Regulation of Cardiovascular Functions by Eicosanoids and Other Lipid Mediators

Docosahexaenoic acid induces ciap1 mRNA and protects human endothelial cells from stress-induced apoptosis

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Pfrommer, Claudia A., Wolfgang Erl, and Peter C. Weber. Docosahexaenoic acid induces ciap1 mRNA and protects human endothelial cells from stress-induced apoptosis. Am J Physiol Heart Circ Physiol 290: H2178–H2186, 2006. First published February 10, 2006; doi:10.1152/ajpheart.00933.2005.—Induction of apoptosis represents a potential reaction of endothelial cells (ECs) after injury of the vascular endothelium. Beneficial effects of n–3 polyunsaturated fatty acids (PUFAs) in vascular diseases are widely recognized although the responsible mechanisms are not fully understood. Because it is not known whether PUFAs modulate EC apoptosis, we investigated the effects of n–3 and n–6 PUFAs on 4-hydroxynonenal (HNE)-induced EC apoptosis by annexin V staining and caspase-3 activation assays. Pretreatment with the n–3 fatty acid docosahexaenoic acid (DHA) reduced HNE-induced EC apoptosis. DHA-treated cells did not show the pronounced drop in intracellular GSH after HNE exposure seen in vehicle- or n–6 arachidonic acid-treated cells. This is most likely due to increased GSH levels in DHA-treated cells. Furthermore, DHA pretreatment increased ciap1 mRNA levels and transfection of cIAP1 small interfering RNA abolished the protective effect of DHA in HNE-induced apoptosis in HUVECs. Thus pretreatment of HUVECs with DHA reduces HNE-induced oxidative stress and apoptosis, and the protective effects of DHA seem to be dependent on cIAP1. The results provide a possible new mechanism for the atheroprotective effects of n–3 fatty acids in cardiovascular disease; n–3 fatty acids; 4-hydroxynonenal; inhibitor of apoptosis protein; oxidative stress

APOTOPSIS, or programmed cell death, is a fundamental biological process essential for both embryonic development and tissue homeostasis in the adult organism. Dysregulation of apoptotic cell death plays an important role in pathological processes such as oncogenesis, neurodegenerative diseases, or atherosclerosis (28, 32, 38). Indeed, apoptotic endothelial cells have been detected in human atherosclerotic blood vessels especially at sites of disturbed blood flow (2, 5, 6, 25, 39). Further evidence for an involvement of endothelial cell apoptosis in atherogenesis is given by in vitro experiments. Classical risk factors such as oxidized low-density lipoprotein (LDL) particles, increased oxidative stress, or proinflammatory cytokines induce apoptosis in cultured human endothelial cells (32).

Beneficial effects of foods rich in n–3 polyunsaturated fatty acids (PUFAs) mainly from marine sources on cardiovascular diseases are widely recognized. Epidemiological as well as interventional studies indicate that high intake of n–3 PUFAs reduces the risk of cardiovascular mortality (8, 16, 26). The mechanisms through which n–3 fatty acids confer these protective effects are not fully understood but are thought to be attributable to their effects on vascular smooth muscle and endothelial cell functions (20). For example, docosahexaenoic acid (DHA), an n–3 PUFAs, induces apoptosis in vascular smooth muscle cells (9), thereby influencing vascular remodeling and restenosis (42). In addition, DHA has been shown to exert an antitumorigenic effect that was associated with suppression of tumor-mediated angiogenesis (28, 35). Indeed, we could recently demonstrate antitumorigenic properties of DHA in proliferating human endothelial cells (24).

In confluent human umbilical venous endothelial cells (HUVECs), which represent an intact endothelial monolayer, DHA exerts anti-inflammatory effects (43). Furthermore, in vivo studies reported that improved endothelial function especially in stressed endothelium was positively correlated to increased fish or fish oil intake (17, 23, 29).

Risk factors of atherogenesis are known to induce cell stress, which may lead to radical formation and lipid peroxidation (18, 32). In contrast to the wide variety of oxidized substances within oxidized LDL particles, the lipid peroxidation product 4-hydroxynonenal (HNE) represents a clearly defined component. HNE has been detected in oxidized LDL particles, as well as in human atherosclerotic lesions (14, 34, 40). As a highly reactive aldehyde, HNE induces apoptosis in various cell types, including human ECs (19, 30).

In this study, we investigated the effects of different unsaturated fatty acids on HNE-induced endothelial cell apoptosis. We demonstrate that the n–3 PUFA DHA reduces HNE-induced apoptosis of human endothelial cells. Pretreatment with DHA, despite increasing basal GSH levels, does not inhibit the HNE-induced decrease in GSH but reduces HNE-induced superoxide anion formation. In addition, we demonstrate that DHA treatment induces mRNA transcription of the antiapoptotic protein cIAP1 in HUVECs. The cIAP1 protein is a member of the inhibitor of apoptosis protein (IAP) family, which are endogenous caspase inhibitors, and has been shown to be expressed in HUVECs, as well as in endothelial cells of human arteries (22). Transfection of small interfering RNA...
(siRNA) specifically directed against ciap1 mRNA abolishes the protective effect of DHA against HNE-induced apoptosis. We therefore conclude that regulation of the cIAP1 protein by DHA contributes to protective effects in stress-induced endothelial apoptosis.

MATERIALS AND METHODS

**Endothelial cell culture.** Endothelial cells (HUVECs) were prepared from human umbilical cord veins in accordance with Institutional Review Board-approved protocols and cultured as previously described (11). The use of human umbilical cords for research purposes was approved by the ethical committee of the medical faculty (University of Munich). HUVECs from passages 2 or 3 were used for experiments. Confluent cells were detached by trypsin/EDTA and seeded in collagen-coated 6- or 12-well plates (for flow cytometry analyses) or in 10-cm Petri dishes (for Western blot analyses). All experiments were performed in confluent cells. HNE or fatty acid solutions in ethanol were further diluted in ethanol and stored as aliquots at −80°C. Each aliquot was used twice at most to prevent oxidation processes and dissolved in prewarmed medium before addition to the HUVECs. The ethanol concentration in the medium did not exceed 0.1%; controls were incubated with identical amounts of ethanol.

**Chemicals.** Endothelial cell growth medium (ECGM) was from PromoCell (Heidelberg; Germany). 4-Hydroxynonenal, DHA, arachidonic acid (AA), linoleic acid (LA), and oleic acid (OA) were from Cayman Chemicals (Ann Arbor, MI). Annexin V-FITC was from Pharmingen (Heidelberg, Germany). Hydroethidine was purchased from Molecular Probes (Leiden, Netherlands). Anti-active caspase-3, anti-active caspase-9, anti-cytochrome c and anti-cIAP1 antibodies were obtained from Pharmingen or Cell Signaling (Frankfurt, Germany). Anti-Bcl-2 and anti-Bax were from Dako (Hamburg, Germany). Anti-cIAP2 and anti-poly(ADP-ribose)polymerase (anti-PARP) antibodies and the FITC-conjugated anti-rabbit antibody were obtained from Santa Cruz (Taufkirchen, Germany) and Cell Signaling, respectively. All other chemicals were from Sigma (Seelze, Germany).

**Cell viability assays.** Cell viability was determined by Trypan blue exclusion or a modified 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction assay, as described previously (13). Absorbance was measured in an ELISA reader at 570 nm, with the absorbance at 690 nm to correct for background, and viability was expressed as percentage of untreated controls.

**Annexin V binding assay.** Apoptosis was measured by staining cells with FITC-labeled annexin V and subsequent flow cytometry analysis (12). Confluent HUVECs were treated as indicated for up to 24 h. Cells were detached using trypsin/EDTA, resuspended in 100 μl binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl2), and incubated with annexin V-FITC and propidium iodide (PI) for 30 min at 37°C. Ten thousand cells were analyzed by flow cytometry on a FACSscan (Becton Dickinson, Heidelberg, Germany) by using CellQuest software and a single cell gate.

**Flow cytometrical analysis of superoxide anions.** Superoxide anions were measured as described previously (24). Briefly, cells were harvested after treatment and resuspended in 100 μl binding buffer containing hydroethidine (HE; 2 μM). After incubation at 37°C for 15 min, intracellular superoxide anions were measured on a FACSscan.

**Preparation of protein lysates and Western blot analysis.** Cells were scraped and transferred into precooled tubes. After being washed with ice-cold PBS with Ca2+ and Mg2+, cells were lysed in 200 μl lysis buffer (50 mM Tris·HCl, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 1% Triton X-100, 1% Na-deoxycholate, 0.1% SDS, 1 mM DTT, 5 mM Na-orthovannadate, 1 mM PMSF, aprotinin) for 30 min on ice. Equal amounts of proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, probed with appropriate antibodies, and visualized by enhanced chemiluminescence as described previously (24). The films were scanned, and densitometry analysis was performed with Lab Image software (Kapelan, Germany).

**Cytochrome c release.** Cytochrome c release was determined according to Bossy-Wetzel and Green (4). Briefly, cells were grown in Petri dishes and treated as described after reaching confluency. At the indicated times, cells were gently scraped and collected by centrifugation. After cells were washed twice with ice-cold PBS with Ca2+ and Mg2+, they were incubated in 500 μl lysis buffer [250 mM sucrose, 20 mM HEPES-KOH, 10 mM KCl, 1.5 mM Na-EGTA, 1.5 mM Na-EDTA, 1 mM MgCl2, 1 mM DTT, and Complete Mini protease inhibitor mixture (Roche Diagnostics)]. The cell suspensions were incubated on ice for 30 min. Cells were disrupted by passing through a syringe needle, and the homogenates were centrifuged. Supernatants were further centrifuged at 14,000 rpm, and the resulting supernatants were saved as cytosolic extracts.

**GSH measurement.** Intracellular GSH levels were measured by using glutathione assay kit (Calbiochem, Bad Soden, Germany) with slight modifications for microtiter plates. Briefly, cells were harvested by trypsin/EDTA, pelleted by centrifugation, and resuspended in 1 ml ice-cold PBS with Ca2+ and Mg2+. Fifty microliters were used for total cell count. The remaining cell suspension was centrifuged, and the cells were homogenized in 200 μl freshly prepared, ice-cold 5% meta-phosphoric acid (Fluka, Seelze, Germany). After centrifugation, 60 μl of the supernatant was transferred into a microtiter plate, and 120 μl of 200 mM potassium phosphate buffer containing 0.2 mM diethylene triamine pentaacetic acid and 0.025% LUBROL, 20 μl of 30% NaOH, and 20 μl of 12 mM chromogenic reagent were added. The mixture was incubated for 10 min at room temperature in the dark. Absorbance was measured at 405 nm.

**RNA isolation and real-time RT-PCR.** Total RNA was isolated by using AqueaPure RNA Isolation Kit (Bio-Rad, Munich, Germany) and treated with DNase (Promega, Mannheim, Germany). RT-PCRs were conducted by using the QuantiTect SYBR Green RT-PCR Kit (Qiagen, Hilden, Germany) in the iCycler system (Bio-Rad). Primers were as follows: cIAP1, forward 5'-CGGATGAACTCCTGTCCTTT-3' and reverse 5'-AGATGGCCACGGCTGCTT-3'; β-actin, forward 5'-AGATGGCCACGGCTGCTT-3' and reverse 5'-ATTTCGCGT- GACGATGGAG-3'.

**siRNA Experiments.** Three siRNA constructs with different oligonucleotide sequences were selected from the predesigned siRNA database at www.ambion.com for siRNA knockdown of cIAP1 expression. Annealed siRNA constructs were resuspended in RNA-free water and stored at −20°C. Negative control no. 1 siRNA from Ambion (Huntingdon, UK) was used as nonsense control.

**Magnetofection.** HUVECs were seeded at a density of 30,000 cells/cm² in 12- or 6-well plates and allowed to settle overnight. siRNA was diluted in 200 μl (12 well) or 500 μl (6 well) supplement-free ECGM per well, and appropriate volumes of MATra-A reagent (IBA, Göttingen, Germany) were added. After incubation for 20 min at room temperature, plates were prepared for magnetofection by addition of 800 μl or 1.5 ml ECGM per 12-well or 6-well plate, respectively. The siRNA/bead mixture was added to a final concentration of 80 nM. Plates were placed on a magnetic plate as described in the MATra instruction manual for 15 min in the incubator. Cells were incubated with fresh medium immediately after transfection. Transfection rate reached >80% of total cells as determined by transfection of Alexa Fluor 488-labeled negative control siRNA (Qiagen).

**Statistics.** Data are expressed as means ± SE of at least three independent experiments. The statistical significance between means of two independent groups was determined by Student’s t-test. The Mann-Whitney test was used to compare the effects of DHA or AA on GSH levels. All results were considered significant if the P value was ≤0.05.
RESULTS

HNE reduces HUVEC viability and induces apoptosis time and concentration dependently. The effect of HNE on the viability of HUVECs was examined by Trypan blue and MTT assays. HNE treatment led to a concentration-dependent reduction of total cell number after 24 h and a similarly dose-dependent increase of dead endothelial cells (Fig. 1A). A more detailed time-course analysis of HUVEC viability demonstrated a rapid loss of viability in response to 20 or 30 μM HNE (Fig. 1B). The reduction of cell viability was due to a concentration-dependent induction of apoptosis with 20 μM HNE as the most potent concentration to induce apoptosis after 6 h of treatment, which was therefore used for subsequent experiments (Fig. 1C). Activation of caspase-3 was determined by Western blot analysis of PARP cleavage to confirm apoptosis induction (Fig. 1D). PARP cleavage was detectable after 3 h of HNE treatment, and the protein level was further reduced time dependently. To investigate whether HNE-induced mitochondrial stress could be responsible for caspase-3 activation, cytochrome c release was investigated at different time points. As shown in Fig. 1E, a time-dependent release of cytochrome c was detectable in cytosolic extracts after HNE treatment.

DHA pretreatment attenuates HNE-induced apoptosis in HUVECs. To investigate whether PUFAs modulate HNE-induced apoptosis, four different unsaturated fatty acids were compared in initial experiments. As shown in Fig. 2A, among the selected fatty acids only DHA pretreatment resulted in reduced numbers of apoptotic HUVECs after treatment with 20 μM HNE. Within 6 h of HNE exposure, the protective effect of DHA was seen (Fig. 2B). Treatment of HUVECs with DHA reduced HNE-induced caspase-3 activation significantly (Fig. 2C), which was confirmed by Western blot analysis for active caspase-3 (Fig. 2D). The n−6 polyunsaturated fatty acid AA.

Fig. 1. 4-Hydroxynonenal (HNE) reduces cell viability and induces apoptosis in human umbilical vein endothelial cells (HUVECs). A: HUVECs were treated with 10−50 μM HNE or control (C; 0.1% ethanol) for 24 h. Total and dead cell number were determined by Trypan blue exclusion and expressed as % of control; n = 4. B: 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) activity was determined after treatment of HUVECs for 3−24 h with 10−30 μM HNE; n = 4. C: HUVECs were treated with 10−30 μM HNE for 6 h. Apoptotic cells were determined using annexin V-FITC; n = 3. *P < 0.05. D: total poly(ADP-ribose)polymerase (PARP) (112 kDa) and PARP cleavage (85-kDa fragment) were determined in whole cell extracts by using Western blot analysis. A representative blot of 3 experiments is shown. E: Western blot analysis of cytochrome c in cytosolic extracts was used to detect cytochrome c release. A representative blot of 3 experiments is shown. Densitometric analysis of relative cytochrome c levels is shown as bar graph.
acid showed no protective effect or even enhanced HNE-induced apoptosis in HUVECs (Fig. 2, B and C).

Reduced oxidative stress in DHA-treated cells but no effect on HNE-induced cytochrome c release. HNE has been shown to induce oxidative stress as seen especially by a reduction of GSH levels (27). To test whether polyunsaturated fatty acids modify HNE-induced oxidative stress, we performed colorimetric GSH measurements. As shown in Fig. 3A, HNE treatment rapidly reduced the GSH content of HUVECs by ~80% within 2 h of treatment. Although treatment with DHA increased intracellular GSH levels of HUVECs, this increase was not sufficient to prevent the HNE-induced GSH depletion. However, DHA treatment showed a moderate protective effect as GSH levels of DHA-treated cells remained higher during HNE-treatment (Fig. 3A), whereas AA treatment reduced GSH levels even further.

The HNE-induced decrease in intracellular GSH was accompanied by a constant increase of superoxide anions in HNE-treated cells (Fig. 3B). Whereas AA pretreatment showed no effect on the formation of superoxide anions, DHA pretreatment prevented the HNE-induced increase in superoxide anions.

To further investigate whether DHA reduces HNE-induced mitochondrial stress, cytochrome c release and the expression of Bcl-2/Bax were determined. DHA pretreatment of HUVECs resulted in a decreased Bcl-2/Bax ratio (data not shown) and did not decrease cytochrome c levels in the cytosolic extracts (Fig. 3C). In addition, subsequent caspase-9 activation was not inhibited in DHA-treated cells (Fig. 3D).

Increased ciap1 mRNA transcription after DHA treatment. Because DHA treatment reduced HNE-induced caspase-3 activation (Fig. 2C) without inhibiting cytochrome c release (Fig. 3C) or subsequent caspase-9 activation (Fig. 3D), we investigated whether DHA treatment enhances the expression of ciap1 mRNA and protein levels in HUVECs. The ciap1 protein has been demonstrated to be increased on atheroprotective shear stress regimen in HUVECs (22). Recent evidence furthermore suggests that the antiapoptotic effect of ciap1 does not involve the inhibition of caspases (10). Real-time RT-PCR analyses after 4 and 24 h of fatty acid treatment showed a significant increase in ciap1 mRNA transcripts at both time points in DHA-treated cells (Fig. 4A). This effect again seems to be specific for the n-3 fatty acid DHA as HUVECs treated with the n-6 fatty acid AA did not show a

Fig. 2. Effects of docosahexaenoic acid (DHA) on HNE-induced apoptosis. A: HUVECs were incubated for 24 h with 20 μM of different fatty acids or vehicle control and treated for 24 h with 20 μM HNE or control (0.1% ethanol). Apoptosis was detected by annexin V-FITC staining; n = 3. OA, oleic acid; LA, linoleic acid; AA, arachidonic acid. *P < 0.05. B: HUVECs were stimulated with 20 μM HNE for 2 or 6 h after a 24-h pretreatment with 20 μM AA or DHA. Apoptotic cells were determined by using annexin V-FITC; n = 5. *P < 0.05. C: active caspase-3 was measured by flow cytometry after stimulation of fatty acid-pretreated cells with 20 μM HNE for 6 h. A specific antibody preferentially binding to the active form was used; n = 3. *P < 0.05. D: caspase-3 activation was confirmed in whole cell extracts by using Western blot analysis. The cleaved 17-kDa fragment of caspase-3 represents activated caspase-3. A representative blot of 3 experiments is shown. Densitometric analysis of caspase-3 levels relative to procaspase-3 levels is shown as bar graph.
change in ciap1 mRNA expression. Western blot analyses demonstrated a slight increase of cIAP1 protein levels in cells treated for 24 h with DHA (Fig. 4B). Subsequent densitometric analyses of cIAP1 expression relative to β-actin showed no significant increase in cIAP1 levels after DHA treatment.

cIAP1 siRNA reduces ciap1 mRNA and protein expression after 24 h. To further investigate whether cIAP1 is involved in the protection of HUVECs by DHA against HNE-induced apoptosis, we performed knockdown experiments with the use of RNA interference. HUVECs were transfected with three different siRNA constructs directed against ciap1 mRNA. mRNA and protein expression were assessed 24 h after transfection. Transfection of siRNA constructs 1 and 3 into HUVECs resulted in a significant reduction of cIAP1 mRNA transcription after 24 h (Fig. 5A). The cIAP1 protein levels were significantly reduced in cells transfected with cIAP1 siRNA constructs 2 and 3 (Fig. 5B). As effects on closely related proteins are a major concern in RNA interference technology, the expression of cIAP2 was investigated. As shown in Fig. 5C, none of the siRNA constructs affected cIAP2 protein levels.

Knockdown of ciap1 mRNA by RNA interference abolishes the protective effect of DHA pretreatment. To investigate the effects of DHA pretreatment on HNE-induced apoptosis in cIAP1 siRNA-transfected cells, HUVECs were treated as described after transfection and subjected to flow cytometrical analysis of apoptosis induction. Cell death was not increased after transfection with either of the siRNA constructs per se (nonsense siRNA: 8.45 ± 0.74%; cIAP1 siRNA construct 1: 7.30 ± 0.73%, cIAP1 siRNA construct 2: 7.42 ± 0.67%; cIAP1 siRNA construct 3: 7.47 ± 0.62%). As shown in Fig. 6A, DHA pretreatment reduced the percentage of PI-positive cells significantly in nonsense siRNA-transfected cells after HNE stimulation. This reduction was similar to effects observed in nontransfected cells. In contrast, transfection of HUVECs with each of three different cIAP1 siRNA constructs abolished the protective effect of DHA pretreatment in HNE-induced apoptosis. Similarly, DHA-pretreated, cIAP1 siRNA-transfected cells showed only slightly reduced apoptosis induction compared with cells transfected with nonsense siRNA, which displayed the same protective effect as seen in nontransfected cells (Fig. 6B). The loss of DHA-induced protection in cIAP1 siRNA-transfected cells was further confirmed by measurement of caspase-3 activation in HNE-treated cells. Indeed, cells transfected with cIAP1 siRNA constructs 1 and 3 did not show the DHA-induced reduction in caspase-3 activation as found in nonsense siRNA-transfected cells or nontransfected cells (Fig. 6C).

DISCUSSION

In this study, we show that pretreatment with the n–3 fatty acid DHA but not with the n–6 fatty acid AA reduces HNE-induced apoptosis and oxidative stress in vascular endothelial...
In a recent study, we compared the effects of PUFAs on proliferating and confluent HUVECs. While DHA induced apoptosis in proliferating HUVECs in this study, even higher concentrations of DHA as used in the present study did not affect cell viability of confluent HUVEC monolayers (24). Our data indicate that the n–3 fatty acid DHA may induce different effects in both settings, i.e., antiangiogenic effects in proliferating endothelial cells and endothelium-protective effects in confluent endothelial monolayers. Previous studies using comparable concentrations of DHA had shown reduced apoptosis on DHA treatment in mouse neuronal cells stimulated with staurosporine (1) and in rat photoreceptors after paraquat treatment (36). These cell types are characterized by high DHA levels that are essential for their proper function and survival. In agreement with these previous studies, our results presented here suggest that DHA reduces cell stress-induced apoptosis in endothelial cells.

The ability of the reactive lipid peroxidation product HNE to induce apoptosis has been demonstrated in various cell types including neuronal cells (27), colon carcinoma cells (21), and endothelial cells (19, 30). In our study, HNE demonstrated a narrow dose range in HUVECs as reported for other cell types (21, 33). In agreement with the study by Levonen et al. (30), 20 μM HNE was the most potent concentration to induce HUVEC apoptosis. Within 3 h of HNE treatment, activation of caspase-3 and release of cytochrome c were detectable. Similar time courses have been shown in Jurkat and colon carcinoma cells in response to HNE (21, 33).

Functional effects of HNE are based on its high reactivity with cellular components like proteins or lipids. Depletion of intracellular GSH is one prominent effect of HNE as it is conjugated to GSH for detoxification (15). We analyzed intracellular GSH levels to investigate whether DHA or AA treatment affects HNE detoxification. DHA-treated cells did not show the pronounced drop in intracellular GSH seen in vehicle- or AA-treated cells after HNE treatment. This is most likely due to increased GSH levels in DHA-treated cells. Previous studies have demonstrated upregulation of GSH in response to DHA treatment in human lymphocytes (3) and increased glutathione peroxidase activity in DHA-treated endothelial cells (7). Interestingly, HNE-induced formation of superoxide anions was inhibited in DHA-pretreated cells. Although the origin of superoxide anions formed in response to HNE is not clear, our data indicate that DHA reduces HNE-induced oxidative stress in HUVECs.

DHA pretreatment did not inhibit HNE-induced cytochrome c release, indicating that DHA does not prevent mitochondrial stress. As we and others (1) have demonstrated, DHA pretreatment reduces HNE-induced activation of caspase-3, implicating a downstream effect of DHA to reduce caspase-3 activation. Members of the IAP family of antiapoptotic proteins are important endogenous caspase inhibitors that regulate the activity of initiator as well as of effector caspases (31). Therefore, the upregulation of proteins such as cIAP1 could be responsible for the protective effect of DHA. We observed increased...
ciap1 mRNA levels after 4 and 24 h of DHA treatment. Despite upregulated mRNA, we could not detect a significant increase in cIAP1 protein by semiquantitative Western blot analysis. To further investigate whether cIAP1 is involved in reduced apoptosis in DHA-treated cells, we used RNA interference technology. Using three different constructs, we demonstrated that transfection of cIAP1 siRNA abolished the protective effect of DHA against HNE-induced apoptosis in HUVECs. Notably, construct 3, which led to a significant downregulation of ciap1 mRNA as well as protein, exhibited the most potent inhibition of the protective DHA effect. These data strongly suggest an involvement of cIAP1 in the reduction of HNE-induced apoptosis in DHA pretreated cells.

Until recently, cIAP1 has been thought to function mainly as a cytosolic caspase inhibitor. In light of several recent publications, the function and subcellular localization of this protein need to be reconsidered. Two independent studies demonstrated a nuclear localization of cIAP1 in tumor cell lines (37, 41). The exact function of cIAP1 nuclear localization in apoptosis is still unclear, but nuclear export of cIAP1 on apoptosis induction has been shown in HeLa cells (37). Furthermore, cIAP1 has been shown not only to be expressed cell cycle dependently (44) but also to be involved in the cell cycle regulation as has been concluded from overexpression experiments (37). Perhaps the most important new information stems from the study of Eckelman and Salvesen (10), who demonstrated that although cIAP1 binds to activated caspases...
it is not able to inhibit them. Further studies are therefore needed to define the mechanism by which cIAP1 exerts its antiapoptotic effects.

In summary, the evidence presented in this study shows that pretreatment of HUVECs with the n–3 fatty acid DHA reduces HNE-induced oxidative stress and apoptosis. Increased GSH levels in DHA-treated cells may in part account for the protective effect. Our results further suggest that the antiapoptotic properties of DHA also involve cIAP1 activation. Such functions of DHA may contribute to the cellular atheroprotective effects of n–3 fatty acids.

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