Stabilizing the Inhibitor-

Arachidonic acid (AA), through its myriad metabolites, is involved in inflammation in a number of ways. AA is produced and released by several cell types, including endothelial cells (EC), and acts on a variety of cells. EC activation plays a key role in inflammation presumably by modulating the immune response through up- or down-regulation of several genes. We have previously shown that AA and its nonmetabolizable analogue, 5,8,11,14-eicosatetraynoic acid (ETYA), inhibit up-regulation of proinflammatory genes in EC. In the present study we identify a mechanism to explain the inhibitory effects: AA and ETYA both inhibit the translocation of nuclear factor-κB (NF-κB) to the nucleus by blocking the degradation of the inhibitor of NF-κB (IκB) and thus stabilizing the IκB/NF-κB complex. To investigate the mechanism whereby AA inhibits up-regulation of genes encoding proinflammatory mediators, we examined the ability of ETYA to inhibit tumor necrosis factor-α (TNF-α) mediated phosphorylation and degradation of IκBα. Western blot analysis revealed that preincubation of EC with ETYA for 40 min prior to stimulation with TNF-α inhibits the phosphorylation and degradation of IκBα. These findings establish a mechanism by which AA inhibits nuclear translocation of NF-κB and thereby explains its modulatory role in the induction of proinflammatory genes.

Studies of arachidonic acid (AA) have focused primarily on metabolites of AA and their role in inflammation, including thrombosis and clinical disorders such as asthma. While AA metabolites can have potent proinflammatory effects, prostacyclin and certain AA metabolites have also been shown to have antiinflammatory actions. LITTLE is reported on the effects mediated by AA itself. Endothelial cells (EC), which both release AA and serve as the possible targets of AA released by EC and other cells, play a prominent role in inflammation and hemostasis and organ rejection. The activation of EC results in the up-regulation in EC of genes encoding adhesion molecules, procoagulant factors, cytokines and other molecules, which can participate in the inflammatory-thrombotic responses characterizing a number of conditions including graft rejection. Stimulated by reports demonstrating a significant improvement in graft survival of animals treated with linoleic acid and other precursors of AA, we have previously demonstrated (1) that AA itself can inhibit the up-regulation in EC of several key genes/molecules involved in inflammation and rejection, including E-selectin, ICAM-1, and IL-8. Furthermore, using E-selectin as a prototype gene, we showed that inhibition is at the transcriptional level. Transcriptional up-regulation of proinflammatory genes involved in EC activation is strongly dependent on activation of NF-κB, which involves phosphorylation and degradation of IκB leading to translocation of the NF-κB heterodimer to the nucleus. The NF-κB/IκBα system has been shown to exert transcriptional regulation on proinflammatory genes involved in EC activation (2). Most genes encoding adhesion molecules, cytokines, and other proinflammatory genes have functional NF-κB binding elements in their promoter regions. In the cytoplasm of quiescent EC, IκBα forms a complex with NF-κB and thereby prevents migration of NF-κB into the nucleus (3). One mechanism by which an agent can inhibit NF-κB activation is by preventing the phosphorylation and thus degradation of IκBα, referred to as "IκB stabilization." We show in this paper that the inhibitory effect of AA on EC activation is due to interference at this level of transcriptional regulation.

**EXPERIMENTAL PROCEDURES**

Preparation of Porcine Aortic Endothelial Cells—Porcine EC were isolated and passaged in our laboratory as described previously (4). Briefly, EC were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 4.5 g/liter glucose and supplemented with 10% heat-inactivated fetal bovine serum (Hyclone), L-glutamine and 50 units/ml penicillin/streptomycin. Experiments were performed in DMEM under serum-free conditions. Before incubating EC with polyunsaturated fatty acids (PUFA), cells were washed three times with prewarmed serum-free DMEM. Tumor necrosis factor-α (TNF) was diluted in serum-free medium and added to the EC; in cases where lipopolysaccharide (LPS) was used as a stimulus, fetal calf serum was added simultaneously with LPS to a final concentration of 5%. Human umbilical vein endothelial cells were a gift from Dr. R. Ewenstein, Brigham Women’s Hospital, Boston, MA. Pig EC from passages 3 to 7 were used in these experiments.

Stimulants and Reagents—The following reagents were purchased from Sigma: leupeptin, antipain, aprotinin, benazemide, chymostatin, pepstatin, TLCK, TPKC, phenylmethylsulfonyl fluoride, LPS serotype 055:B5, phorbol 12-myristate 13-acetate, indomethacin, arachidonic acid, and glib switched. Radiolabeled arachidonic acid, [5,6,8,9,11,12,14,15-3H]arachidonic acid, was purchased from NEN Life Science Prod-ucts. 6,9,12-Octadecatrienoic acid, dihomo-y-linoleic acid (8,11,14-eico-satrienoic acid), oleic acid (9-octadecenoic acid), 5,8,11,14,17-eicosapentaenoic acid, 4,7,10,13,16,19-docosahexaenoic acid, mead acid...
Electrophoretic Mobility Shift Assay—Nuclear extracts from porcine and human EC were prepared as described (5). The double-stranded oligonucleotides used in all experiments were end-labeled using T4 polynucleotide kinase and \( \gamma ^{32} P \)ATP. After labeling, 5 \( \mu g \) of nuclear extract was incubated with 100,000 cpm of labeled probe in the presence of 3 \( \mu g \) of poly(dI-dC) at room temperature for 30 min followed by separation of this mixture on a 6% polyacrylamide gel in Tris/glycine/EEDA buffer at pH 8.5. For specific competition, 7 \( \mu l \) of unlabeled NF-\( \kappa B \) oligonucleotides was included; and for nonspecific competition, 7 \( \mu l \) of the double-stranded mutant \( \kappa B \) oligonucleotides 5'-AGCTTA-GATTTTACTTTCCGGAGGA-3' and 7 \( \mu l \) of pig E-selectin CRE-like were used. For supershift assays, 1 \( \mu l \) of the monoclonal anti-NF-\( \kappa B \) antibody (Boehringer Mannheim) was added to the nuclear extract simultaneously with the labeled probe.

Western Blot Analysis—Cytosolic extracts were prepared as described (5) except that further protease inhibitors, TPCK, TLCK, apro tinin, leupeptin, antipain, apro tin, benzamidine, chymostatin, and pepstatin, were added. Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis (12%), transferred to an Immobilon-P polyvinylidene difluoride membrane using a semi-dry transfer cell (Bio-Rad) and probed with the rabbit polyclonal antibody C21 (Santa Cruz, Biotechnology) directed against I\( \kappa B \)A subunit antibody (Boehringer Mannheim) was added to the nuclear extract simultaneously with the labeled probe.

RESULTS AND DISCUSSION

We reported earlier (1) that AA and a nonmetabolizable AA analog, ETYA, inhibit the expression of several proinflammatory genes in EC. We studied E-selectin as an example of these genes and demonstrated by run-off analysis that the up-regulation of this gene is blocked at the transcriptional level. We studied E-selectin as an example of these genes and demonstrated by run-off analysis that the up-regulation of this gene is blocked at the transcriptional level. We now report that this inhibitory effect of AA and ETYA in EC is based on the stabilization of I\( \kappa B \)α by these 20:4 fatty acids, therefore blocking nuclear translocation of NF-\( \kappa B \), a transcription factor known to be essential for the up-regulation of several genes that characterize EC activation (6). Fig. 1 represents an electrophoretic EMSA, showing a time course of TNF-induced NF-\( \kappa B \) translocation. EC were stimulated with TNF for 0, 10, 30, 60, and 120 min with or without pre-treatment with 55 \( \mu M \) ETYA. After 10 min stimulation of EC with TNF, NF-\( \kappa B \) is present in the nuclear extract, and its level increases with time. Precoculation of EC with ETYA for 30 min completely blocks the TNF-induced translocation of NF-\( \kappa B \) into the nucleus. Incubation of EC with 55 \( \mu M \) ETYA only (for 0, 15, 30, or 45 min) did not lead to activation of NF-\( \kappa B \) (first four lanes).

Exogenous AA also suppresses TNF-induced NF-\( \kappa B \) translocation. Stimulation of EC with 5 \( ng/ml \) TNF for 60 min leads to strong activation of NF-\( \kappa B \) (Fig. 2, lane TNF 65 \( ng \)). When these cells were incubated with 35 \( (lane \ AA \ 35 \ + \ TNF) \) or 65 \( \mu M \) AA (lane AA 65 + TNF) for 45 min prior to stimulation with 5 \( ng/ml \) of TNF, the TNF effect on NF-\( \kappa B \) translocation was completely abolished. Incubation of EC with increasing amounts of AA, without blocking the enzymes involved in the metabolism of AA, leads to a weak but significant and dose-dependent augmentation of the levels of NF-\( \kappa B \) found in the nucleus. Lanes AA 35 and AA 65 in Fig. 2 show the results of an experiment in which EC were incubated for 105 min with 35 or 65 \( \mu M \) AA before the nuclear proteins were extracted and an EMSA was carried out. Incubating EC with 35 \( \mu M \) exogenous AA (lane AA 35) results in weak activation of NF-\( \kappa B \); increasing the amount of AA to 65 \( \mu M \) (lane AA 65) results in a stronger translocation of NF-\( \kappa B \) into the nucleus. These results are not surprising as several of the AA metabolites are known to be involved in cell activation (7). In this and similar experiments on human umbilical vein cells (data not shown), 35 \( \mu M \) AA consistently demonstrated a stronger net inhibitory effect on NF-\( \kappa B \) activation. Taken together, these data demonstrate that AA can inhibit the TNF-induced activation of NF-\( \kappa B \) and might explain the phenomenon, seen in other experiments, that higher doses of AA do not lead to a correspondingly greater inhibition of TNF-induced gene up-regulation, as higher amounts of AA result in increasing amounts of AA metabolites that activate NF-\( \kappa B \). Thus the AA effect on NF-\( \kappa B \) activation reflects the net effect of inhibition and activation. The hypothesis that some AA metabolites might be responsible for activation, but that AA itself inhibits NF-\( \kappa B \) activation, is further supported by the fact that the stable and nonmetabolizable AA analog, ETYA, does not activate NF-\( \kappa B \).

Also shown in Fig. 2 are the results of an experiment in which two doses of ETYA (35 and 65 \( \mu M \)) were added to EC 45 min before stimulating these EC with either 5 \( ng/ml \) TNF (lanes E 35 + TNF and E 65 + TNF) or medium alone (lanes E 35 and E 65) for 1 h. These experiments demonstrate that 35 \( \mu M \) of ETYA is sufficient to completely block the TNF-induced NF-\( \kappa B \) activation and that neither 35 \( \mu M \) nor 65 \( \mu M \) of ETYA...
Arachidonic Acid Inhibits NF-κB Translocation

**Fig. 2.** Exogenous AA suppresses TNF-induced NF-κB translocation. Stimulation of EC with 5 ng/ml TNF for 60 min leads to strong activation of NF-κB (lane TNF 5 ng). When these cells were incubated with 35 or 65 μM AA for 45 min prior to stimulation with 5 ng/ml of TNF, the TNF effect on NF-κB translocation was completely abolished. Incubation of EC with increasing amounts of AA for 105 min and without blocking AA metabolism leads to significant translocation of NF-κB into the nucleus (lanes AA 35 and AA 65). Neither 35 μM (lane E 35) nor 65 μM (lane E 65) of the nonmetabolizable AA analog ETYA leads to translocation of NF-κB. Both doses of ETYA completely blocked the TNF-induced NF-κB transmigration (lanes E 35 + TNF and E 65 + TNF).

**Fig. 3.** EMSA demonstrating that the AA precursor γ-linolenic acid suppresses the TNF-induced translocation of NF-κB. A comparison of the inhibitory effect of ETYA and γ-linolenic acid is shown. Although the inhibition by γ-linolenic acid is less pronounced than the one seen with ETYA (lane ETYA 105'), there is nevertheless significantly less NF-κB in the nuclear extract as compared with cells treated with the same amount of TNF only. ETYA treatment of EC for 15, 45, 75, or 285 min does not induce NF-κB translocation.

We reported earlier (1) that the precursors of AA, linoleic acid and γ-linolenic acid inhibited the TNF, LPS, or phorbol 12-myristate 13-acetate-induced up-regulation of several genes although, compared with AA or ETYA, higher doses of these unsaturated fatty acids had to be used to get comparable inhibition. We tested whether the inhibitory effect of the AA precursor, γ-linolenic acid, is due to the same mechanism as that seen with AA. Fig. 3 shows the effect of γ-linolenic acid on TNF-induced activation of NF-κB. Compared with TNF-induced NF-κB activation (lane: TNF 5 ng), pre-incubation of EC with γ-linolenic acid (100 μM) for 45 min leads to significant inhibition of NF-κB translocation when these pre-treated cells are stimulated with 5 ng/ml TNF (lane γ-LIN + TNF) for 1 h. Despite the inhibitory effect of γ-linolenic acid on TNF-induced activation of NF-κB, adding this polyunsaturated fatty acid alone to EC leads to a weak up-regulation of NF-κB (lane γ-LIN) compared with control cells (lane MED), which is consistent with the findings of others (8). Whether further metabolism of linoleic acid and γ-linolenic acid to AA is necessary to exert the inhibitory effect, or whether these reagents are inhibitory by themselves, is not yet clear as specific inhibitors for desaturase and elongase are not available. Again, treatment of EC for up to 285 min with ETYA (60 μM) only does not lead to activation of NF-κB (lanes ETYA 15' ETYA 285').

The main objective of this work has been to expand our insight into the surprising effect of AA on gene regulation in endothelial cells as reported earlier (1). Nevertheless, to gain a better understanding of possible effects that polyunsaturated fatty acids might play in the regulation of inflammatory responses, we tested a panel of saturated, mono-, and polyunsaturated fatty acids for their effectiveness as inhibitors of NF-κB.

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activation by TNF, prostaglandin or lipoxigenase metabolites play essentially no role. In accordance with our conclusions are the results of experiments (data not shown) where NS-398, a potent inhibitor of cyclooxygenase (14) was used without any significant effect on TNF (5 ng/ml) induced NF-κB activation. Similarly, treating EC with indomethacin (10⁻⁴ and 10⁻⁵ M, respectively) prior to stimulation with TNF has no influence on TNF (5 ng/ml) induced NF-κB translocation. The polyunsaturated fatty acid, 8,11-eicosadiynoic acid is another nontoxic acetylenic fatty acid that has been shown to inhibit eicosanoid biosynthesis at several stages (15). This unsaturated fatty acid failed to influence TNF-induced NF-κB up-regulation in our experiments on EC. Lastly, we tested 9,12-octadecadiynoic acid a polyunsaturated fatty acid and irreversible inhibitor of cyclooxygenase and lipoxigenase, which is, compared on a mol to mol ratio, a stronger inhibitor of cyclooxygenase than ETYA (16, 17). Our data indicate that incubation of EC with 10, 25, and 50 μM of 9,12-octadecadiynoic acid prior to stimulation with TNF (5 ng/ml) for 1.5 h had significantly less suppressive effect on the TNF-induced NF-κB activation than treatment with similar amounts of the “weaker” cyclooxygenase and lipoxygenase inhibitor ETYA (data not shown). These data support the concept that it is indeed the structural similarities between AA and ETYA that are responsible for the similarities seen on NF-κB inhibition and that AA metabolites are not involved in the events leading to AA/ETYA induced suppression on TNF-activated NF-κB translocation.

Our working concept is based on the hypothesis that upon cell activation free AA is released intra- or extracellularly to keep inflammatory events localized or as a feedback mechanism to control the extent of inflammatory responses. For these reasons, the amount of free AA has to be tightly controlled. One method commonly used in experiments involving AA and other fatty acids is the binding of these compounds to carrier proteins such as albumin, where such binding seems to render AA inactive, as experiments performed in the presence of albumin demonstrated little or no influence on TNF-induced E-selectin up-regulation, a gene which has been shown to be NF-κB dependent (data not shown). Due to their highly hydrophobic properties, fatty acids are difficult to keep evenly imersed in cell culture solutions. Binding of fatty acids to albumin facilitates the dispersion of AA in culture medium and therefore minimizes the loss due to binding of fatty acids to tubes as well as tissue culture material. For the above mentioned reason, this method was not appropriate in our experiments. To better understand the amount of AA being taken up by EC in our experiments performed under serum-free conditions, EC were incubated with 60 μM AA pulsed with ³H-AA for 45 min. Subsequently, the amount of ³H-AA associated with EC was compared with ³H-AA in culture medium. In these experiments, an equivalent of 9 μM (15 ± 4%) of the added 60 μM AA was bound to EC.

An important mechanism in controlling NF-κB activation is provided by a protein-protein interaction involving members of the IκB family with NF-κB. As demonstrated by others (18), IκB binds to and forms a complex with the subunits (p65 and p50) of NF-κB, thereby inhibiting transmigration of NF-κB into the nucleus. Upon stimulation of cells with a wide array of reagents including TNF, IκB is phosphorylated (19), ubiquitinated (20) and subsequently degraded. We tested the hypothesis that AA might influence the activation/translocation of NF-κB by preventing the degradation of IκB, which would result in stabilization of the IκB-NF-κB complex. Fig. 4 shows the result of a Western blot, where cytoplasmic extracts of porcine EC were separated on a 12% polyacrylamide gel, transferred to a polyvinylidene difluoride membrane and stained with an anti-IκB antibody as described under “Experimental Procedures.” As shown in this figure and demonstrated by others (21), stimulation of EC with 5 ng/ml TNF leads to rapid proteolysis of IκBα, as virtually all the IκBα present in the cytoplasm is degraded within 10 min. In contrast to the nearly nondetectable levels of IκBα in the cytoplasm of cells stimulated with TNF only for 10 and 30 min (Fig. 4A, lanes TNF 10’ and TNF 30’) PAEC pre-treated with 55 μM ETYA for 30 min prior to addition of TNF for 10 or 30 min (Fig. 4A, lanes ETYA + TNF 10’ and E + TNF 30’), show no changes in the amount of IκBα present in the cytoplasm. We achieved identical results when we used human umbilical vein endothelial cell instead of porcine EC to study the effect of ETYA on IκBα stabilization (data not shown).

The first step leading to IκBα degradation requires phosphorylation of IκBα at serines 32 and 36 (22). The inducible phosphorylation of IκBα is readily detected in Western blots as a slight decrease in protein mobility as demonstrated by us (23) and others (24, 25). As shown in Fig. 4A, treatment of EC with ETYA (ETYA 30’) only, or with ETYA followed by TNF (E + TNF 10’), did not lead to changes in the mobility of IκBα. Gliotoxin has been shown to allow IκBα phosphorylation but prevents its degradation (24). We performed identical control experiments using EC pretreated with gliotoxin (1 μg/ml) followed by okadac acid (OA) (0.5 or 1 μM, respectively). The results of an experiment, shown in Fig. 4B, are consistent with the hypothesis that ETYA prevents the phosphorylation of IκBα and therefore inhibits IκBα degradation leading to the inhibition of NF-κB translocation.

The controls for the EMSA are shown in Fig. 5. In the first lane (M), nuclear extracts of nonstimulated cells were separated and compared with nuclear extracts isolated from cells treated with 5 ng/ml TNF (lanes 2–5) for 1 h. The specificity of binding to the consensus NF-κB element is demonstrated by the inability of unlabeled NF-κB (cold) and the failure of a mutated NF-κB element (mut) and a consensus CRE-element (CRE) to bind competitively to NF-κB. The last line in this figure (p65) demonstrates the presence of p65 in the complex bound to the NF-κB element in induced EC. When a monoclonal anti-p65

![Fig. 4. A](image-url)
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In summary, we have demonstrated that AA, independent of its metabolites, can play a key role in EC gene regulation. AA stabilizes the IκB/NF-κB complex and therefore suppresses NF-κB translocation; given the key role of NF-κB in the up-regulation of proinflammatory genes with EC activation, this results in inhibition of gene induction. Despite the large amount of data collected on AA and its metabolites, the relationship of AA uptake and AA release to the production of metabolic compounds and their subsequent biological impact still remains obscure (26).

In the past, AA was merely seen as a precursor for bioactive eicosanoids. More recently it has emerged that AA itself can influence cellular communication systems at several levels (8, 27–31). Our findings strongly support and help to further establish the critical role of AA in intracellular signaling. Our results also bolster the development and use of AA analogs such as ETYA as antiinflammatory drugs. Whether our findings might provide further insight into the antiinflammatory mechanisms of actions of drugs such as aspirin, ibuprofen, and others, remains a matter of speculation. For a long time, aspirin, the most widely used nonsteroidal antiinflammatory drug, has been thought to act solely through inhibition of prostaglandins and thromboxanes. Only recently has it become clear that aspirin can block the translocation of NF-κB, the key transcription factor in the activation of most proinflammatory genes (14). It seems likely, however, that these drugs, which block either cyclooxygenase, lipoxygenase, or both, may counteract inflammation by increasing intracellular levels of free AA, subsequently suppressing NF-κB. This hypothesis, supported by our data, would offer yet another appealing explanation for the beneficial effect and the “mode of action” of these compounds.

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FIG. 5. Control experiments demonstrating the specificity of the EMSA for NF-κB. The second through fifth lanes show nuclear extracts isolated from EC treated with 5 ng/ml TNF for 1 h. The second lane shows nuclear extract incubated with the consensus NF-κB element only; third lane, extract incubated with the labeled consensus element plus unlabeled (COLD) NF-κB consensus element. Fourth lane represents extract incubated with the consensus plus a mutated NF-κB element, and fifth lane represents extract incubated with the consensus NF-κB and an unlabeled CRE element. The last lane, labeled p65, demonstrates the presence of p65 in the complex bound to the NF-κB element in induced EC. A monoclonal anti-p65 antibody incubated with the nuclear extract blocked the formation of the upper, slower migrating complex. In the first lane, nuclear extract of noninduced cells was loaded.

antibody was incubated with the nuclear extract, it blocked the formation of the upper, slower migrating complex.

In summary, we have demonstrated that AA, independent of its metabolites, can play a key role in EC gene regulation. AA stabilizes the IκB/NF-κB complex and therefore suppresses NF-κB translocation; given the key role of NF-κB in the up-regulation of proinflammatory genes with EC activation, this results in inhibition of gene induction. Despite the large amount of data collected on AA and its metabolites, the relationship of AA uptake and AA release to the production of metabolic compounds and their subsequent biological impact still remains obscure (26).