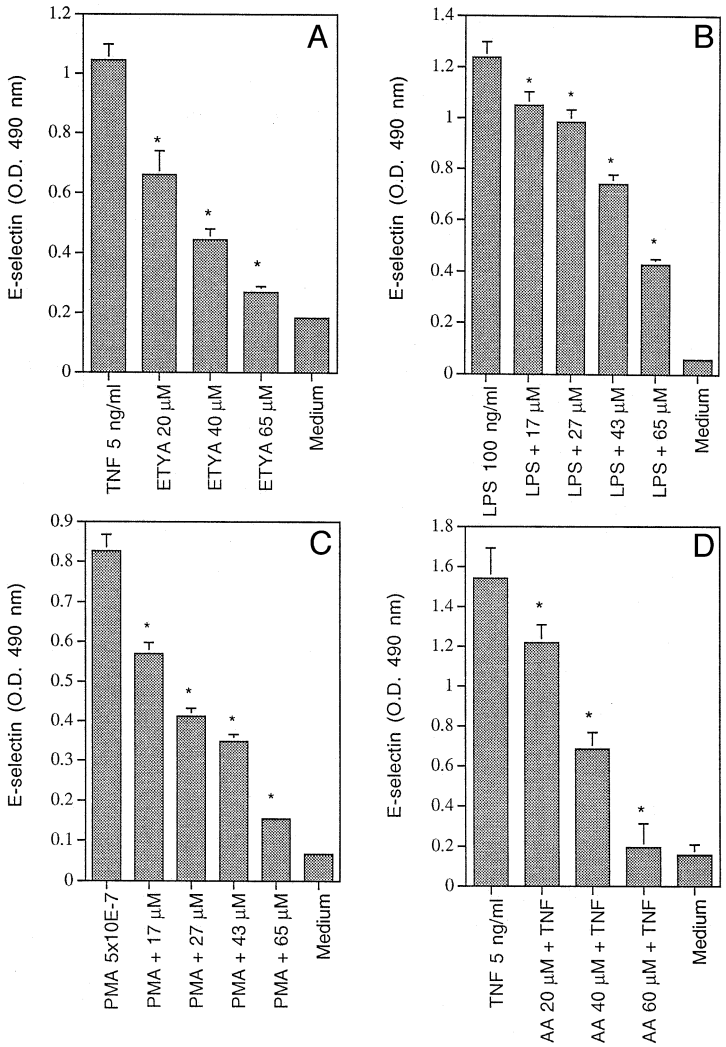


Fig. 1. Inhibition of adhesion molecules up-regulation by ETYA. Pig endothelial cells were pre-incubated with the indicated amounts of ETYA for 30 min followed by 5 ng/ml TNF, 100 ng/ml lipopolysaccharide or PMA (5×10^{-7} M) for 4 h. Increasing amounts of ETYA suppress the up-regulation of E-selectin induced by TNF (A), lipopolysaccharide (B) or PMA (C). In the experiment shown in panel D, arachidonic acid was used instead of ETYA under otherwise identical conditions.

vein endothelial cells. As shown in Fig. 2, pre-incubation of human umbilical vein endothelial cells with 65 μM ETYA leads to 95% lower levels of ICAM-1 expression following stimulation by 5 ng/ml TNF (Fig. 2A); to reduced expression of PMA-induced up-regulation of ICAM-1 (Fig. 2B); and to approximately 80% of inhibition of VCAM-1 up-regulation stimulated with TNF (5 ng/ml) (Fig. 2C). When PMA was used to stimulate human umbilical vein endothelial cells, even stronger inhibition of VCAM-1 was achieved (Fig. 2D). Ethanol, used as a solvent for ETYA at the final concentration of $\leq 0.1\%$ did not influence the up-regulation of the adhesion molecules tested (data not shown).

3.2. The ETYA effect is not due to toxic effects on endothelial cells

Although ETYA has been used in experiments with endothelial cells at higher doses without apparent cytotoxicity, we used two different assays, the (3-4,5-dimethylthizol-2-yl)-2,5-diphenyltetrazolium bromide conversion assay and the lactate-dehydrogenase release assay, to confirm that the inhibition seen in our experiments with ETYA was not due to non-specific damage to endothelial cells. Both viability assays as well as visual inspection under a phase microscope demonstrated the integrity of the endothelial cells pre-treated with ETYA (data not shown).

3.3. ETYA influences the coagulation time of activated endothelial cells

Tissue factor is known to be up-regulated by a variety of inflammatory reagents including TNF, lipopolysaccharide or PMA, as used in our experiments to induce the expression of E-selectin, VCAM-1 and ICAM-1. To gain better insight into the pattern of genes inhibited by ETYA,

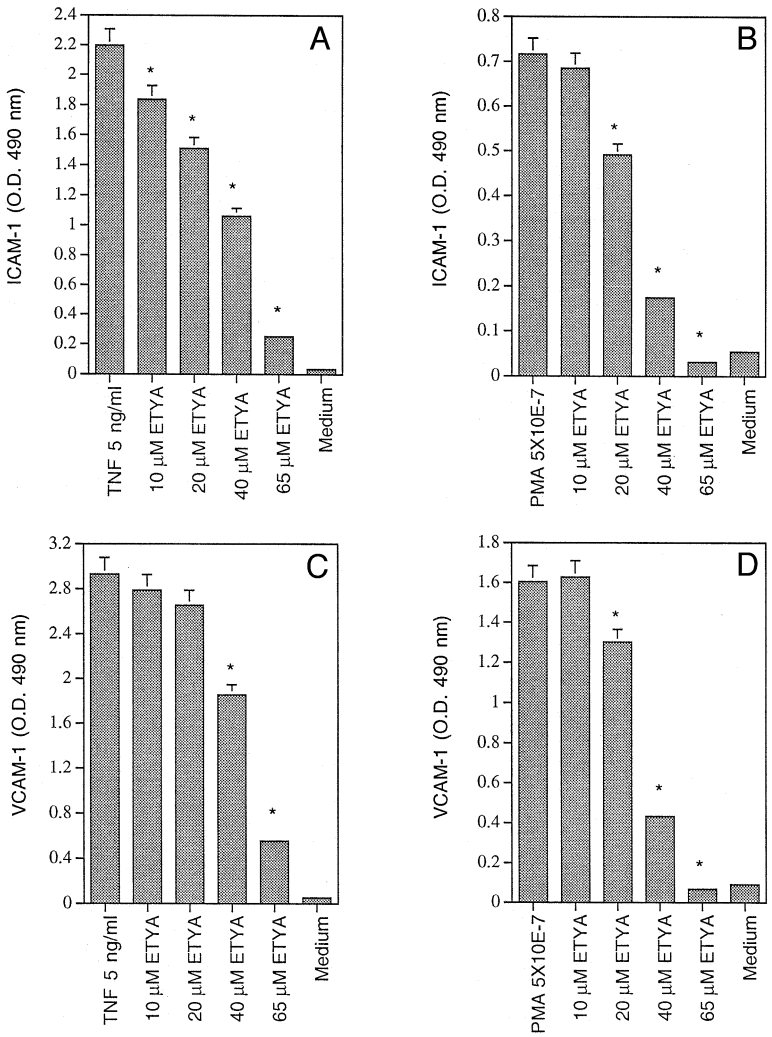


Fig. 2. Pre-incubation with ETYA inhibits the up-regulation of ICAM-1 and VCAM-1 on human umbilical vein endothelial cells. Cells were incubated with the indicated amounts of ETYA and afterwards stimulated with either TNF (A) or PMA (B) for 6 h. With 65 μM ETYA, TNF-induced up-regulation of ICAM-1 expression is suppressed by 95% and PMA-induced ICAM-1 expression by 100%. VCAM-1 expression was measured after 6 h exposure to TNF (C) or PMA (D). Pre-treatment of these cells with 65 μM ETYA leads to 81% inhibition of TNF-induced VCAM-1 expression and to 100% suppression of PMA-induced VCAM-1 up-regulation.

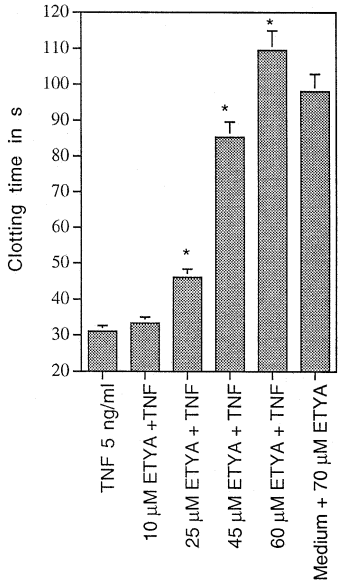


Fig. 3. ETYA influences the TNF-induced clotting time. Pig endothelial cells were pre-incubated with ETYA for 30 min and then with 5 ng/ml TNF for 8 h. Whereas 10 μM ETYA hardly influences the TNF-induced decrease in clotting time, pre-treatment with 60 μM ETYA completely (100%) abolishes the TNF-induced shortened clotting time.

we investigated the effect of varying doses of ETYA for its ability to inhibit the lipopolysaccharide- or TNF-induced pro-coagulation, which we have shown is related to tissue factor up-regulation on endothelial cells (Hofer et al., 1994).

A clotting assay was performed where harvested pig endothelial cells are incubated with plasma. The time it takes to form a clot is generally thought to reflect the amount of tissue factor expressed on the pig endothelial cells, although other factors might influence the clotting time as well. Fig. 3 shows a comparison of clotting times of cells stimulated with TNF alone versus those treated with ETYA prior to TNF stimulation. Cells were exposed to TNF for a total of 8 h. Pre-incubation of pig endothelial cells with ETYA for 30 min abolishes the TNF-induced clotting time in a dose-dependent fashion. In all these experiments, pre-treatment of pig endothelial cells with doses ≥ 50 μM of ETYA completely (100%) abolished the TNF-induced shortened clotting time.

3.4. ETYA inhibits the adhesion of monocytes to activated endothelial cells

Of significance for the practical use of ETYA as an anti-inflammatory reagent is whether ETYA can decrease the number of monocytes that adhere to activated pig endothelial cells. The results of a representative experiment testing this are shown in Fig. 4. Following pre-incubation with various concentrations of ETYA for 30 min, pig endothelial cells were activated for 4 h with TNF (5 ng/ml). Pre-treatment of pig endothelial cells with ETYA diminishes the binding of U937 cells to TNF-activated pig endothelial cells to a very significant degree ($98 \pm 5\%$ at the highest dose used).

3.5. Northern blot analysis reveals that gene suppression by ETYA is selective

To further investigate the inhibition of endothelial cell activation by ETYA, we investigated to what degree the steady-state levels of messenger RNA of various genes are influenced by pre-treatment of pig endothelial cells with ETYA. In a first set of experiments, pig endothelial cell were pre-incubated with 5, 15 and 45 μM ETYA, with appropriate controls, for 45 min. After this pre-incubation time, 5 ng/ml TNF were added for 2 h and total RNA isolated. The results, based on Northern blots, are presented in Fig. 5A. ETYA suppresses the induction of messenger RNA for the adhesion molecules E-selectin, ICAM-1 and VCAM-1, as well as messenger RNA for MCP-1 and to a lesser degree ECI-6. Whereas E-selectin expression is inhibited by nearly 90% at the highest dose (45 μM) used in these experiments, there is a weaker inhibition of PAI-1 expression and hardly any influence on messenger RNA accumulation for the gene encoding interleukin-8.

In a second set of experiments higher doses of ETYA were used to test whether E-selectin messenger RNA

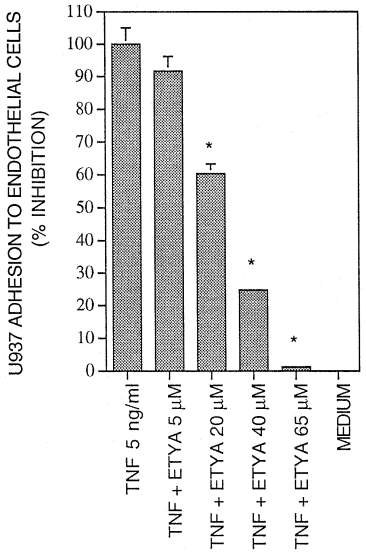
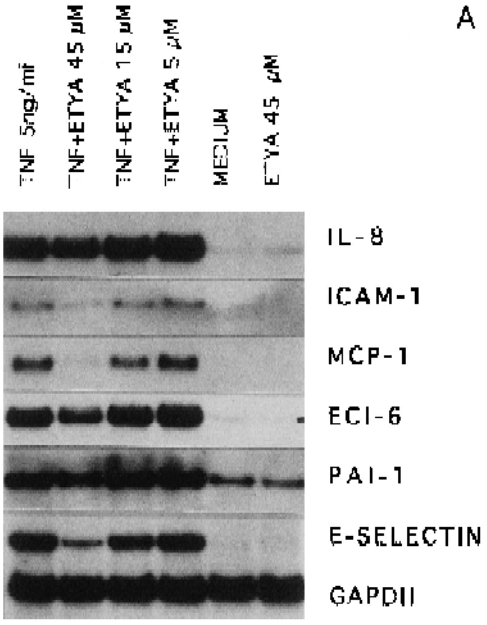


Fig. 4. Pig endothelial cells were treated with increasing amounts of ETYA prior to stimulation with TNF for 4 h. Untreated U937 cells were added and the amount of cells bound to endothelial cells determined. The adhesion to TNF-stimulated endothelial cells was considered to be 100% and cells bound to unstimulated endothelial cells represented zero percent. Pre-treatment of pig endothelial cells with ETYA diminishes the binding of U937 cells to TNF-activated pig endothelial cells to a very significant degree ($98 \pm 5\%$ at the highest dose used).



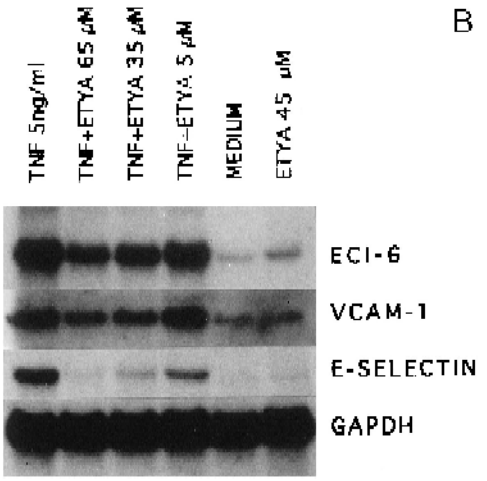


Fig. 5. Northern analysis showing the inhibitory effect of ETYA. Pig endothelial cells were pre-treated with 5, 15 and 45 μM (A) and 5, 35 and 65 μM ETYA (B) for 30 min followed by 5 ng/ml TNF for 2 h. The controls, 5 ng/ml TNF (first line) medium or 45 μM ETYA alone are indicated. The blot was hybridized with complementary DNA for: pig interleukin-8, human ICAM-1, human MCP-1, pig ECI-6, mink PAI-1, pig E-selectin, human VCAM-1 and pig GAPDH. On each lane 20 μg of total RNA was loaded.

accumulation can be completely blocked by ETYA and to see whether higher doses of ETYA might suppress the steady-state messenger RNA levels for ECI-6 even further. Fig. 5B shows the results of these experiments. E-selectin up-regulation is completely blocked, but, despite the higher dose of ETYA used, there is no further suppression of ECI-6 messenger RNA. This figure also shows that messenger RNA accumulation for VCAM-1 is blocked by 54%.

3.6. ETYA inhibits E-selectin up-regulation at the transcriptional level

A runoff assay was performed to further define the role of ETYA in the regulation of E-selectin. Pig endothelial cells were incubated with 45 μM ETYA for 45 min followed by 5 ng/ml TNF for 2 h. The nuclei were harvested and the runoff experiment was performed. The results, shown in Fig. 6A, clearly demonstrate that at least 85% of the inhibition of E-selectin by ETYA is due to inhibition at the transcriptional level. We do not attempt to interpret the results of the runoff assay for other genes tested (PAI-1, interleukin-8), as the assay conditions were not optimized for these genes. Fig. 6B shows a quantification of the runoff assay for E-selectin (dots were measured and quantitated on a densitometer (Molecular Dynamics) and corrected for a housekeeping gene (GAPDH).

In order to gain insight into the underlying mechanism by which ETYA inhibits certain genes but does not influ-

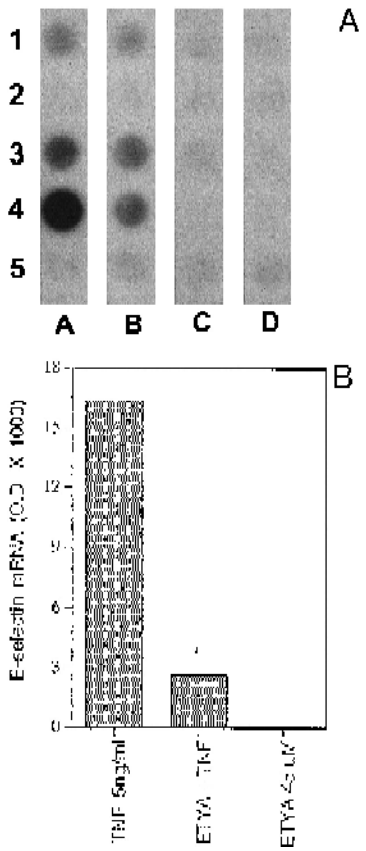


Fig. 6. (Panel A) Nuclear runoff analysis for E-selectin. Complementary DNA for PAI-1 (lane 1), an irrelevant vector (lane 2), interleukin-8 (lane 3), E-selectin (lane 4) and GAPDH (lane 5) were bound to the membrane and probed with RNA isolated from pig endothelial cells treated with 5 ng/ml TNF only (A), cells pre-treated with 45 μM ETYA followed by 5 ng/ml TNF for 2 h (B), 45 μM ETYA only (C) and untreated cells (D). (Panel B) The quantification of the runoff experiment on a densitometer. Dots representing E-selectin messenger RNA were measured and corrected for GAPDH.

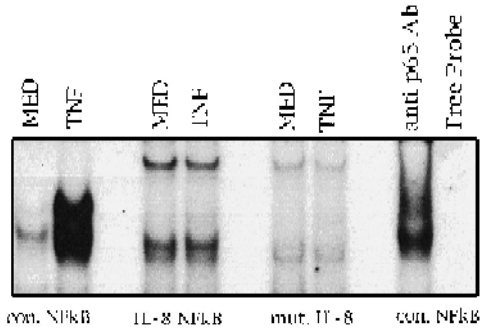


Fig. 7. Shown is an electrophoretic mobility shift assay. Oligonucleotides representing a consensus NF-κB binding site (con. NFκB), as well as one synthesized according to the NF-κB sequence from the interleukin-8 promotor (IL-8 NFκB), and a third oligonucleotide with mutations outside the NF-κB sequence (mut. IL-8), were incubated with nuclear extracts from endothelial cells stimulated for 90 min with TNF (5 ng/ml) or untreated cells. The right panel labeled 'anti p65 Ab' and 'Free Probe' indicates the position of the p65 bound to the consensus NF-κB element and demonstrates that the bands originate from proteins in the nuclear extract.

ence others, we chose to investigate the binding of the transcription factor NF-κB on a sequence in the interleukin-8 promoter considered to represent a NF-κB binding site (Mukaida et al., 1990). Fig. 7 shows the result of an electrophoretic mobility shift assay. Oligonucleotides representing a consensus NF-κB binding site (con. NF-κB), as well as one synthesized according to a published sequence from the interleukin-8 promoter (IL-8 NF-κB), and a third oligonucleotide with mutations outside the NF-κB sequence (mut. IL-8), were incubated with nuclear extract from endothelial cells left unstimulated or stimulated for 90 min with TNF (5 ng/ml). The results demonstrate that stimulation of endothelial cells with TNF leads to strong binding of NF-κB to the consensus NF-κB binding site, but that binding of transcription factors to the IL-8 NF-κB binding site is not altered by TNF treatment; moreover, mutations of the IL-8 sequence outside the so-called IL-8 NF-κB element lead to even lower levels of bound protein. Interestingly, the binding of p65, the subunit of NF-κB, to the IL-8 NF-κB element is not detectable whereas high amounts of this protein bind to the consensus NF-κB element. The right panel labeled 'anti p65 Ab' and 'Free Probe' shows two controls. The position of p65 is indicated by the shifted upper band in the control panel where nuclear extract of endothelial cells was incubated with the consensus NF-κB element in the presence of an anti-p65 antibody.

4. Discussion

Several studies have demonstrated that treatment with polyunsaturated fatty acids prolongs the survival of skin and heart grafts (Kort et al., 1979; MacHugh et al., 1977; Mertin, 1976; Mertin and Hunt, 1976; Perez et al., 1987; Ring et al., 1974; Storm and Carpenter, 1983) and that diets rich in polyunsaturated fatty acids can be employed as adjuncts to conventional immunosuppressive therapy to reduce rejection of human kidney grafts. However, with the advent of modern immunomodulatory drugs like cyclosporin A, interest in the beneficial effects of polyunsaturated fatty acids in transplantation has mostly been lost. We were interested in its effects on a cellular level. We have shown in these studies that the synthetic polyunsaturated fatty acid, ETYA, selectively inhibits the up-regulation of genes in endothelial cells.

As endothelial cells play a critical role in inflammation and organ rejection, we were interested whether polyunsaturated fatty acids might be helpful to control endothelial cell activation. We found earlier (Stuhlmeier et al., 1996a) that arachidonic acid itself might selectively prevent up-regulation of endothelial cell adhesion molecules in response to stimuli like TNF, interleukin-1, lipopolysaccharide or PMA. The obvious disadvantage of using arachidonic acid in investigating the potential of polyunsaturated fatty acids as an immunosuppressive is its rapid conversion into a variety of metabolites (prostaglandins, thromboxanes, lipoxins, leukotrienes) with diverse functions (Kunkel et al., 1986a,b; Lewis et al., 1983; Samuelsson, 1991). This also excludes arachidonic acid from practical use, as several of the metabolites have been shown to be involved in cell activation. ETYA, a synthetic analog of arachidonic acid and a potent inhibitor of lipoxygenase, cyclooxygenase and cytochrome P-450 epoxygenase, seems ideally suited as an agent that is not converted to these metabolites.

In this paper, we demonstrate that ETYA inhibits endothelial cell activation by preventing the induction of a number of genes, the products of which are key participants in inflammation and thrombosis (Gerritsen and Bloor, 1993). Genes that are suppressed by ETYA include the adhesion molecules E-selectin, ICAM-1 and VCAM-1, as well as other genes such as MCP-1, PAI-1 and ECI-6. The inhibition seen with the highest doses tested varied from total inhibition of E-selectin and MCP-1, to almost no suppression of the cytokine interleukin-8. ETYA also inhibits the pro-thrombotic response of endothelial cell to activating stimuli, presumably by preventing the induction of tissue factor. This pattern of inhibition, and the fact that there is no suppression of the transcription of a housekeeping gene (GAPDH), make the inhibition specific.

Why we observed differences in the pattern of inhibition between ETYA and arachidonic acid is not yet clear. Several explanations are possible. Arachidonic acid has an extreme short half-life whereas ETYA is rather stable to oxidation (Tobias and Hamilton, 1979). Addition of ETYA to cells does not result in conversion to the known arachidonic acid metabolites, whereas exogenous arachidonic acid can lead to metabolites known to be involved in gene up-regulation as well as in gene suppression. Neither changes in cell membrane fluidity, as has been reported for

several polyunsaturated fatty acids (Brown et al., 1992), nor direct effects upon membrane-associated receptors, seem to be responsible for the inhibition seen. TNF induced E-selectin up-regulation is completely blocked; however, the same stimulus, in the presence of ETYA, still leads to normal levels of interleukin-8 expression. The finding that ETYA inhibits the same genes when PMA, which functions by interacting directly with protein kinase C isomers (Heller and Kronke, 1994), is used to activate the endothelial cells is also consistent with the suggestion that ETYA does not act by modifying receptor function.

The role for arachidonic acid as a secondary messenger is now well established (Blobe et al., 1995; Khan et al., 1995; Vanderzee et al., 1995). Whether ETYA can mimic arachidonic acid as a second messenger has yet to be tested, as does what signaling pathways are activated by ETYA. Elevated cyclic adenosine monophosphate level were shown to be involved in inhibition of E-selectin (Pober et al., 1993) as well as other genes. Although we did not test the effect of ETYA in this regard, it is interesting that others have reported that ETYA does not induce elevated cyclic adenosine monophosphate levels whereas arachidonic acid does (Long and Pekala, 1996).

It has been shown that polyunsaturated fatty acids and ETYA can influence mitochondrial respiration and oxidative phosphorylation (Arsian et al., 1984; Ingraham et al., 1982). That such rather general effects are involved in gene suppression demonstrated here is unlikely. Several lines of evidence support the conclusion that ETYA functions by inhibiting at the transcriptional level. Using E-selectin as a marker gene, we found that all tested stimuli leading to up-regulation of E-selectin are affected by ETYA treatment. This excludes interruption or interference at a very early step in the signal transduction pathway as an explanation for gene suppression by ETYA. On the other hand, our runoff experiments for E-selectin demonstrated that this gene is suppressed at the transcriptional level, making it likely that ETYA interferes at the point were the signals of such diverse stimuli as PMA, lipopolysaccharide and TNF converge.

Transcriptional up-regulation of pro-inflammatory genes involved in endothelial cell activation is strongly dependent on activation of NF-κB (Collins et al., 1995). The NF-κB/IκBα system has been shown to have near-universal impact on the transcriptional regulation of many proinflammatory genes (Leonard and Baltimore, 1989). We demonstrated that arachidonic acid and ETYA inhibit the translocation of NF-κB to the nucleus (Stuhlmeier et al., 1996b; and data not shown). These findings offer yet one other possible explanation for the differential effect seen with ETYA, namely that genes which are stronger NF-κB dependent are stronger suppressed as well, whereas genes like interleukin-8, whose NF-κB binding site in the promoter seems to bind a different set of proteins (Fig. 7) (compared to genes with the consensus NF-κB element), are less influenced. Although there is also speculation on the existence of a so-called 'arachidonic acid-responsive element' in the promoter region of genes (Tebbey and Buttke, 1992) which might be involved in gene regulation, their existence has yet to be established. Although it is appealing to speculate about similar mechanisms involved in ETYA-induced gene suppression, further investigations are required.

Interestingly, others studying the effect of polyunsaturated fatty acids (De Caterine et al., 1994; Weber et al., 1995), including arachidonic acid on endothelial cell activation, reported no effects of ω−6 fatty acids. Out of several polyunsaturated fatty acids tested the only fatty acid demonstrating moderate inhibition of endothelial cell activation was docosahexaenoic acid – an ω−3 fatty acid. One possible explanation for the apparent discrepancy in these studies might be that we, keeping the short biological half-life (several minutes) of unsaturated fatty acids in mind, pre-incubated endothelial cells for only 10−60 min, whereas in the studies mentioned above endothelial cells were incubated for 24−72 h prior to stimulation.

We have shown that ETYA inhibits several genes associated with endothelial cell activation. These findings might help to explain the beneficial effect of this substance as well as of other polyunsaturated fatty acids as antipyretic and antiinflammatory drugs. Several studies have shown that ETYA is not grossly toxic (which was also demonstrated in clinical studies (Johnson et al., 1995) where ETYA in doses of up to 1000 mg/day/person was administered for the study period of one year). It would be of interest to see whether ETYA can exert antiinflammatory effects in vivo as well. Furthermore, ETYA seems to be a valuable new tool to study endothelial cell gene regulation and signaling pathways in endothelial cells by polyunsaturated fatty acids, arachidonic acid and other substances, as the pattern of inhibition seen differs substantially from the observations made in former studies (Kapiotis et al., 1991; Pober et al., 1993; Vannier et al., 1992).

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