HIGHLY PURIFIED EICOSAPENTAENOIC ACID ATTENUATES TISSUE DAMAGE IN EXPERIMENTAL MYOCARDIAL INFARCTION

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We examined the effects of dietary supplementation with eicosapentaenoic acid (EPA) on experimental myocardial infarction in dogs. Twenty-five dogs were fed standard diets, 10 of which were supplemented with EPA-ester (100 mg/kg body weight/day) for 8 weeks, while 15 served as controls. After ingesting EPA for 8 weeks, the ratio of EPA to arachidonic acid (AA) in platelet cell membranes significantly increased (from 0.033 to 0.105; p<0.01). The chemotactic response of neutrophils to leukotriene B4 (LTB4) was reduced in the EPA group (34% reduction at 10^-6 M LTB4, p<0.01). Also in the EPA group, the amount of 12-hydroxeyicosatetraenoic acid, one of the chemotactic products of AA in infarcted myocardium, was reduced to 40% (p<0.05). EPA treatment resulted in significant reduction in the ultimate size of the infarcted area. Contractile function of infarcted myocardium was well-preserved in the EPA group. Myeloperoxidase activity, an indication of the infiltration of neutrophils into the infarcted myocardium, was less in the EPA group than in the controls (0.68 ± 0.25 U/0.1 gr. vs 1.22 ± 0.55 U/0.1 gr., p<0.05). Therefore, we conclude that dietary supplementation with EPA attenuates ischemic myocardial damage through inhibition of neutrophilic infiltration into the infarcted myocardium.

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MYOCARDIAL infarction can be regarded as a type of inflammation. Neutrophils infiltrate the infarcted area, dispose of infarcted tissue, and promote processes of tissue repair. However, it has also been recognized that infiltrating neutrophils produce oxygen- and hydroxy-radicals and various proteinase enzymes which can exacerbate the tissue injury in the marginal area of the infarction!

Activated neutrophils metabolize arachidonic acid (AA) via a lipoxigenase pathway and produce various substances which are deleterious for myocardium. Some of these products enhance vascular permeability and others are related to neutrophilic chemotaxis and/or activation.

To understand the process of myocardial infarction as it compares to inflammation, we previously examined the roles played by AA lipoxygenase products in this process. In that study, we found that the levels of hydroxyeicosatetraenoic acids (HETE), and especially 12-HETE, a chemotactic product of AA, increased in infarcted myocardium prior to the significant infiltration of neutrophils.

On the other hand, it has been well established that eicosapentaenoic acid (EPA), an

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n-3 polyunsaturated fatty acid (PUSFA), competes with AA to be incorporated into phospholipids in plasma membranes. After an appropriate stimulation, phospholipids which have incorporated EPA produce lipoxygenase and cyclooxygenase products of EPA. However, the biological activities of the lipoxygenase and cyclooxygenase products of EPA are much less than those of the respective products of AA. Attempts have been made to use EPA to prevent the onset of myocardial infarction through reduction of coronary atherosclerosis and/or of thrombogenesis in the atherosclerotic lesion.

We performed the present series of experiments to determine how EPA affects the development of myocardial infarction and its inflammatory aspects. To our knowledge, this is the first study which used highly-purified EPA, instead of fish oils.

**MATERIALS AND METHODS**

**Feeding diets**

Adult mongrel dogs (10–12 kg) were fed a standard diet (30 g/kg body weight/day) prepared by Oriental Yeast Co.. EPA content in the diet was negligible. The 10 dogs in the EPA group were fed the same standard diet which had been supplemented with EPA ester (100 mg/kg body weight/day), kindly supplied by Nihon Suisan-Mochida Pharmaceutical Co.. Dogs were fed for 8 weeks before coronary ligation.

**Inducing experimental myocardial infarction**

Dogs were anesthetized by intravenous injection of thiopental (20 mg/kg), intubated, and artificially ventilated with room air via Harvard respirator. Under ECG monitoring, thoracotomy was performed at the fifth inter-costal space, the pericardium was opened and the heart was exposed. The circumflex coronary artery was isolated from the fat and ligated 0.5 cm distal to its origin. After confirming ST segment elevation in ECG, the thoracic cage was closed. ECG monitoring was maintained until the dogs recovered consciousness. The dogs then lay in the recovery room until they were sacrificed, and their hearts were excised, 72 h after coronary ligation. The entire protocol followed the local ethical standards for the treatment of animals of our hospital.

**Fatty acid composition of platelet cell membrane**

Since purified membrane factions can be easily obtained from platelets, and because EPA is believed to be incorporated into cardiomyocyte and leukocyte membranes, as well as those of platelets, we used to estimate the incorporation of EPA into cell membranes. Blood was drawn from the femoral vein before coronary ligation. Platelets were separated from whole blood and cell membrane fractions were obtained by the method employed by Minkes. The lipid component of the membranes was extracted with Folch’s solution and estimated by analyzing the fractions by gas chromatography.

**Estimation of the chemotactic activities of neutrophils**

To estimate the chemotactic activities of neutrophils, blood was drawn from the femoral vein before and 72 h after coronary ligation. Neutrophils were isolated by the Ficoll gradient method using Histopaque 1077 and 1119. Neutrophils were then suspended in Hanks balanced salt solution (pH 7.4) at a concentration of 3 × 10⁶/ml. Chemotactic activities of neutrophils were measured with a blind well chamber by the method of Boyden with minor modifications. As a chemotaxin, 200 µl of LTB₄ was placed in the lower chamber at a concentration of 10⁻¹⁰ to 10⁻⁶ M, and 200 µl of the suspended neutrophils was placed in the upper chamber. A millipore filter (pore size: 5 µm) was inserted between the chambers and incubated at 37°C for 60 min. After the filter was fixed and stained with hematoxylin, we estimated the number of neutrophils which had migrated to the lower surface of the filter.

**Quantification of ultimate infarct size**

The ischemic or infarcted area was measured by the method employed by Fishbein et al. Briefly, after 72 h of coronary ligation, 30 ml of 0.5% Evans blue dye was injected through the femoral vein to stain the non-ischemic area. Left ventricles of excised hearts were horizontally sectioned from the cardiac base to the apex in 1 cm intervals.
Each section was subdivided into 2 horizontal sections. The apical half of the subdivided section was used for measurement of biochemical parameters, and the basal half was used for evaluation of the ultimate infarct size.

The basal halves of the myocardial slices were incubated with 1.5% triphenyl tetrazolium chloride at 37°C for 10 min to stain the non-infarcted area. After transferring the sections to tracing paper, the areas of the infarcted and ischemic portions, and of the left ventricular wall were calculated using a planimeter.

Measurement of amount of AA lipoxygenase products, especially hydroxyeicosanoid, in myocardium

We measured the amount of hydroxyeicosatetraenoic acids (HETE) to estimate AA lipoxygenase products in infarcted myocardium. Pieces of infarcted myocardium were obtained from the above described area and pieces of non-infarcted myocardium were obtained from the root of the anterior papillary muscle. The myocardial pieces (0.50 gram, wet weight) were homogenized in 5 volumes of 50 mM potassium-phosphate buffer (pH 7.0), with prostaglandin B2 (PGB2, 100 ng in 0.2 ml of methanol) added as an internal standard. The eicosanoids were extracted with ethylacetate by shaking, followed by centrifugation. The homogenate was centrifuged at 3,300 g for 5 min. The supernatant was concentrated to less than 50 μl by evaporation under a stream of nitrogen gas and was then dissolved in 100 μl of pure methanol. Aliquots were analyzed for HETE and PGB2 by reverse-phase liquid chromatography (RP-HPLC) using a column packed with Nucleosil C18 (4.6×150 mm)17. The solvent system consisted of methanol-water-acetic acid (65:35:0.1 v/v). Detection was performed with an ultraviolet detector operating at 235 nm for HETE, and at 280 nm for PGB2. The amount of HETE was calculated from the ratio of the peak height to that of PGB2. Respective peaks corresponding to 12-, 5-, and 15-HETE were separated by high performance liquid chromatography and identified by gas chromatography mass spectrometry (GC-MS). Since it was always present in the greatest amounts, the level of 12-HETE was used to represent the lipoxygenase products of AA in infarcted myocardium.

Measurement of myeloperoxidase activity of infarcted myocardium

To evaluate the extent of neutrophil infiltration into infarcted myocardium, we measured the neutrophil specific myeloperoxidase (MPO) activities by an adaptation of the method of Bradley et al18 according to the procedure of Bednar et al19. Briefly, the serial myocardial pieces (0.20 gram) were homogenized in 10 vol of 50 mM phosphate buffer (pH 6.8) to which hexadecyltrimethyl ammonium bromide had been added to 0.5%. The mixture was then centrifuged at 100,000 g for 15 min at 4°C. A 100 μl aliquot of the supernatant was added to 2.9 ml of 50 mM phosphate buffer containing 0.167 mg/ml o-dianisidine dihydrochloride and 0.0005% H2O2. The reaction was analyzed spectrophotometrically at 460 nm. The results were expressed as units (U) of MPO/0.1 gram wet tissue, where 1 unit of MPO activity is defined as that which degrades 1 μmol of peroxide/min at 25°C.

Estimation of ischemic myocardial injury

The extent of ischemic myocardial injury was estimated by measuring the activities of creatine kinase (CK) remaining in the myocardium at the center of the ischemic area. Myocardial pieces (0.25—0.50 gram) obtained from infarcted and non-infarcted areas were suspended in 25 vol of extraction solution (0.25 M sucrose, 1 mM EDTA, 0.1 mM beta-mercaptoethanol) and homogenized with a Polytron homogenizer. After centrifugation at 16,000 g, the CK activity of the supernatant was then assayed spectrophotometrically with a commercial kit (Iatron Laboratories).

Measurement of dp/dt in left ventricle

In order to estimate the contractile function of the left ventricle, dp/dt was measured with a tip-transducer which was inserted through the left carotid artery just before sacrifice and connected to a polygraphic analyzer (Nihon-Koden Co.).

Histological examination

After measuring the infarcted and non-infarcted areas, myocardial slices were fixed in
TABLE I  FATTY ACID COMPOSITION OF PLATELET PHOSPHOLIPID

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Control</th>
<th>EPA group</th>
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<tbody>
<tr>
<td>C14:0</td>
<td>0.53±0.13</td>
<td>0.85±0.65</td>
</tr>
<tr>
<td>C16:0</td>
<td>16.56±0.88</td>
<td>16.85±2.19</td>
</tr>
<tr>
<td>C18:0</td>
<td>17.57±0.18</td>
<td>18.64±1.35</td>
</tr>
<tr>
<td>C18:1</td>
<td>10.02±1.30</td>
<td>9.92±1.41</td>
</tr>
<tr>
<td>C18:2</td>
<td>15.22±1.24</td>
<td>16.27±1.27</td>
</tr>
<tr>
<td>C20:2+</td>
<td>0.46±0.13</td>
<td>0.55±0.20</td>
</tr>
<tr>
<td>C20:3+</td>
<td>2.06±0.80</td>
<td>1.30±0.47**</td>
</tr>
<tr>
<td>C20:4+</td>
<td>19.85±2.83</td>
<td>14.73±0.96**</td>
</tr>
<tr>
<td>C20:5#</td>
<td>1.80±0.67</td>
<td>7.99±1.54**</td>
</tr>
<tr>
<td>C22:5#</td>
<td>0.65±0.19</td>
<td>1.83±0.72**</td>
</tr>
<tr>
<td>C22:6#</td>
<td>0.87±0.38</td>
<td>0.75±0.81</td>
</tr>
<tr>
<td>Others</td>
<td>11.15±1.46</td>
<td>11.29±1.24</td>
</tr>
</tbody>
</table>

Fatty acid are represented by chain length and number of double bonds.
*: n-6 fatty acid, #: n-3 fatty acid
*p<0.05, **p<0.01, Control vs EPA group

10% formalin and stained with hematoxylin and eosin. The extent of neutrophil infiltration and tissue lysis were then compared with the ratio of EPA/AA in the platelet membrane.

Data analysis
An unpaired t-test was used to test the significance of the difference between dp/dt, the lipid contents of platelet cell membranes, and the levels of 12-HETE and MPO activity in the infarcted myocardia of the control and EPA-treated groups. An analysis of variance (ANOVA) was used to test the change in the chemotactic response of neutrophils. Mortality rate was tested by a chi-square test for independence. Data were expressed as mean±standard error, and a p value <0.05 was considered statistically significant.

RESULTS

Mortality rate
Twenty-five dogs were initially used to evaluate the effect of dietary supplementation with EPA on infarct size resulting from 72 h of permanent coronary occlusion (15 controls and 10 EPA-treated dogs). However, 5 dogs in the control group died due to ventricular fibrillation soon after coronary ligation, while ventricular fibrillation did not occur in the EPA-supplemented dogs. Thus, the mortality rate during coronary occlusion was significantly lower in the EPA-treated group (p<0.05, chi-square test for independence). The remaining 20 dogs (10 controls and 10 EPA-treated dogs) were used in the final data analysis.

Fatty acid composition of platelet cell membrane
We examined the fatty acid composition of the platelet membranes to ensure that dietary supplementation with EPA significantly replaces AA. The results are shown in Table I. After 8 weeks of EPA, the levels of AA and EPA in platelet lipids were 14.7±0.96% and 8.0±1.5% of total lipids, respectively. However, in control dogs, the level of AA was significantly higher (19.8±2.8%; p<0.01) and the level of EPA was about one-fourth (1.8±0.7%; p<0.01) of the experimental values. In the EPA group, docosapentaenoic acid (n-3 PUSFA) levels also increased (0.65±0.2% vs 1.83±0.7%, control vs EPA group, p<0.01). Consequently, the n-3/n-6 ratio was significantly increased in the EPA group (0.139±0.056 vs 0.422±0.28, control vs EPA group; p<0.01). This finding suggests that supplemental dietary EPA efficiently competes with AA to be incorporated into cell membranes.

Chemotactic activities of neutrophils
Fig. 1 shows the changes of the chemotactic activities of neutrophils before and 72 h after coronary ligation. The chemotactic activities of neutrophils were examined various concentrations of LTB4. The data show that (a) the chemotactic activities of the neutrophils in both groups increased as the concentration of LTB4 increased from 10−10M to 10−9M; (b) in both groups, the chemotactic activities of neutrophils were enhanced after coronary ligation; (c) the maximal activity was observed in neutrophils from control dogs at 72 h after coronary ligation, while the minimal activity was observed in neutrophils from EPA-treated dogs before surgery; and (d) the chemotactic activity of neutrophils from EPA-treated dogs was significantly lower than that of neutrophils from control dogs at all chemotaxin concentrations, both before and after coronary ligation.
Chemotactic response of neutrophils to leukotriene B4. Neutrophils were isolated from the femoral vein before and after 72 h of coronary ligation. Chemotactic activities of neutrophils were measured as stated in the “Method”.
Each bar represents the mean±SEM
EPA (−): control group; EPA (+): EPA-treated group; pre-ope: neutrophils isolated before coronary ligation; post-ope: neutrophils isolated 72 h after coronary ligation.

+ p<0.05, * p<0.02, ** p<0.01.

### TABLE II RATIO OF AREA AT RISK (AR) AND INFARCT (IA) TO LEFT VENTRICULAR MASS (LV) OF CONTROL AND EPA GROUP AT 72 H PERMANENT CORONARY LIGATION

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>EPA group</th>
</tr>
</thead>
<tbody>
<tr>
<td>AR/LV</td>
<td>49.3±2.1</td>
<td>43.2±6.9 #</td>
</tr>
<tr>
<td>IA/LV</td>
<td>29.2±0.5</td>
<td>17.6±4.0 *</td>
</tr>
<tr>
<td>IA/AR</td>
<td>59.3±9.9</td>
<td>40.4±5.8 *</td>
</tr>
</tbody>
</table>

*Data were expressed as percent
# NS, *p<0.01 Control vs EPA group

(e.g., after coronary ligation, the neutrophil activity in the EPA-treated group was about 66% of that in the control at an LTB4 concentration of $10^{-6}$M; p<0.01). These results suggest that after treatment with EPA, the chemotactic activities of neutrophils are significantly suppressed regardless of the ligation of the coronary artery.

### Area at risk and infarct size

Table II shows the effect of supplemental dietary EPA on the size of the area at risk and of the myocardial infarct. Dietary EPA significantly reduced the ultimate size of the infarcted area (IA), as compared to the control, whether expressed as a percent of the entire left ventricle (IA/LV; 29.2±0.5% vs 17.6±4.0%, control vs EPA group, p<0.01) or as a percent of the area at risk (LA/AR; 59.3±9.9% vs 40.4±5.8%, control vs EPA group, p<0.01). However, the size of the area at risk did not significantly differ between the 2 groups (expressed as a percent of the entire left ventricle, 49.3±2.1% vs 43.2±6.9%, control vs EPA group). These
findings suggest that EPA supplementation inhibits the progression of ischemic myocardial injury.

CK and 12-HETE content, and MPO activity in the infarced myocardium

Fig. 2 shows the CK content (as an indication of myocardial damage), 12-HETE content, and the MPO activity in infarced myocardium in the control and EPA-treated groups.

In the control group, the amount of CK remaining in infarcted myocardium was 17.0±6.4% of that found in non-infarcted myocardium, which was significantly lower than that of the EPA-treated group (43.8±30.7%, p<0.05). This suggests that CK release was suppressed in the EPA-treated group as compared to the control.

The 12-HETE content in infarced myocardium in the control group was 48.9±32.0 ng/mg protein, which was significantly higher than that in the EPA-treated group (20.0±19.0 ng/mg protein, p<0.05).

Fig. 2. CK content (A), 12-HETE content (B) and MPO activities (C) in infarced myocardium of the control group and the EPA-treated group at 72 h after coronary ligation.
C: control group, E: EPA-treated group
Each value represents the mean±SEM. p<0.05.

Fig. 3. Representative histological pictures in the ischemic center (I) and marginal area (M) at 72 h after permanent coronary ligation in the EPA-treated group, compared with the ratio of AA and EPA levels in the platelet membranes. The EPA/AA ratio of the platelet membranes were 0.16 in (1), 0.23 in (2), and 0.63 in (3).
This suggests that EPA reduces the production of 12-HETE (and other lipoxygenase products of AA) in infarced myocardium. The MPO activity in infarced myocardium in the control group were 1.22±0.55 U/0.1 gram wet tissue, which was significantly higher than that found in the EPA-treated group (0.68±0.25 U/0.1 gram wet tissue, p<0.05). This suggests that EPA reduces the infiltration of neutrophils into the infarcted myocardium.

The representative relationship between myocardial injury produced by ischemia associated with the infiltration of neutrophils, and the EPA/AA ratio in platelet membrane is shown in Fig. 3. The infiltration of neutrophils and tissue lysis were both suppressed as the EPA/AA ratio increased.

**Contractile function of the left ventricle**

Contractile functions of the left ventricles in both groups were estimated by measuring the dp/dt of the left ventricle. The positive peak dp/dt in the EPA-treated group was 1408±156 mmHg/sec², which was significantly higher than that in controls (933±115 mmHg/sec²; p<0.01). This suggests that post-ischemic left ventricular systolic function was preserved better in the EPA-treated group than in the control group.

**DISCUSSION**

The initial epidemiological reports of Dyerberg20–22 promoted interest in the effects of EPA in the prevention of, and therapy for, atherosclerosis and thromboembolic diseases23. As the anti-inflammatory role of EPA became evident, EPA-rich fish oils have been used clinically to treat acute and chronic inflammatory diseases with encouraging results24. However, all of these previous studies used crude extract instead of highly-purified EPA. Furthermore, the effects of highly-purified EPA have not previously been evaluated in experimental infarction. We performed the present series of investigations to determine whether dietary supplementation with highly-purified EPA, instead of fish oils, could attenuate the myocardial damage after coronary ligation.

The data demonstrate that 8 weeks of supplemental dietary EPA resulted in a significant reduction in ischemic myocardial damage induced by 72 h of coronary ligation, as evidenced by a higher content of CK in infarced myocardium, a smaller size of the infarcted area, and a higher value of the peak positive dp/dt. This result confirms and adds to the report of Hock et al25 who examined the effects of fish oil on ischemic damage in rat hearts.

In the EPA-treated group, the incidence of ventricular fibrillation was significantly reduced, and the survival rate after coronary occlusion was greater than in the control group. Although the exact mechanism by which EPA supplementation decreases the incidence of ventricular fibrillation is not known, it may be related to changes in membrane phospholipid composition. It has been suggested that an increase of n-3 PUSFA in the plasma membrane has a beneficial effect on membrane stability by increasing the ratio of n-3/n-6 PUSFA in the membrane26. McLennan27,28 showed that long-term (6–7 to 12 month) feeding with fish oils reduced the incidence of ventricular fibrillation in rat hearts. Our data suggest that even a relatively short period (8 weeks) of supplementation with highly-purified EPA can reduce the incidence of ventricular fibrillation induced by ischemia. Based on these data, we suggest that the mortality rate after coronary occlusion may be lower in subjects whose diets are supplemented with EPA; a hypothesis which deserve serious evaluation.

Our data also showed that after feeding with EPA (1) infiltration of neutrophils into the infarced myocardium was suppressed (Fig. 2-C and Fig. 3); (2) the amount of 12-HETE, a chemotactic and/or neutrophil activating factor produced from AA in ischemic myocardium via a lipooxygenase pathway, was decreased (Fig. 2-B); and (3) the chemotactic activities of neutrophils were depressed (Fig. 1). These data are consistent with an earlier finding which suggested that EPA inhibits the chemotactic activities of neutrophils29.

The role of neutrophils in the promotion of tissue damage in myocardial ischemia attracted interest in the past decade30. Several studies have shown that ischemic myocardial damage is attenuated by depletion of neutrophils31 anti-leukocyte agents32 or free-radical scavengers33 produced by leukocytes. However, no previous experimental study

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examined the effects of EPA on neutrophils in ischemic myocardium. Our data indicate that EPA supplementation produces a decrease in neutrophil infiltration into the ischemic myocardium. This decrease is the result of a reduction in the motility of neutrophils and a decrease in the production of lipoygenase metabolites derived from AA in the infarcted myocardium. These effects may result from an increase of the EPA/AA ratio in the plasma membranes of various cells, including cardiomocytes as well as neutrophils. Although this does not imply that the decreased tissue damage after EPA supplementation results solely from the decreased infiltration of neutrophils, it strongly suggests that the inhibition of neutrophilic function has a favorable influence on myocardial ischemia. These considerations may be supported by the findings shown in Fig. 3.

Fish oil has been used clinically to reduce restenosis after percutaneous transluminal coronary angioplasty with controversial results. Epidemiological study has indicated that fish consumption is related to a decreased mortality rate in coronary heart diseases. Our results suggest that EPA reduces the mortality rate of coronary heart diseases, at least in part, by inhibiting the inflammatory component of tissue damage after coronary occlusion.

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