Alterations of myocardial NOS activity and connexin-43 expression in rats suffering from hypertension and hypertriglyceridemia are attenuated by omega-3 fatty acids

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Abstract. *Introduction:* Our previous and others studies´ suggest that NO bioavailability and cell-to-cell coupling protein, connexin-43 (Cx43), are abnormal in diseased hearts of humans as well as experimental animals. Consequently, it can contribute to deterioration of heart function and occurrence of life-threatening arrhythmias.

Objective: We tested our hypothesis that the cardioprotective compounds, omega-3 PUFA, may be beneficial in hypertensive and hypertriglyceridemic rats in respect to disease-related abnormalities in NO production and Cx43 expression.

Methods: Myocardial nitric oxide synthase (NOS) activity and Cx43 expression were investigated in young and old spontaneously hypertensive rats (SHR), adult hereditary hypertriglyceridemic (HTG) rats, and age-matched healthy rats without and with omega-3 PUFA supplementation (30 mg/day/100g b.w) for 2 months.

Results: When compared to healthy rats myocardial NOS activity was significantly increased in young SHR (8.2 ± 1.16 vs. 1.37 ± 0.67 pmol/min/mg) as well as old SHR (3.21 ± 0.75 vs. 2.22 ± 0.56 pmol/min/mg) and to a much lesser extent in HTG rats, i.e., 1.87 ± 0.42 vs. 1.34 ± 0.1 pmol/min/mg. In parallel, there was a significant decline of total and phosphorylated forms of Cx43 in both groups of SHR while not in HTG rat hearts, in which the phosphorylated form of Cx43 was increased. Elevated NOS activity was suppressed ($p<0.05$) in young and old SHR supplemented with omega-3 PUFA and it was associated with up-regulation of Cx43. In contrast to SHR, elevation of NOS activity in HTG rat hearts was not affected by treatment with omega-3 PUFA. However, increase of phosphorylated form of Cx43 was suppressed.

Conclusions: There is an inverse relationship between myocardial NOS activity and Cx43 expression in SHR, though not HTG, rat hearts, and omega-3 PUFA modulate both NOS activity and Cx43 expression. Whether over-expression of inducible NOS might account for down-regulation of myocardial Cx43 and whether its up-regulation is associated with an increase of endothelial NOS should be explored by further study. Results point out the impairment of Cx43 mediated myocardial cell-to-cell communication due to hypertension and hypertriglyceridemia and its possible protection by compounds affecting the expression of Cx43. In addition, improvement of intercellular communication and consequently myocardial synchronization and diseased heart function is a major task in clinical practice. Tab. 3, Fig. 7, Ref. 42, Online full text (Free, PDF) www.cardiology.sk

Key words: heart – nitric oxide synthase – connexin 43 – omega-3 polyunsaturated fatty acids – spontaneously hypertensive rats – hereditary hypertriglyceridemic rats

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Radošinská J, Bačová B, Bernátová I, Navarová J, Beňová T, Tribulová N. **Omega-3 mastné kyseliny zmierňujú zmeny v aktivite NOS a expresii konexínu-43 v srdci hypertenzných a hypertriglyceridemických potkanov.** Cardiology Lett. 2011;20(4):301–310

Abstrakt. *Úvod:* Naše predchádzajúce štúdie a práce iných poukazujú na abnormality v tvorbe NO a expresii proteínu medzibunkových kanálov, konexínu-43 (Cx43), v chorom srdci u ľudí, ako aj u experimentálnych zvierat. Tieto zmeny majú vplyv na zhoršenie funkcie srdca a na vznik život ohrozujúcich arytmií.
Cieľ: Naším zámerom bolo skúmať, či kardioprotektívne látky, omega-3 polynenasýtené mastné kyseliny (omega-3 PUFA) ovlyvnia abnormality tvorby NO a expresie Cx43 v srdci hypertenzných a hypertriglyceridemických potkanov.
Metódy: Aktivita syntázy oxidu dusnatého (NOS) a expresia Cx43 boli vyšetrované v myokarde mladých a starých spontánne hypertenzných potkanov (SHR), ako aj dospelých hereditárne hypertriglyceridemických (HTG) potkanov a rovnako starých zdravých zvierat bez a po suplementácii s omega-3 PUFA (30 mg/deň/100g) počas dvoch mesiacov.
Výsledky: Myokardiálna aktivita NOS bola v porovnaní so zdravými potkanmi významne zvýšená u mladých (8,2 ± 1,16 vs. 1,37 ± 0,67 pmol/min/mg), ako aj starých SHR (3,21 ± 0,75 vs. 2,22 ± 0,56 pmol/min/mg) a v menšej miere u HTG potkanov, t j. 1,87 ± 0,42 vs. 1,34 ± 0,1 pmol/min/mg. Paralelne s tým sa zistilo významné zníženie celkovej a fosforylovanej formy Cx43 u oboch SHR skupín, nie však v srdci HTG potkanov, ktoré mali zvýšenú fosforylovanú formu Cx43. Zvýšená aktivita NOS bolo potlačená ($p < 0,05$) u mladých a starých SHR supplementovaných omega-3 PUFA a to bolo spojené so zvýšenou expresiou Cx43. V protiklade s SHR, v srdci HTG potkanov nebolo zvýšenie NOS aktivity ovplyvnené omega-3 PUFA, ale zvýšenie fosforylovanej formy Cx43 bolo potlačené.
Závery: Zistil sa inverzný vzťah medzi aktivitou NOS a expresiou Cx43 v srdci SHR, nie však HTG potkanov, pričom omega-3 PUFA modulujú oba, aktivitu NOS a expresiu Cx43. Ďalšie štúdie by mali objasniť, či zhoršená regulácia myokardiálneho Cx43 je v dôsledku zvýšenej expresie indukovateľnej NOS a či zlepšenie regulácie Cx43 sa spája so zvýšením endoteliálnej NOS. Výsledky poukazujú na poruchu medzibunkovej komunikácie, sprostredkovanej Cx43 v dôsledku hypertenzie a hypertriglyceridémie a na jej možnú ochranu látkami ovplyvňujúcimi expresiu Cx43. Zlepšenie medzibunkovej komunikácie a tým myokardiálnej synchronizácie a funkcie chorého srdca má kľúčový význam aj v klinickej praxi. Tab. 3, Obr. 7, Lit. 42, Online full text (Free, PDF) www.cardiology.sk

Kľúčové slová: srdce – syntáza oxidu dusnatého – konexín 43 – omega-3 polynenasýtené mastné kyseliny – spontánne hypertenzný potkan – hereditárne hypertriglyceridemický potkan

Gap junction connexin (Cx) channels play important roles in synchronizing the myocardium, allowing impulse propagation from pacemaker cells along the conduction system and throughout the atria and ventricles. The channels, in addition, are permeable to ions and small molecules up to 1 kD and are opened and closed (gated) by various treatments (1). Impaired intercellular communication due to disease-related alterations in myocardial Cx distribution and/or expression promotes development of life-threatening arrhythmias and contractile dysfunction (2 – 4). The properties of Cx channels constitute the possibilities to search for compounds that might be potentially useful in therapeutic manipulation of myocardial intercellular communication and synchronization, and thereby heart function.

Nitric oxide (NO) is one of the most potent vasodilating substances, which participates in modulation of vascular tone and thus in blood pressure (BP) regulation (5). This small gas molecule is produced from L-arginine by one of four isoforms of nitric oxide synthase (NOS), which have been distinguished until recently, by either constitutive or inducible expression of enzyme (6 – 8). This mediator is of special interest because it is involved not only in vasodilatation, but also in cell proliferation and inflammation. Moreover, NO, in addition to its known vasomotor effects, has a novel role in controlling intercellular coupling. NO has been reported to alter Cx expression in endothelial cells (9). Though the role of NO in the regulation of gap junctional coupling has been examined in some cell types (10, 11), so far little is known about the influence of this signaling molecule on coupling between cardiac muscle cells in healthy and diseased hearts. Nevertheless, NO bioavailability is altered in hypertension, hypertgriglyceridemia (12, 13), and pathophysiological conditions associated with endothelial dysfunction, whereby inflammation and oxidative stress play a key role in this process. Reactive oxygen species (ROS) interact with different vasoactive factors and NO is particularly sensitive to superoxide anion, which reduces its bioavailability (12, 14) due to production of peroxynitrite, a highly reactive nitrogen species (RNS). ROS/RNS serve as signal molecules at low concentration, but evoke harmful and pernicious effects if they are produced in too great amounts (15). It appears that the modulation of their production is an important target in the treatment of inflammatory-related diseases (16, 17).

Polyunsaturated fatty acids (PUFAs) of omega-3 and omega-6 groups are considered to be the most powerful intracellular and intercellular mediators, precursors for the synthesis of eicosanoids and modulators of the cell signaling network and cell membrane fluidity. This action of PUFAs can lead to various effects on inflammatory processes (16). Recently, it has been reported that PUFAs can participate in the regulation of enzymes responsible for reactive species production (16). Moreover, the findings of Champeil-Potokar (18) suggest that the high omega-3: omega-6 PUFA ratio that occurs naturally in astrocyte membranes is needed for optimal gap junction coupling in these cells. Our recent findings (19, 20) indicate that omega-3 PUFA can affect cardiovascular Cx43 expression and phosphorylation, and thus connexin channels function. In this context it is important to note that sudden cardiac death (SCD) presumably due to ventricular fibrillation (VF) can occur as a first manifestation of heart disease, while omega-3 PUFA reduce cardiovascular diseases and the incidence of sudden arrhythmic death in clinical (21 – 23) as well as experimental settings (20, 24). However, definite mechanisms of their cardioprotective and antiarrythmic effects are not yet elucidated.

The purpose of this study was to investigate myocardial NOS activity that indicates NO production and Cx43 expression that points to intercellular coupling in spontaneously hypertensive rats (SHR) and hereditary hypertriglyceridemic rats (HTG), as well as to examine whether supplementations of rats with omega-3 PUFA will affect both investigated parameters.

Material and Methods

All animal experiments were performed in accordance with the rules issued by the State Veterinary Administration of the Slovak Republic, legislation No 289/2003 and with the regulations of the Animal Research and Care Committee of Institute for Heart Research.

Experiments were conducted on SHR, a model that mimics human essential hypertension and is suitable to investigate early (until 6 months) hypertension-induced myocardial changes, and changes during transition from compensated left ventricular hypertrophy to heart failure in aging (> 1 year-old rats) (25). Male young (3 month-old) and aged (12 month-old) SHR as well as their age-matched non-hypertensive Wistar Kyoto (WKY) and Lewis (LEW) rats (n = 32) were used in this study. Animals were divided into two groups (n = 8 per each group), untreated rats fed with standard laboratory food and rats supplemented with omega-3 PUFA for two months (Vesteralens, Norway, 30 mg/day/100g b.w.). Moreover, HTG rats (male young, 3-month-old), were used as an experimental model mimicking human hypertriglyceridemia accompanied by mild hypertension (13), and as controls served their healthy Wistar (WIS) counterparts (n = 32). Half of the animals were fed with omega-3 PUFA (Vesteralens, Norway, 30 mg/day/100g b.w.) for two months.

Animals monitoring and samples collections

BP was measured noninvasively by tail-cuff plethysmography using the Statham Pressure Transducer P23XL (Hugo Sachs, Germany) at the beginning and the end of experimentation and body weight (BW) was monitored as well. Fasting blood was used for blood glucose, triglycerides (TG) and cholesterol (CH) assay using blood glucose test meter and commercial kit from Biolab Diagnostics. The hearts were rapidly excised into ice-cold saline, from the ether-anaesthesized rats, whereby whole heart and left ventricular weights were registered. The middle part of the left ventricle was used for NOS activity assay and the rest of the tissue was rapidly frozen in liquid nitrogen, kept at -80 C, and used for immunofluorescence and western blotting of the main myocardial gap junction protein Cx43.

NO synthase activity

Total NO synthase (NOS) activity was analyzed in 20% crude homogenates of the samples from the left ventricle by conversion of [^{3}H]-L-arginine (Amersham, UK) to [^{3}H]-L--citrulline, as described previously (26). NO synthase activity was expressed as pmol of L-citrulline /min/mg of proteins.

Immunofluorescent distribution of Cx43

Ventricular cryostat sections were used for in situ immunodetection of major myocardial gap junction channel protein Cx43 using mouse monoclonal antiCx43 antibody (Chemicon, Int., Inc. USA) and secondary FITC conjugated goat anti-mouse antibody, as described previously (27). Myocardial immunolabeling was examined with the Axiostar fluorescence microscope (Carl Zeiss Jena, Germany).

Western blot analysis

Frozen tissue from the left ventricle was powdered and solubilized in SB20 solution (20% SDS, 10 mmol/l EDTA, 0,1 mol/l TRIS, pH 6,8) by the sonicator UP 100H (Hielscher, Germany). An equal amount of protein in each sample was separated on 10% SDS-PAGE and transferred electrophoretically to a PVDF membrane. The membrane was incubated with primary rabbit antibodies (Anti-Connexin 43, C 6219, Sigma-Aldrich) overnight at 4°C, followed by further incubation for 1h at room temperature with the secondary donkey antibody (peroxidase-labeled anti-rabbit imunoglobulin, Amersham Biosciences). Bound antibodies were detected by the ECL method. The optical density of individual bands was analyzed by PCBAS 2.08e software and normalizing to GAPDH (anti-GAPDH antibody) as an internal control.

Table 1 Main characteristics of young SHR rats

	WKYc	WKYo3	SHRc	SHRo3
BP (mmHg)	105.1 ± 6.09	107 ± 8.32	219.86 ± 11.01**	222.75 ± 11.51
BW (g)	337.5 ± 10.61	341.67 ± 29.3	298.33 ± 23.63	283.75 ± 23.23
HW (g)	0.899 ± 0.112	0.901 ± 0.038	1.321 ± 0.085**	1.216 ± 0.096
HW/BW (mg/g)	2.68 ± 0.55	2.46 ± 0.13	4.32 ± 0.67**	4.03 ± 0.03
LVW (g)	0.642 ± 0.023	0.637 ± 0.037	0.99 ± 0.032**	0.863 ± 0.035##
LVW/BW (mg/g)	1.88 ± 0.09	1.87 ± 0.05	3.02 ± 0.14***	3.05 ± 0.15

Values are means ± SD of 8 rats in each group. WKYc – Wistar control rats. WKYo3 – Wistar rats fed with omega-3 fatty acid. SHRc – SHR control rats. SHRo3 – SHR rats fed with omega-3 fatty acid.
BP – blood pressure. BW – body weight. HW – heart weight. LVW – left ventricular weight. Significant difference from WKYc: ** p<0.01. *** p<0.001. Significant difference from SHRc: ## p<0.01.

Table 2 Main characteristics of old SHR rats

	LEWc	LEWo3	SHRc	SHRo3
BP (mmHg)	96.54 ± 5.86	87.58 ± 6.29	212.3 ± 12.66**	182.8 ± 11.36#
BW (g)	500 ± 34.64	507.50 ± 42.72	320 ± 28.28**	322.5 ± 45
HW (g)	1.172 ± 0.105	1.146 ± 0.075	1.511 ± 0.134**	1.601 ± 0.366
HW/BW (mg/g)	2.43 ± 0.38	2.35 ± 0.61	4.81 ± 0.22***	4.93 ± 0.18
LVW (g)	0.797 ± 0.065	0.799 ± 0.070	1.176 ± 0.096**	1.247 ± 0.275
LVW/BW (mg/g)	1.6 ± 0.23	1.58 ± 0.41	3.76 ± 0.08***	3.89 ± 0.26
BG (mg/dl)	5.6 ± 0.80	4.93 ± 0.97	5.8 ± 0.4	5.55 ± 0.55
TG (mmol/l)	0.616 ± 0.217	0.819 ± 0.008	0.622 ± 0.152	0.404 ± 0.144#
CH (mmol/l)	1.257 ± 0.073	1.557 ± 0.065	1.72 ± 0.113**	1.523 ± 0.012#

Values are means ± SD of 8 rats in each group. LEWc – Lewis control rats. LEWo3 – Lewis rats fed with omega-3 fatty acid. SHRc – SHR control rats. SHRo3- SHR rats fed with omega-3 fatty acid. BP – blood pressure. BW – body weight. HW – heart weight. LVW – left ventricular weight. BG – blood glucose. TG – triglycerides. CH – cholesterol. Significant difference from LEWc: * p<0.05. ** p<0.01. *** p<0.001. Significant difference from SHRc: # p<0.05.

Statistical analysis

The data are expressed as means ± standard deviations (SD). One-way ANOVA followed by Duncan's post-hoc test was used to analyze statistical significance between means. Values were considered to differ significantly when $p<0,05$.

Results

Main characteristics of omega-3 PUFA treated and untreated rats

There was a marked increase in systolic BP in both young and old SHR and mild but significant increase in HTG rats when compared to healthy counterparts. Supplementation with omega-3 PUFA significantly decreased BP in HTG and old SHR while not in young SHR. In comparison to healthy rats the BW was significantly lower in untreated as well as treated HTG and old SHR. Left ventricular weights were significantly increased in all SHR regardless of the treatment while they decreased in untreated as well as treated HTG rats. There were no significant changes in blood glucose among the groups. However, there was significant suppressive effect of omega-3 PUFA on both serum triglycerides and cholesterol in HTG and old SHR. All data are presented in **Tables 1, 2** and **3**.

Table 3 Main characteristics of young hypertriglyceridemic rats

	WISc	WISo3	HTGc	HTGo3
BP (mmHg)	123.4 ± 3.049	123.6 ± 2.607	130.4 ± 5.594*	118.2 ± 3.420##
BW (g)	403 ± 22.2	426 ± 29.9	338 ± 26.3*	336 ± 31.1
HW (g)	1.11 ± 0.17	1.353 ± 0.278	0.99± 0.18	0.942 ± 0.025
HW/BW (mg/g)	2.54 ± 0.34	3.37 ± 0.31	2.82 ± 0.16	2.83 ± 0.23
LVW (g)	0.78 ± 0.12	0.96 ± 0.2	0.69 ± 0.09	0.68 ± 0.03
LVW/BW (mg/g)	1.95 ± 0.09	2.25 ± 0.24	2.14 ± 0.22	2.02 ± 0.31
BG (mg/dl)	6.36 ± 0.24	7.7 ± 0.927*	5.5 ± 1.22	6.45 ± 1.003#
TG (mmol/l)	0.390 ± 0.132	0.3670 ± 0.158	0.955 ± 0.266*	0.540 ± 0.178#
CH (mmol/l)	1.880 ± 0.081	1.295 ± 0.189*	1.776 ± 0.077	1.418 ± 0.093#

Values are means ± SD of 8 hypertriglyceridemic rats in each group. WISc – Wistar control rats. WISo3 – Wistar rats fed with omega-3 fatty acid. HTGc – hypertriglyceridemic control rats. HTGo3 – hypertriglyceridemic rats fed with omega-3 fatty acid. BP – blood pressure. BW – body weight. HW – heart weight,. LVW – left ventricular weight. BG –blood glucose. TG – triglycerides. CH – cholesterol. Significant difference from WISc: * p<0.05.
Significant difference from HTGc: # p<0.05. ## p<0.01.

NO synthase activity

In comparison to non-hypertensive rats the activity of NOS was markedly increased in left ventricles of young SHR (8,2 ± 1,16 *vs* 1,37 ± 0,67 pmol/min/mg), **Figure 1** and to a lesser extent in old SHR (3,21 ± 0,75 *vs* 2,22 ± 0,56 pmol/min/mg), **Figure 2**. By contrast, NOS activity was suppressed (p<0,05) in both the groups of SHR rats that were supplemented with omega-3 PUFA. Mild but significant elevation of NOS activity was detected in HTG rats when compared to WIS rats (1,87 ± 0,42 *vs* 1,34 ± 0,1 pmol/min/mg) and omega-3 PUFA did increase it to 2,14 ± 0,53 pmol/min/mg **(Figure 3)**. Furthermore, supplementation of healthy WKY, WIS and LEW rats with omega-3 PUFA resulted in an increase of NOS activity, although the difference was not significant **(Figures 1 – 3)**.

Immunofluorescent distribution of Cx43

In situ immunolabeling of Cx43 revealed uniform distribution of Cx43-positive gap junctions with predominant localization at the intercalated discs in healthy rat hearts, ventricles **(Figure 4)**.

In contrast, abnormal localization of Cx43 was detected in all diseased rats group. Besides intercalated discs-related Cx43 there was a marked increase of Cx43 immunofluorescence signal on lateral surfaces of the cardiomyocytes. Moreover, myocardial localization of Cx43 was markedly disordered in the areas of fibrosis in old SHR and HTG rats. This pattern of Cx43 distribution was not eliminated by treatment of diseased rats with omega-3 PUFA. Representative pictures are shown in **Figure 4**.

Western blot analysis of Cx43 expression

Western blot analysis revealed in all rats three forms of Cx43, corresponding to the phosphorylated and non-phosphorylated forms of this protein. Two higher molecular forms of Cx43 were eradicated after treatment with alkaline phosphatase (not shown). We found a significant decline in expression of total and phosphorylated forms of Cx43 in both young and old SHR heart ventricles, while the ratio of phosphorylated forms to total Cx43 expression did not differ from non-hypertensive controls **(Figures 5, 6)**. Unlike SHR hearts the phosphorylated forms of Cx43, as well as the ratio of phosphorylated forms to total Cx43 expression, were increased in left ventricle of HTG compared to WIS rats but total Cx43 expression was not changed **(Figure 7)**. Supplementation of young and old SHR with omega-3 PUFA resulted in up-regulation of Cx43 **(Figures 5, 6)**, i.e. there was a significant increase of phosphorylated forms of Cx43 as well as ratio of phosphorylated forms to total Cx43 expression in both SHR groups. Moreover, total Cx43 protein level was significantly increased in old SHR hearts. In contrast, supplementation of HTG rats with omega-3 PUFA resulted in a significant suppression of both elevated phosphorylated forms of Cx43 and ratio of phosphorylated forms to total Cx43 expression, while it did not affect total Cx43 expression (**Figure 7**).

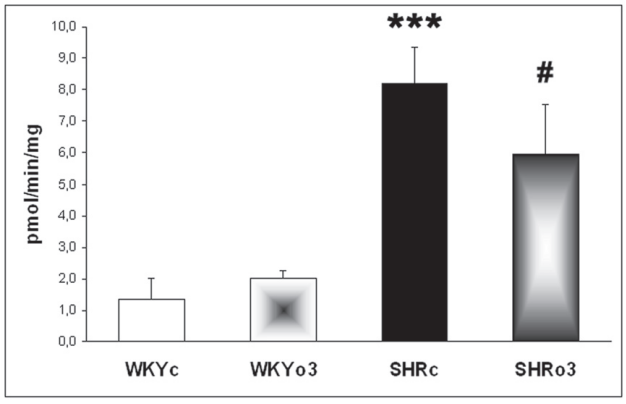

Figure 1 NOS activity in left ventricles of young untreated and omega-3 PUFA treated SHR and WKY rat hearts determined as pmol of L-citrulline/min/mg of protein
WKYc – untreated WKY rats, WKYo3 – WKY rats treated with omega-3 PUFA, SHRc – untreated SHR, SHRo3 – SHR treated with omega-3 PUFA.. Results are mean ± SD. ***p <0,001 vs. untreated WKY, # p<0,05 vs. untreated SHR.

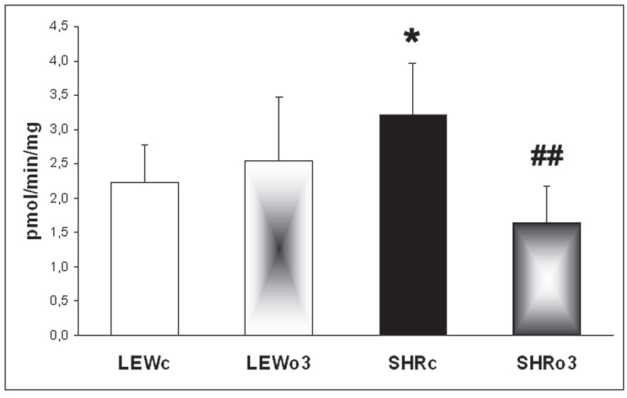

Figure 2 NOS activity in left ventricles of old untreated and omega-3 PUFA treated SHR and LEW rat hearts determined as pmol of L-citrulline/min/mg of protein
LEWc – untreated LEW rats, LEWo3 – LEW rats treated with omega-3 PUFA, SHRc – untreated SHR, SHRo3 – SHR treated with omega-3 PUFA. Results are mean ± SD. *p <0,05 vs. untreated LEW, ## p<0,01 vs. untreated SHR.

Discussion

In this study we have found for the first time that an increase of myocardial NOS activity in both young and old SHR was associated with down-regulation of Cx43, whereas suppression of this enzyme activity due to supplementation of SHR with omega-3 PUFA was associated with up-regulation of Cx43. Interestingly, supplementation of HTG rats with omega-3 PUFA did not suppress elevated NOS activity and did not affect total Cx43 expression while it suppressed its elevated phosphorylated forms. This suggests involvement

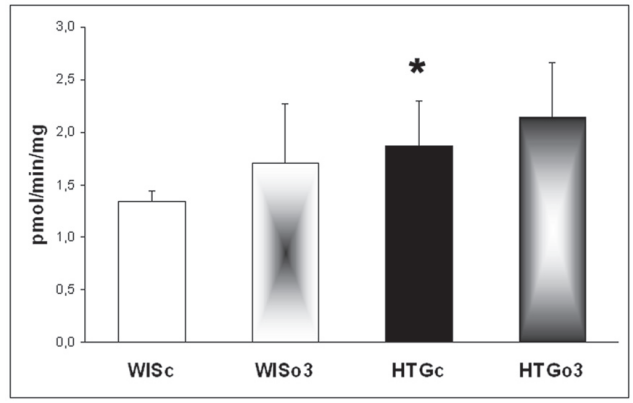

Figure 3 NOS activity in left ventricles of young untreated and omega-3 PUFA treated HTG and WIS rat hearts determined as pmol of L-citrulline/min/mg of protein
WISc – untreated WIS rats, WISo3 – WIS rats treated with omega-3 PUFA, HTGc – untreated HTG, HTGo3 – HTG treated with omega-3 PUFA. Results are mean ± SD. *p <0,05 vs. untreated WIS rats.

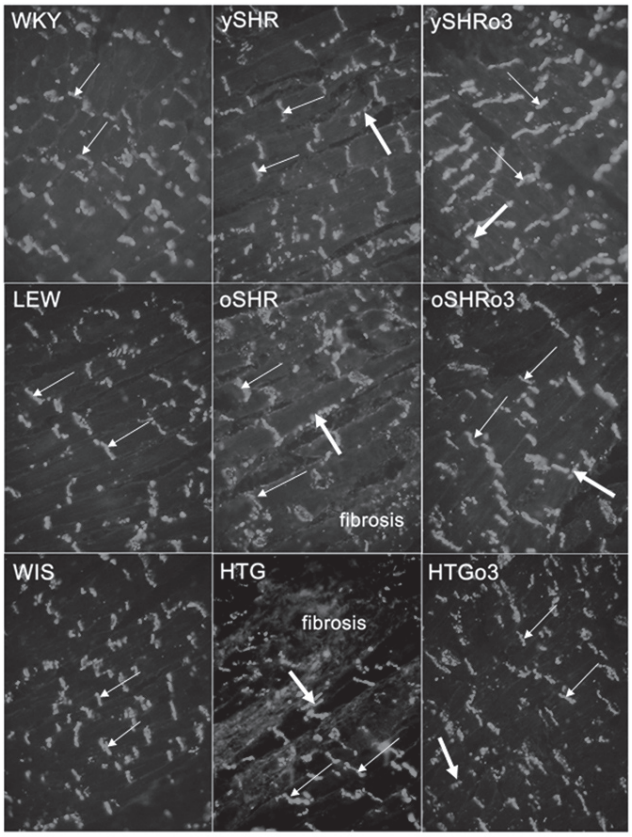

Figure 4 Representative pictures of immunofluorescent myocardial distribution of Cx43 in the left ventricles of healthy WKY, LEW and WIS rats as well as in untreated young and old SHR and young HTG rat hearts. Note: conventional distribution of Cx43--positive gap junctions at the intercalated discs (thin arrows) and enhanced expression of Cx43 on lateral surfaces (thick arrows) of the cardiomyocytes in diseased rat hearts (ySHR – young SHR, oSHR – old SHR, HTG) that was not eliminated by treatment with omega-3 PUFA (ySHRo3 – young SHR, oSHRo3 – old SHR, HTGo3 – HTG treated with omega-3 PUFA). Magnification, objective 40x.

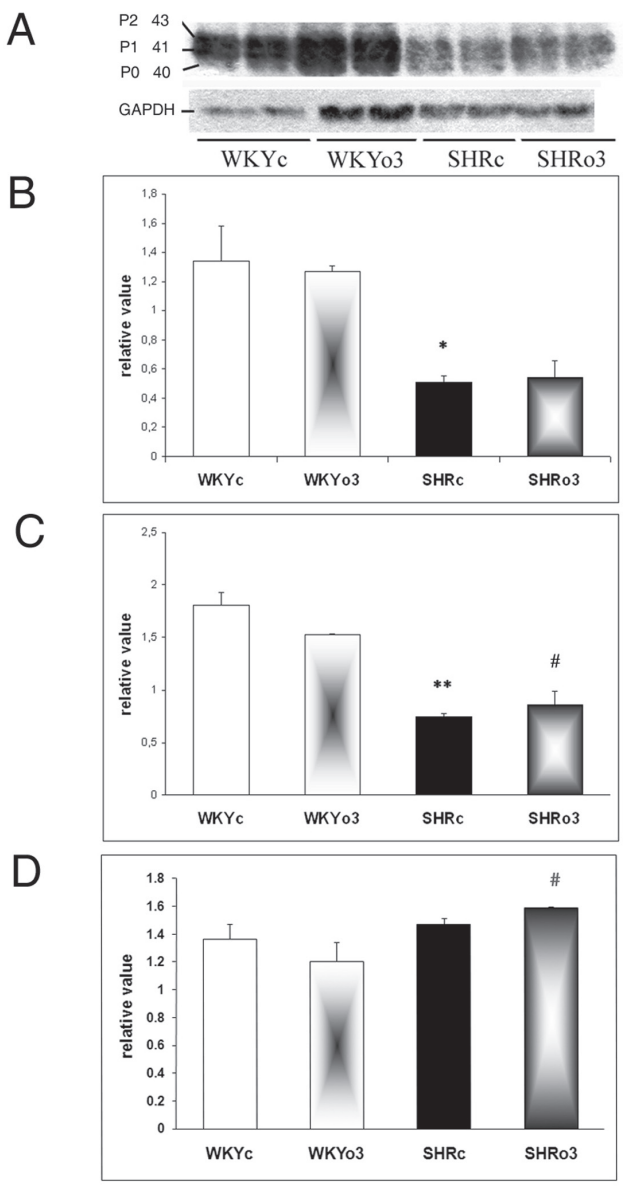

Figure 5 Representative immunoblot (A) showing three forms of Cx43 and densitometric quantification of total Cx43 expression (B), its phosphorylated form (C) and ratio of phosphorylated form to total Cx43 (D) normalized to GAPDH in left ventricles of young untreated and omega-3 PUFA treated SHR and WKY rat hearts

P0 – unphosphorylated, P1, P2 – phosphorylated forms of Cx43, WKYc – untreated WKY rats, WKYo3 – WKY rats treated with omega-3 PUFA, SHRc – untreated SHR, SHRo3 – SHR treated with omega-3 PUFA. Results are mean ± SD. *p <0,05, **p <0,01 vs. untreated WKY, # p<0,05 vs. untreated SHR.

of disease-related distinct pathways or signalling in omega-3 PUFA-induced cardioprotection.

Western blot analysis revealed in all rat-heart ventricles three forms of Cx43 corresponding to phosphorylated forms and to the non-phosphorylated form of this protein. This conventional pattern of Cx43 expression has been previously reported in our (28, 29) and other studies (1, 2). It should be noted that there was a significant reduction in total and phosphorylated forms of Cx43 expression in both young and old SHR heart ventricles that may contribute to impairment of cell-to-cell communication and increased propensity of the heart to arrhythmias (28). However, the ratio of phosphorylated forms to total Cx43 expression did not differ from non-hypertensive controls. It points out that phosphorylation of Cx43 itself is not altered in SHR hearts, i.e. that the function of existing Cx43 channels is most likely preserved. It is noteworthy that the treatment of SHR with omega-3 PUFA resulted in a significant increase of ratio of phosphorylated forms to total Cx43 expression, suggesting

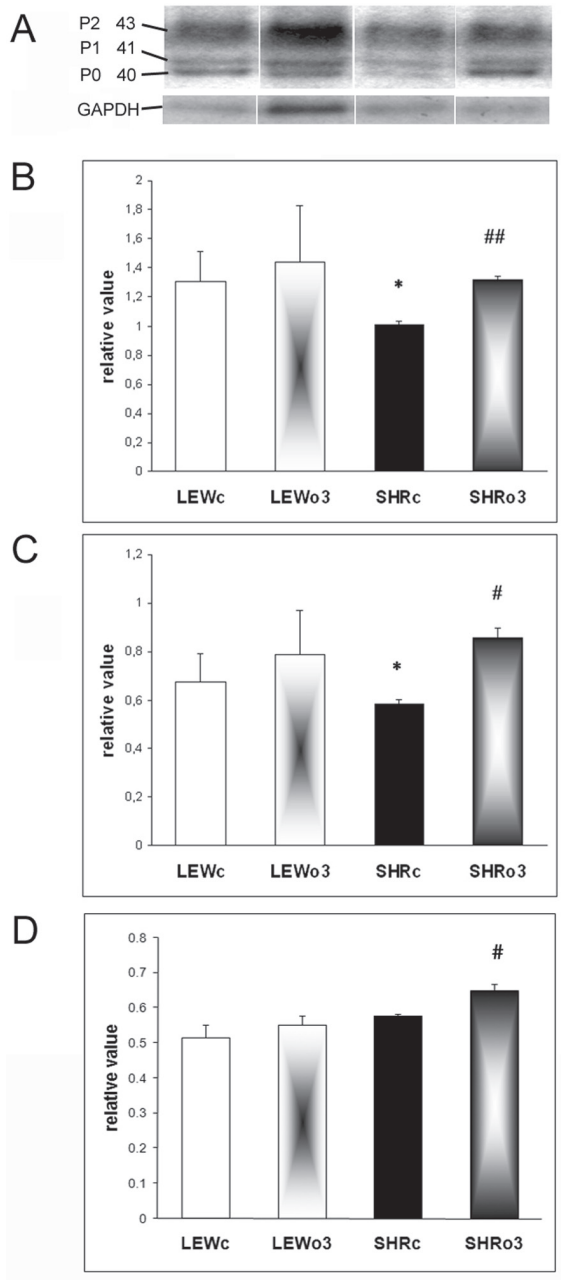

Figure 6 Representative immunoblot (A) showing three forms of Cx43 and densitometric quantification of total Cx43 expression (B), its phosphorylated form (C) and ratio of phosphorylated form to total Cx43 (D) normalized to GAPDH in left ventricles of old untreated and omega-3 PUFA treated SHR and LEW rat hearts

P0 – unphosphorylated, P1, P2 – phosphorylated forms of Cx43, LEWc – untreated LEW rats, LEWo3 – LEW rats treated with omega-3 PUFA, SHRc – untreated SHR, SHRo3 – SHR treated with omega-3 PUFA. Results are mean ± SD. *p <0,05, vs. untreated WKY, # p<0,05, ## p<0,01, vs. untreated SHR.

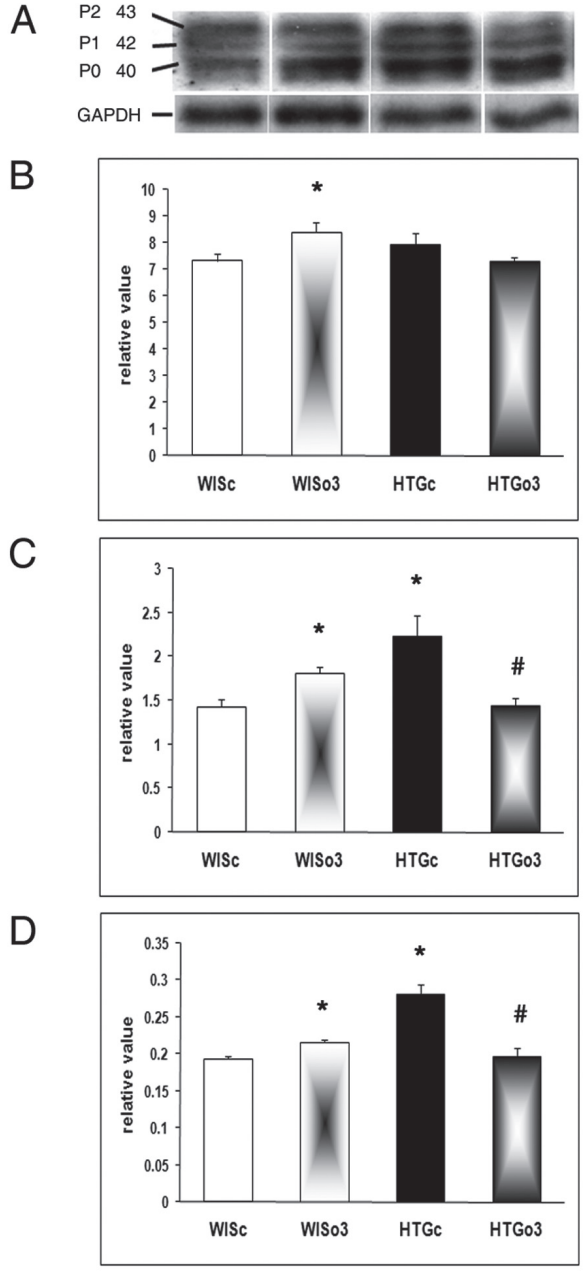

Figure 7 Representative immunoblot (A) showing three forms of Cx43 and densitometric quantification of total Cx43 expression (B), its phosphorylated form (C) and ratio of phosphorylated form to total Cx43 (D) normalized to GAPDH in left ventricles of untreated and omega-3 PUFA treated HTG and WIS rat hearts

P0 – unphosphorylated, P1, P2 – phosphorylated forms of Cx43, WISc – untreated WIS rats, WISo3 – WIS rats treated with omega-3 PUFA, HTGc – untreated HTG, HTGo3 – HTG treated with omega-3 PUFA. Results are mean ± SD. *p <0,05, vs. untreated WIS, # p<0,05 vs. untreated HTG.

enhanced phosphorylation. Moreover, the treatment caused a significant increase of total Cx43 expression in old SHR hearts. In parallel, these changes were accompanied by marked suppression of elevated NOS activity due to omega-3 PUFA

supplementation. It suggests that most likely there is some relationship between NOS activity and Cx43 expression and/or phosphorylation. Nevertheless, this issue should be investigated in more detail. In comparison to SHR, phosphorylated forms of Cx43 and ratio of phosphorylated to total Cx43 were increased in HTG rat ventricles. This suggests disease-related differences. However, it is not clear yet whether enhanced (or hyper-) phosphorylation of Cx43 is beneficial or detrimental for the heart. While the phosphorylated form only is linked with functional Cx43 channels (30) dephosphorylation of Cx43 due to ischemia has been shown to be associated with its altered distribution and impairment of cell-to cell communication (2). Likewise, marked reduction of Cx43 phosphorylation in the left ventricle of SHR hearts was accompanied with disordered distribution (remodeling) of Cx43-positive gap junctions. Although remodeling of Cx43 in SHR and HTG rats was not eliminated by omega-3 PUFA, the attenuation of this process can not be ruled out.

In contrast with the known enzymology of NOS, rather less is known about the regulation of its expression. This is of particular importance in the case of inducible NO synthase (iNOS), which - unlike endothelial NOS (eNOS) and neuronal NOS (nNOS) - is not expressed constitutively but induced by the influence of inflammatory mediators during disease (31). Earlier studies (32) have demonstrated evidence for increased reactive oxygen species, enhanced NOS expression, and elevated NO production in SHR, while the **t**herapy that ameliorated hypertension mitigated the up-regulation of iNOS (33). These findings support the role of oxidative stress in the genesis and/or maintenance of hypertension and compensatory up-regulation of the expression of eNOS and particularly iNOS in SHR**.** It was proposed (34) that the development of a more specific and potent inhibitor of iNOS might be beneficial in preventing pathological conditions such as essential hypertension. Taken together we suggest that marked increase of myocardial NOS activity in our study can be attributed to iNOS and that this specific isoform most likely accounts for down--regulation of Cx43 in SHR hearts. Although it is clear that we should test it by further study, this assumption is supported by recent findings (35) showing that increased NO production from iNOS contributed to the decreased myocardial Cx43 content two weeks after myocardial infarction in mice. Moreover, the suppression of myocardial NOS activity by omega-3 PUFA in SHR hearts that was associated with up-regulation of Cx43 (i.e. an increase of total and phosphorylated forms of Cx43 as well as ratio of phosphorylated forms of Cx43 to total) can be very likely attributed to suppression of iNOS expression. Indeed, high concentrations of PUFAs have been shown to inhibit ROS and RNS formation as well as the expression of iNOS in LPS-stimulated macrophages (16). It seems that omega-3 PUFA inhibit iNOS expression in part due to suppression of inflammation. Nevertheless, this specific issue needs to be elucidated in more detail.

Interestingly, in contrast to SHR hearts, the omega-3 PUFA did not suppress elevated myocardial NOS activity in HTG rats. Moreover, an increase of NOS was not associated with decline (shown in SHR) but up-regulation of myocardial Cx43 in these rats. It strongly points out the implication of eNOS in the increase of total NOS activity in omega-3 PUFA-treated HTG rat hearts. This view is supported by recent studies showing that statins up-regulate eNOS expression and decrease BP in hypertensive animals (36). All above-mentioned data invite an investigation whether over-expression of inducible NOS might account for down-regulation of myocardial Cx43 and whether its up-regulation is associated with an increase of endothelial NOS.

Most of the physiological actions of NO are brought about by its activation of the soluble guanylate cyclase (31). Since an activation of NO/cGMP/PKG signaling cascade mediates antihypertensive effects (37), we suggest that this pathway can be involved in omega-3 PUFA-related decline of BP in old SHR and HTG rats. In addition, it has been reported that NO is a potent modulator of gap-junctional coupling in endothelial cells (9). The NO donor SNAP (S--nitroso-N-acetylpenicillamine) enhanced gap-junctional coupling in HUVECs by about 40%, which was linked with enhanced incorporation of new Cx40 into the gap junctions. The NO-induced increase in cell coupling was elicited by a corresponding rise of cGMP. An increased coupling in omega-3 PUFA-enriched cells was associated with a more functional distribution of Cx43 at the cell--to-cell interfaces and with a specific enhancement of the main phosphorylated electrophoretic band of the protein in cultured astrocytes (18). Further studies, however, should clarify functional consequences of omega-3 PUFA-related modulation of Cx43 phosphorylated forms that has been found in our experiments. Nevertheless, it should be stressed that effects of omega-3 PUFA on Cx43 expression resulted in significant suppression of malignant arrhythmia occurrence in SHR as well as HTG rats (38, 39). It is noteworthy that the antiarrhythmic actions of omega-3 FA were associated with an improvement of Cx43 phosphorylation in SHR and suppression of elevated Cx43 phosphorylation in HTG rat hearts. On the other hand, both deficient (SHR) and hyper-phosphorylation (HTG) of Cx43 can be proarrhythmic, since both SHR and HTG rat hearts are indeed more prone to VF than healthy rats (38, 39). Interestingly, it has been reported that the anti-arrhythmic protection resulting from sodium nitroprusside infusion in the anaesthetized dog may, in part, be associated with the modulation of gap junctional function by NO (40). Moreover, exogenous NO can prevent Ca^{2+}overload (41), which is known to inhibit cell-to-cell coupling and facilitate occurrence of malignant arrhythmias (42).

Conclusion

Our findings have shown that there is an inverse relationship between NOS activity and Cx43 expression in SHR while not HTG rat hearts, and that both examined parameters are modulated by omega-3 PUFA. It points out a possible link between myocardial NOS and Cx43. We hypothesize that over-expression of iNOS is most likely implicated in down-regulation, while enhanced eNOS with that of up-regulation of myocardial Cx43: an idea that should be explored in further study to provide a clear conclusion about the role of NOS in Cx43 regulation.

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