RESEARCH PAPER

Connexin 43 and metabolic effect of fatty acids in stressed endothelial cells

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Abstract Changes in the inner mitochondrial membrane potential ($\Delta\psi$) may lead either to apoptosis or to protective autophagy. Connexin 43 (Cx43), a gap junction protein, is suggested to affect mitochondrial membrane permeability. The aim of our study was to analyze *Cx43* gene expression, Cx43 protein localization and mitochondrial function in the human endothelial cells stressed by dietary-free fatty acids (FFA) and TNFα. Human endothelial cells (HUVECs) were incubated with (10–30 uM) palmitic (PA), oleic (OA), eicosapentaenoic (EPA) or arachidonic (AA) acids for 24 h. TNFα (5 ng/ml) was added at the last 4 h of incubation. The *Cx43* gene expression was analyzed by the quantitative real-time PCR. The Cx43 protein concentrations in whole cells and in the isolated mitochondria were measured. Changes in $\Delta\psi$ and Cx43 localization were analyzed by flow cytometry or fluorescence microscopy. Generated ATP was measured by a luminescence assay. TNFα, PA and OA significantly decreased $\Delta\psi$, while AA ($P = 0.047$) and EPA ($P = 0.004$) increased $\Delta\psi$ value. Preincubation with EPA or AA partially prevented the TNFα-induced decrease of $\Delta\psi$. Incubation with AA resulted in up-regulation of the *Cx43* gene expression. AA or PA significantly increased Cx43 protein content; however, presence of TNFα in general aggravated the negative effect of FFA. Only EPA was found to increase ATP generation in HUVECs. The fatty acid-specific induction of changes in Cx43 expression and protein concentration as well as the normalization of $\Delta\psi$ and increase of ATP generation seem to be the separate, independent mechanisms of FFA-mediated modulatory effect in the human endothelial cells pathology.

Keywords HUVEC · FFA · Cellular stress · Cx 43 · Mitochondrial membrane potential · TNFα · ATP

Introduction

Intercellular communication, mediated by special channels named gap junctions (GJ), plays a crucial role in the regulation of the local signals' transmission and spreading (Mroue et al. 2011). Such a local way of communication is also important in essential cellular processes such as proliferation, differentiation as well as apoptosis (Mroue et al. 2011). One of the main GJ forming proteins is connexin family (Cxs) (Mroue et al. 2011). Connexin 43 (Cx43) is expressed mainly in the heart muscle and endothelial cells (Mroue et al. 2011; Brisset et al. 2009). Several observations demonstrated important Cx43 role in changes that affect cellular (including endothelium) fate. Cx43 translocated from the cellular to mitochondrial membranes during ischemic stress pointing to the participation of this connexin type in the mitochondria-driven cellular response (Alex et al. 2005; Li et al. 2002). Decrease of Cx43 expression in mice resulted in much more severe and extensive necrotic changes of the myocardium during ischemic events (Schwanke et al. 2002). The above results suggest that Cx43 may affect some kind of a multi-protein complex that forms mitochondrial channels, which control mitochondrial inner membrane permeability. In such a case, Cx43 may participate in the cytoprotective effects

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similar to another mitochondrial potassium channel regulated by ATP, mitoK-ATP channel (Rodriguez-Sinovas et al. 2007).

The endoplasmic reticulum (ER) is the site of protein synthesis, their folding, their redistribution to either other intracellular compartments or elimination (Zhang 2010). All processes interfering with ER functions may result in the accumulation of unfolded proteins and induction of ER stress accompanied by toxic-free radicals generation (Zhang 2010). If an adaptation process such as lysosomal proteolysis/autophagy is insufficient, then an immuno-inflammatory reaction is activated and the affected cells are eliminated by apoptosis (Zhang 2010; Tsai and Weissman 2010). Several publications suggested a functional link between ER stress and GJ in cells, i.e., myocardial and cancer ones (Zhang 2010). Cooperation of these two mechanisms may regulate cell/tissue growth (remodelling) and increase cellular resistance to stress conditions caused by hypoxia, anticancer drugs or radiation (Zhang 2010; Autsavapromporn et al. 2011; Huang et al. 2009). A correlation between ER stress and GJ dysfunction was previously shown (Huang et al. 2009). Incubation of the mesangial cells with ER stress-inducing agents resulted in a decrease of Cx43 expression at mRNA and protein levels due to both decreased activation of the *Cx43* gene promoter as well as acceleration of the Cx43 protein degradation (Huang et al. 2009). Reduced amount of the Cx43 protein, due to ER stress, was demonstrated in the mesangial, human hepatoma cells as well as in human umbilical vein endothelial cells (HUVECs) (Huang et al. 2009).

Hypoxia, tumor necrosis factor alpha (TNFα) as well as metabolic substrate overload (some free fatty acids, glucose) are known stressors affecting cellular ER and mitochondrial function (Morgan and Liu 2010; Koopman et al. 2010; Honda et al. 2005). The initial phase of cellular dysfunction is marked by changes of the inner mitochondrial membrane permeability and mitochondrial membrane potential ($\Delta\psi$) (Morgan and Liu 2010; Koopman et al. 2010; Honda et al. 2005; Poyton et al. 2009). A decrease in $\Delta\psi$ is connected with disturbances in the respiratory chain function and increased generation of reactive oxygen species (ROS), which may lead to cell death by mechanisms of apoptosis or necrosis (Morgan and Liu 2010; Poyton et al. 2009).

ROS are important regulators of gene expression, by activating redox-sensitive transcription factors such as hypoxia inducible factor -1 (HIF-1) or nuclear factor kappa B (NFκB) (Gwinn and Vallyathan 2006). *Cx43* gene expression is also regulated by oxidative stress (Liu et al. 2009). It is suggested that increased Cx43 expression and intensified intercellular communication induce an anti-proliferative effect in the cancer cells (Liu et al. 2009).

The aim of the presented study is to analyze *Cx43* gene expression, Cx43 protein localization and mitochondrial function in the human endothelial cells stressed by dietary-free fatty acids (FFA) as well as TNFα.

Materials and methods

Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords by collagenase digestion as previously described (Jaffe et al. 1973) and were grown for 2–4 days in EBM medium (Sigma) in the presence of 2% BSA and 2 nM of vascular endothelial growth factor (VEGF) according to a previously described protocol (Kiec-Wilk et al. 2005). The cells were incubated with nontoxic, physiological blood concentrations of the free fatty acids, 30 μM of palmitic acid (PA), oleic acid (OA), eicosapentaenoic acid (EPA) or 10 μM of arachidonic acid (AA) for 24 h. To induce stress, TNFα (5 ng/ml) was added to the cell culture for the last 4 h of incubation with each FFA (Morgan and Liu 2010; Grieger et al. 2005). The cytotoxic effect was evaluated by the lactate dehydrogenase (LDH) measurement method (CytoTox 96 NonRadioactive Cytotoxicity Assay, Promega).

Monitoring of the mitochondrial membrane potential ($\Delta\psi$)

The mitochondrial membrane potential was monitored in the cells incubated with FFA/TNFα by flow cytometry (FACSCanto, Becton–Dickinson) using JC-1 staining (Cossarizza 1993). The cells were then exposed to 2 mM JC-1 dye solution (MitoProbe Assay Kit M34152, Invitrogen) and incubated in the dark for 45 min at 37°C. The cells were washed and diluted in 500 μl of PBS and analyzed by FACS using 488 nm excitation with 530/30 nm (FL1, green) and 585/42 nm (FL2, orange) emission filters.

Fluorescence signals generated by 10,000 cells were collected in a single analysis. The data were analyzed using the FacsDIVA software (Becton–Dickinson). The ratio of red/green fluorescence intensities reflected changes in the mitochondrial inner membrane potential. This ratio was the result of the $\Delta\psi$ only, without influence of other factors such as mitochondrial size, shape, or density. A known uncoupling agent, carbonyl cyanide 3-chlorophenylhydrazone (CCCP, 50 μM) was used as a positive control.

Intracellular ATP concentration

Following the treatment described above, a batch of 2×10^5 HUVECs was used to measure intracellular ATP

concentration with the use of ATPliteTM Luminescence ATP Detection Assay System (Perkin Elmer). ATP-dependent luminescent reaction with added luciferase enzyme (from *Photinus pyralis*) and D-luciferin was monitored (GENios TECAN Reader) in accordance to the manufacturer's protocol/guidelines. Results were calculated with Magellan 6 software as nmol ATP, then adjusted for protein content (measured by Lowry's method) and presented as nmol ATP/mg of protein.

Analysis of the gene expression

Total cellular mRNA was isolated using the TRIzol® method (Invitrogen Life Technologies) after HUVECs were incubated with the investigated factors. The analysis of *Cx43* expression was performed using a quantitative real-time PCR (qRT-PCR) with specific primers: Cx43-f 5′-TCAATCACTTGGCGTGACTTCA-3′, Cx43-r 5′-GCG CTCCAGTCAACCCATGT-3′ and QuantiTect SYBR Green PCR (Qiagen), DNA Engine Opticon II (MJ Research). *GAPDH* served as the reference gene. Relative gene expression was calculated as a normalized C_T difference between a sample incubated with a selected compound and its corresponding control probe; then adjusted for gene amplification efficiency relative to the expression level of the housekeeping gene, *GAPDH*. The formula used for calculations was according to Pfaffl et al. (2001).

Detection of Cx43 protein distribution in HUVECs

Connexin 43 protein was visualized by using a 1:100 dilution of primary antihuman rabbit polyclonal antibodies (Santa-Cruz) and 1:1,000 dilution of secondary anti-rabbit antibodies labeled with Alexa Fluor 594 (Invitrogen). Imaging studies were performed in 96-well plates (BD Falcon) using the BD Pathway 855 Bioimager microscope (BD Biosciences). All the imaging data were analyzed with Attovision software package.

Estimation of the Cx43 protein amount in HUVECs and isolated mitochondria

Mitochondria were isolated from HUVEC's by the Mitochondrial Isolation Kit for Cultured Cells with Halt™ Protease Inhibitor Cocktail, EDTA-free (PIERCE). Protein content was estimated using Cell Harvesting Buffer (Sigma) and the Bradford method. Immunoblot analyses were performed using the Laemmli method (Penna and Cahalan 2007). Protein expression of Cx43 was estimated using the 1:100 dilution of the specific antihuman rabbit polyclonal antibodies (Santa-Cruz) and the secondary horseradish peroxidase-conjugated anti-rabbit antibodies (NEB). Electrochemiluminescence reagent (ECL) was used

for the protein final detection. Enhanced chemiluminescence, performed according to the manufacturer's instructions/guidelines (Amersham), was used to demonstrate positive bands that were visualized after exposure on a transparent medical X-ray film. Cyclophilin D (CyPD), a protein typically expressed in the mitochondria, served as the reference protein in the analysis of the isolated organelle, while actin-beta was used as the reference protein in the analysis of whole cell content.

Statistical analysis

Data were analyzed by one-way ANOVA and unpaired t-test for comparisons of quantitative variables. The cut off for statistical significance was set at $P < 0.05$. The statistical analysis was performed with Statistica 6 for Windows from Statsoft.

Results

None of the used concentrations of factors (FFA, TNFα) demonstrated toxic effects in the used HUVECs (results not presented).

The extent of the cellular stress was evaluated by analysis of changes in the mitochondrial membrane potential ($\Delta\psi$) after HUVECs incubation with the investigated compounds as well as the known mitochondrial uncoupling agent, CCCP (Morgan and Liu 2010; Koopman et al. 2010; Honda et al. 2005; Poyton et al. 2009; Grieger et al. 2005). FACS analysis confirmed generation of stressful conditions by a significant decrease of $\Delta\psi$ after incubation with CCCP ($P < 0.0001$), TNFα ($P = 0.003$) as well as with PA ($P = 0.042$) and OA ($P = 0.002$) (Fig. 1). On the contrary, incubation of HUVECs with polyunsaturated FFA, either EPA ($P = 0.004$) or AA ($P = 0.047$) caused significant increase of the mitochondrial membrane potential ($\Delta\psi$) and partially ameliorated the negative effect of TNFα on $\Delta\psi$ ($P = 0.007$ for EPA, and $P = 0.051$ for AA) (Fig. 1).

Incubation with FFA resulted in significant up-regulation of *Cx43* gene expression following AA only ($P = 0.0031$) (Fig. 2). However, pre-incubation with examined FFAs and subsequently with TNFα resulted in the aggravation of the TNFα effect. This was significant for AA and PA (Fig. 2).

The quantitative analysis of Cx43 protein expression under different incubation conditions by Western blot confirmed low but significant increase of Cx43 protein after incubation with AA ($P = 0.039$) in HUVECs and its mitochondria (Fig. 3a, b). PA ($P = 0.021$) induced elevation of Cx43 protein level in whole cells, but not in mitochondria (Fig. 3a, b).
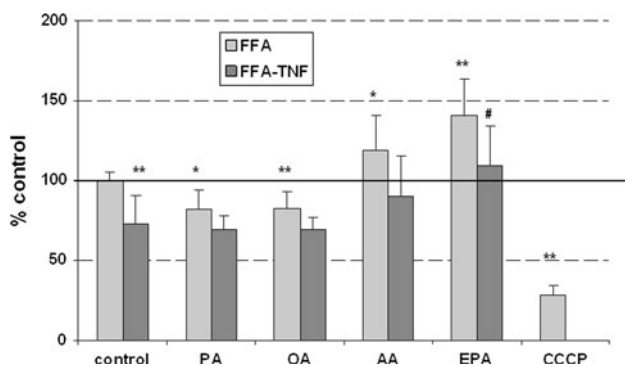
Fig. 1 The FACS quantitative analysis of changes in the inner membrane mitochondrial potential in HUVECs after their incubation with FFAs with or without TNFα. Results are presented as ratios of *red* to *green* fluorescence signals from each sample that reflect mitochondrial metabolism following cells incubation with investigated factors, compared to the status of control sample (percent of control). *AA* 0 μM arachidonic acid, *OA* 30 μM oleic acid, *EPA* 30 μM eicosapentoenoic acid, *PA* 30 μM palmitic acid, *TNF* 5 ng/ml TNFα, Results are mean of 3 experiments done in triplicate, ±SD. $P < 0.05$ versus control, $**P < 0.003$ versus control, $^{\#}P < 0.004$ versus control-TNF
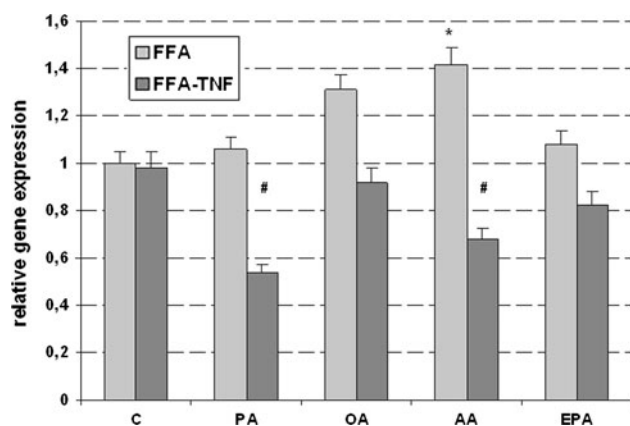

Fig. 2 The quantitative (qRT-PCR) analysis of *connexin 43* gene expression after 24 h-long incubation with investigated fatty acids or FFA and TNFα. Results are mean of 3 experiments done in triplicate, ±SD; $*P < 0.05$ versus control; $^{\#}P < 0.05$ FFA versus FFA + TNF. *C* control, *AA* 10 μM arachidonic acid, *OA* 30 μM oleic acid, *EPA* 30 μM eicosapentoenoic acid, *PA* 30 μM palmitic acid, *TNF* 5 ng/ml TNFα

The elevation of Cx43 protein in HUVEC cytoplasm after their incubation with AA ($P = 0.043$), OA ($P = 0.027$) and no effect of TNFα was also demonstrated by a quantitative analysis by fluorescence microscopy BD Pathway 855 Bioimager (Fig. 4). Incubation with FFAs followed by the addition of TNFα resulted also in decrease of Cx43 protein, in particular after EPA ($P = 0.046$) (Fig. 4).

Saturated PA significantly decreased ATP generation in HUVECs ($P = 0.038$). A similar tendency was observed

after TNFα ($P = 0.029$) (Fig. 5). Preincubation with EPA significantly increased cellular ATP concentration and protected against the inhibitory effect of TNFα on ATP generation (Fig. 5a, b).

Discussion

There is a growing interest in search for mechanisms that would explain cellular dysfunction characteristic for the metabolic syndrome. At the molecular level, such intracellular changes are mostly related to metabolic substrate overload, cellular apoptosis, but they are also connected to the activation of cellular protective mechanisms such as endoplasmic reticulum stress and/or mitochondrial increased ATP biosynthesis, induction of autophagy (Rodriguez-Sinovas et al. 2007; Zhang 2010; Morgan and Liu 2010).

In our study, we observed in in vitro model, a decrease of the mitochondrial membrane potential ($\Delta\psi$) induced by TNFα as well as by nontoxic, physiological concentrations of nutritional PA and OA, which remains in agreement with other reports describing FFA and TNFα as potent cellular stressors, also in the endothelial cells (Morgan and Liu 2010; Grieger et al. 2005). Our results indicated that the observed effects were fatty-acid specific (Shaw et al. 2007), since only polyunsaturated AA and EPA under the same conditions increased $\Delta\psi$ and ameliorated the negative effect of TNFα.

The saturation-dependent effects of FFA on the endothelial cell functions are well known (Shaw et al. 2007; Azekoshi et al. 2010; Moreno 2009; Fuentez et al. 2001). Saturated fatty acids have been shown to cause endothelial dysfunction (Azekoshi et al. 2010), monounsaturated fatty acids exert a neutral or modestly beneficial effect (Moreno 2009), while reported results of incubation with long-chain n-3 and n-6 polyunsaturated fatty acids give inconclusive results (Grieger et al. 2005). The effects of exogenous FFAs complexed with serum albumin appear to depend on several factors such as the type of FFA, duration of incubation, presence of pro-inflammatory cytokines (TNFα), ischemia, ROS, etc. (Shaw et al. 2007). Lipotoxicity observed in the endothelial cells is characteristic for obesity and micro- and macrovascular complications associated with metabolic syndrome and diabetes (Dahlman et al. 2006).

Our results confirm that selected PUFAs can moderate endothelial cell metabolism, in response to metabolic stress condition (Suematsu et al. 2003). We also point out that incubation with the albumin-bound polyunsaturated FFA (AA and EPA) at so-called physiological concentrations, which are typically observed post-prandially in humans, is associated with beneficial elevation of the mitochondrial
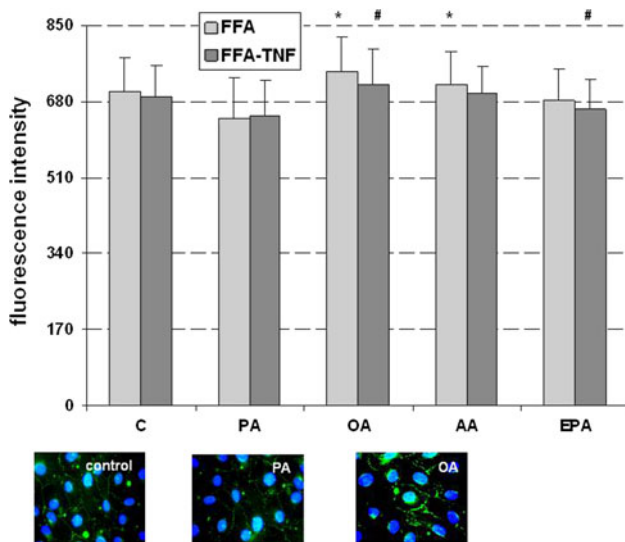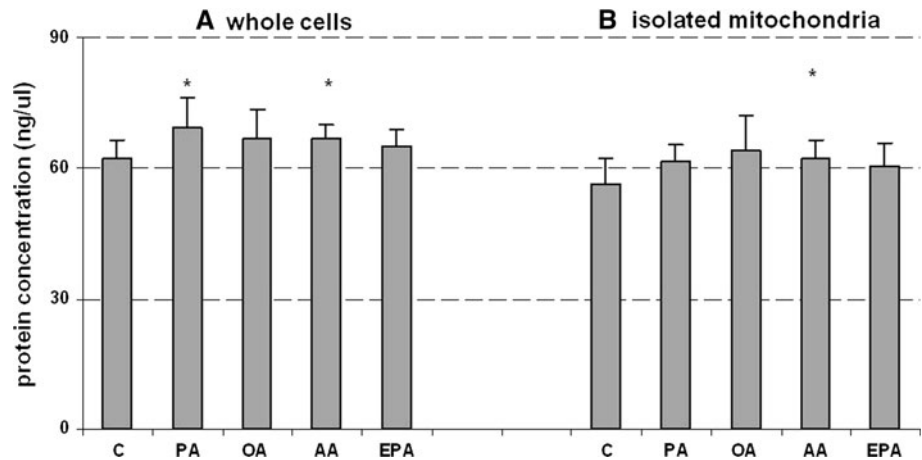
Fig. 4 The quantitative analysis of Cx43 expression and Cx43 localization in HUVECs after incubation with FFAs with or without TNFα as visualized in fluorescent imaging study. Results are mean of 4 experiments done in triplicate; *P < 0.05 versus control #P < 0.05 FFA versus FFA + TNF. *C* control, *AA* 10 μM arachidonic acid, *OA* 30 μM oleic acid, *EPA* 30 μM eicosapentenoic acid, *PA* 30 μM palmitic acid, *TNF* 5 ng/ml TNF

membrane $\Delta\psi$ confirming the protective effect of the polyunsaturated FFA on the endothelial function (Sutherland et al. 2010; von Schacky 2006). However, study also demonstrates that the observed $\Delta\psi$ changes are not associated with the significant variability in ATP production (Korge et al. 2008). Increased mitochondrial generation of ATP in in vitro model was found only in the presence of EPA, but not AA. That might be one of mechanisms of positive effect of PUFAs on endothelial cells metabolism.

The observed changes in Cx43, the gap-junction protein, expression may also contribute to the cellular protection (Hutnik et al. 2008). Our previous results have shown that the CpG island methylation of *Cx43* gene promoter contribute in the regulation of *Cx43* expression, in HUVEC, by selected nutrients (Kiec-Wilk et al. 2011). We demonstrated that most of the investigated fatty acids up-regulated *Cx43* gene expression in HUVECs, out of which AA-induced changes reached statistical significance. Analyses of the Cx43 protein concentration in the whole cells seemed to be closely related to the used FFA, since saturated PA increased the Cx43 protein content in mitochondria. Our observations appear to verify a previous
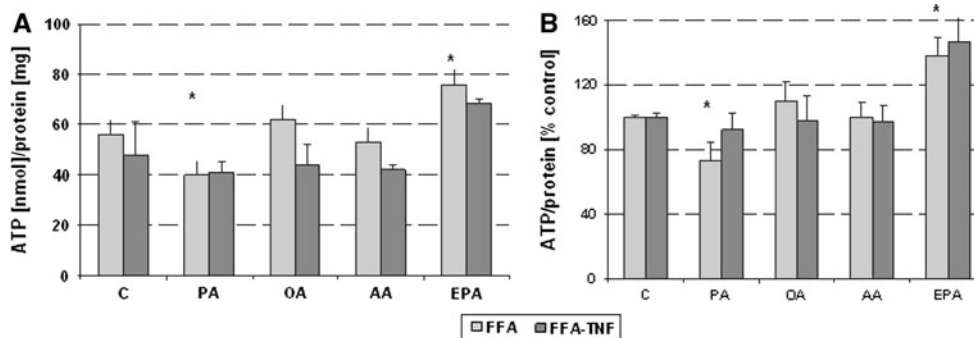

Fig. 5 Intracellular concentration of ATP in HUVECs exposed for 24 h to different FFAs (with or without TNFα for 4 h at the end). Data are expressed as mean of 8 experiments +SD (**a**) or as the % of control (**b**). *P < 0.05 versus control, untreated cells. *C* control, *AA* 10 μM arachidonic acid, *OA* 30 μM oleic acid, *EPA* 30 μM eicosapentenoic acid, *PA* 30 μM palmitic acid, *TNF* 5 ng/ml TNF

report that demonstrated the Cx43 over-expression and its increased translocation into the mitochondria in the endothelial cells under stress conditions (Li et al. 2002). On the other hand, a correlation between decreased *Cx43* gene and protein expressions and ER stress in the cell has already been reported (Huang et al. 2009).

Overall, our study demonstrates that different FFAs may exert a variety of specific effects on the expression of this GJ gene and protein in TNFα stressed HUVECs. Incubation of the endothelial cells with one of the two fatty acids, AA or EPA at low, nontoxic concentrations resulted in a significant up-regulation of *Cx43* gene expression as well as elevation of Cx43 protein content (confirmed by Western blot and confocal microscopy) in not stressed HUVECs. It is interesting to note that Cx43 was increased parallel to the significant rise in the mitochondrial membrane $\Delta\psi$ by AA and EPA.

The up-regulation of Cx43 was suggested to improve intercellular transport and normalize mitochondrial function (Schwanke et al. 2002; Rodriguez-Sinovas et al. 2007). Thus, we believe that enhancement of the mitochondrial function ($\Delta\psi$) in HUVECs after their incubation with the selected, polyunsaturated FFAs promotes cellular trafficking and may involve Cx-mediated modification of GJ function (Rodriguez-Sinovas et al. 2007). One should notice that the positive effect of selected FFAs seems to be inhibited and in some cases inverted in stress conditions (incubation with TNFα).

The fatty acid-specific induction of changes in Cx43 expression and protein concentration as well as the normalization of $\Delta\psi$ and increase of ATP generation seem to be the separate, independent mechanisms of FFA-mediated modulatory effect in the human endothelial cells pathology.

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