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Research Article

Omega-3 Fatty Acids Inhibit Endoplasmic Reticulum (ER) Stress in Human Coronary Artery Endothelial Cells

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The biological effects of fatty acids differ by their structure. Saturated fatty acids and trans-fatty acids are recognized as promoters of coronary artery disease (CAD), while monounsaturated and omega-3 fatty acids may have salutary effects. Since cellular stress is recognized as a fundamental driver of CAD, the effect of these fatty acids on endoplasmic reticulum (ER) stress in human coronary artery endothelial cells (HCAECs) was measured using the ER stress-responsive alkaline phosphatase (ES-TRAP) assay. Docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and eicosapentaenoic acid ethyl ester (EPA-EE) suppressed ER stress induced with pharmacologic (tunicamycin) and physiologic (high-dextrose concentration) ER stress inducers. In tunicamycin-treated cells, DHA reduced the expression of unfolded protein response (UPR) markers such as phosphorylation of inositol requiring enzyme 1α (IRE1α) and protein kinase R-like endoplasmic reticulum kinase (PERK) and increased activating transcription factor 6 (ATF6) and glucose regulated protein 78 (GRP78) expression. Similarly, treatment with both oleic acid and arachidonic acid, but not elaidic acid (a trans-fatty acid), suppressed both tunicamycin and high-dextrose-induced ER stress while treatment with saturated fatty acids (C14 : 0, C16 : 0, and C18 : 0) enhanced both tunicamycin and high-dextrose-induced ER stress. The latter fatty acids at higher concentrations caused cytotoxicity. These results indicate that omega-3 fatty acids as well as select unsaturated fatty acids and arachidonic acid suppress ER stress in HCAEC.

1. Introduction

Elevated blood unesterified fatty acids (FAs) are common in people with diabetes and metabolic syndrome [1, 2] and can cause cellular dysfunction, particularly in pancreatic β-cells [3–5] as well as vascular endothelial cells [6, 7]. In endothelial cells, both excess glucose and selected FAs increase superoxide (SO) production [8], while high concentrations of glucose can also cause endoplasmic reticulum (ER) stress in human coronary artery endothelial cells (HCAECs) [6, 7]. In human umbilical vein endothelial cells (HUVECs), treatment with myristic acid (C14 : 0), palmitic acid (C16 : 0), and stearic acid (C18 : 0) increases SO generation [8]. Similar results were observed with unsaturated fatty acids as well as two of the three trans-FAs tested [8]. Though high glucose (27.5 mM) has been shown to increase SO generation and ER stress in HCAEC [6, 7], no studies have examined the effects of various classes of FAs on ER stress in this particular cell type [9].

The ER stress is mediated by three unfolded protein response (UPR) mediators [10–13]. Inositol requiring enzyme 1α (IRE1α), protein kinase RNA-like endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (ATF6) are all localized to the ER membrane and associate with glucose regulated protein 78 (GRP78), an ER-resident molecular chaperone [10]. The accumulation of unfolded proteins in the ER induces the release of GRP78 from IRE1α, PERK, and ATF6, stimulating their activity. After the release of GRP78, IRE1α and PERK form homodimers, and after undergoing autophosphorylation, both IRE1α and PERK phosphorylate unique sets of substrates. Once activated, IRE1α also possesses RNA splicing activity and is essential

for the activation of the transcription factor X-box binding protein-1 (XBP-1) [11]. The conversion of XBP-1 mRNA from the long (XBP-1L) to XBP-1 short (XBP-1S) by IRE1α-mediated splicing promotes ribosome association with the XBP-1 mRNA and translation of the transcription factor [11]. The XBP-1 translocates to the nucleus where it regulates the expression of XBP-1-dependent genes [11]. When detached from GRP78, ATF6 translocates to the Golgi where it is released from the organelle by two site-specific proteases [12]. Once released from the Golgi, ATF6 translocates to the nucleus where it also regulates various genes coding for ER-resident molecular chaperones as well as other ER-resident enzymes involved in enhancing protein synthesis [10]. However, if the stress is too severe or sustained for an excessive length of time, the UPR induces a mitochondria-dependent apoptotic response via changes in C/EBP homologous protein (CHOP) expression and c-*jun* N-terminal kinase-1 (JNK-1) activation [14].

The UPR activation has been shown to play important roles in diabetes and cardiovascular disease [15, 16], while ER stress inhibition may prove beneficial in ameliorating both conditions [16]. To examine the effects of various types of FAs on ER stress in HCAEC, we performed a systematic analysis of the effects of the three omega-3 FAs, docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and eicosapentaenoic acid ethyl ester (EPA-EE), on ER stress. We also examined the effect of three saturated FAs (C14 : 0, C16 : 0, and C18 : 0), a monounsaturated FA (oleic acid), a trans-FA (elaidic acid), and a polyunsaturated FA (arachidonic acid) on tunicamycin and high-dextrose-induced ER stress. This is the first report examining the effects of omega-3 FAs on ER stress in HCAEC. Likewise, the effects of long-chain saturated fatty acids, trans-FAs, monounsaturated FAs, and arachidonic on ER stress in HCAEC have not been reported previously.

2. Materials and Methods

2.1. Materials. Antibodies to ATF6 (MA1-25358), phospho-IRE1α (PA1-16927), IRE1α (PA1-46027), phospho-PERK (PA5-40294), PERK (PA5-38811), GRP78 (PA5-22967), and HALT protease/phosphatase inhibitor cocktail were purchased from Thermo Scientific (Pittsburg, PA). Tunicamycin, C18 : 0, C16 : 0, and C14 : 0 were purchased from Cayman Chemical (Ann Arbor, MI). Immobilon-P was purchased from Millipore Sigma (Burlington, MA). An antibody to tubulin (CP06) was purchased from Calbiochem (San Diego, CA). Horseradish peroxidase conjugated goat-anti-rabbit (4010-05) and goat-anti-mouse (1030-05) secondary antibodies, conjugated to horseradish peroxidase (HRP), were purchased from Southern Biotech (Birmingham, AL). Plasmid DNA purification reagents were purchased from Qiagen (Hiden, Germany). DHA, EPA, EPA-EE, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Saint Louis, MO). Newborn calf serum (NCS) was purchased from Hyclone (Logan, UT). Lipofectamine was purchased from Invitrogen (Waltham, MA), and the plasmid pSEAP2.control (expressing secreted alkaline phosphatase (SAP)) and the chemiluminescent SAP substrate disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2′-(5′-chloro)tricyclo [3.3.1.13,7]decan}-4-yl)phenyl phosphate (CSPD) were purchased from Clone Tech (Palo Alto, CA). All the other reagents were purchased from either Thermo Fisher Scientific or Sigma-Aldrich.

2.2. HCAEC Culture. HCAECs were purchased from American Type Culture Collection (Manassas, VA) and maintained in a endothelial cell growth medium containing 2% fetal bovine serum, 5 ng/ml recombinant epidermal growth factor, 10 μg/ml ascorbic acid, 1 μg/ml hydrocortisone hemisuccinate, 0.75 units/ml heparin sulfate, 0.2% bovine brain extract, 10 mM glutamine, 10 units/ml penicillin, and 10 μg/ml streptomycin. The cells were maintained in a dedicated humidified cell culture incubator at 37°C and 5% CO_2. Cells between passages 2 and 7 were used in all experiments.

2.3. Measurement of ER Stress Using the Endoplasmic Reticulum Stress-Responsive Alkaline Phosphatase (ES-TRAP) Assay. ER stress was measured using the ES-TRAP assay [17]. HCAECs were transfected with 1 μg of the plasmid pSEAP2.control, and 24 hours later, the cells were treated as described in Figure 1. All FAs were dissolved in FA-free bovine serum albumin (BSA) dissolved in Hank's balanced salt solution (HBSS), and control cells were treated with an equivalent amount of BSA in HBSS, with or without tunicamycin (dissolved in DMSO), 5.5 mM (100 mg/dl) dextrose, or 27.5 mM (500 mg/dl) dextrose. Twenty-four hours later, the conditioned medium was collected and SAP activity was measured using the chemiluminescent substrate CSPD and cell viability was measured in the remaining cells using the MTT assay.

2.4. The MTT Assay. Cell viability was measured using the MTT assay [18]. Cells were treated as described in each figure, and MTT (5 mg/ml in phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4 \cdot 2H_2O$, 2 mM KH_2PO_4, and pH 7.4) was added to a final concentration of 0.5 μg/ml and incubated for 2 hours at 37°C. The media was aspirated, and the formazan crystals were dissolved in 500 μl of 0.1 M HCl, 10% Triton X-100 in isopropanol. After 15 minutes, the absorbance was measured at 570 nm with background correction at 690 nm, with a Bio-Tek ELx800 microplate spectrophotometer (Winooski, VT).

2.5. Western Blotting. After each treatment, HCAECs were washed in HBSS three times and then lysed in 200 μl of electrophoresis sample buffer (50 mM tris-(hydroxymethyl)-aminomethane-hydrochloride (Tris-Cl) (pH 7.4), 1% sodium dodecylsulfate (SDS), 1 mM ethylenediaminetetraacetic acid (EDTA), and HALT protease/phosphatase inhibitor cocktail). The bicinchoninic acid (BCA) assay [19] was used to measure protein content in each sample, and 50 μg of protein was fractionated by electrophoresis on a 10% SDS-polyacrylamide gel. After transfer to Immobilon-P transfer membrane and blocking with Tris-buffered saline-

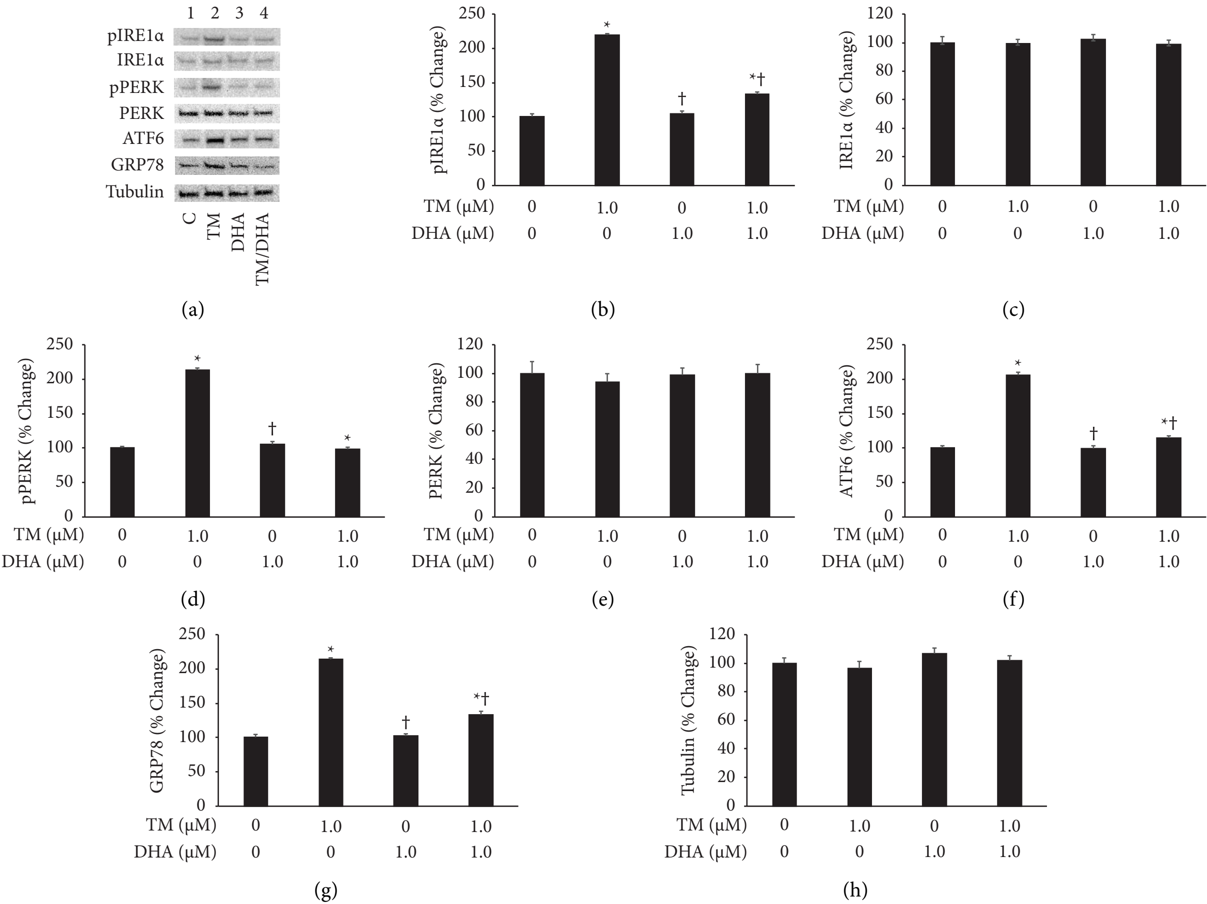

Figure 1: The effect of DHA on UPR marker expression and activation. HCAECs were treated with the solvent DMSO, 1.0 μM tunicamycin (TM), 1.0 μM DHA, or tunicamycin plus DHA (1.0 μM each) (TM/DHA) for 24 hours and protein extracts were prepared. Representative blots are shown in panel A and quantified in panel B through H phospho-IRE1α (b), IRE1α (c), phospho-PERK (d), PERK (e), ATF6 (f), GRP78 (g), and tubulin (h) expression were measured by Western blot. TM increased phospho-IRE1α and phospho-PERK levels and increased ATF6 and GRP78 expression, while treatment with DHA had no effect. In cells treated with TM and DHA, phospho-IRE1α and phospho-PERK levels decreased and ATF6 and GRP78 expression decreased relative to TM-treated cells. IRE1α, PERK, and tubulin expression did not change with any treatment. (a) Phospho-IRE1α. $N = 6$; $^{*}p < 0.0001$ and $p < 0.007$, respectively, relative to control cells; $^{\dagger}p < 0.0001$ and $p < 0.01$, respectively, relative to TM-treated cells. (b) IRE1α; $N = 6$. (c) Phospho-PERK; $N = 6$; $^{*}p < 0.0002$ and $p < 0.0001$, respectively, relative to control cells. $^{\dagger}p < 0.05$ relative to TM-treated cells. (d) PERK; $N = 6$. (e) ATF6; $N = 6$; $^{*}p < 0.0002$ and $p < 0.03$, respectively, relative to control cells. $^{\dagger}p < 0.0003$ and $p < 0.0004$, respectively, relative to TM-treated cells. (f) GRP78; $N = 6$; $^{*}p < 0.0001$ and $p < 0.0005$, respectively, relative to control cells. $^{\dagger}p < 0.05$ relative to TM-treated cells. (g) Tubulin; $N = 6$.

Tween 20 (50 mM Tris-Cl (pH 8.0), 150 mM NaCl, and 0.1% Tween 20) (TBST) containing 10% NCS (TBST/NCS), the membranes were incubated with antibodies to phospho-IRE1α, IRE1α, phospho-PERK, PERK, ATF6, and GRP78 (all diluted 1 : 1,000 in TBST/NCS) overnight at 4°C. After antibody exposure, the membranes were washed with TBST four times, five minutes each wash, and then incubated with a secondary antibody diluted 1 : 4,000 in TBST/NCS. After 45 minutes, membranes were washed four times with TBST, twice with Tris-buffered saline (TBS), five minutes each, and binding was detected with enhanced chemiluminescence (ECL) on a Protein Simple Fluor Chem E imaging system (Biotechne, San Jose, CA). Images were analyzed using Alpha View software (Biotechne, San Jose, CA) and quantified by densitometry using ImageJ (https://imagej.nih.gov/). After removing the primary/secondary antibodies, the stripped blots blocked with TBST/NCS and incubated with an antibody to measure tubulin expression.

2.6. Data Analysis and Statistics. The data are presented as the mean ± standard deviation. Statistical significance was assessed by analysis of variance (ANOVA) and Student's t-test for independent variables using Microsoft Excel. The Neuman–Keuls procedure for subgroup analysis and a Bonferroni correction for post hoc analysis were performed with Statistica for Windows (Statsoft Inc., Tulsa, OK). EC_{50} values were calculated with GraphPad Prism 10 (San Diego, CA). A $p < 0.05$ was considered significant.

3. Results

3.1. The Effect of DHA, EPA, and EPA-EE on Tunicamycin- and High-Dextrose-Induced ER Stress in HCAEC. HCAECs were transfected with 1 μg of pSEAP2.control and 24 hours later treated with either 5.5 mM (normal dextrose), 27.5 mM dextrose (high-dextrose), or 1.0 μM tunicamycin, with 0.001, 0.01, 0.1, 1.0, and 10 μM of DHA, EPA, or EPA-EE. After 24 hours, the conditioned medium was collected and the SAP activity was measured. Cell viability in the remaining cells was measured using the MTT assay. Tunicamycin suppressed the SAP activity by $35.2 \pm 7.3\%$ ($p < 0.0004$) relative to control cells (Table 1). HCAEC treatment with 0.001, 0.01, 0.1, 1.0, and 10 μM DHA increased SAP activity by $1.7 \pm 8.5\%$, $15.3 \pm 5.0\%$, $21.7 \pm 6.6\%$, $35.6 \pm 4.1\%$, and $36.7 \pm 5.7\%$, respectively (NS, $p < 0.007$, $p < 0.005$, $p < 0.0003$, and $p < 0.0007$, respectively).

In cells treated with 27.5 mM (high) dextrose, the SAP activity decreased by $27.7 \pm 5.5\%$ ($p < 0.002$ relative to control cells) (Table 1). The addition of 0.001, 0.01, 0.1, 1.0, and 10 μM DHA to cells exposed to 27.5 mM dextrose increased SAP activity in a dose-dependent manner ($-3.8 \pm 2.5\%$, $-2.8 \pm 5.1\%$, $10.3 \pm 2.1\%$, $28.5 \pm 5.8\%$, and $28.4 \pm 3.5\%$, respectively, in cells treated with DHA) (NS, NS$p < 0.003$, $p < 0.001$, $p < 0.001$, and $p < 0.0003$, respectively, relative to cells exposed to 27.5 mM dextrose alone) (Table 1).

Treatment with 0.001, 0.01, 0.1, 1.0, and 10 μM EPA increased the SAP activity by $-2.6 \pm 10.3\%$, $2.5 \pm 10.8\%$, $13.5 \pm 5.0\%$, $25.0 \pm 3.2\%$, and $31.2 \pm 7.5\%$, respectively, relative to cells treated with tunicamycin (NS, NS, $p < 0.02$, $p < 0.001$, and $p < 0.003$, respectively, relative to tunicamycin-treated cells).

In HCAEC exposed to 27.5 mM dextrose, the SAP activity decreased by $34.2 \pm 4.8\%$ ($p < 0.0006$ relative to cells exposed to 5.5 mM dextrose) (Table 1). In cells exposed to 27.5 mM dextrose and 0.001, 0.01, 0.1, 1.0, and 10 μM EPA, the SAP activity increased by $2.7 \pm 5.3\%$, $1.0 \pm 5.3\%$, $15.0 \pm 4.4\%$, $22.6 \pm 2.5\%$, and $25.8 \pm 3.9\%$ (NS, NS, $p < 0.007$, $p < 0.0006$, and $p < 0.0008$, respectively, relative to cells exposed to 27.5 mM dextrose).

As observed with DHA and EPA, EPA-EE increased the SAP activity in tunicamycin-treated cells in a dose-dependent manner (Table 1). In cells treated with tunicamycin, the SAP activity increased by $-3.7 \pm 5.4\%$, $0.9 \pm 5.0\%$, $16.4 \pm 4.5\%$, $24.3 \pm 3.9\%$, $24.5 \pm 2.9\%$, respectively, in cells treated with 0.001, 0.01, 0.1, 1.0, and 10 μM EPA-EE (NS, NS, $p < 0.002$, $p < 0.0005$, and $p < 0.0002$, respectively, relative to tunicamycin-treated cells). Similarly, in cells exposed to 27.5 mM dextrose, EPA-EE increased the SAP activity by $-3.2 \pm 3.8\%$, $-2.2 \pm 2.7\%$, $8.7 \pm 2.8\%$, $25.5 \pm 3.1\%$, and $23.1 \pm 2.7\%$, respectively (NS, NS, NS,$p < 0.0003$, and $p < 0.0004$, respectively, relative to cells exposed to 27.5 mM dextrose) (Table 1). In the absence of tunicamycin or high-dextrose concentrations, DHA, EPA, or EPA-EE had no effect on SAP activity (all NS relative to control cells) (Table 1). There were no changes in cell viability in any of these experiments (data not shown).

3.2. The Effect of DHA and Tunicamycin on UPR. HCAECs were treated with 1.0 μg/ml tunicamycin and 1.0 μM DHA for 24 hours, and protein extracts were prepared. Representative Western blots are shown in Figure 1(a) and quantified in Figures 1(b) to 1(h). Treatment with tunicamycin increased IRE1α phosphorylation (Figure 1(b)) and PERK phosphorylation (Figure 1(d)) $119.5 \pm 2.1\%$ and $113.4 \pm 3.0\%$, respectively ($p < 0.0001$ and $p < 0.0001$, respectively, relative to control cells) but had no effect on total IRE1α and PERK levels (Figures 1(c) and 1(e)). DHA treatment alone had no effect on IRE1α and PERK phosphorylation. However, DHA inhibited tunicamycin-induced phospho-IRE1α and phospho-PERK levels (86.9% and 115.7%, respectively, relative to tunicamycin-treated cells) ($p < 0.0001$ and $p < 0.0001$, respectively, relative to tunicamycin-treated cells).

In HCAEC treated with tunicamycin, ATF6 and GRP78 levels (Figures 1(f) and 1(g), respectively) increased $106.3 \pm 3.7\%$ and $113.9 \pm 2.6\%$, respectively ($p < 0.0002$ and $p < 0.0001$, respectively, relative to control cells). DHA treatment alone had no effect on ATF6 levels and GRP78 expression. DHA treatment, however, decreased ATF6 levels by 91.2% and GRP78 expression by 80.3% in tunicamycin-treated cells ($p < 0.0004$ and $p < 0.0005$, respectively, relative to tunicamycin-treated cells). These results as well as those described above suggest that DHA inhibits ER stress by suppressing the activities of key UPR mediators. There were no changes in tubulin expression (Figure 1(h)).

3.3. The Effect of Other Select Fatty Acids on SAP Activity in HCAEC. Since omega-3 FAs had such a favorable effect on ER stress initiated by tunicamycin and high dextrose, we examined the effects of other FAs on ER stress using the ES-TRAP assay. Cell viability was measured using the MTT assay. HCAECs were treated with 1.0 μM tunicamycin and 27.5 mM dextrose, with and without 15.6, 31.3, 62.5, 125, and 250 μM C14 : 0, C16 : 0, and C18 : 0 (all saturated FAs), elaidic acid (a trans-FA), oleic acid (a monounsaturated FA), or arachidonic acid (a polyunsaturated FA), and the SAP activity and MTT activity were measured 24 hours later. At higher concentrations, all three saturated FAs suppressed SAP activity (Table 2). In cells treated with C14 : 0, cell viability was unaffected (data not shown). In cells treated with C16 : 0 with and without tunicamycin, cell toxicity was evident; however, there were no changes in cell viability in cells treated with C16 : 0 and high dextrose (data not shown). Cell viability also decreased in cells exposed to elevated C18 : 0 concentrations (data not shown). Elaidic acid suppressed the SAP activity as well as cell viability at higher concentrations similar to the saturated fatty acids (Table 3 and data not shown). In contrast, oleic acid and arachidonic acid (Table 3) both increased the SAP activity but did so only at the higher concentrations. There was no toxicity associated with either oleic acid or arachidonic acid treatment (data not shown).

Table 1: The effect of omega-3 fatty acids on secreted alkaline phosphatase (SAP) activity in human coronary artery endothelial cells (HCAECs) treated with tunicamycin (TM), normal dextrose (5.5 mM), or high dextrose (27.5 mM).

Lipid treatment	Concentration (μM)	SAP activity (%) control	SAP activity (%) TM (1.0 μM)	SAP activity (%) dextrose (5.5 mM)	SAP activity (%) dextrose (27.5 mM)
DHA	0	100.0 ± 3.3	64.8 ± 7.3*	100 ± 5.3	72.3 ± 5.5†
	0.001	101.9 ± 2.7	66.5 ± 8.5*	101.8 ± 3.6	68.5 ± 2.5†
	0.01	101.0 ± 2.2	80.1 ± 5.0*	101.2 ± 2.8	69.5 ± 5.1†
	0.1	99.8 ± 2.7	86.5 ± 6.6*	101.0 ± 3.0	82.6 ± 2.1†
	1.0	102.6 ± 4.6	100.4 ± 4.1	99.9 ± 5.1	100.8 ± 5.8
	10	98.7 ± 4.6	101.5 ± 5.7	97.7 ± 4.3	100.7 ± 3.5
EPA	0	100.0 ± 4.2	68.0 ± 4.0*	100.0 ± 5.0	65.8 ± 4.8†
	0.001	100.9 ± 2.8	65.4 ± 10.3*	98.6 ± 5.0	68.5 ± 5.3†
	0.01	98.7 ± 1.4	70.5 ± 10.8*	98.6 ± 3.0	66.8 ± 5.3†
	0.1	100.0 ± 3.7	81.5 ± 5.0*	98.9 ± 3.9	80.8 ± 4.4†
	1.0	100.4 ± 3.4	93.0 ± 3.2	95.1 ± 5.1	88.4 ± 2.5†
	10	101.1 ± 2.4	99.2 ± 7.5	97.0 ± 6.3	91.6 ± 3.9
EPA-EE	0	100.0 ± 2.5	74.5 ± 2.6*	100.0 ± 3.4	76.3 ± 3.2†
	0.001	100.2 ± 2.1	70.8 ± 5.4*	100.5 ± 2.5	73.1 ± 3.8†
	0.01	101.0 ± 1.4	75.4 ± 5.0*	100.3 ± 4.0	74.1 ± 2.7†
	0.1	99.3 ± 2.0	90.9 ± 4.5	99.2 ± 3.9	85.0 ± 2.8
	1.0	100.1 ± 2.5	98.7 ± 3.9	99.9 ± 1.5	101.8 ± 3.1
	10	101.6 ± 1.8	99.0 ± 2.9	99.9 ± 1.9	99.4 ± 2.7

HCAECs were treated with 1.0 μM tunicamycin, 5.5 mM dextrose (normal dextrose), or 27.5 mM dextrose (high dextrose) with and without the indicated fatty acids for 24 hours, and secreted alkaline phosphatase (SAP) activity and cell viability were measured after 24 hours using the endoplasmic reticulum stress-responsive alkaline phosphatase (ES-TRAP assay) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, respectively. The mean SAP activity for the control and each treatment group and % change was calculated. Statistical significance was calculated from the mean SAP activity by ANOVA followed by Student's t-test for independent variables as described in the methods section. DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; EPA-EE, eicosapentaenoic acid ethyl ester. $N = 6$; $^{*}p < 0.05$ relative to control cells; $^{\dagger}p < 0.05$ relative to cells exposed to 5.5 mM dextrose.

4. Discussion

Previously published studies in HUVEC suggested that both saturated FAs (C18 : 0, C16 : 0, and C14 : 0) and trans-FAs (elaidic acid, linoelaidic acid, and linolenelaidic acid) augment the effects of high dextrose on reactive oxygen species formation [8]. Others have shown that C16 : 0 (palmitic acid) and C18 : 0 (stearic acid) promote both ER stress and programmed cell death by apoptosis in a number of cell types [9, 20–22]. Due to recent observations that endothelial cells derived from various organs have unique tissue-specific properties [23, 24], we chose the HCAEC to examine the effect of select FA classes on ER stress, an essential component of the integrated stress response [25].

In the absence of ER stress, DHA, EPA, and EPA-EE treatment had no effect on SAP activity in HCAEC. However, in cells treated with tunicamycin or exposed to 27.5 mM dextrose, all three omega-3 FAs suppressed ER stress in a dose-dependent manner (Table 1). The EC_{50} values for DHA in HCAEC treated with tunicamycin and high dextrose were 33.3 μM and 12.5 μM, respectively. The EC_{50} values for EPA in HCAEC treated with tunicamycin and high dextrose were 58.3 μM and 66.2 μM, respectively. The EC_{50} values for EPA-EE in HCAEC treated with tunicamycin and high dextrose were 53.2 μM and 61.9 μM, respectively. Importantly, none of these FAs impacted cell viability. Furthermore, DHA not only suppressed SAP activity but also lowered phospho-IRElα and phospho-PERK levels and decreased ATF6 and GRP78 expression in tunicamycin-treated cells (Figure 1). In contrast, C18 : 0, C16 : 0, and C14 : 0 all suppressed SAP activity at high concentrations, though some of the observed effects with the saturated FAs in this study could have been exaggerated due to cytotoxicity (Table 2). Of note, oleic acid and arachidonic acid both increased SAP activity at the highest concentrations examined (Table 3). The EC_{50} values for oleic acid in tunicamycin- and high-dextrose-treated cells were 107 μM and 127 μM, respectively, while the EC_{50} values for arachidonic acid in tunicamycin- and high dextrose-treated cells were 870 μM and 720 μM, respectively.

Five of the FAs tested (DHA, EPA, EPA-EE, arachidonic acid, and oleic acid) suppressed ER stress in HCAECs treated with both tunicamycin and high dextrose (measured using the ES-TRAP assay). Furthermore, treatment with DHA normalized activities and expression of several UPR-related markers (Figure 1). The EC_{50} values for these FAs indicate that the omega-3 fatty acids, DHA, EPA, and EPA-EE, are quite potent ER stress inhibitors, while oleic acid and arachidonic acid are less so. This may be due to the fact that the omega-3 FAs tested can be metabolized (oxidized) by certain cytochrome P450 monooxygenases [26, 27] generating several metabolites that are also ER stress inhibitors [28]. The effects of saturated FAs as well as elaidic acid on the SAP activity required substantially higher concentrations, and calculating meaningful IC_{50} values for these compounds was not possible since there was also cell toxicity associated with exposure to these FAs.

Omega-3 fatty FAs have been shown in multiple studies to reverse cellular dysfunction due to high glucose and hyperlipidemia [29, 30]. In HUVEC, treatment with DHA

Table 2: The effect of saturated fatty acids on secreted alkaline phosphatase (SAP) activity in human coronary artery endothelial cells (HCAECs) treated with tunicamycin (TM), normal dextrose (5.5 mM), or high dextrose (27.5 mM).

Lipid treatment	Concentration (μM)	SAP activity (%) control	SAP activity (%) TM (1.0 μM)	SAP activity (%) dextrose (5.5 mM)	SAP activity (%) dextrose (27.5 mM)
C14 : 0	0	100.0 ± 7.2	45.7 ± 7.3*	100.0 ± 3.5	70.3 ± 6.0†
	15.6	99.5 ± 4.8	52.2 ± 7.2*	99.5 ± 1.9	69.7 ± 2.6†
	31.3	99.6 ± 5.0	52.6 ± 7.3*	100.8 ± 4.1	70.6 ± 4.3†
	62.5	99.4 ± 6.8	52.5 ± 4.7*	99.4 ± 3.0	69.2 ± 4.5†
	125	100.6 ± 5.3	50.4 ± 4.1*	100.5 ± 2.0	69.7 ± 5.7†
	250	84.3 ± 6.4*	41.6 ± 5.8*	93.6 ± 1.7	62.8 ± 4.3†
C16 : 0	0	100.0 ± 2.2	51.7 ± 8.4*	100.0 ± 6.7	69.6 ± 5.9†
	15.6	100.6 ± 2.0	59.0 ± 9.0*	96.7 ± 5.6	71.0 ± 7.8†
	31.3	99.7 ± 2.8	45.1 ± 6.7*	96.4 ± 5.1	71.6 ± 3.4†
	62.5	78.7 ± 6.0*	29.2 ± 10.0*	96.6 ± 3.6	69.0 ± 2.7†
	125	49.6 ± 9.5*	29.8 ± 5.6*	96.9 ± 4.0	69.4 ± 7.5†
	250	34.5 ± 11.1*	22.1 ± 15.1*	81.4 ± 1.9†	58.1 ± 6.7†
C18 : 0	0	100.0 ± 2.7	45.9 ± 6.8*	100.0 ± 3.2	60.7 ± 4.5†
	15.6	100.9 ± 1.7	43.6 ± 9.5*	103.1 ± 5.6	61.3 ± 2.5†
	31.3	100.6 ± 3.5	46.1 ± 9.4*	97.8 ± 4.1	63.1 ± 3.7†
	62.5	78.7 ± 7.0*	39.6 ± 5.7*	101.8 ± 4.0	58.6 ± 3.2†
	125	68.1 ± 2.1*	25.4 ± 8.6*	100.7 ± 4.2	44.1 ± 2.9†
	250	60.0 ± 4.3*	24.1 ± 6.5*	98.1 ± 2.7	41.4 ± 3.5†

HCAECs were treated with 1.0 μM tunicamycin, 5.5 mM dextrose (normal dextrose), or 27.5 mM dextrose (high dextrose) with or without the indicated fatty acids for 24 hours, and secreted alkaline phosphatase (SAP) activity and cell viability were measured using the endoplasmic reticulum stress-responsive alkaline phosphatase (ES-TRAP assay) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, respectively. The mean SAP activity for the control and each treatment group and % change was calculated. Statistical significance was calculated from the mean SAP activity by ANOVA followed by Student's t-test for independent variables as described in the methods section. $N = 6$; $^{*}p < 0.05$ relative to control cells; $^{\dagger}p < 0.05$ relative to cells exposed to 5.5 mM dextrose.

Table 3: The effect of arachidonic acid, elaidic acid, and oleic acid on secreted alkaline phosphatase (SAP) activity in human coronary artery endothelial cells (HCAEC) treated with tunicamycin (TM), normal dextrose (5.5 mM), or high dextrose (27.5 mM).

Lipid treatment	Concentration (μM)	SAP activity (%) control	SAP activity (%) TM (1.0 μM)	SAP activity (%) dextrose (5.5 mM)	SAP activity (%) dextrose (27.5 mM)
Arachidonic acid	0	100.0 ± 4.0	63.5 ± 4.8*	100.0 ± 3.3	72.8 ± 5.7†
	15.6	100.8 ± 3.6	61.6 ± 5.4*	103.6 ± 3.2	71.3 ± 6.1†
	31.3	102.4 ± 3.4	59.7 ± 7.0*	103.3 ± 3.6	71.1 ± 5.7†
	62.5	102.1 ± 3.6	61.1 ± 4.6*	103.6 ± 3.3	69.6 ± 8.1†
	125	101.0 ± 3.3	69.6 ± 5.4*	102.9 ± 2.4	78.1 ± 4.9†
	250	102.6 ± 3.1	75.6 ± 6.2*	102.9 ± 3.1	82.2 ± 3.8†
Elaidic acid	0	100.0 ± 3.7	46.6 ± 7.1*	100.0 ± 3.5	71.5 ± 4.0†
	15.6	99.7 ± 3.4	49.8 ± 5.1*	99.0 ± 2.6	69.2 ± 4.9†
	31.3	101.4 ± 3.3	48.1 ± 4.7*	101.5 ± 3.2	69.5 ± 3.9†
	62.5	88.3 ± 7.9	42.7 ± 11.5*	93.1 ± 5.4	66.3 ± 4.7†
	125	78.3 ± 3.7*	35.7 ± 6.0*	85.5 ± 5.0†	59.5 ± 5.8†
	250	75.5 ± 2.6*	34.2 ± 6.7*	82.6 ± 4.6†	55.1 ± 5.0†
Oleic acid	0	100.0 ± 3.8	56.8 ± 7.1*	100.0 ± 3.5	71.2 ± 5.6†
	15.6	100.3 ± 4.2	59.6 ± 7.0*	99.6 ± 2.4	70.4 ± 3.7†
	31.3	99.4 ± 3.7	58.9 ± 6.4*	98.7 ± 1.7	72.8 ± 4.4†
	62.5	99.5 ± 3.7	60.6 ± 6.5*	92.7 ± 10.6	73.6 ± 6.7†
	125	100.3 ± 3.9	70.8 ± 4.0*	97.2 ± 1.9	86.3 ± 4.5†
	250	101.5 ± 5.1	73.1 ± 3.3*	99.4 ± 3.3	88.8 ± 2.6†

HCAECs were treated with 1.0 μM tunicamycin, 5.5 mM dextrose (normal dextrose), or 27.5 mM dextrose (high dextrose) with or without the indicated fatty acids for 24 hours, and secreted alkaline phosphatase (SAP) activity and cell viability were measured using the endoplasmic reticulum stress-responsive alkaline phosphatase (ES-TRAP assay) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, respectively. The mean SAP activity for the control and each treatment group and % change was calculated. Statistical significance was calculated from the mean SAP activity by ANOVA followed by Student's t-test for independent variables as described in the methods section. $N = 6$; $^{*}p < 0.05$ relative to control cells; $^{\dagger}p < 0.05$ relative to cells exposed to 5.5 mM dextrose.

and insulin suppressed C16:0-induced intracellular lipid accumulation as well as apoptosis [29]. DHA and insulin treatment also suppressed tumor necrosis factor α, interleukin 6, and nuclear factor-κB expression, as well as atherosclerosis-related gene expression [29]. In cardiomyocytes, DHA treatment suppressed hypoxia-induced apoptosis, in part by inducing microRNA-210-39 levels, inhibiting caspase-8-associated protein 2 gene expression [30]. DHA, EPA, and EPA-EE have been shown in a few clinical trials to prevent cardiovascular disease. A recent meta-analysis of the effects of DHA and EPA on various cardiovascular risk factors concluded that DHA and EPA reduce low-density lipoprotein-cholesterol and plasma glucose and insulin levels [31]. A second meta-analysis examined 17 randomized clinical trials of the effects of EPA, EPA plus DHA, mineral oil placebo, corn oil placebo, olive oil placebo and no oil placebo on cardiovascular death, myocardial infarction, and stroke [32]. All three of these outcomes were lower in those receiving EPA relative to those receiving mineral oil, but there were no differences with those receiving the other oils as well as the no oil controls [32]. Revascularization rates were significantly lower in those receiving EPA than in those treated with EPA plus DHA and all the controls [32]. Other studies with mixed EPA/DHA formulations have not proven to be beneficial [33, 34]. EPA-EE received Food and Drug Administration approval after the REDUCE-IT trial [35]. In this trial, the administration of 4 mg/day icosapent ethyl (EPA-EE) reduced triglyceride levels and lowered the incidence of cardiovascular events by 25% in high- and very high-risk patients already on statin therapy. However, the comparator in this trial, a mineral oil-based placebo, may have been problematic. Nevertheless, a recent analysis of commonly used placebos casts doubt on the latter hypothesis [36]. A recent head-to-head trial comparing EPA – EE to EPA/DHA plus free fatty acid (FFA) (EPA + DHA – FFA) demonstrated that the latter treatment elevated plasma EPA and DHA levels more than EPA – EE, likely due to greater intestinal absorption [37]. EPA + DHA – FFA reduced triglyceride and high-sensitivity C-reactive protein levels [37].

The cells used were primary human coronary artery endothelial cells that play a critical role in the progression of atherosclerosis. The results provide important evidence that omega-3 FA-mediated ER stress inhibition may be an important factor in preventing atherosclerosis and cardiovascular disease, in part by inhibiting ER stress.

5. Conclusion

Omega-3 FAs reduce ER stress in coronary artery endothelial cells, and as such, they may reduce the risk of premature CAD. The polyunsaturated fatty acids such as oleic acid and arachidonic acid also suppress ER stress, although at much higher concentrations than omega-3 FAs. In contrast, saturated FAs as well as the trans-FA elaidic acid aggravate ER stress, decreasing endothelial cell survival. This latter observation suggests that high plasma saturated FA levels, either due to disease or dietary factors, may promote endothelial cell dysfunction via prolonged ER stress and apoptosis. In contrast, the three omega-3 fatty acids examined here have beneficial effects, reversing both tunicamycin- and high dextrose-induced ER stress, promoting endothelial cell homeostasis. These novel findings imply that diets rich in omega-3 FAs or certain fish oil supplements or pharmaceuticals may prove useful in preventing CAD.

Data Availability

The data used to support the findings of this study are available upon request from the corresponding author.

Disclosure

This research was performed at the University of Florida-Jacksonville.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

References

[1] J. E. Manson, G. A. Colditz, M. J. Stampfer et al., “A prospective study of obesity and risk of coronary heart disease in women,” *New England Journal of Medicine*, vol. 322, no. 13, pp. 882–889, 1990.

[2] S. Kenchaiah, J. C. Evans, D. Levy et al., “Obesity and the risk of heart failure,” *New England Journal of Medicine*, vol. 347, no. 5, pp. 305–313, 2002.

[3] A. D. Mooradian, “Dyslipidemia in type 2 diabetes mellitus,” *Nature Reviews Endocrinology*, vol. 5, no. 3, pp. 150–159, 2009.

[4] H. Qiu, L. Ma, and F. Feng, “PICK1 attenuates high glucose-induced pancreatic β-cell death through the PI3K/AKT pathway and is negatively regulated by miR-139-5p,” *Biochemical and Biophysical Research Communications*, vol. 522, no. 1, pp. 14–20, 2020.

[5] Y. Wang, T. Xie, D. Zhang, and P. S. Leung, “GPR120 protects lipotoxicity-induced pancreatic β-cell dysfunction through regulation of PDX1 expression and inhibition of islet inflammation,” *Clinical Science*, vol. 133, no. 1, pp. 101–116, 2019.

[6] Y. E. Cho, A. Basu, A. Dai, M. Heldak, and A. Makino, “Coronary endothelial dysfunction and mitochondrial reactive oxygen species in type 2 diabetic mice,” *American Journal of Physiology Cell Physiology*, vol. 305, no. 10, pp. C1033–C1040, 2013.

[7] S. H. Wilson, N. M. Caplice, R. D. Simari, D. R. Holmes Jr, P. J. Carlson, and A. Lerman, “Activated nuclear factor-κB is present in the coronary vasculature in experimental hypercholesterolemia,” *Atherosclerosis*, vol. 148, no. 1, pp. 23–30, 2000.

[8] M. H. Horani, M. J. Haas, and A. D. Mooradian, “Saturated, unsaturated, and trans-fatty acids modulate oxidative burst induced by high dextrose in human umbilical vein endothelial cells,” *Nutrition*, vol. 22, no. 2, pp. 123–127, 2006.

[9] W. Chai and Z. Liu, “p38 mitogen-activated protein kinase mediates palmitate-induced apoptosis but not inhibitor of nuclear factor-κb degradation in human coronary artery endothelial cells,” *Endocrinology*, vol. 148, no. 4, pp. 1622–1628, 2007.

[10] I. M. Ibrahim, D. H. Abdelmalek, and A. A. Elfiky, "GRP78: a cell's response to stress," *Life Sciences*, vol. 226, pp. 156–163, 2019.

[11] S.-M. Park, T.-I. Kang, and J.-S. So, "Roles of XBP1s in transcriptional regulation of target genes," *Biomedicines*, vol. 9, no. 7, p. 791, 2021.

[12] H. Yoshida, T. Matsui, A. Yamamoto, T. Okada, and K. Mori, "XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor," *Cell*, vol. 107, no. 7, pp. 881–891, 2001.

[13] M. J. Haas, V. Feng, K. Gonzales, P. Bikkina, M. Angelica-Landicho, and A. D. Mooradian, "Transcription factor EB protects against endoplasmic reticulum stress in human coronary artery endothelial cells," *European Journal of Pharmacology*, vol. 933, Article ID 175274, 2022.

[14] I. Tabas and D. Ron, "Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress," *Nature Cell Biology*, vol. 13, no. 3, pp. 184–190, 2011.

[15] X. Zheng, Q. W. C. Ho, M. Chua et al., "Destabilization of β cell FIT2 by saturated fatty acids alter lipid droplet numbers and contribute to ER stress and diabetes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 119, no. 11, Article ID e2113074119, 2022.

[16] M. Cnop, "Fatty acids and glucolipotoxicity in the pathogenesis of Type 2 diabetes," *Biochemical Society Transactions*, vol. 36, no. 3, pp. 348–352, 2008.

[17] N. Hiramatsu, A. Kasai, K. Hayakawa, J. Yao, and M. Kitamura, "Real-time detection and continuous monitoring of ER stress *in vitro* and in vivo by ES-TRAP: evidence for systemic, transient ER stress during endotoxemia," *Nucleic Acids Research*, vol. 34, no. 13, p. e93, 2006.

[18] A. A. van de Loosdrecht, R. H. J. Beelen, G. J. Ossenkoppele, M. G. Broekhoven, and M. M. A. C. Langenhuijsen, "A tetrazolium-based colorimetric MTT assay to quantitate human monocyte mediated cytotoxicity against leukemic cells from cell lines and patients with acute myeloid leukemia," *Journal of Immunological Methods*, vol. 174, no. 1-2, pp. 311–320, 1994.

[19] P. K. Smith, R. I. Krohn, G. T. Hermanson et al., "Measurement of protein using bicinchoninic acid," *Analytical Biochemistry*, vol. 150, no. 1, pp. 76–85, 1985.

[20] V. Nemcova-Furstova, R. F. James, and J. Kovar, "Inhibitory effect of unsaturated fatty acids on saturated fatty acid-induced apoptosis in human pancreatic β-cells: activation of caspases and ER stress induction," *Cellular Physiology and Biochemistry*, vol. 27, no. 5, pp. 525–538, 2011.

[21] J. Sramek, V. Nemcova-Furstova, N. Pavlíkova, and J. Kovar, "Effect of saturated stearic acid on MAP kinase and ER stress signaling pathways during apoptosis induction in human pancreatic β-cells is inhibited by unsaturated oleic acid," *International Journal of Molecular Sciences*, vol. 18, no. 11, p. 2313, 2017.

[22] J. Sramek, V. Nemcova-Furstova, and J. Kovar, "Molecular mechanisms of apoptosis induction and its regulation by fatty acids in pancreatic β-cells," *International Journal of Molecular Sciences*, vol. 22, no. 8, p. 4285, 2021.

[23] S. Khan, F. Taverna, K. Rohlenova et al., "EndoDB: a database of endothelial cell transcriptomics data," *Nucleic Acids Research*, vol. 47, no. D1, pp. D736–D744, 2019.

[24] N. Ricard, S. Bailly, C. Guignabert, and M. Simons, "The quiescent endothelium: signalling pathways regulating organ-specific endothelial normalcy," *Nature Reviews Cardiology*, vol. 18, no. 8, pp. 565–580, 2021.

[25] M. Costa-Mattioli and P. Walter, "The integrated stress response: from mechanism to disease," *Science*, vol. 368, no. 6489, Article ID eaat5314, 2020.

[26] B. Fisslthaler, R. Popp, L. Kiss et al., "Cytochrome P450 2C is an EDHF synthase in coronary arteries," *Nature*, vol. 401, no. 6752, pp. 493–497, 1999.

[27] S. Diani-Moore, Y. Ma, S. S. Gross, and A. B. Rifkind, "Increases in levels of epoxyeicosatrienoic and dihydroxyeicosatrienoic acids (EETs and DHETs) in liver and heart *in vivo* by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and in hepatic EET:DHET ratios by cotreatment with TCDD and the soluble epoxide hydrolase inhibitor AUDA," *Drug Metabolism and Disposition*, vol. 42, no. 2, pp. 294–300, 2014.

[28] B. Inceoglu, A. Bettaieb, F. G. Haj, A. V. Gomes, and B. D. Hammock, "Modulation of mitochondrial dysfunction and endoplasmic reticulum stress are key mechanisms for the wide-ranging actions of epoxy fatty acids and soluble epoxide hydrolase inhibitors," *Prostaglandins & Other Lipid Mediators*, vol. 133, pp. 68–78, 2017.

[29] A. E. Abriz, R. Rahbarghazi, A. Nourazarian et al., "Effect of docosahexaenoic acid plus insulin on atherosclerotic human endothelial cells," *Journal of Inflammation*, vol. 18, no. 1, p. 10, 2021.

[30] X. Yu, F. Liu, Y. Liu et al., "Omega-3 fatty acid protects cardiomyocytes against hypoxia-induced injury through targeting MiR-210-3p/CASP8AP2 axis," *Molecular and Cellular Biochemistry*, vol. 476, no. 8, pp. 2999–3007, 2021.

[31] S. Fatahi, M. H. Sohouli, E. I. da Silva Magalhães et al., "Comparing the effects of docosahexaenoic and eicosapentaenoic acids on cardiovascular risk factors: pairwise and network meta-analyses of randomized controlled trials," *Nutrition, Metabolism, and Cardiovascular Diseases*, vol. 33, no. 1, pp. 11–21, 2023.

[32] Y. Yokoyama, T. Kuno, S. X. Morita et al., "Eicosapentaenoic acid for cardiovascular events reduction- systematic review and network meta-analysis of randomized controlled trials," *Journal of Cardiology*, vol. 80, no. 5, pp. 416–422, 2022.

[33] P. L. Myhre, A. A. Kalstad, S. H. Tveit et al., "Changes in eicosapentaenoic acid and docosahexaenoic acid and risk of cardiovascular events and atrial fibrillation: a secondary analysis of the OMEMI trial," *Journal of Internal Medicine*, vol. 291, no. 5, pp. 637–647, 2022.

[34] S. J. Nicholls, A. M. Lincoff, M. Garcia et al., "Effect of high-dose omega-3 fatty acids vs corn oil on major adverse cardiovascular events in patients at high cardiovascular risk: the STRENGTH randomized clinical trial," *JAMA*, vol. 324, no. 22, pp. 2268–2280, 2020.

[35] D. L. Bhatt, P. G. Steg, M. Miller et al., "Cardiovascular risk reduction with icosapent ethyl for hypertriglyceridemia," *New England Journal of Medicine*, vol. 380, no. 1, pp. 11–22, 2019.

[36] B. Olshansky, M. K. Chung, M. J. Budoff et al., "Mineral oil: safety and use as placebo in REDUCE-IT and other clinical studies," *European Heart Journal Supplements*, vol. 22, pp. J34–J48, 2020.

[37] K. C. Maki, H. E. Bays, C. M. Ballantyne et al., "A head-to-head comparison of a free fatty acid formulation of omega-3 pentaenoic acids versus icosapent ethyl in adults with hypertriglyceridemia: the ENHANCE-IT study," *Journal of the American Heart Association*, vol. 11, no. 6, Article ID e024176, 2022.