Perfusion therapy by percutaneous coronary intervention (PCI) reduces infarct size and improves left ventricular (LV) function, with improved clinical outcomes in patients with acute myocardial infarction (AMI). However, reperfusion therapy could also elicit adverse reactions that might limit its beneficial action, leading to irreversible cardiac damage.

In order to reduce and/or prevent those adverse reactions, cardioprotective agents are emerging in patients with AMI. The previous studies demonstrated that high intake of fish oil and n-3 polyunsaturated fatty acids could reduce myocardial infarction and death including sudden cardiac death. Eicosapentaenoic acid (EPA), the major component of fish oil, exerts several beneficial effects in the pathological processes of AMI, including inhibition of thrombus formation and inflammation and stimulation of endothelial production of nitric oxide. EPA also inhibits sphingosylphosphorylcholine-induced Rho-kinase activation, leading to inhibition of vasoconstriction. Rho-kinase has been identified as one of the effectors of the small GTP-binding protein, Rho. Rho-kinase is involved in various cellular functions, including not only contraction of smooth muscle cells but also apoptosis and proliferation of vascular smooth muscle cells, mitogenesis and angiogenesis of endothelial cells, and matrix metalloproteinase/ MMP production in cardiac fibroblasts.

Background: Eicosapentaenoic acid (EPA), the major n-3 fatty acid in fish oil, exerts cardioprotective effects against ischemic heart disease; however, the detailed mechanisms remain to be elucidated. Rho-kinase plays an important role in the pathogenesis of cardiovascular diseases including ischemia-reperfusion (I/R) injury. Thus, the hypothesis that long-term EPA treatment ameliorates myocardial I/R injury through Rho-kinase pathway inhibition in pigs in vivo was investigated.

Methods and Results: Male pigs were treated with either a control chow or EPA (600 mg·kg⁻¹·day⁻¹) for 3 weeks (n=8 each) and were subjected to myocardial ischemia by 90-min occlusion of the left circumflex coronary artery and subsequent 60-min reperfusion. The EPA group had an increased EPA level in red blood cells (4.4±0.3 mol%). The EPA treatment significantly ameliorated myocardial I/R injury, including regional wall motion abnormality (EPA 5.3±3.6 vs. control 35.1±3.8 unit, *P*<0.0001), left ventricular ejection fraction (EPA 43±9% vs. control 32±7%, *P*<0.05), occurrence of ventricular arrhythmias (EPA 18±73 vs. control 389±51 events, *P*<0.0001) and histological accumulation of inflammatory cells (*P*<0.01). Importantly, the EPA treatment significantly inhibited myocardial Rho-kinase activity (assessed by the extent of the myosin-binding subunit phosphorylation) (EPA 0.47±0.11 vs. control 0.77±0.14, *P*<0.05) and preserved myocardial eNOS activity (EPA 0.56±0.13 vs. control 0.23±0.07, *P*<0.01) with a significant correlation noted between them.


Key Words: Eicosapentaenoic acid (EPA); Inflammation; Nitric oxide; Reperfusion

Reperfusion therapy by percutaneous coronary intervention (PCI) reduces infarct size and improves left ventricular (LV) function, with improved clinical outcomes in patients with acute myocardial infarction (AMI). However, reperfusion therapy could also elicit adverse reactions that might limit its beneficial action, leading to irreversible cardiac damage. In order to reduce and/or prevent those adverse reactions, cardioprotective agents are emerging in patients with AMI.

The previous studies demonstrated that high intake of fish oil and n-3 polyunsaturated fatty acids could reduce myocardial infarction and death including sudden cardiac death. Eicosapentaenoic acid (EPA), the major component of fish oil, exerts several beneficial effects in the pathological processes of AMI, including inhibition of thrombus formation and inflammation and stimulation of endothelial production of nitric oxide (NO). EPA also inhibits sphingosylphosphorylcholine-induced Rho-kinase activation, leading to inhibition of vasospasm.

Rho-kinase has been identified as one of the effectors of the small GTP-binding protein, Rho. Rho-kinase is involved in various cellular functions, including not only contraction of smooth muscle cells but also apoptosis and proliferation of vascular smooth muscle cells, mitogenesis and angiogenesis of endothelial cells, and matrix metalloproteinase/ MMP production in cardiac fibroblasts.
vascular smooth muscle cells but also actin cytoskeleton organization, cell adhesion and motility, cytokinesis, and gene expression. Rho-kinase also upregulates pro-inflammatory molecules and downregulates endothelial NO synthase (eNOS), which might play an important role in the pathogenesis of ischemia-reperfusion (I/R) injury.

In the present study, we thus tested our hypothesis that long-term treatment with EPA ameliorates myocardial I/R injury partly through inhibition of the Rho-kinase pathway in pigs in vivo.

**Methods**

The present study conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All procedures were performed according to the protocols approved by the Institutional Committee for Use and Care of Laboratory Animals of Tohoku University (20MdA-46).

**Animals and EPA Treatment**

A total of 16 domestic male pigs (2–3 months old and weighing 20–30 kg) were randomly divided into the following 2 groups: 8 pigs were orally given EPA (600 mg · kg⁻¹ · day⁻¹; EPA ethyl ester of purity >99%; Mochida Pharmaceutical, Tokyo, Japan) for 21 days (EPA group), and the remaining 8 pigs were fed with a standard chow alone (control group).

The present dose and duration of the EPA treatment were determined based on a previous study with rabbits. The fatty acids composition of the plasma, red blood cells (RBC), and homogenized heart tissue extracted from the interventricular septum (100 mg of tissue/ml of saline) was determined by capillary gas chromatography. Total lipids were extracted by Folch’s procedure and then fatty acids were methylated with boron trifluoride and methanol, and then methylated fatty acids were analyzed using a gas chromatograph (Shimadzu GC-17A, Shimadzu Corporation, Kyoto, Japan) and a BPX70 capillary column (0.25 mm in internal diameter × 30 m in length, SGE International Ltd, Melbourne, Australia). Tricosanoic acid, C23:0 was used as an internal standard.

Porcine Model of Myocardial I/R

After the 3-week treatment, the animals were anesthetized with ketamine hydrochloride (20 mg/kg, IM) and sodium pentobarbital (20 mg/kg, IV). Surface ECG, heart rate and arterial blood pressure were continuously monitored by a polygraph recording system (LEG1000, Nihon-Kohden, Tokyo, Japan). We inserted a 7 Fr sheath into the left carotid artery for cardiac catheterization. A bolus of heparin (5,000 IU) was administered intravenously and 2,000 IU was injected every hour. We performed left ventriculography (LVG) and coronary angiography (CAG) in a left oblique view with the use of a cineangiography system (Toshiba Medical, Tochigi, Japan).

LV volume and LV ejection fraction were calculated using

### Table 1. Fatty Acids Composition in the Plasma, Red Blood Cells and the Heart (n=8)

<table>
<thead>
<tr>
<th>Condition</th>
<th>AA</th>
<th>EPA</th>
<th>AA/EPA</th>
<th>DHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7.34±2.12</td>
<td>0.51±0.44</td>
<td>15.25±8.96</td>
<td>1.40±0.77</td>
</tr>
<tr>
<td>EPA</td>
<td>3.94±3.10</td>
<td>13.28±2.92</td>
<td>0.32±0.27</td>
<td>0.49±0.26</td>
</tr>
<tr>
<td>RBC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>4.11±1.39</td>
<td>0.18±0.10</td>
<td>24.31±8.53</td>
<td>1.35±0.61</td>
</tr>
<tr>
<td>EPA</td>
<td>2.61±0.29</td>
<td>4.30±0.63</td>
<td>0.63±0.20</td>
<td>1.08±0.17</td>
</tr>
<tr>
<td>Heart</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>14.70±1.57</td>
<td>1.18±0.44</td>
<td>13.49±3.49</td>
<td>1.34±0.35</td>
</tr>
<tr>
<td>EPA</td>
<td>6.69±1.54</td>
<td>14.46±1.97</td>
<td>0.48±0.17</td>
<td>0.74±0.28</td>
</tr>
</tbody>
</table>

Results (mol% to total fatty acids) are expressed as mean±SD. *P<0.01 vs. Control group.

AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; RBC, red blood cells.

### Table 2. Hemodynamic Changes During Ischemia and Following Reperfusion

<table>
<thead>
<tr>
<th>Condition (min)</th>
<th>Pre</th>
<th>Ischemia</th>
<th>Reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td>HR (1/min)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>120±8</td>
<td>137±9*</td>
<td>139±12*</td>
</tr>
<tr>
<td>EPA</td>
<td>119±20</td>
<td>135±23*</td>
<td>138±23</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>105±6</td>
<td>83±7*</td>
<td>85±8*</td>
</tr>
<tr>
<td>EPA</td>
<td>106±11</td>
<td>81±3*</td>
<td>83±2*</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>77±9</td>
<td>68±6*</td>
<td>65±6*</td>
</tr>
<tr>
<td>EPA</td>
<td>78±9</td>
<td>67±4*</td>
<td>62±3*</td>
</tr>
<tr>
<td>MBP (mmHg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>87±6</td>
<td>73±6*</td>
<td>72±6*</td>
</tr>
<tr>
<td>EPA</td>
<td>87±9</td>
<td>72±3*</td>
<td>69±2*</td>
</tr>
</tbody>
</table>

Results are expressed as mean±SD. *P<0.05 vs. pre. †P<0.05 vs. Control group.

HR, heart rate; EPA, eicosapentaenoic acid; SBP, systolic blood pressure (BP); DBP, diastolic BP; MBP, mean BP.
Simpson’s method. The centerline method was used to assess regional LV contractility. As reported previously, coronary vasomotor responses to serotonin (5-HT, 10 and 100 μg/kg, IC) and then endothelium-independent vasodilating responses to nitroglycerin (10 μg/kg, IC) were examined. The measurements were made in a blinded manner for the left anterior descending coronary artery, at both the large (just proximal to the first diagonal branch) and small (distal portion of branch with a baseline diameter of ~500 μm) coronary arteries. A coronary angioplasty balloon (2.5–3.5 mm in diameter depending on the vessel size) was then introduced into the left circumflex coronary artery (LCX) and inflated to induce myocardial ischemia at the lowest pressure that completely occluded distal coronary flow. After 90 min of myocardial ischemia, reperfusion was made by completely deflating the angioplasty balloon for 60 min. Both CAG and LVG were repeated to assess the patency of LCX and the LV wall motion following I/R, respectively. Finally, the animals were euthanized with a lethal dose of sodium pentobarbital (40 mg/kg, IV). The heart tissues were immediately extracted from the interventricular septum as a non-ischemic area sample and those from the LV posterior wall as an ischemic area sample, frozen in tissue-freezing medium and stored at −80°C for subsequent histological and molecular analyses. A preliminary experiment with Evans blue staining demonstrated that the extent of the risk area relative to the LV was comparable between the 2 groups (EPA 38±2% vs. control 34±2%, NS) (n=4 each).

**Histological Analysis**

Histological analysis was performed in a blinded manner on 5-μm cryosections of the tissue. The sections were examined with a fluorescence microscope, using an NIB filter, and subsequently stained with hematoxylin and eosin for light microscopy study. The number of infiltrating neutrophils was determined by counting the cells in 10 randomly selected high-power fields from various samples of each experiment. The stained sections were examined at a magnification of ×100. The number of neutrophils (mm²) in the interstitium and in vessels in each section was determined in a blinded manner from 25 random fields (0.01 mm² each) and was averaged to give the number of the cells (mm²).

**Western Blot Analysis**

The myocardial tissue was homogenized in a sample buffer that contained 50 mmol/L HCL (PH 7.4), 150 mmol/L NaCl, 10% glycerol, 1% Triton-X, 10 mmol/L sodium pyrophosphate, 10 mmol/L β-glycerophosphate, 1 mmol/L orthovanadate, 10 mmol/L NaF, 1 mmol/L DDT, 5 mmol/L ethylene diamine tetraacetic acid and 1% protease inhibitor cocktail. The tissue lysate was then centrifuged (15,000 rpm, 4°C, 20 min) and the supernatant was collected. The protein concentration was quantified by a bicinechoninic protein assay kit (Pierce Chemical Rockford, IL, USA). The extracted samples (20 μg of protein) were subjected to 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis/immunoblot analysis (100A for 1 h) and subsequently transferred to a polyvinylidene difluoride membrane electrophoretically (100V for...
The membranes were incubated by the specific antibody for Rho-kinase (ROCK) I and II (BD Transduction Laboratories), phosphorylated (p)-myosin binding subunit (MBS) (Thr696; Cosmo Bio Co, Ltd, Tokyo, Japan) and p-eNOS (Ser1177; Cell Signaling, Tokyo, Japan). Anti-mouse IgG was used as a secondary antibody (1:5,000). The ischemic and non-ischemic regions containing proteins were visualized by an electrochemiluminescence Western blotting luminal reagent (RPN2132; GE Healthcare UK Ltd, UK). Immnoreactivity was detected by enhanced chemiluminescence autoradiography (ECL Western blotting detection kit; Amersham Pharmacia Biotechnology, UK). Normalization for loading differences was accomplished using ratios of the densitometry signals for proteins of interest to GAPDH or β-actin. Scanning densitometry was used to quantify signal density from luminograms.

**Statistical Analysis**

Continuous variables are expressed as mean±SD and categorical variables as percentages. An unpaired Student’s t-test was used to analyze differences in continuous variables. One way analysis of variance followed by Bonferroni’s test was used to examine differences among multiple variables. Correlation among variables was determined using linear regression analysis. A linear regression line was calculated by the least-square method to assess the correlation between 2 parameters. Statistical analyses were performed with GraphPad Instat V3.06 for Windows (GraphPad Software Inc, La Jolla, CA, USA) and SigmaStat for Windows version 3.00.0 (SPSS Inc, Chicago, IL, USA). A value of P<0.05 was considered to be statistically significant.

**Results**

**Effects of EPA Treatment on Fatty Acid Components**

The long-term EPA treatment markedly increased the proportion of EPA (mol%) not only in the plasma but also in RBC and cardiac tissue (all P<0.01) (Table 1). In contrast, the EPA treatment significantly decreased the proportion of arachidonic acid (AA), AA/EPA ratio and docosahexaenoic acid (DHA) (mol%) in the plasma, RBC and heart tissue (all P<0.01) (Table 1).

**Coronary Vascular Responses to Serotonin In Vivo**

Between the EPA and the control group (n=8 each), there were no significant differences in hemodynamic variables (heart rate and blood pressure) at any measurement points except at 20 min during ischemia (Table 2). After I/R, the heart rate increased and blood pressure decreased in both groups, but to a similar extent (Table 2).

Figure 1 shows coronary vascular responses to serotonin (5-HT). There was no significant difference in baseline coronary diameter between the 2 groups (data not shown). A low dose of serotonin (10 μg/kg, IC) caused mild and insignificant

**Figure 2.** Eicosapentaenoic acid (EPA) treatment improves ischemia-reperfusion-induced myocardial dysfunction. Although left ventricular (LV) end-diastolic volume was comparable (A, C), LV end-systolic volume was larger in a control pig than in an EPA-treated pig following ischemia-reperfusion (B, D). The quantitative data shows that the EPA treatment significantly ameliorated myocardial ischemia-reperfusion injury, including LV ejection fraction (LVEF) (E) and regional wall motion of the left circumflex territory (F). Results are expressed as mean±SD.
EPA Ameliorates Myocardial I/R Injury

coronary vasodilatation in both groups, whereas a high dose of serotonin (100 μg/kg, IC) caused coronary vasoconstriction in large and small arteries in the control group, but these responses were significantly attenuated in the EPA group (Figure 1).

LV Function and Arrhythmias Following Reperfusion

The baseline LV volumetric data were comparable between the 2 groups (data not shown). Also, myocardial blood flow in the LCX region during ischemia, when measured by colored microspheres, was low and comparable between the 2 groups (EPA 0.10±0.04 vs. control 0.10±0.08 ml·min⁻¹·kg⁻¹, n=4 each). After I/R, both global and regional LV functions were significantly reduced in the control group, whereas the EPA treatment significantly ameliorated the I/R injury, including LV ejection fraction (EPA, 43±9% vs. control, 32±7%, P<0.05) and ischemic regional wall motion abnormality (EPA, 5.3±3.6 vs. control, 35.1±3.8 unit, P<0.0001) (Figure 2). The EPA treatment also suppressed the occurrence of ventricular arrhythmias, including ventricular fibrillation, ventricular tachycardia and premature ventricular contraction, after I/R (EPA, 181±73 vs. control, 389±51 events, P<0.0001) (Figure 3).

Inflammatory Cell Infiltration and Myocardial eNOS and Rho-Kinase Activities

The EPA treatment also significantly attenuated I/R-induced neutrophil infiltration in the ischemic region as compared with the control group (EPA, 69±34 vs. control, 170±107 cells/mm², P<0.001) (Figure S1). In comparison with the non-ischemic myocardium, Rho-kinase activity (as assessed by the extent of MBS phosphorylation) was markedly increased by ~50–100 fold after I/R in both groups (Figure 4). However, the EPA treatment significantly inhibited the I/R-induced Rho-kinase activation (EPA, 0.47±0.11 vs. control, 0.77±0.14, P<0.05) (Figure 4). The EPA treatment also preserved eNOS activity (as assessed by the extent of eNOS phosphorylation) in the ischemic myocardium (EPA, 0.56±0.13 vs. control, 0.23±0.07, P<0.01) (Figure 5). In contrast, myocardial Rho-kinase and eNOS activities in the non-ischemic myocardium were comparable between the 2 groups (Figures 4, 5). Importantly, there was a significant negative correlation between myocardial Rho-kinase activity and myocardial eNOS activity (R=−0.584, P=0.01) (Figure 6). The expression of ROCK-I was not changed by I/R or by the treatment (Figure S2), whereas the expression of ROCK-II was decreased by I/R, but was not affected by the treatment (Figure S3).

Discussion

The major findings of the present study were as follows: (1) the long-term EPA treatment significantly ameliorated myocardial I/R injury, including LVEF and regional wall motion abnormality, occurrence of ventricular arrhythmias and myocardial accumulation of neutrophils; and (2) these beneficial effects of EPA were associated with inhibition of myocardial Rho-kinase activity and preserved myocardial eNOS activity with a significant negative correlation between them.

Previous Clinical Studies of EPA Treatment and Cardiovascular Events

In 1976, it was first demonstrated that the high consumption
of fish oil was associated with reduced cardiovascular risk in Inuit in Greenland. Since then, accumulating evidence has demonstrated the beneficial effects of n-3 fatty acids, specifically those of EPA and DHA, especially for primary and secondary prevention of coronary artery diseases. Dietary supplementation with n-3 fatty acids are now known to exert multiple beneficial effects, including a reduction in lipid levels, blood pressure and arrhythmias, improvement of endothelial and autonomic functions and inhibition of platelet aggregation. In the present study, the EPA treatment markedly increased its concentration, not only in the plasma but also in RBC and heart tissue, while it conversely decreased the concentration of AA and DHA. The EPA levels in the RBC membrane in the present model were comparable with those found in patients treated with 2,400 mg EPA for 2 years. Also, in the present study, highly purified EPA was used, which might have been an advantage as compared with the previous studies where fish oil or a combination of EPA and DHA was used.

Pleiotropic Effects of EPA on I/R Injury
In patients with AMI, the extent of infarct size is crucial because residual LV function determines their prognosis. It has been well established that the most effective strategy for limiting infarct size is early restoration of coronary blood flow to the ischemic myocardium by thrombolysis, PCI or their combination. Despite the success of contemporary reperfusion therapy and the effective restoration of epicardial coronary flow, many patients have a suboptimal flow at the coronary microcirculatory level. This could be due to I/R injury, which is a complex process involving various interrupting factors, such as microthrombus embolization, activated inflammatory cascade and enhanced production of reactive oxygen species. Those patients with impaired coronary microcirculation have an impaired recovery of LV function and poor long-term prognosis.

EPA could exert beneficial effects against myocardial I/R injury. In the present study, although the EPA effect on blood pressure (found only at 20 min during ischemia) seems to be minimized and platelet aggregation activity following EPA treatment was not assessed, the EPA treatment significantly attenuated coronary vasoconstricting responses to serotonin and ameliorated regional LV wall motion abnormality, LV dysfunction and occurrence of ventricular arrhythmias. It has been previously demonstrated that cardioprotective effects of EPA are mediated, in part, by its anti-thrombotic and anti-inflammatory effects and improved ion channel functions. However, the detailed mechanisms of the beneficial effects of EPA remain to be fully elucidated.

Pathological Role of Rho-Kinase Pathway Activation and Its Inhibition by EPA
In the present study, we focused on the Rho-kinase pathway, as we and others have previously demonstrated an involvement of Rho-kinase activation in the pathogenesis of myocardial I/R injury. Rho-kinase is a downstream effector of the
EPA Ameliorates Myocardial I/R Injury

**Figure 5.** Eicosapentaenoic acid (EPA) treatment preserved eNOS activity in the ischemic myocardium. (A) Representative Western blots of myocardial expression of p- and t-eNOS. (B) Quantitative results of the ratio of p-eNOS to t-NOS, indicating eNOS activity. Results are expressed as mean±SD. eNOS, endothelial nitric oxide synthase.

**Figure 6.** Significant negative correlation between Rho-kinase activity and eNOS activity in the myocardium. A significant negative correlation was noted between Rho-kinase activity and eNOS activity in the myocardium. EPA, eicosapentaenoic acid; eNOS, endothelial nitric oxide synthase.
small GTPase Rho and mediates diverse cellular functions, such as smooth muscle cell contraction, cell migration and proliferation. In a previous study, it has been reported that hydroxyfasudil, a specific Rho-kinase inhibitor, could reduce myocardial infarct size after I/R. It has been reported that EPA could inhibit sphingosylphosphorylcholine-induced Rho-kinase activation in vitro. However, to the best of our knowledge, the present study is the first study demonstrating that long-term EPA treatment significantly inhibits Rho-kinase activation in the myocardium subjected to I/R in vivo. The Rho-kinase pathway is activated by inflammatory stimuli, which is likely to be involved in neutrophil accumulation after I/R. Platelet activation with a resultant microthrombus formation might also be associated with Rho-kinase pathway activation by releasing serotonin and platelet-derived growth factors and by interaction with thrombin. It also has been reported that expression and activity of Rho-kinase are enhanced by hypoxia.

The present study also demonstrated that the inhibitory effects of EPA on Rho-kinase activation is accompanied with preserved eNOS activity and that there was a negative correlation between Rho-kinase activity and eNOS activity in the myocardium (Figure 6). Rho-kinase is involved in the regulation of eNOS activity, where activated Rho-kinase reduces eNOS activity through inhibition of protein kinase B/Akt. Conversely, Rho-kinase inhibition leads to a rapid phosphorylation and activation of Akt via PI3-kinase, leading to increased NO production.

There seems to be a discrepancy between Rho-kinase activity (assessed by the extent of the myosin-binding subunit phosphorylation) and its expression in response to I/R injury and EPA treatment. The Rho-kinase was activated following I/R, which was significantly decreased by the EPA treatment. The expression of ROCK-I was not changed by I/R or by the treatment, whereas the expression of ROCK-II was decreased by I/R, but was not affected by the treatment. As reported previously, these findings might be related, in part, to the time-course of the myosin-binding subunit –ROCK interaction or cleavage of ROCK. Further studies are needed to determine the exact mechanism.

Study Limitations
Several limitations should be mentioned for the present study. First, the present study was designed by using a single dose and performed in normal juvenile pigs without pre-existing atherosclerotic coronary plaques or myocardial dysfunction, both of which could affect myocardial responses to ischemia and reperfusion. Second, because of the potential confounding effect of the relatively short (60 min) reperfusion period, more extended follow up is required to evaluate the effect of infarct size reduction in future studies. Finally, despite the comparable area at risk between the 2 groups, we did not directly measure the area of necrosis following a 90 min-LCX occlusion, which could develop infarction in 30–40% of the area at risk, which was examined by triphenyl tetrazolium chloride staining.

Conclusion
In conclusion, the present study demonstrates that long-term treatment with EPA ameliorates myocardial I/R injury partly through the Rho-kinase pathway inhibition in pigs in vivo.

Acknowledgments
The present work was supported, in part, by grants-in-aid [18890018] from the Scientific Research and the global COE project [F02], and grants-in-aid [H22-Shinkin-004] from the Japanese Ministry of Education, Culture, Sports, Science, and Technology, Tokyo, Japan. The authors wish to thank Dr Masayuki Sonomoto (Mochida Pharmaceutical Co, Ltd) and Dr Takatoshi Hizume (Kyushu University Graduate School of Medicine) for their valuable cooperation in the present study, Mochida Pharmaceutical Co, Ltd for providing EPA and Fumie Tatebayashi and Naomi Yamaki for their excellent technical assistance.

Disclosure
Conflict of interest: none.

References
19. Yada T, Shimokawa H, Hiramatsu O, Kajita T, Shigeto F, Tanaka E,


