Research Article

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Chemical composition of hepatic lipids mediates reperfusion injury of the macrosteatotic mouse liver through thromboxane A_2

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Background & Aims: Chemical composition of hepatic lipids is an evolving player in steatotic liver ischemia/reperfusion (I/R) injury. Thromboxane A_2 (TXA_2) is a vasoactive pro-inflammatory lipid mediator derived from arachidonic acid (AA), an omega-6 fatty acid (Ω-6 FA). Reduced tolerance of the macrosteatotic liver to I/R may be related to increased TXA_2 synthesis due to the predominance of Ω-6 FAs.

Methods: TXA_2 levels elicited by I/R in *ob*/*ob* and wild type mice were assessed by ELISA. *Ob*/*ob* mice were fed Ω-3 FAs enriched diet to reduce hepatic synthesis of AA and TXA_2 or treated with selective TXA_2 receptor blocker before I/R.

Results: I/R triggered significantly higher hepatic TXA_2 production in *ob*/*ob* than wild type animals. Compared with *ob*/*ob* mice on regular diet, Ω-3 FAs supplementation markedly reduced hepatic AA levels before ischemia and consistently blunted hepatic TXA_2 synthesis after reperfusion. Sinusoidal perfusion and hepatocellular damage were significantly ameliorated despite downregulation of heme oxygenase-1. Hepatic transcript and protein levels of IL-1β and neutrophil recruitment were significantly diminished after reperfusion. Moreover, TXA_2 receptor blockage conferred similar protection without modification of the histological pattern of steatosis. A stronger protection was achieved in the steatotic compared with lean animals.

Conclusions: Enhanced I/R injury in the macrosteatotic liver is explained, at least partially, by TXA_2 mediated microcirculatory failure rather than size-related mechanical compression of the sinusoids by lipid droplets. TXA_2 blockage may be a simple strategy to include steatotic organs and overcome the shortage of donor organs for liver transplantation.

Keywords: Ischemia/reperfusion; Liver transplantation; Fatty acids; Eicosanoids; Microcirculation.

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Abbreviations: AA, arachidonic acid; DGLA, dihomogammalinolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; Ω-3 FAs, omega-3 fatty acids; FSD, functional sinusoidal density; I/R, ischemia/reperfusion; IVFM, intravital fluorescence microscopy; LA, linoleic acid; LT, leukotreine; MPO, myeloperoxidase; OPS, orthogonal polarization spectral; RBC, red blood corpuscle; VBF, volumetric blood flow; V_{RBC}, velocity of red blood corpuscles.

Introduction

Hepatic steatosis is a highly prevalent obesity-related disorder and remains a difficult challenge in liver surgery and transplantation [1,2]. Patients with hepatic steatosis are at higher risk of postoperative complications after major hepatectomy [3,4]. The use of steatotic grafts for orthotopic liver transplantation (OLT) is associated with a high rate of primary graft dysfunction [5] and decreased graft and patient survival particularly with macrosteatosis [6].

Hepatic steatosis is characterized by excessive accumulation of triglycerides, increased hepatic Ω-6 FAs levels and unphysiologically high Ω-6:Ω-3 FAs ratio [7]. Using *ob*/*ob* mice as a model of hepatic steatosis with predominance of the macrovesicular pattern, we introduced the chemical composition of hepatic lipids, particularly the equilibrium between Ω-6 and Ω-3 FAs as a potential contributor to I/R injury of the steatotic liver [8].

Eicosanoids are derived from a number of Ω-6 and Ω-3 FAs which harbor 20 carbon atoms. The impact of eicosanoids on inflammation, blood coagulation, and viscosity has been extensively emphasized [9–11]. Arachidonic acid (AA:C 20:4) is Ω-6 FA generated from linoleic acid (LA:C 18:2; the precursor of Ω-6 FAs) by series of elongation and desaturation reactions [7]. Under physiological Ω-6:Ω-3 FAs ratio, excessive synthesis of AA is suppressed by normal levels of Ω-3 FAs [10,12]. AA is the precursor of pro-aggregatory and pro-inflammatory eicosanoids, among which TXA_2 is the most powerful [7,10]. TXA_2 receptors have been identified in murine hepatic sinusoidal endothelial cells [13]. Given its potent effect; TXA_2 aggravates I/R injury in the lean liver of mice [14] and humans [15] despite low hepatic content of Ω-6 FAs [8,16].

The increased vulnerability of the steatotic liver to I/R has long been linked with the morphology of steatosis (predominance of

macrosteatosis) and liver injury was attributed to preexisting compression of the sinusoids by large lipid droplets [17]. However, the sinusoidal diameter in the steatotic liver of obese Zucker rats [18] and *ob*/*ob* mice [19] does not differ significantly from that of lean littermates. In the same line, aggravation of microcirculatory impairment after I/R [20] may be explained by excessive synthesis of injurious Ω-6 eicosanoids due to elevation of their intrahepatic precursor levels [8].

In the present study, we investigated the hypothesis that manipulation of hepatic lipids (Ω-6:Ω-3 FAs ratio) modulates I/R injury in the macrosteatotic liver through TXA_2, independent of the histological pattern of steatosis. Initially, we compared TXA_2 synthesis by the steatotic (*ob*/*ob*) *vs.* lean (wild type) mouse liver after I/R. Then, we attempted to decrease TXA_2 synthesis by the *ob*/*ob* mouse liver using dietary Ω-3 FAs. We investigated the influence of Ω-3 FAs supplementation on the conversion rate of LA to Ω-6 FAs with emphasis on AA (precursor of TXA_2 and PGI_2) and dihomo-γ-linolenic acid (DGLA, precursor of PGE_1). We extended our investigations to address the impact of Ω-3 FAs pretreatment on other potential modulators of hepatic I/R injury such as heme oxygenase-1, pro-inflammatory cytokines, inflammatory cell requirement, AA-derived leukotriens (LTs), and oxidative stress. Finally, as an alternative strategy to interfere with TXA_2 action, we selectively blocked its receptors by TXA_2 analog (Ono 3708) [13]. This approach dissects the response of the steatotic liver to I/R injury independent of modulation of the histological pattern from macro- to microsteatosis which occurs with Ω-3 FAs supplementation [8]. The effect of TXA_2 receptor blockage was also tested in lean mice.

Materials and methods

Diets and drug

Diets were purchased from KLIBA, Kaiseraugust, Switzerland. The Ω-3 FAs enriched diet (regular laboratory chow enriched with 10% fish oil) contains high concentrations of the long chain Ω-3 FAs (eicosapentaenoic (EPA): C20:5 and docosahexaenoic acid (DHA): C22:6) as previously described [8]. Ono 3708, the TXA_2 analog [13] which selectively blocks TXA_2 receptors [20] was a generous gift from Ono Pharmaceutical Co., Ltd., Osaka, Japan.

Animals

Male mice were purchased at age of 6–8 weeks (Harlan, Netherlands). A pilot experiment was carried out on obese *ob*/*ob* C57/BL6 mice and lean wild type C57/BL6 controls which were fed regular laboratory chow. Another series of experiments involved feeding of two groups of *ob*/*ob* mice with regular chow or Ω-3 FAs enriched diet for 12 weeks. In a third set of experiments *ob*/*ob* and lean mice on standard laboratory chow were treated with saline or selective TXA_2 receptor blocker. All animals were fed the aforementioned diets and water *ad libitum* until use and kept under constant environmental conditions with 12-h light/dark cycles. All procedures were performed in accordance with the Zurich University Institutional Animal Care and were approved by the local animal ethics committee.

Hepatic Ω-3 and Ω-6 FAs assay

Determination of hepatic contents of Ω-3 and Ω-6 FAs was performed by gas chromatography with a polar column [8]. Each fatty acid was expressed as percentage ratio of the sum of all fatty acids (total is 100%). The results are recalculated to indicate the relative lipid content given as mg/g of mouse total hepatic fat.

Surgical procedure

A model of segmental (70%) hepatic ischemia was applied for 45 min followed by reperfusion for different periods [8]. At the end, the left lobe was harvested and enough tissue was obtained for either preservation in 10% formalin or immediate freezing in liquid nitrogen and storage at −80 °C. All animals in TXA_2 blockage experiments were examined for microvascular perfusion by IVFM. The animal groups in this set of experiments were injected intravenously with Ono 3708 (10 mg/kg) or vehicle (saline) twice (10 min before ischemia and 10 min before reperfusion).

In vivo microscopy and analysis of hepatic microcirculation and leukocyte activation

Hepatic microcirculation was examined by orthogonal polarization spectral (OPS) imaging [20] and intravital fluorescence microscopy (IVFM). Examination of hepatic microcirculation by OPS imaging provides the same image quality obtained by IVFM [21] with the added advantage of performing the test in a short time without injection of fluorescent dye. OPS imaging was carried out by the Cytoscan device (Cytometrics Inc., Philadelphia, PA). The microcirculation sequences were recorded on video (Panasonic AG 7350-SVHS; Tokyo, Japan) and displayed on a television screen (Trinitron PVM-20N5E; Sony, UK) during subsequent off-line analysis. OPS imaging enables determining the sinusoidal diameter, functional sinusoidal density (FSD) and sinusoidal red blood cell velocity (V_{RBC}) indicated in μm/s [20]. IVFM was performed as we previously described [8,22]. Leukocyte activation was investigated in 10 randomly chosen postsinusoidal venules under green filtered light after rhodamine-6G (2 μmol/kg, Molecular Probes, Eugene, Oregon) injection for in vivo staining [20]. FSD was defined as the total length of all perfused sinusoids per observation area (cm/cm^2) [8]. The sinusoidal diameter and V_{RBC} were measured in 10 sinusoids in the midzonal region. The VBF in the sinusoids was calculated from the V_{RBC} and the cross-sectional surface area ($\pi \times r^2$) according to the following equation: VBF = V_{RBC} × cross-sectional surface area indicated in pL/s [22,23]. The FSD and the sinusoidal diameter were measured by a computer-assisted image analysis software system (CapImage; Zeintl Software, Heidelberg, Germany).

Arachidonic acid vasoactive metabolite (TXB_2 and 6-keto $PGF_1\alpha$) levels in hepatic venous blood

TXB_2 and 6-keto $PGF_1\alpha$ levels were measured in blood samples from the hepatic veins immediately after OPS imaging. Blood samples were collected by suprahepatic vena caval venipuncture using a curved needle supplemented with EDTA. With the needle tip placed in the hepatic vein, blood (approximately 600 μl) was drawn before ischemia, 30 and 180 min after reperfusion. Plasma levels of TXB_2 and 6-keto $PGF_1\alpha$ were measured by competitive enzyme immunoassay to reflect the levels of TXA_2 and PGI_2, respectively, because of their transient nature. The assays were performed according to the manufacturer's guide (TXB_2 and 6-keto $PGF_1\alpha$ EIA kits, Cayman Chemical Company Ellsworth Rd. Ann Arbor, MI).

mRNA gene analysis by quantitative reverse transcription– polymerase chain reaction (RT-PCR)

Total RNA was extracted from 50 mg liver tissue using TRIzol reagent (Invitrogen, Paisley, UK). Five micrograms RNA were reverse-transcribed using the Thermoscript RT-PCR kit (Invitrogen, Basel, Switzerland), yielding the complementary DNA template. The quantitative real-time polymerase chain reaction (PCR) amplification and the data analysis were performed using an ABI-Prism 7000 Sequence Detector system. TaqMan gene expression assays (PE Applied Biosystems, Rotkreuz, Switzerland) for heme oxygenase-1 *(Hmox1)* (Mm00516004_m1), interleukin-1β (*Il1b*) (Mm00434228_m1) and F4/80 m (*Emr1*) (Mm00802530_m1) were used to quantify the messenger RNA (mRNA) expression of the respective genes. The mRNA expression levels for each sample were normalized to 18S RNA. The results gained represent fold induction *vs.* baseline levels in the *ob*/*ob* mice on regular diet.

Western blotting

Experiments were carried out as we previously described [24]. Antibodies used were rabbit anti-IL-1β ab 9722 (Abcam plc, Cambridge, UK), mouse anti-beta actin antibody (Sigma–Aldrich, St. Louis, USA), donkey anti-rabbit HRP (GE Healthcare, Glattbrugg, Switzerland) and donkey anti-mouse HRP (Sigma–Aldrich, St. Louis, USA).

Histology

Formalin-preserved liver tissue was subsequently embedded in paraffin and sectioned. Platelet accumulation in the liver was analyzed using monoclonal antibodies to mouse CD61 (BD Biosciences). Immunostaining for heme-oxygenase (HO-1) (rabbit anti-mouse HO-1 antibody, Stressgene, Victoria, BC, Canada), F4/80 (monoclonal rat antibody, BMA biomedicals, August, Switzerland), 4-hydroxy-2-nonenal protein adducts (monoclonal mouse (HNEJ-2) antibody, Abcam, Cambridge, UK) and myeloperoxidase (MPO) (polyclonal rabbit antibody, Neo-Markers, Fremont, CA) according to the manufacturers' instructions and as previously described [23–25].

Lipid peroxidation

Malondialdehyde (MDA) concentration in liver homogenate was spectrophotometrically measured (BIOXYTEC MDA-586, OxisResearch, OXIS Health Products, Inc) as indicator of lipid peroxidation.

Mass spectrometry for arachidonic acid-derived leukotrienes

Liver samples (100–300 mg) were collected and extracted according to Bligh and Dyer [26]. Primary extraction was carried out with a homogenous chloroform–methanol-aqueous buffer solvent mixture followed by a liquid–liquid partition of the lipids into chloroform and the polar compounds into methanol/aqueous buffer mixture. Defined amounts of ribitol, 1,2-dilaurin, and methyl nonadecanoate (Sigma) were spiked to the primary extraction mixture and were used as internal standards. Extracts were diluted in 5 mM ammonium acetate in chloroform/methanol (1:2) prior to the mass spectrometric analyses. Mass spectrometry was performed by direct infusion of the extracted lipids as previously reported [27,28]. Briefly, for all analyses an orthogonal hybrid quadrupole time-of-flight mass spectrometer (QTOF Ultima, Micromass Waters, Manchester, UK) equipped with a chip-based nanoelectrospray source (NanoMate HD, Advion, Ithaca, NY, US) was used. Nanoelectrospray was generated in positive mode at 1.2 kV and 0.2 psi head pressure. Typically 15 µl lipid solution was aspired from a 96-well plate and infused for up to 10 min. The mass spectrometer settings were calibrated and optimized for a mass range of 50–1250 (mass-to-charge ratio) to provide a resolution around 8000 full width at half maximum and a mass accuracy of better than ±50 mDa. The ion source block was kept at 80 °C and the collision energy at 10 eV. The scan rate was set to 1 scan per second. Spectra were acquired in Time of Flight-Mass Spectrometry mode without fragmentation only. The results represent fold increase *vs.* the internal standard (control).

Hepatocyte injury

The extent of hepatocyte injury was assessed by plasma aminotransferase levels in blood samples drawn from the tail vein using serum multiple analyzer (Johnson & Johnson, Ektachem DTSC II multianalyzer).

Statistical analysis

Data were analyzed by GraphPad Prism software and values were expressed as means ± standard deviation. Differences between groups were evaluated using unpaired *t* test and considered statistically significant with *p* value of less than 0.05.

Results

Does I/R injury trigger higher TXB_2 levels in the ob/ob compared with lean mice?

TXA_2 synthesis (as indicated by TXB_2 levels) is regulated by the Ω-6:Ω-3 FAs ratio [7] which is abnormally increased in the *ob*/*ob* mouse liver [8]. In line with our hypothesis, I/R resulted in excessive hepatic synthesis of TXB_2 at 30 min of reperfusion in the steatotic compared with lean animals (Fig. 1A). Of note, we observed increased plasma aspartate aminotransferase (AST)

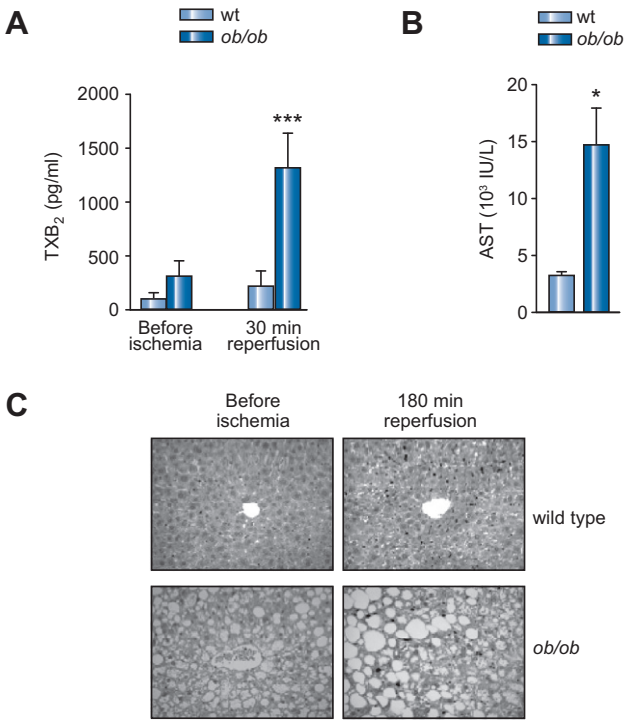

Fig. 1. Effect of I/R on TXB_2 (stable product converted from TXA2) levels, hepatocyte injury, and platelet accumulation in the steatotic (*ob*/*ob*, dark blue bars) and wild type (wt, light blue bars) mouse liver 30 min after reperfusion, n = 3 animals per group. (A) TXB_2 levels, * and **p = 0.0087 and 0.0063 (*vs. ob*/*ob* mice before ischemia and wild type mice after I/R, respectively). (B) AST levels, *p = 0.0034. (C) Representative images of CD61 staining showing platelet accumulation in *ob*/*ob* and wild type mouse liver after reperfusion, original magnification 200×.

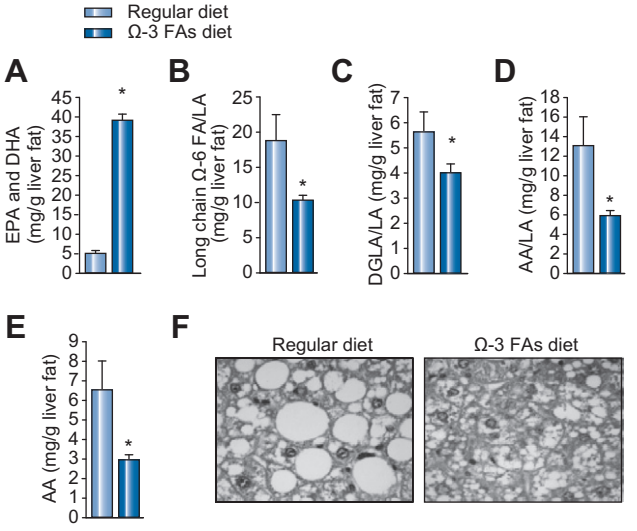

Fig. 2. Effect of Ω-3 FAs enriched diet (dark blue bars) *vs.* regular diet (light blue bars) on metabolism of hepatic Ω-6 FAs, n = 3 animals per group. (A) Hepatic content of EPA and DHA, *p <0.0001. (B-D) Conversion of LA to long chain Ω-6 FAs, hepatic ratios of total Ω-6 long chain FAs/LA (C 20:3 + C20:4 + C22:2/C18:2), DGLA/LA, and AA/LA, *p = 0.0157, 0.0305 and 0.0135, respectively. (E) Hepatic content of AA, *p = 0.0210. (F) Increased hepatic content of Ω-3 FAs (EPA and DHA) was associated with a visible reduction of the extent of macrosteatosis, representative hematoxylin-eosin stained liver sections, original magnification 400×.

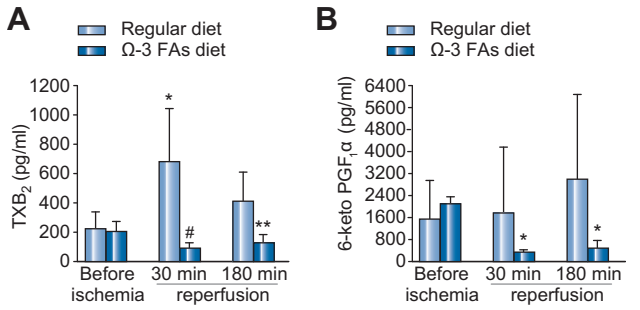

Fig. 3. Effect of Ω-3 FAs enriched diet (dark blue bars) *vs.* regular diet (light blue bars) on TXB_2 and 6-keto $PGF_1\alpha$ (stable product converted from PGI2), n = 5 animals per group. (A) Hepatic venous plasma levels of TXB_2, $^{*}p = 0.0247$ (*vs.* pre-ischemic value), $^{\#}$ and $^{**}p = 0.007$ and 0.0144 (*vs.* regular diet 30 and 180 min after reperfusion, respectively). (B) 6-keto $PGF_1\alpha$ levels, $^{*}p < 0.0001$ (*vs.* pre-ischemic value).

levels and less platelet accumulation in the steatotic animals (Fig. 1B and C).

What are the effects of dietary Ω-3 FAs on hepatic content and metabolism of Ω-6 FAs in the ob/ob mice?

Dietary supplementation with Ω-3 FAs resulted in significant elevation of intrahepatic content of EPA and DHA (39.27 ± 1.2 mg/g) compared with the regular diet group (4.93 ± 0.8 mg/g) (Fig. 2A).

The long chain Ω-6 FAs/LA ratio was markedly decreased (19 ± 3.6) compared with the regular diet group (10.53 ± 0.4) indicating that the increased availability of Ω-3 FAs effectively blunted the elongation and desaturation of LA. This reduction was imposed on AA synthesis since the AA/LA ratio was profoundly reduced from 13.21 ± 2.8 in the regular diet group to 6.09 ± 0.3 in the Ω-3 FAs supplemented animals. The DGLA/LA was also reduced but to a much lesser extent (5.66 ± 0.7 *vs.* 4.05 ± 0.3 in the regular diet fed animals and Ω-3 FAs supplemented animals, respectively) (Fig. 2B–D).

DGLA is the precursor of prostaglandin E1 (PGE_1) [7,10] which exerts a hepatoprotective effect via enhancement of sinusoidal perfusion, inhibition of leukocyte adhesion and reduction of the oxidative stress-induced hepatocyte injury [7]. The dietary supplementation protocol we adopted did not significantly alter the levels of DGLA (3.06 ± 0.4 mg/g) compared with the regular diet (2.46 ± 0.3 mg/g). In contrast, the hepatic content of AA was significantly reduced in the Ω-3 FAs treated animals (7.16 ± 1.6 mg/g) compared with the regular diet group (3.7 ± 0.2 mg/g) (Fig. 2E). The increased level of Ω-3 FAs was associated with a visible reduction of the extent of macrosteatosis (Fig. 2F).

Does Ω-3 FAs supplementation modulate hepatic synthesis of TXB_2 and 6-keto $PGF_1\alpha$?

Before ischemia, TXB_2 and 6-keto $PGF_1\alpha$ levels were not significantly different in the regular diet compared with Ω-3 FAs fed animals. TXB_2 was significantly elevated 30 min after reperfusion in the regular diet group. However, this rise was intensely blunted by Ω-3 FAs with persistence of low TXB_2 levels at 180 min after reperfusion compared with the regular diet group.

Animals on the regular diet did not show significant elevation of PGI_2 (indicated by 6-keto $PGF_1\alpha$ levels) after reperfusion to

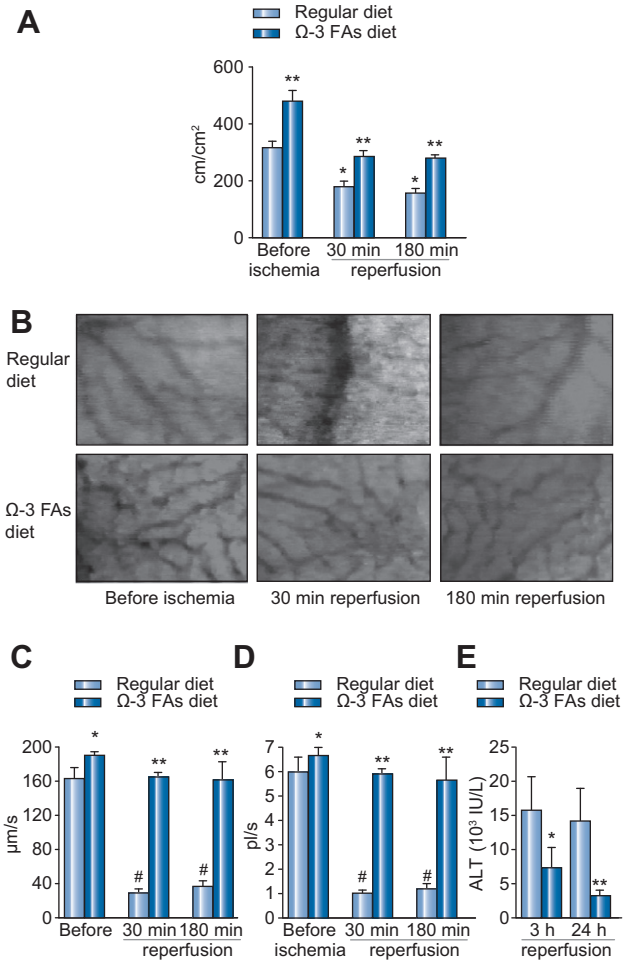

Fig. 4. Influence of Ω-3 FAs enriched diet (dark blue bars) *vs.* regular diet (light blue bars) on sinusoidal microcirculation and hepatocyte injury, n = 5 animals per group. (A) FSD, * and $^{**}p < 0.0001$ (*vs.* pre-ischemic level and corresponding regular diet group, respectively). (B) Representative OPS images, FSD in Ω -3 FAs group before ischemia (lower left panel) and after 30 and 180 min of reperfusion (lower middle and right panels, respectively) *vs.* regular diet group, magnification 570×. (C and D) Velocity of red blood corpuscles (V_{RBC}) and volumetric blood flow VBF, $^{*}p = 0.0022$ and 0.0494 (*vs.* regular diet group, C and D, respectively), $^{\#}p < 0.0001$ (*vs.* pre-ischemic level) and $^{**}p < 0.0001$ (*vs.* corresponding regular diet group). (E) Plasma ALT levels 180 min and 24 h after reperfusion, * and $^{**}p = 0.0105$ and 0.001, respectively.

counteract the influence of increased TXB_2 levels. After reperfusion, 6-keto $PGF_1\alpha$ levels were not significantly different in the regular diet fed mice compared with the Ω-3 FAs group. Furthermore, a significant reduction in 6-keto $PGF_1\alpha$ levels was noted after reperfusion in the Ω-3 FAs supplemented animals (Fig. 3A and B).

Is there a correlation between TXB_2 levels and hepatic microcirculation and hepatocellular damage after I/R?

In the regular diet group, the parameters of sinusoidal perfusion were dramatically reduced compared with the Ω-3 FAs fed animals. For instance, the functional sinusoidal density (FSD) was significantly lower before ischemia and after reperfusion compared with the Ω-3 FAs group. As an indicator of enhanced

Table 1. Sinusoidal diameter (μm) in *ob*/*ob* mice supplemented with Ω-3 FAs *vs.* regular diet.

	Regular diet	Ω-3 FAs enriched diet
Before ischemia	6.770 ± 0.10	6.728 ± 0.01
Reperfusion (30 minutes)	6.430 ± 0.07*	6.724 ± 0.01**
Reperfusion (180 minutes)	6.492 ± 0.05†	6.712 ± 0.03**

*$p = 0.0003$ *vs.* pre-ischemic value.
†$p = 0.0007$ *vs.* pre-ischemic value.
**$p < 0.0001$ *vs.* corresponding regular diet group after reperfusion.

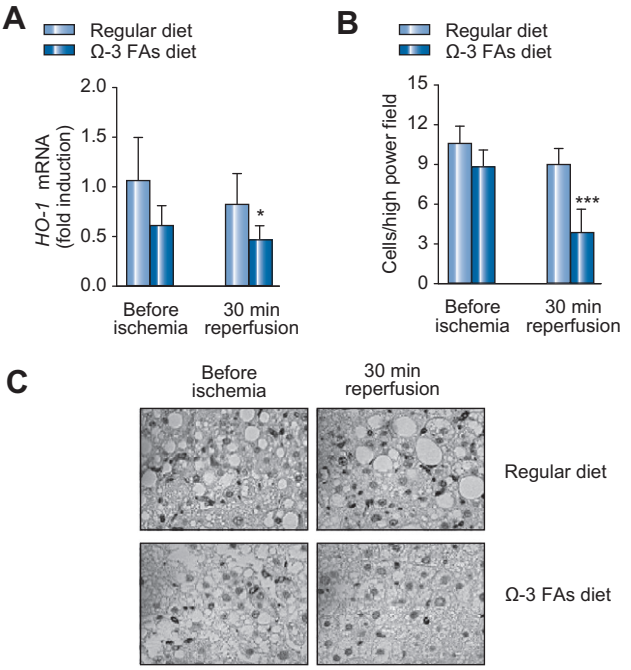

Fig. 5. Effect of Ω-3 FAs supplementation on HO-1 expression in *ob*/*ob* mouse liver. (A) Effect of Ω-3 FAs enriched diet (dark blue bars) *vs.* regular diet (light blue bars) on *HO-1* expression, *$p = 0.0436$ (*vs.* regular diet after reperfusion), n = 5 animals per group. (B) Morphometric determination of HO-1 positive cells by immunostaining, * and **$p = 0.001$ and 0.0007 (*vs.* Ω-3 fed group before ischemia and regular diet group after reperfusion, respectively). (C) Representative images of HO-1 immunostaining, original magnification 400×.

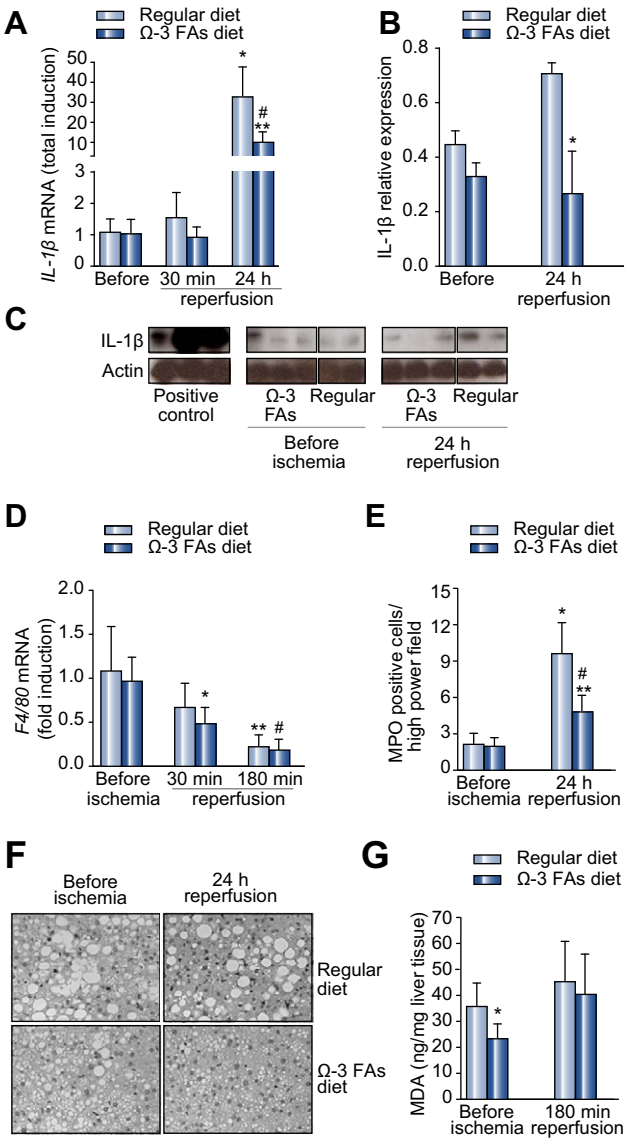

Fig. 6. Hepatic *IL-1β* and *F4/80* mRNA expression, IL-1β protein levels, MPO immunostaining and MDA levels in Ω-3 FAs enriched diet (dark blue bars) *vs.* regular diet (light blue bars) fed mice, n = 5 animals per group (except for IL-1β and F4/80 expression in Ω-3 FAs groups at 180 min and 24 h, n = 4). (A) *IL-1β* expression, *, and **$p = 0.001$ and 0.0193 (*vs.* pre-ischemic values, respectively), #$p = 0.0184$ *vs.* regular diet. (B and C) Quantification of IL-1β levels by Western blotting normalized by loading control (actin), *$p = 0.0336$. (D) *F4/80* expression, *, **, and #$p = 0.0111$, 0.0004, and 0.0061 (*vs.* the corresponding pre-ischemic values, respectively). (E) MPO immunostaining, * and **$p = 0.0003$ and 0.0052 respectively (*vs.* pre-ischemic count), #$p = 0.0072$ (*vs.* regular diet after reperfusion), (F) Representative images of MPO immunostaining, original magnification 200×. (G) MDA levels, *$p = 0.0283$.

resistance to blood flow which correlates with increased TXB_2 levels after reperfusion, we found that the sinusoidal red blood cell velocity (V_{RBC}) was significantly lower in the regular diet fed mice while pronounced improvement was noted upon Ω-3 FAs supplementation at the same time points. Likewise, decreased TXB_2 levels correlated with amelioration of volumetric blood flow (VBF) in the Ω-3 FAs treated mice after reperfusion compared with the regular diet group. Concurrently, alanine aminotransferase (ALT) levels were significantly reduced 3 and 24 h after reperfusion in Ω-3 FAs supplemented animals (7340 ± 2716 and 3180 ± 819 IU/L) compared with the regular diet group (15,612 ± 4855 and 14,060 ± 4737 IU/L) (Fig. 4A–E and Table 1).

Is the Ω-3 FAs-induced amelioration of hepatic microcirculation related to augmentation of heme-oxygenase (HO-1) expression?

A protective effect on hepatic microcirculation in the *ob*/*ob* mouse liver was shown for HO-1 [19]. In our experiments, *HO-1* expression was significantly decreased after reperfusion in the Ω-3 FAs supplemented animals (Fig. 5A). This was further confirmed by HO-1 immunostaining (Fig. 5B and C). Of note, decreased HO-1 expression in the Ω-3 FAs group was not related

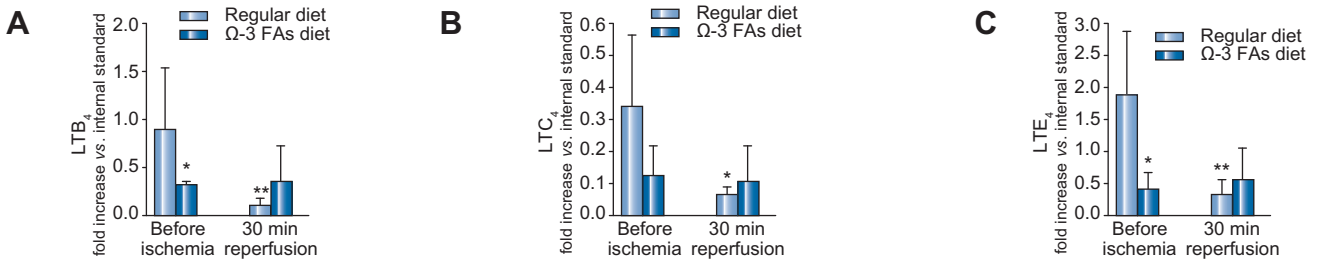

Fig. 7. Hepatic AA-derived leukotrienes (LTs) in the Ω-3 FAs enriched diet (dark blue bars) *vs.* regular diet groups (light blue bars) determined by mass spectrometry. (A) LTB4, *, and **p = 0.0245 and 0.0408, respectively (B) LTC4, *p = 0.073 (C) LTE4, * and **p = 0.01 and 0.0136, respectively (*vs.* pre-ischemic level in regular diet group).

to reduction of oxidative stress since immunostaining for 4-hydroxy-2-nonenal protein adducts showed no remarkable difference between the standard diet and Ω-3 FAs fed animals before ischemia and after reperfusion (Supplementary Fig. 1).

Does Ω-3 FAs supplementation modulate the expression of pro-inflammatory genes in this model?

In the next step, we investigated whether the anti-inflammatory properties of Ω-3 FAs [7] contributed to the reduction of hepatocellular damage after I/R. *IL-1β* was upregulated only 24 h after reperfusion compared with pre-ischemic values in both groups with significantly lower level in Ω-3 FAs supplemented animals (Fig. 6A). Similarly, protein levels of IL-1β were significantly reduced in Ω-3 FAs compared with regular diet group at 24 h after reperfusion (Fig. 6B and C).

Does dietary Ω-3 FAs feeding decrease post-reperfusion activation of macrophages and neutrophils?

We investigated the expression of *F4/80* as a marker of macrophages. *F4/80* was significantly downregulated in the Ω-3 FAs fed animals at 30 min of reperfusion and in both groups after 180 min compared with the pre-ischemic levels (Fig. 6D). After 24 h of reperfusion, *F4/80* levels did not significantly differ from pre-ischemic values in both groups. In liver sections, the number of F4/80 positive macrophages was significantly raised after reperfusion in regular diet and Ω-3 FAs supplemented mice with no remarkable difference between both groups (data not shown).

After reperfusion, an increased number of rolling and adherent neutrophils to the endothelium of post sinusoidal venules was observed in both groups compared with pre-ischemia counts. No significant reduction was seen in Ω-3 FAs fed mice (2.5 ± 0.64 cells/mm/s and 73.6 ± 15.75 cells/mm^2, respectively) in comparison with regular diet fed animals (2.6 ± 0.65 cells/mm/s and 74.4 ± 16.82 cells/mm^2, respectively) at 180 min of reperfusion.

An increased number of MPO-positive neutrophils was observed in both groups 24 h after reperfusion. However, dietary Ω-3 FAs partially prevented inflammatory cell recruitment after reperfusion (4.8 ± 1.5 cells/HPF) compared with the regular diet group (9.6 ± 2.6 cells/HPF) (Fig. 6E and F).

Does Ω-3 FAs supplementation alter the oxidative stress in this model?

To test whether Ω-3 FAs protect against I/R injury by amelioration of oxidative stress, we measured MDA, an established marker of lipid peroxidation [29]. MDA levels were significantly lower in Ω-FAs compared with regular diet fed animals before ischemia (23.85 ± 5.6 and 35.74 ± 8.8 ng/mg, respectively); however this effect was not sustained after reperfusion (39.68 ± 15.7 and 44.95 ± 15.1 ng/mg, respectively), (Fig. 6G).

Does Ω-3 FAs supplementation affect hepatic synthesis of AA-derived leukotrienes?

AA-derived LT levels were noticeably decreased after reperfusion in the regular diet group. Ω-FAs supplementation significantly reduced the AA-derived LTs (LTB_4, LTC_4, and LTE_4) before ischemia with no significant increase after reperfusion (Fig. 7A–C).

What are the effects of TXA2 antagonist on hepatic microcirculation and hepatocellular injury after I/R?

To test whether TXA_2, the derivative of Ω-6 FAs, is directly responsible for I/R injury, we selectively blocked TXA_2 receptors in regular diet fed *ob/ob* mice. Pretreatment of *ob/ob* mice with TXA_2 receptor blocker yielded comparable protection with that obtained with Ω-3 FAs supplementation but without conversion of the histological pattern from predominantly macro- to microsteatosis. FSD was significantly improved before ischemia and after reperfusion in the TXA_2 receptor blocker compared with the saline treated animals. Moreover, the TXA_2 receptor blocker-treated *ob/ob* mice showed significant enhancement of V_{RBC} and VBR compared with the saline controls before ischemia and after reperfusion (Fig. 8A–D and Table 2). ALT levels were significantly lowered by TXA_2 receptor blockage compared with the saline treatment at 180 min (6200 ± 1055 and 20,528 ± 4367 U/L, respectively) and 24 h (1936 ± 901 and 11,380 ± 5707 U/L, respectively) of reperfusion. In lean animals, blockage of TXA_2 receptor resulted in significant reduction of ALT levels compared with saline treatment at 180 min of reperfusion (2220 ± 115 and 5110 ± 858 U/L, respectively), however this effect was lost after 24 h (1860 ± 924 and 1210 ± 317 U/L, respectively), (Fig. 8E).

Discussion

In a mouse model of hepatic steatosis with predominance of the macrovesicular pattern, we demonstrated a new strategy to ameliorate I/R injury via manipulation of the chemical composition of hepatic lipids. We showed that elevated TXA_2 (as reflected by TXB_2) levels triggered by high hepatic content of AA impairs sinusoidal perfusion and augments inflammation and hepatocellular injury after I/R. These events were prevented by preconditioning

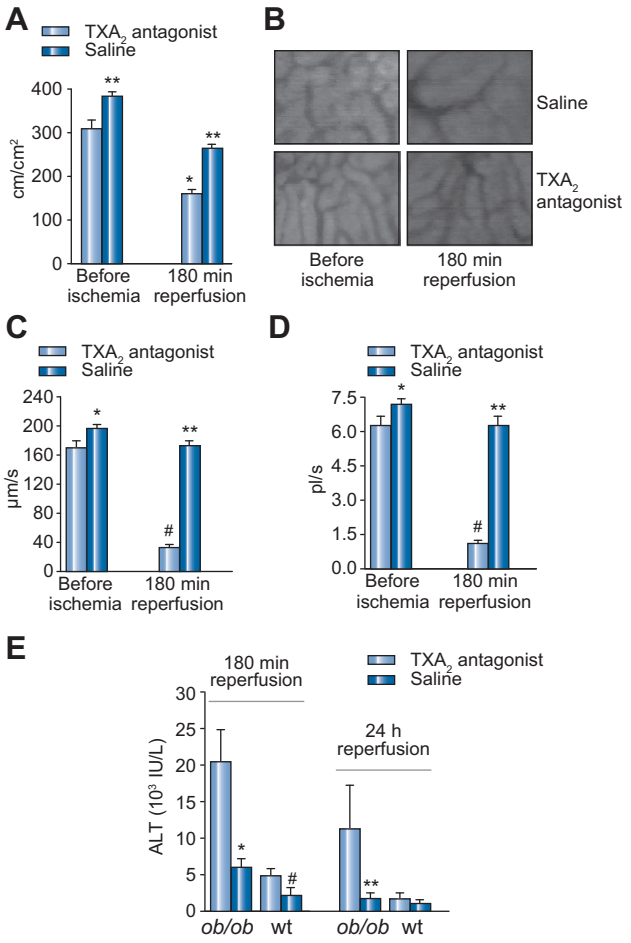

Fig. 8. Effect of treatment with selective TXA2 receptor blocker (Ono 3708) (dark blue bars) *vs.* saline control (light blue bars) on sinusoidal microcirculation and hepatocyte injury. (A) FSD of the *ob*/*ob* mouse liver, * and **p <0.0001 (*vs.* pre-ischemic value and corresponding saline control group, respectively). (B) Representative IVFM images showing FSD in TXA2 receptor blocker treated *ob*/*ob* mice before ischemia (lower left panel) and after 180 min of reperfusion (lower right panel) compared with saline controls, magnification 800×. (C–D) Velocity of red blood corpuscles (V_{RBC}) and volumetric blood flow VBF of the *ob*/*ob* mouse liver, *p = 0.0003 and 0.0012 (*vs.* saline control, C and D, respectively), #p < (*vs.* pre-ischemic level) and **p <0.0001 (*vs.* corresponding saline control group). (E) Plasma ALT levels 180 min and 24 h after reperfusion in the *ob*/*ob* and wild type (wt) mice after treatment with TXA2 receptor blocker (black bars) or saline (open bars), *, #, and **p <0.0001, = 0.002, and = 0.0065, respectively.

Table 2. Sinusoidal diameter (µm) in *ob*/*ob* mice treated with TXA_2 receptor blocker (Ono 3708) *vs.* saline (control).

	Vehicle saline	TXA_2 blocker
Before ischemia	6.826 ± 0.07	6.834 ± 0.08
Reperfusion (180 minutes)	6.490 ± 0.09*	6.746 ± 0.04**

*p = 0.0002 *vs.* pre-ischemic value.
**p = 0.0005 *vs.* saline control group 180 min after reperfusion.

with dietary Ω-3 FAs. Similar protection was achieved by selective blockage of TXA_2 receptors without the Ω-3 FAs-induced modulation of the histological pattern of steatosis. Therefore, this study presents the chemical composition of hepatic lipids and TXA_2 as new contributors to I/R injury of the steatotic liver.

Increased TXA_2 levels in liver of wild type mice after I/R correlates with pronounced sinusoidal perfusion failure and hepatocellular damage [30]. Likewise, inhibition of TXA_2 protects the lean liver against I/R injury in rats [31]. This effect may be attributed to the powerful pro-inflammatory properties of TXA_2 that amplifies leukocyte adhesion and clogging of the sinusoids [30]. In patients undergoing liver resection, TXA_2 synthase inhibitor reduced postoperative hepatocellular damage without influencing the levels of other AA-derived eicosanoids [15]. In the first set of experiments, we compared TXA_2 release from the steatotic *vs.* lean liver. Conforming with the higher Ω-6:Ω-3 FAs ratio that we previously reported in the steatotic liver [8], TXA_2 levels were significantly higher in the *ob*/*ob* mice. In the lean liver of mice, Khandoga *et al.* [32] showed increased platelet accumulation after I/R [32]. Increased TXA_2 levels after reperfusion were associated with recruitment of neutrophils and platelets and enhancement of inflammation [30]. However, we have convincingly demonstrated that the role of platelets is this setting is rather negligible since depletion of platelets did not alleviate hepatocellular damage [33]. To explore the potential contribution of platelets to increased TXA_2 synthesis by the steatotic liver, we examined platelet accumulation in liver sections [23] and observed less platelet recruitment into the steatotic liver. In essence, this was expected since platelet count in the *ob*/*ob* mouse blood is significantly lower than that of the wild type mice [34]. This observation further supports our assumption that the enhanced TXA_2 levels in the steatotic liver is related to the high hepatic content of AA, which is released after I/R by the action of phospholipase A_2 on cell membranes, mainly of Kupffer cells, and subsequently utilized for eicosanoid synthesis [7,35].

Dietary Ω-3 FAs reduces lipid accumulation and improves microcirculation of the steatotic mouse liver [8,36]. In patients with fatty liver, oral administration of Ω-3 FAs results in pronounced decrease in AA acid and TXA_2 levels with enhancement of hepatic blood flow [37]. Moreover, Ω-3 FAs ameliorate the clinical outcome after OLT partly by improvement of renal blood flow [38,39]. Thus, we attempted to break the pathway of TXA_2 production at the root (LA) to block the synthesis of the substrate (AA) of the final product (TXA_2). We supplemented the *ob*/*ob* mice with dietary Ω-3 FAs since this approach normalizes the Ω-6:Ω-3 FAs ratio and interferes with the enzymatic activity of desaturases and elongases on Ω-6 FAs series [7,8,40]. Dietary Ω-3 FAs resulted in significant hepatic accumulation of EPA and DHA compared with the control diet. In the next step, we investigated whether accumulation of EPA and DHA in the liver would hinder the elongation and desaturation of LA [7]. We found that the rate of conversion of LA to long chain Ω-6 FAs was significantly decreased. In particular, both AA/LA and DGLA/AA ratios were diminished. AA content was profoundly reduced; raising the possibility of modulation of its eicosanoid products [7]. DGLA levels did not show significant change; therefore we focused on the strong AA-derived vasoactive eicosanoids TXA_2 and PGI_2. Blood sampling from the hepatic vein through a needle inserted in the suprahepatic vena cava has been used in rats and dogs for determination of hepatic eicosanoid levels [31,41]. We found that TXA_2 was significantly increased after 30 min of reperfusion with persistent elevation at 180 min. This rise was not neutralized by concomitant increase in PGI_2 (as indicated by 6-keto $PGF_1\alpha$ levels). FSD dramatically deteriorated early (30 min) after

reperfusion with significant narrowing of the sinusoids correlating with increased TXA_2. V_{RBC} was remarkably decreased, suggesting increased resistance to blood flow [42]. Wunder *et al.* [43] reported similar changes after hind limb I/R in wild type mice [43]. We found a strong decrease in the sinusoidal VBF which might have prompted hepatocellular failure. In accordance with a previous study in septic rats [44] we found that dietary Ω-3 FAs substantially blunted the production of TXA_2 and PGI_2. FSD was significantly ameliorated before ischemia and after reperfusion. Ω-3 FAs abolished the vasoconstrictor effect of TXA_2 after reperfusion. Furthermore, Ω-3FAs significantly increased sinusoidal V_{RBC} before ischemia and protected against massive drop after reperfusion. Similarly, adequate sinusoidal VBF has been maintained despite the significant decrease of PGI_2 levels. This observation further enforces the assumption that an increased TXA_2 level accounts for provocation of microcirculatory failure and hepatocellular damage after reperfusion in this model.

We further extended our analyses to include other mechanisms that could be implicated in Ω-3 FAs-related beneficial effects. For instance, HO-1 has been shown to protect the *ob*/*ob* mouse liver against microcirculatory failure after I/R [19]. Nonetheless, Ω-FAs treated animals showed significantly reduced transcript levels of HO-1 compared with regular diet controls. HO-1 positive cells in liver sections were also decreased in the Ω-3 FAs group. These data are consistent with the reduction of Kupffer cell activity after Ω-3 FAs treatment [8]. Therefore, a role for HO-1 in the Ω-3 FAs induced improvement of microcirculation in this model can be excluded.

Then, we studied the potential impact of the anti-inflammatory effects of Ω-3 FAs on the response of our liver steatosis model to I/R. Enhanced *IL-1β* gene expression was observed in both groups with significantly lower mRNA levels in Ω-3 FAs supplemented animals. Furthermore, protein levels of IL-1β were significantly reduced in Ω-3 FAs treated mice 24 h after reperfusion. Contrarily, *F4*/*80* transcript levels were reduced in both groups 3 h after reperfusion. Hepatic recruitment of inflammatory cells increased after reperfusion in both groups, with significantly lower accumulation in Ω-3 FAs mice. As an evidence of reduced oxidative stress, Ω-3 FAs fed mice showed decreased lipid peroxidation before ischemia. However, this protective effect did not persist after reperfusion. It should be emphasized that we focused on IL-1β or F4/80 since the synthesis of other known pro-inflammatory cytokines such as tumor necrosis factor-α and interferon-γ could be altered due to impaired interleukin-12 production in the leptin-deficient *ob*/*ob* mouse liver [45,46]. AA is the source of powerful pro-inflammatory leukotrienes which may be implicated in hepatic I/R injury [7]. Mass spectrometry showed a significant reduction of LTB_4 and LTE_4 after reperfusion in the regular diet group. This observation conforms with the available literature that does not show strong evidence of involvement of LTs in hepatic I/R injury [7]. Furthermore, I/R did not significantly influence the hepatic levels of LTB_4, LTC_4, and LTE_4 in the Ω-3 FAs fed animals. Taken together, the protective effect of Ω-3 FAs was mainly related to amelioration of sinusoidal perfusion, with minor anti-inflammatory effect.

To validate our assumption, we selectively blocked TXA_2 receptors by TXA_2 analog (Ono 3708) [13,31,47]. This maneuver does not alter other eicosanoid levels [31,47]. Ono 3708 resulted in an amelioration of all parameters of hepatic microcirculation comparable to the supplementation with Ω-3 FAs. ALT levels were remarkably reduced; indicating significant defense against hepatocellular damage. The protection conferred by TXA_2 blocker further suggests that a reduction of I/R injury in the macrosteatotic liver can be achieved without modulation of the histological pattern from macro- to microsteatosis. Of note, TXA_2 blockage resulted in more remarkable and sustained hepatocellular protection in the steatotic compared with the lean mice.

In conclusion, we demonstrated that the composition of hepatic lipids and particularly TXA_2 levels are principal modulators of I/R injury in the steatotic liver, while the size and morphology of lipid droplets are insignificant. The study proposes treatment with TXA_2 receptor blocker as a potentially useful strategy to rescue the steatotic liver from I/R injury during liver resection, and to overcome the lack of organs for liver transplantation.

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Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2011.04.019.

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