Eicosapentaenoic Acid Protects Endothelial Cells Against Anoikis Through Restoration of cFLIP

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Abstract—Dietary supplementation with eicosapentaenoic acid (EPA) improves the prognosis of chronic inflammatory diseases, including atherosclerosis. The mechanism underlying these beneficial effects, however, remains to be elucidated. Here we show that EPA protects endothelial cells from anoikis through upregulation of the cellular FLICE (Fas-associating protein with death domain-like interleukin-1–converting enzyme)-inhibitory protein (cFLIP), an endogenous inhibitor of caspase-8. EPA-induced upregulation of cFLIP expression was partially suppressed by the phosphatidylinositol-3-kinase inhibitor wortmannin. Conversely, treatment with insulinlike growth factor-1 (IGF-1), an activator of phosphatidylinositol-3-kinase/Akt signaling, or infection with an adenoviral construct expressing the constitutively active Akt gene induced upregulation of cFLIP expression. In addition, pretreatment of endothelial cells with either EPA or IGF-1 protected them from anoikis, suggesting that EPA-induced protection against anoikis is partially mediated through activation of Akt. On the other hand, when endothelial cells were already detached, treatment of these cells with EPA but not with IGF-1 protected them against anoikis. Importantly, EPA restored cFLIP expression without activating Akt signaling in detached endothelial cells, whereas IGF-1 had no effect. Additionally, exogenously restored expression of cFLIP by the tetracycline-regulated adenovirus system protected endothelial cells against anoikis. Furthermore, EPA was protective against the loss of endothelium in an organ culture of rat aortas. These findings suggest that EPA protects against endothelial cell anoikis through restoration of cFLIP expression, which might contribute to the mechanism underlying the beneficial effects of EPA in patients with hypertension. (Hypertension. 2003;42:342-348.)

Key Words: fish oils • endothelium • apoptosis • anoikis • tumor necrosis factor • protein kinase

Apoptosis is an important physiologic mechanism for eliminating damaged or unwanted cells. However, unregulated and excessive apoptosis of normal cells has been implicated in many human diseases. Several lines of evidence suggest that loss of endothelial cells (ECs) by apoptosis might result in the endothelial dysfunction that is known to contribute to the development of atherosclerosis, inflammation, and end-organ damage in patients with hypertension. Notably, atherogenic factors such as oxidative stress and inflammatory cytokines induce EC apoptosis, whereas antiatherogenic factors such as estrogen and shear stress inhibit EC apoptosis. Fas, which belongs to the tumor necrosis factor-α receptor family, mediates apoptosis by cross-linking to its ligand, FasL. Ligation of Fas with FasL induces recruitment of the adaptor protein Fas-associated death domain and caspase-8, which in turn results in the activation of downstream caspases that lead to apoptosis. ECs are normally resistant to Fas-mediated apoptosis. These cells, however, are sensitized to this apoptotic pathway when expression of cellular FLICE (Fas-associating protein with death domain-like interleukin-1–converting enzyme)-inhibitory protein (cFLIP), an inhibitor of Fas-induced apoptosis, is downregulated under pathologic conditions, such as exposure to oxidized LDL, hypoxia, and loss of matrix adhesion. Phosphatidylinositol-3 (PI3)-kinase/Akt signaling is of central importance in EC survival. It was shown that cFLIP is downregulated under conditions that lead to diminished PI3-kinase/Akt signaling, whereas activation of PI3-kinase/Akt signaling induces upregulation of cFLIP expression in ECs. However, the molecular mechanism for regulation of cFLIP expression in ECs has not been fully elucidated.

Previous studies have shown that cardiovascular events are less frequent among populations consuming fish oils. Accumulating evidence indicates that administration of eicosapentaenoic acid (EPA), a polyunsaturated fatty acid of the n-3 series extracted from fish oils, has antiatherogenic and cardioprotective effects. Although the precise mechanisms of these beneficial effects of EPA remain to be clarified, several possible mechanisms have been reported. For example, EPA induces augmentation of endothelium-dependent vasodilatation in patients with coronary artery disease, inhibition of serotonin-induced smooth muscle cell prolifera-
lation, \(^{14}\) enhancement of nitric oxide production, \(^{15}\) reduction of thrombosis, \(^{16}\) inhibition of cytokine synthesis, \(^{17}\) inhibition of superoxide generation, \(^{18}\) and suppression of leukocyte-endothelium interaction. \(^{19}\) Interestingly, EPA has been shown to improve insulin sensitivity in hepatoma cells through upregulation of the intracellular insulin signaling pathway including Akt, which is known as a survival signal in many cell types, including ECs. \(^{20}\) In the present study, we attempted to determine whether EPA protects ECs against apoptosis through upregulation of Akt-mediated signaling pathways. We found that EPA protects ECs against detachment-induced apoptosis (anoikis) through restoration of cFLIP expression via both Akt-dependent and Akt-independent pathways.

### Methods

#### Cell Culture and Reagents

Human umbilical vein ECs were cultured in endothelial basal medium 2 (Sanko Junyaku) supplemented with 2% fetal calf serum. For suspension cultures, \(10^6\) cells were suspended in serum-free medium with or without reagents and placed in a sterile centrifuge tube for 8 hours at 37°C in a CO₂ incubator. Thus, in this study, the responsiveness of ECs to detachment-induced apoptosis (anoikis) was examined under serum starvation. EPA and wortmannin were obtained from Sigma. Insulinlike growth factor-1 (IGF-1) from R&D Systems. Mouse monoclonal antibody against human cFLIP (FLIP-L) was generously provided by Dr. Kenneth Walsh (Whitaker Cardiovascular Institute, Boston University School of Medicine, Boston, Mass.). To examine the role of cFLIP in Fas-mediated apoptosis of ECs, a tetracycline-inducible cFLIP expression system was developed by using a binary-defective adenovirus strategy. The first replication-defective adenovirus encoded the transgene, either FLIP-L or LacZ, under transcriptional control of 7 tetracycline operator sites (AdTet-FLIP). The second vector expressed a chimeric transcription factor composed of a mutant tetracycline repressor fused to the VP16 trans-activator domain from the cytomegalovirus promoter/enhancer (AdCMV-rtTA). This factor does not efficiently trans-activate tetracycline operator sites under basal conditions, but the addition of Dox, an analogue of tetracycline, results in maximal transgene expression.

#### Adenoviral Constructs

Replication-defective adenovirus vectors expressing the constitutively active form of murine Akt (Adeno-myrcAkt) from the cytomegalovirus promoter and adenoviral vectors of AdTet-FLIP were provided by Dr. Kenneth Walsh (Whitaker Cardiovascular Institute, Boston University School of Medicine, Boston, Mass.). To examine the role of cFLIP in Fas-mediated apoptosis of ECs, a tetracycline-inducible cFLIP expression system was developed by using a binary-defective adenovirus strategy. The first replication-defective adenovirus encoded the transgene, either FLIP-L or LacZ, under transcriptional control of 7 tetracycline operator sites (AdTet-FLIP). The second vector expressed a chimeric transcription factor composed of a mutant tetracycline repressor fused to the VP16 trans-activator domain from the cytomegalovirus promoter/enhancer (AdCMV-rtTA). This factor does not efficiently trans-activate tetracycline operator sites under basal conditions, but the addition of Dox, an analogue of tetracycline, results in maximal transgene expression.

#### Western Blot Analysis

Protein extract (20 \(\mu\)g) was fractionated on sodium dodecyl sulfate– polyacrylamide gel electrophoresis gels and transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore). The membrane was blocked with T-phosphate-buffered saline (PBS; 1× PBS, 0.3% Tween 20) containing 3% dry milk and incubated with primary antibody (anti–phospho-Akt [Cell Signaling], anti–\(\alpha\)-tubulin [Calbiochem], and anti–caspase-8 [Medical Biological Laboratories]) overnight at 4°C. The immune complexes were detected by chemiluminescence methods (ECL, Amersham International plc).

#### Detection of Cell Viability by Annexin V/PI Staining

Cells (10\(^3\)/mL) were incubated with 1 \(\mu\)L annexin V–fluorescein isothiocyanate (Fluos, Boehringer Mannheim) in the provided solution and 0.5 \(\mu\)L propidium iodide (PI 10 mg/mL; Sigma) and subsequently analyzed by fluorescence-activated cell sorting methods (FACSort, Becton Dickinson). PI was added to distinguish between early apoptotic (annexin V+/PI−) and late apoptotic or necrotic (annexin V+/PI+) cells. Data analysis was performed with Cell Quest software.

#### Organ Culture

Rat aortic rings were cultured in Dulbecco’s modified Eagle medium in the presence or absence of 10% fetal bovine serum or EPA (10 \(\mu\)mol/L) for 24 or 48 hours at 37°C, 5% CO₂. The rings were washed in PBS and snap-frozen in OTC compound (Sakura). ECs were stained with an anti-CD31 monoclonal antibody (BD Bioscience Pharmingen). Apoptotic nuclei were detected with an in situ detection kit for apoptosis (Medical Biological Laboratories). Nuclei were counterstained with PI and mounted for fluorescence. Specimens were examined and photographed on a laser confocal microscope (Axioplan 2, Carl Zeiss).

#### Statistical Analysis

Statistical analysis was performed by 1-way ANOVA. Results are expressed as mean±SEM. A value of \(P<0.05\) was considered significant.

### Results

#### EPA Induces Upregulation of cFLIP Expression in ECs

We first examined whether EPA modulates the levels of cFLIP expression in ECs. As shown in Figure 1, serum...
deprivation of ECs for 24 hours resulted in a decrease in cFLIP protein expression. Incubation with EPA (10 to 50 μmol/L) restored the levels of cFLIP expression. A time-course study showed that a significant increase in the levels of cFLIP expression was observed from 3 hours after stimulation with EPA (Figure 1B).

**EPA-Induced Upregulation of cFLIP Expression Is Partially Mediated Through Activation of Akt**

Recently, it has been reported that a serine/threonine protein kinase Akt promotes EC survival through upregulation of cFLIP expression. We next examined whether Akt activation is involved in this mechanism. As shown in Figure 2A, incubation with the PI3-kinase inhibitor wortmannin partially inhibited the ability of EPA to upregulate cFLIP expression. IGF-1 that activates Akt signaling also induced upregulation of cFLIP expression. To further elucidate the role of Akt signaling in cFLIP expression, ECs were transfected with adenovirus vectors expressing the constitutively active Akt (Adeno- myrAkt). Infection of ECs with Adeno-myrAkt induced upregulation of cFLIP expression, whereas Adeno-LacZ had no effect (Figure 2B). These data suggest that activation of Akt signaling is partially involved in the mechanism by which EPA induces upregulation of cFLIP expression in ECs.

**Figure 2.** EPA-induced restoration of cFLIP expression is partially mediated via Akt signaling. A, ECs were incubated for 24 hours with serum-free medium in the presence or absence of EPA (10 μmol/L) or wortmannin (100 nmol/L). B, ECs were incubated for 24 hours with serum-free medium in the presence or absence of EPA (10 μmol/L) or IGF-1 (50 ng/mL). Where indicated, cells were infected with adenovirus vector expressing the constitutively active Akt (myrAkt) at a multiplicity of infection of 50. cFLIP expression was analyzed by Western blotting. cFLIP expression was quantified by densitometric analysis as described in the legend to Figure 1. *P<0.05, significantly different from control ECs cultured in the absence of EPA or serum. **P<0.05, significantly different from ECs treated with EPA.

**Figure 3.** Pretreatment with EPA or IGF-1 protects ECs against anoikis. ECs were pretreated for 24 hours with EPA (10 μmol/L) or IGF-1 (50 ng/mL) and then cultured for 3 or 8 hours in serum-free medium under suspension conditions. Caspase-8 activation was analyzed at 3 hours by Western blotting, and cell viability was analyzed at 8 hours by fluorescence-activated cell sorting analysis for annexin V and PI staining. A, Representative data are shown. Percentages indicate the portion of cells in the respective quadrant. S indicates survival cells; EA, early apoptotic cells, and LA, late apoptotic cells. Attached cells served as a positive control. B, Graph shows percentages of dead cells (EA+LA), and values are mean±SEM from 4 experiments. *P<0.05, significantly different from control ECs cultured in suspension.

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Pretreatment of ECs With EPA or IGF-1 Protects Against EC Anoikis

To define the physiologic significance of EPA on upregulation of cFLIP, an endogenous inhibitor of caspase-8, we next examined whether EPA modulates EC anoikis. As shown in Figure 3A and 3B, pretreatment with EPA partially but significantly protected ECs against anoikis. IGF-1, an activator of Akt, also protected ECs against anoikis. In addition, pretreatment with EPA or IGF-1 suppressed levels of the active form of caspase-8, a downstream signal for Fas-mediated apoptosis induced by cell detachment (Figure 3C).

Treatment of Detached ECs With EPA but Not IGF-1 Protects ECs Against Anoikis via Akt-Independent Pathways

We next examined whether EPA can protect ECs against anoikis when these cells were already detached. Interestingly, EPA partially but significantly protected ECs against anoikis even when treatment was applied to detached ECs, whereas IGF-1 had no effect (Figures 4A and 4B). As shown in Figure 5A, cFLIP expression levels in ECs were significantly decreased when they were cultured in suspension. Importantly, EPA but not IGF-1 restored the levels of cFLIP expression in detached ECs (Figure 5A). Moreover, although EPA induced activation of Akt in attached ECs, neither EPA nor IGF-1 induced activation of Akt signaling in detached cells (Figure 5B), suggesting that EPA induces restoration of cFLIP expression via Akt-independent pathways as well. To determine whether EPA-induced restoration of cFLIP expression is directly related to protection against EC anoikis, we next examined the effects of upregulation of cFLIP by infecting ECs with AdTet-FLIP at a multiplicity of infection of 2 in the presence of AdCMV-rTA at a multiplicity of infection of 10 for 12 hours, followed by incubation with serum-free medium or 50 ng/mL IGF-1 for 8 hours. The results showed that EPA but not IGF-1 restored the levels of cFLIP expression in detached ECs (Figure 5A). Moreover, although EPA induced activation of Akt in attached ECs, neither EPA nor IGF-1 induced activation of Akt signaling in detached cells (Figure 5B), suggesting that EPA induces restoration of cFLIP expression via Akt-independent pathways as well. To determine whether EPA-induced restoration of cFLIP expression is directly related to protection against EC anoikis, we next examined the effects of upregulation of cFLIP by infecting ECs with AdTet-FLIP at a multiplicity of infection of 2 in the presence of AdCMV-rTA on EC anoikis, because high levels of cFLIP have been reported to be cytotoxic per se without the need for stimulation of Fas. Overexpression of cFLIP by AdTet-FLIP exogenously protected ECs against anoikis, whereas the control vector had no effect (Figure 4A and 4B). These data indicate that EPA-induced restoration of endogenous cFLIP expression is related to protection against EC anoikis.

EPA Protects Against EC Apoptosis in an Organ Culture of the Rat Aorta

Next, we examined whether EPA can protect ECs against apoptosis in vessels by using an organ culture of rat aorta. Incubation with serum-free medium induced losses of endo-
thelium by 48 hours (Figure 6A). EC apoptosis was also detected in aortic rings that were incubated with serum-free medium for 24 hours, as assessed by the terminal dUTP nick end-labeling method (Figure 6B). Consistent with observations in cultured cells, apoptosis and loss of endothelium were inhibited by treatment with EPA.

Discussion

The endothelium plays important roles in the regulation of vascular homeostasis. Recent evidence has shown that its dysfunction is closely related to the development of cardiovascular events. It is well known that EC survival is critical in the maintenance of endothelial function, as well as in the regulation of angiogenesis and vessel integrity. In this study we demonstrated that EPA promotes detached-EC survival through restoration of the expression of cFLIP, an endogenous inhibitor of Fas-mediated apoptosis. Expression of Fas has been detected in both normal and diseased vessel walls. Although ECs are generally resistant to Fas-mediated apoptosis, these cells can be sensitized to this death process by many pathologic stimuli, including anoikis. Similarly, recent studies have shown that activated T cells, FasL-expressing cells, mediate endothelial injury by loss of ECs in acute coronary syndromes and in chronic graft-versus-host disease. Our previous data also demonstrated that plasma levels of the soluble form of FasL are elevated in patients with acute myocardial infarction and unstable angina pectoris. Furthermore, it has been proposed that Fas-mediated apoptosis of vascular cells is a feature of atherogenesis, plaque vulnerability, and allograft arteriopathy. These findings suggest the importance of Fas-mediated EC apoptosis in the pathogenesis of endothelial dysfunction and subsequent tissue damage in patients with hypertension.

Previous studies have shown that matrix attachment induces activation of the PI3-kinase/Akt and mitogen-activated protein kinase/extracellular signal–regulated kinase pathways in many cell types, whereas cell detachment downregulates these signals. Aoudjit and Vuori recently reported that the Fas/FasL signaling pathway is activated in detached ECs through downregulation of cFLIP and contributes to the induction of anoikis. The results of this study demonstrate for the first time that EPA inhibits EC anoikis through restoration of cFLIP expression. It should be noted that EPA treatment was able to protect against EC anoikis even when these cells

- **Figure 5.** EPA but not IGF-1 restores cFLIP expression in detached ECs without activation of Akt signaling. ECs were cultured in serum-free medium with or without EPA (10 μmol/L) or IGF-1 (50 ng/mL) under normal conditions or suspension conditions. Where indicated, cells were previously infected with Ad-myrAkt at a multiplicity of infection of 50. Western blot analyses of cFLIP expression (A) and phospho-Akt levels (B) were performed on 20 μg lysate. cFLIP expression was quantified by densitometric analysis as described in the legend to Figure 1. *P<0.05, significantly different from attached ECs in control culture. C indicates control. **P<0.05, significantly different from detached ECs in control culture.

- **Figure 6.** EPA protects against EC apoptosis in an organ culture of rat aorta. Rings from rat aortas were incubated with culture medium in the presence or absence of serum with or without EPA (10 μmol/L). A, Aortas harvested at 48 hours were stained for CD31, an EC marker, with the ABC method (Dako). Endothelium is indicated by arrows. Bar=100 μm. B, Aortic rings harvested at 24 hours were stained by terminal dUTP nick end-labeling (TUNEL; green) to detect fragmented chromatin and PI (red) to detect total chromatin and examined by laser confocal microscopy. Apoptotic nuclei are shown as yellow, with a filter for rhodamine and fluorescein. Internal elastic lamina containing autofluorescent elastin is visible with a filter specific for fluorescein. Bar=25 μm.
were already detached, whereas IGF-1, an activator of Akt signaling, had no effect (Figures 5A and 5B). This anchorage dependence of the activation of PI3-kinase/Akt signaling in ECs has been reported previously. Importantly, restoration of cFLIP expression exogenously by transfection with AdTetFLIP directly inhibits EC anoikis. Taken together, these findings suggest that the level of endogenous cFLIP expression is an important determinant of susceptibility to EC anoikis. The data showing that wortmannin, an inhibitor of PI3-kinase, partially suppressed cFLIP expression induced by EPA and that transduction of constitutively active Akt directly induced upregulation of cFLIP expression suggest involvement of the PI3-kinase/Akt pathway in this upregulation by EPA. On the other hand, it should be noted that EPA induced restoration of cFLIP expression without activating the Akt signaling pathway in detached ECs (Figures 5A and 5B). Although the precise mechanism by which EPA induces upregulation of cFLIP in detached ECs is not clear at present, these findings suggest that EPA induces upregulation of cFLIP expression via both Akt-dependent and Akt-independent pathways.

Recent evidence has demonstrated that inflammatory cells such as activated lymphocytes induce loss of EC adhesive-ness to extracellular matrix, independent of cytolytic damage. Although the physiologic significance of this phenomenon remains to be defined, loss of EC adhesion might compromise vessel integrity and tissue perfusion. In addition, focal loss of ECs might be a feature of robust inflammatory infiltrates, such as in allograft rejection and unstable lesions of the atherosclerotic plaque. Shed ECs have actually been detected in patients with a variety of pathologic conditions, such as acute myocardial infarction and active systemic lupus erythematosus. Conversely, loss of adhesiveness of ECs to the extracellular matrix might be important in several physiologic settings. For example, angiogenesis requires EC migration at the budding end of the newly forming capillary. In all settings, the finding that EPA protects detached ECs through restoration of cFLIP might be important, because detached ECs are susceptible to Fas-mediated apoptosis. However, further studies are needed to determine whether EPA stimulates angiogenesis in vivo.

In conclusion, we have demonstrated for the first time that EPA promotes EC survival through restoration of the expression of cFLIP, an endogenous caspase-8 inhibitor. The restoration of cFLIP expression by EPA appears to be important in modulating cell susceptibility to anoikis and therefore provides some insight into the mechanism underlying the beneficial effects of EPA.

Perspectives
EPA protects ECs from anoikis through restoration of the levels of the cFLIP, an endogenous inhibitor of caspase-8, which might provide some insight into the mechanism underlying the beneficial effects of EPA.

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