Modulation of platelet aggregation-related eicosanoid production by dietary F-fucoidan from brown alga *Laminaria japonica* in human subjects

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Abstract

Laminaria japonica is traditionally eaten in Japan as a beneficial food for thrombosis. The alga contains two specific ingredients, a xanthophyll fucoxanthin (FX) and a polysaccharide, F-fucoidan (FD). The aim of the present study was to investigate whether FX or FD exhibited anti-thrombotic effects. For this purpose, three types of capsules, containing 1 mg FX, 400 mg fucoidan, and both, were prepared from the alga and administered to volunteers for 5 weeks. The dose of FD or FD + FX significantly shortened lysis time (LT) of the thrombus measured by a global thrombosis test in the blood, but FX did not. Examining the mechanism, dietary FD increased H_2O_2 and the secretion of prostacyclin (PGI_2), a potent inhibitor of platelet aggregation, in the blood, although FD was under the detection limit in the blood, determining with its monoclonal antibody. Furthermore, in mouse experiments, dietary FD was totally excreted into the faeces and was not incorporated into the blood. We then employed a co-culture system of a Caco-2 cell monolayer with fresh human blood. The addition of FD to Caco-2 cells stimulated the expression of NADPH oxidase 1 (*NOX1*) and dual oxidase 2 (*DUOX2*) mRNA and secreted H_2O_2 onto the blood side accompanied by a significant increase in serum PGI_2 production. These effects were invalidated by the combined addition of FD with its monoclonal antibody. The results suggested that dietary FD stimulated the expression of H_2O_2-producing enzymes in intestinal epithelial cells and released H_2O_2 into the blood, which played a signalling role to increase PGI_2 production and then shortened LT for thrombi.

Key words: F-fucoidan: Prostacyclin: Thromboxane A$_2$: Hydrogen peroxide: Anti-thrombosis

Thrombosis prevention is an important issue since thrombosis causes various abnormal conditions in the circulatory organs, such as vascular occlusion, transient ischaemia and infarction, which are closely associated with the induction of various degenerative diseases[1]. Thrombus formation is induced by platelet activation, and the ability of platelets to participate in both normal haemostasis and atherothrombosis depends on their adhesive properties and their capacity to be activated very quickly in response to various stimuli[2]. When activated, platelets secrete biologically active ligands, including ADP, serotonin and thromboxane A_2 (TxA_2), to induce thrombus formation[3]. Platelets also release arachidonic acid from their plasma membranes when subjected to stimuli such as shear stress, and produce TxA_2 and prostacyclin (PGI_2) via the arachidonic cascade[4]. TxA_2 is a potent platelet agonist and vasoconstrictor that activates resting platelets, resulting in thrombus formation, while PGI_2 is a potent vasodilator

and inhibitor of platelet aggregation[5]. Patients with atherosclerotic disease or pulmonary hypertension display high TxA_2 levels and reduced PGI_2 levels[6,7]. On the other hand, H_2O_2 has been reported to cause vasodilatation by several mechanisms, including cyclo-oxygenase and cyclic AMP in canine cerebral arteries[8], and phospholipase A_2 in porcine coronary microvessels[9]. Exogenous H_2O_2 also causes vasodilatation by opening several K channels, including K_{ATP} channels in cat cerebral[10] and rabbit mesenteric arteries[11]. Furthermore, H_2O_2 can diffuse in tissues and cross cell membranes[12], making it an interesting candidate for signalling involved in the immune response.

Fucoidan is a polysulphated polysaccharide found in various species of brown sea algae. The Japanese traditionally eat a large amount of sea algae and their life expectancy is the highest in the world[13]. Daily fucoidan intake from sea algae is estimated to be about 400 mg/capita[14,15]. Fucoidan

has been reported to have various biological effects, such as anti-tumour activity, by enhancing the immune response of hosts[16], anti-allergy activity by preventing NF-κB p52-mediated pathways through CD40[17] and anti-thrombosis activity in cell and animal experiments[18–20]; however, there is no information about the effect of dietary fucoidan on human thrombosis, and the mechanism is unclear.

In the present study, a dietary dose of fucoidan, prepared from *Laminaria japonica*, was orally administered to healthy participants to assess its anti-thrombotic effects. Furthermore, the anti-thrombotic mechanism of fucoidan was investigated using a Transwell co-culture system, the Caco-2 intestinal epithelial model and human blood. The present study provides new insight into the mechanism of fucoidan as an anticoagulant in human subjects.

Materials and methods

Preparation of capsules

The brown alga *L. japonica* was cultured in Hakodate city, Japan, and extracted with absolute ethanol. To use the ethanol extract and its residues for the human study, the fucoidan and lipid fractions were encapsulated after the following preparation. Briefly, the extract was dried, de-salted and re-extracted with hexane. After removing hexane, the material was composed of 46·5 % fucoxanthin (FX), 8·01 % lipids and 45·4 % carbohydrates of mostly cellulose. It was then mixed with cyclodextrin to make FX capsules. The residue of the ethanol extract was suspended in thirty volumes of 0·1 M-acetic acid, which was then filtered through no. 2 filter paper, neutralised with 0·1 M-NaOH, precipitated with 70 % ethanol and centrifuged at 7500 g. According to a determination using an antibody to F-fucoidan (FD)[15], the precipitate was composed of 63 % FD and 37 % of various other polysaccharides, mainly including alginic acid. The salt and arsenic contents were 0·02 % and 0·9 parts per million, respectively. This precipitate was made for FD capsules. The FX+FD capsule was made from combining the two fractions. Each capsule contained 0·2 mg FX and/or 80 mg fucoidan. In the following human study, five capsules (1·0 mg FX and/or 400 mg fucoidan)/participant per d were employed.

Purification of F-fucoidan

For cell culture experiments, FD in the FD capsules was further purified as follows: the components of the FD capsule were dissolved in 20 mM-sodium acetate buffer (pH 4·6) and applied to a Toyopearl-DEAE 650M column (2·0 × 20 cm; TOSOH). The column was washed with 0·5 M-NaCl to wash out alginic acid and fucoidan was eluted with 1·0 M-NaCl. FD was then precipitated with ethanol in a final concentration of 67 % and washed twice with 70 % ethanol. The purity of FD was >95 % according to the determination of total sugar by the phenol-sulphate method[21]. TLC for FD hydrolysates prepared by 1 % trifluoroacetic acid degradation at 100°C for 1 h indicated that FD was composed of fucose and sulphated fucose in a molar ratio of approximately 1:1. No appreciable

amounts of galactose and uronic acid were detected in TLC. The sulphate content of FD was determined to be less than 45 % by the rhodizonate method[22]. The apparent molecular size of FD was estimated to be 300 kDa by 1 % agarose gel electrophoresis using DNA molecular marker (500 bp DNA ladder; Takara). These analyses indicated that fucoidan extracted from *L. japonica* was classified as FD of fucose-rich fucan.

Study design

The present study involved five experiments, all of which were approved by the Ethics Committee of Kobe University Graduate School of Medicine (permission no. 615). Expt 1 involved a 5-week intake of the FX+FD capsule, FX capsule or FD capsule, and the effects of the FD treatments on the thrombosis status of twenty-four healthy participants were evaluated. Expt 2 tested the effect of stress on eicosanoid production using the fresh blood of healthy volunteers who had not been administered any test samples. Expt 3 examined eicosanoid production, H_2O_2 levels and FD amount in the blood of thirteen healthy participants after the 5-week intake of FD capsules. In addition, the effects of the low levels of H_2O_2 on eicosanoid production and platelet aggregation were investigated. Expt 4 studied the fate of dietary FD in the mouse digestive tract. Expt 5 involved *in vitro* examinations of the blood of healthy volunteers who had not been administered any capsules, aiming to elucidate the mechanism by which FD modulates eicosanoid secretion into the blood. To clarify this description, a flow diagram is presented in Fig. 1.

Participants

Participants were recruited from employees and students of Kobe University who were assessed as healthy in the regular health check at the health management centre, and were excluded if they were taking medicine for hyperlipidaemia, hypertension, diabetes or hyperuricaemia; if they had diet-controlled diabetes; if they were pre- or postmenopausal women, or used oral contraceptives or hormone replacement therapy; if they were smokers or had a bleeding disorder, an allergy to algae, or a history of any gastrointestinal or cerebro-vascular disease; or if they were using any dietary supplements. Informed consent was obtained from all eligible participants.

In vivo *evaluation of the effects of fucoidan on thrombosis status (Expt 1)*

First, thirty-three participants were randomly assigned to one of three groups containing eleven participants each, the FX + FD, FX and FD groups. Participants and investigators were blinded to which of the three groups each participant had been assigned to until the end of the study. At the base-line examination, blood was taken from participants and subjected to regular biochemical analysis (SRL, Inc.). After the analysis, four participants were excluded because they were outside the normal range of 3·88–5·66 mmol/l of total
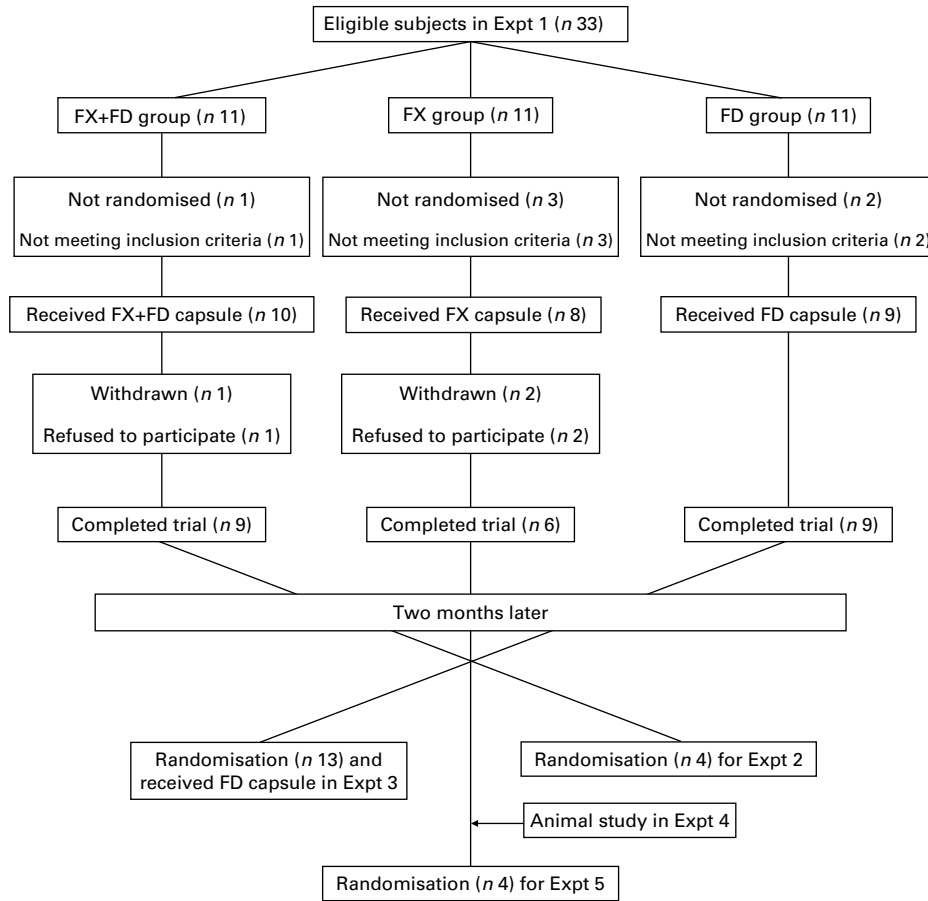
Fig. 1. Flow diagram of the study design. FX, fucoxanthin; FD, F-fucoidan.

cholesterol (6·02, 6·41, 6·56 or 3·28 mmol/l), and two because they had caught a cold and were taking medicine. After the twenty-seven participants had started to receive five capsules per d for 5 weeks, three withdrew as they refused to participate because of the difficulty of taking five capsules daily. Finally, thirteen male and eleven female participants were eligible and completed the study: nine in the FX + FD

group (five male, four female), six in the FX group (three male, three female) and nine in the FD group (five male, four female), as illustrated in Fig. 1. Their characteristics are shown in Table 1. Adherence to the intervention, which was evaluated by counting the remaining capsules in the bottle returned to the investigators and by examining a self-recorded dose diary, was >98 %. The participants continued their usual

Table 1. Basal and final characteristics of the participants in Expt 1

(Mean values and standard deviations)

Group*	FX+FD (n 9)				FX (n 6)				FD (n 9)			
	Basal		Final		Basal		Final		Basal		Final	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Age (years)	27·3	6·9			24·0	2·4			22·2	1·5		
Glucose (mmol/l)	4·9	0·7	4·9	0·4	4·9	0·1	5·0	0·2	4·8	0·2	4·8	0·3
Total cholesterol (mmol/l)	5·03	0·89	4·71	0·63	4·54	1·01	4·38	0·89	4·48	1·00	4·37	0·91
Aspartate transaminase (μkat/l)	0·32	0·06	0·33	0·08	0·35	0·01	0·34	0·02	0·35	0·13	0·34	0·07
Alanine transaminase (μkat/l)	0·25	0·10	0·26	0·10	0·25	0·07	0·25	0·04	0·26	0·17	0·26	0·17
Haematocrit (litres/l)	0·48	0·02	0·45	0·04	0·48	0·02	0·46	0·03	0·43	0·04	0·45	0·03
Erythrocyte count (10^6/l)	0·49	0·04	0·50	0·04	0·52	0·02	0·52	0·04	0·48	0·03	0·49	0·04
Platelets (10^9/l)	208	46	234	43	223	16	229	22	223	35	229	25
Hb (g/l)	152	15	152	13	155	10	153	13	149	14	151	13
Creatinine (μmol/l)	60	10	60	10	70	10	70	10	60	10	60	10
Leucocyte count (10^6/l)	5·2	0·8	5·0	1·2	5·3	1·1	5·5	0·5	5·8	1·1	5·7	1·6

FX, fucoxanthin; FD, F-fucoidan.
* Blood was drawn after a 12 h fast.

eating habits without any bias of eating algae during the 5-week experimental period.

At the baseline and final examinations, blood was carefully drawn from the forearm by venepuncture through a twenty-one-gauge butterfly cannula into a plastic syringe without using anticoagulants. The first 2 ml were discarded because the first blood had been subjected to stress by being churned in the syringe and would disturb the global thrombosis test (GTT)[23,24]. The next 3 ml were used within 5 s for the GTT, and another 10 ml were subjected to regular biochemical analysis.

Global thrombosis test

The GTT was performed as described previously[25] to determine the occlusion time as a measure of platelet reactivity and lysis time (LT) as a measure of thrombolytic activity. Briefly, the above fresh blood was flowed spontaneously by gravity at 37°C into a tube with a narrow end, which included two gaps formed by two steel balls, an initial large gap and a small point at the end. The blood undergoes shear force when passing through the gap made by the large ball, activates platelets to induce coagulation around the small ball and then its flow is arrested. Drops of blood were detected optically with a light emitter and the recorded time when drops had not been detected for 15 s was referred to as occlusion time to the start of blood coagulation. When blood flow was later restored with thrombolysis, the time of the first drop was referred to as LT to the start of lysis.

Estimation of the effects of shear stress on eicosanoid secretion (Expt 2)

To estimate the effects of stress on eicosanoid secretion, four participants were randomly selected and their fresh blood was taken 2 months after Expt 1. After discarding the first 2 ml, 1 ml blood was subjected to shear stress by agitating it for 5 s with a vortex mixer or by incubating it for 40 min at 37°C. After blood collection, serum samples were prepared by centrifugation and used for the determination of eicosanoid levels.

Measurement of eicosanoids

TxA_2 and PGI_2 have been well recognised to be immediately converted to thromboxane B_2 (TxB_2) after secretion and generally determined as equal products, TxB_2 and 6-keto-$PGF_{1\alpha}$, respectively[26,27]. In the present study, 50 μl of the above serum were analysed with the general method using an enzyme immunoassay kit (Cayman Chemical)[28].

The metabolite of TxA_2 was also determined in the urine. Urine was collected from the participants in the morning before their breakfast at the baseline and final examinations, and was subjected to analysis after removing impurities with centrifugation at 3000 g for 5 min at 4°C. The level of 11-dehydro-TxB_2, a metabolite of TxA_2, in the urine was determined with an ELISA kit (Cayman Chemical) according to the manufacturer's instructions[6].

In vivo determination of eicosanoid production (Expt 3)

At 2 months after Expt 1, thirteen participants, seven male and six female, were randomly selected from the participants of Expt 1. They were instructed to avoid brown sea algae-containing foods for the duration of the experiment and administered 400 mg FD capsules for 5 weeks, and then blood samples were taken at the baseline and final examinations. After discarding the first 2 ml, the next 2 ml blood samples were centrifuged at 3000 g for 3 min at 4°C, and the eicosanoid levels and fucoidan amount were measured in the serum. Also, H_2O_2 was determined with the following method.

Determination of hydrogen peroxide levels

The serum level of H_2O_2 was determined using the scopoletin fluorescence method[29]. A 100 μl aliquot of serum was added to 100 μl Krebs–Ringer phosphate buffer at pH 7·4 containing 5·5 mM-glucose, 17 μM-scopoletin and 0·44 units of horse-radish peroxidase, and incubated at 37°C for 30 min, before being subjected to analysis with fluorescence at 460 nm and excitation at 350 nm. The concentration of H_2O_2 was calculated using a standard calibration curve.

Effect of low hydrogen peroxide on human platelet aggregation

Platelet aggregation was determined by the published method[30]. Briefly, human blood in 3·2 % of tri-sodium citrate was centrifuged at 200 g for 15 min and separated into platelet-rich plasma. After confirming normal platelet numbers to be $2–4 \times 10^8$, the platelet-rich plasma was mixed with 2·5 μM-H_2O_2 at 37°C for 30 min and then placed in the wells of ninety-six-well plates at a volume of 100 μl. After the addition of platelet agonists (Sigma-Aldrich Company), 3·0 mg collagen/l, 0·5 μM–arachidonic acid or 0·1 μM-adrenalin, the ninety-six-well plate was immediately placed in a microplate reader (SH-9000Lab; Corona Electric Company), shaken vigorously at 37°C and absorbance monitored at 595 nm every 15 s for 15 min. In addition, a small part of the platelet-rich plasma was subjected to centrifugation at 15 000 g for 5 min and separated into platelet-poor plasma. The platelet-poor plasma was treated similarly to the platelet-rich plasma and was referred to as a control.

Study on the fate of dietary F-fucoidan in the mouse digestive tract (Expt 4)

The animal treatments were approved by the Institutional Animal Care and Use Committee (permission no. 20-5-11) and carried out according to the Guidelines for Animal Experiments at Kobe University. Male ICR mice (7 weeks old, 28–30 g) were obtained from Japan SLC, Inc. The animals were acclimatised at 23°C with a 12 h light–12 h dark cycle for 1 week and were allowed *ad libitum* access to food (Rodent labo EQ 5L37; Japan SLC) and drinking water. Food was composed of 21 % vitamin-free casein, 43·6 % dextrin, 15 % sucrose, 5 % maize oil, 3 % cellulose, 5 % lard, 0·2 %

choline chloride, 0·2 % DL-methionine, 5 % mineral mix and 2 % vitamin mix. Mice received a single oral administration of 5·0 mg/mouse of FD after a 12 h fast and were then killed at 2, 4, 8, 16 and 32 h after administration. Blood and contents of the stomach, small intestine and large intestine, and faeces were collected and subjected to an ELISA inhibition assay for the measurement of the FD amount.

Co-culture system for evaluating eicosanoid secretion and hydrogen peroxide production (Expt 5)

To elucidate the modulation mechanisms of dietary FD on the production of eicosanoid in the blood, an intestinal epithelial model was employed as described previously[31]. Human intestinal epithelial cell line Caco-2 cells were cultured in the upper well of a Transwell after being fully differentiated, which was measured by the method of Hidalgo *et al.*[32] with a Millicell-ERS (Millipore Company) exceeding $1200\,\Omega \times cm^2$ at transepithelial electrical resistance. Then, 0·8 ml fresh blood from healthy volunteers (*n* 4) was placed on the basolateral side and co-cultured with Caco-2 cells in inserts. After 2 h co-culture, the medium in the inserts was replaced with a medium containing 0, 125, 250 and 500 mg/l of fucoidan and incubated for another 3 h. Blood was collected and the levels of H_2O_2 and eicosanoid were determined in the serum. The Caco-2 cells in the insert were washed with PBS and subjected to real-time RT-PCR to evaluate the effects of fucoidan on mRNA expression.

Real-time RT-PCR

Total RNA in Caco-2 cells was purified with a Mini RNA Isolation Kit using a capped column (Zymo Research Company), as described previously[31]. Briefly, complementary DNA was synthesised using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time PCR was performed using the TaqMan Fast Universal PCR Master Mix (Applied Biosystems), according to the manufacturer's instructions. TaqMan primers (assay ID: Hs0024659_m1, Hs00204187_m1 and Hs02758991_g1; Applied Biosystems) were used to amplify human NADPH oxidase 1 (*NOX1*), dual oxidase 2 (*DUOX2*) and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), respectively. A real-time PCR 7500 fast system was used for real-time PCR according to the manufacturer's instructions.

Statistical analysis

Data are reported as means and standard deviations. In Expt 1 and 3, comparisons were made between the baseline and final examinations in each group using paired Student's *t* tests. All other data were analysed using unpaired Student's *t* tests. All statistical analyses were performed using SPSS 13.0 (SPSS Inc.). $P < 0·05$ was considered to be significant.

Results

Oral administration of F-fucoidan showed thrombolytic activity (Expt 1)

No participants displayed any abnormalities or had any complaints at the end of the 5-week study period, and the background characteristics of the participants were not significantly different between the baseline and final examinations in any group (Table 1). Occlusion time did not significantly differ between the baseline and final examinations in any of
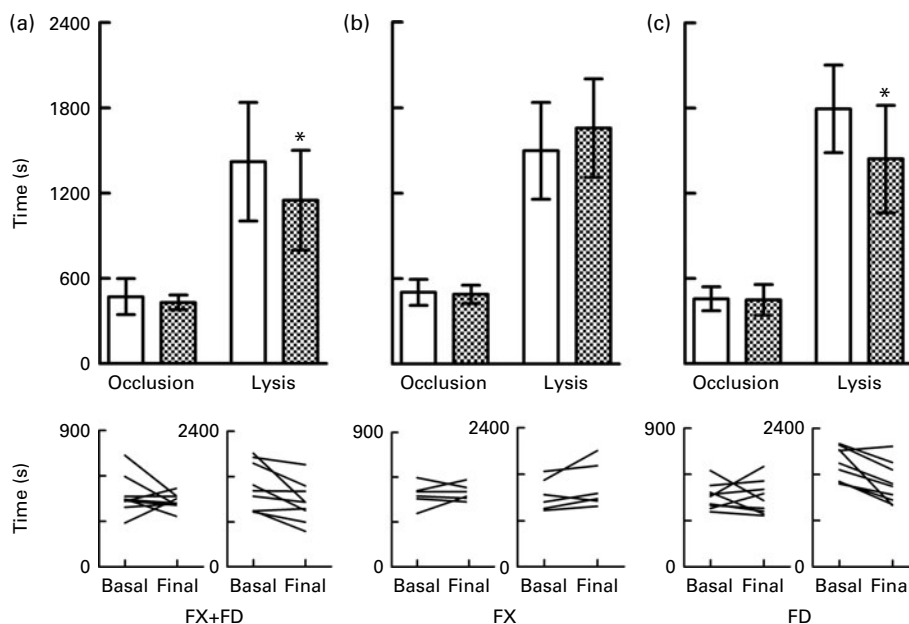

Fig. 2. Occlusion time and lysis time of blood at the baseline (☐) and final examinations (▨) in a 5-week F-fucoidan (FD) ingestion study (Expt 1). (a) Fucoxanthin + FD (FX+FD) capsule group (*n* 9), (b) FX group (*n* 6) and (c) FD group (*n* 9). Values in the upper panels are means, with standard deviations represented by vertical bars, and those in the lower panels are individual items of raw data. * Mean value was significantly different from that at baseline (*P* < 0·05).

the three groups, nor did LT in the FX group (Fig. 2). In contrast, LT in the FD and FX + FD groups was significantly shortened in the final examination compared with that at baseline. As LT reflects the level of thrombolytic activity induced to de-aggregate platelets that have been occluded in blood vessels[23], the oral administration of FD might be effective in preventing thrombosis.

Inducing production of thromboxane B_2 and 6-keto-PGF$_{1\alpha}$ by shear stress (Expt 2)

The aforementioned GTT assay evaluates platelet aggregation induced by shear stress on the blood when it passes through the gap made by the large ball[23]. Shear stress has been reported to induce the secretion of TxA_2, a potent platelet agonist and vasoconstrictor, and PGI_2, a potent vasodilator and inhibitor of platelet aggregation[5,33]. The half-lives of TxA_2 and PGI_2 have been reported to be very short, 30 s and 5 min, respectively, and metabolised to stable TxB_2 and 6-keto-PGF$_{1\alpha}$[26,27]. In Expt 2, the change in serum levels of TxB_2 and 6-keto-PGF$_{1\alpha}$ was determined when fresh blood had undergone agitation for 5 s with a vortex mixer or had been left to stand at 37°C, as shown in Fig. 3. Stress by vortexing significantly increased the level of TxA_2 from 32 (SD 11) to 94 (SD 43) ng/l but 6-keto-PGF$_{1\alpha}$ remained unchanged (Fig. 3(a)). Leaving to stand at 37°C markedly increased TxA_2 with time from 61 (SD 12) to 587 (SD 24) ng/l after standing for 10 min, and 6-keto-PGF$_{1\alpha}$ increased slightly but significantly with time (Fig. 3(b)). Thus, subjecting blood to stress increased the secretion of the platelet aggregator TxA_2 from platelets or leucocytes.

Oral administration of F-fucoidan increased the serum level of 6-keto-PGF$_{1\alpha}$ and hydrogen peroxide (Expt 3)

To examine the relevance of the FD dose to eicosanoid production in the blood, FD capsules were administered to thirteen participants again for 5 weeks. At the end of the administration, their blood was analysed with a monoclonal
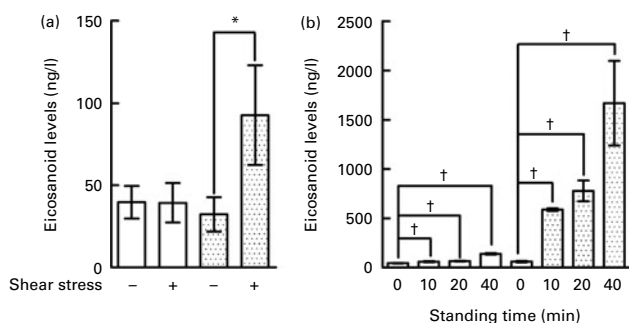

Fig. 3. Effects of stress on the production of thromboxane B_2 (TxB$_2$,▨) and 6-keto-PGF$_{1\alpha}$ (▢) in serum (Expt 2). (a) Fresh blood was treated with or without agitating and the serum levels of TxB$_2$ and 6-keto-PGF$_{1\alpha}$ were compared. (b) Fresh blood was left to stand at 37°C to determine the serum levels of TxB$_2$ and 6-keto-PGF$_{1\alpha}$. Values are means (n 4), with standard deviations represented by vertical bars. * Mean value was significantly different from that of stress (P<0·05). † Mean value was significantly different from that of 0-time (P<0·05).
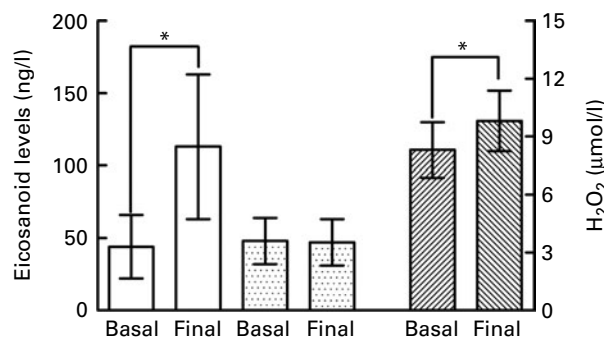

Fig. 4. Eicosanoid secretion and hydrogen peroxide production in serum after oral administration of F-fucoidan (Expt 3). Thirteen healthy participants received 400 mg F-fucoidan/d for 5 weeks, and the serum levels of 6-keto-PGF$_{1\alpha}$ (▢), thromboxane B_2 (TxB$_2$,▨) and hydrogen peroxide (▨) were compared in the basal and final experiments. Serum level of TxB$_2$ was compared after leaving the blood to stand at 37°C for 40 min. Values are means (n 13), with standard deviations represented by vertical bars. * Mean value of final was significantly different from that of basal (P<0·05).

antibody and FD was found to be under the detection limit ($<2\cdot5\,\mu$g/l) in the serum (data not shown); however, the level of 6-keto-PGF$_{1\alpha}$ had significantly increased in the serum from 44 (SD 22) ng/l at baseline to 113 (SD 50) ng/l (Fig. 4(a)). TxB_2 remained unchanged between the baseline and final examinations and has been reported to be excreted in the urine after metabolisation to 11-dehydro-TxB$_2$[6]. Morning urine was collected and analysed at the baseline and final time points. 11-Dehydro-TxB$_2$ was similar (P=0·42) between the baseline and final time points (1426 (SD 624) and 1452 (SD 847) ng/l, respectively). On the other hand, H_2O_2 is thought to induce vasodilatation through a mechanism including cyclo-oxygenase[8]. We therefore determined the serum level of H_2O_2 and detected a significant increase from 8·31 (SD 1·43) to 9·76 (SD 1·51) μmol/l. These results indicated that H_2O_2 may be involved in eicosanoid production.

Effect of hydrogen peroxide on platelet aggregation

To examine the effects of H_2O_2 on the production of TxB$_2$ and 6-keto-PGF$_{1\alpha}$, H_2O_2 was added to the fresh blood, which was stressed by leaving to stand at 37°C for 10 min or by agitating with 5 s vortexing, as shown in Fig. 5. The addition of H_2O_2 increased 6-keto-PGF$_{1\alpha}$ in the stressed blood in a dose-dependent manner, and TxB_2 remained unchanged (Fig. 5(a)). Production of both was inhibited by 100 μM-aspirin, indicating that eicosanoid production is involved in cyclo-oxygenase[34] (Fig. 5(a)). H_2O_2 (2·5 μmol/l) also increased 6-keto-PGF$_{1\alpha}$ in the blood that had undergone shear stress (Fig. 5(b)). These results indicated that H_2O_2 modulated eicosanoid secretion in the blood.

Platelet aggregation has been reported to be dependent on the production of the platelet agonist TxA_2 in platelets[30]. In Fig. 5, H_2O_2 stimulated the production of the platelet aggregation inhibitor PGI_2 at a concentration of more than 1·0 μmol/l. The effects of H_2O_2 are examined in Fig. 6. H_2O_2 suppressed platelet aggregation dose dependently (Fig. 6(a)) and significantly inhibited aggregation induced by agonists[30] such as
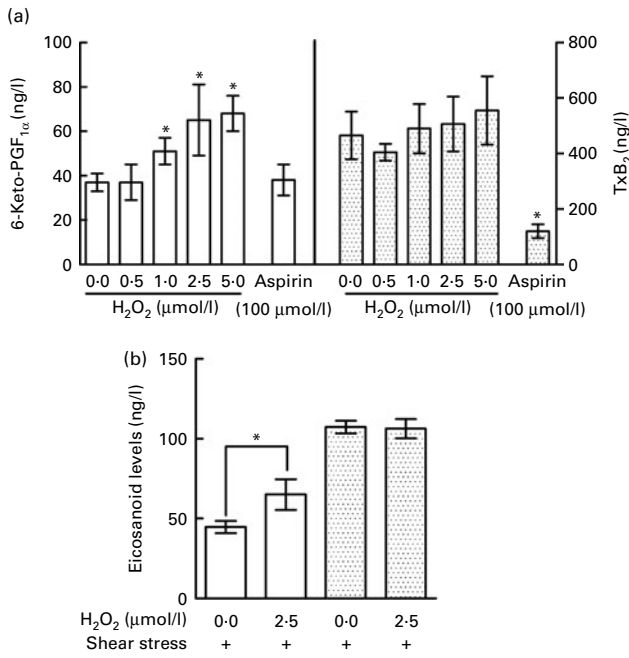
Fig. 5. Effect of hydrogen peroxide on the production of 6-keto-PGF$_{1\alpha}$ (□) and thromboxane B$_2$ (TxB$_2$, □) in the blood. The indicated concentration of hydrogen peroxide or aspirin was added to the fresh blood and subjected to stress by (a) leaving to stand at 37°C for 10 min or (b) agitating by 5 s vortexing, and then TxB$_2$ and 6-keto-PGF$_{1\alpha}$ levels were determined in serum. Values are means (n 4), with standard deviations represented by vertical bars. * Mean value was significantly different from that of 0 μM-hydrogen peroxide (P<0·05).

collagen, adrenalin and arachidonic acid (Fig. 6(b)–(d)). Thus, H$_2$O$_2$ plays an inhibitory role in platelet aggregation at a low concentration of around 2·5 μmol/l.

Fate of dietary F-fucoidan in the mouse digestive tract (Expt 4)

To verify whether FD could be incorporated into the blood, 5·0 mg/mouse of pure FD were orally dosed and its fate was examined in the digestive tract using a monoclonal antibody (Fig. 7). Dietary FD in the stomach moved to the small and large intestine, and then all of FD were excreted in the faeces 32 h after dosing. In the blood, FD was below the detection limit (<2·5 μg/l) at every determination time. The results clearly show that dietary FD was not digested and was not incorporated into the blood.

F-fucoidan modulates eicosanoid secretion by stimulating Caco-2 cells to produce hydrogen peroxide (Expt 5)

The above results indicated that dietary FD increased H$_2$O$_2$ and modulated the secretion of TxA$_2$ and PGI$_2$ in blood serum, although FD was not incorporated into the blood. To investigate the mechanisms of the effect of FD, we then employed an intestinal epithelial model of Caco-2 cells in the upper well and fresh blood in the lower well[35]. Before the investigation, the permeability of FD through the Caco-2 monolayer was examined (data not shown). When FD was
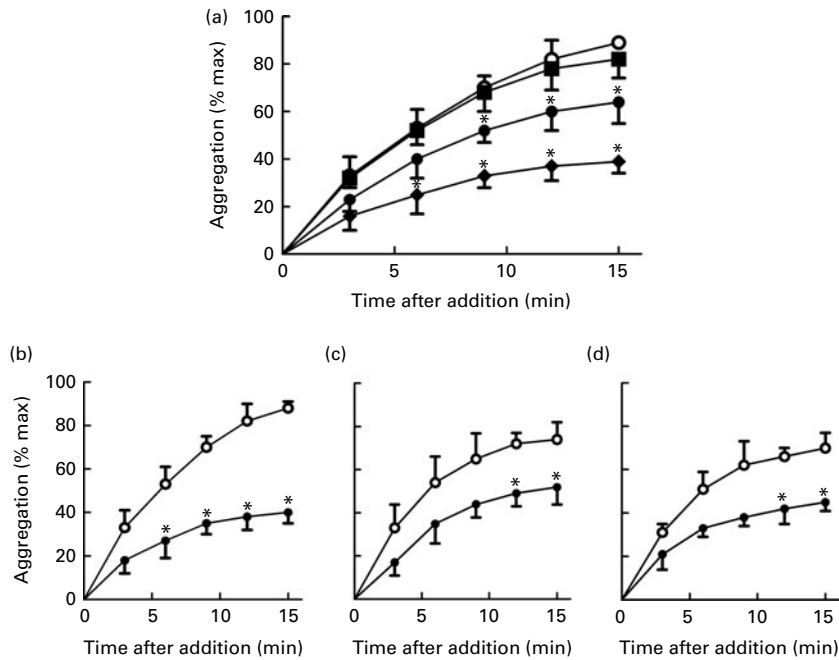

Fig. 6. Effect of hydrogen peroxide on platelet aggregation. Platelet-rich plasma (PRP) prepared from the participants' blood was treated with 2·5 μM-hydrogen peroxide at 37°C for 30 min, and mixed (b)–(d) with or (a) without platelet agonists, (b) 3·0 mg collagen/l, (c) 0·1 μM-adrenalin and (d) 0·5 μM-arachidonic acid, and absorbance was immediately monitored at 595 nm every 15 s for 15 min. For the blank control, platelet-poor plasma was separated from some of the PRP by centrifugation and treated similarly to PRP. Values are means (n 4), with standard deviations represented by vertical bars. * Mean value was significantly different from each point without agonist or 0 μM-hydrogen peroxide (P<0·05). ○, 0 μM-Hydrogen peroxide; ●, 1 μM-hydrogen peroxide; ■, 0·5 μM-hydrogen peroxide; ◆, 2·5 μM-hydrogen peroxide in Fig. (a); -●-, hydrogen peroxide-treated; -○-, non-treated in Fig. (b)–(d).
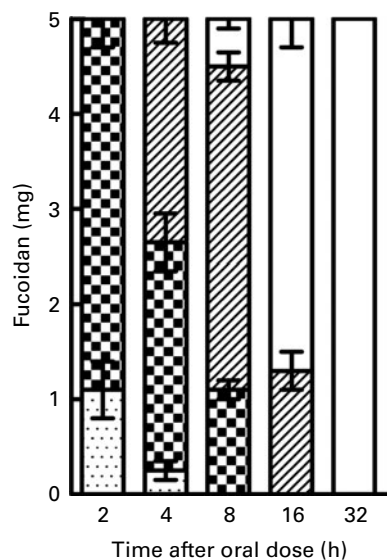
Fig. 7. Fate of dietary F-fucoidan in the mouse digestive tract (Expt 4). F-fucoidan 5·0 mg/mouse was orally dosed with a stomach sonde and traced in the digestive tract and blood with an ELISA inhibition assay at the indicated time after dosing. In the blood, F-fucoidan was below the detection limit ($< 2·5 \mu g/l$). Values are means (n 6), with standard deviations represented by vertical bars. ☐, Stomach; ▪, small intestine; ▨, large intestine; ☐, faeces.

added to the upper well for 3 h, it remained unchanged on the apical side and was below the detection limit ($< 2·5 \mu g/l$) on the basolateral side by the detection with its antibody. Thus, FD did not transfer to the blood side of the lower well.

The addition of 500 mg/l of FD to Caco-2 cells for 3 h significantly stimulated the mRNA expression of H_2O_2-producing enzymes, NOX1[36] and DUOX2[37], in Caco-2 cells 1·62 (SD 0·30)-fold (Fig. 8(a)) and 1·28 (SD 0·05)-fold (Fig. 8(b)), respectively. Also, FD increased the concentration of H_2O_2 on the blood side dose dependently (Fig. 8(c)), and 500 mg/l of FD significantly elevated the level of 6-keto-PGF$_{1\alpha}$ in the serum while TxB$_2$ remained unchanged (Fig. 8(d)). These events were invalidated when FD was added after mixing with its antibody (Fig. 8(a)–(d)), indicating that FD itself directly affected Caco-2 cells. These results indicated that dietary FD increased the production of H_2O_2 by stimulating the expression of *NOX1* and *DUOX2* in intestinal epithelial cells and then modulated PGI$_2$ production in the blood.

Discussion

The present study demonstrated that the intake of FD from *L. japonica* shortened the LT of the thrombus by elevating the secretion of PGI$_2$ as a result of increasing H_2O_2 production in the blood. This is the first study to show that dietary FD modulates platelet aggregation anti-thrombotically in human subjects.

Expt 1 was a 5-week randomised and double-blind study aimed at evaluating whether FD has anti-thrombotic effects. Dietary fibre such as FD is generally regarded to be neither digested nor absorbed into the body, and this has been

suggested to involve the immune system in the intestinal epithelium[31,38]. Assuming that a relatively long time will be required for the epithelium to respond to the action of dietary fibre, we decided that the period of oral administration should be 5 weeks. From *L. japonica*, three types of capsules (FX, FD and FX + FD) were prepared and dosed. FX is free of fucoidan and FD is free of FX. Thus, FX is a placebo against FD, and FD is a placebo against FX.

The GTT assay can evaluate coagulating conditions of platelets when the blood undergoes shear stress that mimics the blood flow conditions induced by atherosclerotic plaque[24]. Under pathological conditions such as the rupture of atherosclerotic plaque or luminal stenosis, shear stress and flow disturbance account for most arterial thrombus formation[39]. Strony *et al.*[40] reported that shear stress is a risk factor for cardiovascular disease development because high stress occurs in 90 % of stenosed coronary arteries. Thus, a shorter LT is closely associated with anti-thrombotic activity. In the present study, the oral administration of fucoidan (400 mg/d) for 5 weeks significantly reduced LT (Fig. 2), indicating that fucoidan may possess anti-thrombotic activity.

However, the signals that modulate the coagulation of platelets and their de-aggregation remain unclear. Thrombus formation involves three functionally independent pathways that mediate the interactions between circulating blood cells and the cells of the vessel wall: eicosanoids (PGI$_2$ and TxA$_2$); biological gases (NO and CO); ectonucleotidase (CD39)[4]; Ca ions[41]. Numerous reports have recognised that platelets play a pivotal role in arterial thrombus formation through TxA$_2$ and PGI$_2$ secretion[4–6,26,27]. Cheng *et al.*[42] have provided compelling evidence for the anti-thrombotic role of PGI$_2$ *in vivo*. The present study therefore focused on the eicosanoid pathway. Increasing production of eicosanoids was detected when shear stress in the GTT was reproduced *in vitro* using human blood (Fig. 3). FD administration was performed again for 5 weeks in Expt 3 and a significant increase was found in the serum level of 6-keto-PGF$_{1\alpha}$, a metabolite of PGI$_2$ (Fig. 4). Thus, the intake of fucoidan was considered to shorten LT by inducing PGI$_2$ production and would probably prevent cardiovascular diseases related to thrombosis.

The next question was which signals induced PGI$_2$ production. H_2O_2 is a reactive oxygen species and can react with various cellular targets, thereby producing toxicity that causes cell damage or even cell death. On the other hand, a low concentration of H_2O_2 is well known to play physiological roles, such as stimulating corneal epithelial cell adhesion to promote wound healing[43], having beneficial effects on post-ischaemic myocardial recovery[44] and strongly enhancing the inhibitory effect of NO on platelet aggregation[45]. Sabetkar *et al.*[46] reported that H_2O_2 at biologically relevant concentrations inhibited platelet function through increasing the serine phosphorylation of vasodilator-sensitive phosphoprotein. Shimokawa & Morikawa[47] reported that H_2O_2 played an important role as a redox signalling molecule, causing vasodilation as well as cardioprotection. This indicates that H_2O_2 produces toxicity at a high concentration and plays a signalling role at a lower concentration, bordering on a specific threshold
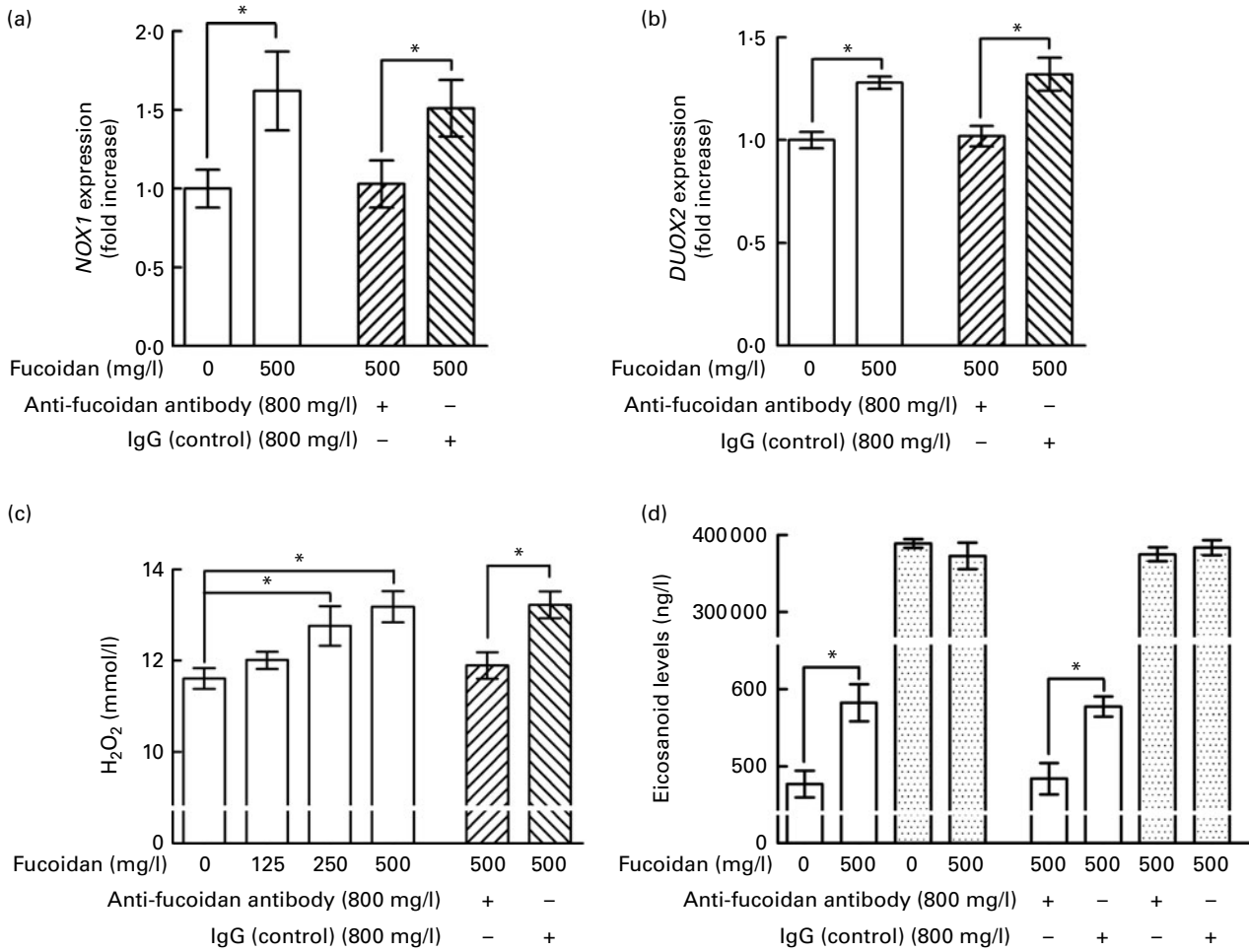
Fig. 8. Stimulating the expression of NADPH oxidase 1 (*NOX1*) and secretion of eicosanoid and hydrogen peroxide in Caco-2 cells after the treatment with F-fucoidan (Expt 5). Using the Transwell, Caco-2 cells in the upper well and fresh blood in the lower well were cultured for 2 h, and then the medium in the upper well was changed to the medium containing the indicated concentration of F-fucoidan or F-fucoidan combined with its antibody. After another 3 h culture, the expression of (a) *NOX1* and (b) dual oxidase 2 (*DUOX2*) in Caco-2 cells, and the levels of (c) hydrogen peroxide and (d) thromboxane B_2 (TxB$_2$, □) and 6-keto-PGF$_{1\alpha}$ (□) in the blood serum of the lower wells were determined. Values are means (n 4), with standard deviations represented by vertical bars. * Mean value was significantly different from that of 0 mg F-fucoidan ($P < 0.05$).

concentration. In the present Expt 3, we detected an increase of H_2O_2 in the blood after the 5-week ingestion of FD (Fig. 4). Then, we added a lower concentration of H_2O_2 to the fresh blood and found that H_2O_2 increased PGI$_2$ in both non- and stressed blood (Fig. 5). Furthermore, the addition of H_2O_2 reduced platelet aggregation induced by the agonists (Fig. 6). These results indicate that H_2O_2 is a signal to modulate the secretion of the inhibitor of platelet aggregation, PGI$_2$, in the blood.

An interesting question is how dietary FD affects PGI$_2$ secretion in the blood. In Expt 3, FD was not detected in the participants' blood after the 5-week ingestion of FD. In Expt 4, the mouse study showed that dietary FD was not incorporated into the blood and was totally excreted in the faeces (Fig. 7). A relatively lager amount of FD (175 mg/kg body weight) was dosed compared with the amount in the human study (6·7 mg/kg). Even with this large amount, the animals did not have diarrhoea and did not incorporate FD into the blood. Several kinds of fucoidan have been

reported to undergo digestion and to be partly incorporated into the blood. FD used in the present study was composed of fucose-rich fucan and was clearly a different type from G-fucoidan (galactose-containing fucan) and U-fucoidan (uronic acid-containing fucan) reported in other brown algae[48−51]. We therefore employed an intestinal epithelial model to elucidate the mechanisms of the effects of unincorporated FD. FD stimulated the expression of *NOX1* and *DUOX2* in Caco-2 cells and simultaneously secreted H_2O_2 and increased PGI$_2$ in the blood (Fig. 8). NOX1 and DUOX2 are enzymes producing H_2O_2, and DUOX2 is expressed throughout the digestive tract[52]. H_2O_2 has been recognised to diffuse in tissues and cross cell membranes[12]. Thus, these findings clearly demonstrate that dietary FD acts on intestinal epithelial cells and produces H_2O_2 to transmit signals to modulate eicosanoid production in platelets.

Dietary *Laminaria* FD markedly shortened LT in human blood. Daily intake of FD might contribute to the prevention of cardiovascular diseases by suppressing thrombus formation.

Acknowledgements

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