

RESEARCH PAPER

Protective effects of n-6 fatty acids-enriched diet on intestinal ischaemia/reperfusion injury involve lipoxin A₄ and its receptor

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BACKGROUND AND PURPOSE

Long-term intake of dietary fatty acids is known to predispose to chronic inflammation, but their effects on acute intestinal ischaemia/reperfusion (I/R) injury is unknown. The aim of this study was to determine the consequences of a diet rich in n-3 or n-6 polyunsaturated fatty acids (PUFA) on intestinal I/R-induced damage.

EXPERIMENTAL APPROACH

Mice were fed three different isocaloric diets: a balanced diet used as a control and two different PUFA-enriched diets, providing either high levels of n-3 or of n-6 PUFA. Intestinal injury was evaluated after intestinal I/R. PUFA metabolites were quantitated in intestinal tissues by LC-MS/MS.

KEY RESULTS

In control diet-fed mice, intestinal I/R caused inflammation and increased COX and lipoxygenase-derived metabolites compared with sham-operated animals. Lipoxin A₄ (LxA₄) was significantly and selectively increased after ischaemia. Animals fed a high n-3 diet did not display a different inflammatory profile following intestinal I/R compared with control diet-fed animals. In contrast, intestinal inflammation was decreased in the I/R group fed with high n-6 diet and level of LxA₄ was increased post-ischaemia compared with control diet-fed mice. Blockade of the LxA₄ receptor (Fpr2), prevented the anti-inflammatory effects associated with the n-6 rich diet.

CONCLUSIONS AND IMPLICATIONS

This study indicates that high levels of dietary n-6, but not n-3, PUFAs provides significant protection against intestinal I/R-induced damage and demonstrates that the endogenous production of LxA₄ can be influenced by diet.

Abbreviations

6kPGF_{1α}, 6-keto-prostaglandin F_{1α}; 7-MaR1, 7(S)-maresin; AA, arachidonic acid; ACN, acetonitrile; DHA, docosahexaenoic acid; DiHETE, dihydroxy-eicosatetraenoic acid; EET, epoxyeicosatrienoic acid; EPA, eicosapentaenoic acid; FA, fatty acid; Fpr2, LxA₄ receptor; HDoHE, hydroxy docosahexaenoic acid; HEPE, hydroxyeicosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid; I/R, ischaemia/reperfusion; LTb₄-d₄, deuterated LTb₄; LOD, limit of detection; LOQ, limit of quantification; LxA₄, lipoxin A₄; LxA₄-d₅, deuterated lipoxin A₄; LxB₄, lipoxin B₄; LOX, lipoxygenase; MeOH, methanol; MPO, myeloperoxidase; NEC, neonatal necrotizing enterocolitis; oxo-ETE, oxoeicosatetraenoic acid; PDX, 10(S),17(S)-protectin; PUFA, polyunsaturated fatty acids; RvD1, resolvin D1; RvD2, resolvin D2; SMA, superior mesenteric artery; UHPLC, ultra HPLC

Tables of Links

TARGETS
Enzymes^a
COX
LOX, lipoxygenase
GPCR^b
Fpr2, LxA ₄ receptor

LIGANDS	
5-HETE	CXCL1
8-HETE	DHA, docosahexaenoic acid
12-HETE	LTB ₄
15-HETE	LxA ₄
AA, arachidonic acid	PGE ₂
CCL2	

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (^{a,b}Alexander *et al.*, 2013a,b).

Introduction

There is growing evidence, which indicates bioactive lipid mediators derived from n-3 or n-6 polyunsaturated fatty acids (PUFA) play a pivotal role in initiating and sustaining the inflammatory response or engaging proresolving/anti-inflammatory pathways in several cardiovascular and inflammatory pathologies. These include atherosclerosis, thrombosis, arrhythmia and ischaemia reperfusion (I/R) injury (De Caterina, 2011). The recent development of new techniques of high sensitivity LC-MS/MS allows a better comprehension of disease-associated lipidic metaboloma detecting simultaneously the presence of a number of PUFA metabolites in tissues. In our previous study, we characterized, by LC-MS/MS, the tissue profile of n-6 and n-3 PUFA metabolites in an experimental model of murine intestinal I/R (Gobbetti *et al.*, 2013). We identified temporal and quantitative differences in n-3 and n-6 PUFA metabolite production, which correlated with inflammatory damage. However, it is unclear whether modulating the n-3/n-6 ratio composition in membranes could be used as therapeutic tool to modify the resistance of the gut to I/R injury.

Acute mesenteric ischaemia is a life-threatening pathological event associated with a number of diseases including vessel occlusion, hernias, septic shock, major cardiovascular surgery, necrotizing enterocolitis and small bowel transplantation (Cerqueira *et al.*, 2005; Eltzschig and Eckle, 2011; Vollmar and Menger, 2011). Intestinal ischaemia remains a clinical challenge because of difficulty in diagnosis and especially to the lack of established pharmacological treatments. Therefore, the need for new therapeutic strategies is urgent. The intestinal mucosa is particularly prone to I/R injury because of the anatomical and physiological characteristic of the villus microcirculation (Vollmar and Menger, 2011). The temporary interruption of the blood leads to a lack of oxygen supply to the tissue (ischaemia), which in turn causes cellular dysfunction, protease and phospholipases activation (Otamiri *et al.*, 1987; Vollmar and Menger, 2011; Gobbetti *et al.*, 2012). Paradoxically, the restoration of blood flow and the consequent tissue re-oxygenation (reperfusion) aggravates the local (epithelial/endothelial damage) and systemic inflammatory response, leading to bacterial translocation and multiple organ failure (Cerqueira *et al.*, 2005).

The main objective of this study was to identify if the modification of n-6/n-3 PUFA status induced by the modification of PUFA metabolite biosynthesis associated with diet changes might improve intestinal resistance to I/R injury. We performed a dietary intervention by exposing mice to a diet rich in n-3, n-6 PUFA or to a balanced diet (Kelavkar *et al.*, 2006; Ducheix *et al.*, 2013) and we evaluated the importance of n-3 and n-6 PUFA metabolites on intestinal I/R injury. Dietary intervention significantly changed the abundance of hepatic PUFA and intestinal PUFA metabolites. Intestinal inflammation was decreased in the group fed with a high n-6 diet and the level of lipoxin A₄ (LxA₄) after ischaemia was increased compared with the group fed with a balanced diet. Early systemic pharmacological blockade of LxA₄ receptor, formyl peptide receptor (Fpr)2, prevented the anti-inflammatory effects attained by the n-6-rich diet. This study indicates that early mobilization of LxA₄ leads to significant protection against intestinal I/R-induced damage.

Methods

Animals

All animal care and experimental procedures were in accordance with the Guide for the Care and Use of Laboratory Animals of the European Council and were approved by the Animal Care and Ethics Committee of US006/CREFE (CEEA-122) with permit No. MP/01/64/09/12. All animal experiments were reported in accordance with the ARRIVE guidelines (Kilkenny *et al.*, 2010a,b; McGrath *et al.*, 2010). A total of 160 mice were included in this study. C57 BL/6 male mice (3 weeks old) were purchased from Janvier (Le Genest Saint Isle, France). Animals were maintained in ventilated cages (four mice per cage) in a specific pathogen-free room at 20–24°C and relative humidity (40–70%) with a 12 h light/dark cycle and given free access to food and water.

Study design and surgical procedure

One hundred and twenty mice were randomly assigned to one of three isocaloric diets containing 5% fat (w/w) for 9 weeks. A balanced diet providing both n-3 and n-6 fatty acid (FA) was used as a control, against two different PUFA-

enriched diets, providing either high n-3 or high n-6 intake. Mice were weighed once a week during the diet and the weight gain determined. After 9 weeks, a randomized complete block design has been used to split the experiment into a number of five ‘mini-experiments’. The days of the surgery, each diet group were split in four different experimental groups: (i) sham ischaemia, (ii) ischaemia, (iii) sham I/R and (iv) I/R (*n* = 10 per group). For each surgery day, two animals of the three different diet conditions have been subject to the surgical protocol (24 animals per day for 5 days).

Mice were anaesthetized by an i.p. injection of sodium pentobarbital (50 mg·kg⁻¹). Animals were kept under anaesthesia during all periods of surgery. The adequacy of anaesthesia was monitored with a toe pinch testing to check the animal’s reaction. Following abdominal laparotomy, the small bowel was retracted to the left and the superior mesenteric artery (SMA) was temporarily occluded using a microvascular clip (Harvard Apparatus, Les Ulis, France) to cause ischaemia. Small bowel and SMA were replaced in mice abdomen and the incision was protected with a gauze soak in saline solution (NaCl 0.9%). After 50 min, the clip was gently removed allowing reperfusion. Mice of the ischaemia groups were killed immediately after the ischaemic period. Mice of I/R groups were killed 5 h after reperfusion. Sham-operated animals, in which abdominal laparotomy and artery isolation were performed without occlusion of the vessel, served as controls and were killed just after the corresponding ischaemic (sham ischaemia) or I/R (sham I/R) time. After the surgical procedure, two-layer sutures closed the midline incision of the abdominal wall. During the surgical procedure, mice were placed on a warming plate (32°C). An independent experiment was performed to determine the effect of Fpr2 endogenous activation during intestinal I/R. Eighty mice were fed with the diet providing high n-6 intake. After 9 weeks, animals were randomized in four different subgroups of 20 animals: sham ischaemia, sham I/R, ischaemia and I/R, as previously described. Ten mice per group were treated by i.v. injection of the tail vein with Boc2 (500 µg kg⁻¹). Animals were killed at the end of the study for the purpose of sample collection by a lethal overdose of pentobarbital i.p. followed by cervical dislocation.

Fatty composition of diets

The three diets were isocaloric and contained 5% fat (w/w) and were designed as previously described (Ducheix *et al.*, 2013). Pellets were prepared by UPAE (unité de préparation des aliments expérimentaux) (INRA, Jouy en Josas, France). Oils used for experimental diets preparation were grape seed and colza oils (50/50) for the Control diet (Ctrl), grape seed oil (n-6) and colza oil/fish oils (80/20) for a long-chain n-3 FA-enriched diet (n-3). The eicosapentaenoic acid (EPA)-enriched fish oil that was used in this study was obtained from Polaris (Quimper, France). The full composition of the diet is given in Table 1. The FA composition expressed in percentage of total PUFA is provided in Table 2. Pellets were stored under vacuum at -20°C. The diets were changed twice a week in each animal cage to avoid oxidative degradation of lipids.

Assessment of inflammation

Specimens of the ileum were embedded in paraffin. Sections (5 µm) were stained with hematoxylin and eosin. Micro-

Table 1

Composition in percentage (%) of the three isocaloric diets

	Control	n-6	n-3
Cellulose	2	2	2
Casein	22	22	22
Starch	43.6	43.6	43.6
Methionine	0.2	0.2	0.2
Sucrose	21.8	21.8	21.8
Minerals	4.5	4.5	4.5
Vitamins	1.0	1.0	1.0
Grape seed oil	2.5	5	0
Colza oil	2.5	0	4
Fish oil	0	0	1

Table 2

Composition in percentage (%) of PUFA of the three isocaloric diets containing 5% fat (w/w)

	Control	n-6	n-3
C12:0	0.0	0.0	0.0
C14:0	0.0	0.0	0.0
C16:0	6.0	6.5	4.5
C18:0	2.5	4.2	0.7
C18:1n-9	38.8	18.4	47.4
C18:2n-6	47.4	70.0	19.9
C18:3n-3	5.2	1.0	7.6
C20:5n-3	0.0	0.0	17.0
C22:6n-3	0.0	0.0	3.0
n-6/n-3 ratio	10	70	0.72

scopic histological damage was scored, without knowledge of the treatments and was based on a semi-quantitative scoring system in which the following features were graded: extent of destruction of normal mucosal architecture, presence and degree of cellular infiltration, extent of muscle thickening, presence or absence of crypt abscesses and presence or absence of goblet cell depletion. The scores for each feature were summed with a maximum possible score of 11 as previously described (Cattaruzza *et al.*, 2006).

Myeloperoxidase (MPO) activity was assessed in the ileum tissue and was used as an index of granulocyte infiltration as previously described (Motta *et al.*, 2012). The chemokines CXCL1 and CCL2 were measured in intestinal tissue by cytometric bead array (BD Biosciences, Le Pont de Claix, France) as previously described (Motta *et al.*, 2012).

Immunohistochemistry

Intestines were cryoprotected, cut into 5 µm sections in a cryostat and mounted on a Superfrost slide (Thermo Fisher

Scientific, Illkirch, France). Slides were washed in PBS, 0.5% Triton X-100 and 1% BSA solution (Sigma, Saint-Quentin Fallavier, France) and incubated overnight at 4°C with the primary antibody directed against Fpr2 (1/100, Santa Cruz, Heidelberg, Germany) along with anti-cytokeratin 18 (1/500, Santa Cruz), anti-CD45 (1/500, R&D Systems, Lille, France) or anti-Ly-6B.2 (1/400, Bio-Rad AbD Serotec GmbH, Colmar, France). After washes, slides were incubated with appropriate secondary antibody (Alexa Fluor 488 and 555; Invitrogen, Cergy Pontoise, France) for 2 h. Slides were mounted with Prolong® containing DAPI (Invitrogen). As control procedures, reactions were performed with omission of primary or secondary antibodies. Images were acquired using Zeiss LSM-710 confocal microscopes with 63X objective in the inverted configuration (Carl Zeiss MicroImaging, Inc., Göttingen, Germany). Images of stained and control slides were collected and processed identically. The quantification of fluorescence was determined using Metamorph software (Molecular Devices, Berkshire, UK).

Real-time PCR analysis

Total RNA from small intestine was isolated with TRIzol reagent (Invitrogen, Paisley, UK) and 1 µg of the total RNA was then reverse-transcribed with random hexamer oligonucleotides and SuperScript III (Invitrogen), according to the manufacturer's instructions. Amplification was performed with a LightCycler 480 using a SYBR Green I Master kit (Qiagen, Ltd., Manchester, UK) and one of the following mouse primer: *fpr2* (QT00171514, Qiagen, Ltd.), *alox12b* (QT00173551), *alox15* (QT00111034) or *alox5* (QT00258622). Relative expression of the target gene was normalized to expression of the *Hprt* gene, using the $\Delta\Delta C_t$ method (Boue *et al.*, 2011).

Hepatic and intestinal FA profiling

Hepatic FAs were analysed as described previously (Zadravec *et al.*, 2010). Briefly, following homogenization of tissue samples in methanol/5 mM EGTA (2:1, v/v), lipids corresponding to an equivalent of 2 mg of tissue were extracted in the presence of glyceryl triheptadecanoate as an internal standard. The lipid extract in 1 mL of heptane was transmethylated with 1 mL of boron trifluoride in methanol (MeOH, 1:20, v/v) for 150 min at 100°C, evaporated to dryness, and the fatty acid methyl esters (FAMES) were extracted with hexane/water (3:1). The organic phase was evaporated to dryness and dissolved in 50 µL ethyl acetate. One microliter of FAME was analysed by gas-LC on a 5890 Hewlett-Packard system (Hewlett-Packard, Palo Alto, CA, USA) using a Famewax fused-silica capillary column (30.0 × 0.32 mm i.d., 0.25 µm film thickness; Restek, Belfast, UK). Oven temperature was programmed from 110 to 220°C at a rate of 2°C per min and the carrier gas was hydrogen (0.5 bar). The injector and the detector were at 225 and 245°C respectively.

Intestinal lipid extraction

Mouse small intestines were crushed with a FastPrep®-24 Instrument (MP Biomedical, Illkirch, France) in 500 µL of HBSS (Invitrogen) and 5 µL of internal standard mixture (LxA₄-d₅, LTB₄-d₄ and 5-HETE-d₈ at 400 ng mL⁻¹ in MeOH). After two crush cycles (6.5 m s⁻¹, 30 s), 10 µL is withdrawn for protein quantification and 300 µL of cold methanol was

added. Samples were centrifuged at 2000×g for 15 min at 4°C. Supernatants were collected, completed to 2 mL in H₂O and submitted to solid-phase extraction using HRX-50 mg 96-well (Macherey Nagel, Hoerd, France). Briefly, after plate conditioning, the sample was loaded at flow rate of 0.1 mL min⁻¹. After complete loading, the plate was washed with H₂O/MeOH (90:10, 2 mL) and lipid mediators were eluted with MeOH (2 mL). Solvent was evaporated under nitrogen and samples were dissolved with MeOH and stored at -80°C for LC-MS/MS measurements.

LC-MS/MS measurements

By this technique, we performed the quantification of 6kPGF_{1α}, TXB₂, PGE₂, PGA₁, 8-isoPGA₂, PGE₃, LxA₄, LxB₄, RvD1, RvD2, 7-MaR1, LTB₄, LTB₅, PDX, 18-HEPE, 5,6-DiHETE, 15-HETE, 12-HETE, 8-HETE, 5-HETE, 17-HDoHE, 14-HDoHE, 14,15-EET, 11,12-EET, 8,9-EET, 5,6-EET and 5-oxo-EETE in mouse intestinal tissue (Le Faouder *et al.*, 2013). To simultaneously separate 27 lipids of interest and three deuterated internal standards, LC-MS/MS analysis was performed on ultra HPLC system (UHPLC, Agilent LC1290 Infinity) coupled to Agilent 6460 triple quadrupole MS (Agilent Technologies, Les Ulis, France) equipped with electrospray ionization operating in negative mode. Reverse-phase UHPLC was performed using Zorbax SB-C18 column (Agilent Technologies) with a gradient elution. The mobile phases consisted of water, acetonitrile (ACN) and formic acid (75:25:0.1; v/v/v) (A) and ACN, formic acid (100:0.1, v/v) (B). The linear gradient was as follows: 0% B at 0 min, 85% B at 8.5 min, 100% B at 9.5 min, 100% B at 10.5 min and 0% B at 12 min. The flow rate was 0.35 mL min⁻¹. The autosampler was set at 5°C and the injection volume was 5 µL. Data were acquired in multiple reaction monitoring mode with optimized conditions. Peak detection, integration and quantitative analysis were performed using Mass Hunter Quantitative analysis software (Agilent Technologies). For each standard, calibration curves were built using 10 solutions at concentration ranging from 0.95 to 500 ng mL⁻¹. A linear regression with a weight factor of 1/X was applied for each compound. The limit of detection (LOD) and the limit of quantification (LOQ) were determined for the 27 compounds using signal to noise ratio. The LOD corresponded to the lowest concentration leading to a signal to noise over 3 and LOQ corresponded to the lowest concentration leading to a signal to noise over 10. All values under the LOQ were not considered. Importantly, blank samples were evaluated and their injection showed no interference (no peak detected) during the analysis. Hierarchical clustering and heat-map were obtained with R (www.r-project.org). PUFA metabolite quantities were transformed to z-scores and clustered based on one Pearson correlation coefficient as distance and the Ward algorithm as agglomeration criterion.

Data analysis

Data are presented as mean ± SEM. Analyses were performed using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). All data were normally distributed. Between-group comparisons were performed by Student's unpaired two-tailed *t*-test. Multiple comparisons within groups I/R were performed by repeated-measures one-way ANOVA followed by Tukey's procedure. Statistical significance was accepted at $P \leq 0.05$.

Materials

N-tert-butoxycarbonyl-L-Phe-D-Leu-L-Phe-D-Leu-L-Phe (Boc₂; Bachem, Bubendorf, Switzerland) was dissolved in dimethyl sulfoxide (1%)/sodium chloride (NaCl) 0.9%. 6-keto-prostaglandin F_{1α} (6kPGF_{1α}), thromboxane B₂ (TXB₂), PGE₂, PGA₁, 8-isoPGA₂, PGE₃, LXA₄, lipoxin B₄ (LxB₄), lipoxin A₄ deuterated (LxA₄-d5), resolvin D1 (RvD1), resolvin D2 (RvD2), 7(S)-maresin (7-MaR1), leukotriene B₄ (LTB₄), leukotriene B₅ (LTB₅), leukotriene B₄ deuterated (LTB₄-d4), 10(S),17(S)-protectin (PDx), 18-hydroxyeicosapentaenoic acid (18-HEPE), dihydroxy-eicosatetraenoic acid (5,6-DiHETE), 15-hydroxyeicosatetraenoic acid (15-HETE) and 12-HETE, 8-HETE, 5-HETE, 5-HETE-d8, 17-hydroxy-docosahexaenoic acid (17-HDoHE) and 14-HDoHE, 14,15-epoxyeicosatrienoic acid (14,15-EET) and 11,12-EET, 8,9-EET, 5,6-EET, 5-oxoeicosatetraenoic acid (5-oxo-ETE) were purchased from Cayman Chemicals (Ann Arbor, MI, USA).

Results

Effect of dietary intervention on the abundance of FAs

In order to investigate the effect of the dietary intervention on the lipid composition, intestinal and hepatic FAs were profiled by gas chromatography analysis. Compared with the control group, the ratio between arachidonic (AA) and docosahexaenoic (DHA) acids increased threefold in the liver of animals receiving the n-6 diet (Figure 1). Conversely, animals receiving the n-3 diet had a significantly decreased ratio. Even if the total intestinal FA content was the same, the dietary intervention had a major impact on the relative FA composition of the gut (Table 3). In the intestine of mice fed with n-6 diet, linoleic acid (LA, C18:2 n-6), C20:2 n-6 and AA (C20:4 n-6) were significantly increased compared with the control diet group (Table 3). In the intestine of mice fed on n-3 diet, α-LA (C18:3 n-3), EPA (C20:5 n-3), C22:5 n-3 and

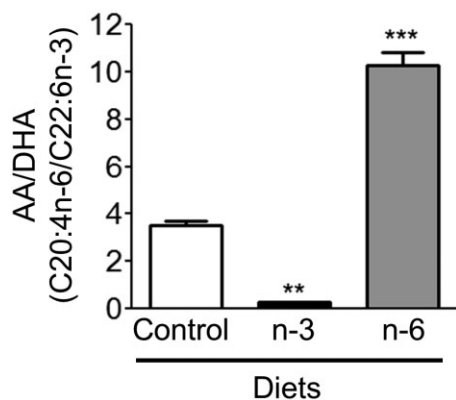


Figure 1

Effect of dietary intervention on the hepatic FAs composition. Total AA (C20:4 n-6) and DHA (C22:6 n-3) ratio in liver of mice submitted to control, n-3 or n-6 PUFA diet for 9 weeks. Values are mean ± SEM, $n = 6-8$ per group. ** $p < 0.01$, *** $p < 0.001$, significantly different from control diet group.

DHA (C22:6 n-3) were significantly increased compared with the control diet. On the other hand, in this group, C20:2 n-6, C20:3 n-6 and AA were significantly decreased. The ratio between AA and DHA mirrored the changes in the relative abundance of intestinal PUFA. During dietary intervention, weight gain was weekly assessed and it was found to be similar regardless of diet (Supporting Information Fig. S1).

Long-term n-6 diet prevents intestinal I/R injury

In animals fed with the control diet, 50 min of ischaemia followed by 5 h of reperfusion resulted in acute inflammation. This was characterized by microscopic damage: fragments of mucosa and red blood cells were found in the lumen. Villi were completely denuded or severely damaged, together with loss of crypt architecture. MPO activity and CXCL1/CCL2 expressions were also markedly increased in I/R versus sham operation in control diet animals (Figure 2). These inflammatory markers were unchanged in animals receiving a high n-3 diet and then subjected to I/R. Conversely, the microscopic damage score, MPO activity and CXCL1/CCL2 intestinal expression were significantly decreased in the I/R group fed with a high n-6 diet (Figure 2) compared with control diet group. Taken together, these data demonstrate that the administration of a long-term n-6 diet, but not of n-3 diet, protects the gut from the proinflammatory damaging consequences of I/R injury.

Effects of diet on the production of intestinal n-6 and n-3 metabolites after ischaemia or ischaemia followed by reperfusion

We questioned how much the dietary FAs might influence the eicosanoid and docosanoid profiles likely to influence I/R-induced damage. RvD1, RvD2, 7-MaR1, 14,15-EET, 11,12-EET, 8,9-EET and 5,6-EET were not detectable and PGA₁, 8-isoPGA₂ and 5,6-DiHETE were not quantifiable in intestinal tissue. PUFA metabolites hierarchical clustering was used to reveal the main differences (Figure 3A). The columns represented the average of PUFA metabolite quantity (pg·mg⁻¹ of proteins) of the different groups of mice fed Ctrl, n-3 or n-6 diet. PUFA metabolites formed four different clusters. The first cluster was composed by product of AA metabolism by COX- (PGE₂, 6kPGF_{1α}, TXB₂) and lipoxygenase (LOX)-derived metabolites (LTB₄, LxB₄, 5/8/15/12-HETE). The principal characteristic of this cluster was that the quantity of eicosanoids was maximal following I/R in groups of mice submitted to control diet (Figure 3A). 8/15/12-HETE, PGE₂, 6kPGF_{1α}, TXB₂, LTB₄ and LxB₄ were significantly increased only in control diet group submitted to I/R compared with sham I/R (Supporting Information Fig. S2). 5-HETE was significantly increased in control diet group submitted to ischaemia compared with sham ischaemia (Supporting Information Fig. S2). The second cluster was represented only by LxA₄; its quantity was maximal in the group of mice submitted to n-6 diet and to ischaemia (Figure 3A). 5-oxo-ETE and 15dPGj₂ formed the third cluster characterized by a decrease of these two metabolites by ischaemia independently of the diet. The last cluster regrouped all n-3 PUFA metabolites. They were increased in animals submitted to n-3 diet, independently of ischaemia or I/R (Figure 3A).

Table 3

Concentration of FA (nmol·mg⁻¹ of protein) in mouse intestine

	Diet		
	Control	n-3	n-6
10:0	0.00	0.00	0.00
12:0	0.00	0.00	0.00
14:0	0.00	5.39 ± 2.57	1.02 ± 1.02
15:0	0.00	0.00	0.00
16:0	286.85 ± 43.95	349.08 ± 75.12	329 ± 79.41
18:0	185.97 ± 23.68	147.24 ± 21.21	127.53 ± 42.45
20:0	0.00	0.00	0.00
21:0	0.00	0.00	0.00
22:0	0.00	0.00	0.00
14:1 n-5	0.00	0.00	0.00
15:1 n-5	0.00	0.00	0.00
16:1 n-9	0.00	0.00	0.00
16:1 n-7	150.83 ± 13.09	157.22 ± 38.09	146.05 ± 14.37
17:1 n-7	0.00	0.00	0.00
18:1 n-9	413.22 ± 68.93	450.11 ± 49.71	445.18 ± 65.70
18:1 n-7	100.73 ± 17.17	93.00 ± 11.58	94.35 ± 19.73
20:1 n-9	0.00	0.00	0.00
22:1 n-9	0.00	0.00	0.00
24:1 n-9	0.00	0.00	0.00
18:2 n-6 (LA)	84.56 ± 18.38	69.76 ± 10.07	209.05 ± 23.64*
18:3 n-6	0.00	0.00	0.00
18:3 n-3 (ALA)	0.00	15.65 ± 1.65*	0.00
20:2 n-6	3.41 ± 1.50	0.00*	6.97 ± 0.92*
20:3 n-3	0.00	0.00	0.00
20:3 n-6	18.29 ± 1.03	8.56 ± 0.86*	13.47 ± 1.34
20:4 n-6 (AA)	39.95 ± 2.34	13.81 ± 1.19*	60.37 ± 5.70*
20:5 n-3 (EPA)	0.00	10.78 ± 1.86*	0.00
22:5 n-3	0.00	5.69 ± 0.78*	0.00
22:2 n-6	0.00	0.00	0.00
22:4 n-6	0.00	0.00	0.00
22:5 n-6	0.00	0.00	0.00
22:6 n-3 (DHA)	1.88 ± 1.21	19.87 ± 3.14*	0.93 ± 0.52
20:4 n-6/22:6 n-3	9.51 ± 0.75	0.73 ± 0.07*	44.09 ± 11.34*
Total SAFA	472.82 ± 67.64	501.71 ± 98.91	457.98 ± 122.88
Total MUFA	664.78 ± 99.19	700.33 ± 99.38	685.58 ± 99.80
Total PUFA	148.08 ± 24.46	144.50 ± 19.93	290.78 ± 32.30*
Total FA	1284 ± 151	1345 ± 162	1432 ± 114

Mice were submitted to 9 weeks of control, n-3 or n-6 diet before FA metabolite extraction. Data are express as mean ± SEM, $n = 10$. MUFA, monounsaturated fatty acid; SAFA, saturated fatty acid. * $P < 0.05$ versus control diet group.

Because of the protective effect of n-6 diet on I/R injury, we focused our attention on LxA₄. In mice receiving the control diet, the anti-inflammatory mediator LxA₄ was significantly increased compared with sham after ischaemia (Figure 3B). In mice submitted to n-6 diet, after ischaemia,

LxA₄ was significantly increased compared with sham and also compared with mice submitted to control diet and ischaemia (Figure 3B). Moreover, in this group of mice, we quantified a significant increase in *alox5*, *alox12* and *alox15* mRNA expression compared with mice receiving the control

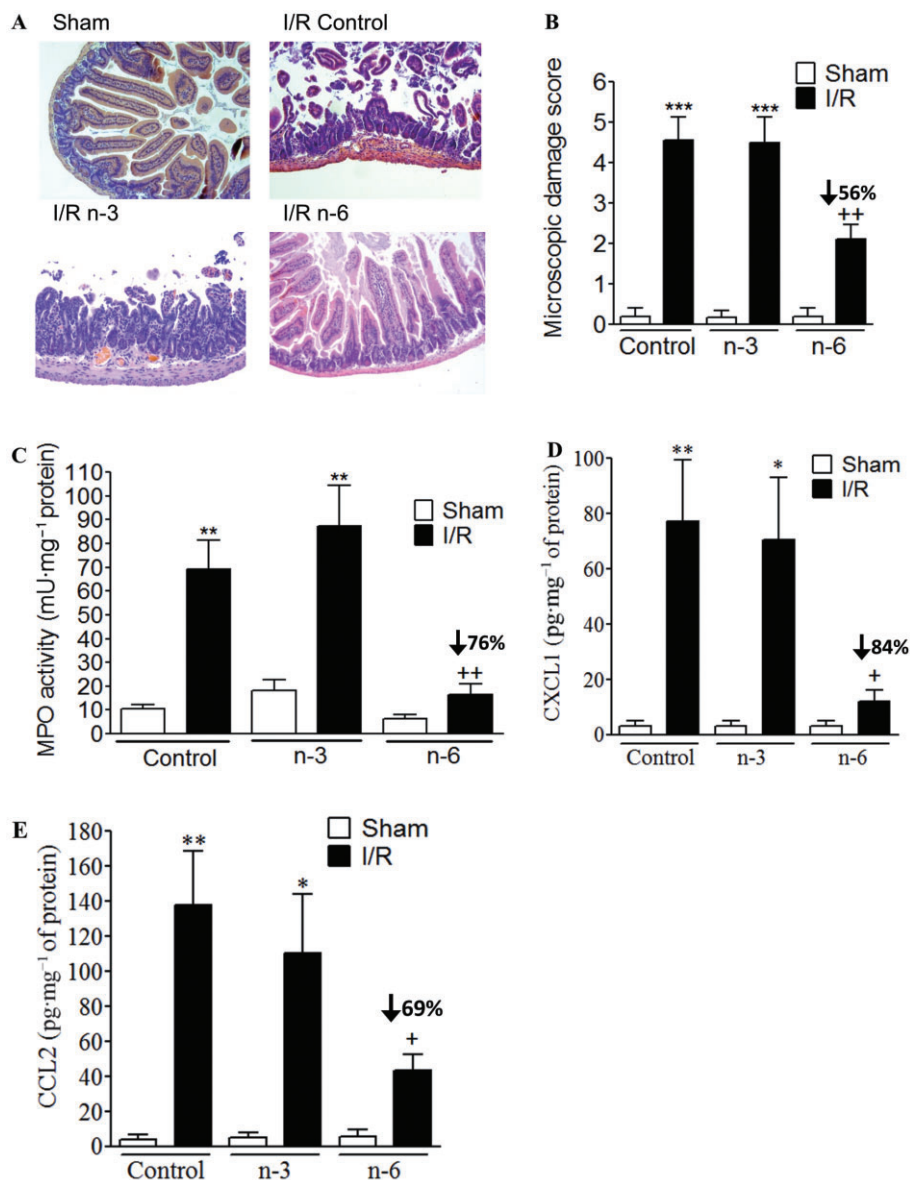


Figure 2

Inflammation of the intestine induced by I/R. (A) Representative hematoxylin and eosin-stained histological sections of small intestine from sham-operated mouse or mouse submitted to I/R and receiving control, n-3 or n-6 PUFA diet. Different inflammatory parameters were followed: microscopic damage scores (B), MPO activity (C) in sham or I/R mice exposed to control, n-3 or n-6 PUFA diet for 9 weeks. (D–E) CXCL1 and CCL2 expressions were quantified in I/R mice versus corresponding sham, exposed to control, n-3 or n-6 PUFA diet. Values are mean \pm SEM, $n = 6–8$ per group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, significantly different from sham; + $P < 0.05$, ++ $P < 0.01$, significantly different from control diet group.

diet after ischaemia (Supporting Information Fig. S3). These findings suggest that diet supplementation is able to change the PUFA metabolites quantity in colonic tissue after ischaemia or I/R by an increase of LOX expression. Interestingly, after ischaemia, the increased LxA₄ in n-6 diet versus control diet suggests a possible protective role for this anti-inflammatory lipid in the protection offered by this diet.

Fpr2 is expressed in mouse small intestine

Because the tissue levels of LxA₄ were modified in some of the groups studied, we also investigated the expression of the LxA₄ receptor Fpr2 in the same tissues. Ischaemia did not

modify *fpr2* expression compared with sham (Figure 4A). We observed an increase of *fpr2* mRNA after I/R in control and n-3 diet groups compared with corresponding sham (Figure 4A). Following I/R, the expression of *fpr2* mRNA was not different in mice that received the n-6 diet compared with sham (Figure 4A). By immunohistochemistry, we observed that Fpr2 was expressed in the mucosa of murine small intestine (Figure 4B). Fpr2-immunoreactivity colocalized with cytokeratin 18, Ly-6B.2 and partially with CD45-immunoreactivity meaning that Fpr2 was expressed on epithelial cells, neutrophils and on 44% of nucleated hematopoietic cells respectively (Figure 4B). In those different cell

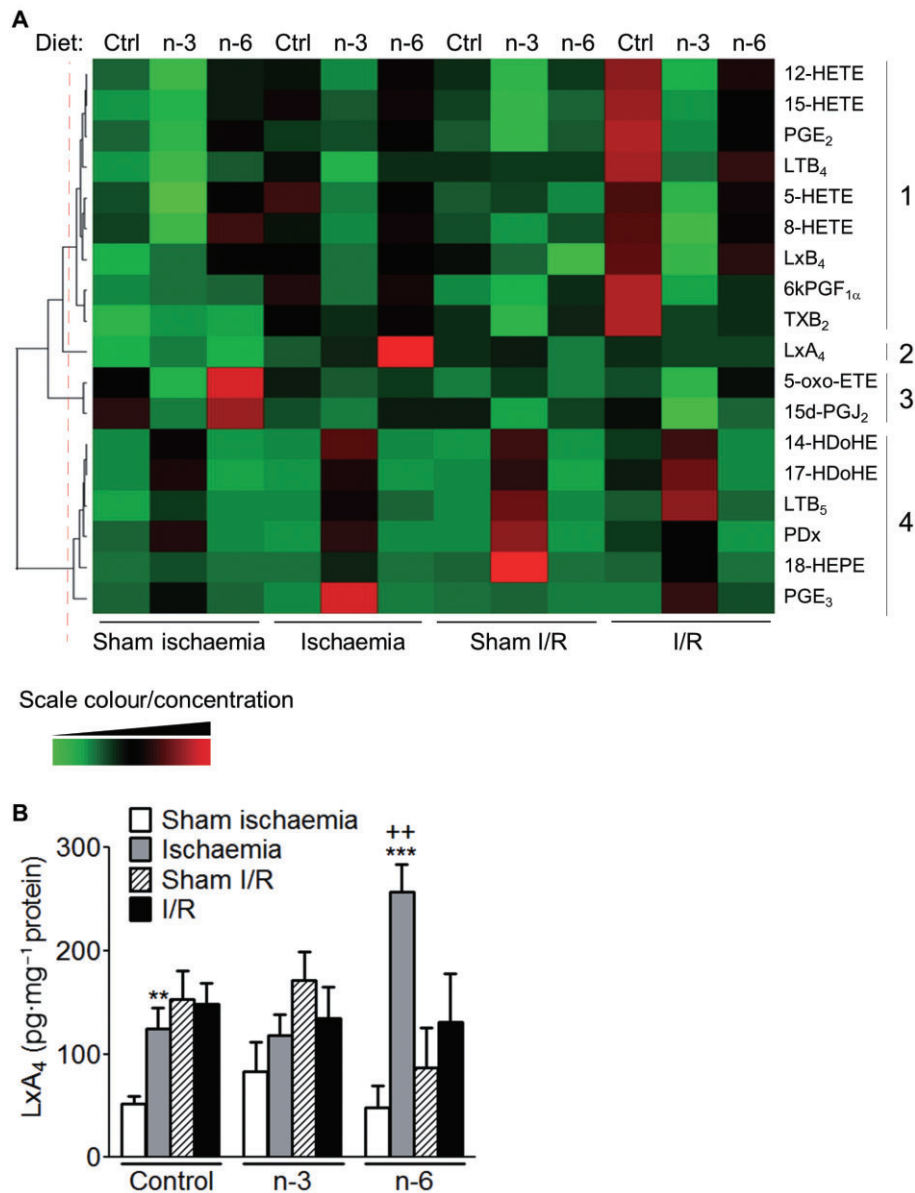


Figure 3

Quantification of PUFA metabolites in intestine of mice exposed to I/R and to the three different diets. (A) Heat-map and clustering of quantified PUFA metabolites by LC-MS/MS. The heat-map represents a color coding of average z-scores obtained for the different PUFA metabolites (in row) and the groups of mice (in column). A red colour indicates a quantity greater than the mean of the row, a green colour indicates a quantity lower than the mean and a black colour indicates a quantity close to the mean. The dendrogram reflects the correlation of the PUFA metabolites. The dotted red line represents the clusters. (B) Synthesis of LxA₄ was measured by LC-MS/MS in sham ischaemia, sham I/R animals and following 50 min of ischaemia or 50 min of ischaemia and 5 h of reperfusion (I/R). Data are expressed in pg·mg⁻¹ protein and represent means ± SEM of six to eight animals per group. **P < 0.01, ***P < 0.001, significantly different from corresponding sham; ++P < 0.01, significantly different from ischaemia control diet group.

types, no difference of Fpr2 fluorescence intensity was observed (Supporting Information Fig. S4A) between the different groups or diets. In correlation with MPO activity (Figure 2B), Ly-6B.2 expression was increased following I/R in control and n-3 diet groups compared with I/R n-6 diet group (Supporting Information Fig. S4B). Thus, in these two groups, the overexpression of *fpr2* could be the consequence of the infiltration of granulocytes expressing Fpr2. Pictures of

immunocytochemistry control procedures are shown in Supporting Information Fig. S4C.

Pharmacological blockade of Fpr2 prevented the anti-inflammatory effects obtained by the n-6 rich diet

LxA₄ is an effector of endogenous anti-inflammation acting via its receptor, Fpr2. In mice receiving a high n-6 diet,

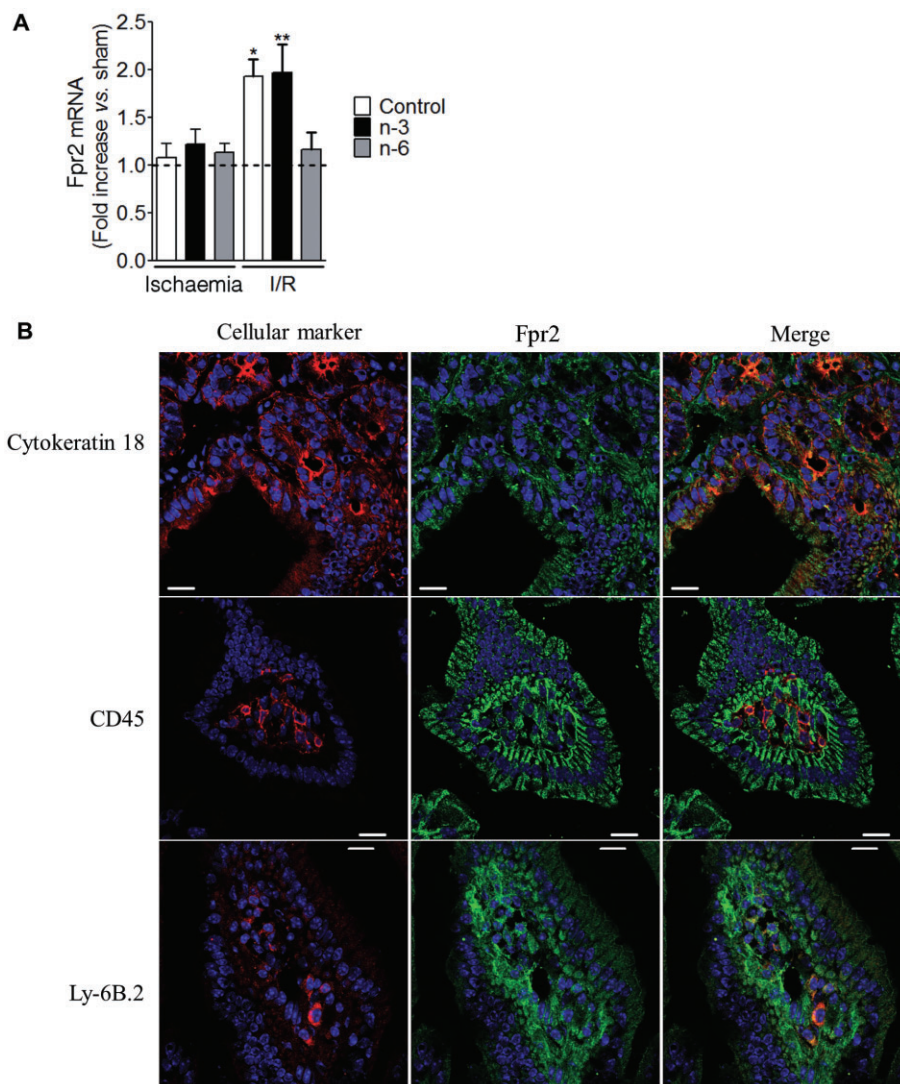


Figure 4

Expression of Fpr2 in mouse small intestine. (A) Fpr2 mRNA expression was quantified in I/R mice versus corresponding sham exposed to control, n-3 or n-6 PUFA diet. (B) Pictures of Fpr2, cytokeratin 18, CD45 and Ly-6B.2 immunoreactivity detected in the small intestine of mice exposed to sham and to n-6 PUFA diet (scale: 50 μ m). Values are mean \pm SEM, $n = 6-8$ per group. * $P < 0.05$, ** $P < 0.01$, significantly different from corresponding ischaemia group.

administration of the Fpr pan antagonist, Boc2 (500 μ g kg^{-1} before ischaemia i.v.) abolished the inhibition of microscopic damage score, MPO activity, CXCL1 and CCL2 expression induced by n-6 diet following I/R (Figure 5A). Administration of the Fpr2 antagonist also prevented the inhibition of 15-HETE, PGE₂, 6kPGF_{1 α} and TXB₂ induced by the n-6-enriched diet (Figure 5B). Administration of Boc2 had no effect on the increase of LxA₄ induced by ischaemia in n-6 diet group (Figure 5C). These findings show that the blockade of the LxA₄ receptor (Fpr2) annulled the anti-inflammatory effects obtained by the n-6-rich diet and that n-6 supplementation-induced protection after I/R is indeed mediated by Fpr2 activation.

Discussion

In the present study, we evaluated the effect of dietary intervention based on different n-6 and n-3 PUFA intake, on injury associated with intestinal I/R. Our data indicate that n-6 PUFA afforded significant protection against I/R-induced tissue injuries, while n-3 PUFA diet had no effect on intestinal damage as determined in these settings. Specifically, in mice subjected to intestinal I/R, n-6 PUFA supplementation in diet decreased mucosal damage, granulocyte recruitment and chemokine expression compared with animals fed with a control balanced diet. Protection against I/R injury was associated with an increase of *alox5*, *alox12* and *alox15* mRNA

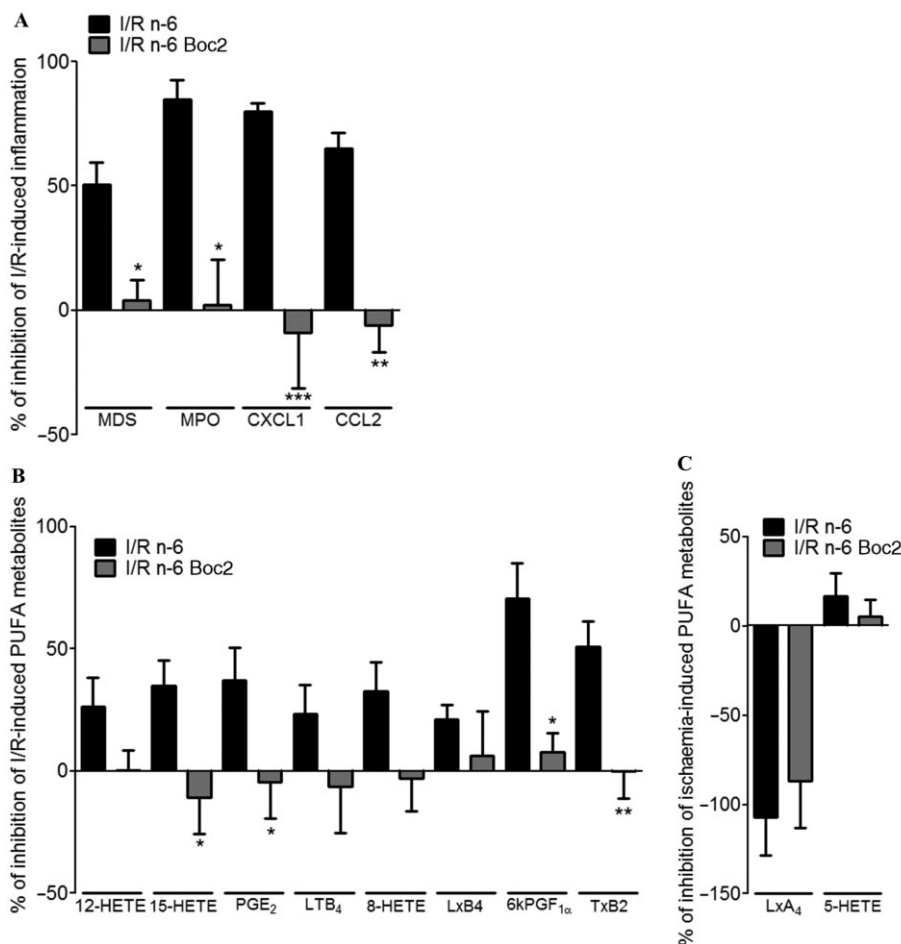


Figure 5

Role of Fpr2 antagonist in n-6 PUFA diet anti-inflammatory effects. (A) Different inflammatory parameters were followed: MPO activity, microscopic damage scores, CXCL1 expression and CCL2 expression, in sham or I/R mice treated with the Fpr2 antagonist (Boc2, 500 $\mu\text{g kg}^{-1}$ before ischaemia i.v.) and exposed to n-6 PUFA diet for 9 weeks. (B) n-6 PUFA-derived metabolites in sham or I/R mice treated with the Fpr2 antagonist (Boc2, 500 $\mu\text{g kg}^{-1}$ before ischaemia i.v.) and exposed to n-6 PUFA diet for 9 weeks. Data are expressed as percentage of inhibition of I/R-induced effect in control diet group. Values are mean \pm SEM, $n = 6-8$ per group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, significantly different from I/R n-6 vehicle group.

expression and consequently of LxA₄ production following ischaemia in intestinal tissues from n-6-enriched FA-treated group compared with control diet-fed group. *In vivo* experiments demonstrated that the LxA₄ receptor Fpr2 was involved in the protective effects associated with n-6 PUFA-enriched diet. Collectively, these data make us propose that early during ischaemia, an endogenous protective pathway mediated by LxA₄ and its receptor could be triggered by changes in dietary FA.

Our results further confirmed the essential role that AA-derived lipid mediators could play in resolving inflammatory response *in vivo* (Serhan and Samuelsson, 1988). This notion has been well developed by a number of studies and AA is now considered not only as a precursor of proinflammatory bioactive lipids, but also of potent anti-inflammatory mediators named lipoxins (Morris *et al.*, 2006; Schwab and Serhan, 2006; Wallace, 2006; Dufton and Perretti, 2010). Indeed, in our experimental conditions, we observed that

ischaemia and ischaemia followed by reperfusion led to an increase of proinflammatory bioactive lipids or pathway marker derived from AA metabolism by COX (PGE₂, 6kPGF_{1α}, TxB₂) and LOX (5-, 8-, 12-, 15-HETE, LTB₄). Simultaneously, potent anti-inflammatory mediators derived from the AA metabolism, LxA₄ and LxB₄, were also increased. These two lipoxins are differentially expressed in intestinal tissues over time, LxA₄ being produced from the ischaemic period and LxB₄ (its positional isomer) increased only after 5 h reperfusion. In our study, increased level of LxA₄ was significantly higher in n-6 diet group after ischaemia compared with control diet group. However, levels of LxB₄ [whose receptor(s) is yet to be discovered] were the same in both groups. We have recently reported a mechanism activated by neutrophil/platelet aggregates to increased circulating levels of LxA₄ at the end of 30 min mesenteric ischaemia (Brancaleone *et al.*, 2013). This is in agreement with our current data and supports the notion that LxA₄ is an early metabolite of ischaemic

conditions. The effect of LXA₄/Fpr2 system in the protective effects of n-6 diet suggests a minor role for LxB₄ in our experimental conditions.

LxA₄ was identified by Serhan *et al.* in 1984 as a short-lived lipid produced principally by transcellular synthesis (Serhan *et al.*, 1984). LxA₄ is described as a potent endogenous anti-inflammatory bioactive lipid mediator (Serhan and Savill, 2005). Acting through its receptor, Fpr2 (Lavigne *et al.*, 2002; Perretti *et al.*, 2002; Ye *et al.*, 2009), a GPCR belonging to the Fpr family, LxA₄ is able to halt leukocyte migration (Chiang *et al.*, 2006; Perretti and D'Acquisto, 2009) and promote macrophage phagocytosis of infectious agents and apoptotic neutrophils (Godson *et al.*, 2000; Maderna *et al.*, 2005). In agreement with our results, several studies have demonstrated a protective role for pharmacologically delivered lipoxins or stable lipoxin analogues in I/R (Bannenberg *et al.*, 2004; Sun *et al.*, 2009; Wu *et al.*, 2012).

Cell–cell interaction is one of the routes by which lipoxins are endogenously biosynthesized by cooperation between 15- and 5-LOX (Serhan *et al.*, 2008). Primarily described in granulocytes and later in airway epithelial cells and dendritic cells, metabolism of AA by 15-LOX produces 15S-hydroperoxyeicosatetraenoic acid or the reduced alcohol form 15S-hydroxyeicosatetraenoic acid (15S-HETE) (Serhan, 1989; Levy *et al.*, 1993; Serhan *et al.*, 2008). These metabolites are further transformed into epoxytetraene intermediates by the action of leukocyte 5-LOX yielding LxA₄ and LxB₄ (Serhan, 1989; Levy *et al.*, 1993; Serhan *et al.*, 2008). In the intestine, resident tissue cells such as intestinal epithelial cells expressed 15-LOX and could further contribute to LxA₄ generation by the production of 15S-H(p)ETE (McMahon *et al.*, 2001). Indeed, LxA₄ is synthesized in the human gut by colonic mucosa (Mangino *et al.*, 2006). Interestingly, in pathological conditions such as ulcerative colitis, LxA₄ endogenous production is significantly reduced compared with control patients (Mangino *et al.*, 2006). This decrease was coupled to a lower level of 15-LOX in the mucosa of ulcerative colitis patients compared with tissues from healthy controls (Mangino *et al.*, 2006). In our experimental conditions, where *alox5* and *alox15* expression and LxA₄ were increased after ischaemia before granulocyte infiltration, epithelial cells could be the principal source of 15S-H(p)ETE or 15S-HETE metabolized into LxA₄ by 5-LOX-bearing cells (polymorphonuclear leukocytes, macrophages and mast cells that physiologically populate the gut). LxA₄ acts on a specific target receptor termed Fpr2/lipoxin A₄ receptor (FPR2/ALX) in humans and Fpr2/3 in mice (Perretti *et al.*, 2002; Ye *et al.*, 2009). FPR2 not only transduces the proresolving properties of LXA₄, but also of the glucocorticoid regulated protein Annexin A1 and humanin (Dufton and Perretti, 2010). Increasing evidence suggests that members of the FPR family and in particular FPR2 might have an important role in the pathophysiology of I/R injury (Gavins, 2010). In a recent study, the use of *fpr2/3*-deficient mice highlighted the importance of this receptor in I/R; in the absence of Fpr2/3, an aberrant inflammatory response characterized by prolonged neutrophil adhesion and infiltration was observed (Brancaleone *et al.*, 2013). Furthermore, exogenous delivery of LxA₄ attenuated I/R-mediated inflammation in wild-type but not in Fpr2/3-deficient animals (Brancaleone *et al.*, 2013). In mice receiving a high n-6 diet, administration of

the Fpr2 antagonist before ischaemia reverted the protective anti-inflammatory effect of this diet. Our data indicate that the protective association between n-6 supplementation and the prevention of post-ischaemic damage was due, at least in part, to the interaction between LxA₄ (produced in higher amount in the n-6 group) and its receptor, Fpr2. Human FPR2/ALX was first identified on neutrophils and monocytes (Fiore *et al.*, 1992; Takano *et al.*, 1997). Here, we observed that the murine receptor was expressed also in intestinal epithelial cells, confirming a previous *in vitro* study that had identified Fpr2 in intestinal epithelial cell lines (Goh *et al.*, 2001). Interestingly, *in vitro* studies have identified Fpr2 expression preferentially on the basolateral side of intestinal epithelial cells (Kucharzik *et al.*, 2003). LxA₄ produced on the serosal side of the epithelium by leukocyte–epithelial cell interaction could rapidly signal by activating the receptor on the basolateral side, resulting in potential attenuation of inflammation. LxA₄ promotes anti-inflammatory signals not only by regulating innate immune cells, but also by acting on intestinal epithelial cells. Upon stimulation with bacteria (*Salmonella typhimurium*) (Gewirtz *et al.*, 1998), TNF- α (Gronert *et al.*, 1998; Goh *et al.*, 2001) or LPS (Kure *et al.*, 2010), human intestinal epithelial cells release the proinflammatory chemokine IL-8: this response could be inhibited by cell exposure to LxA₄. In accordance with these data, we observed that after I/R the n-6-enriched diet was associated with a decrease in CXCL1, the murine equivalent of human IL-8. Other proinflammatory signals, such as the transcription factor NF- κ B can be modulated by LxA₄ in epithelial cells (Gewirtz *et al.*, 1998). Such inhibition is also downstream of Fpr2 engagement by LxA₄ (Gewirtz *et al.*, 2002). There is conflicting data regarding how much n-6 PUFA (i.e. LA) should be consumed in a healthy diet. Some claim that high LA intake promotes inflammation through accumulation of tissue AA and subsequent production of proinflammatory lipid mediators. Human studies indicate that high LA in the diet or circulation is not associated with higher *in vivo* or *ex vivo* proinflammatory responses. Several studies have shown that individuals consuming the highest level of LA have the lowest inflammatory status (Fritsche, 2008). In our experimental animal study, we did not detect any 'proinflammatory phenotype' of the n-6 diet group that underwent a sham operation compared with the n-3 or control diet. Conversely, the n-6 but not the n-3-enriched diet displayed anti-inflammatory properties indicating that the consumption of high n-6 diets may work in specific intestinal scenarios. Exploiting this n-6-dependent endogenous anti-inflammatory loop described here could be of therapeutic impact in diseases in which intestinal ischaemia contributes to the pathogenesis, such as neonatal necrotizing enterocolitis (NEC) (Berman and Moss, 2011). It has been shown that the type and composition of infant diet can regulate the outcome of this pathology. In a clinical study, Carlson *et al.* found a lower incidence of NEC in infants fed formula containing higher amounts of PUFA compared with controls (Carlson *et al.*, 1998). In the rat NEC model, supplementation of the formula with AA has been shown to reduce the degree of intestinal injury (Lu *et al.*, 2007). Thus, in this pathology, delivery of LxA₄, shown to be the most important AA metabolite in our model, could be even more effective than AA-based treatment.

In conclusion, these data prompt us to propose that the endogenous anti-inflammatory circuit activated by a selective Fpr2 agonist, LxA₄, could be harnessed by boosting the n-6 PUFA content of the diet, as a viable therapeutic approach for the prevention of intestinal I/R injuries.

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Author contributions

T. G. contributed in the acquisition, analysis and interpretation of data and in the redaction of the manuscript. S. D. contributed in the acquisition and analysis of data. P. L. F. contributed in the acquisition and analysis of data and in the redaction of the manuscript. J. B. contributed in the acquisition and analysis of data. J. B.-M. contributed in the analysis and interpretation of data. M. D. contributed in the interpretation of data and in the redaction of the manuscript. H. G. contributed in the conception and design of the study, interpretation of data and in the redaction of the manuscript. M. P. contributed in the conception and design of the study and in the redaction of the manuscript. N. V. contributed in the conception and design of the study and in the redaction of the manuscript. N. C. contributed in the conception and design of the study, acquisition, analysis and interpretation of data and in the redaction of the manuscript. T. G. and N. C. take responsibility for the integrity of the work as a whole, from inception to the published article.

Conflict of interest

The authors have no potential conflicts (financial, professional or personal) that are relevant to the manuscript.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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Figure S1 Effect of different PUFA diets on weight gain. Body weight gain in male C57BL/6J mice given n-3, n-6 or control diet for 9 weeks. Values are mean \pm SEM, $n = 6-8$ per group.

Figure S2 Concentration of PUFA metabolites in intestine of mice exposed to sham, ischaemia or I/R and to the three different diets. PUFA concentration in sham ischaemia (white bar), sham I/R animals (striped bar) and following 50 min of

ischaemia (gray bar) or 50 min of ischaemia and 5 h of reperfusion (I/R, black bar). Data are expressed in $\text{pg}\cdot\text{mg}^{-1}$ protein and represented as mean \pm SEM of six to eight animals per group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, significantly different from corresponding sham.

Figure S3 Expression of LOX isoforms in mouse small intestine. Arachidonate 15 lipoxygenase (ALOX15; A), arachidonate 12 lipoxygenase (ALOX12; B) and arachidonate 5 lipoxygenase (ALOX5; C) mRNA expression were quantified in ischaemia and I/R mice versus corresponding sham, exposed to control (white bar), n-6 (black bar) or n-3 (gray bar) PUFA diet. Values are mean \pm SEM, $n = 6-8$ per group. ** $P < 0.01$, significantly different from control diet/ischaemia group.

Figure S4 Quantification of FPR2 and Ly-6B.2 expression in mouse small intestine. (A) Quantification of FPR2 fluorescence intensity in the different cell types of the small intestine of mice exposed to sham, ischaemia, sham I/R and I/R and to the control, n-3 or n-6 diet. (B) quantification of the area positive for Ly-6B.2 immunoreactivity in the small intestine of mice exposed to sham, ischaemia, sham I/R and I/R and to the control, n-3 or n-6 diet. Values are mean \pm SEM, $n = 5-6$ per group. * $P < 0.05$, ** $P < 0.01$, significantly different from n-6 diet and I/R group. (C) Pictures of immunohistochemistry control procedures: reaction performed with omission of primary (488 donkey anti-rabbit Ig + 555 donkey anti-goat IgG or 488 donkey anti-rabbit IgG + 555 donkey anti-rat IgG) or secondary antibodies (rabbit anti-FPR2 + rat anti-LY-6B.2 + goat anti-CD45 + goat anti-CK18).