Eicosapentanoic acid inhibits hypoxia-reoxygenation-induced injury by attenuating upregulation of MMP-1 in adult rat myocytes

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Abstract

Background: Myocardial hypoxia-reoxygenation (H-R) is associated with upregulation of metalloproteinases (MMPs). Upregulation of MMPs is associated with cell injury. Previous studies have shown that fish oil can protect myocardium from injury induced by H-R. This study was designed to examine the effect of eicosapentanoic acid (EPA), one of the major components in fish oil, on the modulation of MMP-1 expression in response to H-R in cultured adult rat myocytes. Methods and results: Myocytes isolated from adult Sprague–Dawley rat hearts were cultured with or without EPA or arachidonic acid (AA) (10 and 50 μM) and exposed to 24 h of hypoxia followed by 3 h of reoxygenation (H-R). H-R resulted in myocyte injury (measured on LDH release), increase in p38MAPK phosphorylation (Western analysis), augmentation of lipid peroxidation, and upregulation of MMP-1 activity (zymography) and expression (RT-PCR and Western analysis) (all \( P<0.01 \) vs. control, \( n=5 \)). Pretreatment of myocytes with EPA, but not AA, resulted in a reduction in LDH release, and attenuation of p38MAPK phosphorylation and MMP-1 activity and expression in response to H-R (all \( P<0.05 \) vs. H-R alone). Pretreatment of myocytes with EPA also reduced lipid peroxidation in myocytes exposed to H-R (\( P<0.05 \) vs. H-R alone). A high concentration of EPA (50 μM) was more potent than the lower concentration of EPA (10 μM). Conclusions: These observations suggest that EPA attenuates an increase in MMP-1 following H-R, which may be a basis of protection of myocytes from the adverse effects of H-R. p38MAPK phosphorylation may be an important signaling event in this process.

Keywords: EPA; Arachidonic acid; Hypoxia-reoxygenation; MAP kinase; MMP-1; Myocytes

1. Introduction

Myocardial hypoxia-reoxygenation (H-R) is associated with upregulation of a number of endogenous enzymes, including the matrix metalloproteinases (MMPs), which degrade extracellular matrix [1,2], cause cellular injury and degradation [3,4]. MMPs secreted from myocardial cells can induce apoptosis with subsequent exacerbation of cardiac dysfunction [5]. Inhibition of expression and/or activity of MMPs can protect myocardium from the adverse effects of acute and chronic ischemia [6,7]. Although some studies did not show a protective role of dietary fish oil on coronary artery disease [8], most experimental studies and clinical intervention trials have shown a cardioprotective effect of dietary fish and fish oil intake [9–13]. The cardioprotective effects of fish consumption have been attributed to the modulation of lipid and lipoprotein metabolism [14,15], regulation of blood pressure [16], improvement in vascular endothelial function [17], enhancement of vascular reactivity and compliance [18], reduction of neutrophil and monocyte cytokine production [19], inhibition of thrombogenesis and
inflammatory response [20,21], and an anti-arrhythmic effect [22]. There is evidence that docosahexanoic acid (DHA), one of the major ω-3 polyunsaturated fatty acids (PUFAs) in fish, can suppress the activity of MMPs [23,24]. DHA is also thought to modulate activation of the extracellular signaling pathways, including protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) [25–27]. Other studies have suggested that eicosapentanoic acid (EPA), another major ω-3 PUFAs in fish, can protect myocardium from H-R injury [10,11]. In contrast, arachidonic acid (AA), a major ω-6 PUFAs, does not display these tissue-protective effects [28,29].

Cardiac myocytes are a potent source of MMP-1. A recent study from our group showed that exogenous MMP-1 causes myocyte injury and death [4]. Inhibition of this MMP has been shown to be associated with improved cardiac hemodynamics [7].

The present study was designed to examine the modulation of MMP-1 expression and activity by EPA in cultured rat myocytes in response to H-R. We also examined the role of MAPK pathway in the effect of EPA on MMP-1.

2. Methods

2.1. Myocyte isolation and culture

Calcium tolerant myocytes were obtained as detailed earlier [30]. Briefly, adult male Sprague–Dawley rats weighing 200–250 g were given heparin (1000 U/kg, i.p.) and anesthetized with sodium pentobarbital (60 mg/kg). The chest cavity was opened, and the heart was removed and placed into ice-cold Ca2+-free Krebs–Henseleit (K–H) buffer (perfusion medium, composition: NaCl 118 mM, KCl 4.7 mM, KH2PO4 1.2 mM, MgSO4 1.2 mM, NaHCO3 25 mM, and glucose 11 mM, pH 7.4). Within 1 min, the heart was transferred to a perfusion apparatus and perfused via aorta with oxygen-saturated (95% O2 and 5% CO2) Ca2+-free K–H buffer at 37 °C and a rate of 5–6 ml/min for 5 min. Then the heart wasperfused with 1 mg/ml of crude type I collagenase (Worthington, Lakewood, NJ) in the same medium for 15–20 min to rinse out the intervascular space.

Following perfusion, the heart was removed, and atria and large vessels were dissected off. The ventricles were minced into small pieces, then shaken in 10 ml of perfusion medium containing 2% bovine serum albumin at 37 °C for 5 min, and the released cells were collected and centrifuged at 10×g for 5 min. The cell pellet was then washed repeatedly. The cells were resuspended in cell culture medium containing 5% fetal bovine serum and antibiotics.

Myocytes from each rat heart were divided into 10-cm dishes containing 10 ml of Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 0.1 mg/ml streptomycin (about 106 cells in each dish) and cultured under 95% air and 5% CO2 at 37 °C. Culture medium was changed every other day.

The cultured myocytes were divided into the following groups: (1) control, myocytes were incubated in 95% air and 5% CO2; (2) H-R, myocytes were exposed to 24 h of hypoxia (95% N2 and 5% CO2, PO2<30 mmHg) followed by 3 h of reoxygenation (95% air and 5% CO2); (3) EPA (10 mM) plus H-R, myocytes were incubated with 10 mM of EPA for 4 h followed by exposure to H-R; (4) EPA (50 mM) plus H-R, myocytes were incubated with 50 mM of EPA for 4 h followed by exposure to H-R; (5) EPA plus normoxia, myocytes were incubated with 50 mM of EPA in 95% air and 5% CO2. This degree of H-R has been shown to result in myocyte injury, including apoptosis and necrosis [31]. As control, AA in the same concentrations as EPA was also incubated with cultured myocytes. Culture medium was collected for the determination of lactate dehydrogenase (LDH) and MMP-1 activity.

2.2. Determination of LDH in the culture media

A spectrophotometric method based on the oxidation of lactate (Sigma) was used to measure LDH release. LDH activity was expressed as units per ml medium [30].

2.3. Determination of MMP-1 activity in culture media

The activated MMP-1 in cultured media released by myocytes was determined by zymography. Aliquots of conditioned medium (1 µg/lane with volumes adjusted according to protein content) were subjected to non-denaturing SDS–PAGE at a constant voltage of 125 V. The gel was then washed in 2.5% Triton X-100 solution with gentle agitation for 6 h at room temperature, followed by replacement with the developing buffer (50 mM Tris, 5 mM CaCl2, 0.02% NaN3, pH 7.6). The gel was stained with 0.5% Coomassie blue, destained in destaining solution containing 5% methanol and 7% acetic acid, photographed, and dried for permanent record.

2.4. Determination of MMP-1 protein in myocardium

The method for Western blot has been described previously [30]. The protein lysate from cultured myocytes (20 µg/lane) was separated by 10% SDS–PAGE. The primary monoclonal antibody to MMP-1 (Calbiochem) was used at a 1:1000 dilution. The bands were detected with the
enhanced chemiluminescence (ECL) system, and the relative intensity of the bands of interest was analyzed.

2.5. Determination of MMP-1 mRNA in myocytes

The methodology for mRNA determination has been described earlier [30]. Briefly, one microgram of total RNA was reverse transcribed with oligo-dT (Promega) and M-MLV reverse transcriptase (Promega) at 37 °C for 1 h. RT material (1.5 μl) was amplified with Taq DNA polymerase (Promega) using a primer pair specific to MMP-1 (forward primer: 5’-TTGTGTGCCTCATGAGCTT-3’, reverse primer: 5’-ACTTTTGTGCCCAA-TTCAGG-3’ [32]. PCR product was 639 base pairs. For PCR, 30 cycles were used at 95 °C for 45 s, 60 °C for 45 s, and 72 °C for 2 min. The RT-PCR amplified samples were visualized on 1.2% agarose gels using ethidium bromide. A primer pair rat β-actin was used as control.

2.6. Determination of p38MAPK and its phosphorylation

p38MAPK and its phosphorylation were determined by Western analysis, as described earlier [33]. The primary polyclonal antibodies to p38MAPK and phosphorylated p38MAPK (pp38MAPK) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and used at a dilution of 1:1000. The secondary antibody was obtained from Amersham.

2.7. Determination of MDA levels in myocytes

Malondialdehyde (MDA) levels in the myocytes were measured as an index of lipid peroxidation (OXIS, Portland, OR). The final MDA levels in myocytes were expressed as μmol/g protein.

2.8. Data analysis

Data are presented as mean±S.E.M. Statistical significance was determined in multiple comparisons among independent groups of data in which ANOVA and the Student–Newman–Keuls test indicated the presence of significant differences. A P value <0.05 was considered statistically significant.

3. Results

3.1. Effect of EPA on myocardial injury induced by hypoxia-reoxygenation

H-R caused a marked increase in LDH release in the supernatants of myocytes, indicating myocardial injury (P<0.01, vs. control group, n=5). Treatment of cultured myocytes with EPA before H-R attenuated LDH release in response to H-R (P<0.05, vs. H-R group, n=5). A high concentration of EPA was more potent than the lower concentration of EPA. Notably, EPA also exerted a mild inhibitory effect on LDH release from myocytes cultured under normoxic conditions, but the difference was not significant (Fig. 1).

3.2. Effect of EPA on activity and expression of MMP-1 in myocytes

As shown in Fig. 2, MMP-1 activity was markedly upregulated in the medium of myocytes exposed to H-R (P<0.01, vs. control). Pretreatment of myocytes with EPA reduced this enhanced MMP-1 activity during H-R (P<...
A high concentration of EPA was more potent than the lower concentration of EPA. EPA did not affect MMP-1 activity in the medium of myocytes kept under normoxic conditions.

As shown in Fig. 3, MMP-1 expression (protein and mRNA) was also increased in myocytes exposed to H-R (P<0.01, vs. control). Treatment of myocytes with EPA attenuated the increase in MMP-1 expression during H-R in a dose-dependent manner (both P<0.05 vs. H-R alone). EPA did not affect MMP-1 expression in myocytes kept under normoxic conditions.

3.3. Effect of EPA on p38MAPK and its phosphorylation in myocytes

H-R did not affect p38MAPK protein levels in cultured myocytes; however, phosphorylation of p38MAPK (pp38MAPK) increased during H-R (P<0.01 vs. control group). Treatment with EPA reduced the increased levels of pp38MAPK in cultured myocytes during H-R (P<0.05 vs. H-R alone) (Fig. 4). A high concentration of EPA was more potent than the lower concentration of EPA. EPA did not affect p38MAPK or pp38MAPK levels in myocytes kept under normoxic conditions.

3.4. Effect of EPA on lipid peroxidation in myocytes

H-R markedly enhanced lipid peroxidation in the cultured myocytes as indicated by MDA measurement (P<0.01 vs. control). Treatment with EPA reduced the increased MDA levels in cultured myocytes during H-R (P<0.05 vs. H-R alone). A high concentration of EPA was more potent than the lower concentration of EPA. EPA did not significantly affect MDA levels in myocytes kept under normoxic conditions (Fig. 5).

3.5. Effect of AA on myocytes exposed to H-R

The myocytes were pretreated with AA before exposure...
Fig. 6. Summary of the effects of arachidonic acid (AA) on H-R-induced injury in myocytes. (A) LDH release in the supernatants of myocytes. (B) Activity of MMP-1 in cultured myocytes. (C) MMP-1 expression in cultured myocytes determined by Western blot. (D) Expression and phosphorylation of p38MAPK in cultured myocytes determined by Western blot. H-R resulted in LDH release, upregulation of MMP-1 activity and protein expression, and increase in p38MAPK phosphorylation. Pretreatment of myocytes with AA caused a further small increase in these indices. AA alone induced a modest increase in LDH release, MMP-1 activity and expression, and p38MAPK protein and phosphorylation.

to H-R as control for pretreatment with EPA. As shown in Fig. 6, H-R resulted in LDH release, increased MMP-1 activity and protein expression and pp38MAPK. Pretreatment of cells with AA slightly, but not significantly, further increased LDH release, MMP-1 activity and protein expression and pp38MAPK. The presence of AA alone caused a small degree of cell injury, as indicated by LDH release.

4. Discussion

Most studies on the cardioprotective effects of fish oil were based on clinical observations and in vivo or ex vivo animal hearts exposed to ischemia-reperfusion. Hallaq et al. [33] showed that both EPA and DHA can prevent the toxic effect of high concentrations of cardiac glycoside ouabain in isolated neonatal rat cardiac myocytes. The same group [22] subsequently reported anti-arrhythmic effect of EPA in neonatal rat cardiac myocytes. We now extend these observations on the effect of EPA and show that this PUFAs can protect adult rat myocytes from H-R-induced injury. Importantly, we demonstrate potent inhibitory effects of EPA on MMP-1 expression and activity and pp38 MAPK. A high concentration (50 μM) of EPA was more potent than the low concentration (10 μM) in these effects. In contrast a ω-6 PUFAs, AA, did not show the same protective effect against H-R-induced injury. As a matter of fact, there was a suggestion of direct toxic effect of AA on myocytes, which is consistent with previous studies which showed that exogenous AA injures vascular endothelium and other tissues [29,34].

Increasing evidence indicates that MMPs are activated in early stages of myocardial ischemia [1,2]. Almost all cell types in ischemic-reperfused area, such as, leukocytes, vascular endothelial cells, smooth muscle cells, fibroblasts and myocytes, can synthesize and secrete MMPs [2]. MMPs released from leukocytes degrade vascular ECM, breakdown basement membrane, increase vascular permeability, and enhance leukocyte migration outside the vascular lumen [2]. MMPs secreted from cardiomycocytes may lead to cell injury and death and apoptosis and potentially cardiac rupture [3–5]. Recent studies have shown elevated levels of MMPs in plasma in patients with acute myocardial infarction [35,36]. It has, therefore, been proposed that inhibition of MMPs by deletion of target mRNA or by chemical inhibitors may prevent myocardial injury [6,7,9]. Our study is novel in this regard. First, we show that H-R upregulates MMP-1 (both activity and expression) in rat myocytes, and second, treatment with EPA, but not AA, blunts the H-R-induced upregulation of MMP-1. In recent unpublished studies, we have observed direct toxic effects of recombinant MMP-1 on cardiac myocytes, and the effects of MMP-1 can be inhibited by a specific chemical inhibitor. It is conceivable that the inhibition of MMP-1, observed in the present study, is one of the mechanisms of cardioprotective effect of EPA.

Several investigators have looked at the intracellular mechanisms of action of fish oil, including protein kinase pathways, but the precise signaling pathway role remains unclear [25–27,37]. Diep et al. [37] showed that DHA induces activation of protein kinase C (PKC) and MAPK. Others [25–27] have suggested that both EPA and DHA can diminish activation of MAPK in the isolated myocytes. Our observations indicate that H-R increases the activity of p38MAPK and concurrently upregulates expression of MMP-1 expression and activation. Pretreatment of the cultured myocytes with EPA inhibited the H-R-mediated upregulation of pp38MAPK, and this was associated with a decrease in MMP-1 expression and attenuation of myocyte injury. On the other hand, pretreatment myocytes with AA before H-R further enhanced p38MAPK, and this was associated with additional increase in MMP-1 expression and myocyte injury. Activation of p38MAPK pathway has previously been shown to participate in cell injury [38], and inhibition of p38MAPK activation attenuates H-R-mediated cell injury [39]. Our data, in concert with these studies [38,39], would suggest that the inhibition of MMP-1 via p38MAPK phosphorylation is one of the mechanisms of the reduction in H-R-induced myocyte injury by EPA.

Myocardial ischemia-reperfusion injury is always accompanied with lipid peroxidation [40], and antioxidants decrease lipid peroxidation and reduce myocardial injury [41]. Reduction in lipid peroxidation with EPA pretreatment observed in this study is consistent with the results of previous studies, which show EPA-induced reduction in
H-R injury as well as myocardial lipid peroxidation [41–44]. Oxidative stress is thought to be an important inducer of vascular MMP activity [37]. The upregulation of MMP-1 in association with lipid peroxidation is in concert with the observation of Uemura et al. [45]. The potent antioxidant effect of EPA [40–44] could be another basis of decrease in MMP-1 expression and activity in the cultured myocytes exposed to H-R.

In conclusion, the present study shows that EPA, a major component in dietary fish oil, protects cultured rat myocytes from H-R-induced injury. The tissue protection may relate to the inhibition of MMP-1 expression and activation. Inhibition of p38MAPK activity and lipid peroxidation may be key steps in the effect of EPA.

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References


