Iranian Journal of Basic Medical Sciences

ijbms.mums.ac.ir

IJBMS

The effect of omega- 3 polyunsaturated fatty acids on endothelial tight junction occludin expression in rat aorta during lipopolysaccharide-induced inflammation

Jakub Krizak [1], Karel Frimmel [1], Iveta Bernatova [2], Jana Navarova [3], Ruzena Sotnikova [3], Ludmila Okruhlicova [1]*

[1] Institute for Heart Research, Slovak Academy of Sciences, Bratislava, Slovakia
[2] Institute of Normal and Pathological Physiology, Slovak Academy of Sciences, Bratislava, Slovakia
[3] Institute of Experimental Pharmacology and Toxicology, Slovak Academy of Sciences, Bratislava, Slovakia

ARTICLE INFO

Article type:
Original article

Article history:
Received: Aug 28, 2015
Accepted: Jan 7, 2016

Keywords:
Aorta
Endothelium
Lipopolysaccharide
Occludin
Omega-3 polyunsaturated fatty acids

ABSTRACT

Objective(s): Occludin is essential for proper assembly of tight junctions (TJs) which regulate paracellular endothelial permeability. Omega-3 polyunsaturated fatty acids (Ω-3 PUFA) protect endothelial barrier function against injury.

Materials and Methods: We examined anti-inflammatory effect of Ω-3 PUFA intake (30 mg/kg/day for 10 days) on expression and location of occludin in the aorta of adult Wistar rats after a single dose of bacterial lipopolysaccharide (LPS, *Escherichia coli*, 1 mg/kg). The ultrastructure of TJs after LPS administration was also investigated. We measured plasma levels of C-reactive protein (CRP), Malondialdehyde (MDA) and CD68 expression and determined the total activity of NO synthase (NOS) in the aortic tissue.

Results: LPS induced a significant decrease of occludin expression accompanied by structural alterations of TJs. Levels of CRP, MDA, CD68 and NOS activity were elevated after LPS injection compared to controls indicating presence of moderate inflammation. Ω-3 PUFA supplementation did not affect occludin expression in treated inflammatory group. However they reduced CRP and MDA concentration and CD68 expression, but conversely, they increased NOS activity compared to inflammatory group.

Conclusion: Our results indicate that a single dose of LPS could have a long-term impact on occludin expression and thus contribute to endothelial barrier dysfunction. 10-day administration of Ω-3 PUFA had partial anti-inflammatory effects on health of rats without any effect on occludin expression.

► *Please cite this article as:*
Krizak J, Frimmel K, Bernatova I, Navarova J, Sotnikova R, Okruhlicova L. The effect of omega-3 polyunsaturated fatty acids on endothelial tight junction occludin expression in rat aorta during lipopolysaccharide-induced inflammation. Iran J Basic Med Sci 2016; 19:290-299.

Introduction

Epidemiological and experimental studies suggest that bacterial infection can represent one of risk factors for atherosclerosis and its related clinical diseases. Gram-negative organisms colonize the human gastrointestinal, genitourinal and respiration tract and release virulent endotoxin (lipopolysaccharide- LPS), which is a unique glycolipid forming a majority of their outer wall not only during evident infection, but also during ordinary subclinical and chronic conditions, such as periodontal diseases or bronchitis (1).

It has been demonstrated that exposure of the vascular endothelium to LPS causes endothelial hyperpermeability contributing to development of atherosclerosis (2).

Endothelial permeability is regulated by intercellular junctions, which are formed by a network of transmembrane adhesion proteins stitched together by the glycocalyx (3). Their configuration and location is unique and corresponds with their function. Depending on a type of blood vessel, there are three different types of intercellular junctions in endothelium: gap junctions (GJs), adherens junctions (AJs) and tight junctions (TJs) (4). TJs are considered to be raft-like membrane compartments essential in regulation of paracellular permeability (5, 6). TJ is formed by membrane, cytosolic and cytoskeletal proteins (7). The transmembrane proteins define a passage for designated substances and are responsible for TJ permeability and paracellular transport (8). This group of proteins contains occludin, claudins and junctional adhesion molecules (JAMs) (9). The cytoplasmic proteins (including ZO-1, ZO-2 and ZO-3) provide the link of transmembrane proteins with actin cytoskeleton (10). The occludin molecule passes four times through membrane forming two extracellular loops, one intercellular loop and cytoplasmic NH_2 and COOH ends.

*Corresponding author: Ludmila Okruhlicova. Institute for Heart Research, Slovak Academy of Sciences, Bratislava, Slovakia. Tel: +421-254774405; Fax: +421-254776637; email: ludmila.okruhlicova@savba.sk

The extracellular domains of occludin on adjacent cells bind and thus form TJ (10). Occludin plays a special role in TJ - it is responsible for the TJ formation and integrity (10). Therefore, its abnormal expression induced by various pathophysiological conditions represents a key factor in TJ assembly and/or presence respectively in cellular membrane. Defective TJs cause an increase of endothelial permeability and paracellular flux for undesirable substances to subendothelium (11), contributing to a development of vascular wall diseases including atherosclerosis. LPS- and/or cytokines-induced endothelial barrier dysfunction due to abnormal occludin expression has been demonstrated in intestine (12), blood-brain barrier (13-15), kidneys (16) as well as in cultured endothelial and/or epithelial cells respectively (17). However, until now, there is no data about the effect of LPS on occludin expression in endothelium of the aorta.

It is well-known, that regular intake of fish oil or isolated Ω-3 polyunsaturated fatty acids (Ω-3 PUFA) has beneficial effects on health. Ω-3 PUFA are regarded as strong intracellular and intercellular mediators (18). They have multifunctional beneficial effects – they reduce lipid levels and blood pressure, inhibit thrombosis, modulate NO production and suppress production of reactive species (19). They are also modulators of cell signalling network and membrane fluidity what can lead to reduction of the inflammation processes (20). Our previous studies demonstrated positive effects of Ω-3 PUFA supplementation on expression of connexin proteins of endothelial gap junctions in rat aorta (21-24) as well as in the rat heart (25-27). Regulation of tight junction permeability associated with modulation of occludin expression by Ω-3 PUFA were observed in intestine epithelial cells (28-30). All these data indicate involvement of both endothelial junctions as further therapeutic targets of Ω-3 PUFA. According to our knowledge, the effect of LPS and Ω-3 PUFA on occludin expression in the aorta has not yet been studied.

Therefore, the aim of our work was to examine the effect of bacterial endotoxin on occludin expression and on the ultrastructure of the endothelial tight junctions in the rat aorta. We also examined the modulation of occludin expression with Ω-3 PUFA after LPS application in the endothelial cells of the aorta.

Materials and Methods

Animal model

The experiments performed on animals were made in accordance with the Ethics Committee of the Institute for Heart Research SAS and protocols approved by the State Veterinary and Food Administration of the Slovak Republic. In the experiment we used three-months-old normotensive Wistar male rats (Dobra Voda, Slovakia). They were kept in controlled conditions with light:dark cycle (12:12), at 22–24 ˚C, humidity of 50-60%, food (standard dietary mixture) and water were available *ad libitum*. The animals were divided into four groups (n=6/group), the same as in our previous study (22): 1, C – the control healthy rats; 2, P – the control rats fed with a diet enriched with Ω-3 PUFA (30 mg/kg/day) for 10 days (57% eicosapentaenoic acid and 43 % docosahexaenoic acid, commercial nutritional supplement MaxiCor, SVUS Pharma, Czech Republic); 3, LPS – the control rats injected with a single dose of LPS (1 mg/kg, IP) from *E. coli* (055:B5 serotype; Sigma Aldrich, Germany). The experimental tissue was removed 10 days after LPS administration. 4, LPS-P – the rats infected with LPS were fed with a diet enriched by Ω-3 PUFA for 10 days. LPS was dissolved in sterile 0.9 % NaCl solution. The same volume of the NaCl solution was injected to the rats in the groups 1) and 2).

At the end of the experiment, the animals were anesthetized and the thoracic aorta was excised and cleaned. Tissue samples were frozen and prepared to subsequent SDS-PAGE and Western blot analysis of occludin and CD68 (marker of macrophages/ monocytes). The second part of the aorta was used to monitor the distribution of occludin by immunofluorescent method. Electron microscopy was used to analyse the ultrastructure of TJ in endothelium of the aorta after LPS administration.

Basic characteristics

Blood pressure was measured at the beginning and at the end of the experiment by a method of non-invasive plethysmography according to Dlugosova *et al* (21). Body weight (BW) was measured at the same periods of the experiment. At the end of the experiment, the heart was excised from the anesthetized animals, weighed (HW) and the relative weight of the heart (HW/BW) was calculated. The blood plasma was separated from collected blood samples to determine the hs-CRP level (marker of acute inflammation), and the MDA levels (index of lipid peroxidation) (31). The levels of hs-CRP were measured using Cholestech LDX System (California, USA). The concentration of MDA was determined spectrophotometrically by measuring colour produced in the reaction of MDA with thiobarbituric acid according to Draper and Hadley (32). We also determined the total activity of NO synthase (NOS) in the aortic tissue as described by Bernatova *et al* (33).

Western blot of occludin and CD68

Frozen samples of the aorta were homogenized with sonifier (Dr Hielscher, Germany) in SB20 solution (20% SDS, 10 mmol/l EDTA, 0.1 mol/l TRIS, pH 6.8). The total protein content of the samples was determined by the Bradford method (34). The proteins were separated on 10% sodium dodecyl sulphate

polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Advantec, Japan). To study the occludin expression, the membrane was incubated in the primary rabbit polyclonal anti-occludin antibody (Invitrogen Corporation, USA; 3 µg/ml) at a room temperature for 2 hr, followed by the incubation in the secondary goat anti-rabbit antibody labelled with horseradish peroxidase (Cell Signaling Technology, USA; 1:2,000). Expression of CD68 was detected using the primary monoclonal mouse anti-CD68 antibody (Millipore, USA; 1:1,000) and the secondary goat anti-mouse antibody labelled with horseradish peroxidase (GE Healthcare, Great Britain; 1:2,000). The optical density of specific bands was detected by "Kodak *In-Vivo* Multispectral Imaging System Fx Pro" (Carestream Health, Inc., USA) using "Carestream MI SE" software. The optical density of each band was tested in relation to the optical density of GAPDH as the endogenous control.

Immunofluorescence of occludin

Indirect immunofluorescent detection of occludin was performed on the frozen cross-sections of the non-fixed aortic rings of all experimental groups. Series of the sections were fixed in 4 % paraformaldehyde, incubated in 0.3 % Triton X-100, blocked by 10 % BSA and incubated with the primary polyclonal rabbit anti-occludin antibody (Invitrogen Corporation, USA; 15 µg/ml) over night at 4°C, followed by the incubation in the secondary goat anti-rabbit IgG antibody conjugated with fluorescein isothiocyanate (FITC) (Jackson Immunoresearch Laboratories Inc., USA; 1:200) for 2 hr at a laboratory temperature. Omission of the primary antibody during incubation served as the control of the antibody specificity. The aortic sections were potted into Vectashield with DAPI (4', 6-diamidino-2-phenylindole) H-1200 (Vector Labora- tories, Inc., USA). The immunofluorescent signal was observed by the microscope Zeiss Apotome 2 (Jena, Germany).

Transmission electron microscopy

The aorta of the control rats and the LPS rats (n=4 for each group) was cut into 3 mm rings. These specimens were immersion fixed in 2.5 % glutaraldehyde in 0.1 mol/l cacodylate buffer (pH 7.4) for 3 hr, then rinsed in the buffer, postfixed in 1% OsO_4, dehydrated in ethanol and embedded in Epon 812 (35). Ultrathin sections were stained with uranyl acetate and lead citrate and examined by TESLA 500 electron microscope (Brno, Czech Republic).

Statistical analysis

The values were expressed as mean±SEM. One-way analysis of variance (ANOVA) with post-tests: the Student-Newman-Keuls test (CRP, MDA, NOS) and Dunn test (occludin, CD68) were used for statistical evaluation. The results were statistically significant when $P<0.05$.

Results

Biometric parameters and inflammatory markers

The inflammatory group (LPS) did not have any significant changes in body weight gain (BWG), systolic blood pressure and relative heart weight (HW/BW) compared to the controls (C). 10-day administration of Ω-3 PUFA to the LPS rats (LPS-P) did not affect the measured parameters when compared to the inflammatory group (Table 1).

LPS significantly increased the plasma levels of hs-CRP compared to the controls (Table 2). The levels of hs-CRP in the LPS-P group were significantly lower than in the LPS group. However, they were still significantly higher than in the control group. Administration of Ω-3 PUFA to the controls had no effect on plasma levels of hs-CRP.

MDA concentration in plasma was significantly higher in the LPS group when compared to the C group (Table 2). The application of Ω-3 PUFA to the LPS rats resulted in significant decrease of MDA concentration when compared to the inflammatory group, to the value of the control group. Ω-3 PUFA administered to the control group had no effect on levels of MDA.

A single dose of LPS significantly increased CD68 expression in the aortic tissue comparing to the controls (Figure 1). Subsequent treatment of the LPS rats with Ω-3 PUFA caused significant decrease of CD68 expression comparing to the LPS group. However, the levels of CD68 were still higher than in the control group. Ω-3 PUFA had no effect on CD68 expression in the controls.

The NOS activity of the thoracic aorta in the LPS group was significantly increased when compared to the C group (Figure 2). In the LPS-P group the NOS activity was still increased when compared not only to the C group, but also to the LPS group (Figure 2). Ω-3 PUFA administered to the healthy rats had no effect on the NOS activity (Figure 2).

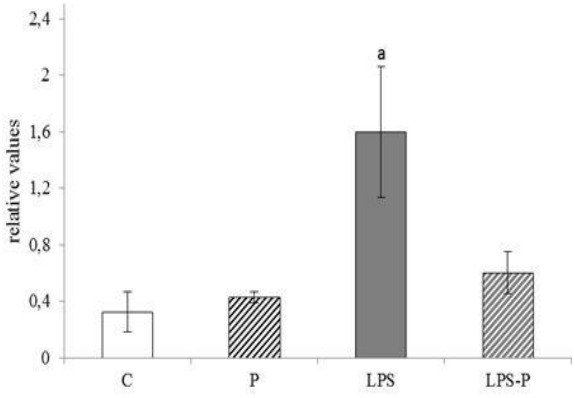

Figure 1. CD68 protein expression in endothelium of the rat thoracic aorta normalized to GAPDH. C - the control group; P - the control group fed with Ω-3 PUFA; LPS - the inflammatory group; LPS-P - the inflammatory group fed with Ω-3 PUFA. Results are expressed as mean±SEM. [a]$P<0.05$ vs. C; [c]$P<0.05$ vs. LPS

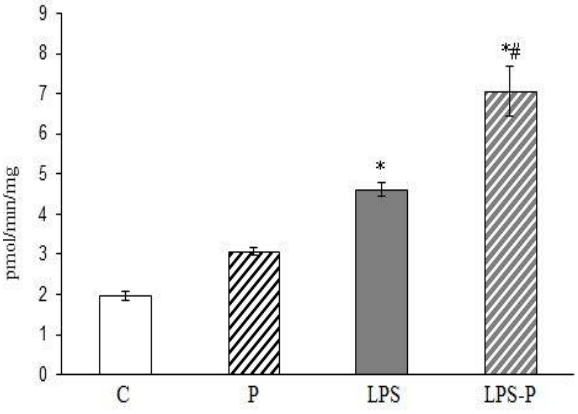

Figure 2. The NO synthase activity in the rat thoracic aorta tissue. The control group (C), the inflammatory group (LPS), the control group fed with Ω-3 PUFA (P) and the inflammatory group fed with Ω-3 PUFA (LPS-P). Results are expressed as mean ± SEM. $^{*}P<0.05$ vs. C; $^{\#}P<0.05$ vs. LPS

Occludin expression

We observed significantly lower occludin expression in the aorta of the LPS group than in the control group (Figure 3).

The diet enriched by Ω-3 PUFA administered to the LPS rats did not affect the occludin expression in the aorta when compared to the LPS group. We rather observed the tendency to decrease in the expression. Surprisingly, we also demonstrated significantly (P=0.049) decreased occludin expression when compared with the C group.

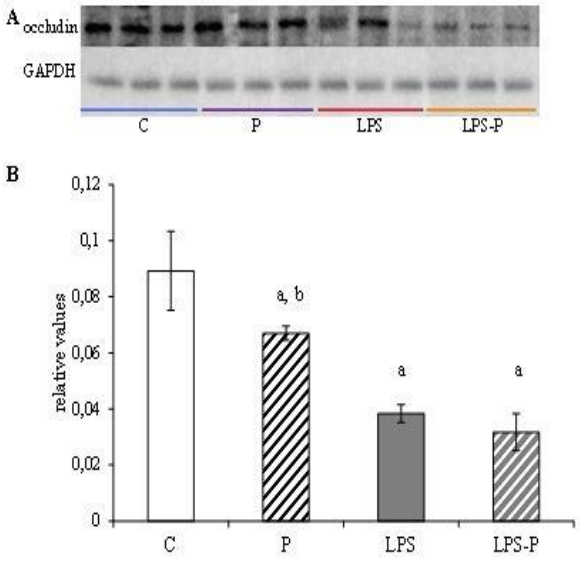

Figure 3. A) Representative Western blot of occludin and GAPDH expression in the rat thoracic aorta. B) Expression of occludin protein normalized to GAPDH in the rat thoracic aorta of the control group (C), the inflammatory group (LPS), the control group fed with Ω-3 PUFA (P) and the inflammatory group fed with Ω-3 PUFA (LPS-P). Results are expressed as mean ± SEM. $^{a}P<0.05$ vs. C; $^{b}P<0.001$ vs. LPS/LPS-P

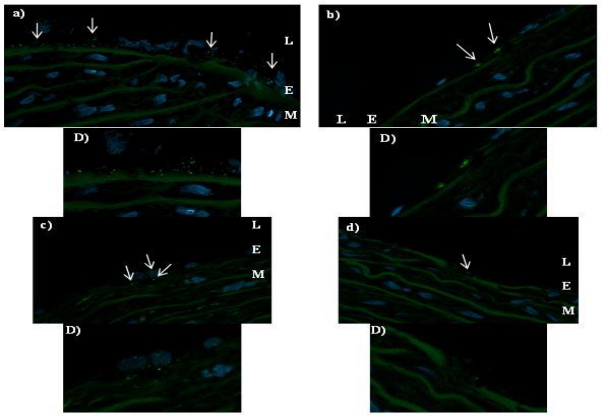

Figure 4. Representative images of immunofluorescent detection of occludin in the aortic endothelium of a) the control group (C); b) the control group fed with Ω-3 PUFA (P); c) the inflammatory group (LPS); d) the inflammatory group fed with Ω-3 PUFA (LPS-P); D) Detail of the endothelial layer with specific occludin reaction. Nuclei were visualized by DAPI. Arrow - immunofluorescent reaction of occludin; L - lumen; E - endothelium; M - tunica media. Original magnification 63x

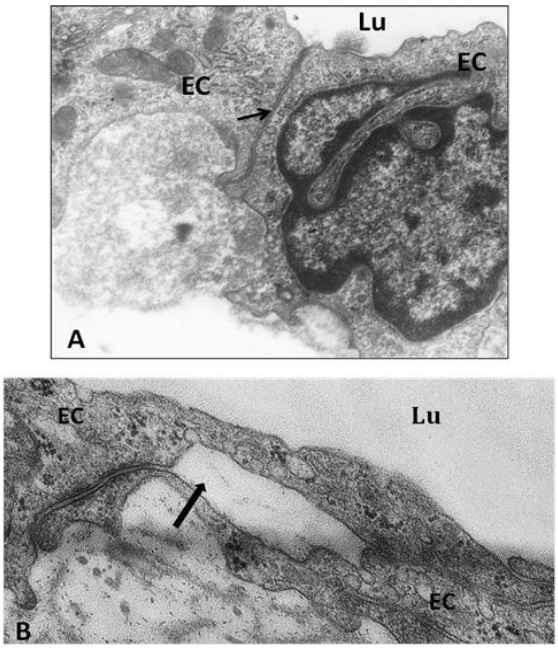

Figure 5. Representative electronograms of intercellular tight junctions of rat aortic endothelium. A) The control group with intact TJ; B) the inflammatory group with defective TJ. Lu - lumen; EC - endothelial cell; thin arrow - intact TJ; thick arrow - disturbed TJ. Original magnification: A – 10 000x, B – 10 000x

Immunofluorescence of occludin

Using the indirect immunofluorescence, we demonstrated the specific reaction of occludin distribution in endothelium of the aorta of all four groups (Figure 4). The immunolabeling was relatively regularly distributed within endothelium of the control group (Figure 4a), while heterogeneous distribution of occludin was found in endothelium of the rats treated with LPS or Ω-3 PUFA (Figures 4b-d).

Table 1. Biometric parameters of rats. BWG - body weight gain, SBP - systolic blood pressure and HW/BW - relative heart weight. C - the control group; P - the control group fed with Ω-3 PUFA; LPS - the inflammatory group; LPS-P - the inflammatory group fed with Ω-3 PUFA Results are expressed as ± SEM

experimental group	BWG (g)	systolic blood pressure (mm Hg)	HW/BW
C	27 ± 5	100 ± 5.40	3.2×10^{-3}
P	24 ± 6	99 ± 1.53	2.6×10^{-3}
LPS	18 ± 5	89 ± 3.86	2.8×10^{-3}
LPS-P	17 ± 4	91 ± 1.09	2.9×10^{-3}

Table 2. The measured biomarkers in plasma. hs-CRP - high sensitive C-reactive protein; MDA – malondialdehyde. C - the control group; P - the control group fed with Ω-3 PUFA; LPS - the inflammatory group; LPS-P - the inflammatory group fed with Ω-3 PUFA. Results are expressed as ± SEM. Results are expressed as ± SEM. **P*<0.05 vs. C, #*P*<0.05 vs. LPS, ***P*<0.05 vs. LPS-P

exerimental group	hs-CRP (mmol/l)	MDA (µg/mg)
C	0.30 ± 0.04**	1.780 ± 0.087
P	0.28 ± 0.06	1.764 ± 0.085
LPS	1.86 ± 0.12*	2.417 ± 0.193*
LPS-P	0.92 ± 0.07#	1.760 ± 0.103#

Tight junction electron microscopy

Electron microscopy revealed a standard architecture of the aortic endothelial cells in the C group. They were mutually connected by intact intercellular junctions including TJs (Figure 5a). We locally observed widened TJs and irregular clefts of endothelium in apical surface of endothelium in the rat aorta of the LPS group (Figure 5b). The ultrastructural abnormalities of endothelial TJs represent the structural marker of the endothelial barrier dysfunction. Moreover, endothelial cells of the aorta of the LPS group contained very high amount of ribosomes. Locally, the presence of pinocytic vesicules was suppressed in endothelium of the aorta of the LPS group.

Discussion

The vascular endothelium due to its location and structure works as a permeable barrier between circulating blood and vascular wall. As we already mentioned above, the paracellular permeability of endothelium is regulated by TJs (8). Disruption and/or loss of TJ function respectively due to various pathophysiological conditions lead to uncontrolled transport of substances into the interstitial environment resulting in a development of pathological processes in a vascular wall.

People often underestimate the treatment of moderate bacterial inflammation which might represent risk for cardiovascular system diseases. We designed our experimental model in order to study, if several days after a single non-lethal dose of LPS, in our case after 10 days, when immune system of healthy animals would be able to suppress moderate inflammation, there may be present changes in the mechanisms regulating endothelial integrity and permeability in the aorta.

The inflammation induced by bacterial LPS has been used as an experimental model of infection and chronic inflammation. The latter, due to its detrimental effects on endothelium, belongs to risk atherosclerotic factors. Numerous studies have demonstrated that the effects of LPS depend on a way of its application, used concentration and duration of inflammation (36). In our experiments, we injected healthy adult normotensive Wistar rats with LPS in the concentration 1 mg/kg IP Such amount is sufficient for the development of moderate inflammation, but it is not lethal (37). No mortality of the rats after LPS administration in our experiment corresponds with the mentioned data. LPS affected neither body weight of the rats, their systolic blood pressure, nor relative heart weight comparing to the healthy rats. However, six-fold increase of CRP levels and two-fold elevation of MDA concentration in plasma 10 days after LPS injection indicate the presence of inflammation accompanied by increased oxidative stress. During inflammation, endothelium is a major target of generated pro-inflammatory mediators as well as oxygen and nitrogen free radicals, which together destroy endothelial function and can affect cell-cell junctions between endothelial cells as well. That leads to widening of intercellular space and uncontrolled transendothelial flux.

We observed that LPS caused down-regulation of occludin expression in endothelium. As it is mentioned above, occludin is significantly involved in the TJ sealing function and formation-folding, thus, down-regulation of its expression refers to TJ dysfunction and/or local absence respectively that might contribute to

endothelial barrier dysfunction. In order to verify, if the changes in occludin expression influenced the ultrastructure of endothelial TJs, we used the electron microscopic analysis. It revealed the locally widened TJs, which represent the ultrastructural substrate of the occludin-related endothelial hyperpermeability. In addition, irregular shapes of clefts of endothelial cells in their luminal surface also indicate changes in integrity and permeability of endothelium. Increased CD68 expression suggesting more macrophages and/or their activation respectively in the aortic tissue of the LPS rats corresponds with the damage of the endothelial barrier function. Moreover, the ultrastructure of the aortic endothelium of the LPS rats demonstrated the local presence of the cells containing reduced amount of pinocytic vesicules which are known for their regulation of transcellular endothelial permeability. Our results thus indicate that hyperpermeability of the aortic endothelium during inflammation might result mainly from damaged and/or absent TJs. In contrast, Krueger *et al* (38) demonstrated that disturbances in occludin expression were associated with increased transendothelial vesicle trafficking in rat microvascular blood-brain barrier after ischemia. The results indicate blood vessel type- and size-dependent differences in mechanisms regulating endothelial permeability. Very high amount of ribosomes observed in the aortic endothelium after LPS administration points to a high proteosynthetic activity supporting increased generation of proinflammatory mediators in endothelium (39).

Our finding correlates with other studies. LPS-induced decreased occludin expression associated with TJ dysfunction has been demonstrated in the intestine (12), the kidney (16) as well as in the cell cultures (17, 40), independently on the used dose of LPS and time of inflammation. Proinflammatory cytokine TNF-α-induced decrease of occludin expression and damage of endothelial integrity has been found in blood-brain barrier (41) and cultured human brain microvascular endothelial cells (14).

Expression of occludin during inflammation can be modulated by various substances and pathways. The NOS system is one of fundamental factors affecting endothelial permeability (42). In our experiment, the LPS-induced decrease of occludin expression was accompanied with the increased aortic NOS activity. According to numerous publications, it has been attributed to the iNOS isoform (43-45). The iNOS was reported to be associated with reduced occludin expression and TJ hyperpermeability in intestinal epithelium (46, 30), diabetic retinopathy of mice (47) and blood-brain barrier (48). TJ opening mediated by NO was demonstrated by Takizawa *et al* (49) during early phase of intestinal ischemia/reperfusion. The LPS-induced increased NOS activity in the aorta could therefore contribute to the modulation of occludin expression in our experiment. Oxidative stress, accompanying inflammation, represents further potential modulator of occludin expression. Increased oxidative stress reduced occludin expression in rat skin (50) and the kidneys during experimental diabetic nephropathy (51) as well as in rat blood-brain barrier (13). Lochhead *et al* (52) demonstrated that oxidative stress caused a movement of occludin away from TJ, that was associated with alterations in the structures and localization of occludin. Jamaluddin *et al* (53) provided a proof of evidence that ROS reduced occludin expression (at the level of transcription and translation) and endothelial barrier function in human endothelial cells of coronary artery. In accordance with that, the elevated plasma levels of both CRP and MDA in our experiment, supporting the presence of inflammation and increased oxidative stress, could also be involved in downregulation of occludin expression in the aorta.

It has also been found that TJ function is closely associated with other types of interendothelial connections including the gap junction (GJ) (54). GJs consist of connexin proteins (Cx) and modulate intracellular homeostasis and synchronization of cellular functions along a vascular wall (20). Expression of proteins of both types of junctions is regulated by actual needs of endothelium (21). In addition, their mutual interactions can affect function and aggregation of both junctions (31-33). Our recent work, using the same LPS model, demonstrated upregulation of Cx40 expression in endothelium of the rat aorta associated with its impaired relaxation (22). Our present results showed downregulation of occludin expression in endothelium of the aorta after LPS administration. Therefore, it could be possible that the expression of both proteins might be mutually related and contribute to endothelial barrier dysfunction. The issue requires further research using different experimental approaches. However, the breakdown of TJ barrier function can be associated not only with abnormal expression of occludin, but also with changes of expression of other TJ proteins - ZO-1 and claudin (55) as well as Toll-like receptor 4 (39).

Regular chronic Ω-3 PUFA supplementation is well known for its reduction of several key risk factors causing cardiovascular diseases. Ω-3 PUFA decrease levels of lipids and blood pressure, attenuate thrombosis, modulate NO production and suppress the ROS production (19, 56), altogether contributing to the protection of endothelial function as well. Our previous studies have demonstrated the positive effects of chronic Ω-3 PUFA diet on expression of two connexin isoforms: Cx43 in the myocardium and endothelium of the aorta of hypertriglyceridemic and hypertensive rats (21, 24, 26) that was associated with protection of heart and aorta function. Our recent studies reported that 10-days-term Ω-3 PUFA diet modulated Cx40 expression in aortic endothelium and kidney Na, K-ATPase activity of the rats of LPS-P group (22, 23). Therefore, in the

present study, we expected protective effect of 10- day Ω-3 PUFA diet on occludin expression in the aorta of the LPS-P group. Surprisingly, Ω-3 PUFA diet had no effect on the occludin expression when compared to the LPS group. Our finding suggests the potential persistence of TJ dysfunction which might increase endothelial permeability. However, it contrasts with the reduced expression of CD68 in the aortic tissue that indicates the reparation of endothelial barrier function. The latter corresponds with Ω-3 PUFA-induced reduction of inflammation and oxidative stress. Data indicate that short-term PUFA diet might specifically influence different mechanisms regulating endothelial function and permeability.

Our results indicate that 10-day intake of Ω-3 PUFA could protect the function of the aorta. Ω-3 PUFA administration to the LPS rats (LPS-P group) did not improve the NO-dependent relaxation of aorta (22), nor protected the NOS activity, which actually incresed. The results do not enable the elucidation of this situation. However, Ω-3 PUFA are known with their various (healthy and toxic) effects and their co-existence could occur (57, 58). It was reported that the administration of Ω-3 PUFA prior a pathological insult for chronic period bettered the signs of inflammation and protected against the effects of endotoxin in some animal models (59). We applied Ω-3 PUFA at the same day as a pathological insult (LPS). Ω-3 PUFA are highly unsaturated and highly susceptible to the oxidation to form toxic oxidation products (60-62). At the end of the experiment we demonstrated partial reduction of inflammatory markers levels in the LPS-P group. It suggests the presence of a low inflammation, products of which might still affect Ω-3 PUFA to produce oxidative form products contributing to the local deterioration of endothelial function which is associated with elevated iNOS activity. That might partially explain increased aortic NOS activity in the LPS-P group. The results may also depend on the type of the used artery, the age of experimental animals (21, 63, 64), on the dose and the period of Ω-3 PUFA supplementation (65, 66) and the timing of Ω-3 PUFA application. The mechanisms underlying the effects of Ω-3 PUFA on the occludin expression and aortic function remain to be elucidated. Moreover, the farnesoid X receptors may represent another potential mechanism of Ω-3 PUFA involved in the modulation of NOS activity in the thoracic aorta (67).

Interestingly, Ω-3 PUFA reduced occludin expression in the aorta of the rats of P group as well. Now we are not able to explain this change but we suppose that it could be caused by an adaptation of aortic endothelial cells to the presence of Ω-3 PUFA. The mechanisms underlying the effects of Ω-3 PUFA on the occludin expression and aortic function remain to be elucidated.

In contrast to our results, protective effects of Ω-3 PUFA on TJ permeability and occludin expression were observed in human vascular endothelial cell line ECV304 (28) and intestinal monolayer cells (68). Similarly, fish oil protected intestinal barrier function damage by LPS through expression of occludin and TLR4 signalling pathway (29, 30).

At present we cannot explain our results completely. One of pathways which could modulate occludin expression in the aorta of the LPS-P group is the NOS system. It has been shown that the activation of iNOS isoform can reduce occludin expression (46). Further, Ω-3 PUFA form a part of cellular membrane, affecting its barrier properties as well (69-71). Li *et al* (72) demonstrated that incorporation of Ω-3 PUFA into membrane of epithelial cells, followed by TNF-α and IFN-γ incubation, reduced paracellular permeability of cells and prevented distortion of TJ morphology. Our results rather correspond with the data of Beguin *et al* (73), who observed, that incorporation of Ω-3 PUFA into the plasma membrane did not affect the presence of occludin in TJ complexes. Protective effects of Ω-3 PUFA on occludin expression have been demonstrated by Jiang *et al* (28) in human vascular endothelial cell, by Coquerel *et al* (30) in intestine after endotoxic shock or endothelial and/or epithelial cell cultures respectively, but not in the aorta. The aorta is a large conductive blood vessel and the reactivity and the sensitivity of its endothelial cells to Ω-3 PUFA treatment can differ from endothelial cells of smaller resistant arteries and/or cultured endothelial cells respectivelly. Different regulation could depend on *in vitro* and *in vivo* experiments, too. The effect of Ω-3 PUFA on occludin expression might depend on used concentration (56), time of their application and their adverse effects as well (74, 75). The results could also reflect an individual sensitivity. The exact mechanisms explaining the effect of Ω-3 PUFA and LPS on occludin expression need to be further elucidated.

Conclusion

Our results demonstrate that a single intraperitoneal injection of LPS to healthy Wistar rats had a long-term impact on the production of occludin protein in endothelium of rat aorta, indicating the involvement of occludin in the regulation of endothelial paracellular permeability of the aorta during the inflammation. Changes in occludin expression induced by LPS could have a novel prognostic value for aortic disease. 10-day-term Ω-3 PUFA diet of the rats of the LPS group did not affect the occludin expression, despite their partial anti-inflammatory effects. Data indicate that short-term PUFA diet might specifically influence different mechanisms regulating endothelial function and permeability.

Acknowledgement

This study was supported by VEGA grant no. 2/0022/16.

References

1. De Nardin E. The role of inflammatory and immunological mediators in periodontitis and cardiovascular disease. Annals of Periodontology. 2001; 6: 30–40.
2. Magrone T, Jirillo E. The impact of bacterial lipopolysaccharides on the endothelial system: pathological consequences and therapeutic countermeasures. Endocr Metab Immune Disord Drug Targets 2011; 11: 310-325.
3. Rubin LL. Endothelial cells: adhesion and tight junctions. Curr Opin Cell Biol. 1992; 4: 830-833.
4. Simionescu N, Simionescu M. Endothelial transport macromolecules: transcytosis and endocytosis. Cell Biology Rev 1991; 25: 5-80.
5. Li Q, Zhang Q, Wang M, Zhao S, Xu G, Li J. n-3 polyunsaturated fatty acids prevent disruption of epithelial barrier function induced by proinflammatory cytokines. Mol Immunol 2008; 45: 1356-1365.
6. Nusrat A, Parkos CA, Verkade P, Foley CS, Liang TW, Innis-Whitehouse W, *et al*. Tight junctions are membrane microdomains. J Cell Sci 2000; 113: 1771-1781.
7. Gumbiner BM. Cell adhesion: tha molecular basis of tissue architecture and morphogenesis. Cell 1996; 84: 345-357.
8. Fanning AS, Mitic LL, Anderson JM. Transmembrane proteins in the tight junction barrier. J Am Soc Nephrol 1999; 10: 1337-1345.
9. Tsukita S, Furuse M, Itoh M. Multifunctional strands in tight junctions. Nat Rev Mol Cell Biol 2001; 2: 285-293.
10. Turner JR. Putting the squeeze" on the tight junction:understanding cytoskeletal regulation. Semin Cell Dev Biol 2000; 11: 301-308.
11. Chai Q, He WQ, Zhou M, Lu H, Fu ZF. Enhancement of blood-brain barrier permeability and reduction of tight junction protein expression are modulated by chemokines/cytokines induced by rabies virus infection. J Virol 2014; 88: 4698-4710.
12. Hou Y, Wang L, Zhang W, Yang Z, Ding B, Zhu H, *et al*. Protective effects of N-acetylcysteine on intestinal functions of piglets challenged with lipopolysaccharide. Amino Acids 2012; 43: 1233-1242.
13. Zhou T, Zhao L, Zhan R, He Q, Tong Y, Tian X, *et al*. Blood-brain barrier dysfunction in mice induced by lipopolysaccharide is attenuated by dapsone. Biochem Biophys Res Commun 2014; 453:419-424.
14. Rochfort KD, Cummins PM. Cytokine-mediated dysregulation of zonula occludens-1 properties in human brain microvascular endothelium. Microvasc Res 2015; 100: 48-53.
15. Dohgu S, Fleegal-DeMotta MA, Banks WA. Lipopolysaccharide-enhanced transcellular transport of HIV-1 across the blood-brain barrier is mediated by luminal microvessel IL-6 and GM-CSF. J Neouroinflammation 2011; 8: 167-178.
16. Eadon MT, Hack BK, Xu C, Ko B, Toback FG, Cunningham PN. Endotoxemia alters tight junction gene and protein expression in the kidney. Am J Renal Physiol 2012; 303: F821-F830.
17. Sheth P, Delos Santos N, Seth A, LaRusso NF, Rao RK. Lipopolysaccharide disrupts tight junctions in cholangiocyte monolayers by a c-Src-, TLR4-, and LBP-dependent mechanism. Am J Physiol Gastrointest Liver Physiol 2007; 293:G08-G318.
18. Ambrozova G, Pekarova M, Lojek A. Effect of polyunsaturated fatty acids in the reactive oxigen and nitrogen speciesproduction by raw 254.7 macrophages. Eur J Nutr 2010; 49:133-139.
19. Demaison L, Moreau D. Dietary n-3 polyunsaturated fatty acids and coronary heart disease-related mortality: a possible mechanism of action. Cell Mol Life Sci 2002; 59: 463-477.
20. Das UN. Infection, inflammation, and polyunsaturated fatty acids. Nutrition 2011; 27:1080-1084.
21. Dlugosova K, Okruhlicova L, Mitasikova M, Sotnikova R, Bernatova I, Weisman P, *et al*. Modulation of connexin-43 by omega-3 fatty acids in the aorta of old spontaneously hypertensive rats. J Physiol Pharmacol 2009a; 60: 63-69.
22. Frimmel K, Vlkovicova J, Sotnikova R, Navarova J, Bernatova I, Okruhlicova L. The effect of omega-3 fatty acids on expression of connexin-40 in wistar rat aorta after lipopolysaccharide administration. J Physiol Pharmacol 2014; 65:83-94.
23. Mézešová L, Jendruchová-Javorková V, Vlkovičová J, Okruhlicová Ľ, Frimmel K, Navarová J, *et al*. Supplementation with n-3 polyunsaturated fatty acids to lipopolysaccharide-induced rats improved inflammation and functional properties of renal Na,K-ATPase. Nutr Res 2013; 33:772-779.
24. Dlugosova K, Weisman P, Bernatova I, Sotnikova R, Slezak J, Okruhlicova L. Omega-3 fatty acids and atorvastatin affects connexin 43 expression in the aorta of hereditary hypertriglyceridemic rats. Can J Physiol Pharmacol 2009b; 87:1074-1082.
25. Bacova B, Radosinska J, Knezl V, Kolenova L, Weismann P, Navarova J, *et al*. Omega-3 fatty acids and atorvastatin suppress ventricular fibrillation inducibility in hypertriglyceridemic rat hearts: implication of intercellular coupling protein, connexion-43. J Physiol Pharmacol 2010; 61:717-723.
26. Radosinska J, Bacova B, Bernatova I, Navarova J, Zhukovska A, Shysh A, *et al*. Myocardial NOS activity and connexion-43 expression in untreated and omega-3 fatty acids-treated spontaneously hypertensive and hereditary hypertriglyceridemic rats. Mol Cell Biochem 2011; 347:163-173.
27. Radosinska J, Bacova B, Knezl V, Benova T, Zurmanova J, Soukup T, *et al*. Dietary omega-3 fatty acids attenuate myocardial arrhythmogenic factors and propensity of the heart to lethal arrhythmias in a rodent model of human essential hypertension. J Hypertens 2013; 31:1876-1885.
28. Jiang WG, Bryce RP, Horrobin DF, Mansel RE. Regulation of tight junction permeability and occludin expression by polyunsaturated fatty acids. Biochem Biophys Res Commun 1998; 244:414-420.
29. Liu Y, Chen F, Odle J, Lin X, Jacobi SK, Zhu H, *et al*. Fish oil enhances intestinal integrity and inhibits TLR4 and NOD2 gignaling pathways in weaned pigs after LPS challenge. J Nutr 2012; 142:2017-2024.
30. Coquerel D, Kušíková E, Mulder P, Coëffier M, Renet S, Dechelotte P, *et al*. Omega-3 polyunsaturated fatty acids delay the progression of endotoxemic shock-

induced myocardial dysfunction. Inflammation 2013; 36:932-940.
31. Nielsen F, Mikkelsen BB, Nielsen JB, Andersen HR, Grandjean P. Plasma malondialdehyde as biomarker for oxidative stress: reference interval and effects of life-style factors. Clin Chem 1997; 43:1209-1214.
32. Draper HH, Hadley M. Malondialdehyde determination as index of lipid peroxidation. Methods Enzymol 1990; 186:421-431.
33. Bernatova I, Pechanova O, Babal P, Kyselá S, Stvrtina S, Andriantsitohaina R. Wine polyphenols improve cardiovascular remodeling and vascular function in NO-deficient hypertension. Am J Physiol 2002; 282:H942-H948.
34. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72:248-254.
35. Dlugosová K, Mitasíková M, Bernátová I, Weismann P, Okruhlicová L. Reduced connexion-43 expression in the aorta of prehypertensive rats. Physiol Res 2008; 57:S23-S29.
36. Morris M, Li L. Molecular mechanisms and pathological consequences of endotoxin tolerance and priming. Arch Immunol Ther Exp 2012; 60:13-18.
37. Steib CJ, Hartmann AC, Hesler C, Benesic A, Hennenberg M, Blitzer M, *et al*. Intraperitoneal LPS amplifies portal hypertension in rat liver fibrosis. Lab Invest 2010; 90:1024-1032.
38. Krueger M, Härting W, Reichenbach A, Bechmann I, Michalski D. Blood-brain barrier breakdown after embolic stroke in rats occurs without ultrastructural evidence for disrupting tight junctions. PLoS ONE 2013; 8:e56419.
39. Wen B, Combes V, Bonhoure A, Weksler BB, Couraud PO, Grau GER. Endotoxin-induced monocytic microparticles have contrasting effects on endothelial inflammatory responses. PLoS ONE 2014; 9:e91597.
40. Guo S, Al-Sadi R, Said HM, Ma TY. Lipopolysaccharide causes an increase in intestinal tight junction permeability in vitro and in vivo by inducing enterocyte membrane expression and localization of TLR-4 and CD14. Am J Pathol 2013; 182:375-387.
41. Abdullah Z, Bayraktutan U. NADPH oxidase mediates TNF-α-evoked in vitro brain barrier dysfunction: roles of apoptosis and time. Mol Cell Neurosci 2014; 61:72-84.
42. Sánchez FA, Rana R, González FG, Iwahashi T, Durán RG, Fulton DJ, *et al*. Functional significance of cytosolic endothelial nitric-oxide synthase (eNOS). J Biol Chem 2011; 286:30409-30414.
43. Muller B, Kleschyov AL, Gyorgy K, Stoclet JC. Inducible NO synthase activity in blood vessels and heart: new insight into cell origin and consequences. Physiol Res 2000; 49:19-26.
44. Chauhan SD, Seggara G, Vo PA, Macallister RJ, Hobbs AJ, Ahluwalia A. Protection against lipopolysaccharide-induced endothelial dysfunction in resistance and conduit vasculature of iNOS knockout mice. FASEB J 2003; 17:773-775.
45. Kang WS, Tamarkin FJ, Wheeler MA, Weiss RM. Rapid up-regulation of endothelial nitric-oxide synthase in a mouse model of Escherichia coli lipopolysaccharide-induced bladder inflammation. J Pharmacol Exp Ther 2004; 310:452-458.
46. Han X, Fink MP, Yang R, Delude RL. Increased iNOS activity is essential for intestinal epithelial tight junction dysfunction in endotoxemic mice. Shock 2004; 21: 261-270.
47. Leal EC, Manivannan A, Hosoya K, Terasaki T, Cunha-Vaz J, Ambrósio AF, *et al*. Inducible nitric oxide synthase isoform is a key mediator of leukostasis and blood-retinal barrier breakdown in diabetic retinopathy. Invest. Ophthalmol Vis Sci 2007; 48: 5257-5265.
48. Yang S, Chen Y, Deng X, Jiang W, Li B, Fu Z, *et al*. Hemoglobin-induced nitric oxide synthase overexpression and nitric oxide production contribute to blood-brain barrier disruption in the rat. J Mol Neurosci 2013; 51:352-363.
49. Takizawa Y, Kishimoto H, Kitazato T, Tomita M, Hayashi M. Effects of nitric oxide on mucosal barrier dysfunction during early phase of intestinal ischemia/reperfusion. Eur J Pharm Sci 2011; 42:246-252.
50. Feng Y, Hu L, Xu Q, Yuan H, Ba L, He Y, *et al*. Cytoprotective role of alpha-1 antitrypsin in vascular endothelial cell under hypoxia/reoxygenation condition. J Cardiovasc Pharmacol 2015; 66:96-107.
51. Molina-Jijón E, Rodríguez-Munoz R, Namorado MC, Pedraza-Chaverri J, Reyes JL. Oxidative stress induces claudin-2 nitration in experimental type 1 diabetic nephropathy. Free Radic Biol Med 2014; 72:162-175.
52. Lochhead JJ, McCaffrey G, Quigley CE, Finch J, DeMarco KM, Nametz N, *et al*. Oxidative stress increases blood-brain barrier permeability and induces alterations in occludin during hypoxia-reoxygenation. J Cereb Blood Flow Metab 2010; 30:1625-1636.
53. Jamaluddin MS, Yan S, Lü J, Liang Z, Yao Q, Chen Ch. Resistin increases monolayer permeability of human coronary artery endothelial cells. PLoS ONE 2013; 8:e84576.
54. Nagasawa K, Chiba H, Fujita H, Kojima T, Saito T, Endo T, *et al*. Possible involvement of gap junctions in the barrier function of tight junctions of brain and lung endothelial cells. J Cell Physiol 2006; 208:123-132.
55. Zhang Y, Li J. Carbachol ameliorates lipopolysaccharide-induced intestinal epithelial tight junction damage by down-regulating NF-κβ and myosin light-chain kinase pathways. Biochem Bioph Res Co 2012; 428:321-326.
56. Loustarinen R, Saldeen T. Dietary fish oil decreases superoxide generation by human neutrophils: relation to cyclooxygenase pathway and lysosomal enzyme release. Prostaglandins Leukot Essent Fatty Acids 1996; 55:167-172.
57. Di Nunzio M, Valli V, Bordoni A. Pro- and anti-oxidant effects of polyunsaturated fatty acid supplementation in HepG2 cells. Prostaglandins Leukot Ess Fatty Acids 2011; 85:121-127.
58. Serini S, Fasano E, Piccioni E, Cittadini AR, Calviello G. Dietary n-3 polyunsaturated fatty acids and the paradox of their health benefits and potential harmful effects. Chem Res Toxicol 2011; 24:2093-20103.
59. Calder PC. Dietary modification of inflammation with lipids. Proc Nutr Soc 2002; 61:345-358.
60. Esterbauer H. Cytotoxicity and genotoxicity of lipid oxidation products. Am J Clin Nutr 1993; 57 (Suppl. 5): 779-785.

61. Halvorsen BL, Blomhoff R. Determination of lipid oxidation products in vegetable oils and marine omega-3 supplements. Food Nutr Res 2011; 55: doi: 10.3402/fnr.v55i0.5792.
62. Awada M, Soulage CO, Meynier A, Debard C, Plaisancié P, Benoit B, *et al*. Dietary oxidized n-3 PUFA induce oxidative stress and inflammation: role of intestinal absorption of 4-HHE and reactivity in intestinal cells. J Lipid Res 2012; 53: 2069-2080.
63. Gourdin MJ, Ponchau O, Jamart J, De Kock M. Ageing influences the effect of pre-hypoxic administration of clonidine, an alpha2-adrenoceptor agonist, on post-hypoxic vasomotricity. J Physiol Pharmacol 2012; 63: 165-171.
64. Sotnikova R, Okruhlicova L, Dlugosova K, Bernatova I, Bezek Š, Navarova J, *et al*. Effect of n- 3 PUFA on endothelium-dependent relaxation of the 93 superior mesenteric artery. In: Tribulova N, Okruhlicova L, Slezak J, editors. Proceedings of Experimental Approaches in Basic Research and Diagnostic of Diseases: Tailoring the Treatment. Bratislava: VEDA 2008. pp.127-134.
65. de Lima TM, de Sa Lima L, Scavone C, Curi R. Fatty acids control of nitric oxide production by macrophages. FEBS Lett 2006; 580: 3287-3295.
66. Mullen A, Loscher CE, Roche HM. Anti-inflammatory effects of EPA and DHA are dependent upon time and doseresponse elements associated with LPS stimulation in THP- 1-derived macrophages. J Nutr Biochem 2010; 21: 444-450.
67. Zhang R, Ran HH, Zhang YX, Liu P, Lu CY, Xu Q, *et al*. Farnesoid X receptor regulates vascular reactivity through nitric oxide mechanism. J Physiol Pharmacol 2012; 63: 367-372.
68. Usami M, Muraki K, Iwamoto M, Ohata A, Matsushita E, Miki A. Effect of eicosapentaenoic acid (EPA) on tight junction permeability in intestinal monolayer cells. Clin Nutr 2001; 20:351-359.
69. Coyne CB, Kelly MM, Boucher RC, Johnson LG. Enhanced epithelial gene transfer by modulation of tight junctions with sodium caprate. Am J Respir Cell Mol Biol 2000; 23:602-609.
70. Roche HM, Terres AM, Black IB, Gibney MJ, Kelleher D. Fatty acids and epithelial permeability: effect of conjugated linoleic acid in Caco-2 cells. Gut 2001; 48: 797-802.
71. Sawai T, Drongowski RA, Lampman RW, Coran AG, Harmon CM. The effect of phospholipids and fatty acids on tight-junction permeability and bacterial translocation. Pediatr Surg Int 2001; 17:269-274.
72. Li Q, Zhang Q, Zhang M, Wang C, Zhu Z, Li N, *et al*. Effect of n-3 polyunsaturated fatty acids on membrane microdomain localization of tight junction proteins in experimental colitis. FEBS J 2008; 275:411-420.
73. Beguin P, Errachid A, Larondelle Y, Schneider YJ. Effect of polyunsaturated fatty acids on tight junctions in a model of the human intestinal epithelium under normal and inflammatory conditions. Food Funct 2013; 4:923-931.
74. Di Nunzio M, Valli V, Bordoni A. Pro- and anti-oxidant effects of polyunsaturated fatty acid supplementation in HepG2 cells. Prostaglandins Leukot Essent Fatty Acids 2011; 85:121-127.
75. Serini G, Bussolino F, Maione F, Giraudo E. Class 3 semaphorins: physiological vascular normalizing agents for anti-cancer therapy. J Intern Med 2013; 273: 138-155.