Free Radical Research, August 2010; 44(8): 854–863

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Protection against *in vivo* liver ischemia-reperfusion injury by *n-3* long-chain polyunsaturated fatty acids in the rat

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(Received date: 4 March 2009; In revised date: 6 April 2010)

Abstract

N-3 polyunsaturated fatty acids (n-3 PUFA) affect inflammatory processes. This study evaluated the effects of dietary supplementation with fish oil on hepatic ischemia-reperfusion (IR) injury in the rat. Parameters of liver injury (serum transaminases and histology) and oxidative stress (serum 8-isoprostanes and hepatic GSH and GSSG), were correlated with NF-κB DNA binding and FA composition and inflammatory cytokine release. N-3 PUFA supplementation significantly increased liver n-3 PUFA content and decreased n-6/n-3 PUFA ratios. IR significantly modified liver histology and enhanced serum transaminases, 8-isoprotanes and inflammatory cytokines, with net reduction in liver GSH levels and net increment in those of GSSG. Early increase (3 h) and late reduction (20 h) in NF-κB activity was induced. All IR-induced changes were normalized by n-3 PUFA supplementation. In conclusion, prevention of liver IR-injury was achieved by n-3 PUFA supplementation, with suppression of oxidative stress and recovery of pro-inflammatory cytokine homeostasis and NF-κB functionality lost during IR.

Keywords: *Ischemia-reperfusion injury, n-3 polyunsaturated fatty acid, liver preconditioning, oxidative stress, pro-inflammatory cytokines, NF-κB activation*

Introduction

Liver ischemia-reperfusion (IR) injury is triggered upon prolonged oxygen deprivation followed by restoration of oxygen delivery, in conditions such as tissue resection, transplantation or trauma, involving hepatocyte and endothelial cell death [1]. These effects are the consequence of a failure of microcirculatory perfusion after reperfusion, primarily due to sinusoidal occlusion triggered by endothelial cell swelling, vasoconstriction, leukocyte entrapment and platelet aggregation [1,2]. Additional contributory factors include Kupffer-cell and infiltrating neutrophil and lymphocyte activation, reactive oxygen species (ROS) generation and release of proteases and cytokines [1,2]. Activation of Kupffer cells plays a central role in IR liver injury due to their ability to produce ROS and inflammatory cytokines, such as tumour necrosis factor (TNF)-α and interleukin (IL)-1, through ROS-mediated activation of nuclear factor (NF)-κB and activator protein (AP)-1 [1,3,4]. Activated Kupffer cells are also able to release nitric oxide (NO), thromboxanes and leukotrienes and recruit neutrophils to the liver [1,3]. Consequently, due to the importance of IR injury as a pathogenic mechanism underlying liver transplant and surgical procedures, hepatic pre-conditioning strategies reducing IR-induced damage have been extensively explored [5].

Polyunsaturated fatty acids (PUFA) are important dietary components with key roles in several physiological functions. Both n-6 and n-3 PUFAs are structural components of cellular lipids as well as substrates

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2010 Informa UK Ltd.
DOI: 10.3109/10715762.2010.485995

for the synthesis of physiological mediators. α-Linolenic acid (C18:3n-3), a minor component in human cells, is the predominant dietary n-3 PUFA and the precursor for the longer-chain n-3 PUFAs eicosapentaenoic acid (C20:5n-3; EPA) and docosahexaenoic acid (C22:6n-3; DHA) [6]. Both EPA and DHA have been reported as effective anti-inflammatory and tissue protective mediators [6], being hypolipidemic doses of EPA able to enhance hepatic antioxidant defences [7]. Evidence also suggests reduction of prostaglandin D2 production upon incorporation of dietary EPA into cell membranes and decreased production of pro-inflammatory cytokines after n-3 PUFA consumption [6,8,9]. In addition to these effects, eicosanoid metabolism plays a key role in the anti-inflammatory effects of n-3 PUFA, due to conversion of EPA and DHA to E-resolvins and D-resolvins by the cyclooxygenase-2/5-lipoxygenase pathway, respectively, lipid mediators produced in the resolution phase following acute inflammation [10,11]. In addition to resolvins, cytochrome P450 NADPH-dependent epoxygenases may also contribute to the anti-inflammatory effects of n-3 PUFA, through the production of epoxyeicosaquatraenoic acid and epoxydocosapentaenoic acid derivatives [11].

Considering that EPA and DHA are highly concentrated in fish oils, several studies have been conducted to evaluate the potential therapeutic effects of fish oils in diverse clinical conditions having inflammation as a key component of their pathology [6]. Fish oil n-3 PUFAs may directly alter intracellular signalling pathways associated with the activation of NF-κB and peroxisome proliferators activated receptors (PPAR), thus regulating several inflammatory genes. In fact, cell culture studies revealed that EPA and DHA inhibit the production of inflammatory cytokines by monocytes, macrophages and endothelial cells, whereas fish oil feeding elicited a decrease in the *ex vivo* production of TNF-α, IL-1β and IL-6 by rodent macrophages [12]. Taking into account that EPA and fish oil also diminish lipopolysaccharide-induced activation of NF-κB in cultured human monocytes, these observations suggest that n-3 PUFAs may directly affect inflammatory gene expression via inhibition of NF-κB DNA binding activity. Although decreased IκB phosphorylation and enhanced IκB expression seem to occur, PPAR-α-mediated inhibition of NF-κB p65 sub-unit has been suggested as one of the underlying mechanisms for the anti-inflammatory effects of fish oil [12–15]. In view of the protective effects associated to EPA and DHA, the objective of this study was to test the hypothesis that dietary n-3 PUFAs administration to rats protects the liver against IR injury. For this purpose, we studied the effects of dietary supplementation with fish oil on subsequent hepatic IR injury, the results of which were correlated with parameters of liver oxidative stress and tissue injury, NF-κB DNA binding and inflammatory cytokine release.

Materials and methods

Animal preparation and model of partial ischemia-reperfusion injury

Weaning male Sprague-Dawley rats (Bioterio Central, ICBM, Faculty of Medicine, University of Chile) were allowed free access to a specially formulated diet (20% casein, 10% n-6 PUFAs, lipo/hydrosoluble vitamins and minerals, Department of Nutrition, Faculty of Medicine, University of Chile). Animals received water *ad libitum* and were housed on a 12-h light/dark cycle. At day 15, the n-3 PUFA groups were supplemented for 7 days with encapsulated fish oil (General Nutrition Corp., Pittsburg, PA) and the control groups received isovolumetric amounts of saline, thus comprising four experimental groups: (a) Control-Sham, (b) Control-IR, (c) (EPA+DHA)-Sham and (d) (EPA+DHA)-IR. In these conditions the n-3 PUFA groups received EPA (270 mg/kg) and DHA (180 mg/kg). At day 8 after EPA plus DHA supplementation, rats were anaesthetized with intraperitoneal (1 ml/kg) zolazepam chlorhydrate (25 mg/ml) and tiletamine chlorhydrate (25 mg/ml) (Zoletil 50; Virbac S/A, Carros, France) and IR was induced by temporarily occluding the blood supply to the left and median lobes of the liver by means of a Schwartz clip (Fine Science Tools, Vancouver, BC, Canada) for 1 h, followed by up to 20 h of reperfusion, as previously described [16]. Control animals were subjected to anaesthesia and sham laparotomy. At indicated times during the reperfusion period, blood samples were obtained by cardiac puncture for serum AST, ALT, 8-isoprostanes and cytokines assessment. At indicated times during the reperfusion period, liver samples were taken from the medial lobes, frozen in liquid nitrogen (NF-κB assessment) or fixed in phosphate-buffered formalin, embedded in paraffin and stained with haematoxylin-eosin (morphology assessment). In parallel groups, livers were perfused *in situ* with a cold solution containing 159 mM KCl and 5 mM Tris (pH 7.4) to remove blood, for measurement of liver reduced (GSH) and oxidized (GSSG) glutathione and fatty acid composition. Experimental animal protocols and animal procedures complied with the Guide for the Care and Use of Laboratory Animals (National Academy of Sciences, NIH Publication 86-23, revised 1985).

Measurements of serum transaminases, cytokines and 8-isoprostanes

AST and ALT levels (units/l) were measured using specific diagnostic kits (Biomerieux SA, Marcy

l'Etoile, France). ELISA kits were used for assessment of serum levels (pg/mL) of TNF-α, IL-6 and IL-1β (Biosource International, Camarillo, CA) and 8-isoprostanes (Cayman Chemical, Ann Arbor, MI).

Fatty acid analysis and glutathione assays

For fatty acid analysis the liver samples were homogenized in distilled water and the lipid components were extracted with a 1:2 chloroform:methanol solution, followed by centrifugation (2000 g, 10 min, room temperature). After extraction of the chloroformic phase, the solvent was allowed to evaporate and the samples were stored at −20°C [17]. Previous to the gas-liquid chromatography assay, fatty acids from liver phospholipids were methylated by incubation (100°C) with BF_3 methanol (14%) and the fatty acid methyl esters (FAME) were extracted with hexane. After evaporation with nitrogen and resuspension in dichloromethane, the samples were stored at −20°C until the gas-liquid chromatography assay [18]. A Hewlett Packard gas chromatograph (model 7890A series II plus), equipped with a capillary column (J and W DB-FFAP, 30 m × 0.25 mm; I.D. 0.25 μm), authomatic injector and flame ionization detector, was used for FAME separation and detection. Identification of FAME was carried out by comparison of their retention times with those of individual purified standards and values were expressed as g/100g FAME.

GSH and GSSG contents (μmol/g liver) were measured using a specific glutathione assay kit (Cayman Chemical, Ann Arbor, MI).

NF-κB DNA binding

Nuclear protein extracts from liver samples were prepared according to Deryckere and Gannon [19] and subjected to electromobility shift assay using the NF-κB probe 5'-GAT CTC AGA GGG GAC TTT CCG AG-3' (Invitrogen Life Technologies, Carlsbad, CA), labelled with α-^{32}PdCTP using the Klenow DNA Polymerase Fragment I (Invitrogen Corp., Carlsbad, CA) as described previously [20]. The specificity of the reaction was determined by a competition assay using 100-fold molar excess of unlabelled DNA probe. The sub-unit composition of DNA binding protein was confirmed by supershift assay using specific antibodies from goat and rabbit IgG raised against NF-kB p50 and p65 (Santa Cruz Biotechnology, Santa Cruz, CA). Samples were loaded on non-denaturating 6% polyacrylamide gels and run until the free probe reached the end of the gel; NF-κB bands were detected by autoradiography and quantified by densitometry using Scion Image (Scion Corp., Frederick, MD).

Statistical analyses

Values shown represent the mean ± SEM for the number of separate experiments indicated. Student's *t*-test for unpaired data or one-way ANOVA and the Newman-Keuls test assessed the statistical significance of differences between mean values, as required. A *p*-value of less than 0.05 was considered significant.

Results

Control rats subjected to IR exhibited a liver fatty acid pattern similar to that of control-sham operated animals (Table I). N-3 PUFA supplementation enhanced the hepatic content of EPA plus DHA by 100% ($p < 0.05$), with a 43% diminution in the n-6/n-3 PUFA ratio ($p < 0.05$) over control values, parameters that were not further modified by IR (Table I). However, when the net effects of IR were estimated, it significantly decreased both n-3 PUFA (Figure 1A) and n-6 PUFA (Figure 1B) in the liver

Table I. Fatty acid composition of liver total lipids in control rats and animals subjected to EPA plus DHA supplementation under the influence of ischemia-reperfusion (IR).

	Groups			
	(a) Control -Sham	(b) Control-IR	(c) (EPA+DHA) -Sham	(d) (EPA+ DHA)-IR
SAFA	1.97 ± 0.15	2.35 ± 0.20	2.38 ± 0.24	2.05 ± 0.09
MUFA	0.55 ± 0.07	0.76 ± 0.10	0.69 ± 0.16	0.54 ± 0.02
PUFA	2.98 ± 0.27	2.25 ± 0.16	3.86 ± 0.60	3.31 ± 0.11
n-6 PUFA	2.63 ± 0.25	2.85 ± 0.14	3.14 ± 0.54	2.23 ± 0.08
n-3 PUFA	0.35 ± 0.03	0.40 ± 0.03	0.72 ± 0.08[a,b]	0.58 ± 0.04[a,b]
EPA + DHA	0.28 ± 0.03	0.30 ± 0.01	0.57 ± 0.06[a,b]	0.48 ± 0.02[a,b]
n-6/n-3 PUFA ratio	7.60 ± 0.79	7.07 ± 0.45	4.35 ± 0.58[a,b]	3.81 ± 0.27[a,b]

Values, expressed as g/100 g fatty acid methyl esters, represent mean ± SEM for five-to-seven rats per experimental group. Significant differences between the groups are indicated by the letters identifying each group ($p < 0.05$; one-way ANOVA and the Newman-Keuls' *t*-test). Saturated fatty acids (SAFA) are 12:0, 14:0, 16:0, 18:0, 20:0, 22:0 and 24:0. Mono-unsaturated fatty acids (MUFA) are 14:1,n-7, 16:1,n-7, 18:1,n-9, 20:1,n-9, 22:1,n-9 and 22:1,n-9. Poly-unsaturated fatty acids (PUFA) are 18:2,n-6, 18:3,n-6, 18:3,n-3, 20:2,n-6, 20:3,n-6, 20:3,n-3, 20:4,n-6, 20:5,n-3 (eicosapentaenoic acid, EPA), 22:5,n-3 and 22:6,n-3 (docosahexaenoic acid, DHA).

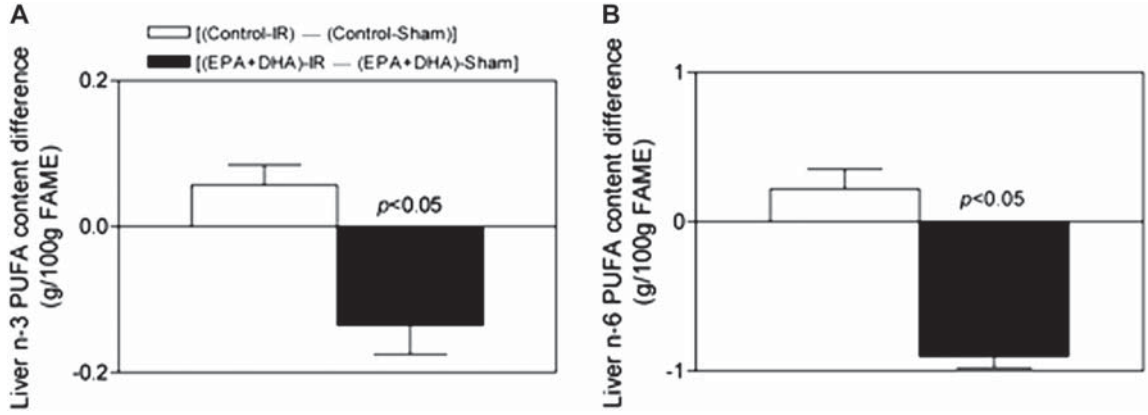

Figure 1. Net changes in liver n-3 PUFA content induced by ischemia-reperfusion (IR) in non-supplemented rats and animals subjected to EPA plus DHA supplementation. Data was calculated by subtracting the mean value in the control-Sham group from individual values in the control-IR group and the mean value in the (EPA plus DHA)-Sham group from individual values in the (EPA plus DHA)-IR group. Values shown correspond to the means ± SEM for five-to-seven rats per experimental group. Significance studies ($p < 0.05$) were carried out by Student's *t*-test for unpaired data.

of rats given EPA plus DHA over non-supplemented animals.

Plasma 8-isoprostane levels in control-sham rats and in EPA plus DHA supplemented-sham operated animals were comparable during the 20 h period equivalent to reperfusion conditions (Figure 2). In the (EPA + DHA)-IR group, plasma 8-isoprostanes were 50% and 120% higher ($p < 0.05$) than (EPA + DHA)-sham and control-IR groups at 1 h reperfusion, respectively, with 116% and 78% ($p < 0.05$) enhancements being found at 2 h reperfusion, respectively (Figure 2). Under condition of IR, plasma

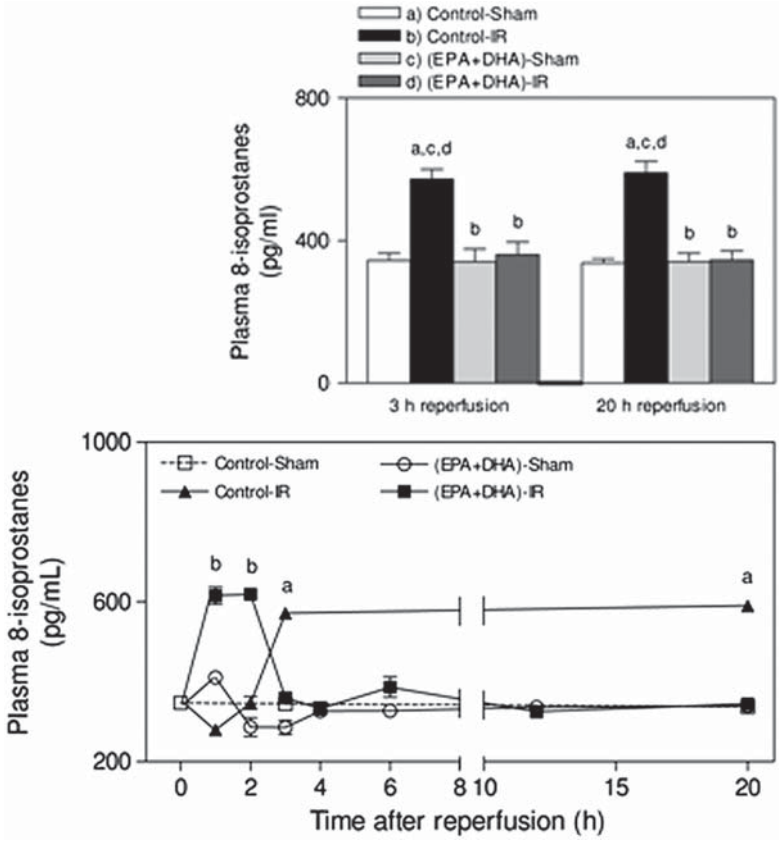

Figure 2. Effect of EPA plus DHA supplementation on the plasma levels of 8-isoprostanes after hepatic ischemia-reperfusion (IR) as a function of the reperfusion time. Values shown correspond to the means ± SEM for three-to-six rats per experimental group. [a]$p < 0.05$ vs values at time 0, 3 and 20 h in the control-IR group; [b]$p < 0.05$ vs values at time 0, 3, 4, 6, 12 and 20 h in the (EPA+DHA)-IR group. Inset: Plasma 8-isoprotanes values in the studied groups after 3 h and 20 h reperfusion, with significance shown by the letters identifying each experimental group ($p < 0.05$). All statistical analyses were done by one-way ANOVA and the Newman-Keuls' test.

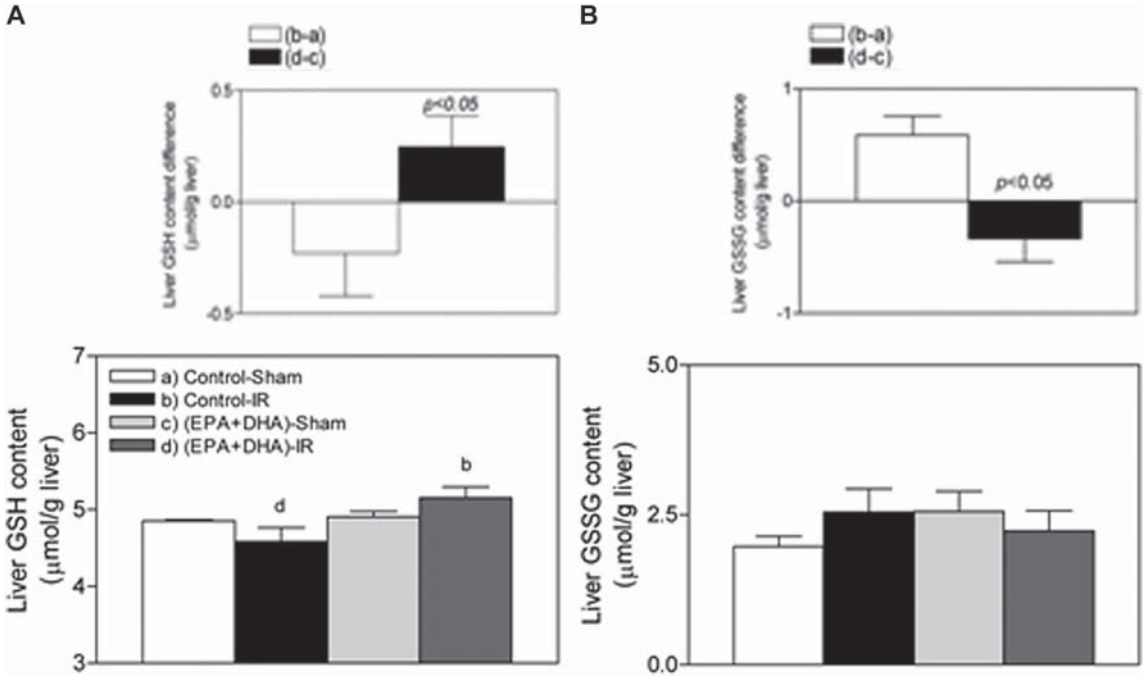

Figure 3. Effect of EPA plus DHA supplementation on liver (A) GSH and (B) GSSG contents after hepatic ischemia (1 h)–reperfusion (20 h) (IR). Values shown correspond to the means ± SEM for four rats per experimental group and significance assessed by one-way ANOVA and the Newman-Keuls' test ($p < 0.05$) is shown by the letters identifying each experimental group. Net changes in GSH (Inset A) and GSSG (Inset B) after IR in non-supplemented rats and animals subjected to EPA plus DHA supplementation were calculated as described for Figure 1 and significance studies ($p < 0.05$) were carried out by Student's *t*-test for unpaired data.

8-isoprostane levels showed an enhancement after 3 h reperfusion up to the end of reperfusion, compared to values found in control-sham animals (Figure 2), an effect that was abolished by EPA plus DHA supplementation at 3 and 20 h reperfusion (Figure 2, inset). The content of hepatic reduced glutathione (GSH) was diminished by IR in control rats compared to values observed after IR with EPA plus DHA pre-treatment ($p < 0.05$) (Figure 3A), with a net enhancement in liver GSH in the latter group over that in non-supplemented animals ($p < 0.05$) (Figure 3A, inset). Liver glutathione disulphide (GSSG) levels were comparable in the studied groups (Figure 3B); however, IR in EPA plus DHA treated animals achieved a net diminution in GSSG levels ($p < 0.05$) compared to those after IR in non-supplemented rats (Figure 3B, inset).

IR in control rats led to extensive liver injury, as shown by a 9.9- (Figure 4A) and 15.9-fold (Figure 4B) increase ($p < 0.05$) in serum AST and ALT, respectively, compared to control-sham-operated animals. In EPA plus DHA supplemented rats, serum AST and ALT values (Figure 4A and B) remained comparable to those in control animals, thus eliciting a net diminution of 88% (Figure 4A, inset) and 90% (Figure 4B, inset), respectively, in relation to the non-supplemented group. Histological assessment of the liver from the studied groups showed that control-Sham (Figure 5A) and EPA plus DHA-Sham (Figure 5C) groups exhibited normal liver morphology. Non-supplemented animals subjected to IR presented substantial degenerative changes and apoptosis, with extensive areas of hepatocyte necrosis (Figure 5B). On the contrary, the livers of EPA plus DHA-IR group showed normal architecture, with minimal-to-moderate necrosis (Figure 5D).

The levels of TNF-α (Figure 6A), IL-1β (Figure 6B) and IL-6 (Figure 6C) were increased by 72%, 26% and 47% ($p < 0.05$) by IR, respectively, over values in control-sham rats, effects that were reduced by 97% (Figure 6A, inset), 102% (Figure 6B, inset) and 75% (Figure 6C, inset) in EPA plus DHA supplemented animals subjected to IR over non-supplemented rats.

Control rats subjected to IR showed a biphasic effect on liver NF-κB DNA binding in relation to that in sham-operated controls (Figure 7A), as assessed by EMSA including supershift analysis confirming the presence of NF-κB p50 and p65 (Figure 7B). In fact, IR increased NF-κB DNA binding by 100% after 3 h reperfusion, whereas a 54% diminution was observed following 20 h reperfusion, being both effects suppressed by EPA plus DHA supplementation (Figure 7C).

Discussion

Data presented indicate that the liver IR protocol used involving 20 h reperfusion after 1 h of warm

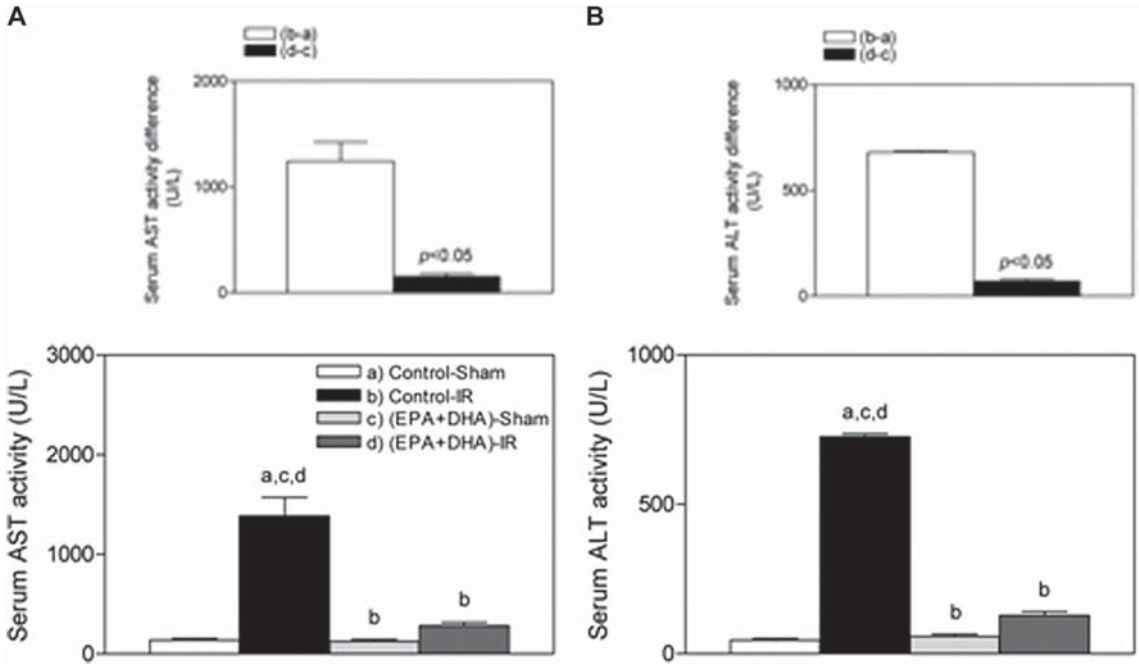

Figure 4. Effect of EPA plus DHA supplementation on serum (A) AST and (B) ALT levels after hepatic ischemia (1 h)–reperfusion (20 h) (IR). Values shown correspond to the means ± SEM for four-to-15 rats per experimental group and significance assessed by one-way ANOVA and the Newman-Keuls' test ($p < 0.05$) is shown by the letters identifying each experimental group. Net changes in AST (Inset A) and ALT (Inset B) after IR in non-supplemented rats and animals subjected to EPA plus DHA supplementation were calculated as described for Figure 1 and significance studies ($p < 0.05$) were carried out by Student's *t*-test for unpaired data.

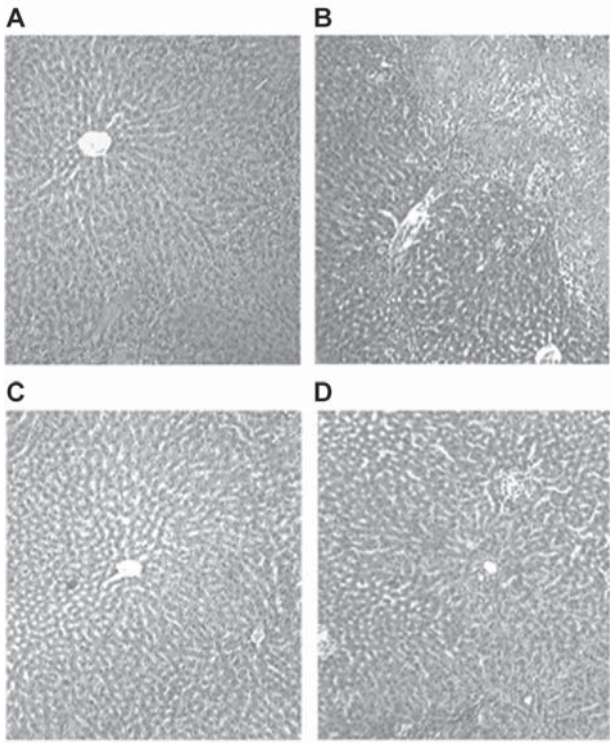

Figure 5. Effect of EPA plus DHA supplementation on liver histology after hepatic ischemia (1 h)–reperfusion (20 h) (IR). Representative liver sections from (A) control-Sham rat, (B) a control-IR animal, (C) a (EPA plus DHA)-sham rat and (D) a (EPA plus DHA)-IR animal (haematoxylin-eosin liver sections from a total of five animals per experimental group; original magnification ×40).

ischemia induced major changes in parameters related to oxidative stress, pro-inflammatory cytokine signalling and liver injury, in agreement with previous studies [16,21]. IR-induced liver injury is related to the drastic increase in oxidative stress status developed, triggering an early (2–3 h) enhancement in the DNA binding capacity of NF-κB with up-regulation of TNF-α, IL-1β and IL-6 expression at the Kupffer-cell level [21]. Furthermore, the late phase of IR liver injury (20 h) is associated with reduction in NF-κB activation, which has been related to loss of cytoprotective functions such as the acute-phase response [16] or lack of changes in inducible nitric oxide synthase expression and activity [22]. In agreement with these findings, Kupffer-cell inactivation by gadolinium chloride ($GdCl_3$) given alone [23] or combined with α-tocopherol [24] protects the liver from IR injury, which was related to a mechanism reducing oxidative stress-dependent lipid peroxidation. In this context, α-tocopherol suppresses release of TNF-α and IL-6 from Kupffer cells and diminishes liver leukocyte recruitment by inhibiting hydroxyalkenal-stimulated chemotaxis [25].

Seven-day EPA plus DHA supplementation to rats eliciting a significant enhancement of the hepatic content of the n-3 PUFAs with diminution in the n-6/n-3 PUFA ratio achieved protection against IR liver injury. N-3 PUFA pre-conditioning against liver IR, evidenced by normalization of the serum levels of AST and ALT with minimal morphological alterations

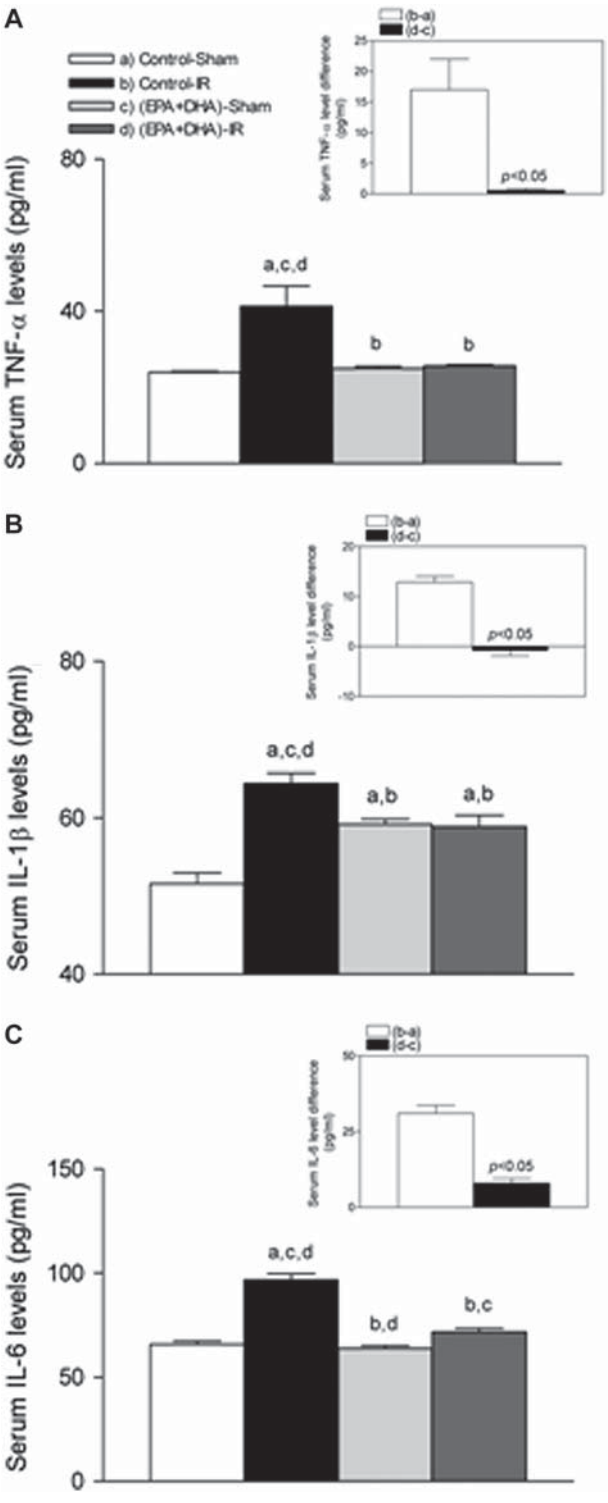

Figure 6. Effect of EPA plus DHA supplementation on serum (A) TNF-α, (B) IL-1β and (C) IL-6 levels after hepatic ischemia (1 h)–reperfusion (20 h) (IR). Values shown correspond to the means ± SEM for four-to-11 rats per experimental group and significance assessed by one-way ANOVA and the Newman-Keuls' test ($p < 0.05$) is shown by the letters identifying each experimental group. Net changes in TNF-α (Inset A), IL-1β (Inset B) and IL-6 (Inset C) after IR in non-supplemented rats and animals subjected to EPA plus DHA supplementation were calculated as described for Figure 1 and significance studies ($p < 0.05$) were carried out by Student's *t*-test for unpaired data.

in liver parenchyma, is associated with return of the serum levels of 8-isoprostanes at 3–20 h reperfusion toward control values and normalization in the net changes induced by IR on the content of hepatic GSH and GSSG found at 20 h reperfusion. Interestingly, recovery of IR-induced oxidative stress by n-3 PUFA supplementation occurs concomitantly with normalization in liver NF-κB DNA binding capacity, which is significantly enhanced at early times (3 h) and reduced at latter times (20 h) of reperfusion. NF-κB has prominent transcriptional control over the expression of the pro-inflammatory cytokines IL-6 and TNF-α in Kupffer cells [26,27], being IL-1 expression significantly up-regulated by TNF-α and IL-1 itself [28]. Consequently, EPA plus DHA supplementation led to the re-establishment of pro-inflammatory cytokine homeostasis, as shown by the suppression of IR-induced excessive TNF-α, IL-6 and IL-1β responses observed in non-supplemented animals. In agreement with these results, administration of n-3 PUFA rich oil resulted in reduced plasma levels of TNF-α and IL-6 following 2 h reperfusion after 25 min ischemia, with alleviation of liver injury, partial recovery of sinusoidal perfusion rate and diminution in leukocyte infiltration of the liver [29]. Improvement of hepatic microcirculation in a low flow, reflow liver perfusion in the rat is also achieved by a fish oil diet thus protecting against IR injury [30], whereas exacerbation of IR-induced damage in a model of macrosteatosis in the mouse liver compared to lean and microsteatotic livers was suppressed by dietary supplementation with n-3 PUFAs, an effect involving improvement in microcirculation with reduction in Kupffer cell activity [31]. Although the impact of n-3 PUFA on IR liver injury in humans has not been investigated [32], prolonged n-3 PUFA supplementation in overweight non-alcoholic fatty liver disease patients improves biochemical, ultrasonographic and haemodynamic features of liver steatosis [33], a condition underlying a significant 53–63% depletion in hepatic n-3 PUFA content [34,35].

Although EPA and DHA supplementation can protect the liver against IR injury, a process underlying severe oxidative stress, the molecular mechanisms responsible for this effect are beginning to be understood. Considering that IR elicited a net decrease of 0.09 g of n-3 PUFA/100 g FAME (from Figure 1) in the liver of EPA plus DHA supplemented rats over those in non-supplemented animals, protection may be related to a direct antioxidant action of n-3 PUFAs, which are highly susceptible to free-radical attack [36]. However, due to the greater availability of n-6 PUFA compared to n-3 PUFA observed in the liver and the total calculated decline of 0.78 g of n-3 plus n-6 PUFAs/100 g FAME (from Figures 1A and B), the possible consumption of n-3 PUFA by IR-induced ROS production after EPA plus DHA supplementation represents a minor (11%) contribution. In agreement

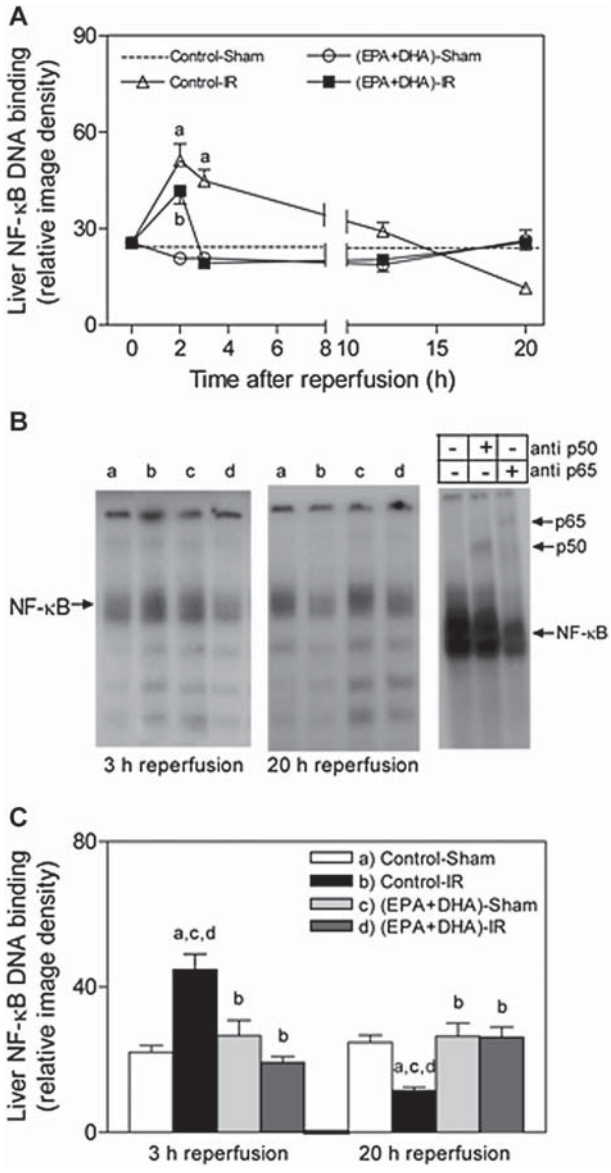

Figure 7. Effect of EPA plus DHA supplementation on liver NF-κB DNA binding after hepatic ischemia (1 h)–reperfusion (20 h) (IR). (A) Liver NF-κB DNA binding as a function of the reperfusion time. Values shown correspond to the means ± SEM for three-to-nine rats per experimental group; [a] $p < 0.05$ vs values at time 0, 12 and 20 h in the control-IR group; [b] $p < 0.05$ vs values at 0, 3, 12 and 20 h in the (EPA plus DHA)-IR group. (B) Autoradiographs representing lanes loaded with 8 μg nuclear protein from an animal of each experimental group, both at 3 h and 20 h reperfusion, and supershift analysis of a sample from a control-IR rat at 3 h reperfusion incubated with the labelled probe for NF-κB and with antibodies specific for NF-κB p50 (anti-p50) and NF-κB p65 (anti-p65). (C) Bar graphs corresponding to densitometric quantification of relative NF-κB DNA binding; significance was assessed by one-way ANOVA and the Newman-Keuls' test ($p < 0.05$) is shown by the letters identifying each experimental group.

with this view, supplementation with EPA plus DHA competes with the n-6 PUFA arachidonic acid (AA) at the level of incorporation into cell membrane phospholipids [37], implying n-6 PUFA replacement by n-3 PUFA [29], which may increase n-6 PUFA availability for interaction with IR-induced ROS generation. Thus, indirect mechanisms mediating n-3 PUFA pre-conditioning must be considered. Data reported show that n-3 PUFA supplementation attaining higher hepatic levels of n-3 PUFAs led to an early (3 h) suppression of IR-induced oxidative stress and late (20 h) recovery of NF-κB activation to basal levels, which may allow recovery of the expression of NF-κB-controlling genes encoding for antioxidant, anti-apoptotic and/or acute-phase response proteins [38]. The early n-3 PUFA-dependent suppression of IR-induced oxidative stress may involve activation of NF-E2-related factor-2 (Nrf2) by the pro-oxidant condition developed at 1 and 2 h reperfusion, considering that Nrf2 is rapidly activated by low levels of oxidative stress [39], with up-regulation of genes coding for antioxidant proteins [40]. This contention is supported by data showing activation of Nrf2 by EPA and DHA oxidation products, arising from the reaction between free radicals and the n-3 PUFAs [41]. This process leads to the formation of F_3-isoprostanes reacting directly with the negative regulator of Nrf2, Keap1, thereby activating the Nrf2-controlled expression of genes of the antioxidant enzymes heme-oxygenase-1 and the catalytic sub-unit of glutamate cysteine ligase (GCLC) [41]. Up-regulation of GCLC by n-3 PUFA is in line with the 61% increase in the hepatic content of GSH induced by the administration of 1g of EPA/kg for 10 days to mice, with concomitant enhancement in the activity of catalase, glutathione peroxidase, glutathione reductase and glutathione-S-transferase [7].

Collectively, data reported here support a role for EPA plus DHA supplementation in prevention of liver injury induced by IR, as previously suggested [29,30]. In addition, our results indicate that n-3 PUFA liver pre-conditioning is associated with (i) a minor direct antioxidant effect suggested by the IR-induced decrease in n-3 PUFA levels; (ii) suppression of IR-induced oxidative stress observed at 3–20 h reperfusion after enhancement at 1 and 2 h, a mechanism that may underlie liver Nrf2 activation; and (iii) recovery of NF-κB DNA binding activity at 3 and 20 h of reperfusion, with attainment of pro-inflammatory cytokine homeostasis.

Declaration of interest: This work was supported by grant 1080039 from FONDECYT (to V.F.), Chile. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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This paper was first published online on Early Online on 18 May 2010.