Myocardial protection using an omega-3 fatty acid infusion: Quantification and mechanism of action

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Supplemental material is available online.

Objectives: Omega-3 fatty acids exhibit anti-inflammatory, antithrombotic, and antiarrhythmic properties. We investigated the extent and underlying mechanism of protection conferred by a pre-emptive omega-3 infusion in a model of regional cardiac ischemia-reperfusion injury.

Methods: New-Zealand White rabbits received either the omega-3 infusion or a control infusion of 0.9% saline ($n = 14$ in each group). The large marginal branch of the left coronary artery was occluded for 30 minutes, cardiac function was assessed during 3 hours of reperfusion, and infarct size was measured. Pretreatment-induced alterations in myocardial membrane fatty acid composition and intramyocardial heat shock protein 72 were additionally assessed ($n = 5$ in each group). Serum markers of myocardial membrane oxidative stress, malonaldehyde and 8-isoprostane, were also determined. Results are expressed as means $\pm$ standard error of the mean and significance was tested with analysis of variance.

Results: Pretreatment increased myocardial membrane omega-3 fatty acid content 5-fold, from $0.94\% \pm 0.07\%$ in controls to $5.38\% \pm 0.44\%$ in the omega-3 group ($P < .01$), and it produced a 225% elevation of levels of heat shock protein 72 ($P = .019$) before ischemia-reperfusion. This was associated with a 40% reduction in infarct size ($P < .01$). Whereas the reperfusion-induced rise in malonaldehyde levels was higher with omega-3 pretreatment, $10.2 \pm 1.5 \mu\text{mol/L}$ versus $6.1 \pm 0.7 \mu\text{mol/L}$ in controls ($P = .04$), 8-isoprostanes showed a 9-fold reduction, $679 \pm 190 \text{ pg/mL}$ in controls vs $74 \pm 45 \text{ pg/mL}$ in the omega-3 group ($P = .0077$).

Conclusions: A pre-emptive omega-3 infusion significantly reduces infarct size through the dual mechanisms of upregulation of heat shock protein 72, a key preconditioning protein, and a dramatic increase in the omega-3 content of myocardial membranes, which appears to facilitate a shift in oxidant ischemia-reperfusion injury. Further study to optimally shorten the pretreatment regimen for this potentially acceptable infusion will now be pursued.

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More than 25 years have passed since Buckberg and associates¹ defined the benefits of cardioplegia in attenuating myocardial oxygen consumption, and our understanding of myocardial preservation has moved from the simple paradigm of the balance between supply and demand to tackling the multidimensional pathology of ischemia-reperfusion injury. Complex combined

Abbreviations and Acronyms

HSP	= heat shock protein
LVEDP	= left ventricular end-diastolic pressure
LVESP	= left ventricular end-systolic pressure
$dP/dt_{\max}$	= maximum rate of change of systolic and diastolic pressures
$dP/dt_{\min}$	= minimum rate of change of systolic and diastolic pressures

valve-graft procedures, redo procedures, more diffuse coronary disease, and surgery in those with hypertrophic or dilated hearts challenge current methods of myocardial protection with cardioplegia.² More recently, off-pump surgery potentially limits protection against regional ischemia. The infant's heart appears to be more vulnerable to injury during surgery,² in which there has been a shift from palliation to earlier primary anatomic correction. In all cases, the lack of physiologic reserve to cope with inadvertent intraoperative ischemia-reperfusion injury is becoming more prevalent.³

It is widely established experimentally that long-chain omega-3 fatty acids protect against and can terminate ischemic ventricular arrhythmias.^{4,5} Their anti-inflammatory effects, which include attenuation of leukocyte-endothelial interactions and production of less biologically active prostaglandins and leukotrienes, could also be beneficial in cardiac ischemia-reperfusion injury. Previous observations have shown that omega-3 pretreatment induces heat shock proteins (HSP) in the brain, protecting against cerebral infarction, suggesting a preconditioning phenomenon.⁶ We sought to delineate whether a long-chain omega-3 fatty acid infusion normally used as part of parenteral nutrition, and so approved for clinical use, might confer myocardial protection when administered in the acute setting. In eliciting the possible mechanism of any observed protection, we focused on the effects of incorporating the fatty acids into the myocardial membrane, a process shown to be important in their antiarrhythmic effect.⁵ We further hypothesized that enhanced levels within the membrane would favorably alter the pattern of myocardial membrane oxidant ischemia-reperfusion injury. The presence of a preconditioning effect was also studied through measurement of HSP72 levels.

Materials and Methods

All animal experiments were performed under a license obtained from the Department of Health of Ireland under the Cruelty to Animals Act of 1876. The protocol was approved by the animal research ethics committee of The Royal College of Surgeons in Ireland. Animal care was conducted in conformity with institutional guidelines in compliance with international laws and policies.

Experimental Procedure

Male New Zealand White rabbits (weight 2.5–3.5 kg) were acclimated in a quarantine room with access to standard pellet diet and water ad libitum for 10 days without any stresses. Rabbits were assigned alternatively ($n = 14$ in each group) to receive either an eicosapentanoic acid–docosahexanoic acid lipid emulsion approved for clinical use as part of parenteral nutrition (Omegaven; Fresenius Kabi, Bad Homburg, Germany) at a dose of 5 mL/kg or an equal volume of 0.9% saline. The infusion was given daily for 4 days over 4 hours and again 2 hours before the procedure via the marginal ear vein. The anesthetized in situ rabbit model of regional cardiac ischemia-reperfusion injury was used as described previously.⁷ In brief, anesthetized rabbits (ketamine 35 mg/kg and xylazine 5 mg/kg) were ventilated via a tracheostomy (100% oxygen, isoflurane 2 L/min) maintaining a PCO_2 less than 4.0 kPa. A 2.2F Millar cardiac catheter (SPR-249A; Millar Instruments, Inc, Houston, Tex) was in the left carotid artery for hemodynamic measurements and a 20-gauge cannula in the left external jugular vein for blood sampling, with a temperature of $38.5^\circ\text{C} \pm 0.5^\circ\text{C}$. After left anterolateral thoracotomy, the large marginal branch of the left coronary artery was occluded for 30 minutes and reperfused for 3 hours. Further rabbits ($n = 5$ in each group) underwent infusions, planned death under anesthesia, and cardiac excision. These hearts were preserved for immunohistochemistry and for measurement of myocardial membrane fatty acid composition and HSP72.

Evaluation of infarct size. After 3 hours of reperfusion the excised heart was mounted on a modified Langendorff apparatus, snare reoccluded, and infused with 0.5% copper phthalocyanine dye (Sigma-Aldrich, UK, Poole, Dorset, United Kingdom) to determine the volume “at risk” of infarction. The heart was cut into 2-mm thick slices and stained with 1% triphenyl-tetrazolium dye (Sigma-Aldrich, UK) to determine the infarcted volume. At both stages the slices were scanned into a computer, and “at-risk” and infarct measurements were performed with Scion Image software (Microsoft Corporation, Redmond, Wash). Results were reported as the percentage of the “at-risk” volume infarcted for each heart.⁷

Hemodynamic measurements. Hemodynamic recordings were processed with PowerLab 2/20 (ADInstruments, Oxfordshire, UK) and Chart v4.04. The catheter was temporarily advanced into the left ventricle before thoracotomy and at 3 hours of reperfusion. The left ventricular end-diastolic (LVEDP) and end-systolic (LVESP) pressures and the maximum and minimum rates of change of systolic and diastolic pressures ($dP/dt_{\max}$ and $dP/dt_{\min}$), measures of left ventricular function, were assessed.

Quantification of myocardial membrane fatty acid composition. Myocardial membrane fatty acid composition was assessed by high-performance capillary gas chromatography. In brief, a 150-mg heart sample was homogenized in 1 mL of methanol containing 75 $\mu\text{g/mL}$ of undecanoic acid. Contents were vortex mixed, then 2 mL of chloroform was added, and the contents were vortex mixed again. This was followed by the addition of 1 mL of 2% potassium chloride solution, vortex mixed, contents centrifuged, and the lower lipid containing layer removed and evaporated to near dryness. The extract was reconstituted in 3 mL of methanol saturated with sodium hydroxide and heated at 70°C for 30 minutes. Then 4 mL of a 14% boron trifluoride in methanol

solution was added and further heated at 70°C for 20 minutes. Next, 15 mL of deionized water was added and the contents were allowed to cool. The resultant methyl esters were extracted into 1 mL of hexane and dried over anhydrous sodium sulfate. All reagents were from VWR International (UK). One microliter of each sample was injected into a Shimadzu GC-17AAF gas chromatograph (Shimadzu, Kyoto, Japan). Relative concentrations of the 28 fatty acids that compose the myocardial membrane were reported as percentage of the total myocardial membrane fatty acids.

Measurement of membrane fatty acid oxidation products. Venous blood samples were taken at baseline, 15 minutes, and 3 hours of reperfusion for measurement of membrane fatty acid oxidation products liberated into the circulation during reperfusion as markers of myocardial cell membrane injury. Malonaldehyde, a marker of general polyunsaturated fatty acid oxidation including omega-3 fatty acids, was assessed in plasma samples with a calorimetric assay (437634; Merck Biosciences, UK, Nottingham, United Kingdom), as described previously.⁸ Membrane arachidonic acid oxidation liberates 8-isoprostane into the circulation during early reperfusion.⁹ For 8-isoprostane levels, serum samples underwent affinity purification (516358; Cayman Chemical, Ann Arbor, Mich) and then measurement of 8-isoprostane with the Stat-8-Isoprostane ELISA kit (500431; Cayman Chemical).

Measurement of HSP72 by Western blotting. Myocardial HSP72 levels were examined by Western blotting. In brief, left ventricular samples were thawed, homogenized in cold phosphate-buffered saline solution, centrifuged, supernatants were collected, and protein concentrations were measured with the bicinchoninic acid protein concentration assay (23227; Pierce Biotechnology, Rockford, Ill). Thirty micrograms of each sample in loading buffer (0.6 mmol/L Tris-HCl, pH 6.8, 2% sodium dodecylsulfate, 5% 2-mercaptoethanol, 10% glycerol, 0.01% bromophenol blue) was separated by electrophoresis on a 12% sodium dodecylsulfate gel. Proteins were transferred electrophoretically to nitrocellulose membrane (66485; Pall Life Sciences, East Hills, NY) overnight. The membrane was incubated in 6% nonfat dried milk and 0.2% Tween-20 (BDH Laboratory Supplies, Poole, Dorset, United Kingdom) in phosphate-buffered saline solution, followed by incubation with 1:1000 mouse antihuman HSP72 antibody (SPA-810; Stressgen Biotechnologies Corporation, San Diego, Calif), then 1:2000 horseradish peroxidase-conjugated goat antimouse antibody (SAB-100; Stressgen), and developed with the enhanced chemiluminescence system (34075; Pierce Biotechnology). The membranes were subsequently exposed to stripping solution (0.2 mol/L glycine, 0.1% sodium dodecylsulfate, 1% Tween, pH 2.2) and reprobed with a 1:20,000 dilution of mouse antirabbit beta-actin antibody and a 1:2000 dilution of secondary antibody as before. The developed films were scanned into a computer for densitometry assessment of band intensity, with normalization of HSP72 values with beta-actin used for statistical comparison with controls.

Immunohistochemistry. The samples of left ventricle were cut into 4- μ m sections, deparaffinized, and stained with standard hematoxylin and eosin to assess for any acute inflammation or structural changes in the myocardium after pretreatment. Immunohistochemistry was also performed to identify any changes in macrophage content in the pretreated myocardium. Sections were incubated in 1X Trilogy solution (Cell Marque Corp, Hot Springs, Ark) in a pressure cooker for dewaxing, rehydration, and antigen

retrieval. The DAKO Envision System was used for processing (Dako Cytomation, Carpinteria, Calif). In brief, endogenous peroxidase activity was blocked, tissue sections were probed with 1:100 dilution of mouse antihuman macrophage antibody (MCA874G; Serotec UK, Kidlington, Oxford, United Kingdom), then labeled with horseradish peroxidase polymer antibody, exposed to 3-3'-diaminobenzidine substrate chromagen, and counterstained with hematoxylin. Rabbit spleen was used as a positive control.

Statistical analysis. Results are expressed as means $\pm$ standard error of the mean. Infarct and "at-risk" volumes were analyzed with a Student *t* test. Hemodynamic data were analyzed by a 2-way classification analysis of variance. Mechanism data were analyzed with a 1-way analysis of variance, with post-hoc comparisons performed using the Bonferroni comparison of means as appropriate.

Results

Reduction of Infarct Size

Overall, there was approximately a 40% reduction in infarct size in the omega-3 pretreated group ($P < .01$). The "at-risk" volumes were similar in both groups ($0.78 \pm 0.05 \text{ cm}^3$ in the control group vs $0.82 \pm 0.05 \text{ cm}^3$ in the omega-3 group, $P = .7$), making them comparable, but the percentage infarction of the "at-risk" area was significantly less in the omega-3 pretreated group ($29.1\% \pm 1.9\%$) than that in the control group ($50.4\% \pm 2.1\%$, $P < .01$).

Changes in Left Ventricular Function

Throughout the ischemia-reperfusion period there was no significant difference in hemodynamics, with LVESP ($54.1 \pm 2.1 \text{ mm Hg}$ in the control group vs $56.3 \pm 0.8 \text{ mm Hg}$ in the omega-3 group, $P = .46$) and left ventricular function ($\text{dP/dt}_{\text{max}}$ $1688 \pm 105 \text{ mm Hg/s}$ in the control group vs $1562 \pm 53 \text{ mm Hg/s}$ in the omega-3 group, $P = .38$, and $\text{dP/dt}_{\text{min}}$ $-1717 \pm 81 \text{ mm Hg/s}$ in the control group vs $-1681 \pm 56 \text{ mm Hg/s}$ in the omega-3 group, $P = .7$) comparable after 3 hours of reperfusion (Table 1). The fact that there was no difference in hemodynamics outruled this as a confounding factor that may have influenced infarct size as a result of differences in cardiac perfusion. Also, the lack of an early improvement in hemodynamics despite significant reduction in infarct size has previously been demonstrated with this model.¹⁰ After the omega-3 infusion, a small but significant amount of diastolic dysfunction was seen before thoracotomy. This was evidenced by an increase in LVEDP ($1.0 \pm 0.5 \text{ mm Hg}$ in the control group vs $4.4 \pm 0.8 \text{ mm Hg}$ in the omega-3 group, $P < .01$) and a decrease in diastolic function ($\text{dP/dt}_{\text{min}}$ $-3146 \pm 78 \text{ mm Hg/s}$ in the control group vs $-2532 \pm 146 \text{ mm Hg/s}$ in the omega-3 group, $P < .01$), which persisted during reperfusion (Table 1).

Changes in HSP72 Levels

To assess whether the natural endogenous protective stress response, that is, the phenomenon of preconditioning, was

TABLE 1. Left ventricular function during the ischemia-reperfusion period

	Control group	Omega-3 group	P value
Baseline			
LVESP (mm Hg)	67.9 ± 1.3	68.7 ± 0.8	0.8
dP/dt _{max} (mm Hg/s)	2287 ± 68	2287 ± 142	>.9
LVEDP (mm Hg)	1.0 ± 0.5	4.4 ± 0.8	<.01
dP/dt _{min} (mm Hg/s)	−3146 ± 78	−2532 ± 146	<.01
After 3 hours of reperfusion			
LVESP (mm Hg)	54.1 ± 2.1	56.3 ± 0.8	.46
dP/dt _{max} (mm Hg/s)	1688 ± 105	1562 ± 53	.38
LVEDP (mm Hg)	3.5 ± 0.5	8.3 ± 0.8	<.01
dP/dt _{min} (mm Hg/s)	−1717 ± 81	−1681 ± 56	.7

The reduction in infarct size in the omega-3 pretreated group was not associated with any early improvement in left ventricular function at 3 hours of reperfusion. A small but significant amount of diastolic dysfunction was present in the omega-3 pretreated group after pretreatment as indicated by changes in rate of change of pressure during diastole (*dP/dt_{min}*) and left ventricular end-diastolic pressure (*LVEDP*). *LVESP*, Left ventricular end-systolic pressure; *dP/dt_{max}*, rate of pressure change during systole.

involved in the protection afforded by the omega-3 pretreatment, we looked at changes in myocardial HSP72 levels after pretreatment but before ischemia-reperfusion.¹¹ Western blot assessment showed that HSP72 levels were 225% higher in the omega-3 pretreated group than in controls (*P* = .019, Figure 1).

Altered Composition of the Myocardial Membrane and Pattern of Membrane Fatty Acid Oxidant Injury
The myocardial membranes of the groups of rabbits pretreated with the infusions without undergoing ischemia-reperfusion (*n* = 10) showed marked specific changes in their composition despite only 4 days of pretreatment. Of the 28 fatty acids that compose the myocardial membrane, 2 showed significant changes in their relative content. These were the two long-chain omega-3 fatty acids. There was a 15-fold increase in eicosapentanoic acid (0.10% ± 0.02% of total membrane fatty acids in the control group vs 1.87% ± 0.06% in the omega-3 pretreated group, *P* < 0.0001) and a 7-fold increase in docosahexanoic acid (0.48% ± 0.05% of total membrane fatty acids in the control group vs 3.52% ± 0.39% in the omega-3 pretreated group, *P* < .01) (Figure 2). Despite this, arachidonic acid remained in the same concentration within the membrane (11.01% ± 1.14% of total membrane fatty acids in the control group vs 12.44% ± 0.41% in the omega-3 pretreated group, *P* = .6) (Figure 2). Malonaldehyde levels, which represent polyunsaturated fatty acid oxidant injury, rose in both groups during reperfusion but to significantly higher levels in the omega-3 pretreated group at 3 hours of reperfusion (6.1 ± 0.7 μmol/L in the control group vs 10.2 ± 1.5 μmol/L in the omega-3 pretreated group, *P* = .04). In contrast to malon-

Figure 1. Myocardial heat shock protein 72 (HSP72) levels measured by Western blot after pretreatment. Relative concentrations were standardized with beta-actin levels. A significantly higher (225%) level of HSP72 expression is present in the omega-3 pretreated group (*P* = .019).

aldehyde, at 15 minutes of reperfusion 8-isoprostane levels, a marker of arachidonic acid oxidant injury, were 9-fold lower in the omega-3 pretreated group (679.1 ± 189.6 pg/mL in the control group vs 73.7 ± 44.8 pg/mL in the omega-3 pretreated group, *P* = .0077) (Figure 3).

Histologic Changes

Histologic assessment of the hearts after pretreatment but without ischemia-reperfusion (*n* = 10) showed no inflammatory changes after pretreatment, including tissue macrophage content. Infrequently but consistently in each heart in the omega-3 pretreated group, we noted the presence of small areas of contraction band necrosis (Figure E1), a histologic change seen in the heart under conditions of stress.

Discussion

With increasing complexity of adult and pediatric cardiac surgical procedures, complete myocardial protection is proving challenging. Attempts to provide additive cardioprotection to date have met with little benefit in clinical trials. Most new therapies, such as the sodium/hydrogen exchange inhibitors or leukocyte depletion of cardioplegic solutions, have focused on a single aspect of the pathologic ischemia-reperfusion injury, with minimal benefit. Recent

Figure 2. Eicosapentaenoic acid, docosahexanoic acid, and arachidonic acid concentrations in the myocardial cell membrane after pretreatment, expressed as a percentage of total membrane fatty acid content.

observations of a reduction in sudden death both in normal healthy individuals and in those who received oral omega-3 fatty acids after a myocardial infarct suggested that there may be a conserved natural phenomenon of protection against arrhythmias associated with higher omega-3 fatty acid levels.^{12,13} Confirmation of an antiarrhythmic effect in animal models and delineation of the mechanism in isolated cardiomyocytes pointed to the common essential fact that the additional omega-3 fatty acids reside either partitioned or incorporated into the myocardial cell membrane to mediate their effect.¹⁴ We hypothesized that acute intravenous pretreatment with omega-3 fatty acids might rapidly allow their incorporation into the myocardial membrane to provide anti-infarct protection through a dual effect: protection of the intracellular environment through induction of a natural HSP stress response (that is, preconditioning) and protection of the cell membrane against oxidant reperfusion injury through the changes in membrane composition. We used the in situ rabbit model of regional cardiac ischemia-reperfusion injury to assess the potential to attenuate infarct development. This model has been used widely to examine potential cardiac preconditioning agents and to assess the mechanism of protection with preconditioning.⁷

This study demonstrates for the first time that acute intravenous supplementation with an omega-3 emulsion, which is normally used as part of parenteral nutrition regimens, is associated with a significant reduction in myocardial ischemia-reperfusion injury. This protection is preceded by an upregulation of HSP72 in the heart in response to omega-3 pretreatment. HSPs function as molecular chap-

erones in all cells, guiding the folding of newly formed proteins, maintaining protein conformation, and enhancing the elimination of damaged proteins to prevent their toxic accumulation.¹¹ HSP induction has been used as a marker of induction of the phenomenon of preconditioning, where exposure to a nonlethal stimulus activates the natural endogenous cellular stress response to protect against a subsequent more lethal injury. The finding of higher HSP72 levels in the heart in response to omega-3 pretreatment and the subsequent reduction in infarct size in this study is supported by previous studies demonstrating a direct correlation between the amount of HSPs induced (particularly HSP72) and the degree of myocardial protection ($r = 0.97$, $P = .37$).¹¹ Intriguing relationships between preconditioning and the omega-3 content of the cell membrane have been suggested. Increased omega-3 content in the cell membrane reduces myocardial oxygen demand and attenuates acidosis and lactate accumulation in the ischemic heart.¹⁵ Ischemic preconditioning actually increases the content of the omega-3 fatty acid docosahexanoic acid in the myocardial membrane in advance of a further injury.¹⁶ Also similar to our findings, thermal preconditioning 24 hours in advance of cardiac ischemia-reperfusion injury produces lower circulating levels of arachidonic acid and improved functional recovery, again illustrating that enhanced membrane stability during reperfusion is a key mediator of preconditioning cardioprotection,¹⁷ as suggested in this study. Although induction of HSP72 in this study was associated with a reduction in infarct size, it did not improve early hemodynamic recovery from ischemic injury, a widely described observation with ischemic precondition-

Figure 3. Malonaldehyde levels, a marker of polyunsaturated fatty acid oxidant injury, rose as is normal during reperfusion but were higher in the omega-3 pretreated group at 3 hours of reperfusion ($P = .04$). Conversely, 8-isoprostane levels, a marker of arachidonic acid oxidant injury, were 9 times lower in the omega-3 pretreated group during reperfusion ($P = .0077$).

ing. Therefore, to delineate whether pretreatment is activating preconditioning, further work is ongoing to assess other aspects involved in preconditioning protection, such as the activation state of the inflammatory transcription factor nuclear factor kappa B, and measurement of other proteins such as cyclooxygenase-2 and inducible nitric oxide synthase, known to be upregulated by preconditioning.

We also found that the protection was preceded by a significant increase in the omega-3 content of the cell mem-

brane and subsequently changes in the pattern of oxidant reperfusion injury; that is, an increase in the amount of polyunsaturated fatty acid oxidation (malonaldehyde levels) and a marked reduction in the amount of arachidonic acid oxidation (8-isoprostane levels). This suggests a shift in the oxidant injury away from arachidonic acid, which is structurally and functionally important to the myocardial membrane, and instead toward polyunsaturated fatty acids such as the additional omega-3 fatty acids that have partitioned into the membrane. Polyunsaturation within a fatty acid refers to the number of double bonds within the fatty acid. The more double bonds a fatty acid has, the more readily it is oxidized. Of the 28 fatty acids that compose the myocardial membrane, the omega-3 fatty acids, eicosapentanoic acid and docosahexanoic acid, have 5 and 6 double bonds respectively, making them the most polyunsaturated molecules within the membrane. Arachidonic acid has only 4 double bonds, so it is likely that the additional omega-3 fatty acids that have been incorporated into the membrane as a result of pretreatment will be preferentially oxidized over arachidonic acid. Additionally, the majority of the 28 fatty acids that compose the membrane are relatively unsaturated. A much larger quantity would be required to absorb a given oxidant injury, whereas only a smaller quantity of highly polyunsaturated fatty acids would be needed to absorb the same oxidant injury owing to the greater amount of double bonds in the polyunsaturated fatty acids. It may be that by increasing the omega-3 fatty acid content of the myocardial membrane, we not only protect against arachidonic acid oxidant injury but also likely limit the overall degree of membrane injury by, in effect "mopping-up" the excessive reactive oxygen species during reperfusion.

It is widely known that oxidant damage to the heart is closely linked to cardiac dysfunction.¹⁸ In this study, malonaldehyde levels are higher in the omega-3 pretreated group during reperfusion, but 8-isoprostane levels are lower, indicating less arachidonic acid oxidation. Lipid hydroperoxides from arachidonic acid oxidation cause loss of cardiomyocyte membrane integrity, electromechanical alterations, and enzyme leakage, leading to cell destruction.¹⁹ Arachidonic acid supplementation has previously been shown to enhance reactive oxygen radical injury to neonatal rat cardiac myocytes.²⁰

The enhanced omega-3 content of the myocardial membranes that we observed may also confer other well-established beneficial traits for ischemic myocardium. Prolonged oral supplementation with omega-3 fatty acids produces similar myocardial membrane fatty acid changes to those seen in our study, which are associated with reduced myocardial oxygen consumption and increased coronary perfusion reserve; after ischemia, creatine kinase, lactate, and acidosis within the heart are reduced, with improvements in contractile recovery during reperfusion.¹⁵ When rats are supple-

mented for 8 weeks with oral omega-3 fatty acids, there is a reduction in infarct size of 48%, similar to our findings.²¹ This benefit is seen only after 8 weeks of oral supplementation, as oral supplementation for a single week does not produce any reduction in infarct size.²¹ Acute intravenous supplementation may be able to circumvent this mandatory long pretreatment period, as our study suggests.

The presence of contraction band necrosis and mild diastolic dysfunction in this study after pretreatment also suggests that a stress response has been induced within the heart. The phenomenon of contraction band necrosis and associated myocardial dysfunction has been shown to be a transient effect not apparently associated with further deleterious consequences.²²

This study has some limitations, particularly related to the use of a regional myocardial ischemia-reperfusion injury model. The reduction in infarct size was not associated with improvements in hemodynamics at 3 hours of reperfusion, a finding that has previously been demonstrated in this model with ischemic preconditioning,¹⁰ although it is widely known in other experimental models that ischemic preconditioning improves functional recovery. This initial study was to investigate whether intravenous short pretreatment with omega-3 fatty acids could protect against infarction. Clearly, a 4-day pretreatment period is not clinically relevant, but this prolonged pretreatment was chosen to ensure incorporation of omega-3 into the myocardial membrane. The marked increase in eicosapentanoic and docosahexanoic acid levels in the membrane that we observed suggest that a shorter pretreatment period may also be effective. Finally, although this model more likely parallels myocardial injury in off-pump surgery, the mechanism of protection would clearly be additional to cardioplegia, but further studies using a global myocardial ischemic model with cardioplegic arrest are necessary to define this further.

Patients with a more limited physiologic reserve are particularly vulnerable to any further small diminution in cardiac function as an inadvertent consequence of the operative process. This study shows that an omega-3 emulsion should not be considered only as a nutritional supplement but also as a potentially clinically safe cardioprotective agent that may act in part through pharmacologic induction of preconditioning and modulation of myocardial cell membrane structure and function. This strategy warrants further investigation with optimization and shortening of pretreatment regimens to be more clinically applicable.

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Discussion

Dr Vivek Rao (Toronto, Ontario, Canada). Thank you, Dr McGuinness, for quite an exhaustive presentation of a lot of data. I would like to summarize some of your findings for the benefit of the audience, as you allowed me to read your manuscript as well as to hear your presentation.

You found that a preliminary administration of omega-3 fatty acids resulted in improvement in the membrane composition of these important molecules, which led to protection against infarction in a regional model of ischemia-reperfusion. Interestingly, you did not find significant differences in left ventricular function. I will come back to that in my questions to you.

You also postulate that the mechanism of benefit is stabilization of membrane fatty acids, preventing oxidant injury and therefore leading to preservation model of ischemia-reperfusion.

I have several questions for you.

In the past, several antioxidant-type strategies have looked at protection during ischemia-reperfusion both in a regional and a global ischemic model. Although several of these strategies have been proven to be beneficial, very few have been actually used clinically. I'd like you to comment on the feasibility of clinical application in the type of patients in whom we probably want to use this strategy, that is, our high-risk, urgent patients in the setting of an acute infarct. Is it feasible to give them a 4-day pretreatment of omega-3 or does this require further investigations into the subcellular mechanism of action.

Second, you implicate a preconditioning-type phenomenon in this process with the upregulation of HSP. Can you again describe to us how you interrelate a preconditioning-type effect with omega-3?

Last, we all know that the heart is an omnivore: it will eat anything that you give it. What you have not actually shown us in this study is how the administration of a fatty acid composition alters normal homeostasis in myocardial energy metabolism. Did you inhibit normal glucose oxidation during reperfusion and thereby lose the benefits of aerobic metabolism on ventricular function?

Dr McGuinness. Thank you for your comments.

First of all, yes, certainly many antioxidants have been described as being potentially beneficial but in the clinical scenario have been absolutely useless. In this case we have shown an antioxidant's beneficial effect. However, I think that the effect of omega-3 lies much more beyond an antioxidant effect. To say yes,

this is an antioxidant effect, this is how it's going to work, is very simplistic.

Omega-3 fatty acids have multiple actions. They have been known to be antiarrhythmic. They inhibit leukocyte endothelial interactions. They affect prostaglandin and leukotriene production. As we have shown here, they may have antioxidant effects, and they may act in a preconditioning manner. So by giving this infusion, we are not looking at one particular aspect. But the advantage of it is that we are looking at multimodal therapy for a particular type of injury.

In regard to the fact that we did not actually show any improvements in hemodynamics in response to the omega-3 infusion, although there was a reduction in infarct size, we were not actually surprised to see this at all. This open chest rabbit model is the well-established model that is used by Dr Bolli's and Dr Downey's groups in the investigation of preconditioning with cardiac ischemia-reperfusion injury. It is widely known, and Dr Downey has written a paper on this, that although ischemic-preconditioning does result in early infarct reduction at 3 hours of reperfusion, there is no functional improvement within the heart at this early 3-hour reperfusion phase. This is probably due to a stunning effect within the heart. So it is perhaps too early to see hemodynamic benefits.

When you evaluate survival models in which you use the same rabbit model, but you keep the animal alive out to about 6 weeks and do echos and measure ejection fraction, you do actually see improvements in ejection fraction and so on at that stage corresponding to the reduction in infarct size. Therein lies the benefit.

The other question concerned the potential for HSP induction and how that feeds into omega-3 fatty acids. A number of studies with long-term oral supplementation with omega-3 fatty acids have been conducted. To get the kind of changes in membrane fatty acid content that we've shown here takes 8 to 16 weeks with oral supplementation. That's the advantage of using it intravenously.

In rat studies, supplements have been used for that period of time and then hemodynamic studies have been conducted. These have shown that by pretreating and increasing the omega-3 content within the cell membrane, you reduce myocardial oxygen consumption, you improve the coronary perfusion reserve, and you induce a kind of hibernation-type state within the heart, which preconditioning has been aligned with in recent times. We do plan to do further studies to look at the activation state of nuclear factor kappa B and other preconditioning factors such as cyclooxygenase 2, inducible nitric oxide synthase, and so on. That may further elucidate the mechanism.

Control Heart

Omega-3 Pretreated Heart

Figure E1. Infrequently but consistently small areas of contraction band necrosis (*black arrows*) were seen within the omega-3 pretreated hearts on hematoxylin-eosin staining of the left ventricle.