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ORIGINAL CONTRIBUTION

Effect of docosahexaenoic acid on hypoxia/reoxygenation injury in human coronary arterial smooth muscle cells

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Abstract
Purpose Hypoxia and reoxygenation (H/R) occur in a wide variety of important clinical conditions such as myocardial infarction. H/R injury is a complex phenomenon involving not only intracellular damage processes but also an injurious inflammatory response. Docosahexaenoic acid (DHA), an n-3 polyunsaturated fatty acid, has long been proved to be protective against several types of cardiovascular disease. However, its beneficial effect during H/R is inconclusive. In this study, we employed an in vitro model to examine whether DHA is protective against H/R-induced cell damage in human coronary artery smooth muscle cells (HCASMCs).
Methods HCASMCs in the absence or presence of DHA (1, 3, 10, and 30 μM) were subjected to control or H/R treatment using a modular incubator chamber to create hypoxic condition. Cell viability was evaluated by MTT assay. Spectrophotometric and spectrofluorometric assays were used to measure the generation of nitric oxide (NO) and reactive oxygen species (ROS), respectively. Inflammatory cytokines were determined by enzyme-linked immunosorbent assay. Intracellular calcium mobilization was estimated microfluorimetrically using calcium indicator dye, fura 2-acetomethyl ester.
Results Hypoxia/reoxygenation caused significant injury in cultured HCASMCs. DHA at low concentrations (1, 3, and 10 μM) did not afford protection, whereas at 30 μM, it caused deleterious effects, presumably by enhancing the production of NO, ROS, IL-1β, and IL-6 and altering the intracellular calcium dynamics.
Conclusions Our results do not support the protective function of DHA in H/R-injured coronary arterial smooth muscle cells.

Keywords Hypoxia/reoxygenation · Coronary artery · Smooth muscle cells · Docosahexaenoic acid

Introduction

The heart is highly vulnerable to restriction of blood supply, which usually occurs in the setting of atherosclerotic coronary artery disease. Reduced blood flow produces hypoxia in the tissues downstream of the lesion, and complete occlusion leads to severe hypoxia that threatens the viability of the myocardium and ultimately cardiac function. Subsequent reoxygenation may subject cells to further damages [1]. The hypoxia/reoxygenation (H/R)-induced cell injury is a complex, multifactorial pathophysiological process, which mainly involves the actions of nitric oxide (NO), reactive oxygen species (ROS), and various cytokines such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α [2, 3].

Dietary lipids have been well known to modulate the incidence and severity of cardiovascular diseases. Although not without controversy, numerous experimental

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studies and clinical intervention trials have demonstrated a protective role of dietary fish oils and n-3 polyunsaturated fatty acids (PUFAs) on coronary artery diseases [4–6]. The beneficial effects have been attributed to the improvement of vascular endothelial cell function, enhancement of vascular reactivity and compliance, modulation of lipid metabolism, reduction of inflammatory cytokine production, etc. [7–9]. Docosahexaenoic acid (DHA, C22:6n-3), a major n-3 PUFA, is generally acknowledged as a physiologically active component of these effects. The cellular and molecular mechanisms underlying the beneficial effects of DHA on various cell types, especially cardiomyocytes, vascular endothelial, and smooth muscle cells, have been extensively studied. Compared to endothelial cells, vascular smooth muscle cells have drawn much less attention, despite their vital roles in the vascular pathophysiology. Furthermore, the finding of endothelium-independent vascular effects of DHA indicates that the protective effects might also be attributable to their direct actions on vascular smooth muscle cells [8].

The benefits of n-3 PUFAs on ischemia/reperfusion (I/R)- or H/R-induced cell injury have been supported by substantial evidence [4, 10, 11]. Nevertheless, an adverse effect of DHA on postischemic functional recovery in perfused rat hearts was noted [12]. Also, long-term supplementation with DHA did not protect cardiomyocytes from H/R-induced injury [13], an observation which is similar to that reported by other investigators [14]. These inconclusive data, together with the vital role of coronary vascular smooth muscle cells in the pathophysiology of cardiovascular diseases, prompted us to examine whether DHA exerts protective actions on human coronary artery smooth muscle cells (HCASMCs) in response to H/R insult.

Materials and methods

Chemicals

All chemicals were purchased from Sigma Chemical Company, St. Louis, MO, USA.

Cell culture and treatment

HCASMCs were purchased form Cell Applications, Inc. (San Diego, CA, USA) and cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics. Cells were seeded in 24- or 48-well plates and allowed to grow to confluence. Prior to the start of experiment, cells were incubated overnight in 2% FBS-supplemented DMEM, and then the medium was replaced with fresh medium containing various concentrations (0, 1, 3, 10, and 30 μM) of DHA. DHA (sodium salt) was dissolved in 95% ethanol to prepare 0.1 M stock solution, and the maximum final concentration of ethanol in the culture medium did not exceed 0.03% (v/v). The cultures were placed into a modular incubator chamber (Billups-Rothenberg, Del Mar, CA, USA) that was sealed and flushed, for 30 min, with 95% N_2/5% CO_2 at a rate of 2 L/min. The hypoxic chamber was then sealed tightly and placed in an incubator (at 37 °C in humidified 5% CO_2/95% air) for 48 h. Using this method, the O_2 concentration of the culture media was measured to be 0.8–1.0% with a Dissolved Oxygen Meter (Model 5509, Lutron, Taiwan), significantly lower than that of the normoxic cultures (5–6%). At the completion of hypoxic exposure, half of the cultures were removed from the chamber and processed for analysis as described below. For the "hypoxia/reoxygenation" treatment, the other half of the cultures were removed from the chamber and maintained in the regular incubator under normal incubation conditions for 72-h reoxygenation period. "Normoxia" cultures were incubated under normoxic conditions (95% ambient air–5% CO_2) for the corresponding periods. H48 indicates hypoxia for 48 h. H48/R72 represents hypoxia for 48 h with additional 72 h of reoxygenation. N48 and N(48 + 72) are the corresponding normoxic controls of H48 and H48/R72, respectively.

Cell counting

Cells were harvested and counted in an improved Neubauer hemacytometer chamber using the trypan blue exclusion stain.

3-(4,5-Dimethylthianol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) reduction assay

Cell viability at the end of each experiment was analyzed by a MTT reduction assay. This assay is based upon the capacity of mitochondrial enzymes to transform MTT to MTT formazan. Briefly, MTT stock solution (5 mg/mL in PBS) was added to each well of the multi-well-plate containing cells to a final concentration of 0.5 mg/mL. Following incubation at 37 °C for a period of 2 h, the medium was aspirated, and an equal volume of dimethyl sulfoxide (DMSO) was added to each well in order to dissolve the reduced MTT formazan crystals. The absorbance of the color product at 570 nm against the 630 nm reference was measured using a microplate reader. All experiments were performed in triplicate.

Measurement of nitrite level

NO production by cultured cells was determined by the measurement of total nitrite, a stable oxidation product of

NO. The level of total nitrite was determined in serum-free media by means of a spectrophotometric assay based upon the Griess reaction. Griess reagent consists of 0.1% *N*-(1-naphthyl) ethylene diamide dihydrochloride, 1% sulfanilic acid, and 2.5% phosphoric acid. Duplicate aliquots (100 μL) of culture medium were removed and mixed with an equal volume of Griess reagent and allowed to stay 30 min at room temperature. The absorbance was measured at 550 nm. Nitrite concentration was determined by using sodium nitrite as standard.

Measurement of IL-1β, IL-6, and TNF-α levels

Levels of IL-1β, IL-6, and TNF-α in the culture media were measured by use of enzyme-linked immunosorbent assay (ELISA) kits (R&D Inc., CA, USA). This assay was based upon the quantitative sandwich-enzyme-immunoassay principle using antibodies directed against rat IL-1β, IL-6, and TNF-α, respectively.

Determination of intracellular ROS production

The production of ROS was measured spectrofluorometrically by using the probe of 2′,7′-dichlorodihydrofluorescein-diacetate (H_2DCF-DA). Cells were washed and then incubated at 37 °C for 30 min with 100 μM H_2DCF-DA, which is converted to H_2DCF by intracellular esterase. The H_2DCF was then oxidized by ROS to the highly fluorescent DCF. Fluorescence was measured by assaying in a microplate reader using excitation/emission wavelengths of 485/530 nm (FluoroskanAscent FL, ThermoFisher, MA, USA).

Ca^{2+} measurement

Cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_i$) were measured by the microfluorimetric technique, as described by Jin et al. [15]. Briefly, cells cultured on coverslips were washed twice with a loading buffer containing (in mM) 150 NaCl, 5 KCl, 5 glucose, 1 $MgCl_2$, 2.2 $CaCl_2$, and 10 HEPES (pH 7.4) and then incubated with 2.5 μM fura 2-acetomethyl ester (fura 2-AM) in the same buffer at 37 °C for 40 min. After loading, cells were washed to remove the excess dye and incubated at 37 °C for 30 min. The coverslip was then mounted in a modified Cunningham chamber attached to the stage of an inverted microscope. The fluorescence of the fura 2-loaded cells was monitored using a micro-photoluminescence measurement system. Cells were excited alternatively with 340- and 380-nm light, and the emitted fluorescence measured at 510 nm was collected. The fluorescence ratio obtained at 340 and 380 nm (F340/F380) was used as an index of $[Ca^{2+}]_i$. A pharmacological agent, angiotensin II (200 nM), was administered to induce the mobilization of intracellular calcium. All experiments were performed using 10–15 cells.

Data analysis

All data are expressed as the mean ± SEM. Statistical significance was assessed with analysis of variance (ANOVA) test followed by Bonferroni's *t* test for multiple comparisons. Student's *t* test was used when two groups were compared. A value of $P < 0.05$ was considered to be statistically significant.

Results

Cell count and viability

In the absence of DHA, the total number of cells that survived after 48 h of hypoxic exposure was significantly less than that of corresponding normoxic control cells [$(14 \pm 0.3) \times 10^3$/mL vs. $(17 \pm 0.7) \times 10^3$/mL, $P < 0.05$] and was further decreased during the 72-h reoxygenation period (Fig. 1a, b). This reduction in survival cells was consistent with the result of MTT reduction assay (Fig. 1c, d). On the other hand, the presence of DHA did not improve the cell viability in response to H/R insult; instead, a marked decrease was detected in cells treated with high-concentration (30 μM) DHA.

NO release

The nitrite levels in the normoxic cultures not treated with DHA were not significantly different (N48 subgroup 0.21 ± 0.04 μM vs. N(48 + 72) subgroup 0.37 ± 0.04 μM) (Fig. 2). At the end of hypoxic exposure, the nitrite level in the hypoxic culture medium was significantly higher than that in normoxic medium (0.49 ± 0.01 μM vs. 0.21 ± 0.04 μM, $P < 0.05$); reoxygenation further increased it (1.09 ± 0.16 μM vs. 0.49 ± 0.01 μM, $P < 0.05$). DHA at concentrations lower than 30 μM did not influence the NO production in each group, while a significant enhancement was observed at 30 μM.

ROS generation

The ROS levels in normoxic cells, either in the presence or absence of DHA, were similar in groups (Fig. 3). Treatment with hypoxia alone or combined with low-concentration (<30 μM) DHA did not alter the ROS generation, in contrast to a marked induction by 30 μM DHA. Subsequent reoxygenation elevated the cellular ROS level and DHA at the concentration of 30 μM further extended the increase.

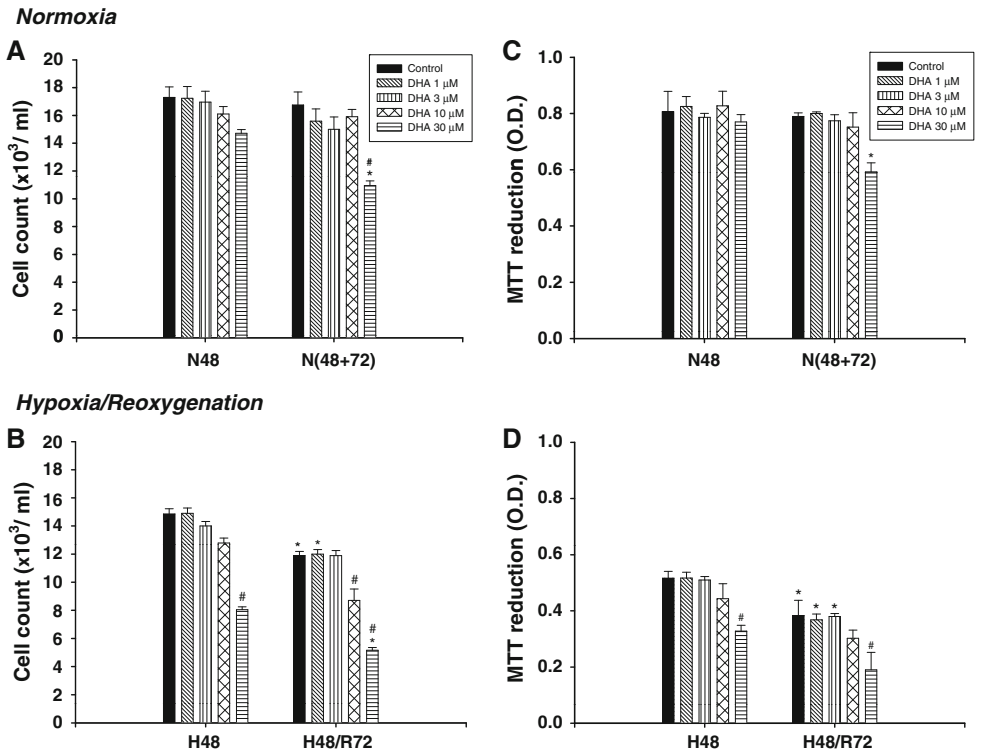

Fig. 1 Effect of DHA on the cell count (**a**, **b**) and MTT reduction (**c**, **d**) in human coronary artery smooth muscle cells in response to hypoxia/reoxygenation. Cells in the presence of various concentrations of DHA (0–30 μM) were subjected to 48 h hypoxia with or without a following 72 h of reoxygenation. Viable cells were counted in hematocytometer using trypan blue exclusion method. MTT reduction assay was used to determine the cell viability. Values are means ± SEM of at least three independent experiments. $^{\#}P < 0.05$ compared with control (DHA 0) within each subgroup (N48, N(48 + 72), H48 and H48/R72). $^{*}P < 0.05$ compared with the treatment of same DHA concentration in the corresponding subgroup (N(48 + 72) vs. N48; H48/R72 vs. H48)

Levels of inflammatory cytokines

Under normoxic condition, no significant variations in the levels of IL-1β, IL-6, and TNFα were observed in cells with or without the DHA treatment (Fig. 4a, c, e). During the 48-h hypoxic period, DHA generally did not alter the release of all these three cytokines from cells, except that a significant increase in IL-1β level was detected in 30 μM DHA-treated cells (Fig. 4b, d, f). Reoxygenation induced mild but significant elevation in IL-1β and IL-6 release, and DHA at higher concentrations (>10 μM) markedly augmented the production of both cytokines (Fig. 4b, d). The release of TNFα, in contrast, was unaltered by either reoxygenation or the presence of DHA (Fig. 4f).

Intracellular Ca^{+2} mobilization

No significant difference in the intracellular calcium concentration was observed among normoxic cultures, except that a reduction was induced by 30 μM DHA (Fig. 5). In the absence of DHA, cells exposed to hypoxia for 48 h resulted in a substantial increase (H48 subgroup 2.78 ± 0.28 vs. N48 subgroup 1.79 ± 0.15 F340/F380, $P < 0.05$) in $[Ca^{2+}]_i$ and reoxygenation reversed it (H48/R72 subgroup 2.09 ± 0.26 vs. N(48 + 72) subgroup 1.85 ± 0.05 F340/F380, $P > 0.05$). Furthermore, DHA concentration-dependently decreased the $[Ca^{2+}]_i$ in each experimental group and reached the statistical significance at 30 μM.

Discussion

The present study demonstrates that hypoxia/reoxygenation causes significant injury in cultured HCASMCs and high-concentration (30 μM) DHA exacerbates the damage.

The selected time course for hypoxia and reoxygenation in this experimental model was based on the results of our pilot studies (data not shown). HCASMCs were subjected to hypoxia for different time periods (6, 24, 48, 72, and 96 h), and viability was determined by cell count and MTT assay. Significantly reduced viability in hypoxic cells, compared with that in normoxic controls, was observed at 48 h and the subsequent time points. We thus chose 48 h as the hypoxia exposure time. Cells exposed to this fixed period of hypoxia were then treated with various durations of reoxygenation (6, 24, 48, 72, and 96 h), and a further

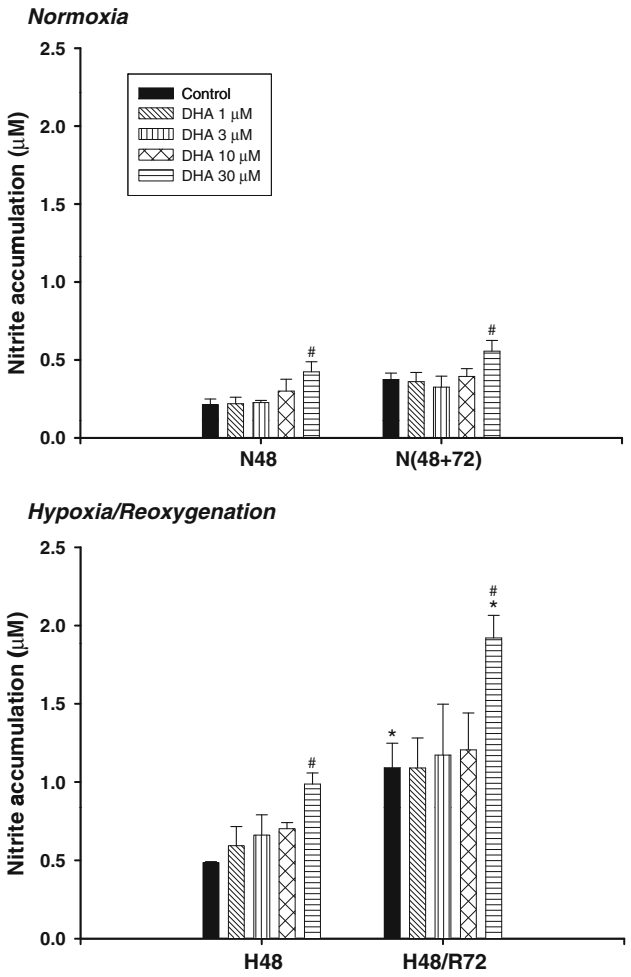

Fig. 2 Effect of DHA on the nitrite accumulation in human coronary artery smooth muscle cells in response to hypoxia/reoxygenation. Cells in the presence of various concentrations of DHA (0–30 μM) were subjected to 48 h hypoxia with or without a following 72 h of reoxygenation. Aliquots of culture medium were collected and mixed with equal volume of Griess reagent to measure the nitrite levels. Values are means ± SEM of at least three independent experiments. $^{\#}P < 0.05$ compared with control (DHA 0) within each subgroup (N48, N(48 + 72), H48 and H48/R72). $^{*}P < 0.05$ compared with the treatment of same DHA concentration in the corresponding subgroup (H48/R72 vs. H48)

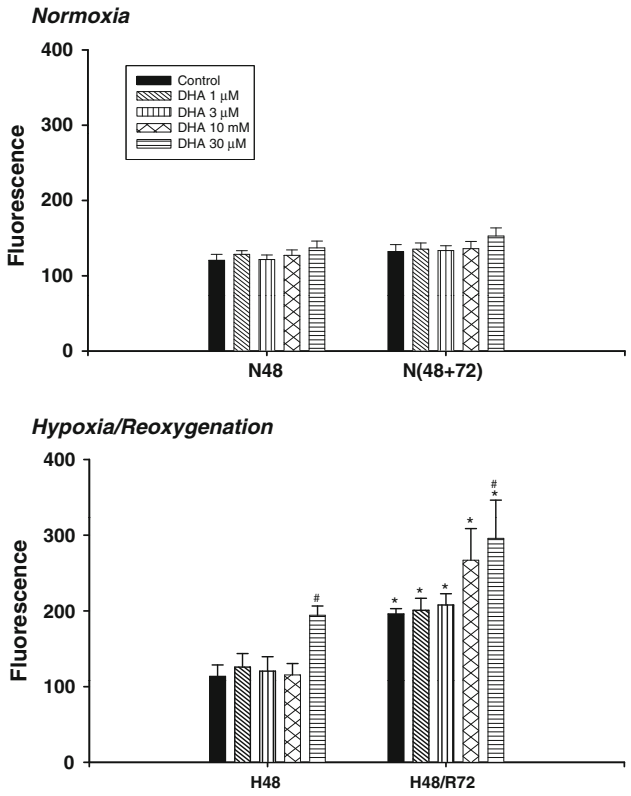

Fig. 3 Effect of DHA on the ROS levels in human coronary artery smooth muscle cells in response to hypoxia/reoxygenation. Cells in the presence of various concentrations of DHA (0–30 μM) were subjected to 48 h hypoxia with or without a following 72 h of reoxygenation. The intracellular ROS levels were measured fluorometrically by using the probe of 2′,7′-dichlorodihydrofluorescein-diacetate (H_2DCF-DA). Values are means ± SEM of at least three independent experiments. $^{\#}P < 0.05$ compared with control (DHA 0) within H48 or H48/R72 subgroup. $^{*}P < 0.05$ compared with the treatment of same DHA concentration in the corresponding subgroup (H48/R72 vs. H48)

decrease in viability was detected at 72 and 96 h. Consequently, treatment with hypoxia for 48 h followed by reoxygenation for 72 h was selected for this study.

The plasma level of total DHA in normal humans is approximately between 100 and 300 μM [16–18] with a great proportion bound to carriers (e.g., albumin) and only about 0.5–3% in free fatty acid form [19, 20]. In this in vitro study, DHA was introduced to cells, mostly as a solubilized lipid rather than carrier bound as in vivo. The concentrations 1, 3, and 10 μM used are within physiological range, while 30 μM is much higher than the normal plasma level of free DHA. Since a relatively low number of cells were exposed to high amounts of DHA in this study, the observed in vitro effects of DHA at 30 μM, for instance, might possibly correspond to the in vivo effects of DHA at a higher concentration.

The observation that the viability was significantly reduced in hypoxic cells versus normoxic cells indicates that 48 h hypoxia per se can induce the death of HCASMC, a cell type that is considered to be hypoxic tolerant [21]. The hypoxia-induced cell injury has been postulated to be associated with alterations in cellular enzyme activities, mitochondrial function, cytoskeletal structure, membrane transport, and antioxidant defenses [22]. The worsening effect of reoxygenation, as evidenced by aggravated cell damage during reoxygenation in this study, has also been reported by numerous in vitro and in vivo studies, and factors such as ROS, intracellular calcium overload, and inflammatory cytokines might all be involved [1, 22–24]. DHA at concentrations used in this study did not offer

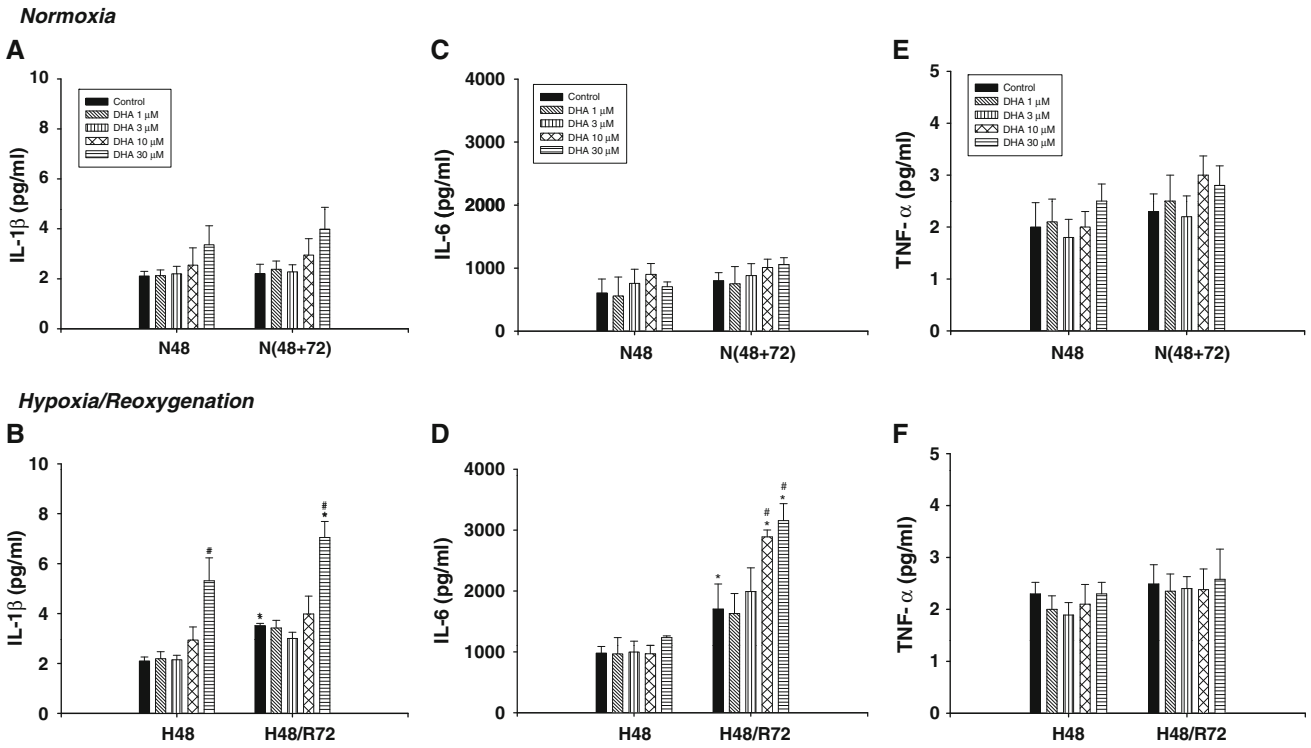

Fig. 4 Effect of DHA on the levels of cytokines (**a**, **b** IL-1β, **c**, **d** IL-6, **e**, **f** TNFα) in human coronary artery smooth muscle cells in response to normoxia or hypoxia/reoxygenation. Cells in the presence of various concentrations of DHA (0–30 μM) were subjected to 48 h hypoxia with or without a following 72 h of reoxygenation. Culture media were collected and assayed for cytokines using commercial enzyme-linked immunosorbent assay kits. Values are means ± SEM of at least three independent experiments. $^{\#}P < 0.05$ compared with control (DHA 0) within H48 or H48/R72 subgroup. $^{*}P < 0.05$ compared with the treatment of same DHA concentration in the corresponding subgroup (H48/R72 vs. H48)

protective action; instead, it augmented the cell death at high concentrations.

The aberrant production of NO in cells in response to hypoxia/reoxygenation has been acknowledged to be a key factor leading to cell damage. In our study, hypoxia caused a slight but significant increase in the nitrite level, and reoxygenation resulted in a further enhancement. Similar observations have been described in other studies, and the mechanisms involved might be related to alterations in intracellular pH, ion channel potential and ATP storage, ROS generation, and activation of inflammatory response [25, 26]. DHA at low concentrations (1–10 μM) had no influence on the nitrite levels in both normoxic and H/R groups, while at high concentration (30 μM) elicited an increasing effect. The altered NO production is presumably due to the modulation effect of DHA on NF_kB, a transcription factor that plays an important role in the regulation of inflammatory process [27].

Oxygen free radicals are produced in excess during reoxygenation and have been known to play a crucial role in the pathogenesis of reoxygenation injury. However, cellular ROS production also may increase in the hypoxic state, as evidenced by the increased ROS in pulmonary artery smooth muscles cells, cardiomyocytes, and several other cell types [28, 29]. In our present study, no significant increase in ROS production was observed in cells incubated in hypoxia for 48 h, which presumably reflecting the low level of oxygen substrate available for ROS production. During reoxygenation, a significant increase in ROS levels was detected, as expected. Studies regarding the effects of DHA or other n-3 PUFAs on the ROS generation are controversial, as both inhibitory and stimulating actions in various cell types have been reported [30]. DHA, at all concentrations used in this study, did not influence the ROS levels in normoxic cells, whereas at 30 μM significantly increased the ROS production in both hypoxic and reoxygenated cells. This observation suggests that the DHA modulation of ROS generation in H/R-injured cells is dose dependent. Precise mechanisms underlying the stimulating effect of high-concentration DHA on ROS generation remain unidentified, although a mechanism via regulation of enzymes responsible for ROS production has been proposed [31].

IL-1β, IL-6, and TNF-α are key cytokines responsible for H/R-induced injury in a wide variety of tissues. In our experiment, hypoxia per se did not alter both the IL-1β and

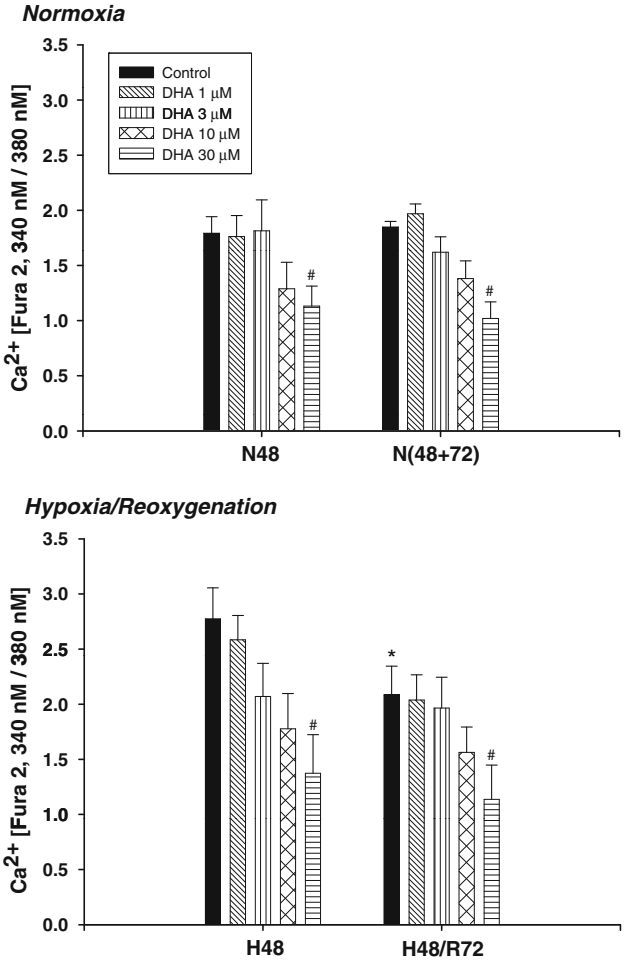

Fig. 5 Effect of DHA on the intracellular Ca^{+2} mobilization in human coronary artery smooth muscle cells in response to normoxia or hypoxia/reoxygenation. Cells in the presence of various concentrations of DHA (0–30 μM) were subjected to 48 h hypoxia with or without a following 72 h of reoxygenation. Cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_i$) were measured by the microfluorimetric technique using calcium indicator dye, fura 2-acetomethyl ester. Values are means ± SEM ($n = 10$–15 cells). $^{\#}P < 0.05$ compared with control (DHA 0) within each subgroup (N48, N(48 + 72), H48 and H48/R72). $^{*}P < 0.05$ compared with the treatment of same DHA concentration in the corresponding subgroup (H48/R72 vs. H48)

IL-6 levels in the culture medium, whereas a significant increase in the release of both cytokines from reoxygenated cells was observed. The levels of TNF-α, a cytokine which is also produced by human VSMC [32], were low, and no difference between groups was found. Similar observation was reported [33]. The reasons for the low level of TNF-α released from hypoxic or hypoxia/reoxygenated cells are unknown. One possible explanation is that the effect of hypoxia on TNF-α production may be cell type specific. Thus, TNF-α is unlikely to mediate the damaging effect of hypoxia/reoxygenation in cultured human vascular smooth muscle cells. The supplementation of 10 and 30 μM DHA increased the release of both IL-1β and IL-6 in hypoxia/reoxygenated cells, contradictory to numerous reports that DHA is capable of inhibiting the production of inflammatory cytokines [34, 35]. The discrepancy might result from cell types used, cell injury models employed, and forms of fatty acid administered.

The hypoxia-induced calcium overload and reversed calcium concentration during reoxygenation observed in this study are consistent with the previous studies in literature [36]. The increased intracellular calcium concentration is proposed to result from the hypoxia-activated anaerobic glycolysis leading to cellular acidosis and insufficient energy production which favors the accumulation of calcium. This elevated cytosolic calcium concentration rapidly returns to physiological level when coronary blood flow resumes [36]. In the present study, DHA concentration-dependently decreased the intracellular calcium concentration during normoxia, hypoxia, and H/R. This effect has been attributed to the generation of prostanoids that activate K^{+}_{ATP} channels and the inhibition of intracellular Ca^{2+} release and Ca^{2+} channels in vascular smooth muscle cells, thus contributing to the beneficial vasorelaxant effects of DHA [37]. Nevertheless, the abnormally low levels of intracellular calcium concurred with marked cell death induced by 30 μM DHA might suggest the cell-damaging effect of DHA at high concentrations.

The cytotoxic effect of DHA at high concentration (30 μM) observed in this present study was similarly demonstrated in several cell types, particularly in tumor cell lines. For example, DHA at 30 μM induced apoptosis in human breast cancer cells [38]. In neuroblastoma cell cultures, the IC_{50} of DHA is 12–15 μM, whereas 17-HpDHA, an oxidized DHA metabolite, was even more potent in inducing cell death with an IC_{50} of 3–6 μM [39]. Also, the pro-apoptotic effect was observed in lung cancer cells treated with DHA (30, 50 μM) [40]. This cytotoxic effect on tumor cells makes DHA a highly potential agent for clinical application.

In summary, our study showed that H/R induced significant damage in cultured HCASMCs. DHA, at low concentrations, offered no significant protective functions, whereas at high concentration (30 μM), it exacerbated the cell injury, presumably by augmenting the generation of NO, ROS, and inflammatory cytokines. Also, an altered intracellular calcium dynamics might be involved. These results thus do not support the protective function of DHA in coronary arterial smooth muscle cells in response to H/R injury.

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