OMEGA-3 FATTY ACIDS AND ATORVASTATIN SUPPRESS VENTRICULAR FIBRILLATION INDUCIBILITY IN HYPERTRIGLYCERIDEMIC RAT HEARTS: IMPLICATION OF INTERCELLULAR COUPLING PROTEIN, CONNEXIN-43

B. BACOVÁ, J. RADOSINSKA, V. KNEZL, L. KOLENOVÁ, P. WEISMANN, J. NAVAROVÁ, M. BARANCIK, M. MITASIKOVA, N. TRIBULOVA

OMEGA-3 FATTY ACIDS AND ATORVASTATIN SUPPRESS VENTRICULAR FIBRILLATION INDUCIBILITY IN HYPERTRIGLYCERIDEMIC RAT HEARTS: IMPLICATION OF INTERCELLULAR COUPLING PROTEIN, CONNEXIN-43

INTRODUCTION

Hypertriglyceridemia (also known as hypertriacyl glycerolemia) is an independent risk factor for coronary artery disease and it participates in the development of atherosclerosis and hypertension in human (1-3). The Prague hereditary hypertriglyceridemic (HTG) rat was developed as a model of human hypertriglyceridemia. Numerous studies have been performed on this model investigating genotype, humoral, metabolic and cardiovascular system alterations including structural remodelling (4). The latter likely contributes to increased propensity of the HTG rat heart to malignant arrhythmias, as shown in our pilot study (5). This seems very probably, since myocardial structural remodeling has been found to be associated with intercellular gap junctions remodelling (in experimental as well as clinical setting), which is highly arrhythmogenic (6-10). Cardiomyocytes are tightly interconnected by gap junctions, which are composed of multiple intercellular connexin (Cx) channels. These channels ensure direct cell-to-cell communication and electrical coupling in the heart allowing electrical and molecular signal propagation for proper function of the heart (11, 12). Cardiac ventricles express mainly Cx43. There is a strong evidence that impaired electrical coupling and non-uniform conduction due to alterations in the spatial distribution, expression and/or phosphorylation of Cx43 has a causative role in the genesis of arrhythmias associated with different chronic and acute pathophysiological conditions, e.g. hypertension, diabetes, ischemia or electrolyte disorders (10, 13-19).

It has been recently reported that statins in addition to the prevention of coronary heart disease and atherosclerosis exhibit antiarrhythmic properties in clinic (20, 21). Statins act by inhibiting the enzyme 3-hydroxy-3-methylglutaryl-CoA reductase, the rate-limiting enzyme in endogenous cholesterol biosynthesis. Inhibition of this enzyme has proven to be effective for lowering plasma total cholesterol, low-density lipoprotein-cholesterol, and triglyceride levels in humans and can therefore...
be useful to treat atherosclerotic and dyslipidemic disorders (1-3). However, there is still lack of knowledge about whole spectrum of mechanisms by which statins protect the heart. It can be expected that statins by modulation of cholesterol levels might affect membrane composition and properties, hence influencing the transmembrane proteins, including Cx channels.

Likewise to statins lipid lowering compounds, such as omega-3 fatty acids (omega-3 FA), have been shown in numerous epidemiological studies and clinical trials to reduce the incidence of cardiovascular disease (22) and sudden cardiac death (SCD) presumably by preventing life threatening arrhythmias (23, 24). Antiarrhythmic properties of omega-3 FA have been reported in animal models as well (23, 25, 26). Several mechanisms have been proposed and studied to explain anti-arrhythmic actions of omega-3 FA (27) but no definite mechanism has been validated. Nevertheless, these fatty acids have been shown to alter the physical structure of plasma membranes (28, 29) and modulate cell membrane ion currents (30).

Taking into account above mentioned facts, our goal was to examine whether myocardial cell-to-cell coupling protein Cx43 is involved in increased propensity of HTG rat hearts to ventricular fibrillation (VF) and whether the treatment with omega-3 FA and atorvastatin may affect susceptibility of the heart to VF as well as expression or distribution of Cx43.

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. This study was conducted on male 3-month-old HTG (n=36) and healthy Wistar (WIS) rats (n=36) that were treated either with omega-3 FA (eicosapentaenoic and docosahexaenoic acids, Vesteralens, Norway, 30 mg/100 g/day) or atorvastatin (Zentiva, Slovakia, 0.5 mg/100 g/day) for 2 months and compared with untreated ones (n=12 per each group).

Animals monitoring and samples collections

Blood pressure (BP) was measured noninvasively by tail-cuff plethysmography using the Statham Pressure Transducer P23XL (Hugo Sachs, Germany) at the beginning and the end of experiment and body weight (BW) was monitored as well. Fasting blood was collected at the end of experiment and used for blood glucose (BG), triglycerides (TG) and cholesterol (CHOL) assay using BG test meter and commercial kit from Biolab Diagnostics. The rats were ether-anesthesized and the hearts were rapidly excised into ice-cold saline, whereby whole heart (HW) and left ventricular weights (LVW) were registered. Thereafter, middle part of the left ventricle was rapidly frozen in liquid nitrogen, kept in freezer at -80°C until use for immunofluorescence staining and western blotting of Cx43. Small blocks of transmural ventricular tissues were processed for transmission electron microscopy as described elsewhere (31).

Transmission electron microscopy examination to detect qualitative structural alterations

Small (1-2 mm) transmural ventricular heart tissue samples (n=6 per each group) were fixed in buffered 2.5% glutaraldehyde, postfixed in 1% OsO4, dehydrated in series, infiltrated with propylene oxide, embedded in Epon 812 and routinely processed for transmission electron microscopy as described elsewhere (31).

VF susceptibility examination

The hearts of six rats from each group (rapidly excised under ether anesthesia) were perfused via canulated aorta in Langendorff mode with oxygenated Krebs-Henseleit solution at constant pressure of 80 mmHg and temperature of 37°C. Left ventricular pressure (LVP) was measured isolumetrically by a water-filled latex balloon, which was introduced into the left ventricle through the mitral orifice and connected to a pressure transducer. Bipolar ECG, LVP and the coronary flow were continuously monitored. Upon 20 min of equilibration, threshold for VF was tested by starting with 1 sec burst of electrical rectangular pulses (100 pulses/sec), 1 ms in duration at 10 mA (Electrostimulator ST-3ª, Hungary) delivered via stimulating electrodes attached to the epicardium of the right ventricle. When sustained (two minutes-lasting) VF was not induced after repetitive 5 stimuli, the stimulus intensity was increased in 5 mA steps.

Statistical analysis

The data are expressed as means ± standard deviations (S.D.). One-way ANOVA followed by Duncan’s post hoc test was used to analyze statistical significance between means.
Comparison between two groups was performed by the two-tailed Student’s t-test. Values were considered to differ significantly when $p<0.05$.

**RESULTS**

**General characteristics of animals**

HTG rats exhibited moderate but significant increase in BP and decrease in BW when compared to Wistar rats. Elevated BP was suppressed by the treatment either with omega-3 FA or atorvastatin, while BW was not affected. TG were significantly increased in HTG rats and reduced by both compounds. Moreover, omega-3 FA and atorvastatin significantly reduced CHOL in HTG rats. There were no significant changes in HW and LVW among the groups. Interestingly, BG was moderately increased in both Wistar and HTG rats upon feeding with omega-3 FA. All data are summarized in Table 1.

**Cx43-immunolabelling of left ventricle**

In ventricular tissues of all animal groups (Fig. 1), the anti-Cx43 antibody mainly stained at intercalated disk-related gap

<table>
<thead>
<tr>
<th></th>
<th>WISc</th>
<th>WISo3</th>
<th>WISa</th>
<th>HTGc</th>
<th>HTGo3</th>
<th>HTGa</th>
</tr>
</thead>
<tbody>
<tr>
<td>BP (mmHg)</td>
<td>123.4 ± 3.049</td>
<td>123.6 ± 2.607</td>
<td>117.4 ± 5.85</td>
<td>130.4 ± 5.594*</td>
<td>118.2 ± 3.420##</td>
<td>116.8 ± 1.92##</td>
</tr>
<tr>
<td>BW (g)</td>
<td>403 ± 22.2</td>
<td>426 ± 29.9</td>
<td>408 ± 28</td>
<td>338 ± 26.3*</td>
<td>336 ± 31.1</td>
<td>328 ± 25</td>
</tr>
<tr>
<td>HW (g)</td>
<td>1.11 ± 0.17</td>
<td>1.353 ± 0.278</td>
<td>1.626 ± 0.217</td>
<td>0.99± 0.18</td>
<td>0.94± 0.025</td>
<td>0.925 ± 0.153</td>
</tr>
<tr>
<td>LVW (g)</td>
<td>0.78 ± 0.12</td>
<td>0.96 ± 0.2</td>
<td>0.88 ± 0.14</td>
<td>0.69± 0.09</td>
<td>0.68 ± 0.03</td>
<td>0.63 ± 0.1</td>
</tr>
<tr>
<td>HW/BW (mg/g)</td>
<td>4.380 ± 0.774</td>
<td>5.433 ± 1.063</td>
<td>5.004 ± 0.907</td>
<td>3.469 ± 0.407</td>
<td>3.461 ± 0.482</td>
<td>3.294 ± 0.55612</td>
</tr>
<tr>
<td>BG(mg/dl)</td>
<td>6.36 ± 0.24</td>
<td>7.7 ± 0.927*</td>
<td>7.46 ± 1.32</td>
<td>5.5 ± 1.22</td>
<td>6.45 ± 1.003#</td>
<td>5.38 ± 0.64</td>
</tr>
<tr>
<td>TG (mmol/l)</td>
<td>0.390 ± 0.132</td>
<td>0.3670 ± 0.158</td>
<td>0.376 ± 0.116</td>
<td>0.955 ± 0.266*</td>
<td>0.540 ± 0.178#</td>
<td>0.686 ± 0.203#</td>
</tr>
<tr>
<td>CHOL (mmol/l)</td>
<td>1.880 ± 0.189</td>
<td>1.295 ± 0.189*</td>
<td>1.722 ± 0.254</td>
<td>1.776 ± 0.077</td>
<td>1.418 ± 0.093#</td>
<td>1.53 ± 128 #</td>
</tr>
</tbody>
</table>

Values are means ± S.D. of 8 hypertriglyceridemic rats in each group. WISc- Wistar control rats, WISo3- Wistar rats fed with omega-3 fatty acid, WISa - Wistar rats fed with atorvastatin, HTGc- Hypertriglyceridemic control rats, HTGo3- Hypertriglyceridemic rats fed with omega-3 fatty acid, HTGa- Hypertriglyceridemic rats fed with atorvastatin, BP- Blood pressure, BW- body weight, HW- heart weight, LVW- left ventricular weight, BG- blood glucose, TG- triglyceride, CHOL- cholesterol. * Significantly different from WISc ( P<0.05), # Significantly different from bHTGc ( P<0.05), ##P<0.001.

**Fig. 1.** Distribution of myocardial Cx43 in untreated and omega-3 FA and atorvastatin-treated HTG and Wistar rat heart left ventricles. Note conventional immunofluorescence labeling of Cx43 at the gap junctions (thin arrows) and on lateral surfaces of the cardiomyocytes (thick arrows) that is enhanced in HTG rats compared to Wistar rats regardless the treatment. Bar-50 µm.
junctions as a bright punctate staining with very little on the longitudinal cardiomyocyte borders in Wistar rats, while enhanced in HTG rat. In the latter, in addition, markedly disorganized distribution of Cx43 was detected in the area of fibrosis (not shown). The alterations in distribution of Cx43-positive gap junctions including enhanced lateralization was attenuated but not fully eliminated by the treatments either with omega-3 FA or atorvastatin. In parallel, HTG-treated rats exhibited higher labeling levels compared to un-treated once that was confirmed by quantitative image analysis, while anti-Cx43 immunofluorescence signal was lower in HTG compared to Wistar rat heart ventricles (Fig. 2).

Western blot analysis of Cx43

Western blot analysis revealed in all rats three forms of Cx43 corresponding to two phosphorylated forms and to an un-phosphorylated form of this protein. There was no significant change in total Cx43 expression between untreated HTG and Wistar rats (Fig. 3A). However, phosphorylated form of Cx43 (Fig. 3B) as well as ratio of phosphorylated to an un-phosphorylated form were increased in left ventricle of HTG rats (p<0.05) compared to Wistar (Fig. 3C). Supplementation of HTG rats with omega-3 FA or treatment with atorvastatin resulted in significant suppression of this elevation. On the other hand, both compounds increased significantly phosphorylated status of Cx43 in Wistar rat heart ventricle (Fig. 3).

Qualitative subcellular alterations

There were abnormalities of extracellular matrix, cardiomyocytes ultrastructure and gap junction distribution in HTG rat ventricles. Major changes (Fig. 4) consisted of activation of fibroblasts and increased extracellular matrix, proteins resulting in focal fibrotic areas, decreased electrondensity of some mitochondria, myealinisation of sarcolemma, dehiscence of fascia adherence junctions and increased number of lateral gap junctions as well as cytoplasmic annular profiles of internalized intercalated dis-related gap junctions (not shown). Treatment of HTG rats either by omega-3 FA or atorvastatin caused of improvement of fine structure of the cardiomyocytes and integrity their junctions (Fig. 4).

Vulnerability to VF and some characteristics of Langendorff-perfused hearts

As it is demonstrated on Fig. 5A, VF threshold of HTG rat hearts was less than in normal Wistar rats (15 mA vs. 25 mA, p<0.05). Omega-3 FA supplementation and atorvastatin treatment resulted in a significant increase of VF threshold in Wistar and to greater extent in HTG rat hearts (i.e. to 40 mA and 45 mA respectively). Moreover, in three omega-3 FA fed HTG rats only transient VF could be induced using repetitive 45 mA test stimulus. Furthermore, the treatment with either compounds significantly supressed elevated heart rate in HTG rats (Fig. 5B). LVP was also significantly increased in omega-3 FA-treated HTG rat heart and to lesser extent in atorvastatin-treated (Fig. 5C). There were no significant changes in coronary flow (CF) at baseline perfusion between Wistar and HTG rat hearts, i.e. 13.57±1.10 vs. 12.07±0.87 ml/min. Omega-3 FA and atorvastatin significantly increased CF in Wistar rat hearts to 17.75±1.50 and 16.98±0.58 ml/min, while the treatment did not significantly afffect CF in HTG rat hearts (14.07±1.68 and 13.87±0.58 ml/min upon omega-3 FA and atorvastatin respectively).
We have shown in this study for the first time that HTG rats are more vulnerable to potentially lethal arrhythmia, such as VF, because of lower threshold for VF. It points out that dyslipidemia itself may be a risk factor to increase propensity of the heart to life-threatening arrhythmias. In this context it is important to note that HTG rat hearts prone to VF exhibited both, abnormal myocardial Cx43-gap junctions topology (i.e. enhanced lateralization and disordered distribution at area of fibrosis) and abnormal Cx43 protein expression (i.e. increased phosphorylated forms of Cx43). In general, acute and chronic forms of heart disease caused by diverse etiologies are associated with changes in the expression of Cx43 and remodeling of gap junctions. Such remodeling may have both adaptive and maladaptive consequences and contribute to major clinical events, as they are heart failure (mechanical dysfunction) and sudden cardiac death (due to VF) (9). It has been shown by numerous studies, including ours, (6, 9, 10) that change in gap junctions distribution (i.e. Cx43 remodeling) is one of the key factors underlying arrhythmia substrate in ischemic and nonischemic forms of heart diseases or in aged hearts, most likely due to abnormalities in electrical impulse conduction (6, 9, 14). Subcellular alterations pointing out impairment of cardiomyocyte plasma membranes integrity (myelinization) indicate that hypertriglyceridemia may affect cell membrane compositions and consequently assembly of Cx43 into gap junction plaques. It can explain, at least partially, the decrease of immunostaining of Cx43 (Fig. 2) and presence of cytoplasmic annular gap junctions in HTG rat hearts. The more that decrease of plasma TG in HTG rats treated with atorvastatin and omega-3 FA was accompanied with enhanced Cx43 immunostaining and preservation of subcellular structure. The more that decrease of plasma TG in HTG rats treated with atorvastatin and omega-3 FA was accompanied with enhanced Cx43 immunostaining and preservation of subcellular structure. Western blot analysis revealed that compared to Wistar rats there were no significant changes in total myocardial Cx43 protein expression, whereas phosphorylated form of Cx43 was significantly increased in HTG rats. Question arises, what does it mean in respect of Cx43 channel function and arrhythmogenesis? It has been established that phosphorylation of Cx43 is a prerequisite for proper channel function, while both
deficient and hyper-phosphorylation indicate that myocardial cell-to-cell electrical coupling ensured by these channels might be impaired (12). Data in literature (14, 16, 32) and our previous studies (10, 19) suggest, that reduced Cx43 expression and/or phosphorylation (e.g. due to ischemia, hypertension, hyperthyroidism, or aging) in experimental as well as clinical settings facilitate occurrence of malignant arrhythmias. Accordingly, it can be expected that increased expression and/or phosphorylation of Cx43 in such conditions should improve cell-to-cell coupling and decrease cardiac susceptibility to VF. Indeed, up-regulation of Cx43 (i.e. increase of both, total expression and phosphorylation) in old male and female spontaneously hypertensive rats (SHR) supplemented with omega-3 FA was associated with significant suppression of inducible VF (26). Unlike to SHR, increased propensity to VF in HTG rat hearts was associated with hyper-phosphorylation of Cx43 and treatment with either omega-3 FA or atorvastatin normalized Cx43 phosphorylation as well as significantly reduced VF-inducibility. Altogether, it indicates the implication of Cx43 alterations, particularly these related to Cx43 phosphorylation, in cardiac susceptibility to malignant arrhythmias. Furthermore, enhanced immunostaining of Cx43 in HTG rat hearts due to treatment with atorvastatin and omega-3 FA likely reflects the attenuation of Cx43 mislocalization and an improvement of the integrity of the cardiomyocytes and intercellular junctions revealed by ultrastructure examination.

The results strongly point out that omega-3 FA and atorvastatin can prevent or attenuate the maladaptive consequences of hypertriglyceridemia on the heart, such as hyper-phosphorylation of Cx43 and subcellular alterations. It has been reported that omega-3 FA by regulation of transcription factors, such as sterol-regulatory-element binding proteins and peroxisome proliferator-activated receptors (PPAR), modulate the expression of genes, including these controlling lipid homeostasis (33). This can explain their lipids lowering actions demonstrated also in our study. However, it should also be noted that omega-3 FA and atorvastatin treatment resulted in an increase of threshold for VF in healthy Wistar rat hearts as well and it was associated with up-regulation of Cx43, i.e. its expression as well as phosphorylation. It points out a possible differential signaling mechanisms and pathways implicated in regulation of Cx43 channel function resulting in arrhythmias protection in healthy and diseased hearts.

According to the literature the cardioprotective and antiarrhythmic effects of omega-3 FA seem to be mediated through effects on several pathways within cardiomyocytes. These may include direct interaction in changing the composition of cell membranes and membrane function, activating or suppressing signaling molecules (such as NO and PKCepsilon, which are known to affect Cx43), direct interaction with DNA as well as with proteins that affect transcription factors, and affecting enzyme activities (including antioxidants as well as protein kinases) and sarcoplasmic-Golgi trafficking (33). It seems very probably that some of these actions may be involved in modulation of Cx43 expression, trafficking and assembly into gap junctions as well as in phosphorylation and Cx43 channels function in rat hearts. However, specific action related directly to Cx43 alteration remains to be elucidated. Likewise to omega-3 FA, statins were shown to be ligands of PPAR, hence affecting gene transcription in addition to inhibition of cholesterol biosynthesis (34). Indeed, statins up-regulate endothelial nitric oxide synthase (eNOS), activate antioxidant defense enzymes and cellular protective PI3K-Akt and p38MAPK-HSP27 signaling pathways (34). Whether omega-3 FA and statins may regulate Cx43 gene expression is not elucidated yet. Both compounds have been reported to modulate Cx43 protein expression and/or intercellular communication in mesangial cells (35), atherosclerotic aorta (36) as well as in aorta and heart tissue of hypertensive rats (26, 37) Moreover, lipophilic compounds, as they are unsaturated fatty acids (e.g. omega-6 FA) and some statins (e.g. atorvastatin) have been reported directly affect cell-to-cell coupling (12).

In conclusion, treatment of HTG and healthy rats with omega-3 FA and atorvastatin resulted in significant suppression of inducible VF. Modulation of Cx43 protein expression and/or phosphorylation by these compounds may be, at least partially, involved in their antiarrhythmic ability. Nevertheless, further studies are needed to investigate how omega-3 FA and atorvastatin affect Cx43 phosphorylation and perhaps Cx43 gene transcription in impaired as well as healthy heart.

Acknowledgements: This study was realized by support of VEGA 2/0049/09, APVV 51-059505 and APVV SK-UA-0022-09 grants. Omega-3 FA was generous gift of Vesteralens Company, Norway and atorvastatin was provided by Zentiva Company, Slovakia.

Conflict of interests: None declared.

REFERENCES


Received: September 9, 2010
Accepted: December 20, 2010

Author’ address: Dr. Narcisa Tribulova, Institute for Heart Research, Slovak Academy of Sciences, 9 Dubravská cesta Street, PO Box 104, 840 05 Bratislava, Slovakia; Phone: 004212 5466405; Fax: 004212 54776637; E-mail: narcisa.tribulova@savba.sk