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Influence of ezetimibe on ADMA-DDAH-NO pathway in rat liver subjected to partial ischemia followed by global reperfusion

Małgorzata Trocha[1], Anna Merwid-Ląd[1], Tomasz Sozański[1], Ewa Chlebda-Sieragowska[1], Andrzej Szuba[2], Piotr Dzięgiel[3,4,5], Małgorzata Pieśniewska[1], Lidia Fereniec-Gołębiewska[1], Joanna Kwiatkowska[1], Agnieszka Gomułkiewicz[3], Łucja Cwynar-Zając[3], Renata Brykner[3], Adam Szeląg[1]

[1]Department of Pharmacology, Wroclaw Medical University, Mikulicza-Radeckiego 2, PL 50-345 Wrocław, Poland

[2]Department of Internal Medicine, Wroclaw Medical University & 4th Military Hospital in Wroclaw, Weigla 5, PL 50-981 Wrocław, Poland

[3]Department of Histology and Embryology, Wroclaw Medical University, Chałubińskiego 6a, PL 50-368 Wrocław, Poland

[4]Department of Histology and Embryology, Poznan Medical University, Święcickiego 6, PL 60-781 Poznań, Poland

[5]Department of Physiotherapy, Wroclaw University School of Physical Education, Paderewskiego 35, PL 51-612 Wrocław, Poland

Correspondence: Małgorzata Trocha, e-mail: malgorzata.trocha@gmail.com

Abstract:
Background: We evaluated effect of ezetimibe on selected parameters determining NO level in rat liver subjected to ischemia-reperfusion (IR).
Methods: Rats received ezetimibe (5 mg/kg) (groups E0 and EIR) or saline solution (groups C0 and CIR) intragastrically for 21 days. Then, the livers of CIR and EIR underwent ischemia (60 min) and reperfusion (4 h). Blood samples were obtained before surgery to estimate activities of aminotransferases, and just before ischemia and during reperfusion to estimate asymmetric and symmetric dimethylarginine (ADMA, SDMA) and arginine (Arg) levels. After IR, dimethylarginine dimethylaminohydrolase (DDAH) activity and endothelial nitric oxide synthase (eNOS) protein concentration were measured in liver homogenates. DDAH and protein arginine methyltransferase (PRMT) mRNA were quantified by real-time PCR in liver tissue samples.
Results: In CIR, the ADMA level was significantly higher compared to all other groups in 30 min and to E0 group in 120 min of reperfusion. In EIR, ADMA was low, compared to non-ischemic groups. At 30 and 120 min of reperfusion, in non-ischemic groups the level of Arg and Arg/ADMA ratio were significantly higher than in ischemic groups and E0 was the group with the highest levels of those parameters of all. In CIR, eNOS protein concentration was significantly lower than in ezetimibe-treated groups. Activity of DDAH was significantly higher in E0 than in non-treated groups. In ischemic groups, DDAH mRNA expression was significantly higher than in non-ischemic ones and PRMT mRNA expression was significantly higher in C0 than in all other groups.
Conclusions: Influence of ezetimibe on ADMA/DDAH/NO pathway demonstrated in this work may suggest protective properties of this drug on rat livers injured by IR and, to a lower extent, on livers non-subjected to IR.

Key words:
ezetimibe, liver, ischemia/reperfusion, eNOS, ADMA, rat

Abbreviations: ADMA – asymmetric dimethylarginine, ALT – alanine aminotransferase, Arg – arginine, AST – asparagine aminotransferase, CKD – chronic kidney disease, DDAH – dimethylarginine dimethylaminohydrolase, ELISA – enzyme-linked immunosorbent assay, eNOS – endothelial nitric oxide synthase, HPLC – high-performance liquid chromatography, iNOS – inducible nitric oxide synthase, IR – ischemia/reperfusion, L-NMMA – N^G-monomethyl-L-arginine, NADPH – nicotinamide adenine dinucleotide phosphate, nNOS – neuronal nitric oxide synthase, NO – nitric oxide, NPC1L1 – Niemann-Pick C1-like protein, PRMT – protein arginine methyltransferase, ROS – reactive oxygen species, RT-PCR – real time polymerase chain reaction, SDMA – symmetric dimethylarginine, TNFα – tumor necrosis factor α

Introduction

Ischemia/reperfusion (IR) is considered to be the main cause of cell damage in various organs. IR conditions are associated, for example, with liver transplantation procedures, that have become a method of choice in the end-stage disease of that organ. Liver damage occurring during transplantation is a double-stage process: initially it is caused by ischemia that is later aggravated by reperfusion of the organ. IR injury in the liver involves an early acute phase (3–6 h after reperfusion), associated with the generation of free radicals and nitric oxide (NO), and with T-lymphocyte and Kupffer cell activation, followed by a subacute phase (18–24 h after reperfusion), characterized by neutrophil infiltration leading to a continuous oxidant, cytokine, and chemokine production [7, 11].

Experimental data suggest that NO is one of the components of IR injury and is able to modulate reactive oxygen species (ROS) metabolism by reaction with free radicals such as superoxide in the formation of peroxynitrite [7, 33]. On the other hand, NO evokes relaxation of hepatic stellate cell and maintains the normal vascular tone within the sinusoids. Endothelial nitric oxide synthase (eNOS) is responsible for the basal production of NO [37]. Increased level of NOS inhibitors such as asymmetric dimethylarginine (ADMA) may influence NO level in the liver. ADMA released from the ischemic organ during the reperfusion phase competes with arginine (Arg) for the binding site in the active center of NOS [23, 47]. In IR injury activity of protein arginine methyltransferases (PRMTs) – enzymes responsible for protein methylation, and dimethylarginine dimethylaminohydrolase (DDAH) – enzyme that metabolizes ADMA, are also important [16]. Correlation between methylarginine derivatives concentrations and liver function and survival after liver transplantation was observed [23]. Therefore, it is reasonable to learn which drugs may decrease the level of ADMA and what the mechanism of that action is.

If extent of damage in liver preserved for transplantation largely depends on ADMA/DDAH/NO pathway, then it is logical to ask which drugs have a protective effect on NO level in IR conditions. Ezetimibe belongs to a new class of 2-azetidinones, which are selective inhibitors of cholesterol absorption at the brush border of the intestine [40]. It interacts with the sterol transporter – Niemann-Pick C1-like protein (NPC1L1) [10]. In recent years the usage of this drug has become more frequent and many patients who may become liver donors are treated chronically in this way.

Contrary to statins, pleiotropic effects of ezetimibe are still not well known and results of some studies are conflicting [2, 15]. Some properties of that substance may be revealed only in stressful conditions such as IR. The aim of the study was to evaluate protective properties of ezetimibe on selected parameters determining NO level in livers subjected or non-subjected to IR.

Materials and Methods

Animals

The study was carried out on Wistar male rats obtained from the Animal Laboratory of the Department of Pathological Anatomy, Medical University in Wroclaw. Animals were housed individually in chambers with a 12:12 h light-dark cycle, temperature maintained at 21–23°C. Before the experiment, animals had free access to standard food and water. The experiment was performed with consent of the I Local Ethics Commission for Experiments on Animals in Wrocław.

Chemicals

Ezetimibe (Ezetrol – tabl. 10 mg; MSD, UK), heparin (Heparinum WZF – amp. 25000 U/5 ml, Polfa Warszawa, Poland), ketamine hydrochloride (Biokетan, Vetoquinol Biowet, Poland), medetomidine hydrochloride (Domitor, amp. 1 mg/ml, Orion Pharma, Finland), 0.9% sodium chloride solution (Polpharma S.A., Poland), and Ringer solution (Polfa Lublin S.A., Poland) were used in this study.

Experimental design

After adaptation rats were randomly divided into four groups. Rats in groups C0 (n = 8) and E0 (n = 8) were not subjected to IR conditions. Rats in groups CIR (n = 9) and EIR (n = 8) were subjected to 60 min of warm ischemia followed by 4 h of reperfusion. Rats in groups E0 and EIR rats were treated with ezetimibe intragastrically (5 mg/kg), once a day for 21 days. After 3 weeks of drug administration, blood samples (0.8 ml) were obtained from tail vein to determine the initial activities of alanine and asparagine aminotransferase (ALT, AST).

Preparation of the liver IR injury model

Rats were weighed and anesthetized with intramuscular injection of ketamine (7 mg/kg) with medetomidine (0.1 mg/kg) and underwent midline laparotomy. In groups CIR and EIR a 70% liver ischemia (left lateral and median lobes) was achieved by occlusion of branches of the portal vein and the hepatic artery using a microvascular clip. Rats were administered heparin (200 U/kg) to prevent blood coagulation. After 60 min of ischemia, the clip was removed to allow reperfusion for 4 h. The abdomen was subsequently closed and rats were observed during reperfusion. At 30, 120 and 240 min of reperfusion, blood samples (0.8 ml) were collected from tail vein, to determine ADMA and symmetric dimethylarginine (SDMA) as well as Arg levels. When the experiment was terminated, livers were weighted and ischemic lobes were isolated.

In E0 and C0 groups animals were anesthetized in the same way as those in ischemic groups. After midline laparotomy, branches of the portal vein and the hepatic artery were isolated but not occluded. After 60 min, the abdomen was closed and the rats were being observed for 4 h. Blood samples were obtained at the same points of time as in case of ischemic groups. After 4 h, left lateral and median lobes of livers were isolated to be compared with corresponding lobes obtained from ischemic livers.

Part of the liver was prepared for real-time PCR procedure. The liver tissue was placed in the RNA*later* RNA Stabilization Reagent (Qiagen, Germany) immediately after collection. Remaining part of liver was homogenized on ice using lysis buffer (140 mM NaCl, 10 mM EDTA, 10% glycerol, 1% NP40, 20 mM Tris base, pH 7.5). Then, homogenized tissues were centrifuged at 14,000 rpm for 25 min at 4°C and supernatants were collected [28].

Blood enzyme, arginine and its derivatives, and tissue NO synthase analyses

In liver homogenates, DDAH activity was estimated using the colorimetric method (spectrophotometer MARCEL S350 PRO) and was expressed per gram of protein. The method is based on L-citrulline production rate. For that purpose, liver homogenate was mixed with phosphate buffer, pH = 6.5. 1 mM ADMA was added to each sample and samples were incubated at 37°C for 45 min. After the reaction was stopped by addition of 4% sulfosalicylic acid, samples were centrifuged. Oxime (diacetic monooxime (0.08% w/v) in 5% acetic acid) mixed with antipyrine (antipyrine (0.5% w/v) in 50% sulfuric acid) was added to samples at the next stage. Following 110 min of incubation at 60°C and 10 min of cooling on ice, L-citrulline was determined at 466 nm wavelength. Obtained values were subtracted from corresponding values in blind samples (no ADMA). Standard values were prepared as aliquots of L-citrulline. DDAH activity was presented as μm of L-citrulline/gram of protein/minute at 37°C.

The protein level for eNOS was determined in supernatants using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Quantikine, R&D Systems, Mineapolis, USA). The method utilizes highly purified, monoclonal antibodies for eNOS coated on a solid phase (a well in a titration plate). Tested protein contained in homogenates was incubated and bound to antibodies in the solid phase. Washing removed all unbound reagents. Next, polyclonal antibodies specific for eNOS were added to wells. Following another washing, a solution with specific substrate were added. It is a color reaction leading to visualization of test protein. Color intensity was proportional to quantity of the protein. Color intensity was measured colorimetrically using ELISA readers at appropriate wavelength (450 nm). All samples and standards were performed in duplicates.

Arg, ADMA, and SDMA concentrations were measured simultaneously by high-performance liquid chromatography (HPLC) with fluorescence detection [1, 32]. Plasma samples and standards were extracted on a solid-phase extraction cartridge with SCX 50 columns (Varian, Palo Alto, USA). Analytes were derivatized with o-phthaldialdehyde and separated by isocratic reversed-phase chromatography on a Symmetry C18 column (150 × 4.6 mm, 5 μm particle size; Waters Corp., Milford, MA, USA). Potassium phos-

phate buffer (50 mM, pH 6.6) containing 12% v/v acetonitrile was used as the mobile phase at a flow rate of 1.1 ml/min and a column temperature of 35°C. Fluorescence detection was performed at the excitation and emission wavelengths of 340 and 450 nm, respectively.

The serum activities of ALT and AST and the concentration of protein in the homogenates were assayed with a commercial enzymatic method in a certified laboratory. Activities of ALT and AST were expressed per gram of liver.

RNA isolation and reverse transcription

Total RNA was isolated from tissue samples using the RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. RNA products were quantified by measurement of absorbance at 260 nm with spectrophotometer NanoDrop1000 (NanoDrop Technologies, USA). First-strand cDNA was synthesized using the QuantiTect Reverse Transcription Kit (Qiagen, Germany).

Real-time PCR

Relative amounts of DDAH1 and PRMT1 mRNA were determined by quantitative real-time PCR with 7900HT Fast Real-Time PCR System and TaqMan Gene Expression Master Mix (Applied Biosystems, USA) according to the manufacturer's protocols. GAPDH was used as reference gene. Applied primers and TaqMan probes were: DDAH1 Rn00574200_m1, PRMT1 Rn00821202_g1, GAPDH Rn99999916_m1 (Applied Biosystems, USA). Reactions were performed under the following conditions: activation of polymerase at 50°C for 2 min, initial denaturation at 94°C for 10 min followed by 40 cycles of denaturation at 94°C for 15 s, annealing and elongation at 60°C for 1 min. The relative mRNA expression of DDAH1 and PRMT1 was calculated with the ΔΔCt method.

Statistical analysis

Data were expressed as the mean values ± SD. Statistical analysis of the effect of the drug and IR on eNOS levels as well DDAH activity and DDAH and PRMT mRNA expression was performed using two-way analysis of variance (ANOVA). Influence of the drug administration on initial values of ALT, AST was analyzed by one-way ANOVA. Statistical analysis of the effect of the drug and the time of reperfusion on Arg, ADMA, and SDMA levels and the Arg/ADMA ratio was performed using MANOVA with repetitions. Specific comparisons were made using a contrast analysis. Hypotheses were considered positively verified if $p < 0.05$.

Results

ADMA, SDMA, Arg, and Arg/ADMA

After 3 weeks of ezetimibe administration, no significant differences in ADMA0, Arg0, SDMA0, and the Arg0/ADMA0 ratio were found between the groups (p = NS in all cases) (Fig. 1).

In the non-treated and subjected to IR group the highest increase of ADMA concentrations were observed between 0 and 30 min. (CIR0 *vs.* CIR30, $p < 0.001$). Those levels decreased significantly in the next period of time (and CIR30 *vs.* CIR120, $p < 0.005$). At 30 min of reperfusion, the difference between that group and other groups were significant (CIR *vs.* C0, E0, and EIR, $p < 0.01$ in all comparisons). At 120 min of reperfusion, the difference between that group and ezetimibe-treated group non-subjected to IR was also significant (CIR *vs.* E0, $p < 0.05$). In non-IR groups treated with ezetimibe the level of ADMA increased significantly at the end of the experiment (E0 120 *vs.* E0 240, $p < 0.05$) but differences in concentrations of ADMA between ezetimibe-treated and non-treated groups were not significant in all examined points of time (Fig. 1A).

Differences in concentrations of SDMA between groups of rats were not significant. SDMA level increased significantly between 120 and 240 min in non-ischemic group treated with ezetimibe (E0 120 *vs.* E0 240, $p < 0.05$). In the group non-treated and subjected to IR after 240 min of reperfusion, the SDMA level was the lowest and the difference between those groups was on the edge of significance (CIR *vs.* E0, $p = 0.051$) (Fig. 1B).

In both groups subjected to IR, Arg levels decreased the most between 0 min and 30 min of reperfusion (CIR 0 *vs.* CIR 30 and EIR0 *vs.* EIR30, $p < 0.001$ in both cases). The level of Arg/ADMA ratio decreased in the same way (CIR 0 *vs.* CIR 30 and EIR0 *vs.* EIR30, $p < 0.01$ in both cases). In group E0, the Arg level was the highest at 30 min of perfusion and differences be-

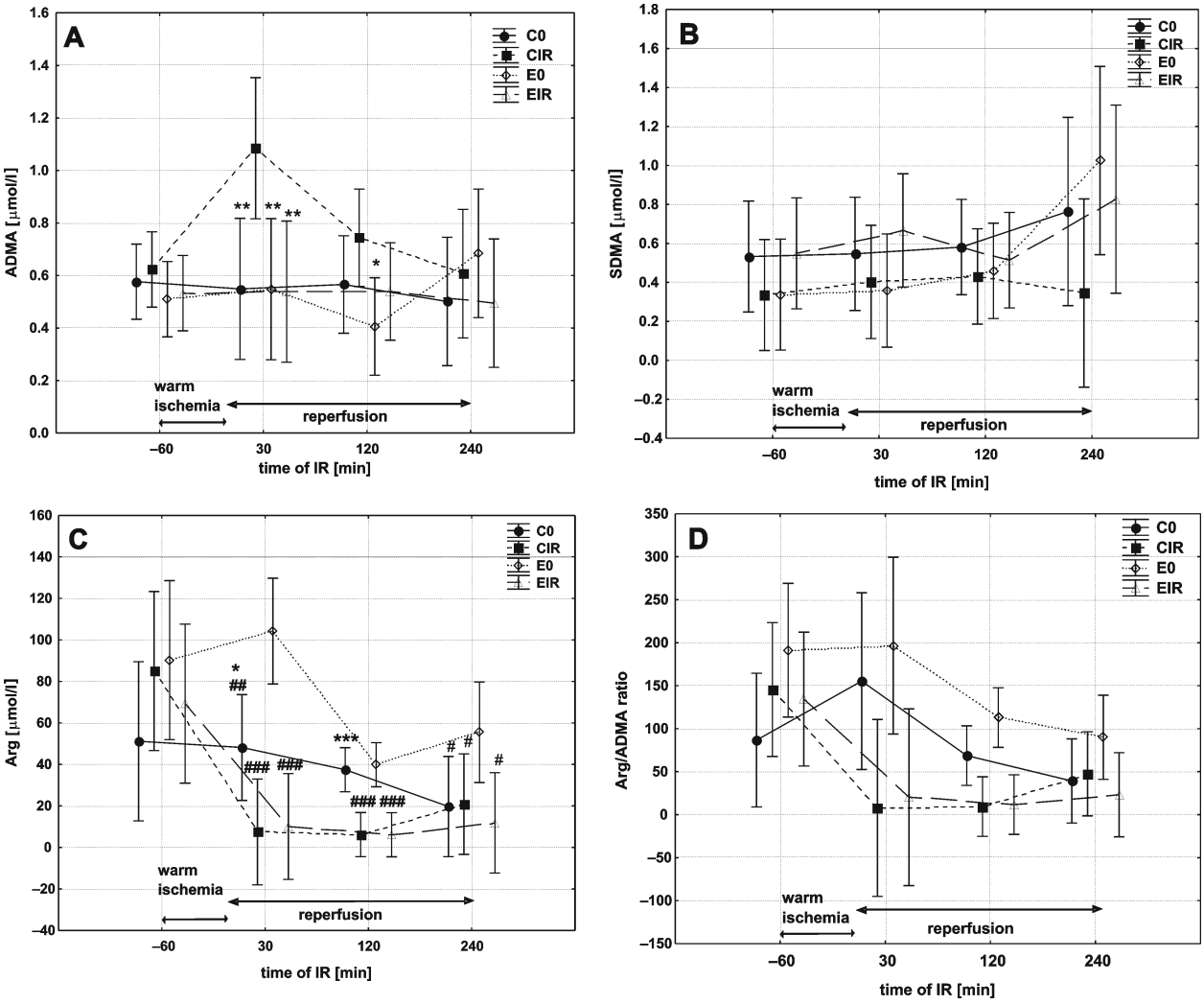

Fig. 1. Influence of IR and ezetimibe treatment on levels of ADMA (**A**), SDMA (**B**), Arg (**C**) and Arg/ADMA ratio (**D**). Values are presented as the mean ± SD. Group C0 – non-treated and non-subjected to IR, group CIR – non-treated and subjected to IR, group E0 – ezetimibe-treated and non-subjected to IR, group EIR – ezetimibe-treated and subjected to IR. Specific comparisons: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.005$ (compared to CIR); # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.005$ (compared to E0)

tween that group and all other groups were significant (E0 *vs.* C0, $p < 0.005$ and E0 *vs*. CIR, EIR, $p < 0.001$ in both comparisons). The difference between non-treated groups was also significant (C0 *vs.* CIR, $p < 0.05$). At that point of time the value of Arg/ADMA ratio was significantly higher in non-ischemic groups (E0 *vs*. EIR and C0 *vs*. CIR, $p < 0.05$ in both cases). However, between 30 and 120 min of perfusion the Arg level and Arg/ADMA ratio decreased significantly (E0 30 *vs.* E0 120, $p < 0.001$ in case of Arg and $p < 0.05$ in case of Arg/ADMA ratio). The Arg/ADMA ratio decreased significantly in non-treated group as well (C0 30 *vs.* C0 120, $p < 0.05$). At 120 min of reperfusion, the Arg level and Arg/ADMA ratio in groups subjected to IR were lower than corresponding values in group non-subjected to IR and differences between ezetimibe-treated groups were significant (E0 *vs*. EIR, $p < 0.001$ in both cases). Differences in Arg level and Arg/ADMA ratio observed between non-treated groups were also significant (C0 *vs.* CIR, $p < 0.001$ in case of Arg and $p < 0.05$ in case of Arg/ADMA ratio). At the end of the experiment, the Arg concentration was the highest in non-ischemic ezetimibe-treated group and differences between that group and all other groups were significant (E0 *vs.* EIR, C0 and CIR, $p < 0.05$ in all comparisons) (Fig. 1C, 1D).

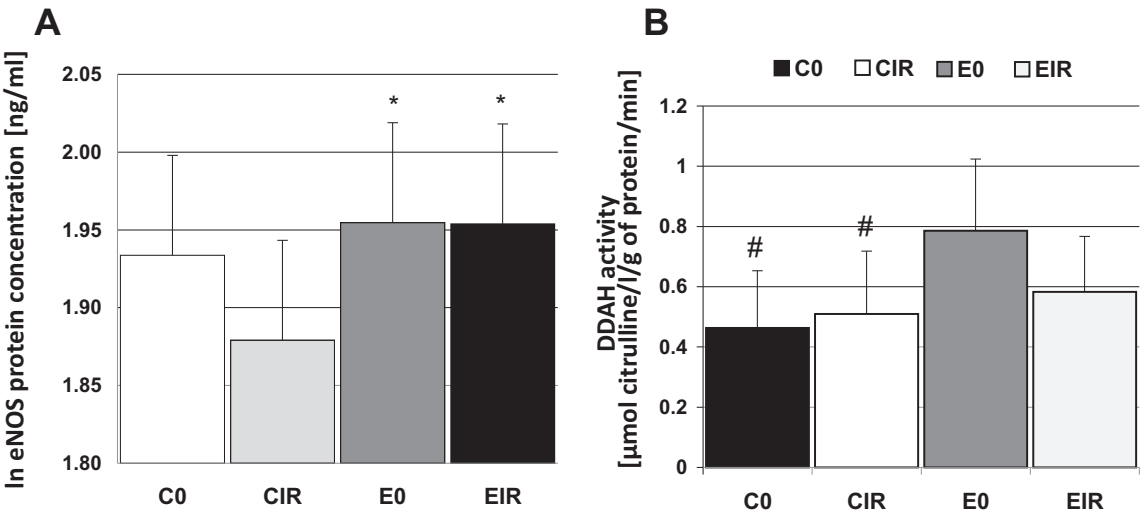

Fig. 2. Influence of IR and ezetimibe treatment on eNOS protein concentration (**A**) and DDAH activity (**B**). Values are presented as the mean ± SD. Group C0 – non-treated and non-subjected to IR, group CIR – non-treated and subjected to IR, group E0 – ezetimibe-treated and non-subjected to IR, group EIR – ezetimibe-treated and subjected to IR. Specific comparisons: * $p < 0.05$ (compared to CIR), # $p < 0.05$ (compared to E0)

BIOCHEMICAL ANALYSES

eNOS

In the non-treated and subjected to IR group eNOS protein concentration was the lowest and differences between that group and both groups of rats treated with ezetimibe were significant (CIR *vs.* E0 and EIR, $p < 0.05$ in both comparisons) (Fig. 2A).

DDAH activity

Activity of DDAH was the highest in the group of rats treated with ezetimibe and non-subjected to IR and differences between that group and both non-treated groups were significant (E0 *vs.* C0 and E0 *vs.* CIR, $p < 0.05$ in both comparisons) (Fig. 2B).

Aminotransferases

After 3 weeks of treatment no significant changes in liver aminotransferase (ALT and AST) activities were observed in all examined groups (p = NS in all comparisons) (Tab. 1).

Tab. 1. Values of ALT and AST activity after 3 weeks of treatment by ezetimibe 10 mg/kg intragastrically (E0 and EIR groups) or saline solution (C0, CIR groups). Values are presented as the mean ± SD, p = NS in all comparisons

Groups	ALT (U/l/g of liver)		AST (U/l/g of liver)	
	mean	± SD	mean	± SD
C0 (n = 8)	4.09	1.66	11.54	2.67
CIR (n = 9)	3.64	2.49	14.52	11.45
E0 (n = 8)	4.01	1.5	16.13	6.01
EIR (n = 8)	4.97	1.94	18.55	5.99

DDAH and PRMT mRNA expression

In groups subjected to IR the expression of DDAH mRNA was higher than in groups non-subjected to IR. Differences between those groups were significant (C0 *vs.* CIR and EIR, as well E0 *vs.* CIR and EIR, $p < 0.05$ in all comparisons). No differences between drug-treated and non-treated groups were observed (p = NS) (Fig. 3A).

In non-treated and non-subjected to IR group PRMT mRNA expression was the highest, and differences between that group and other groups were significant (C0 *vs.* CIR, EIR and E0, $p < 0.05$ in all comparisons) (Fig. 3B)

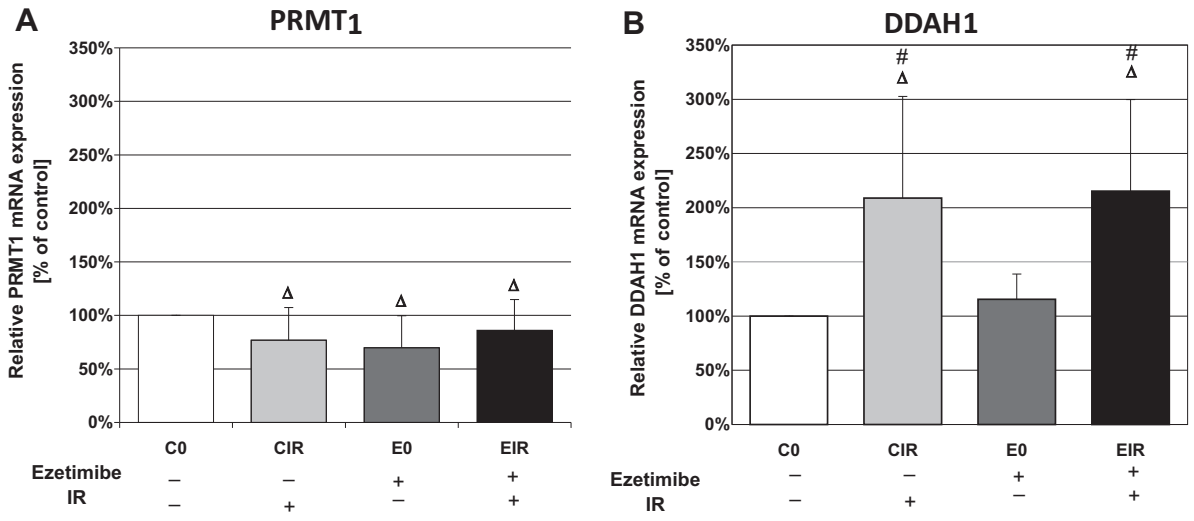

Fig. 3. Influence of IR and ezetimibe treatment on relative expression of PRMT1 (**A**) and DDAH1 (**B**) mRNA. Data are expressed as percent of control values and are the mean ± SD. Group C0 – non-treated and non-subjected to IR, group CIR – non-treated and subjected to IR, group E0 – ezetimibe-treated and non-subjected to IR, group EIR – ezetimibe-treated and subjected to IR. Specific comparisons: Δ $p < 0.05$ (compared to C0), # $p < 0.05$ (compared to E0)

Discussion

Because of conflicting data concerning the influence of ezetimibe on endothelial function in oxidative stress conditions and lack of experimental and clinical data concerning the action of this drug on ADMA-DDAH-NO pathway in liver subjected to IR, our experiment was designed to assess the effect of the drug on some parameters determining nitric oxide level after IR. Results of this study indicate that the preventive action of ezetimibe on the liver may be observed under both normal and IR conditions. More important conclusions suggesting that effect of ezetimibe demonstrated in this work are as follows:

1) similarly low concentration of ADMA in ezetimibe-treated group subjected and non-subjected to IR. In non-treated, IR-subjected group ADMA concentration increased significantly after 30 min of reperfusion;
2) in CIR group also eNOS protein concentration is significantly lower than in ezetimibe-treated groups both non-subjected and subjected to IR;
3) increased levels of Arg and Arg/ADMA ratio in first 90 min of experiment, higher concentration of Arg in all examined points of time and the highest concentration of that compound in the end of experiment in the ezetimibe-treated non-ischemic group;
4) higher activity of DDAH in non-ischemic ezetimibe-treated groups than in both non-treated groups;
5) lower expression of PRMT1 mRNA in ezetimibe-treated non-subjected to IR group than in the non-treated group and higher expression of DDAH1 mRNA in groups subjected to IR.

NO is synthesized mainly from Arg by NOS and its level is determined by various factors and diseases. However, increased NO concentration is, at least partly, NOS-independent and involves a pathway with S-nitrosothiol, nitroprotein, and nitrosylhemoglobin formation [19]. Low NO levels after ischemia may be related to a low intracellular level of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) and tetrahydropterin (eNOS cofactors), limited oxygen support, increased activity of arginase that removes Arg required for NO synthesis, and finally to increased release of NOS inhibitors from the ischemic organ during reperfusion [23]. In our work, the level of ADMA – inhibitor of eNOS, was estimated before ischemia and during reperfusion and no important changes of ADMA after three weeks of ezetimibe administration were observed. We noticed that under IR conditions in ezetimibe-treated group the ADMA concentration was lower than in groups non-subjected to IR. Conversely, in non-treated, IR-

subjected group ADMA concentration increased significantly after 30 min of reperfusion. After 240 min of reperfusion, concentration of ADMA was similar in all groups, which indicated that monitoring of changes of ADMA level during the time of experiment is more valuable than a single measurement at the end of reperfusion.

In many other works increased plasma levels of ADMA are associated with impaired liver functions, such as: decompensated alcoholic cirrhosis [21], acute alcoholic hepatitis [27], portal hypertension evoked by bile duct ligation [14, 38], cholestatic cirrhosis induced through bile duct excision [34, 48], and IR injury [23]. Conversely, no elevation of ADMA levels was observed in drug-evoked cirrhosis, as thioacetamide-induced liver injury [14], or CCl_4-induced cirrhosis [48], which shows some differences among various diseases and suggests that the pathogenesis of impaired NO production may differ and depend on the etiology of the liver injury. Under IR conditions, extensive production of ADMA could be responsible for deterioration of the balance between NO and endothelin and the resulting vasoconstriction affecting the liver function [14].

Influence of ezetimibe on ADMA level in organ injury was also presented in other works. In patients with chronic kidney disease (CKD) and dyslipidemia, ezetimibe administered for 6 months alone [30] or with pivastatin [13] also decreased ADMA level and improved renal injury partly in cholesterol-independent manner. But those clinical works are difficult to compare with our study because of different kinds of oxidative stress and not identical pharmacokinetics of ADMA in humans and rats [45].

Not only ADMA concentration but also SDMA, Arg and Arg/ADMA ratio may influence NO bioavailability. Under pathological conditions, the relationship between ADMA and Arg levels is very important and, therefore, not only ADMA levels, but also the Arg/ADMA ratio needs to be assessed. L-arginine is the main substrate for NO synthase, and deficiency of this compound causes a drop in NO synthesis [9]. When arginine or a cofactor for eNOS – tetrahydrobiopterin (BH4) is deficient, eNOS assumes an uncoupled form and acting as an oxidase becomes responsible for reaction with molecular oxygen instead of Arg, which results in production of peroxide, instead of NO [42]. It seems that various effects of L-Arg depend on initial ADMA level and administration of L-Arg is justified in patients with low L-Arg : ADMA concentration ratio. Arg administration results in normalization of the endothelium-dependent relaxation in patients with hypercholesterolemia [1] or in patients with chronic heart failure. That kind of effect was not observed in patients with low ADMA levels [12]. In our work, not only significant increase in ADMA concentration but also the decrease in Arg concentration were revealed in IR groups, similarly to other reports in which an increase in ADMA and a decrease in Arg in various pathological states have been reported [6]. In our work, as in the previous one [44], the influence of IR injury was observed, because in both groups subjected to IR the highest decrease of Arg level and Arg/ADMA ratio was observed during the first 90 min of the experient. At the same time, values of those parameters were higher in non-ischemic groups. In spite of the decrease of Arg level and Arg/ADMA ratio during the next period of reperfusion, values of those parameters remained on higher level in all examined points of time in non-ischemic groups. The drug-treated non-ischemic group appears to be the best protected because the level of Arg and Arg/ADMA ratio were the highest during the first 90 min of the experiment and the concentration of Arg was the highest until the end of the experiment.

NOS is responsible for NO synthesis. It was also shown that endothelial (eNOS, type III) and neuronal (nNOS, type I) nitric oxide synthases are predominantly inhibited by ADMA [18], but the inducible isoform (iNOS, type II) is inhibited to a lesser extent [46]. Physiologically, ADMA inhibition of eNOS activity is only 10%. Under such pathological conditions as blood vessel injury, the concentration of methylated Arg derivatives is 3–9 times higher and eNOS activity inhibition reaches 30–70% [3]. In our work, IR injury caused the decrease in eNOS protein level in non-treated group. Protective effect of ezetimibe appeared as the increase in eNOS protein concentration in drug-treated groups of rats irrespective of IR conditions what correlated with lower concentration of ADMA and higher concentration of Arg in first 90 min of the experiment in those groups. Similarly to our results, other studies also showed that IR liver injury may decrease eNOS level [25, 50] and the treatment with ezetimibe increased eNOS in obese and diabetic db/db mice [8] and eNOS mRNA expression in aorta of ApoE-deficient mice [29]. However, those latter results are difficult to compare with ours because of different kinds of experimental conditions

of oxidative stress. Moreover, changes in NO synthase protein concentrations may not directly reflect increased or decreased enzyme activity. Hines et al. [11] suggested that a reduction in NO synthesis during reperfusion may occur through inhibition of enzyme function, not its concentration.

PRMTs is a family of enzymes containing eight isoforms with different substrate specificity that are able to catalyze reactions of monomethylation leading to the synthesis of N^G-monomethyl-L-Arg (L-NMMA) or dimethylation in an asymmetric and a symmetric configuration, leading to ADMA and SDMA production, respectively [22]. PRMTs can also be divided into two groups: type I producing ADMA and type II producing SDMA. Both types are responsible for production of L-NMMA [43, 47]. With IR injury, protein methylation is upregulated to remove altered proteins [43]. Increased protein degradation has also been reported in stress, ischemic heart disease, diabetes, and during fasting or infections [5, 43]. However, in our experiment PRMT I mRNA expression was the highest in non-treated and non-subjected to IR group. In other studies expression of PRMT1 mRNA was raised in such conditions as hypertension or diabetes [24]. That discrepancy may be associated with different type of oxidative stress. Of interest, our study revealed for the first time that in ezetimibe-treated group expression is lower than in non-treated group. For better understanding those results should be in the future supplemented by assessment of PRMT activity and protein level.

With altered liver function, ADMA concentration is usually increased also due to not only increased synthesis but also inhibited degradation [31]. DDAH metabolizes L-NMMA or ADMA to citrulline and methylamine or dimethylamine, respectively [18, 43, 47]. That reaction is present in both liver endothelial cells [35] and hepatocytes [26]. DDAH is divided into two types. Type I is found, among others, in liver and is mainly responsible for ADMA degradation [14, 17].

Many factors are able to modulate DDAH activity or change the gene expression of the enzyme. Sulfhydryl group of cysteine in the active site predisposes that enzyme for easy oxidation or nitrosation with subsequent lost of its activity [16]. Such cardiovascular risk factors as a high level of glucose or homocysteine could modulate DDAH activity by a ROS-sensitive manner and some antioxidants may preserve DDAH activity [20, 39]. A growing body of evidence suggests that in hypertension, hypercholesterolemia, hyperglycemia, and hyperhomocysteinemia, oxidative stress is the main factor affecting DDAH activity leading to the increased ADMA concentration [1, 20, 49]. However, there are papers that do not confirm such effect of the oxidative stress [4]. Results of our study have shown for the first time that ezetimibe treatment increased activity of DDAH in rat livers. In livers subjected to IR that effect disappeared. In those groups expression of DDAH mRNA is relatively higher than in non-ischemic groups. In various kinds of oxidative stress expression of PRMT1 mRNA was rather lower than in controls [24]. This is difficult to explain with currently available data that are rarely related to livers subject to IR.

In summary, in our study we have demonstrated for the first time the influence of ezetimibe on ADMA-DDAH-NO pathway in rat livers subject to IR. Protective effect of that drug was shown in both livers non-subjected and subjected to IR. It is worth noting that in non-ischemic livers that were also subject to a gentle *in situ* organ manipulation [36], ezetimibe evoked higher activity of DDAH, higher level of Arg and Arg/ADMA ratio and lower expression of PRMT mRNA. In the ischemic group, the effect of ezetimibe was also noticeable and occurred as a comparably low concentration of ADMA as non-ischemic group and a higher eNOS protein concentration compared to the non-treated group.

It is important to determine the mechanism of liver action of drugs commonly used by patients whose liver is exposed to IR during, for example, a transplantation procedure. The protective effect of ezetimibe on liver subjected to IR may be independent from the main hypolipemic activity of the drug. The impact of ezetimibe on NO production is associated with changes in ADMA and Arg concentrations as well as eNOS and DDAH activities, but the exact mechanism is still unknown. A possible mechanism of that action is the influence of ezetimibe on oxidative stress parameters. It was shown that ezetimibe decreased 8-OHdG (8-hydroxydeoxyguanosine) (indicator of oxidative stress) urine excretion in patients with CKD [30]. Oxidative stress causes DDAH inactivation [45], which may be a reason for increased ADMA concentration and subsequently decreased NO level and endothelial dysfunction. The discussion on the pleiotropic effects of ezetimibe also its potential antiinflammatory activity should be also taken into consideration [41]. That effect may, directly or indirectly (due to influence on oxido-redox state),

modulate the ADMA-DDAH-NO pathway. However, as it is emphasized by the authors, it is not clearly proven that the anti-inflammatory action of ezetimibe is independent from its effect on LDL cholesterol level or could be a result of hypolipemic action of ezetimibe. Changes in expression of mRNA for DDAH and PRMT may indicate the effect of the drug on the level of protein synthesis, but that hypothesis requires further confirmatory studies.

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