

Omega-3 Fatty Acids Reduce Lipopolysaccharide-Induced Abnormalities in Expression of Connexin-40 in Aorta of Hereditary Hypertriglyceridemic Rats

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Summary

Omega-3 fatty acids (Ω 3FA) are known to reduce hypertriglyceridemia- and inflammation-induced vascular wall diseases. However, mechanisms of their effects are not completely clear. We examined, whether 10-day Ω 3FA diet can reduce bacterial lipopolysaccharide-induced changes in expression of gap junction protein connexin40 (Cx40) in the aorta of hereditary hypertriglyceridemic (hHTG) rats. After administration of a single dose of lipopolysaccharide (LPS, 1 mg/kg, i.p.) to adult hHTG rats, animals were fed with Ω 3FA diet (30 mg/kg/day) for 10 days. LPS decreased Cx40 expression that was associated with reduced acetylcholine-induced relaxation of aorta. Ω 3FA administration to LPS rats had partial anti-inflammatory effects, associated with increased Cx40 expression and improved endothelium dependent relaxation of the aorta. Our results suggest that 10-day Ω 3FA diet could protect endothelium-dependent relaxation of the aorta of hHTG rats against LPS-induced damage through the modulation of endothelial Cx40 expression.

Key words

Lipopolysaccharide • Omega-3 fatty acids • Connexin-40 • Hypertriglyceridemia • Rat aorta

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Introduction

Vascular intercellular gap junctions are transmembrane channels allowing passage of ions and small metabolites and molecules (<1 kDa) directly between two adjacent cells synchronizing cell functions along vascular wall (Figuroa and Duling 2009). Gap junctions are formed by two hemichannels (connexons). Each connexon consists from six connexin proteins (Cx) and can exist in an unpaired state as well (Perkins *et al.* 1998). Vascular cells express four types of Cx isoforms (of the 21-member family of Cxs) (Söhl and Willecke 2004). Their expression is dependent on the type of blood vessels and cells as well as animal species (Van Kempen and Jongsma 1999). Pathophysiological conditions-induced disturbances in expression of Cx isoforms reported in many studies (Burnier *et al.* 2009, Dluhošová *et al.* 2009a,b, Laird 2010) underline participation of gap junctions in a development of vascular wall injury and their potential role in treatment of vascular disorders. Cx40 is a major connexin expressed in endothelial cells (Brisset *et al.* 2009). Abnormalities in its expression observed during hypertriglyceridemia (Okruhlicová *et al.* 2012) as well as during atherosclerotic plaque development (Kwak *et al.* 2002) signify the role of Cx40-channels in a development of endothelial dysfunction which is generally accepted as a key step in development of vascular wall damage (Okumura *et al.* 2005).

Atherosclerosis is recognized as a multifactorial vascular disease, associated with the inflammation during all its phases. It was reported that Gram-negative bacteria might represent one of the risk factors involved in the initiation and/or progression of atherosclerosis (Al-Banawi *et al.* 2011, Libby and Simon 2001). The response of body to bacteria or bacterial lipopolysaccharide (LPS) is known to be associated with overproduction of various inflammatory mediators and oxidative stress which cause endothelial dysfunction (Guzik *et al.* 2003, Sprague and Khalil 2009). There are numerous studies, including ours, which demonstrate that LPS and/or inflammatory mediators influenced expression of Cx isoforms and GJ function in vascular wall (Rignault *et al.* 2005, 2007, Frimmel *et al.* 2014) and circulating leukocytes, as well (Oviedo-Orta *et al.* 2002, Wong *et al.* 2004). All data emphasize the participation of Cx-channels in modulation of vascular wall response during inflammation. Moreover, it was reported that LPS administration can increase plasma levels of triglycerides (TG) and TG-rich lipoproteins (Uchiumi *et al.* 2004). Hypertriglyceridemia (HTG) represents an independent risk factor for atherosclerosis, therefore LPS-induced elevation of TG can represent additional risk for atherosclerosis in subjects with inherited and/or acquired dyslipidemia (Feingold *et al.* 1992).

Hereditary HTG (hHTG) rats were selectively bred as an experimental model for study of mechanisms of human HTG (Vrana and Kazdova 1990). In this model, elevated levels of TG are associated with a moderate hypertension, metabolic disorder of glucose and cholesterol as well as impaired function and structure of cardiovascular system (Zicha *et al.* 2006). The hHTG-related abnormalities in expression of Cx40 and Cx43 isoforms found in the rat aorta and the myocardium (Dlugošová *et al.* 2009b, Bacova *et al.* 2010, Radosinska *et al.* 2011, Okruhlicova *et al.* 2012) indicate the involvement of Cx-channels in the above mentioned disturbances of cardiovascular system of hHTG subjects. However, according to our knowledge, the effects of LPS on Cx expression in a vascular system of HTG subjects have not been studied yet.

Beneficial effects of TG lowering in reduction of atherosclerosis and cardiovascular diseases have been observed in numerous clinical trials. Lifestyle influences, including omega-3 fatty acids (Ω 3FA) rich diet, play primary strategies in effective reduction in the incidence of cardiovascular diseases. Ω 3FA are well-known for their multiple protective effects, e.g. reducing of plasma lipid levels, inflammation and oxidative stress, improving

of NO production, endothelial function (Calder 2012) and renal Na/K-ATPase activity (Mézešová *et al.* 2013) as well as modulating of physical structure of cellular membranes and their ion currents (Pirillo and Catapano 2013). Our previous studies showed that chronic Ω 3FA supplementation caused modulation of Cx43 expression in the aorta and myocardium of hHTG rats (Dlugošová *et al.* 2009b, Bacova *et al.* 2010, Radosinska *et al.* 2011). Our recent work showed that also short-term Ω 3FA diet can affect LPS-induced disturbances of Cx40 expression in the aorta of rats (Frimmel *et al.* 2014). The results together indicate the involvement of Cx-channels in cardioprotective mechanisms of Ω 3FA. However, it has not been studied yet if short-term Ω 3FA supplementation of hHTG rats can protect Cx40 expression in a vascular system after LPS challenge. Therefore, the aim of the present study was to examine the influence of LPS and Ω 3FA on the expression and distribution of Cx40 in the aorta of hHTG rats.

Materials and Methods

Animal model

The experiments were performed in accordance with the rules issued by the State Veterinary Administration of the Slovak Republic, Act No. 377/2012 Coll. and with the rules of the Animal Research and Care Committee of Institute for Heart Research SAS. The animals were anesthetized with thiopental (50 mg/kg, i.p.) and the aorta was excised for functional, biochemical and microscopic studies.

In 10-day lasting experiment, 3-month-old hHTG male rats (270-300 g) were used. They were divided into four groups (n=6): 1) the control group (C) – rats fed a standard diet, 2) the Ω 3 group – rats fed a standard diet enriched with Ω 3FA (30 mg/kg/day), 3) the L group – rats injected with a single dose of LPS (1 mg/kg, i.p.) and 4) the L Ω 3 group – the L rats fed a diet enriched with Ω 3FA. LPS (*Escherichia coli* serotype 055:B5, Sigma Chemical, Germany) was dissolved in sterile 0.9 % NaCl solution. The rats from the groups 1) and 2) were injected with the same volume of sterile 0.9 % NaCl solution. Ω 3FA consisted of 57 % eicosapentaenoic acid and 43 % docosahexaenoic acid (commercial nutritional supplement of MaxiCor, SVUS Pharma, Czech Republic).

Measurement of physiological and biochemical parameters

Body weight and systolic blood pressure were

measured at the beginning and at the end of the experiment. Blood pressure was measured by non-invasive method of tail-cuff plethysmography using the device ML866/P PowerLab4/30 (AD Instruments GmbH, Germany). To reduce the influence of stress on blood pressure, the animals were handled and acclimatized according to Dlugosova *et al.* (2009a). Each measurement was repeated five to six times in one rat.

At the end of the experiment, blood samples were collected for plasma separation which was stored at -82°C until the further analyses. Plasma levels of high sensitive C-reactive protein (CRP, marker of acute inflammation), TG and high-density lipoproteins (HDL) were measured using commercial clinical Cholestech LDX system (Cholestech Corporation, California, USA). The lactate dehydrogenase activity (LDH, marker of cellular and organ damage, cytotoxicity and hemolysis (Peppes *et al.* 2008) as well as acute inflammation) was measured in plasma using Randox kit (Laboratories Ltd., UK). The specific activity of lysosomal N-acetyl- β -D-glucosaminidase (NAGA, marker of lysosomal cellular damage and chronic phase of inflammation) (Krammer *et al.* 2006) was assayed in plasma according to standard method as described previously in Navarova and Nosalova (1994). The concentration of malondialdehyde (MDA, marker of lipid peroxidation) (Draper and Hadley 1989) was measured in liver, too.

Thoracic aorta was quickly excised and washed in a chilled saline, carefully cleaned from connective tissue and fat and used for the relaxation response study. Further part of the aortic tissue was stored at -82°C for immunofluorescent (IF) and Western blotting (WB) analyses and the nitric oxide synthase (NOS) activity measurement.

NO synthase activity and functional responses of the aorta

The activity of NOS was assayed in a homogenate of the aortic tissue by determination of $[3\text{H}]\text{-L-citrulline}$ formation from $[3\text{H}]\text{-L-arginine}$ (MP, Biomedicals) as described previously in Bernatova *et al.* (2002). We studied also functional relaxation of the aorta according to Sotnikova *et al.* (2006).

Immunofluorescent detection of Cx40

Indirect *in situ* immunodetection of Cx40 was performed on frozen cross-sections of the non-fixed aortic rings of the rats of all experimental groups according to Frimmel *et al.* (2014) with a minor modification concerning antibodies concentration. Shortly, fixed

cryostat tissue sections of the aorta were incubated in the primary rabbit anti-Cx40 (Santa-Cruz, USA, 1:300) and the secondary anti-rabbit antibody (Johnson ImmunoResearch, USA, 1:300) labeled with fluorescein isothiocyanate (FITC). Immunofluorescent signal of Cx40 was monitored by confocal microscope Olympus FV1000. Obtained images were evaluated by morphometric analysis (Image J, Olympus). The total area of IF reaction per unit area of the aorta, together with the density of Cx40 spots and their average area were analyzed for demonstration of the spatial expression of Cx40. Seven sections per the rat aorta and eleven fields per the section were visualized and analyzed.

Western blot analysis of Cx40 and CD68

The frozen aortic tissue for Western blot was homogenized in SB20 (1 mg of tissue/10 ml of SB20 solution) and sonicated with a device UP 100H (Hielscher, Germany). The samples were diluted to the desired concentration by electrophoresis sample buffer and run on 10 % polyacrylamide gel. After electrophoresis, the proteins were transferred to a nitrocellulose membrane. It was incubated in the blocking buffer consisting of 5 % milk for 30 min and incubated in the rabbit monoclonal primary antibodies anti-Cx40 and anti-CD68 (marker of macrophage receptors) both at dilution 1:1000, followed by the incubation in peroxidase labeled with secondary polyclonal anti-rabbit antibody at a dilution 1:2000. The detection was performed using luminol system (chromophore), coumaric acid in a ratio of 3:1. Obtained chemiluminescent reaction was recorded by the device "KODAK in vivo FX" and evaluated by "Carestream SE". The result was given as the sum of grayscale intensity of each protein band and was normalized to GAPDH as an endogenous control.

Results

Physiological and biochemical parameters

Our results showed that LPS significantly reduced body weight gain of hHTG rats when compared with the C group (Table 1). 10-day Ω 3FA supplementation had no apparent influence on the body weight gain in the Ω 3 group. There was observed only a tendency towards a decreased weight gain. Ω 3FA diet had no significant effect on body weight gain in the L Ω 3 group compared to the L one. No significant changes were observed in relative heart weight, systolic blood pressure and heart rate among the experimental groups (Table 1).

Table 1. The effect of LPS and Ω 3FA on selected biometric parameters.

	C	Ω 3	L	L Ω 3
BWG (g)	21±9	14±4	6±3*	4±4
HW (g)	1.01±0.01	0.99±0.02	1.002±0.047	0.94±0.01
HW/BW	3.3±0.2	3.7±0.4	3.5±0.2	3.3±0.2
SBP α (mm Hg)	114.0±12.4	117.5±3.9	124.5±9.9	110.7±6.7
SBP ω (mm Hg)	121.0±4.6	112.7±4.5	116.8±6.1	103.1±3.0
HR ω (bpm)	305±30	291±32	332±14	286±21

Results are expressed as mean \pm SEM, n=6 animals per group, * p<0.05 vs. C. BWG – body weight gain, HW – heart weight, HW/BW – relative heart weight, SBP α /SBP ω – systolic blood pressure at the start/the end of the experiment, HR ω – heart rate at the end of experiment.

Table 2. The effects of LPS and Ω 3FA on selected biomarkers measured in plasma.

	C	Ω 3	L	L Ω 3
TG (mmol/l)	1.65±0.12	2.17±0.1*	2.01±0.11*	1.84±0.11
HDL (mmol/l)	0.73±0.05	0.66±0.04	0.77±0.05	0.76±0.05
Glu (mmol/l)	11.5±0.2	11.1±0.3	10.8±0.2	10.6±0.2
NAGA (μ g/min/mg protein)	0.059±0.004	0.061±0.005	0.087±0.008*	0.088±0.006*
LDH (U/l)	787±61	1010±230	1188±148*	943±117
CRP (mmol/l)	<0.30	<0.30	<0.30	<0.30

Results are expressed as mean \pm SEM, n=6 animals per group, * p<0.05 vs. C. TG – triglycerides, HDL – high density lipoprotein, Glu – glucose, NAGA – N-acetyl- β -D-glucosaminidase, LDH – lactate dehydrogenase, CRP – C-reactive protein.

Table 3. The effects of LPS and Ω 3FA on the activity of N-acetyl- β -D-glucosaminidase (NAGA) and malondialdehyde (MDA) in liver.

	C	Ω 3	L	L Ω 3
NAGA (μ g/min/mg protein)	12.03±0.25	12.27±0.62	14.04±0.52*	12.76±0.19*#
MDA (μ g/mg protein)	1.33±0.039	1.57±0.183	2.24±0.371*	1.52±0.198#

Results are expressed as mean \pm SEM, n=6 animals per group, * p<0.05 vs. C, # p<0.05 vs. L.

LPS significantly increased TG plasma levels in the L group. Surprisingly, TG concentration in Ω 3 group was also higher than in the controls (Table 2). Ω 3FA treatment in the L Ω 3 group did not markedly affect TG levels when compared to the L group, but there was a trend towards a decrease. No changes in HDL and glucose levels were observed among experimental groups after LPS and Ω 3FA administration. On the other hand, the specific activity of NAGA was higher in the L group than in the C group. Ω 3FA supplementation of the L Ω 3 rats did not change the enzyme activity when compared to the L group. No significant differences in the activity of NAGA were observed between Ω 3 and C groups. LPS resulted in a significant elevation of the LDH activity. No

differences in the enzyme activity were demonstrated between the L and L Ω 3 groups. There was seen a tendency towards a decline in the L Ω 3 one. The measurement of the CRP concentration revealed no differences among the experimental groups (Table 2). In liver, LPS caused increase of the MDA concentration when compared to the C group. Ω 3FA diet suppressed the MDA levels in the rats of the L Ω 3 group (Table 3). Ω 3FA supplementation did not affect MDA in Ω 3 group. LPS resulted in marked increase of the NAGA specific activity in liver (Table 3). Ω 3FA diet reduced the enzyme activity in the L Ω 3 group. Ω 3FA had no effect on the enzymes activity compared to the C group.

NO synthase activity and vascular response of the aorta

LPS caused the significant increase of the NOS activity when compared to the C group (Fig. 1). 10-day Ω 3FA supplementation of the L Ω 3 rats resulted in the additional elevation of the enzyme activity when compared to the L and the C groups. No differences in the NOS activity were found between the Ω 3 group and the C group.

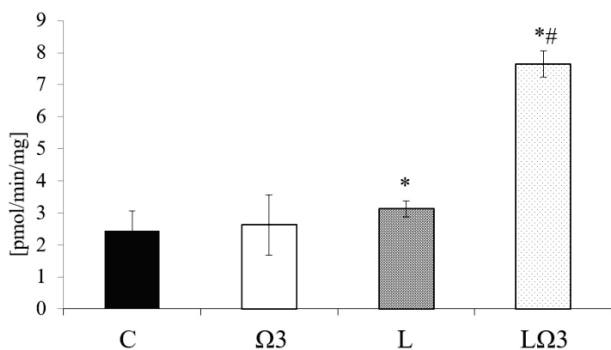


Fig. 1. The NO-synthase activity in the rat aorta. Results are mean \pm SEM, n=6 animals per group, * p<0.05 vs. C, # p<0.05 vs. L. C – control hHTG rats, Ω 3 – hHTG rats treated with Ω 3FA, L – hHTG rats injected with LPS, L Ω 3 – hHTG rats injected with LPS treated with Ω 3FA.

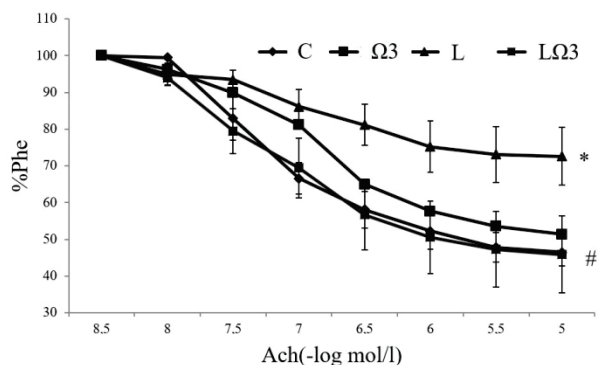


Fig. 2. The effect of LPS and Ω 3FA on the endothelium-dependent relaxation of the aorta. C – control hHTG rats, Ω 3 – hHTG rats treated with Ω 3FA, L – hHTG rats injected with LPS, L Ω 3 – hHTG rats injected with LPS treated with Ω 3FA, Phe – phenylephrine. * p<0.05 vs. C, # p<0.05 vs. L.

LPS administration resulted in the significant impairment of endothelium-dependent relaxation of the aorta compared to the C group (Fig. 2). In contrary, NPS-induced relaxation was not changed (Fig. 3). Ω 3FA diet administered to the L rats caused the evident restoration of the ACh-induced modulation of aortic relaxation to the control level. Ω 3FA had no significant effect on the aortic relaxation response when compared to the C group.

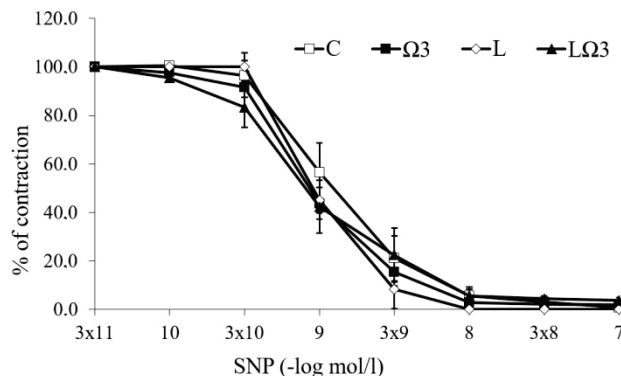


Fig. 3. The effect of LPS and Ω 3FA on the endothelium-independent relaxation of the aorta. C – control hHTG rats, Ω 3 – hHTG rats treated with Ω 3FA, L – hHTG rats injected with LPS, L Ω 3 – hHTG rats injected with LPS treated with Ω 3FA, SNP – sodium nitroprusside.

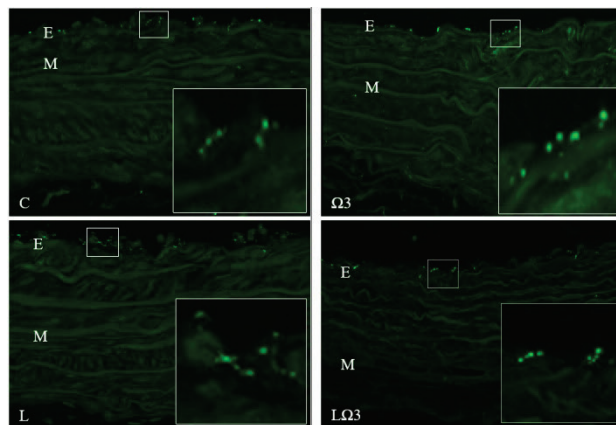


Fig. 4. Representative figures of Cx40 immunolabeling in endothelium (E) of the aorta of the control group (C), the inflammatory group (L), the control treated with Ω 3FA (Ω 3) and the treated inflammatory group (L Ω 3), M – media. n=6 animals per group. Original magnification: x40.

Immunofluorescent detection of Cx40

Immunofluorescence (IF) revealed the distribution of Cx40 in endothelium of the aorta of all rats (Fig. 4). The morphometric analysis demonstrated that LPS reduced both the density (Fig. 5) and the total IF area of Cx40 (Fig. 6) when compared to the C group. Ω 3FA supplementation of the rats injected with LPS increased the spots density and the total IF reaction compared to the L group. However, the values were still lower than in the C group. Ω 3FA diet did not markedly reduce the density of Cx40 spots, but it significantly suppressed the total IF reaction of Cx40 in the Ω 3 group. No differences were observed among the experimental groups concerning the average size of Cx40 spots (Table 4).

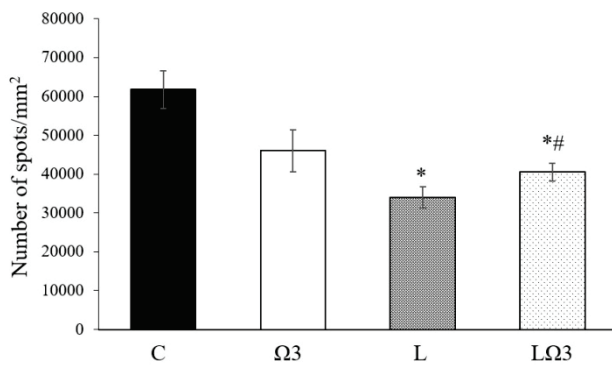


Fig. 5. Morphometric analysis of density of Cx40 spots in endothelium of the aorta control rats (C), treated control rats (Ω3), inflammatory rats (L) and treated inflammatory rats (LΩ3). Results are expressed as mean ± SEM, n=6 animals per group, * p<0.05 vs. C, # p<0.05 vs. L.

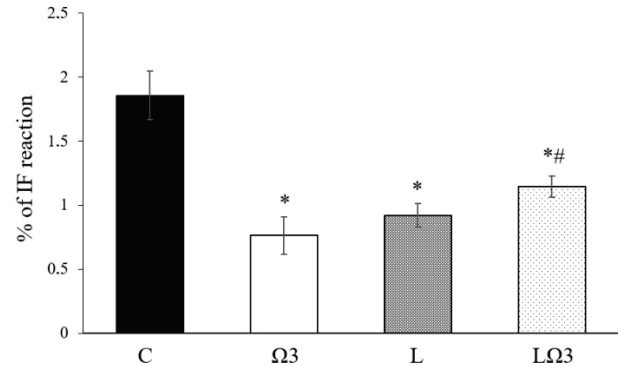


Fig. 6. Morphometric analysis of total immunofluorescent area of Cx40 spots in endothelium of the aorta of experimental rats. Control rats (C), treated control rats (Ω3), inflammatory rats (L) and treated inflammatory rats (LΩ3). Results are expressed as mean ± SEM, n=6 animals per group, * p<0.05 vs. C. # p<0.05 vs. L, Ω3.

Table 4. Average size of single Cx40 spot in the endothelium of the rat aorta of controls (C), treated controls (Ω3), inflammatory group (L) and treated inflammatory group (LΩ3).

	C	Ω3	L	LΩ3
Average size of spots (μm^2)	0.291±0.009	0.273±0.014	0.262±0.008	0.274±0.007

n=6 animals per group.

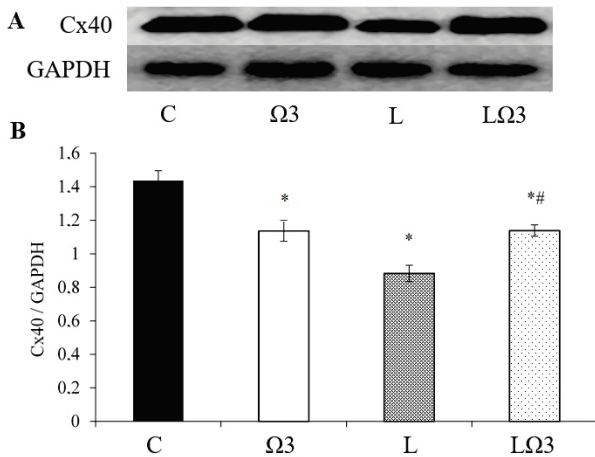


Fig. 7. A) Representative immunoblot of Cx40 in hHTG rat aorta. **B)** Quantitative evaluation of total Cx40 expression normalized to protein GAPDH in the control rats (C), the treated control (Ω3), the inflammatory group (L) and the treated inflammatory group (LΩ3). Results are expressed as mean ± SEM, n=6 animals per group, * p<0.05 vs. C, # p<0.05 vs. L.

Western blot analysis of Cx40 and CD68

Cx40 expression measured in the aortic tissue corresponded with the morphometric evaluation. LPS application resulted in down-regulation of Cx40 expression in the L group when compared to the C group (Fig. 7). Ω3FA treatment of the rats in the LΩ3 group resulted in up-regulation of Cx40 expression compared to the L one, but the levels of Cx40 protein remained

significantly lower than in the C group. In the Ω3 group, Cx40 expression was lower than in the C one.

Western blot revealed significant increase of CD68 expression in the L group (Fig. 8). Ω3FA treatment did not affect CD68 expression in the LΩ3 group compared to the L group. Ω3FA did not change CD68 expression in the Ω3 when compared to the C group.

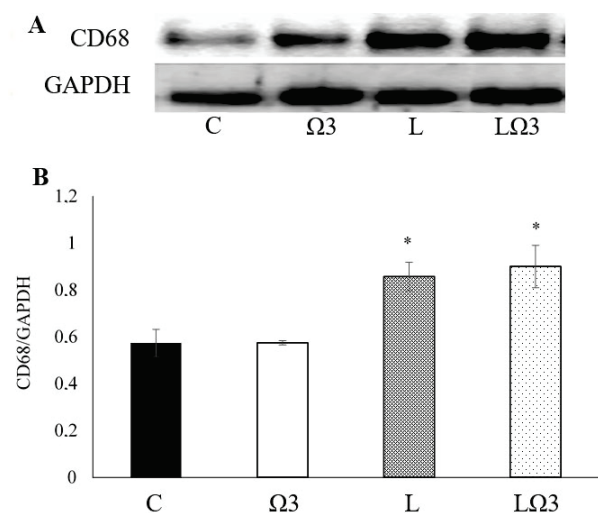


Fig. 8. A) Representative Western blot of CD68 in hHTG rat aorta. **B)** Quantitative evaluation of CD68 macrophage receptor expression normalized to protein GAPDH. C – control hHTG rats, Ω3 – hHTG rats treated with Ω3FA, L – hHTG rats injected with LPS, LΩ3 – hHTG rats injected with LPS and treated with Ω3FA. Results are expressed as mean ± SEM. * p<0.05 vs. C.

Discussion

In the present work we demonstrated that 10-day Ω 3FA supplementation suppressed LPS-induced abnormalities in the aorta of hHTG rats: it improved Cx40 expression associated with endothelium-dependent relaxation of the aorta.

We used the same experimental model as in our other study, in which LPS was applied to healthy Wistar rats (Frimmel *et al.* 2014). The used dose of LPS was lower than the dose inducing sepsis and death (Berczi *et al.* 1966, Thomas *et al.* 2014) and did not cause any mortality of the experimental animals.

LPS did not affect measured physiological parameters (blood pressure, heart rate and heart weight), but it caused reduction of body weight gain (Table 1). The changes are in accordance with data of Valles *et al.* (2000) who demonstrated that also a single exposure of rats to stressors, including LPS, can cause marked and lasting changes in food intake in different ways.

LPS injected to hHTG rats increased TG levels when compared to the control hHTG rats that correspond with data of other studies (Feingold *et al.* 2009). Our results thus support that LPS is an additional risk factor for individuals with HTG. On the other hand, TG and chylomicrons were reported to neutralize the toxic effects of LPS in circulation and significantly improved survival of endotoxemic rats even after lethal LPS dose (Harris *et al.* 1993, Read *et al.* 1995). This ability of TG and chylomicrons might at least partially explain our result concerning no changes of plasma CRP levels observed after LPS application. Oxidative stress represents one of main factors contributing to damage of endothelial function. In order to verify the contribution of oxidative stress in the effects of LPS, we measured levels of MDA. Its enhanced concentration indicates increased lipid peroxidation and oxidative stress which could change endothelial function.

In our experiments, LPS induced impairment of endothelium-dependent relaxation of the aorta, but not endothelium-independent relaxation. Therefore, we suppose that the detrimental effect of LPS is targeted predominantly at the endothelium, in which signaling and metabolic disturbances can contribute to impaired endothelium-mediated vasodilation of the aorta. Increased activity of NOS, found in our experiments, could be a manifestation of endothelial dysfunction as well as macrophage-stimulated iNOS which produces NO involved in damaging endothelium. Moreover, enhanced

NAGA activity and increased values of LDH also point to cell injury including endothelial ones. Above mentioned actual changes of metabolism and inflammation in the LPS treated group can affect different mechanisms modulating endothelial function, including expression of Cx40 protein (Zhao *et al.* 2007, Alonso *et al.* 2010, Radosinska *et al.* 2011, Frimmel *et al.* 2014, Le Gal *et al.* 2015).

Cx-channels, including Cx40-ones, represent one of the key pathways for normal function of blood vessel wall. They allow spread of electrotonic signaling between cells of vascular wall modulating its vasomotion (De Wit and Griffith 2010). Therefore, LPS-induced decrease in expression of Cx40 isoform in the aorta could contribute to disturbances of endothelial cells homeostasis and thus impaired vascular tone of aorta. Cx40 has been suggested to be involved in vasodilation through the NO system (Alonso *et al.* 2010, Le Gal *et al.* 2015) and endothelium-derived hyperpolarizing factors (EDHF) (Looft-Wilson *et al.* 2012). Concerning the aorta, various pathophysiological conditions, including HTG, atherosclerosis and hypertension impaired its NO-dependent relaxation, while EDHF-mediated vasodilation, in contrast to smaller arteries, was not changed (Csanyi *et al.* 2007, De Wit and Wolfle 2007, Bartus *et al.* 2008). Moreover, EDHF signaling may also depend on density of myoendothelial junctions. Concerning the aorta, it was demonstrated that it contained less the junctions than smaller arteries (Shaun *et al.* 2009). In our experiments, LPS-related decrease in Cx40 expression in the aorta of hHTG rats was associated with impaired endothelium-dependent relaxation of the aorta and disturbances of its NO system, whereas endothelium-independent relaxation was unchanged. We did not examine mechanisms involved in EDHF signaling. However, based on all the above mentioned data, we suppose that LPS-induced changes of Cx40 expression in the aorta during HTG might be associated rather with NO-system than EDHF-mediated vasodilation. This remains to be determined in more detail.

Endothelial integrity and permeability depends on mutual interactions of intercellular gap and tight junctions (Nafasawa *et al.* 2006). In correlation, LPS resulted in different expression of Cx40 and occludin in Wistar rat aorta, which were accompanied with subcellular damage of intercellular junctions (Frimmel *et al.* 2014, Krizak *et al.* 2016). Local ultrastructural alterations of intercellular junctions were also seen in

endothelium of hHTG rat aorta (Dlugošová *et al.* 2009b), indicating changes in cell-cell junctions. Therefore, we hypothesize that Cx40 expression in the rats injected with LPS might be at least partially modulated with disturbances of tight junctions, too.

Both, HTG and LPS represent independent risk factors for atherosclerosis and their effects on Cx40 expression can be diverse. In atherosclerotic arteries, reduced Cx40 expression was found in mice (Kwak *et al.* 2002, Chadjichrostos *et al.* 2010), while it was increased in rabbit (Wang *et al.* 2005). Other studies demonstrated LPS-induced Cx40 down-regulation in mouse aorta (Simon *et al.* 2004, Rignault *et al.* 2007) but up-regulation of Cx40 expression in rat aorta (Rignault *et al.* 2005, Frimmel *et al.* 2014). Our previous study showed that HTG increased Cx40 expression when compared to healthy Wistar rats (Okruhlicová *et al.* 2012). Here, we observed LPS-induced decrease of Cx40 expression in the aorta of hHTG rats.

The results thus indicate that Cx40 expression may vary according to the experimental models, type of vessel, animal species, and genetic predisposition as well as various pathophysiological conditions. LPS-induced downregulation of Cx40 expression suggests functional and structural remodeling of endothelial Cx40-GJ clusters in respect to actual metabolic and inflammatory conditions induced with LPS. Precise mechanisms, affecting Cx40 expression remain to be elucidated.

HTG is known to be associated with endothelial dysfunction (Bae *et al.* 2001, Cebova *et al.* 2006), which predispose endothelial cells to be more sensitive to various exogenous factors, including inflammatory factors as well as substances with protective effects.

Chronic dietary Ω 3FA intake is well-known to suppress progression of vascular wall diseases and is strongly recommended in the management of HTG. Ω 3FA possess multifactorial effects including anti-lipidemic and anti-inflammatory ones (Lu *et al.* 2014, Tousoulis *et al.* 2014). In our experiment, the LPS challenged rats were treated with Ω 3FA diet for 10 days to examine its short-term effects. The diet had no protective effect on TG and HDL concentration, therefore we suppose that 10-day Ω 3FA administration is too short to influence lipid metabolic processes in hHTG rats injected with LPS. Surprisingly, Ω 3FA diet caused increase in TG levels in the Ω 3 group as well. Currently, there is no sufficient explanation of this result, but we hypothesize that it might be attributed to short-term diet rich in fats. Beneficial effects of regular consumption of

Ω 3FA on TG levels have been observed approximately 40-60 days later.

Surprisingly, Ω 3FA diet caused elevation of triglyceride levels in the hHTG rats. It might mean that the diet, at this phase of experiment, does not have beneficial effects for hHTG rats. We are not able to explain the results at the present, but we hypothesize that it might be attributed to short-term diet rich in fats during actual metabolic conditions.

Although Ω 3FA diet had no anti-lipidemic effects in our experiment, it significantly decreased values of MDA and NAGA and ameliorated endothelium-dependent relaxation of the aorta. The results point to suppression of oxidative stress- and inflammation-related cellular damage. Nevertheless, the NOS activity in the L Ω 3 group was even higher than in the LPS rats. It indicates the presence of eNOS activity and/or increased availability of NO probably due to high response of endothelial cells to anti-inflammatory and anti-oxidative effects of Ω 3FA. Although Ω 3FA protected endothelial function, they did not reduce CD68 expression in the LPS rats. Elevated CD68 expression and TG levels might contribute to LPS-induced storage of TG in macrophages (Feingold *et al.* 1992), which however, in our case, had no deleterious effects on aorta relaxation.

Ω 3FA-induced protection of endothelium-dependent aortic relaxation in the L Ω 3 group was associated with significant increase of Cx40 protein expression. Because Ω 3FA suppressed values of oxidative stress, affected NO system, whereas did not affect lipid metabolism disorder, we hypothesize that normalization of Cx40 expression can be attributed mainly to Ω 3FA anti-oxidative and anti-inflammatory potencies. Due to already mentioned Cx40-eNOS interaction (Alonso *et al.* 2010, Le Gal *et al.* 2015), it is also possible to speculate that restoration of Cx40 by Ω 3FA diet in LPS affected animals could improve eNOS activity. The results indicate that Cx40 expression can be involved in Ω 3FA-related amelioration of aortic relaxation function. The data are in concordance with our previous studies demonstrating protective effects of Ω 3FA on LPS-induced changes in Cx40 expression in aorta of Wistar rats (Frimmel *et al.* 2014) and hHTG-induced disturbances in Cx43 expression in rat aorta (Dlugošová *et al.* 2009b). The modulation of Cx40 expression by Ω 3FA was also observed by Sarrazin *et al.* (2007) in canine atrial tissue.

Connexins are transmembrane proteins which

very dynamically respond to actual conditions. Ω 3FA are an integral part of cellular membrane, therefore they can influence structure of membrane, determine its susceptibility to oxidative conditions and repair membrane transport properties (Rustan and Drevon 2005) including Cx-channels. Biological effects of Ω 3FA diet can also occur *via* peroxisome proliferator-activated receptors pathway which is involved in lipid metabolism (Lu *et al.* 2014). However, the mechanisms by which Ω 3FA protect endothelial Cx40 expression in the aorta of the hHTG rats after LPS administration, remain still in discussion.

Conclusions

Our results indicate involvement of Cx40

expression in LPS-induced endothelial damage of the aorta in rats with inherited hypertriglyceridemia. The findings also suggest that hHTG rats suffering from LPS-induced injury might benefit from 10-day Ω 3FA therapy because of the protection of endothelial function due to increased Cx40 expression. In this respect, our results suggest that also short-term Ω 3FA diet may be of great importance in some pathology.

Conflict of Interest

There is no conflict of interest.

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