ORIGINAL ARTICLE

Activities of respiratory chain complexes and citrate synthase influenced by pharmacologically different antidepressants and mood stabilizers

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Abstract

OBJECTIVE: Mitochondrial dysfunctions, impaired bioenergetics and dysfunction of neurotrophins are included in many neurodegenerative and psychiatric diseases. We investigated *in vitro* effects of pharmacologically different antidepressants and mood stabilizers on mitochondrial enzymes to discover, which mitochondrial functions could be involved in pathophysiology of mood disorders.
METHODS: *In vitro* effects of eight pharmacologically different antidepressants (desipramine, amitriptyline, imipramine, citalopram, venlafaxine, mirtazapine, tianeptine, and moclobemide) and three mood stabilizers (lithium, valproate, and olanzapine) on the activities of mitochondrial enzymes (citrate synthase and enzymes in electron transport chain, i.e. complexes I, II, IV) were measured in crude mitochondrial fraction isolated from pig brain.
RESULTS: Most of the antidepressants and mood stabilizers inhibited the activities of respiratory electron transport chain complexes, complexes I and IV were the most affected. Statistically significant decrease of the complex I activity was caused by desipramine, amitriptyline, imipramine, citalopram, mirtazapine, valproate and olanzapine. Complex II was significantly inhibited only by amitriptyline, imipramine, citalopram and venlafaxine. Complex IV was significantly inhibited by all tested drugs except for citalopram and moclobemide. Unchanged or slightly increased citrate synthase activity was observed; significant activation of the enzyme was observed after citalopram, tianeptine and olanzapine.
CONCLUSIONS: Our results indicate that antidepressants may act generally as inhibitors of complex I and complex IV of the electron transport chain. These mitochondrial enzymes are suggested as proper candidates in searching of new biological markers of mood disorders, targets of new antidepressants or predictors of response to pharmacotherapy.

Abbreviations:

ATP	- adenosine 5´-triphosphate
Bcl-2	- B-cell CLL/lymphoma 2
BD	- bipolar disorder
DCPIP	- 2,6-dichlorophenolindophenol
DNTB	- 5,5´-dithiobis-(2-nitrobenzoic) acid
ETC	- electron transport chain
GSK-3	- glycogen synthase kinase-3
INT	- iodonitrotetrazolium
MDD	- major depressive disorder
MRS	- magnetic resonance spectroscopy
mtDNA	- mitochondrial DNA
NADH	- reduced nicotinamide adenine dinucleotide

INTRODUCTION

Pathophysiology of mood disorders is not sufficiently elucidated and about 1/3 of patients do not response to pharmacotherapy sufficiently. Recently, attention in the research of biological basis of mood disorders is devoted on an overlapping set of molecular and cellular mechanisms of mood disorders, antidepressant response, neuroplasticity and consequences of chronic stress (Pittenger & Duman 2008). The most recently discussed biological hypotheses of mood disorders are neurotrophic hypothesis of depression (Duman *et al.* 1997; Duman 2002; Zarate *et al.* 2006; Einat *et al.* 2006) and neuroplasticity hypothesis of depressive disorder (Pittenger & Duman 2008). However, the true molecular site and the primary cause of signal transduction disturbance associated with the symptoms of depression or mania is not known. Therefore, changes in the activities of compounds of intracellular signalling pathways are studied with the aim of discovery of new biological markers of mood disorders or predictors of response to antidepressant treatment (Fišar & Raboch 2008; Fišar & Hroudová 2010). It is well-known that mitochondria strongly affect many intracellular processes coupled to signal transduction and neuron survival and plasticity. Mitochondrial dysfunctions are assuming an increasingly important role in hypotheses about mood disorders, bipolar disorder mainly (Stork & Renshaw 2005; Kato 2008; Quiroz *et al.* 2008).

Except a crucial role of mitochondria in energy production, they are involved in other important processes, such as regulation of free radicals and neurotransmitters, calcium buffering, and apoptosis. They are also included in neuronal development – synaptogenesis, synaptic development and plasticity, and programmed cell death. Impaired functions of mitochondria lead to impaired energy metabolism, decrease of adenosine 5´-triphosphate (ATP) production, impaired calcium homeostasis, increased production of free radicals and oxidative stress.

Impaired mitochondrial functions manifest in various ways. Dysfunctions of mitochondria may be related to many psychiatric and neurodegenerative diseases, including bipolar disorder (BD), major depressive disorder (MDD), schizophrenia, psychosis and anxiety (Shao *et al.* 2008; Rezin *et al.* 2009; Jou *et al.* 2009). There is significant evidence, that neurodegenerative diseases, including Parkinson's disease, Alzheimer's disease, Huntington's disease, Friedreich's ataxia, multiple sclerosis and amyotrophic lateral sclerosis, involve production of reactive oxygen species and reactive nitrogen species and are associated with mitochondrial dysfunctions (Orth & Schapira 2001; Kato *et al.* 2003; Stork & Renshaw 2005; Mattson *et al.* 2008; Quiroz *et al.* 2008; Neustadt & Pieczenik 2008).

Evidence of impaired mitochondrial functions comes from studies using different methods of the search – electron microscopy, magnetic resonance spectroscopy (MRS), DNA microarray techniques, etc. DNA microarray technology was used to analyze gene-expression profiles in the *post-mortem* frontal cortex of subjects with BD; downregulation of mitochondrial electron transport chain (ETC) complex I, complex IV and complex V were verified by real-time polymerase chain reaction (Sun *et al.* 2006). Another evidence of mitochondrial dysfunctions in bipolar disorder comes from MRS. According to Stork and Renshaw (2005) impaired mitochondrial functions involve impaired oxidative phosphorylation, final shift to glycolytic production of energy, general decrease of energy (decreased ATP production), changed concentrations of phosphomonoesters and changed lipid metabolism. Mitochondrial DNA (mtDNA) mutations in the brain, associations of mtDNA polymorphisms and bipolar disorder, and changes in gene expression related to mitochondria in the brain were observed (Kato *et al.* 2007; Kato 2008). According to mitochondrial dysfunction hypothesis, mtDNA polymorphisms/mutations or mtRNA deletions caused by nuclear gene mutations can cause mitochondrial dysregulation of calcium leading to symptoms of bipolar disorder (Kato & Kato, 2000; Kato 2007; Kato 2008).

There are evidences that mitochondrial dysfunctions are implicated in etiology of drug-induced toxicities (Maurer & Möller, 1997; Scatena *et al.* 2007; Neustadt & Pieczenik, 2008). However, there is relatively little information about association of antidepressants-induced changes of mitochondrial functions to therapeutic or side effects of these drugs. Recently, very different effects of antidepressants on induction of mitochondrial dysfunction and cytotoxicity were described (Dykens *et al.* 2008). Tricyclic antidepressants may modulate mitochondrial functions indirectly through decrease of nitric oxide production (Hwang *et al.* 2008). Mood stabilizers exert major effects on regulation of mitochondrial functions. Chronic lithium increases concentration of antiapoptotic protein Bcl-2, reduces levels of the proapoptotic protein p53 and inhibits glycogen synthase kinase-3 (GSK-3) (Gould & Manji 2005); GSK-3 inhibition may lead to an increase in maximal metabolic rate in the brain. Lithium induces also changes in activity of the respiratory chain com-

plexes. Activities of complexes I + III and complexes II + III were increased by lithium, activity of succinate dehydrogenase remained unchanged, and activity of complex IV was not affected or decreased (Lambert *et al.* 1999; Maurer *et al.* 2009).

Nowadays, there is no valuable biochemical, genetic, physiological or other test that could enable diagnose depressive disorder and its subtypes or that could predict a successful response to pharmacotherapy. Therapeutic effects of long-term treatment with antidepressants and mood stabilizers are probably related to modulation of synaptic plasticity; however, molecular mechanisms of these processes are not known sufficiently. We hypothesise that there are common effects of antidepressants and mood stabilizers on activity of some mitochondrial enzymes, and activity of these enzymes could be tested as possible biological markers of mood disorders or predictors of response to pharmacotherapy. The first step in verification of this hypothesis was the measurement of *in vitro* effects of a series of pharmacologically different antidepressants and mood stabilizers on activity of various mitochondrial enzymes, such as citrate synthase and complexes I, II and IV of the ETC.

MATERIAL & METHODS

Isolation of brain mitochondria

The mitochondria were isolated from pig brain cortex as described previously (Fišar *et al.* 2010). Briefly, the gray matter was homogenised in ice-cold buffered sucrose (0.32 mol/l sucrose, 4 mmol/l HEPES; pH 7.4) and was centrifuged at 1 000 g for 10 min to remove unbroken cells, nuclei and cell debris. The supernatant was carefully decanted; the pellet was resuspended in buffered sucrose and centrifuged again under the same conditions. Supernatants were collected and recentrifuged at 10 000 g for 15 min. The final pellet containing crude mitochondrial fraction was washed twice with buffered sucrose (10 000 g, 15 min), resuspended to a protein concentration of 20–40 mg/ml, and stored at –70 °C until the assays. Protein concentration was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

Measurement of drug effect on enzyme activity

Crude mitochondrial fraction was resuspended with hypotonic buffer (25 mmol/l potassium phosphate, 5 mmol/l $MgCl_2$, pH 7.2), and suspension was frozen and thaw two times to achieve the maximum of enzyme activities (Kirby *et al.* 2007). Samples were incubated with selected psychopharmaca for 30 minutes at 30 °C. Final drug concentration was 5 mmol/l for lithium and valproate, and 500 µmol/l for desipramine, amitriptyline, imipramine, citalopram, venlafaxine, mirtazapine, tianeptine, moclobemide and olanzapine. Samples were measured at 30 °C and in a total reaction volume of 3 ml; final protein concentration was 150 µg/ml. Activities of respiratory chain complexes and enzymes of citric acid cycle were measured spectrophotometrically using Uvicon XL spectrophotometer (SECOMAM, Alès, France). All chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Enzyme assays used in our study are stated below.

Complex I (NADH dehydrogenase (ubiquinone), EC 1.6.5.3)

NADH dehydrogenase activity was measured as the rotenone-sensitive rate of NADH oxidation at 340 nm. Previously published method was used (Ragan *et al.* 1987, Folbergrová *et al.* 2007) with a slight modification. The reaction medium was composed of 25 mmol/l potassium phosphate (pH 7.2), 5 mmol/l $MgCl_2$, 2.5 mg/ml bovine serum albumin (BSA), 2 mmol/l KCN, and 0.3 mmol/l NADH. The reaction was started by the addition of coenzyme Q_1 (in final concentration 33 µmol/l) and measured for 10 min. Afterwards rotenone was added in final concentration 50 µmol/l and the inhibited rate was measured for further 2 min.

Complex II (succinate dehydrogenase (ubiquinone), EC 1.3.5.1)

The activity of succinate dehydrogenase complex was measured as a decrease of absorbance of 2,6-dichlorophenolindophenol (DCPIP, artificial acceptor of electrons) for 3 minutes at 610 nm. The reaction mixture contained 25 mmol/l potassium phosphate buffer (pH 7.2), 5 mmol/l $MgCl_2$, 20 mmol/l sodium succinate, 50 µmol/l DCPIP, 2 mmol/l KCN, 2 µmol/l antimycin A, and 2 µmol/l rotenone. The reaction was initiated by the addition of coenzyme Q_{11} in final concentration 60 µmol/l (Trounce *et al.* 1996).

Complex IV (cytochrome-*c* oxidase, EC 1.9.3.1)

Cytochrome-*c* oxidase activity was measured as a decrease of absorbance during oxidation of reduced cytochrome *c* at 550 nm. The reaction mixture was consisted of 20 mmol/l KH_2PO_4 (pH 7.0), 0.45 mmol/l lauryl maltoside; reaction was started with reduced cytochrome c (final concentration 25 µmol/l) and was monitored for 1 min (Rustin *et al.* 1994).

Citrate synthase (CS 2.3.3.1)

The activity of citrate synthase was measured as a colour change of 5,5´-dithiobis-(2-nitrobenzoic) acid (DNTB). Incubation medium was composed of 100 mmol/l Tris/HCl (pH 8.1), 0.1% Triton X-100, 0.2 mmol/l DTNB, and 0.3 mmol/l acetyl-CoA. The reaction was initiated by the addition of 0.5 mmol/l oxaloacetate and absorbance was measured at 412 nm for 3 min (Srere *et al.* 1969).

Data analysis and statistics

Enzyme activities were evaluated as a slope of time dependence of absorbance of samples using LabPower Junior software (SECOMAM). Each independent mea-

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surement had a control, i.e. sample containing all components except for the drug. Relative changes of enzyme activities evoked by drugs were determined assuming that the activity of the control sample is equal to 100 %. Residual enzyme activity, i.e. activity at very high drug concentration, was determined in our experiments. The full inhibitory curve was measured only for the effect of desipramine on complex I activity. This inhibition was analyzed using the four-parameter logistic function (SigmaPlot, Systat Software. Inc., Richmond, CA, USA), to establish the half maximal inhibitory concentration (IC_{50}), residual activity and Hill slope (coefficient).

All data presented are expressed as the mean ± standard deviation. Results were analyzed by STATISTICA (data analysis software system, version 9.0, StatSoft, Inc., Tulsa, OK, USA). The Wilcoxon matched pairs test (a nonparametric alternative to the t-test for dependent samples) was used to calculate test statistics in order to compare the enzyme activities in samples with and without the drug.

RESULTS

Activities of respiratory chain complexes were mostly decreased owing to tested antidepressants and mood stabilizers; the most affected was complex I and IV (Figure 1). Statistically significant decrease of complex I activity was found for desipramine, amitriptyline, imipramine, citalopram, mirtazapine and valproate. Activity of complex II was significantly decreased by amitriptyline and imipramine. Activity of complex IV was significantly decreased for desipramine, amitriptyline, imipramine, mirtazapine, tianeptine, lithium, valproate and olanzapine.

Considering very high final drug concentrations in samples the values showed on Figure 1 conform to residual activities of enzyme complexes. The inhibitory curve for inhibition of the complex I by desipramine illustrates the relevance of residual activity (Figure 2).

Potency of tested drug in affecting of citrate synthase activity is summarized in the Table 1. Except for mirtazapine and moclobemide all tested drugs slightly increased citrate synthase activity; however, the increase was statistically significant only for citalopram, tianeptine and olanzapine.

DISCUSSION

Evidences that mitochondrial dysfunctions are included in pathophysiology of psychiatric disorders have been reviewed recently (Shao *et al.* 2008; Rezin *et al.* 2009; Jou *et al.* 2009). They include disturbances in activity of mitochondrial enzymes, impaired calcium signalling and energy metabolism, increased mtDNA deletions, mutations or polymorphisms, and effects of psychotropic drugs on mitochondria. Recent findings provide the evidence that mood-stabilizing drugs are able to prevent dysfunctional mitochondrial ETC-induced oxidative damage (Wang 2007). However, there is almost no data about direct effects of antidepressants and mood stabilizers on mitochondrial functions; we miss studies comparing effects of pharmacologically different antidepressants on the activities of key enzymes of citric acid cycle and the ETC.

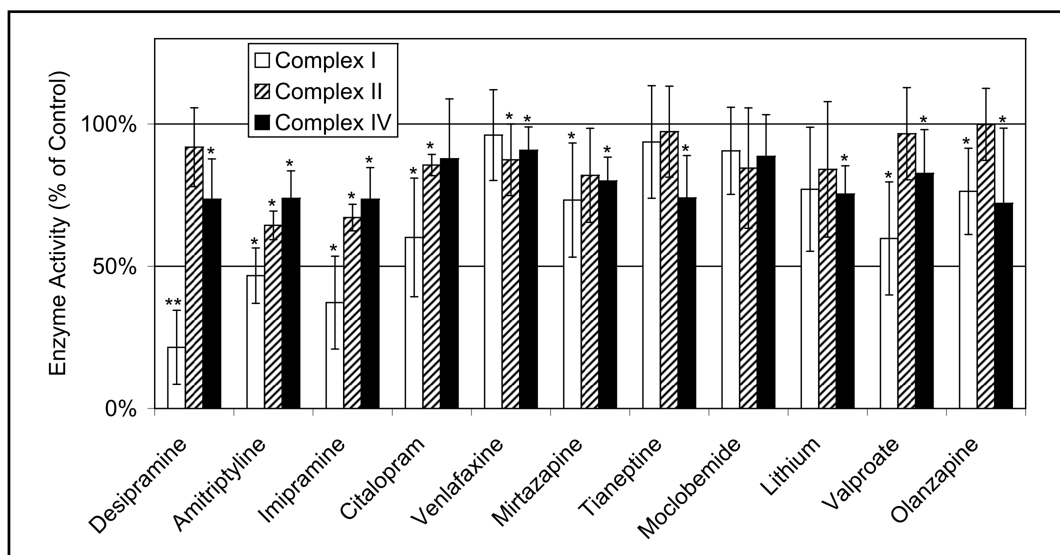

Fig. 1. Effects of antidepressants and mood stabilizers on activities of the respiratory chain complexes I, II, IV in a brain crude mitochondrial fraction. The samples were incubated with drugs at 30 °C for 30 minutes and enzyme kinetics were measured spectrophotometrically as described in the section "Material and Methods". Relative activities are displayed (100% = control sample without the drug). Values are means ± standard deviation of at least five independent measurements. Comparisons between controls and samples with drug were performed using the Wilcoxon matched pairs test (**p*<0.05, ***p*<0.01).

Tab. 1. Effects of antidepressants and mood stabilizers on citrate synthase activity in a brain crude mitochondrial fraction.

Drug	Activity (% of Control)	N
Desipramine	105 ± 13	9
Amitriptyline	109 ± 13	8
Imipramine	106 ± 11	5
Citalopram	*116 ± 12	6
Venlafaxine	115 ± 22	6
Mirtazapine	96 ± 24	6
Tianeptine	*120 ± 21	9
Moclobemide	98 ± 4	5
Lithium	108 ± 12	8
Valproate	109 ± 10	6
Olanzapine	*129 ± 16	8

The samples were incubated with drugs at 30 °C for 30 minutes and the reaction was started by the addition of 0.5 mmol/l oxaloacetate. The control samples (without the drug) were measured simultaneously. The effect of antidepressants and mood stabilizers on the enzyme activity was expressed as percentage of activity of the control sample.
Values are mean ± standard deviation; N = number of independent measurements; *$p<0.05$, i.e. the Wilcoxon matched pairs test was significant at the 0.05 level.

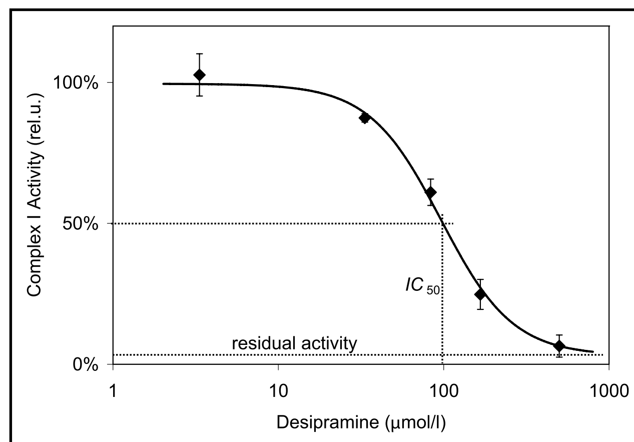

Fig. 2. Inhibition of basal NADH dehydrogenase (complex I) activity by desipramine in a brain crude mitochondrial fraction. Concentration-response curve is displayed as plot of the initial activity of complex I against the desipramine concentration. The samples were incubated with drugs at 30 °C for 30 minutes as described in "Material and Methods". The reaction was started by the addition of 33 µmol/l coenzyme Q_1 and measured for 10 min; afterwards rotenone was added (in final concentration 50 µmol/l) and the inhibited rate was measured for further 2 min. The control samples were measured simultaneously. Values are mean ± standard deviation of three independent measurements. Line represent the best fitted curve using the four-parameter logistic function (median effective concentration IC_{50} = 96.3 ± 9.7 µmol/l, residual activity = 3.05±0.55%, and Hill slope = 2.00±0.35).

Citrate synthase, the first and rate-limiting enzyme of the tricarboxylic acid cycle, plays a decisive role in regulating energy generation of mitochondrial respiration, complex I is a rate-limiting for oxygen consumption in the synapses (Telford *et al.* 2009), and complex IV was suggested as an endogenous metabolic marker for neuronal activity (Wong-Riley 1989). Therefore, we focused on the study of effects of pharmacologically different antidepressants and mood stabilizers on activities of these mitochondrial enzymes.

Effects of desipramine, amitriptyline, imipramine, citalopram, venlafaxine, mirtazapine, tianeptine, moclobemide, olanzapine, lithium and valproate on mitochondrial enzymes were studied *in vitro* using pig brain mitochondria. Pig is a relatively unusual species for the most pharmacological studies; however, pig mitochondria are relatively often used in studies of mitochondrial functions and enzymes. It may be supposed that the pig brain mitochondria are more similar to the human brain mitochondria than rodent mitochondria.

The results show slightly increased or unaffected activity of citrate synthase for all tested antidepressants and mood-stabilizing drugs. Insignificant effect of drugs on succinate dehydrogenase activity was observed (data not shown). Compared to effect on enzymes of citric acid cycle, most of tested drugs induced considerable decrease of activity of respiratory chain complexes. Drug-induced decrease of enzyme activity was found to be statistically significant for complex I and IV; only little effect was observed for complex II.

Our data are consistent with previous data about the role of complex I in mental disorders and in mechanisms of action of psychotropic drugs (Wang 2007). Complex I plays a major role in controlling oxidative phosphorylation and its abnormal activity can lead to defects in energy metabolism and thereby to changes in neuronal activity (Pathak & Davey 2008). Neuroanatomical pattern of complex I pathology parallels the diversity and similarities in clinical symptoms of schizophrenia, MDD and BD (Ben-Shachar & Karry 2008). Reductions were observed in the prefrontal cortex and striatum at patients with schizophrenia; patients with depression showed reductions in the cerebellum, and the bipolar group showed increased expressions in the parieto-occipital cortex. Studies have shown that the polymorphisms of mtDNA coding complex I genes are significantly associated with BD (Kato *et al.* 2001). It can be hypothesised that the mitochondria-controlled process of oxidative damage could be a significant therapeutic target for treatment of BD both with mood stabilizers (Wang 2007) and antidepressants.

Although effect of tested drugs on the activity of citrate synthase was not significant, most of the drugs increased citrate synthase activity. It is consistent with finding that valproate reversed and lithium prevented

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amphetamine-induced citrate synthase inhibition in animal model (Corrêa *et al.* 2007).

Our findings of mitochondrial changes induced by antidepressants and mood-stabilizing drugs support the suggestion that mitochondrial dysfunction could be a primary event in mood disorders. However, it remains to be determined if mitochondrial dysfunction is rather a causal or a consequential event of abnormal signalling, and if effects of antidepressants and mood stabilizers on mitochondrial functions are related rather to therapeutic or to side effects of pharmacotherapy.

Biological markers of depression and predictors of the response on the drug administration are searched on the basis of recently known hypotheses of affective disorders. We come out mostly from stimuli of neurotrophic hypothesis and mitochondrial hypothesis. According to these hypotheses, leading role in pathophysiology of mood disorders and therapeutic effects of antidepressants could have changes in energetic metabolism of cells determined by mitochondria. Mitochondrial dysfunctions and thereby impaired neuronal metabolism can lead to alterations in neuronal function, plasticity and brain circuitry. Supposing that mechanism of action of antidepressants and mood stabilizers is related to processes implicated in pathophysiology of mood disorders our results designate complexes I and IV of respiratory ETC both as targets of these drugs and as potential markers of the illness.

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