Effects of Metformin on Cytochromes CYP3A, CYP2C and CYP2E1 Functioning at Metabolic Syndrome in Rats of Different Age

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Abstract

The aim of our present study was to carry out a complex estimation of metabolic syndrome (MS) and metformin mediated changes in CYP3A, CYP2C, CYP2E1 mRNA expression and corresponding marker enzymes activities, as soon as liver antioxidant system and lipid peroxidation parameters of adult and pubertal rats.

Wistar albino male rats of two age categories (young animals of 21 days age (50-70g) and adults (160-180g)) were divided into 6 groups (8 animals in each): 1 -Control 1 (intact young rats); 2 -Control 2 (intact adult rats); 3 -MS3 (young rats with MS); 4 -MS4 (adult rats with MS); 5-MS3 (young rats with MS)+metformin; 6-MS4 (adult rats with MS)+metformin. The metabolic syndrome model was induced by full replacement of drinking water with 20% fructose solution (200g/l). After 60 days of MS modeling, investigation of rat liver CYP3A, CYP2C and CYP2E1 mRNA expression, their marker enzymes activities, as well as lipid peroxidation parameters were carried out.

It was demonstrated that metformin administration decreased CYP3A mRNA expression and its marker enzyme activity in rats with MS, but could not normalize corresponding indices for CYP2E1 and CYP2C in pubertal animals. Simultaneously glutathione transferase and reductase activities were also normalized under metformin administration with MS. Liver reduced glutathione contents remained greatly decreased in pubertal animals while in adults more pronounced normalizing effect of metformin has been identified with respect to lipid peroxidation. Thus, metformin demonstrated age-dependent effectiveness for normalization of cytochrome P-450 isoforms expression rates as well as glutathione system and lipid peroxidation rates. The lack of information on age-related differences in metformin effects on the CYP450 and glutathione systems states with MS makes these data of considerable importance. Obtained results indicate the need for optimization and individualization of MS pharmacotherapy depending on the age of patients.

Keywords: Metabolic syndrome; Metformin; CYP450; Pubertal; Adult; Rats

Abbreviations: MS: Metabolic Syndrome; CYP: Cytochrome P450; PNP: P-Nitrophenol Hydroxylase; LPO: Lipid Peroxidation; TBARS: Thiobarbituric Acid Reactive Substance; bp: Base Paires

Introduction

The metabolic syndrome (MS) development is accompanied by a substantial shift in the CYP450 system [1], metabolizing the majority of drugs. Simultaneously with MS specific effects on expression and activity of CYP450, medications used for MS treatment also may cause significant impact on these parameters. Thus, metformin (broadly used for MS treatment) is able to inhibit the expression CYP17A1 [2]. It significantly inhibits the expression of steroidogenic regulatory protein (StAR) and 17α-hydroxylase (CYP17A1) [3]. Series of experiments with different CYP450 isoforms inducers and inhibitors allowed clarifying their roles in metformin metabolism in rats [4]. According to the obtained results metformin in rats metabolized mainly via hepatic CYP2C11, 2D1 and 3A1/2. Other authors also have shown that in rat hepatocytes microsomal fraction 2C11, 2D1 and 3A1, but not CYP1A2, 2B1/2 and 2E1 were involved into the metabolism of metformin [5].

Estimation of complex effects of MS and medications for its treatment on different CYP450 isoforms could be of special interest, as it allows defining additional modifying components for metabolic interactions of preparations, attributed at this pathology. However, such information is limited to a few studies on adults [2,3] without taking into account children and adolescents metabolic characteristics. Our previous results indicated the existence of some age-related features in

cytochromes CYP3A, CYP2C and CYP2E1 functioning at metabolic syndrome [6]. The aim of our present study was to carry out a complex estimation of MS and metformin mediated changes in CYP3A, CYP2C, CYP2E1 mRNA expression and corresponding marker enzymes activities, as soon as liver antioxidant system and lipid peroxidation parameters of adult and pubertal rats with MS.

Materials and Methods

A total of 48 Wistar albino male rats of two age categories (young animals of 21 days age (50-70g) and adults (160-180g)) were used in the study. They were kept under a controlled temperature (from 22 °C to 24 °C), relative humidity of 40% to 70%, lighting (12h light-dark cycle), and on a standard pellet feed diet ("Phoenix" Ltd., Ukraine). The study was performed in accordance with the recommendations of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes and approved by the Institutional Animal Care and Use Committee. The model of metabolic syndrome was reproduced according to the protocol of Abdulla et al. [7]. Young and adult animals were divided into 6 groups (8 animals in each group): 1 -Control 1 (intact young rats), 2 -Control 2 (intact adults), 3 -MS3 (young rats with MS), 4 -MS4 (adult rats with MS), 5 -MS3+metformin (young rats with MS and metformin (266,0mg/kg of body weight, per os, 60 days)), 6 -MS4+metformin (adult rats with MS and metformin treatment). Metabolic syndrome was induced by full replacement of drinking water with 20% fructose solution (200g/l). Crystalline D-fructose>99% (Khimlaborreactiv, Ukraine, series 072000897834, batch XW 130105) was used in experiments. 20% fructose was prepared daily and given every day for two month *ad libitum*. In our experiments Metformin (metformin hydrochloride, manufactured by SANDOZ, Lek C.A., Poland, Series CN8407) was used.

After 60 days of 20% fructose solution consumption and metformin treatment rats were sacrificed under a mild ether anesthesia by decapitation. Post mitochondrial and microsomal fractions of livers were obtained by the method of Kamath et al. [8], and aliquots were kept frozen at -70 °C until needed. We investigated changes of rat orthologs of human cytochromes P-450: CYP3A2 instead of CYP3A4 [9] and CYP2C23 instead of CYP2C9 and CYP2C19 [10]. p-Nitrophenol (PNP) hydroxylase activity (a selective enzyme marker for CYP2E1) was determined in microsomal fraction of liver according to the method of Koop et al. [11]. Erythromycin N-demethylase activity (a selective enzyme marker for CYP3A2) was determined in liver microsomal fraction according to the method of Wang et al. [12], diclofenac hydroxylase activity (a selective enzyme marker for CYP2C23) - according to the method of Necrasova et al. [13]. Glutathione-S-transferase activity was determined in liver post mitochondrial fraction according to the method of Habig et al. [14], glutathione reductase activity -in microsomes in accordance with „Current Protocols in Toxicology" [15], reduced glutathione and proteins SH-groups contents -in liver homogenates by method of Sedlak with Ellman's reagent [16]. Protein contents were determined with Total Protein Kit, Micro Lowry, Onishi & Barr Modification (Sigma-Aldrich, Ink., USA).

The rats' livers were used for investigation of cytochrome P-450 isoforms mRNA expression rates by method of reversed transcriptase polymerase chain reaction (rPCR). Isolation of total mRNA was carried out with TRI-Reagent (Sigma, USA). Synthesis of cDNA was carried out with reagents and protocol of Fermentas (Germany). rPCR reaction mixture contents, specific primers for CYP2E1 gene amplification (forward 5'-CTTCGGGCCAGTGTTCAC-3' and reverse 5'-CCCATATCTCAGAGTTGTGC-3'), as well as amplification protocol were chosen according to Lankford et al. [17]. rPCR reaction mixture, amplification protocol and following specific primers -forward 5'-TACTACAAGGGCTTAGGGAG-3' and reverse 5'- CTTGCCTGTCTCCGCCTCTT-3' were used for CYP3A2 gene amplification according to Jager et al. [9]. rPCR reaction mixture, amplification protocol and following specific primers -forward 5'-GATGCTGTCTTCCGTCATGC-3' and reverse 5'-GTAATAGGCTTGATGTCAAG-3' were used for CYP2C23 gene amplification according to Imaoka et al. [10]. PCR with primers of β-actin (sense 5'-GCTCGTCGTCGACAACGGCTC-3' and antisense 5'-CAAACATGAT CTGGGTCATCTTCT-3') was carried out for internal control. All primers were synthesized by «Metabion» (Germany). Thermocycler MyCycler (BioRaD, USA) was used for amplification. Electrophoresis of rPCR products (CYP2E1-744 b.p., CYP2C23-252 b.p., CYP3A2-349 b.p. and β-actin-353 b.p.) was carried out in 2% agarose gels (80V; 1.5h). After electrophoresis gels were stained with ethidium bromide and visualized under a UV transilluminator (BIORAD, USA). Electrophoresis data analysis was carried out with Quantity One Software (USA).

The levels of lipid peroxidation (LPO) in liver microsomes were investigated as the rates of NADPH-dependent thiobarbituric acid reactive substances (TBARS) formation [18]. The obtained data were expressed as the mean±standard error of the mean (M±SEM) and analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test using Origin Pro 7.5 Software. Differences were considered to be statistically significant at $p < 0.05$.
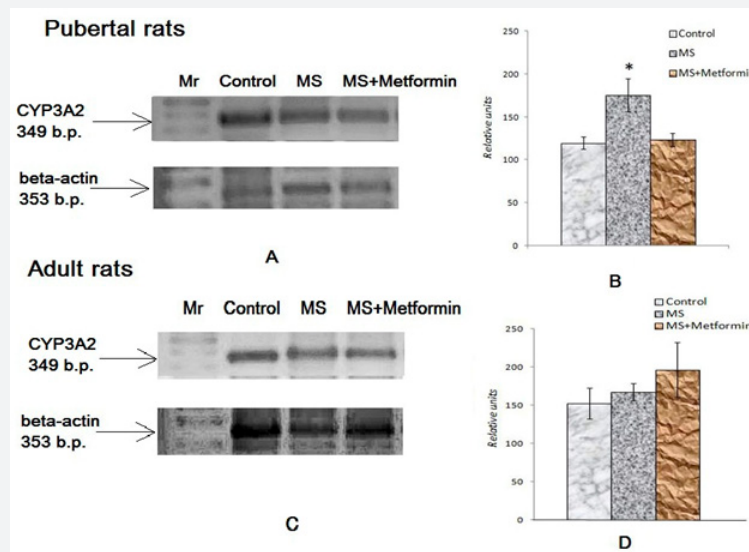
Results and Discussion

CYP2E1 mRNA expression comparative study in the livers of pubertal and adult rats with MS and metformin administration demonstrated more pronounced changes at groups of young rats (Figure 1). CYP2E1 mRNA expression was increased both at group of pubertal animals with MS (1.8 folds compared with the control) and group of pubertal animals with MS and metformin administration (1.7 folds compared with the control). Changes of these parameters in the adult animal groups were not statistically significant.
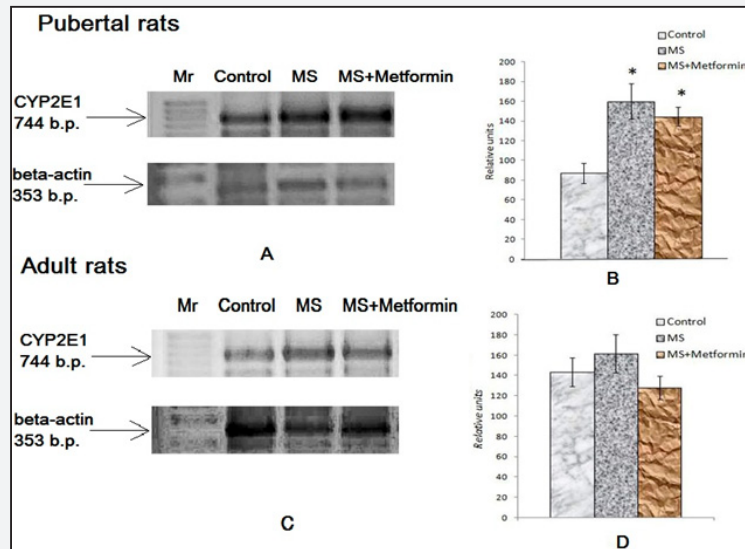
Figure 1: CYP2E1 mRNA content in the liver of pubertal and adult rats with MS and metformin administration: A,C -Representative electrophoregrams of CYP2E1 (744b.p) and reference-gene β-actin (353b.p.) RT-PCR products; B,D-Average rate of CYP2E1 mRNA expression in liver (n =6, Mr -DNA marker, signal intensity of β-actin was taken as 100%).

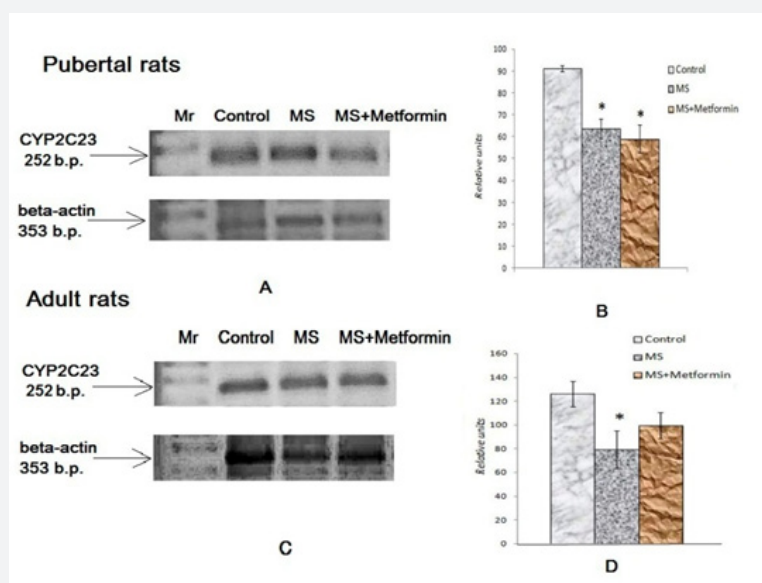*P<0.05 in comparison with control.

Figure 2: CYP3A2 mRNA content in the liver of pubertal and adult rats with MS and metformin administration: A,C -Representative electrophoregrams of CYP3A2 (349b.p) and reference-gene β-actin (353b.p.) RT-PCR products; B,D -Average rate of CYP3A23 mRNA expression in liver (n =6, Mr -DNA marker, signal intensity of β-actin was taken as 100%).

*P<0.05 in comparison with control.

Results of CYP3A2 mRNA expression investigation in the livers of pubertal and adult rats with MS and metformin administration also demonstrated more pronounced changes at groups of young rats (Figure 2). MS caused statistically significant growth of CYP3A2 mRNA expression at group of pubertal animals whereas the metformin administration resulted in this parameter normalization. As in the case of CYP2E1 changes of CYP3A2 gene expression in adult animals groups were not significantly different from control.

In case of CYP2C23 we detected reduction of mRNA expression levels in livers of both age groups with MS as compared with controls: pubertal animals -1.4 folds, adults -1.6 folds (Figure 3). Metformin administration allowed normalizing CYP2C23 mRNA expression rates only in group of adult animals. In group of pubertal animals such metformin effect was absent. We investigated the activity of PNP-hydroxylase in liver microsomes of adult and pubertal rats with MS and metformin administration. Statistically significant growth of PNP- hydroxylase activity with MS was detected both at pubertal (1.6 fold) and adult (1.38 fold) animals groups (Table 1).

Figure 3: CYP2C23 mRNA content in the liver of pubertal and adult rats with MS and metformin administration: A,C -Representative electrophoregrams of CYP2C23 (252 b.p.) and reference-gene β -actin (353 b.p.) RT-PCR products; B, D - Average rate of CYP2C23 mRNA expression in liver (n =6, Mr -DNA marker, signal intensity of β-actin was taken as 100%).

*P<0.05 in comparison with control.

Study of pubertal rats' microsomal PNP-hydroxylase activity with MS and metformin administration also generally matched data on CYP2E1 gene expression. In this group rate of PNP-hydroxylase activity was increased 1.43 fold compared with the control. Changes in PNP-hydroxylase activity in the liver microsomal fraction of adult animals with MS and metformin administration were the same: 1.73 fold growth compared with the control. The investigation of erythromycin-N-demetylase activity showed MS opposite (by nature) effects in groups of pubertal (3 fold reduction) and adult animals (46% increase). But metformin administration caused normalization of erythromycin-N-demetylase activities with MS irrespective of age. Investigation of diclofenac-hydroxylase activity rates (Table 1) demonstrated that MS caused its reduction in rats of both age groups (more pronounced in adults). Effects of metformin administration were opposed to proper MS and caused normalization of diclofenac-hydroxylase activity rate in group of pubertal animals. In adults metformin administration caused increase of diclofenac-hydroxylase activity significantly higher than the levels not only MS4 group, but also Control 2. In our experiments, glutathione-S-reductase (-18.5%), glutathione transferase (-20%) activities and glutathione contents (-43%) were significantly decreased in pubertal rats with MS (Table 2). Metformin administration allowed to normalize glutathione-S-reductase and glutathione transferase activities rates, but glutathione content remained unchanged. In adults MS caused only glutathione content decrease (-42.6%), which was fully normalized by metformin administration.

Table 1: Activities of PNP-hydroxylase, erythromycin-N-demethylase and diclofenac hydroxylase in liver microsomal fraction of pubertal and adult rats (M±SEM, n ≥8).

| Groups	Activity of PNP- Hydroxylase, nmoles×min^{-1}×mg of protein^{-1}	Activity of Erythromycin-N-Demethylase, nmoles×min^{-1}×mg of protein^{-1}	Activity of Diclofenac Hydroxylase, nmoles×min^{-1}×mg of protein^{-1}
Control 1	0.46±0.021	0.96±0.17	552.0±19.1
MS3	0.74±0.051*	0.32±0.09*	380.2±13.8*
MS3+ metformin	0.66±0.039*	0.69±0.17	646.0±169.6
Control 2	0.45±0.018	0.22±0.02	403.6±14.8
MS4	0.62±0.055**	0.32±0.03**	140.8±14.3**
MS4+ metformin	0.78±0.037**	0.34±0.07	1357.0±46.2**,#

*P<0.05 in comparison with control 1

**P<0.05 in comparison with control 2

# P<0.05 in comparison with MS4

Table 2: Contents of reduced glutathione and glutathione transferase and reductase activities in liver of pubertal and adult rats with MS (M±SEM, n ≥8).

Groups	Activity of Glutathione Reductase, nmoles×min^{-1}×mg of protein^{-1}	Activity of Glutathione Transferase, µmoles×min^{-1}×mg of protein^{-1}	Contents of Glutathione, µmoles×g of tissue^{-1}
Control 1	109.8±4.6	1.35±0.09	2.28±0.33
MS3	89.4±5.9*	1.08±0.05*	1.30±0.15*
MS3+ metformin	100.0±5.0	1.09±0.05	1.40±0.13*
Control 2	115.0±5.5	1.25±0.09	2.58±0.27
MS4	114.0±5.0	1.12±0.06	1.48±0.18**
MS4+ metformin	120.3±3.9	1.07±0.062	2.26±0.33
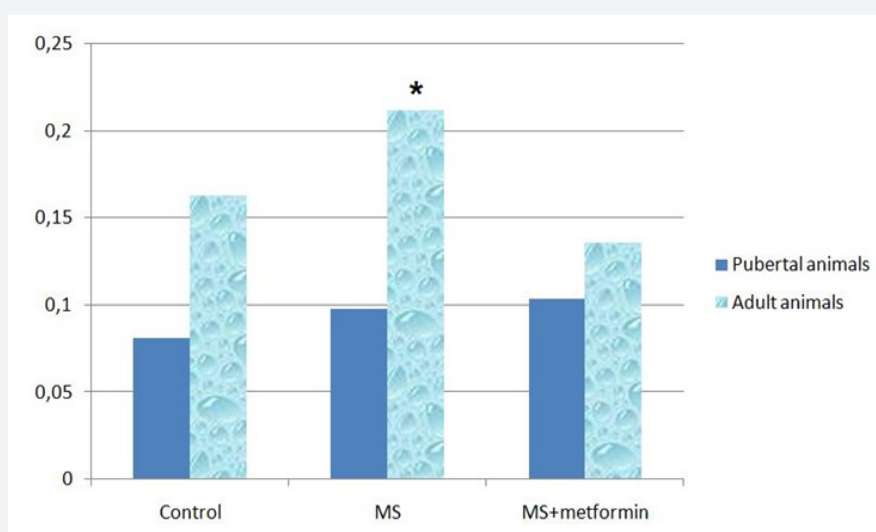
Figure 4: NADPH-dependent LPO in rat liver microsomal fractions with MS and metformin administration (M±SEM; n =6; nmoles×min^{-1} × mg of protein^{-1}).
*P<0.05 in comparison with control 2

Simultaneously with changes in glutathione metabolism, LPO processes in adult rats liver cells were intensified (Figure 4). This was indicated by the increased rates of NADPH-dependent thiobarbituric acid reactive substances production (+30%). In pubertal rats this parameter remained unchanged. Biotransformation is an important part of drugs effects realization on organism's physiological systems [19]. As a result of biotransformation may appear not only biologically active and inactive metabolites, but also cytotoxic reactive intermediates [19]. The main characteristic features of enzymes, involved into biotransformation processes, are not only considerable individual variability of their rates and inducibility under the influence of endogenous and exogenous agents, but also the existence of great variety of their expression regulation mechanisms and isoforms [20].

Among this, pathological processes, which are the subjects to pharmacotherapy, could also significantly change rates of biotransformation processes. Previously we have demonstrated that during MS development levels of certain cytochrome P-450 isoforms expression were greatly violated simultaneously with glutathione system [6]. Changes of investigated parameters were age-dependent and more profound in pubertal rats with MS.

Quite obvious is the fact that for an effective and safe MS pharmacotherapy one should exactly know CYP450 isoforms profiles with this pathologic state. The situation is complicated by the fact that not only different CYP450 isoforms are involved simultaneously, but that most drugs metabolized by several routes, which also involved several isoforms [19,20]. For example, it was demonstrated that metabolism of metformin in rat liver involved CYP2C and CYP3A isoforms [21]. Thus, for optimization of pharmacotherapy, complex estimation of MS and drugs for its medication impacts on the CYP450 2E1, 3A2 and 2C23 isoforms expression profiles are absolutely necessary. Wherein taking into account the profound increase of MS in

pediatric population estimation of these effects' age-related features is of special interest.

Our results on increased CYP2E1 gene expression in pubertal rats both with MS and metformin administration agreed with data of other authors showed greater activity of CYP2E1 in laboratory animals and humans with obesity (one of key MS factors) [22,23]. In pubertal rats metformin administration did not allow CYP2E1 gene expression reducing to a statistically significant level. In case of adult animals with metformin administration -levels of CYP2E1 gene expression remained at control level. It should be noted that in the experiments of other authors the ability of metformin to reduce CYP2E1 mRNA expression in the liver of adult non diabetic animals with nonalcoholic steatohepatosis also has been shown [24].

It must be stressed that changes of PNP-hydroxylase activities (CYP2E1 marker enzyme) in liver microsomal fraction of pubertal rats (both with MS and metformin administration with MS) were in full accordance with our data on CYP2E1 gene expression. Previously we demonstrated age-dependent differences in MS effects on CYP2E1 gene expression rates and CYP2E1 marker enzyme activities [20]. The same situation we noted in case of metformin administration to pubertal and adult rats. Mindful of the large age differences in functioning of CYP450 system [25] and our previous data [20], the weak intensification of CYP2E1 expression followed by statistically significant stimulation of corresponding marker enzyme activity in adult animals (both with MS and metformin administration with MS), were expected. Such changes possibly could be realized via some post-translational mechanisms [26].

Metformin administration effects on the expression of the gene CYP3A2 both in the pubertal and adult rats' livers were fully corresponded with its effects on CYP3A2 marker enzyme activity. As levels of CYP3A2 gene expression in pubertal and adult rats, levels of erythromycin-N-demethylase activities in both abovementioned groups didn't differ from the controls. Such metformin effect on CYP3A2 could be realized due to its influence on PXR, CAR, VDR and GR which regulates CYP3A gene expression [27]. Our assumption is consistent with data obtained by other authors using human hepatocytes, where metformin inhibited the expression of CYP3A4 and PXR targeted gene through regulation SHP [27]. It must be noted, that PXR also regulates the expression of several other drug-metabolizing cytochromes such as CYP2C9 and CYP2C19. In addition, metformin also could inhibit the receptor of vitamin D, glucocorticoid receptor and constitutive androstan receptor mediated induction of CYP3A4 mRNA expression in human hepatocytes [27].

As in case of CYP2E1, metformin administration could not normalize CYP2C23 gene expression level in pubertal rats with MS: in this group we noticed statistically significant reduction of CYP2C23 mRNA content, while in adult animals with MS and metformin administration this parameter did not differ from control. We noticed differences in MS and metformin administration effects on CYP2C23 gene expression rates and CYP2C23 marker enzyme activities, as it was demonstrated in case of CYP2E1 and PNP-hydroxylase activities. Comparatively weak changes of CYP2C23 expression were followed by statistically significant violations of corresponding marker enzyme activity in adult animals with MS and metformin administration. Such differences possibly could be realized via various metformin influences on SHP protein synthesis, VDR, CAR and GR nuclear receptors, coactivated with SRC1 and its ability to cause direct disruption of activated PXR interaction with SRC1 independently of PXR ligand binding pocket, as it was previously demonstrated for CYP3A regulation [27]. Some other post-translational mechanisms also could not be excluded [26].

Observed differences in metformin effects on the gene expression rates of CYP2E1, CYP3A2 and CYP2C23 in rats of different age possibly could be caused not only by previously established age-dependent changes of MS influence [20]. Significant contribution belongs also to complex molecular mechanism of drug-mediated CYP enzymes regulation [27] and the ability of the Cyp3a family to trigger a compensatory regulation of other Cyp genes, as it has been shown previously in Cyp3a-/- mouse studies [28].

Summing up the results on CYP450 isoforms expression and enzymatic activities with MS and metformin administration, greater variability of all isoforms gene expression in pubertal rats compared to adults could be noted. Violations of CYP450 system caused by MS and metformin administration are accompanied by lower productivity of antioxidant defense, which creates conditions for oxidative stress development. Our previous investigations of MS age-dependent effects have shown more profound changes in reduced glutathione contents, as well as glutathione-S-transferase and reductase activities at pubertal animals, while at adults -only glutathione contents decreased [20]. Metformin administration allowed normalizing (to some extent) glutathione-S-transferase and reductase activities at both age groups. But in group of pubertal animals with MS and metformin administration remained reduced glutathione contents. Our results on glutathione system changes are in accordance with other authors' results obtained in experiments *in vitro* and *in vivo* [29-33].

Such influence on glutathione system metformin possibly causes via a glutathione-conjugate transporter RLIP76 [33]. It has been shown that metformin failed to affect glucose and lipid metabolism in RLIP76$^{-/-}$ mice. RLIP76 loss causes profound and global alterations of MS effects [33]. Lower productivity of antioxidant defense creates conditions for oxidative stress. We have previously demonstrated that MS caused oxidative stress and stimulated lipid peroxidation [20]. These processes play important roles in MS development [34]. Metformin administration allowed normalizing MS-induced lipid peroxidation. More pronounced this effect was in group

of adult animals. Our results are in good correspondence with other authors data [30,32,35-38]. Antioxidant effects of metformin were demonstrated in experiments of several authors *in vitro* and *in vivo* on animal models of diabetes and in patients with insulin resistance [30,32,35-38]. Macrophages treated with metformin expressed less reactive oxygen species (ROS) [35]. Metformin-based therapy could reduce oxidative stress, inflammatory cytokine production and shift toward alternative activation of macrophages. In addition, metformin treatment dose-dependently enhanced the activities of catalase (CAT) [36]. At the transcriptional level, in diabetic nephropathy-induced oxidative stress metformin treatment caused significant restoration of mRNA levels (particularly of GSTα, NQO1, and CAT genes), whereas inhibiting TNF-α and IL-6 pro-inflammatory genes [37].

As for pubertal animals, it must be stressed, that our results on little impact of both MS and metformin medication on rates of lipid peroxidation in this age group in bare outlines were in accordance with other authors' data [38].

Conclusion

Thus, metformin demonstrates age-dependent effectiveness towards normalization of cytochrome P-450 isoforms expression levels as well as glutathione system and lipid peroxidation rates. The lack of information on age-related differences in metformin effects on the CYP450 and glutathione systems states with MS, makes these data of considerable importance. Obtained results indicate the need for optimization and individualization of MS pharmacotherapy depending on the age of patients.

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