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## MODULATION OF CONNEXIN-43 BY OMEGA-3 FATTY ACIDS IN THE AORTA OF OLD SPONTANEOUSLY HYPERTENSIVE RATS

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Hypertension alters expression of connexin-43 (Cx43) in cardiovascular system. The aim of the study was to investigate the effect of omega-3 polyunsaturated fatty acids (30 mg/day for 2 months) on expression of Cx43 in the aorta of 1-year-old male spontaneously hypertensive rats (SHR). Spatial distribution and expression of Cx43 in aortic wall of SHR and age-matched Lewis rats were determined by immunofluorescent method and Western blot. NO synthase (NOS) activity and endothelium-dependent relaxation of the aorta were measured as well. Immunofluorescent pattern of Cx43 was identified in endothelial and smooth muscle cells of the aorta of all experimental groups studied. However, local decrease in the number and intensity of fluorescent spots and reduced phosphorylation of Cx43 were observed in SHR in contrast to normotensive LEW. Omega-3 fatty acid diet increased Cx43 immunolabeling in endothelium and media of SHR comparing to untreated ones. Parallel, 3-fatty acids significantly elevated phosphorylation of Cx43 in the aorta of SHR ( $p < 0.001$ ). Despite the omega-3 fatty acids reduced blood pressure and stimulated aortic NOS activity in SHR, endothelium-dependent relaxation of the aorta did not significantly change. Results indicate that the aorta of old SHR might partially benefit from 3-PUFA supplementation due to increased Cx43 phosphorylation, NOS activity and decreased blood pressure.

Key words: *connexin-43, aorta, hypertension, omega-3 fatty acids, microscopy*

### INTRODUCTION

Aging and hypertension represent the important risk factors for the development of atherosclerotic cardiovascular disease that is a major cause of morbidity and mortality in the Western world. Results of clinical trials and experimental studies have shown that consumption of fish oil containing omega-3 polyunsaturated fatty acids (3-PUFA) - eicosapentaenoic and docosahexaenoic acids - protects against several types of cardiovascular diseases such as atherosclerosis and hypertension. Studies have also indicated that diet rich in 3-PUFA reverses the age-associated functional and structural changes of the cardiovascular system. Beside the benefit on arrhythmias (1), protective effects of 3-PUFA are predominantly attributable to the vascular endothelial and smooth muscle cells: 3-PUFA decrease platelet aggregation and inflammation, blood pressure, cause endothelial relaxation and promote arterial compliance (2, 3).

Communication between cells within an organ is necessary to coordinate the behavior of individual cells and to ensure proper organ function. An important communication pathway is direct signaling through the transmembrane channels known as gap junctions. They drive numerous important biological activities between adjacent cells, including rapid transmission of electric

signals to coordinate contraction of smooth muscle cells, the intercellular propagation of  $Ca^{2+}$  waves and thus synchronization of cell activities within a vascular wall (see review 4). The gap junction channels consist of connexin proteins (Cx), the expression and distribution of which may vary depending on the species, position in the vascular tree and pathophysiological conditions (see review 5). Cx43 is one of major gap junction proteins involved in an intercellular communication of the aortic wall (6). Data, concerning its expression during hypertension in large conduit arteries, including the aorta are conflicting: it was increased or decreased, depending on the used model of hypertension (7-10). Because the distensibility and compliance of the arteries differed among the experimental models, it was concluded that alterations in Cx43 expression might represent an adaptive response to functional and structural changes, occurring in the vascular wall during hypertension. In addition, as it was demonstrated by Yeh *et al.* (11), its expression decreased progressively with the age. Despite the numerous data concerning protective effects of 3-PUFA on the cardiovascular system, information whether omega-3 fatty acids can modulate Cx43 expression in the arterial wall *in vivo* is missing. It was reported only that 3-PUFA protected gap junction communication in cultured vascular endothelial cells under hypoxia/reoxygenation associated with improved endothelial

function (12, 13). Therefore, the aim of the present work was to investigate the effect of chronic 3-PUFA supplementation on Cx43 distribution and expression in the aorta of old SHR.

## MATERIAL AND METHODS

### Animals

All procedures were completed in accordance and approved by the State Veterinary and Food Administration of the Slovak Republic, legislation No. 289/2003.

We used 1-year-old spontaneously hypertensive rats (SHR) and age-matched Lewis rats (LEW) as controls. Both groups were divided into rats treated with 3-PUFA (eicosapentaenoic and docosahexaenoic acids, Vesteralens, Norway, 30 mg/day, for 2 months) and untreated ones (n=6 per each group). Animals were anesthetized by thiopental, the thoracic aorta was excised, cleaned of adherent tissue and processed for Cx43 immunofluorescence, Western blot analysis as well as for NO synthase activity measurement and functional responses.

### Functional studies

Blood pressure was measured noninvasively by tail-cuff plethysmography using the Statham Pressure Transducer P23XL (Hugo Sachs, Germany) at the beginning and the end of experiment, after 2-month-lasting 3-PUFA consumption. For accurate measurement, the animals were three-time trained and acclimatized to restraint cages and preheated to 37°C for 10 min, then maintained at that temperature during the test. Each measurement was repeated five to six times in one rat. Body weight of all animals was measured at the start and at the end of experiments, after two months. The endothelium-dependent relaxation of the thoracic aorta was measured according to Sotnikova *et al.* (14). NO synthase (NOS) activity was measured in the homogenate of the aorta by determination of [<sup>3</sup>H]-L-citrulline (L-Cit) formation from [<sup>3</sup>H]-L-arginine (MP Biomedicals, USA) as described previously, with minor modifications (15).

### Immunofluorescence staining of connexin 43

Indirect Cx43 immunolabeling was performed on series of transverse cryostat sections of unfixed aortic tissue (10 µm) (16): sections were incubated with primary monoclonal antibody mouse anti-Cx43 (1:100, Chemicon Int., Inc., USA) for 2 hrs at a room temperature followed by application of secondary antibody goat anti mouse IgG conjugated with fluorescein tiocyanate (1:50, Chemicon). Primary antibody was omitted in negative controls. After washing, aortic sections were rinsed with PBS, mounted into Vectashield mounting medium (Vector Laboratories, USA) and viewed by Axiostar fluorescent microscope (Carl Zeiss, Germany). Pictures were digitized and transferred into PC. 4 slides with 4 sections per rat and 3 high power fields per section were visualized and the intensity of the immunostaining was semiquantitatively described.

### Western blot analysis and quantification of connexin 43 protein

Frozen aortic tissue was processed according to Dlugosova *et al.* (16): it was powdered and solubilized in SB<sub>20</sub> solution (20% SDS, 10 mmol/l EDTA, 0.1 mol/l TRIS, pH 6.8) by sonicator UP 100H (Dr. Hielscher, Germany). Samples were fractionated by electrophoresis in a 12.5% polyacrylamide gel and immunoblotted onto Trans Blot Transfer Medium (Bio Rad, USA) overnight at a constant current 50 mA. Membranes were

incubated for 1 hour at room temperature in 4% solution of dry milk with blocking buffer TRIS-buffered saline - TBS (20 mmol/l Tris, 150 mmol/l NaCl, pH 7.4-7.6) and then incubated for 2 hours with mouse monoclonal antibody to Cx43 (1:1000, Sigma). After membranes were repeatedly rinsed in TBS, they were incubated for 1 hour with donkey anti-mouse IgG antibody coupled with alkaline phosphatase (1:2000, Promega, USA). The bands were visualized with the BCIP-NBT (5-bromo-4-chloro-3 indolyl phosphate/nitro blue tetrazolium) method (Promega).

For the quantification of immunoblot bands, optic density of scanned immunoblot membranes was analyzed using GelPro System (Media Cybernetics, USA), which integrated areas and corrects for background.

### Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Blood pressure and NOS activity were analyzed using one-way ANOVA and followed by Duncan's post-hoc test. Cx43 expression was analyzed using Student t-test. Values were considered to differ significantly when p<0.05.

## RESULTS

### Basic characteristics of experimental rats

Basal blood pressure was significantly increased in SHR comparing to control LEW (213±4.46 mm Hg vs. 96±1.48 mm Hg, p<0.001). The 2-month-treatment by 3-PUFA decreased blood pressure in both rat strains SHR and LEW. In SHR it was reduced from 213 ± 4.46 mm Hg to 182 ± 3.18 mm Hg (p<0.001). In LEW it was suppressed from 96 ± 1.47 mm Hg to 87 ± 1.78 mm Hg (p<0.03) (Fig. 1).

The endothelium-dependent relaxation of the aorta was found to be significantly diminished in SHR as compared with LEW. It was manifested by decreased responses of the preparations to acetylcholine - the maximal relaxation achieved 64 % compared to 34 % of the contraction found in the aortas of LEW. 3-PUFA had no significant effect on the aortic relaxation of LEW and SHR (Fig. 2A).

Contractile responses to phenylephrine (PE) did not differ among experimental groups (not shown). After NOS-blockade, contractile responses of the LEW-aortas to PE were potentiated when compared to control responses (p<0.01). On the contrary,

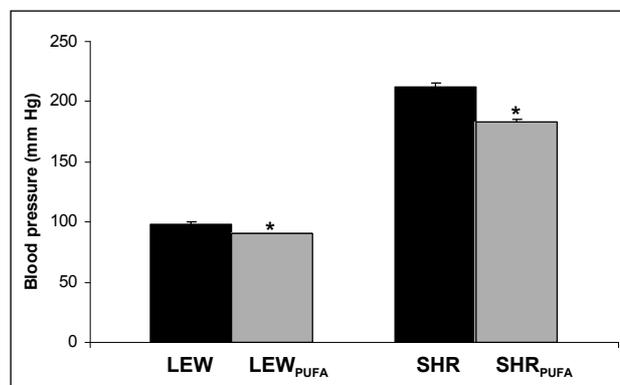
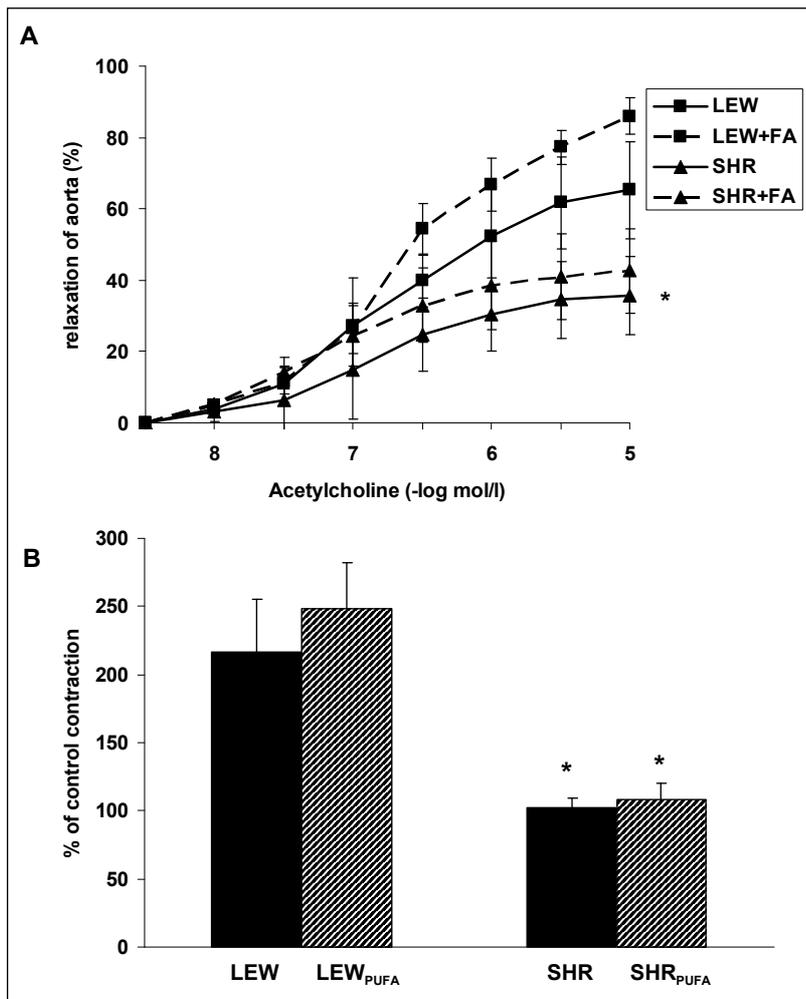
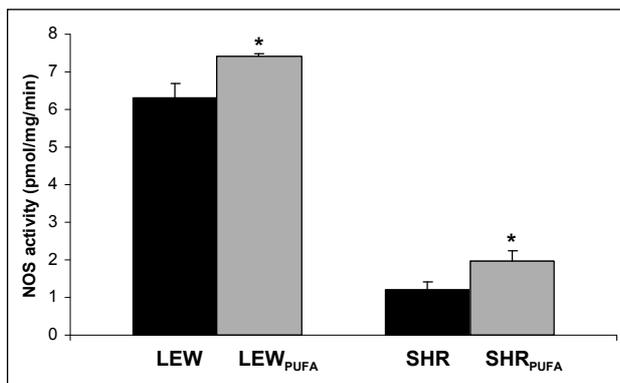


Fig. 1. The hypotensive effect of omega-3 fatty acids (PUFA) in Lewis (LEW) and hypertensive SHR rats. \*p<0.01, SHR<sub>PUFA</sub> vs. SHR, \*p<0.05, LEW<sub>PUFA</sub> vs. LEW. n=6 per each group. Results are expressed as mean ± S.E.M.



*Fig.2. A: The effect of 3-PUFA (FA) on endothelium-dependent relaxation of the aorta of Lewis (LEW) and SHR rats. \*p<0.05 LEW vs. SHR. B: Contraction responses of the aorta of Lewis (LEW) and SHR rats to phenylephrine (PE) after NO synthase blockade with L-NAME. \*p<0.05 LEW vs. SHR. n=6 per each group. Results are expressed as mean  $\pm$  S.E.M.*



*Fig.3. 3-PUFA diet-induced stimulation of the NO synthase activity in the aorta of spontaneously hypertensive rats (SHR) (\*p<0.05, controls vs. treated rats) and Lewis rats (LEW, \*p<0.03, controls vs. treated rats). n=6 per each group. Results are expressed as mean  $\pm$  S.E.M.*

PE-induced contraction of the SHR-aortas was not changed after NOS-blockade in comparison with the control responses. Administration of 3-PUFA did not significantly influence the NO-independent PE-induced contraction of the aorta in f LEW and SHR (*Fig. 2B*).

NOS activity significantly differed between control SHR and LEW (1.10  $\pm$  0.22 pmol/mg/min vs. 6.30  $\pm$  0.11 pmol/mg/min,

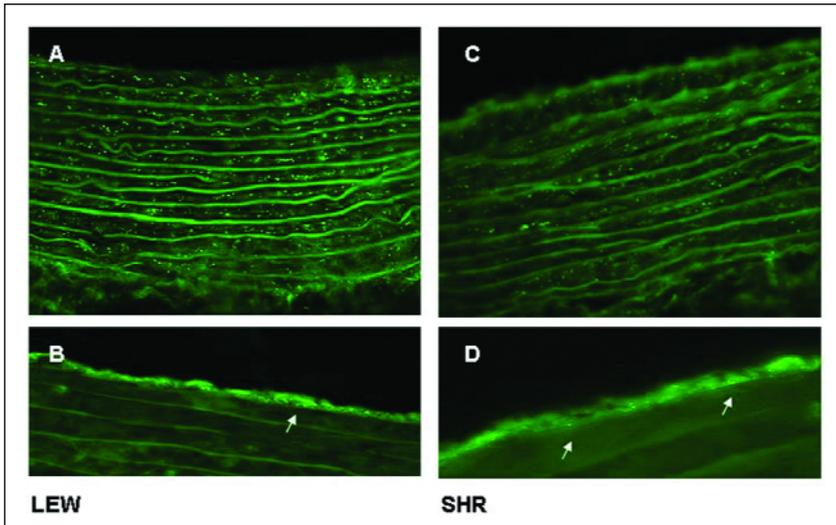
p<0.001). Two months lasting 3-PUFA treatment led to significant increase in the enzyme activity in the aortas of both SHR and LEW by 78% and 17% respectively (p<0.05 vs. untreated group) (*Fig. 3*).

#### *Immunolabeling of Cx43*

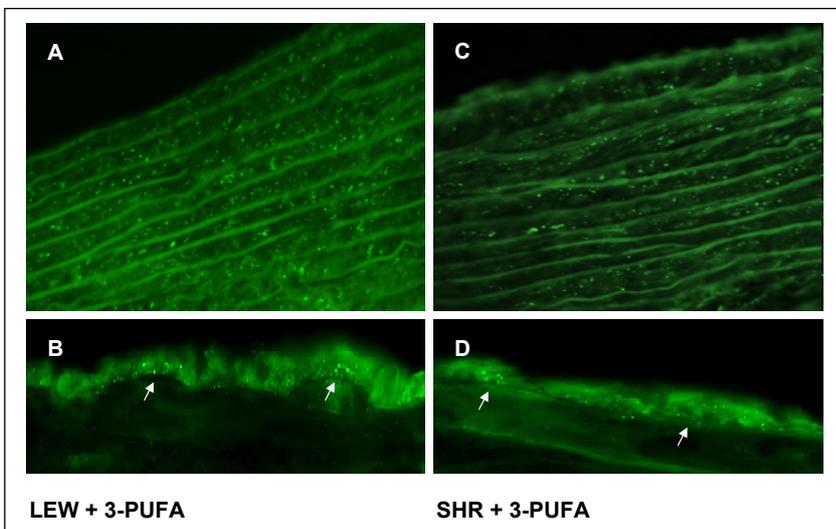
Immunofluorescent pattern of Cx43 was identified in the endothelial and smooth muscle cells of the aorta of all experimental groups studied (*Fig. 4, 5*). However, a local marked decrease in the number and intensity of fluorescent spots was observed in endothelium and media of SHR as compared to LEW (*Fig. 4*). 3-PUFA supplementation resulted in significant increase in Cx43 immunofluorescence throughout endothelial and smooth muscle cells of the aorta of SHR (*Fig. 5*). No differences in the number and pattern of spots were noticed between control and treated LEW rats.

#### *Expression of Cx43*

Western blot demonstrated the presence of two specific bands of Cx43 protein isoforms, corresponding to phosphorylated P1 and non-phosphorylated P0 forms in the aorta of all used experimental rat (*Fig. 6A*). Quantitative analysis showed significant differences in Cx43 expression between LEW and SHR. Total protein expression was markedly lower in the aorta of SHR as compared to LEW (p<0.001) (*Fig. 6B*). The supplementation of 3-PUFA significantly by 23% increased phosphorylation of Cx43 in the aorta of SHR in comparison with



*Fig.4.* Representative immunofluorescence of Cx43 in media and endothelium of the aorta of control Lewis (A, B) and SHR (C, D) rats revealed differences in the amount and intensity of immunolabeling. Arrows show Cx43 spots on endothelium. Original magnification of all pictures: x200.



*Fig.5.* 3-PUFA diet resulted in increased immunolabeling of Cx43 in media and endothelium of the aorta of SHR (C, D), but not in Lewis rats (A, B). Original magnification of all pictures: x200.

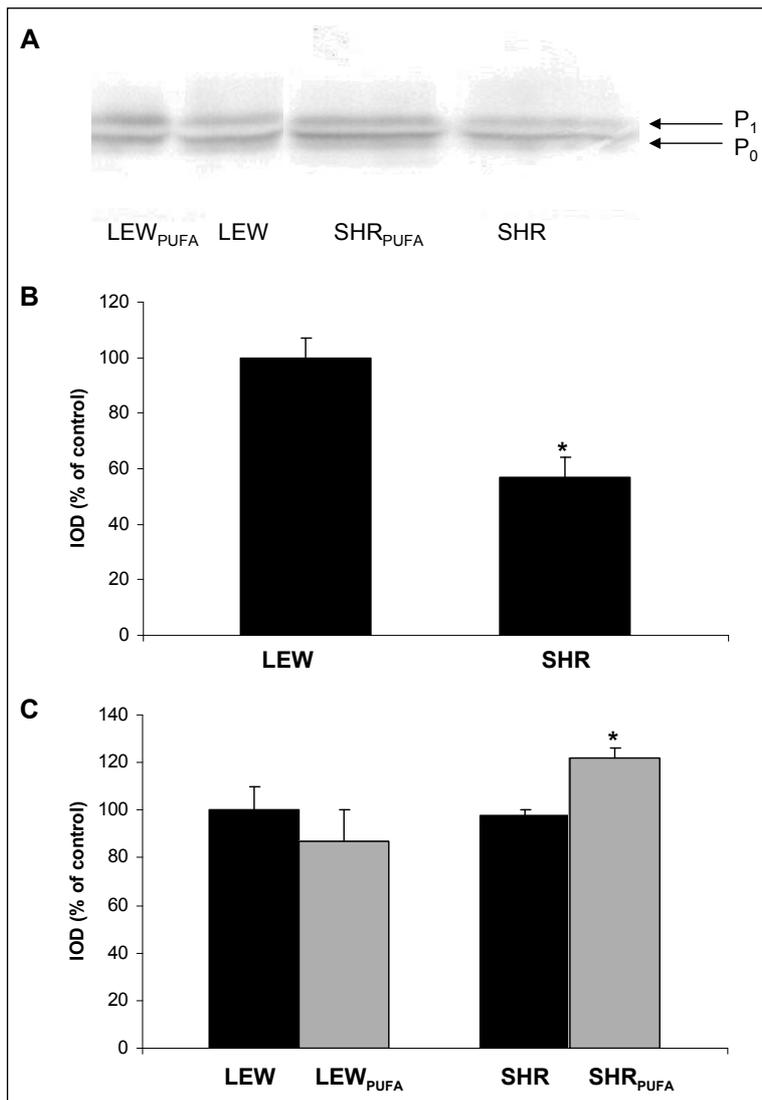
untreated ones ( $p < 0.05$ ), while no marked effect of 3-PUFA on Cx43 expression was observed in the aorta of LEW (*Fig. 6*).

## DISCUSSION

The main goal of the present study was to examine the effect of chronic intake of 3-PUFA on Cx43 expression in the aorta of old genetically hypertensive rats. The results demonstrated that omega-3 fatty acids consumption led to up-regulation of phosphorylated isoform of Cx43 in the aorta of SHR that was accompanied with reduced blood pressure and stimulated NOS activity.

Gap junction communication in the vessel wall allows spreading of electrotonic signaling and may coordinate the mechanical contractions of smooth muscle cells (4). Therefore, the aorta which is a sparsely innervated and electrically quiescent vessel is likely to be particularly dependent on gap junction communication (17). In our experiments we used two inbred rat strains - genetically hypertensive SHR and LEW as normotensive controls, differing each other in the activities of renin-angiotensin-aldosterone system (18, 19), hypothalamo-pituitary-adrenal axis (20, 21), sympathetic nervous system. Additionally, it was reported that hypertension influenced the endocrine cells in rat stomach in contrast to controls (22, 23). As we expected, blood pressure was significantly higher in SHR

than in LEW, correlating with data of other research groups (24). The effect of high blood pressure on Cx43 expression was reported in small and large vessels - as it was mentioned above. Data concerning Cx43 expression in the aorta of hypertensive rats are also controversial (see review 25) - it was elevated in vascular smooth muscle cells of the rat DOCA-salt model and two-kidney, one-clip renal model of hypertension, while reduced in smooth muscle cells of the aorta of NO-deficient hypertensive rats and SHR. Our recent work confirmed decreased Cx43 expression in the aorta of young SHR and even pre-hypertensive rats (16) characterized with endothelial dysfunction (26). Likewise the present study demonstrated down-regulation of Cx43 expression in the aorta of old SHR, confirming the contribution of genetic origin of high blood pressure as well as the age-related influence to Cx43 expression. We did not differentiate protein expression levels in endothelial cells versus in smooth muscle cells. However, immunolabeling was locally reduced till absent in endothelium and media of the aorta of SHR, accompanied with decreased overall phosphorylation of Cx43. Vascular gap junctions have been implicated in a number of vasomotor responses that may regulate vascular tone and blood pressure (17, 27). Additionally, Cx43 may play a role in regulation of smooth muscle cell mitosis (28, 29). The reduced expression of Cx43 in the aorta of SHR rats correlates with disturbances structural and functional



**Fig. 6.** A: Western blot of Cx43 expression in the aorta of untreated and 3-PUFA-treated Lewis (LEW) and SHR rats. P<sub>1</sub> - phosphorylated isoform, P<sub>0</sub> - non-phosphorylated isoform of Cx43. B: Quantification of total Cx43 protein expression in the aorta of LEW and SHR. \*p<.001, LEW vs. SHR. C: Effect of 3-PUFA supplementation on Cx43 phosphorylation in the aorta of SHR and LEW. \*p<.001, SHR vs. SHR<sub>pufa</sub>; p<.05 LEW<sub>pufa</sub> vs. SHR<sub>pufa</sub>. n=6 per each group. IOD - Integrated optical density. Results are expressed as mean ± S.E.M.

changes of a vascular of wall, observed during hypertension. On the other hand, the mean values of blood pressure found herein in LEW ( $96 \pm 1.47$  mm Hg), corresponding relatively with age-matched Wistar rats ( $105 \pm 4.85$  mm Hg), NOS activity and NO tonus, together with acetylcholine-induced aortic relaxation and high levels of Cx43 expression physiologically characterize response of the aorta of old LEW inbred rat strain and support its normal function.

It was reported that localized mechanical forces induced by hypertension represent a major tissue-specific regulator of Cx43 expression in the aortic wall (8). Therefore, antihypertensive treatment would repair vascular Cx43 expression. In agreement, the hypotensive effect of 3-PUFA in our work was associated with up-regulation of aortic Cx43, referring to the direct modulation of connexin expression by blood pressure. Enhanced phosphorylation of Cx43 and its distribution in endothelium and media of the aorta of treated SHR rats might contribute to increased gating of gap junction channels and to improve synchronization of responses in multicellular organism as the aortic wall is. However, 3-PUFA have multifunctional effects, indicating that apart from effect of blood pressure, there can be numerous mechanisms, operating in the modulation of connexin expression. 3-PUFA stimulated NO production (30) and inhibited renin-angiotensin system (31, 32). Both mechanisms, blockade of renin-angiotensin system and increased NO production, beside the

regulation of blood pressure, were shown to alter Cx43 expression (33, 34). Thus, increased NO synthesis in treated SHR suggests a role of NO in *in vivo* modulation of aortic Cx43 in hypertensive rats. Although 3-PUFA reduced blood pressure in SHR, the rats were still hypertensive. Surprisingly, despite the stimulation of the aortic NOS activity, endothelium-dependent relaxation of the aorta of SHR showed nonsignificant, only very slight improvement. One of the explanations of this event could be that in aging tissues overproduction of free radicals results in nitro-oxidative stress, as implicated in the decline of endothelium-dependent relaxant responsiveness of aortic rings (35). Thus enhanced NOS activity in our experiments does not necessarily mean sufficiently increased NO availability. Moreover, previously we observed improved function of the superior mesenteric artery of young Wistar rats after treatment with n-3 PUFA (14). In these experiments a beneficial effect of n-3 PUFA was found also on L-NAME-resistant (EDHF) portion of acetylcholine-induced relaxation, which is indicative of the effect of n-3 PUFA not only on NO but also on EDHF availability. As EDHF is present in smaller arteries and resistant vessels, it is likely that the function of these arteries was improved also in our experiments. The observed systolic pressure decrease reflects a beneficial effect of n-3 PUFA on heart function rather than on vessels, so that pressure decrease does not mirror improved vascular function. Correspondingly, our recent work demonstrated benefit effects of

3-PUFA on the heart function and structure that was associated with up-regulation of Cx43 in the myocardium of old SHR (36).

Furthermore, 3-PUFA form a part of cell membranes, in which they can be easily incorporated during diet modifying the physical properties of the lipid microdomains and proteins and thus cellular function (30). Connexins are dynamic transmembrane proteins, it seems therefore very likely that incorporation of 3-PUFA into cell membranes might modulate Cx43 gap junction channels likewise other channels (37). 3-PUFA could modulate gap junction intercellular communication as well as endothelial dysfunction and vascular injury through the regulation of NADPH oxidase (38, 39) which contributes to the development of cardiovascular disease (40) *via* superoxide anion generation, increasing oxidative stress. As was mentioned above, 3-PUFA have effects on diverse mechanisms, but they favorably influence the blood characteristic - they reduce platelet aggregation and blood viscosity, they exhibit antiinflammatory, antithrombotic and antifibrinolytic activities either *via* elevated NO production or *via* peroxisome proliferators-activated receptors- $\alpha$ . The later might additionally modulate lipid metabolism and redistribution (41, 42) and thus improve viscosity of the vessel wall. We suppose that discrepancies between SHR and LEW might partially be due to strain-dependent and individual variability, but also in asymmetry of 3-PUFA profile in tissues' cellular membranes (43). Therefore, altogether indicate the contribution of 3-PUFA on Cx43 expression, but it is hardly to draw any conclusions concerning the mechanisms how 3-PUFA can effect Cx43 expression. Further analysis is necessary to learn to which extent 3-PUFA can modulate expression and/or phosphorylation and gene transcription of Cx43 and particularly Cx43 channel conduction.

In conclusion, the present study demonstrated for the first time that chronic 3-PUFA diet *in vivo* modulate Cx43 expression and increase phosphorylation of Cx43 in the aorta of old SHR. It was accompanied with decreased blood pressure and increase NOS activity while, nonsignificant changes in the relaxation of the aorta of old SHR. Results indicate that the aorta of old SHR despite the increased Cx43 expression might only partially benefit from 3-PUFA supplementation.

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Conflict of interests: None declared.

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