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RESEARCH PAPER

Aspirin enhances protective effect of fish oil against thrombosis and injury-induced vascular remodelling

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BACKGROUND AND PURPOSE

Although aspirin (acetylsalicylic acid) is commonly used to prevent ischaemic events in patients with coronary artery disease, many patients fail to respond to aspirin treatment. Dietary fish oil (FO), containing ω3 polyunsaturated fatty acids (PUFAs), has anti-inflammatory and cardio-protective properties, such as lowering cholesterol and modulating platelet activity. The objective of the present study was to investigate the potential additional effects of aspirin and FO on platelet activity and vascular response to injury.

EXPERIMENTAL APPROACH

Femoral arterial remodelling was induced by wire injury in mice. Platelet aggregation, and photochemical- and ferric chloride-induced carotid artery thrombosis were employed to evaluate platelet function.

KEY RESULTS

FO treatment increased membrane ω3 PUFA incorporation, lowered plasma triglyceride and cholesterol levels, and reduced systolic BP in mice. FO or aspirin alone inhibited platelet aggregation; however, when combined, they exhibited synergistic suppression of platelet activity in mice, independent of COX-1 inhibition. FO alone, but not aspirin, attenuated arterial neointimal growth in response to injury. Strikingly, a combination of FO and aspirin synergistically inhibited injury-induced neointimal hyperplasia and reduced perivascular inflammatory reactions. Moreover, co-administration of FO and aspirin decreased the expression of pro-inflammatory cytokines and adhesion molecules in inflammatory cells. Consistently, a pro-resolution lipid mediator-Resolvin E1, was significantly elevated in plasma in FO/aspirin-treated mice.

CONCLUSIONS AND IMPLICATIONS

Co-administration of FO and low-dose aspirin may act synergistically to protect against thrombosis and injury-induced vascular remodelling in mice. Our results support further investigation of adjuvant FO supplementation for patients with stable coronary artery disease.

LINKED ARTICLES
This article is part of a themed section on Chinese Innovation in Cardiovascular Drug Discovery. To view the other articles in this section visit http://dx.doi.org/10.1111/bph.2015.172.issue-23

Abbreviations
AA, arachidonic acid; CO, coconut oil; CVD, cardiovascular disease; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FO, fish oil; HDLC, high-density lipoprotein cholesterol; LDLC, low-density lipoprotein cholesterol; PCI, percutaneous coronary intervention; PUFA, polyunsaturated fatty acid; TG, triglyceride

Tables of Links

TARGETS	
Catalytic receptors[a]	**Enzymes**[b]
CD11b (integrin, alpha M subunit)	COX-1
LFA-1 (integrin, beta 2 subunit)	
PDGFRβ	
VLA-4 (integrin α4β1)	

LIGANDS	
α-linolenic acid	IL-6
ADP	IL-10
Arachidonic acid (AA)	Linoleic acid
Aspirin	PDGF
Clopidogrel	Resolvin E1
Docosahexaenoic acid (DHA)	Rose bengal
Eicosapentaenoic acid (EPA)	TNF-α
ICAM-1	TxA_2
	VCAM-1

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

Low-dose aspirin (acetylsalicylic acid, ASA) is effective for the secondary prevention of heart attack and stroke, and for the primary prevention of nonfatal myocardial infarction (Marangoni and Poli, 2013; Gouya *et al.*, 2014). However, patient responses to this therapy are extremely variable (Floyd and Ferro, 2014). The anti-platelet effects of aspirin in patients can vary depending on the assay used, genetic background and dosage of aspirin (Mijajlovic *et al.*, 2013). Poor patient compliance is one of the main causes of aspirin resistance. Additionally, high platelet turnover in some inflammatory diseases, such as atherosclerosis and its complications, can lead to a reduced efficacy of aspirin because of a faster regeneration of platelets (Grove *et al.*, 2011). Thus, resistance to aspirin could be avoided by increasing the daily dosage of aspirin (Capodanno *et al.*, 2011; Dillinger *et al.*, 2012) or by administering other agents, such as fish oil (FO) supplements (Lev *et al.*, 2010).

FO has been studied extensively for its role in the attenuation of cardiovascular disease (CVD), induced by lowering total cholesterol (Astrup, 2014), by modulating platelet function (Marangoni and Poli, 2013) and by reducing vascular inflammation (Harris *et al.*, 2013). The cardiovascular benefits of FO are attributed to its ω3 polyunsaturated fatty acid (PUFA) content, particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Indeed, some of the protective effects of EPA and DHA are believed to occur through a reduction in pro-inflammatory prostaglandins (PGs) induced by its ability to inhibit arachidonic acid (AA) metabolic pathways (Calder, 2014). Low-dose aspirin directly inhibits AA metabolism to PGs, including the powerful platelet agonist TxA_2, through the acetylation of COX-1 on Ser^{529} (Yu *et al.*, 2005). Recently, compared with aspirin alone, a combination treatment with ω3 PUFAs and low-dose aspirin was shown to significantly reduce platelet function and potentiate the platelet response to clopidogrel (Gajos *et al.*, 2010; Lev *et al.*, 2010; Block *et al.*, 2012). However, the underlying mechanism for the synergistic effect of ω3 PUFAs and low-dose aspirin on platelet activity needs to be further investigated.

Arterial restenosis after angioplasty and stent deployment continues to be problematic in coronary intervention treatment, although application of drug-eluting stents has dramatically increased the success rate compared with that of bare metal stents (Inoue and Node, 2009). Aspirin and clopidogrel are commonly used to prevent thrombotic events after percutaneous coronary intervention (PCI). Surprisingly, aspirin does not reduce restenosis post-PCI (Schwartz *et al.*, 1988). Also, aspirin was found to have no effect on vascular neointima formation after denudation injury in rat carotid arteries (Yang *et al.*, 2004). In contrast, ω3 PUFAs were shown to attenuate balloon injury-induced vascular neointima hyperplasia (Faggin *et al.*, 2000), probably through their inhibition of vascular smooth muscle cell proliferation (Pakala and Benedict, 1999). Thus, we hypothesized that co-administration of FO and aspirin could protect against vascular remodelling in response to injury, as well as potently inhibiting platelet function.

Methods

Experimental animals

All animal care and experimental procedures were performed in accordance with the guidelines of the Animal Care and Use Committee of the Institute for Nutritional Sciences, Chinese Academy of Sciences. The total number of mice used in this study was 327. The COX-1^{neo} mice – a neomycin (Neo) resistance cassette inserted in COX-1 intron 10 to ensure hypomorphic expression of COX-1 gene (Yu *et al.*, 2005), and wild-type (WT) littermates were initially produced on a mixed C57BL/6/Sv129 genetic background (50%:50%), and maintained on this hybrid background for over 20 generations. Mice were housed in a temperature-controlled (22 ± 1°C) environment with a 12:12 h light–dark cycle. Mice were fed with a special diet containing 19% FO, 19% coconut oil (CO), with or without aspirin via drinking water (30 mg·L^{-1}). Special diets were provided by Teklad Diets (Harlan Laboratories, Madison, WI, USA) and aspirin was purchased from Sigma (A5376, Saint Louis, MO, USA). After 2 weeks of feeding, BP measurement and thrombotic experiments were conducted and blood, urine and peritoneal macrophages were collected for examination of cell membrane fatty acid and serum lipid, urinary PG metabolites and inflammatory gene expression respectively. For vascular injury experiments, mice were fed specific diets for 2 weeks before surgery and an additional 4 weeks after surgery until the injured arteries were harvested. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Fatty acid analysis

Blood was drawn from the inferior vena cava of 12- to 14-week old mice anaesthetized with chloral hydrate (5 μL g^{-1}, i.p.). The blood was then incubated in a 37°C water bath for 30 min, followed by centrifugation at 900 *g* for 15 min to remove serum. The blood cells collected were stored at –80°C. The fatty acids from blood cells were extracted as described previously (Zhang *et al.*, 2012). First, saturated fatty acid internal standard (21:0, 50 μg) was added to 0.4 mL blood. Next, hexane (6 mL) and isopropanol (4.5 mL) were added, and the sample was mixed. After centrifugation at 400× *g* for 5 min, the upper layer containing fatty acids was removed and dried with nitrogen. The fatty acids were then methylated with methylating reagent (methanol : sulphuric acid = 25:1, 4 mL) and extracted with hexane (2 mL). After being washed twice with water (1 mL), the fatty acids were dried with nitrogen, dissolved in isooctane (2 mL), and prepared for GC-MS analysis.

Serum lipid measurement

Peripheral blood was collected from the retro-orbital plexus of 10- to 12-week-old mice. After incubation in a 37°C water bath for 30 min, blood was centrifuged at 900× *g* for 15 min. Serum was separated and stored at –20°C for later use. Measurement of serum triglycerides (TG), total cholesterol, high-density lipoprotein cholesterol (HDLC) and low-density lipoprotein cholesterol (LDLC) levels was performed with commercial kits, following the manufacturer's protocol (BJKT, Beijing, China) (Yan *et al.*, 2014).

BP measurement

Resting systolic BP was measured in conscious mice (10 to 12 weeks old) using a computerized non-invasive tail cuff system (Visitech Systems, Inc., Apex, NC, USA) as described previously (Cheng *et al.*, 2006). Mice were adapted to the system for 14 days (one 25 min measurement session per day). BP was then recorded daily for 3 consecutive days in the same way. Data were collected and analysed using BP-2000 software (Visitech Systems, Inc., http://www.visitechsystems.com/).

Platelet aggregation

Blood was isolated from the inferior vena cava of anaesthetized 10- to 12-week-old mice using a heparin-containing syringe (15 U·mL^{-1} blood), and then diluted 1:1 with HEPES-Tyrode's buffer. Samples were centrifuged at 150× *g* for 10 min to remove red blood cells. Blood from individual mice in each group was used for aggregation experiments, with the final platelet count adjusted to 2×10^8 cells mL^{-1} with platelet-poor plasma from the same mouse. Aggregation was initiated by adding 2.5 μL of ADP with final concentration of 10 μM or 0.5 μL of collagen with final concentration of 2 μg·mL^{-1} to 250 μL of platelet-rich plasma (PRP). Platelet aggregation was measured using a lumi-aggregometer (Chrono-log Corp., Havertown, PA, USA) and was monitored for 8 min. The area under the aggregation curve was calculated by Image-Pro Plus software (Media Cybernetics, Silver Spring, MD, USA) for statistical analysis as previously described (Hanke *et al.*, 2010).

Models of thrombogenesis

The photochemical-induced carotid artery thrombosis model has been described previously (Yu *et al.*, 2005). Briefly, mice (12–14 weeks old) were anaesthetized with 6% chloral hydrate (5 μL·g^{-1}, i.p.); the depth of anaesthesia was assessed by evaluating rear foot reflexes. A midline cervical incision was made, and the right common carotid artery was isolated. A Doppler flow probe (Model 0.5 VB; Transonic Systems, Inc., Ithaca, NY, USA) connected to a flowmeter (Model T105; Transonic Systems, Inc.) was applied to the artery and measurements were obtained using a computerized data acquisition program (PowerLab; AD Instruments, Castle Hill, New South Wales, Australia). In the photochemical arterial thrombosis model, Rose Bengal (11 mg mL^{-1}; 60 mg kg^{-1}) was diluted in PBS and injected into the jugular vein. Before the injection, a 1.5 mW green light laser (540 nm) (Melles Griot, Carlsbad, CA, USA) was applied to the desired site of carotid artery injury from a distance of 10 cm, and blood flow was monitored for 150 min or until stable occlusion occurred. Those mice still showing blood flow after the allotted time were assigned a value of 150 min for the purpose of statistical analysis (Yu *et al.*, 2005). Stable occlusion was defined as a blood flow of 0 mL min^{-1} for 3 min. In the ferric chloride-induced model of thrombosis (Petrich *et al.*, 2007; Owens *et al.*, 2011), a 1.2 × 1.2 mm piece of filter paper soaked in 7% ferric chloride was applied to the right common carotid artery for 90s and then the vessel was washed thoroughly with saline. Blood flow was monitored for 30 min until stable occlusion occurred. Mice with an occlusion time longer than

30 min were assigned a value of 30 min for the purpose of statistical analyses. The decrease in blood flow rate (BFR) was determined as the initial BFR minus the BFR at 30 min after ferric chloride treatment.

Thromboxane metabolite (Tx-M) analysis

Tx-M was measured in urine collected over the course of 24 h in a metabolic cage and quantified utilizing LC/MS/MS analyses as described previously (Zhang *et al.*, 2013).

Femoral artery injury model

The wire-induced femoral artery injury model was produced as described previously (Zhang *et al.*, 2013). In brief, 10- to 12-week-old male WT mice were anaesthetized with isoflurane (2%, delivered in room air) using an induction chamber during the surgery. Bilateral femoral arteries were exposed and wire placement was carried out using a dissecting microscope (S4E, Leica, Wetzlar, Germany). The distal artery and vein were looped with a 6-0 silk suture and a guide wire (0.38 mm diameter; Cook, Inc., Bloomington, IN, USA) was inserted into the femoral artery. The wire was left in place for 3 min to denude the artery. After this time, the wire was removed and the silk suture was released to restore blood flow. The skin incision was closed with a 5-0 silk suture.

Histopathological analysis

Femoral arteries were harvested 4 weeks after the injury and fixed in 4% formalin overnight. The samples were processed routinely, embedded in paraffin and stained with haematoxylin and eosin and Ponceaou S-Picric acid-Victoria blue routinely. The intimal area was calculated as the area encircled by internal elastic lamina (IEL) minus the lumen area. The medial area was calculated as the area encircled by the external elastic lamina minus intima area. The intima-to-media (I/M) ratio was calculated as the intimal area divided by the medial area. The restenosis index (%) was calculated as intimal area divided by the area encircled by IEL (Zhang *et al.*, 2013). All specimens were analysed by an investigator blinded to the study design.

Immunofluorescence staining

For immunohistochemistry, the distribution of macrophages and neutrophils was analysed using an anti-CD68 antibody (Serotec, Oxford, UK) and an anti-CD11b monoclonal antibody (BD Pharmingen™, Franklin Lakes, NJ, USA), respectively, followed by a secondary antibody. After being washed three times with PBS, samples underwent diaminobenzidine staining. Sections were counterstained with haematoxylin. For immunofluorescence staining, tissue sections were incubated with primary antibodies against CD68, CD11b and CD3 (Alexa Fluor 488 anti-mouse, Biolegend, San Diego, CA, USA). After incubation with Alexa Fluor-conjugated secondary antibodies (1:1000; Invitrogen, Carlsbad, CA, USA), DAPI staining was performed. The samples were mounted on glass slides (ProLong® Gold Antifade Reagent; Invitrogen) and visualized using an inverted fluorescent microscope (Carl Zeiss, Oberkochen, Germany). Subsequent image processing was performed using Photoshop CS2 (Adobe Systems, San Jose, CA, USA). Primary antibodies were used at the following dilutions: CD68, 1:200; CD11b, 1:50; and CD3, 1:200 to identify the inflammatory cells. Six vessel zones (at ×200 view) were selected randomly on four coordinate axes of each stained femoral arterial cross section and three randomly selected sections per mouse were calculated.

RNA extraction and real-time PCR

Total RNA from circulating white blood cells and vascular tissues was extracted using Trizol reagent (Invitrogen, San Diego, CA, USA), according to the manufacturer's instructions. Briefly, total RNA was reverse-transcribed to cDNA by use of a reverse transcription reagent kit (TaKaRa Biotechnology, Co., Ltd. Dalian, China), according to the manufacturer's protocol. Real-time PCR was performed using SYBR Green mix (TaKaRa Biotechnology, Co., Ltd.). Each sample was analysed in triplicate and was normalized to the level of β-actin mRNA. The PCR protocol was as follows: 5 min at 95°C for one cycle followed by 40 cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 30 s, with a final extension at 72°C for 5 min. A dissociation curve was obtained for each PCR product. Relative gene expression was determined using the ΔΔCT method (Livak and Schmittgen, 2001). All the original data were divided by the mean of WT/CO group. The primer sequences are summarized in Table 1.

Resolvin E1 extraction and detection

Resolvin E1 (5*S*,12*R*,18*R*-trihydroxy-6*Z*,8*E*,10*E*,14*Z*,16*E*-EPA) and d_5-Resolvin E1 was prepared by total organic synthesis (Ogawa and Kobayashi, 2009). The structure was confirmed by mass spectral analysis and was consistent with reported properties. The detection of Resolvin E1 generated from dietary FO and aspirin in plasma was performed using a standard LC-MS/MS protocol with minor modifications (Serhan *et al.*, 2000). Briefly, mouse plasma samples were collected after 2 weeks of FO and aspirin administration. Plasma samples were exacted by ethyl acetate with d_5-Resolvin E1 as internal standard. After centrifugation, the supernatants were analysed by TSQ Vantage LC-MS/MS (Thermo Scientific, Waltham, MA, USA) using a UPLC column (Acquity UPLC CSH C8, 100 × 2.1 mm, i.d. 1.7 μm). Mobile phase A was (H_2O : CH_3CN : FA = 63:37:0.1, v $v^{-1}v^{-1}$) and mobile phase B was (MeOH, with the addition of 2 mM ammonium acetate).

Statistical analysis

Results are expressed as mean ± SEM. The number of samples in each group is shown in the figure legends. Statistical significance was determined by use of Student's unpaired *t*-test or one-way ANOVA followed by *post hoc* test. A value of $P < 0.05$ was considered statistically significant.

Results

Dietary FO, but not aspirin, increases the proportion of ω3 PUFAs in erythrocyte membranes and reduces blood TGs in mice

To determine whether FO exerts additional cardiovascular benefits when combined with aspirin, both FO and CO diets (ω6 PUFA-enriched diet as control, Table 2) were fed to WT, WT treated with low-dose aspirin (WT/aspirin), and COX-1^{neo} mice (Yu *et al.*, 2005). As anticipated, FO treatment significantly reduced the proportion of ω6 PUFAs, including linoleic

Table 1

Primers used in this study

Gene	Sense	Anti-sense
IL-6	ACTCACCTCTTCAGAACGAATTG	CCATCTTTGGAAGGTTCAGGTTG
TNF-α	CCTCTCTCTAATCAGCCCTCTG	GAGGACCTGGGAGTAGATGAG
Clec10a	GCTCCGCATACACCTGGATG	GCCGGTCGCATAGTCTGTTC
IL-10	GACTTTAAGGGTTACCTGGGTTG	TCACATGCGCCTTGATGTCTG
MMR	CTACAAGGGATCGGGTTTATGGA	TTGGCATTGCCTAGTAGCGTA
Ym-1	CAGGTCTGGCAATTCTTCTGAA	GTCTTGCTCATGTGTGTAAGTGA
LFA-1	AAGTGACGCTTTACCTGCGAC	AAGCATGGAGTAGGAGAGGTC
VLA-4	CACAACACGCTGTTCGGCTA	CGATCCTGCATCTGTAAATCGC
PSGL-1	CCTGAGTCTACCACTGTGGAG	GCTGCTGAATCCGTGGACA
ICAM-1	AACCGCCAGAGAAAGATCAG	TGTGACAGCCAGAGGAAGTG
VCAM-1	AGTTGGGGATTCGGTTGTTCT	CCCCTCATTCCTTACCACCC
P-selectin	CATCTGGTTCAGTGCTTTGATCT	TGTGCTGTAGTTATAGGTCCACG
β-actin	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT

Table 2

Contents of experimental diets

Ingredient	FO diet ($g \cdot kg^{-1}$)	CO diet ($g \cdot kg^{-1}$)
Casein	240	240
L-cystine	3.6	3.6
Corn starch	189.208	189.208
Sucrose	150	150
Maltodextrin	100	100
Fish oil	190	–
Coconut oil	–	190
Soybean oil	20	20
Cellulose	50	50
Mineral mix, AIN-93G-MX (94046)	42	42
Calcium phosphate, dibasic	3	3
Vitamin mix, teklad (40060)	12	12
TBHQ (antioxidant)	0.042	0.042
Yellow food colour	0.15	–
Blue food colour	–	0.15
Protein (%)	18.9	18.9
Carbohydrate (%)	38.4	38.4
Fat (%)	42.6	42.6

acid (LA) and AA, while increasing levels of ω3 PUFAs, including EPA, docosapentaenoic acid (DPA) and DHA in erythrocyte membranes of all three groups, as compared with CO feeding (Table 3). Among the ω3 PUFAs in the cell membranes, EPA levels in FO-treated mice were increased approximately 9- to 11-fold, as compared with CO-treated mice. Interestingly, the ratio of AA to total EPA, DHA and DPA was completely reversed by FO treatment (Table 3). Importantly, aspirin pretreatment and genetic knockdown of COX-1 alone had no detectable effects on cell membrane fatty acid composition. Thus, FO supplementation could lead to marked increase in membrane incorporation of ω3 PUFAs, particularly EPA.

We next examined the effects of FO intake on lipid metabolism and BP in mice. As shown in Figure 1, the FO diet significantly reduced plasma levels of TG (Figure 1A) and cholesterol (Figure 1B), including HDLC (Figure 1C) and LDLC (Figure 1D), in WT, aspirin-treated and COX-1^{neo} mice, as compared with the CO diet. Moreover, the mice fed with FO diet displayed lower systolic BP than the CO-fed mice (Figure 2). In contrast, inhibition or genetic knockdown of COX-1 had no overt effects on lipid metabolism and BP (Figures 1 and 2).

Aspirin augments the inhibitory effect of FO on platelet activity. To examine the effects of combining FO and low-dose aspirin on platelet function, PRP from treated mice was prepared, and platelet aggregation was examined in response to ADP and collagen. Platelet aggregation could be induced by either ADP or collagen in a dose-dependent manner (data not shown). FO-fed mice displayed reduced platelet aggregation in response to the two agonists, as compared with CO-fed mice (Figure 3A). Similarly, ADP- or collagen-stimulated platelet activation was markedly diminished upon treatment with aspirin (Figure 3B). Platelet aggregation was further suppressed in mice treated with FO and aspirin, as compared with FO or aspirin treatment alone (Figure 3C and D). Therefore, the combination of FO and aspirin enhanced the inhibition of platelet function.

Aspirin inhibits platelet activation through the acetylation and deactivation of platelet COX-1, and the effect of low-dose aspirin is mimicked in COX-1^{neo} mice (Yu *et al.*, 2005). We therefore tested whether the synergistic effect of FO and aspirin is dependent on COX-1 activity. As shown in

Table 3

Effect of FO on fatty acid composition of blood cell membranes

	WT (%)		WT + aspirin (%)		COX-1^{neo} (%)	
PUFAs	**CO**	**FO**	**CO**	**FO**	**CO**	**FO**
ω-6						
18:2 ω-6 (LA)	15.15 ± 0.84	6.49 ± 0.60***	14.49 ± 0.49	8.85 ± 0.64***	14.09 ± 0.56	6.63 ± 0.45***
20:2 ω-6 (EDA)	0.31 ± 0.06	0.69 ± 0.34	0.45 ± 0.20	0.15 ± 0.01	0.59 ± 0.32	0.58 ± 0.35
20:3 ω-6 (DGLA)	2.15 ± 0.27	1.96 ± 0.44	2.16 ± 0.23	1.62 ± 0.38	1.77 ± 0.16	1.23 ± 0.24
20:4 ω-6 (AA)	13.00 ± 0.58	6.80 ± 0.21***	12.97 ± 0.54	6.66 ± 0.66***	13.38 ± 0.89	7.47 ± 0.61***
22:4 ω-6 (DTA)	1.16 ± 0.12	0.71 ± 0.11	1.25 ± 0.06	0.89 ± 0.12	1.42 ± 0.09	0.80 ± 0.13***
22:5 ω-6 (DPA)	0.62 ± 0.06	0.61 ± 0.06	0.63 ± 0.07	0.56 ± 0.06	0.61 ± 0.07	0.56 ± 0.06
Total	32.39 ± 0.62	17.26 ± 0.66***	31.95 ± 0.71	18.73 ± 0.87***	31.87 ± 1.03	17.27 ± 0.98***
ω-3						
18:3 ω-3 (ALA)	0.24 ± 0.02	0.28 ± 0.03	0.31 ± 0.06	0.39 ± 0.11	0.30 ± 0.06	0.22 ± 0.02
20:5 ω-3 (EPA)	0.76 ± 0.08	8.70 ± 0.37***	0.89 ± 0.15	7.44 ± 0.42***	0.76 ± 0.06	7.90 ± 0.29***
22:5 ω-3 (DPA)	0.98 ± 0.15	1.95 ± 0.16***	1.08 ± 0.08	1.83 ± 0.07***	1.02 ± 0.10	2.07 ± 0.09***
22:6 ω-3 (DHA)	7.82 ± 0.60	13.82 ± 0.91***	7.86 ± 0.27	11.98 ± 0.66***	7.53 ± 0.64	13.59 ± 0.82***
Total	9.80 ± 0.79	24.75 ± 1.32***	10.15 ± 0.24	21.65 ± 0.99***	9.61 ± 0.72	23.79 ± 1.10***
Total PUFAs	42.19 ± 0.67	42.01 ± 0.92	42.10 ± 0.85	40.37 ± 1.48	41.48 ± 1.62	41.06 ± 1.42
SFA	42.17 ± 0.53	45.17 ± 1.59	42.89 ± 1.09	44.74 ± 1.02	42.96 ± 0.88	45.39 ± 1.38
MFA	15.59 ± 0.45	12.76 ± 0.78	14.94 ± 0.49	13.86 ± 0.51	15.44 ± 0.04	13.45 ± 0.38
ω-6/ω-3 (of total fractions)	3.40 ± 0.30	0.71 ± 0.06***	3.15 ± 0.08	0.87 ± 0.05***	3.39 ± 0.12	0.73 ± 0.06***
AA/EPA + DHA + DPA	1.38 ± 0.07	0.28 ± 0.01***	1.32 ± 0.05	0.31 ± 0.02***	1.45 ± 0.07	0.32 ± 0.02***

ALA, α-linolenic acid; DGLA, dihomo-γ-linolenic acid; DTA, docosatetraenoic acid; EDA, eicosadienoic acid; LA, linoleic acid; MFA(16:1 + 18:1 + 20:1 + 24:1), monounsaturated fatty acids; SFA(14:0 + 16:0 + 18:0 + 20:0 + 22:0 + 24:0), saturated fatty acids.
Data are given as mean ± SEM (percentage of total fatty acids).
**P < 0.001, ***P < 0.0001 versus CO group; n = 5–6.

Figure 3E and F, the aggregation of platelets from FO and aspirin-treated WT mice was much weaker than that of FO-fed COX-1^{neo} mice (genetic COX-1 knockdown), indicating that the additional effect of FO and aspirin on platelet function is not dependent on COX-1 inhibition.

Enhanced protective effect of FO and aspirin against thrombosis in vivo. As platelets from mice treated with both FO and aspirin exhibited reduced aggregation *in vitro*, we next investigated their response to thrombotic stimuli (Rose Bengal or ferric chloride) *in vivo*. The formation of an occlusive thrombus was monitored by measuring blood flow in the exposed carotid artery with a Doppler probe. In the photochemical-induced carotid artery thrombosis model, as expected, both FO and aspirin treatment prolonged the time to complete occlusion (Figure 4A) in mice. Strikingly, the combination of FO and aspirin significantly delayed photochemical-induced thrombus formation, as compared with FO or aspirin treatment alone (FO + aspirin, 114.7 ± 12.8 min; FO alone, 47.50 ± 7.21 min or aspirin alone, 40.67 ± 4.85 min; P < 0.05; Figure 4A). FO-mediated inhibition of photochemical-induced thrombosis was also detected in COX-1^{neo} mice (Figure 4A). However, the FO-fed COX-1^{neo} mice exhibited a significantly faster occlusion time than WT mice co-treated with FO and aspirin (Figure 4A), suggesting that the enhanced inhibitory effect of FO and aspirin on thrombogenesis is not dependent on COX-1 activity. In the ferric chloride-induced carotid arterial thrombosis mouse model, both FO intake and COX-1 inhibition (aspirin and COX-1^{neo}) prolonged occlusion time (Figure 4B), and co-administration of FO and aspirin significantly improved BFR decrease induced by ferric chloride as compared with each alone (Figure 4C)

We also tested the combined effect of FO and aspirin on TxA_2 synthesis. As previously described, low-dose aspirin and COX-1 knockdown markedly diminished urinary TxA_2 metabolite content (~90% suppression) (Yu *et al.*, 2005). Combined treatment with FO and aspirin resulted in a similar suppression of Tx metabolites as aspirin alone, indicating that the additional effect of FO and aspirin on platelet function did not rely solely on TxA_2 production (Figure 5).

The combination of FO and aspirin confers additional protection against vascular neointima formation in response to injury. Given the beneficial effect of the combined treatment with FO and aspirin on platelet function, we examined the effect of FO and aspirin on mechanical injury-induced arterial restenosis in mice. De-endothelialization of femoral arteries was induced by insertion of a wire into the lumen. Arterial neointima hyperplasia was examined at 28 days post-injury. As shown in Figure 6, wire injury triggered severe arterial neointima

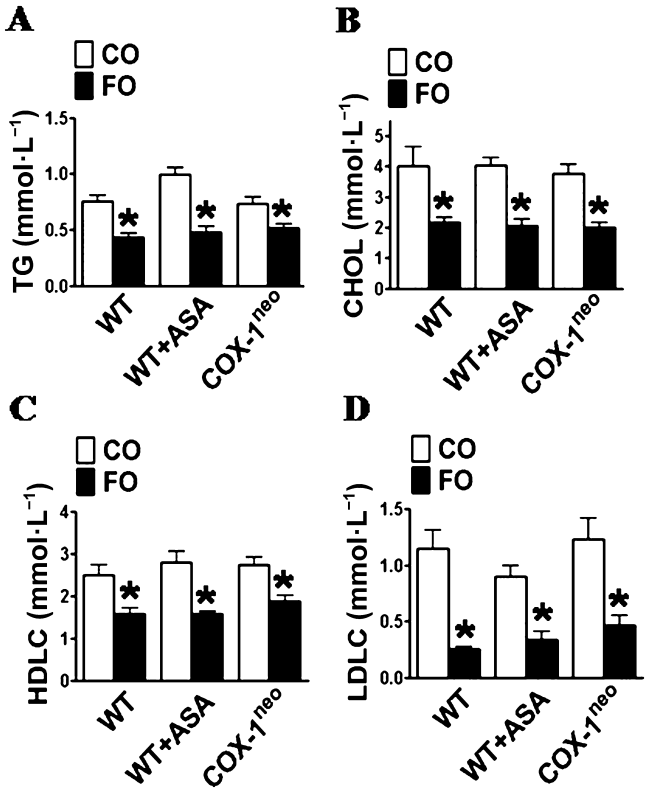

Figure 1

Effect of dietary FO and aspirin (ASA) treatment on serum lipids in mice. WT and COX-1^{neo} mice were fed 19% FO or CO diet for 2 weeks, and aspirin (30 mg·L^{-1}) was administered in drinking water, as indicated. Serum levels of TG (A), total cholesterol (B), HDLC (C) and LDLC (D)were measured. *P < 0.05 versus CO, n = 10–15.

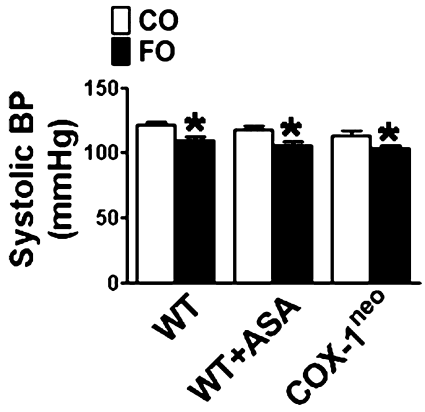

Figure 2

Effect of FO plus aspirin (ASA) on BP. Systolic BP was recorded by tail-cuff measurement. *P < 0.05 versus CO, n = 9–13.

formation and vessel narrowing. Dietary FO intake resulted in a significant reduction in the I/M ratio and in the luminal narrowing, as compared with WT mice fed a CO diet, aspirin-treated WT mice and COX-1^{neo} mice. In contrast, aspirin treatment and COX-1 knockdown had no obvious effect on injury-induced vascular remodelling of the neointima and luminal narrowing (Figure 6C and D), indicating that constitutively expressed COX-1 is not involved in the pathogenesis of mechanical injury-induced vascular remodelling. Interestingly, co-administration of FO and aspirin attenuated vascular neointima formation more than FO intake alone, significantly reducing the I/M ratio (P < 0.05; Figure 6C) and luminal narrowing (P < 0.05; Figure 6D). In addition, the reductions observed in mice treated with both FO and aspirin were significantly greater than those of FO-treated COX-1^{neo} mice (Figure 6C and D), suggesting that the synergistic protective effect of FO and aspirin against injury-induced vascular remodelling is not dependent on COX-1 activity.

Dietary FO and aspirin intake suppress inflammatory reaction in response to injury. FO supplementation has been shown to reduce local inflammatory reactions and enhance resolution (Tomasdottir *et al.*, 2013); and low-dose aspirin exhibits anti-inflammatory properties to a lesser extent (Morris *et al.*, 2009). We further explored the perivascular inflammatory response to wire injury at day 28. As expected, ingestion of FO markedly suppressed both macrophage (CD68$^+$, Figure 7A and B) and neutrophil (CD11b$^+$, Figure 7C and D) infiltration in WT, WT/aspirin and COX-1^{neo} mice. Low-dose aspirin intake tended to decrease inflammatory cell infiltration; however, these reductions did not reach significance (Figure 7B and D). In line with their effects on arterial neointima formation, the combined treatment with FO and aspirin led to a further, significant reduction in the perivascular infiltration of inflammatory cells-CD68$^+$ and CD11b$^+$ cells (Figure 7B and D). In addition, we detected less infiltration of inflammatory cells in WT mice treated with FO and aspirin than in FO-fed COX-1^{neo} mice (Figure 7B and D). At the early stages of acute inflammation (3 days after injury), we observed a similar suppressive pattern of inflammatory cell infiltration, including CD68$^+$ macrophages, CD11b$^+$ neutrophil and CD3$^+$ T lymphocytes, in mice fed FO alone and those treated with FO and aspirin (Figure 8A–F).

Perivascular infiltration by inflammatory cells such as macrophages may contribute to neointimal thickening through the generation of harmful reactive oxygen intermediates and secretion of growth and chemotatic factors (Inoue and Node, 2009). Consistent with previous reports (Oh *et al.*, 2010), FO intake decreased M1 pro-inflammatory gene expression, including IL-6, TNF-α and Clec10a (Figure 9A) in macrophages, and increased M2 anti-inflammatory gene expression, such as IL-10, macrophage mannose receptor (MMR), and Ym-1 (Figure 9B). Aspirin treatment also reduced the expression of some pro-inflammatory markers, such as TNF-α and Clec10a (Figure 9A), but had no effects on M2 anti-inflammatory genes (Figure 9B). Indeed, co-treatment of FO and aspirin further inhibited M1 pro-inflammatory gene expression and elevated M2 anti-inflammatory gene expression, indicating the combination of FO and aspirin had a synergistic inhibitory effect on inflammatory reactions, induced partially by influencing macrophage polarization.

Effect of dietary FO and aspirin intake on expression of endothelium markers and surface adhesion molecules on leukocytes. Leukocyte infiltration into inflamed areas, such as injured vessels, requires a precise sequence of events that involves the

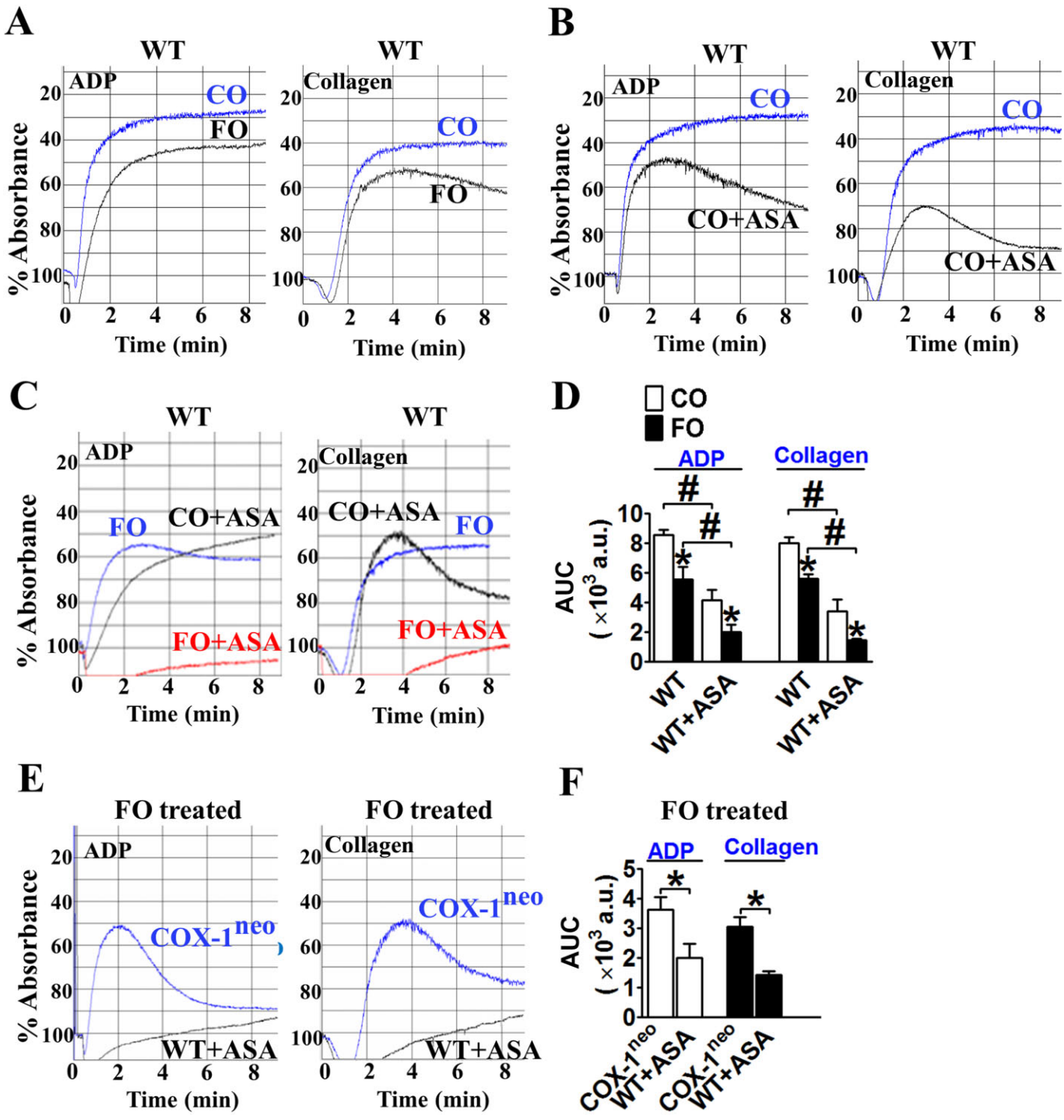

Figure 3

Effect of dietary FO and aspirin (ASA) on inhibition of platelet aggregation. (A) Representative tracings of FO-treated mouse platelet aggregation induced by ADP and collagen; (B) representative tracings of aspirin-treated mouse platelet aggregation induced by ADP and collagen; (C) representative tracings of FO- and aspirin co-treated mouse platelet aggregation induced by ADP and collagen; (D) quantification of the area under platelet aggregation curve for (A–C). *$P < 0.05$ versus CO, #$P < 0.05$ as indicated, $n = 5–7$. a.u. stands for arbitrary unit. (E) Effect of genetic knock-down (COX-1^{neo}) and inhibition (aspirin) of COX-1 on platelet aggregation from FO-treated mice. (F) Quantification of the area under aggregation curve for €. *$P < 0.05$ as indicated, $n = 5–7$.

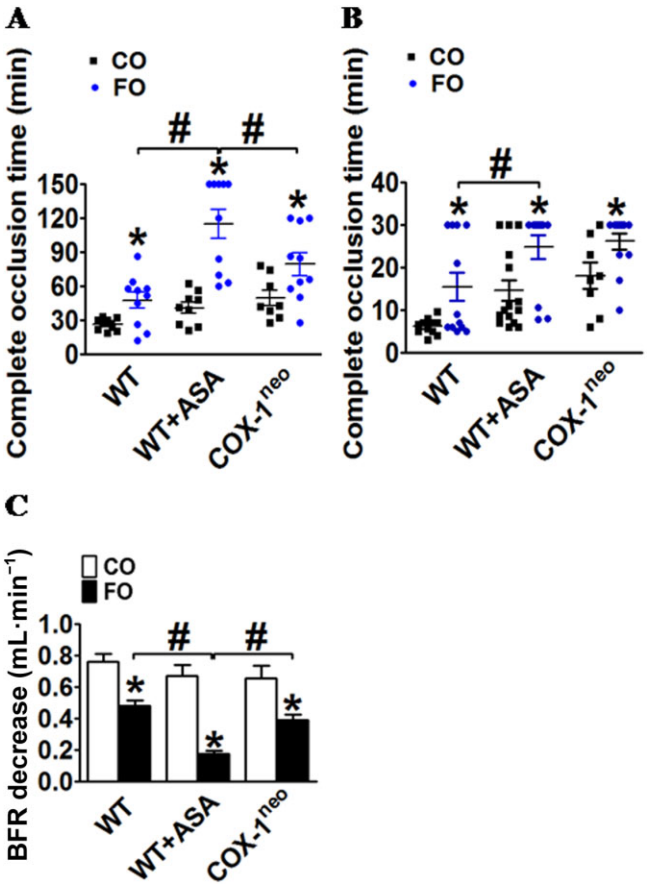

Figure 4

Protective effect of dietary FO plus aspirin against thrombus formation *in vivo*. (A) Effect of FO and aspirin on complete occlusion time in a photochemical-induced thrombosis mouse model. *$P < 0.05$ versus CO, #$P < 0.05$ as indicated, $n = 8–11$; effect of FO and aspirin on complete occlusion time (B) and the decrease in blood flow rate (BFR) in a 30 min period (C) in the ferric chloride-induced thrombosis mouse model. *$P < 0.05$ versus CO, #$P < 0.05$ as indicated, $n = 8–15$.

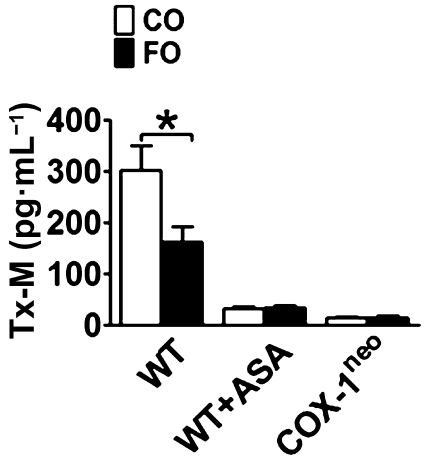

Figure 5

Effect of dietary FO and aspirin (ASA) on TxA_2 metabolite (Tx-M). Urinary Tx-M was measured in a 24 h urine specimen collected in a metabolic cage. *$P < 0.05$, $n = 10–15$.

interaction of leukocytes with activated endothelial cells through modulated expression of surface adhesion molecules (Shah, 2003). The first and most important step for leukocyte recruitment is contact with endothelial cells, rolling and temporal arrest of leukocytes (Butcher, 1991). We examined the expression of surface adhesion molecules on leukocytes, such as lymphocyte function-associated antigen 1 (LFA-1), very late antigen-4 (VLA-4) and P-selectin glycoprotein ligand-1 (PSGL-1). An FO-supplemented diet reduced the expression of LFA-1, VLA-4 and PSGL-1 on leukocytes as compared with a CO-supplemented diet. In contrast, low-dose aspirin had no effect on the expression of these molecules (Figure 10A). Interestingly, we observed that the combination of FO and aspirin significantly diminished the expression of LFA-1, VLA-4 and PSGL-1 on leukocytes, as compared with FO alone.

We next assessed the expression of the corresponding ligands in blood vessels. We observed a similar synergistic inhibition of the vascular expression of LFA-1 ligand-intercellular adhesion molecule (ICAM) and PSGL-1 ligand-P-selectin in mice treated with FO and aspirin (Figure 10B).

Resolvin E1 generation in vivo

Resolvins are a group of anti-inflammatory lipid mediators derived from ω3 PUFAs such as EPA and DHA, and can be generated in the presence of aspirin (Serhan *et al.*, 2008). Hence, we determined plasma resolvin E1 levels in FO- or CO-treated mice by LC-MS/MS (Figure 11A). The formation of resolvin E1 was confirmed by the MS/MS spectrum consistent with synthetic resolvin E1 (data not shown). Indeed, FO or aspirin alone promoted the production of resolvin E1 in mice (Figure 11B). Strikingly, co-treatment with FO and aspirin significantly enhanced resolvin E1 biosynthesis in plasma ($P < 0.05$; Figure 11B).

Discussion and conclusions

In this study, we have shown that dietary FO has broad cardio-protective effects, such as reducing TG and lowering BP. When combined with low-dose aspirin, FO ingestion exerted additional protection against platelet aggregation and thrombosis in mouse models; this effect was not dependent on COX-1 inhibition. Furthermore, we demonstrated that co-treatment with FO and aspirin significantly reduced arterial neointima formation in response to injury, as compared with either FO or aspirin alone, through the suppression of vascular inflammatory reactions and the induction of M2 macrophage polarization. These findings indicate that FO supplementation could be a favourable adjuvant therapy for patients with stable coronary artery disease (CAD).

ω3 PUFAs, either in FO supplements or as prescription ethyl esters, are an accepted therapy for reducing TG levels (Kris-Etherton *et al.*, 2002). These substances, which include EPA and DHA, are believed to reduce TG levels primarily by promoting fatty-acid degradation via peroxisomal

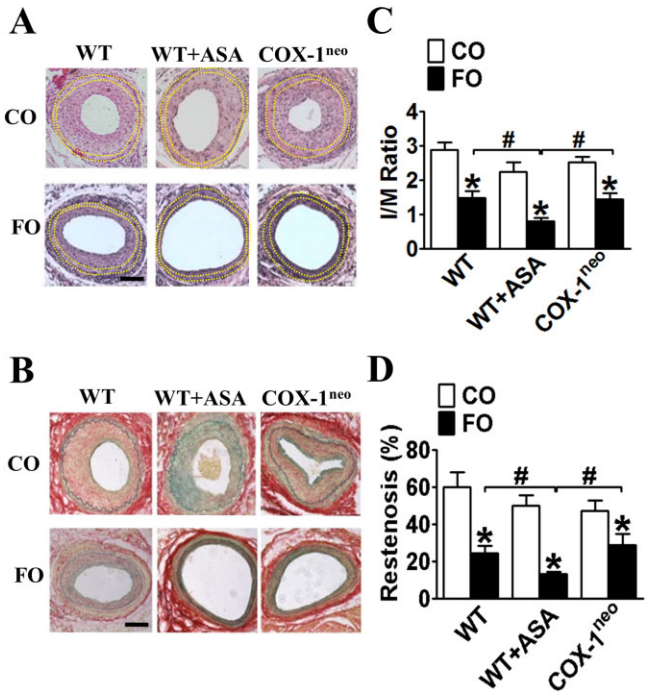

Figure 6

Effect of dietary FO plus aspirin (ASA) on vascular remodelling in response to mechanical injury. Representative haematoxylin and eosin (A) and Ponceaou S-Picric acid-Victoria blue (B) staining of cross sections of wire-injured arteries from WT, aspirin-treated andCOX-1^{neo} mice fed with a FO or CO diet. Scale bar, 50 μm; yellow dashed lines indicate both IEL and eternal elastic lamina. Quantification of I/M ratio (C) and restenosis (D) in arteries post-injury from WT, aspirin-treated and COX-1^{neo} mice fed a FO or CO diet. *P < 0.05 versus CO; #P < 0.05 as indicated, n = 7–11.

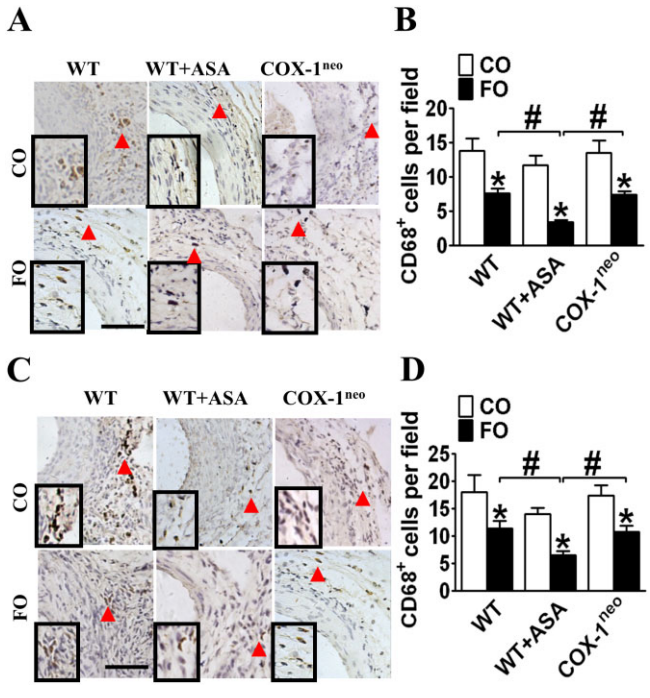

Figure 7

Synergistic effect of dietary FO and aspirin (ASA) on perivascular inflammatory cell infiltration following injury. (A) Representative immunostaining of CD68 in injured artery cross sections from WT, aspirin-treated and COX-1^{neo} mice fed a FO or CO diet. Scale bar, 50 μm; (B) Quantification of CD68^{+} cell for (A). *P < 0.05 versus CO control; #P < 0.05 as indicated, n = 6–12; (C) Representative immunostaining of CD11b in injured artery cross sections from WT, aspirin-treated and COX-1^{neo} mice fed a FO or CO diet. Scale bar, 50 μm; (D) Quantification of CD11b^{+} cells for (C). *P < 0.05 versus CO; #P < 0.05 as indicated, n = 7–11.

β-oxidation, inhibiting hepatic lipogenesis and accelerating the clearance of TG from the plasma (Harris *et al.*, 2008). Both EPA and DHA have similar TG-lowering properties (Grimsgaard *et al.*, 1997). Compared with the well-established TG-lowering effect of ω3 PUFAs, the effects of FO on HDLC and LDLC appear to be weaker (Harris, 1997). Consistently, our data showed dietary FO intake reduced blood levels of TG, cholesterol, HDLC and LDLC in all three groups tested. However, we did not observe any effect of aspirin on lipid profiles in mice. Despite the fact that high levels of LDLC increase the risk for CVD, the Japan EPA Lipid Intervention Study involving 18 645 patients treated with statins showed that the beneficial effects of EPA on CVD were not associated with reductions in LDLC levels (Yokoyama *et al.*, 2007; Sasaki *et al.*, 2012), indicating that other factors may contribute to the cardio-protective effect of EPA, including a lowering of BP and inhibitory effect on platelet activity. Indeed, we observed a mild reduction in BP and suppression of platelet aggregation following FO ingestion, consistent with previous reports (Larson *et al.*, 2013; Miller *et al.*, 2014).

The feeding of FO led to the incorporation of ω3 PUFAs (both EPA and DHA) into cell membranes in mice. The incorporated EPA and DHA can inhibit two-series prostanoid production, including Tx, by competing with AA at the COX enzyme active site. As anticipated, we detected a marked reduction in Tx production in FO-fed mice. This was similar to mice treated with aspirin, which acetylates COX and blocks the metabolism of AA to Tx in platelets. Furthermore, we also observed a suppression of platelet aggregation in response to either ADP or collagen challenge and impaired photochemical- and ferric chloride-induced arterial thrombosis in FO-fed mice. Thus, the combination of aspirin and FO may work in parallel to augment the inhibition of platelet aggregation and thrombosis, partially through shifting membrane phospholipid metabolism towards three-series prostanoid production (Smith, 2005). However, the synergistic effect of aspirin and FO on platelet function is independent of COX-1 inhibition and TxA_2 production. One possible explanation for this is the trans-endothelial generation of EPA-derived resolvins, through the interaction of EPA with aspirin, which has previously been shown to block both Tx-and ADP-induced platelet activation (Dona *et al.*, 2008; Fredman *et al.*, 2010). Another possible explanation is that the combination of FO and aspirin affects the conversion of lysophosphatidic acid to lysophosphatidylcholine, which is strongly correlated with impaired platelet activation induced by AA (Abdolahi *et al.*, 2014). In healthy humans, co-administration of EPA, DHA and low-dose aspirin was

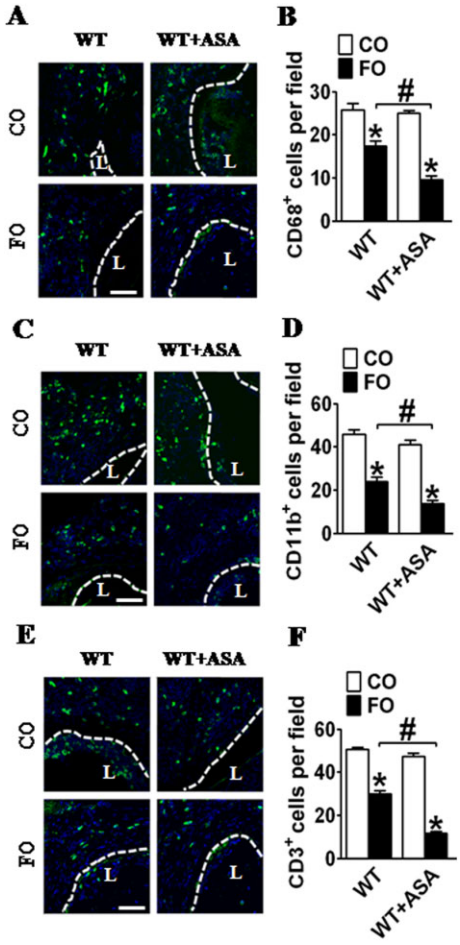

Figure 8

Effect of dietary FO and aspirin (ASA) on early recruitment of inflammatory cells in injured femoral arteries. Representative immunofluorescent staining of CD68(A), CD11b (C) and CD3 (E) in injured femoral artery sections from aspirin alone, FO alone, or FO- and aspirin-treated mice. L, lumen of femoral artery; (B, D, F) Quantification of $CD68^+$, $CD11b^+$ and $CD3^+$ cells in injured femoral artery sections from aspirin alone, FO alone, or FO- and aspirin-treated mice. $*P < 0.05$ versus CO; $\#P < 0.05$ as indicated, $n = 6–7$.

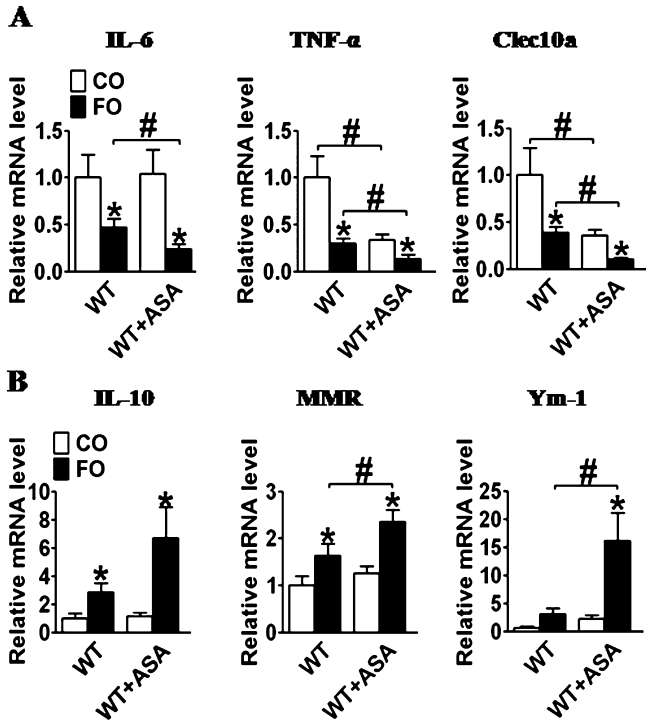

Figure 9

Effect of dietary FO and aspirin (ASA) on mRNA expression of anti- and pro-inflammatory genes in macrophages. (A) IL-6, TNF-α and Clec10a gene expression in macrophages from aspirin alone, FO alone, or FO and aspirin-treated mice; (B) IL-10, MMR and Ym-1 gene expression in macrophages from aspirin alone, FO alone, or FO and aspirin-treated mice. $*P < 0.05$ versus CO; $\#P < 0.05$ as indicated, $n = 6–12$.

recently reported to reduce platelet function when combined, although the individual components did not (Block *et al.*, 2012). A similar synergistic effect of FO and aspirin was also observed in patients with diabetes mellitus (Block *et al.*, 2012; Abdolahi *et al.*, 2014).

Importantly, aspirin resistance or a poor response to aspirin is more common in diabetic patients than in healthy individuals (Pignone *et al.*, 2010). Therefore, the combination of FO and aspirin could be more effective than aspirin alone in reducing platelet aggregation in subjects with cardiovascular risks, particularly those with diabetes mellitus. Despite effectively reducing myocardial infarction and other thrombotic events after PCI, aspirin treatment has failed to inhibit the progression of arterial restenosis (Steinhubl and Berger, 2009). Similar to balloon-induced carotid artery injury (Yang *et al.*, 2004), aspirin had no effect on vascular neointima growth in our femoral artery injury model. In contrast, co-treatment with FO and aspirin markedly suppressed neointima formation as compared with either treatment alone; this effect was independent of COX-1 inhibition, as we did not observe the synergistic effect in COX-1 knockdown mice. Again, these results suggest that aspirin and FO interact biologically, for example, in producing the mediators E- and D-series resolvins, which are generated from EPA and DHA in the presence of aspirin (Spite and Serhan, 2010). Resolvin E1 was detected in the plasma of human subjects, and its levels were negatively correlated with symptomatic peripheral artery disease (Ho *et al.*, 2010). *In vitro*, resolvin E1 blocked PDGF-stimulated migration of human VSMCs by decreasing the phosphorylation of the PDGFRβ (Ho *et al.*, 2010). Indeed, we detected the generation of resolvin E1 in plasma of FO- and aspirin-treated mice (Figure 11). However, the exact roles of EPA-derived resolvin E1 in injury-induced vascular remodelling remain to be determined. Restenosis after stent deployment is considered to be a wound-healing response to mechanical injury. The inflammatory response plays an essential role in transducing signals from the site of vascular injury to ultimately promote neointimal growth (Welt and Rogers, 2002). It has been long known that FO has beneficial anti-inflammatory properties (Calder and Grimble, 2002), which may contribute to its protective effects against vascular remodelling in response to injury (Faggin *et al.*, 2000; de Roos

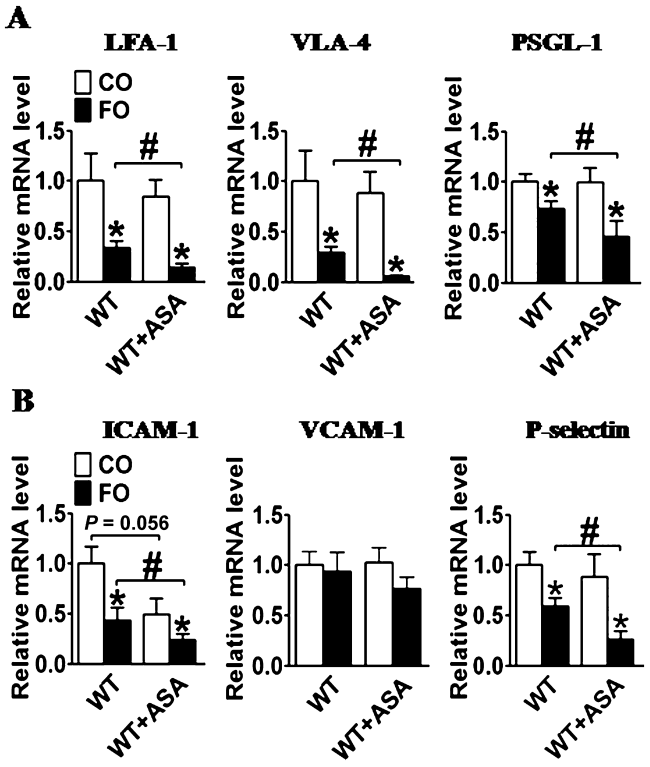

Figure 10

Effect of dietary FO and aspirin (ASA) on the expression of adhesion molecules on leukocytes and endothelium. (A) LFA-1, VLA-4, PSGL-1 gene expression on leukocytes from aspirin alone, FO alone, or FO and aspirin-treated mice;(B) ICAM-1, VCAM-1, and P-selectin gene expression in aortas from aspirin alone, FO alone, or FO and aspirin-treated mice.*$P < 0.05$ versus CO; #$P < 0.05$ as indicated, $n = 5–8$.

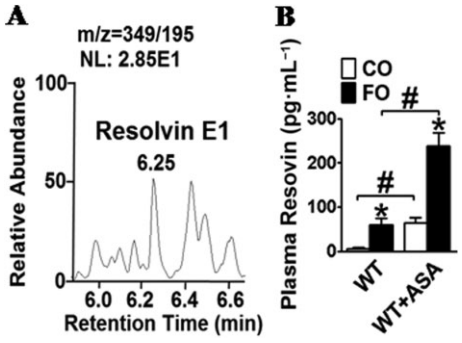

Figure 11

Detection of resolvin E1 in plasma of aspirin (ASA), FO, or FO and aspirin-treated mice. (A) Selected ion monitoring chromatogram of resolvin E1 isolated from freshly collected plasma; (B) quantification of plasma resolvin E1 production in aspirin, FO, or FO and aspirin-treated mice. *$P < 0.05$ versus CO; #$P < 0.05$ as indicated, $n = 8–11$.

et al., 2009). In line with the synergistic suppression of injury-induced neointima growth, the combination of FO and aspirin dramatically attenuated perivascular infiltration of inflammatory cells (macrophages and neutrophils), as compared with FO alone. Moreover, treatment with FO and aspirin markedly reduced the expression of pro-inflammatory genes (IL-6, TNF-α and Celc10a) and enhanced the expression of anti-inflammatory genes (MMR, Ym-1) in macrophages, as well as significantly inhibiting the expression of adhesion molecules on leukocytes, as compared with aspirin or FO alone. D-series resolvins protect against vascular neointima hyperplasia in response to injury (Miyahara *et al.*, 2013). Recently, resolvin E1 has attracted much attention (Buckley *et al.*, 2014); it has been found to ameliorate inflammatory reactions in many conditions such as atopic dermatitis (Kim *et al.*, 2012), allergic airway inflammation (Haworth *et al.*, 2011), colitis (Arita *et al.*, 2005) and acute lung injury (El Kebir *et al.*, 2012). Therefore, the synergistic effect of FO and aspirin on the suppression of injury-induced neointima growth may be attributed to, at least partially, the anti-inflammatory effects of pro-resolving lipid mediators.

In summary, we found that co-administration of FO and aspirin synergistically inhibited platelet aggregation and vascular neointima formation in response to mechanical injury. Our results indicate that FO supplementation could be used as adjuvant therapy in patients with stable CAD, including those undergoing PCI.

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Author contributions

Y. G., M. L., C. D. F. and Y. Y. designed the research associated with the project. Y. G., M. L., L. P., X. L., F. Y., J. Z. and B. X. performed experiments. Q. Z., W. S., H. Y. and L. Z. provided important technical support. Y. G., C. D. F. and Y. Y. wrote the paper.

Conflicts of interest

None.

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