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Induction of energy metabolism related enzymes in yeast *Saccharomyces cerevisiae* exposed to ibogaine is adaptation to acute decrease in ATP energy pool

Roman Paškulin [a,*], Polona Jamnik [b], Nataša Obermajer [c,e], Marija Slavić [d], Borut Štrukelj [e]

[a] *OMI Institute, Trnovska 8, 1000 Ljubljana, Slovenia*
[b] *University of Ljubljana, Biotechnical Faculty, Jamnikarjeva 101, Ljubljana, Slovenia*
[c] *Jožef Stefan Institute, Department of Biotechnology, Jamova 39, Ljubljana, Slovenia*
[d] *Institute for Biological Research "Sinisa Stankovic", Despota Stefana 142, Belgrade, Serbia*
[e] *University of Ljubljana, Faculty of Pharmacy, Aškerčeva 7, Ljubljana, Slovenia*

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ABSTRACT

Ibogaine has been extensively studied in the last decades in relation to its anti-addictive properties that have been repeatedly reported as being addiction interruptive and craving eliminative. In our previous study we have already demonstrated induction of energy related enzymes in rat brains treated with ibogaine at a dose of 20 mg/kg i.p. 24 and 72 h prior to proteomic analysis. In this study a model organism yeast *Saccharomyces cerevisiae* was cultivated with ibogaine in a concentration of 1 mg/l. Energy metabolism cluster enzymes glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, enolase and alcohol dehydrogenase were induced after 5 h of exposure. This is a compensation of demonstrated ATP pool decrease after ibogaine. Yeast in a stationary growth phase is an accepted model for studies of housekeeping metabolism of eukaryotes, including humans. Study showed that ibogaine's influence on metabolism is neither species nor tissue specific. Effect is not mediated by binding of ibogaine to receptors, as previously described in literature since they are lacking in this model.

1. Introduction

Ibogaine, an indole alkaloid present in the root bark of African plant *Tabernanthe iboga* has been extensively studied in the last decades in relation to its anti-addictive properties that have been repeatedly reported as being addiction interruptive and craving eliminative for opiates, stimulants, alcohol and nicotine (Alper et al., 1999; Maciulaitis et al., 2008). Although controlled clinical trials haven't yet been done, both lay and scientific literature suggest a certain level of ibogaine's efficacy (Lotsof, 2007; Alper et al., 2008). Claims are supported with results from *in vitro* studies and proven in animal models of drug addictions (Alper, 2001).

Multiple ligand binding and activity modulation actions of ibogaine on receptors, transporters and enzymes have been described in the scientific literature, in particular: 5-Hydroxytryptamine (5-HT), opioid, nicotinic and N-methyl-D-aspartate (NMDA) receptors, dopaminergic and 5-HT transporters and monoamine oxidase enzyme (MAO) (Alper, 2001; Glick et al., 2002; Leal et al., 2003).

Besides the effects on receptors, transporters and enzymes, the molecular aspects of ibogaine's influence on drug addictions concerning signal transduction and modulation of gene expression are becoming increasingly recognized (Ali et al., 1999; Onaivi et al., 2002). Consequent biochemical, neuroendocrine, structural and functional changes in terms of brain plasticity have been suggested (Ali et al., 1996; He et al., 2005; Carnicella et al., 2008).

Our recent work (Paškulin et al., 2006) showed the stimulating influence of ibogaine at a dose of 20 mg/kg i.p. on rat brain energy metabolism. We have observed changes in proteome at 24 and 72 h after i.p. application with induction of glycolysis and TCA cycle enzymes (glyceraldehyde-3-phosphate dehydrogenase, aldolase A, pyruvate kinase and malate dehydrogenase).

In the present study analysis of changes in proteome using 2-D electrophoresis was done again, this time on yeast *Saccharomyces cerevisiae* in stationary growth phase, at a concentration of 1 mg/l ibogaine in the media, which represents local bioavailability in brain tissue in previous experiment (Mash et al., 2000; Kontrimaviciute et al., 2006).

This cell suspension model doesn't show any cell differentiation, nor organization in tissue, and it lacks the influence on metabolism due to synaptic intercellular communication, as is the case in *in vivo* experiments. In spite of that, yeast is an accepted model for studies of basic metabolic pathways of higher eukaryotes, including mammalian cells (Ma, 2001; Menacho-Marquez and Murguia, 2007).

The aims of this study were to investigate if the effect of ibogaine is species and/or tissue specific and to find the cause for the induction of energy related enzymes.

* Corresponding author. Tel.: +386 41 351 531; fax: +386 1 5497235.
E-mail address: roman.paskulin@siol.net (R. Paškulin).

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2. Materials and methods

2.1. Yeast strain and cultivation

Yeast strain S288C (*MATα mal gal2*) was used in this study. Cells were cultivated in YEPD medium with the following composition: 10 g/l glucose (Kemika), 5 g/l yeast extract (Biolife), and 5 g/l pepton (Oxoid), at 28 °C and 220 rpm to the stationary growth phase. Then cells were centrifuged for 5 min at 4000 rpm, washed once with 50 mM potassium phosphate buffer, pH 7.0 and suspended in the same buffer at a concentration of $1 \cdot 10^8$ cells/ml.

A fresh ibogaine stock solution (10 mg/ml) was added to the cell suspension to reach different concentrations: 0, 1, 4, and 20 mg/l. After 0.25, 0.5, 1, 2 and 5-h incubation at 28 °C and 220 rpm, samples were taken to measure cell energy metabolic status and intracellular oxidation of treated and non-treated cells. Protein profile of yeast cell extract was analyzed only at 5 h of exposure to the lowest concentration of 1 mg/l.

2.2. Two-dimensional electrophoresis

Cells were sedimented by centrifugation from 20 ml samples of the cultures and washed twice with 50 mM potassium phosphate buffer, pH 7.0. 0.1 g of cells (wet weight) was suspended in 0.5 ml extraction buffer (40 mM Tris–HCl, pH 8.0; 2% (w/v) 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 65 mM dithiothreitol (DTT)) containing protease inhibitor cocktail (Complete, Mini; Roche) — 1 tablet per 10 ml of buffer. The cells were disrupted by vortexing with glass beads five times, 1 min each with 1-min intervals for cooling the mixture on ice. The cell homogenate was centrifuged at 20000*g* for 20 min at 4 °C.

The protein content in the cell extracts was determined by the method of Bradford (1976) using bovine serum albumin as standard.

Two-dimensional (2-D) electrophoresis was performed according to Görg (1991) with minor modifications. Samples (150 µg protein) were mixed with rehydration solution (9 M urea, 2% (w/v) CHAPS, 2% (v/v) immobilized pH gradient (IPG) buffer, 18 mM DTT, a trace of bromophenol blue) and applied on 13-cm immobilized pH 3 to 10 gradient (IPG) strips (GE Healthcare). After rehydration (13 h) isoelectric focusing (IEF) as first dimension was carried out at 20 °C on a Multiphor II (GE Healthcare). The following voltage program was applied: 300 V (gradient over 1 min), 3500 V (gradient over 1.5 h), and 3500 V (fixed for 4 h). Prior to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), the IPG strips were equilibrated in SDS equilibration buffer (50 mM Tris–HCl, pH 8.8; 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, a trace of bromophenol blue) containing 1% DTT for 15 min, and containing 4.8% iodoacetamide for an additional 15 min. SDS-PAGE as the second dimension was carried out with 12% running gel on the vertical discontinuing electrophoretic system SE 600 (Hoeffer Scientific Instruments) at constant 20 mA/gel 15 min and then at constant 40 mA/gel until the bromophenol blue reached the bottom of the gel. 2-D gels were stained with Sypro Ruby (Invitrogen). For each sample two 2-D gels were run at the same conditions.

2.3. Protein visualization and image analysis

2-D gels were recorded using the CCD camera G.BOX_HR (Syngene). Gel image analysis was done with the 2-D Dymension software version 2.02 (Syngene) and included spot detection, spot quantification, pattern aligning and matching. For all spot intensity calculations, normalized volume values were used. The results are expressed as a ratio of the normalized volume of protein spot in ibogaine-treated cells divided by normalized volume of matched protein spot in untreated control cells at the same time of exposure. Differences by a fold change >2 between treated and untreated cells were considered as significant.

2.4. Protein identification

The protein spots of interest were excised from the gels and analyzed by LC-MS/MS using an ESI-TRAP instrument. The Mascot software was used to search SwisProt 54.7 database. The following search parameters were applied: *S. cerevisiae* as species; tryptic digest with a maximum number of one missed cleavage. The peptide mass tolerance was set to $\pm$ 1.5 Da and fragment mass tolerance to $\pm$0.5 Da. Additionally, carbamidomethylation and oxidation of methionine were considered as possible modifications. Mascot protein scores greater than 29 were considered statistically significant ($P<0.05$).

2.5. Determination of cell energy metabolic status

Cell energy metabolic status was determined *via* the ATP pool by measuring luminescence with the commercially available kit BacTiter-Glo™ Microbial Cell Viability Assay (Promega) according to the manufacturer's instructions. Briefly, 100 µl of BacTiter-Glo reagent was added to 100 µl sample of cell culture and after 5 min luminescence was recorded using the microplate reader Safire II (Tecan). The results were expressed as a difference in ATP pool regarding the control.

2.6. Estimation of intracellular oxidation

Intracellular oxidation was estimated by using 2′,7′-dichlorofluorescein (H_2DCF), which is able to react with oxidant-reactive oxygen species. It is given as 2′,7′-dichlorofluorescein diacetate (H_2DCFDA), which easily penetrates the plasma membrane and is hydrolysed inside the cells by non-specific esterases. Non-fluorescent H_2DCF is oxidized to fluorescent 2′,7′-dichlorofluorescin (DCF), which is determined fluorimetrically (Jakubowski and Bartosz, 1997).

Cells were sedimented by centrifugation from 2 ml samples of the cultures, washed twice with 50 mM potassium phosphate buffer, pH 7.8, resuspended in the same buffer at a concentration of 1% (v/v) and preincubated at 28 °C for 5 min. H_2DCFDA was added as stock of 1 mM ethanol solution to a final concentration of 10 µM. After incubation (28 °C, 220 rpm, 20 min) 200 µl of cell suspension was transferred to the microplate and fluorescence was measured using the Tecan microplate reader Safire II (excitation and emission wavelengths of 488 and 520 nm, respectively). The results were expressed as a difference in reactive oxygen species level regarding the control.

3. Results

We have repeated our previous *in vivo* experiment with rats, this time on yeast *S. cerevisiae* cell culture in stationary growth phase with 1 mg/l of ibogaine in media, which mirrors peak mammalian brain tissue concentration after 20 mg/kg i.p.

Proteins from 5 h ibogaine-treated and control yeast cells were separated with 2-D electrophoresis and analyzed using the 2-D Dymension software. Of all protein spots that showed a significant change in intensity compared to control samples 12 spots that fall in the category of interest were excised and analyzed by LC-MS/MS, which gave sufficient confirmation of protein identity for five spots.

Proteins that were induced in yeast cells treated with ibogaine relative to control samples were identified as metabolic enzymes involved in glycolysis: glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, enolase and alcohol dehydrogenase (6.3-, 4.6-, 3.8-, and 3.2-fold, respectively); and one as a member of antioxidant defence: superoxide dismutase (2.2-fold) (Fig. 1, Table 1).

To find the background of enzyme induction, ATP pool was measured in dose and time escalation manner. Results showed that immediately after exposure of yeast cells to ibogaine, the ATP pool measured by luciferine/luciferase test significantly falls in a dose dependent manner (Fig. 2A). It reaches the minimum at 30 min and then gradually returns towards control.

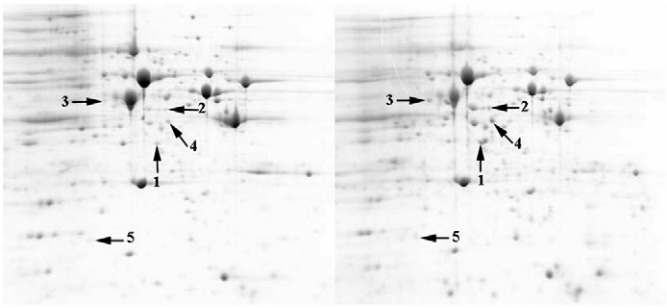

Fig. 1. Representative partial 2-D gel of proteins extracted from *Saccharomyces cerevisiae* cells: left — control cells, right — cells treated with 1 mg/l ibogaine after 5-h exposure. Labeled proteins were found to be upregulated and were identified by LC-MS/MS. Details are listed in Table 1.

Samples grown for 5 h in the presence of ibogaine at 1 mg/l concentration, also used for 2-D proteomic analysis, were washed with phosphate buffer. Eliminating the ibogaine from culture gradually brings ATP levels above the control ones (Fig. 2B).

Reactive oxygen species levels were simultaneously measured and we have observed immediate elevation of reactive oxygen species production after the ibogaine lasting up to 1 h, then inverted to decrease reaching the minimum at 2 h and this was followed by gradual return towards control (Fig. 3).

Additionally, enhanced synthesis of numerous unidentified types of low abundance proteins with relative shift of protein quantity towards low abundance fraction was observed and processed by computer analysis of existing 2-D gels, where a normalized volume value of 0.5% was a distinguishing criterion for a spot to enter low vs. high abundance group. Quantity of low abundance group was relatively enriched by a factor of 1.27 in treated sample compared to control while high abundance group being relatively impoverished. While high abundance group can be considered as a representative of structural proteins being constant at non-growing yeast cells in stationary growth phase, elimination of its relative diminution gives an increase factor of 1.76 for low abundance group (Table 2).

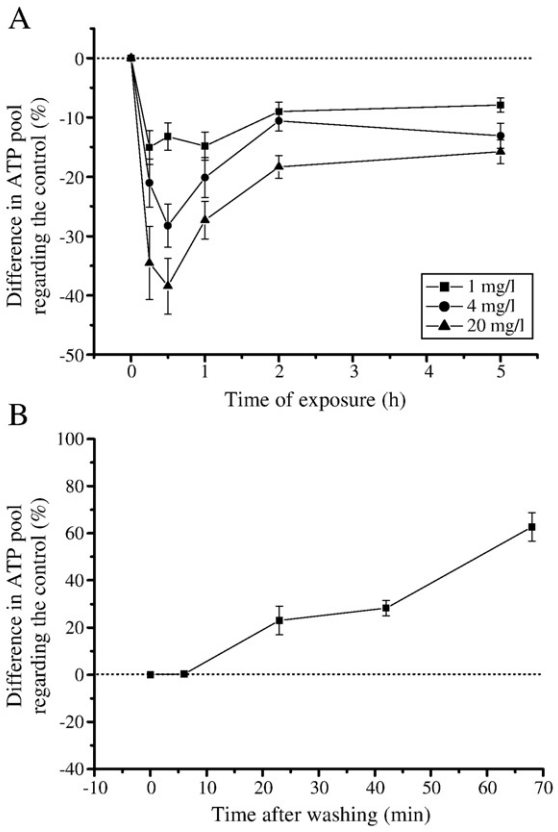

Fig. 2. A. Measuring of ATP pool during exposure of cells to different concentrations of ibogaine. Results are expressed as difference in ATP pool regarding the control and were measured in duplicate from two independent cultures for each concentration. The bars represent the averages ± S.E. B. Measuring of ATP pool after elimination of ibogaine from culture treated for 5 h at a concentration of 1 mg/l. Results are expressed as difference in ATP pool regarding the control and were measured in duplicate from two independent cultures. The bars represent the averages ± S.E.

4. Discussion

Molecular aspects of drug addictions are becoming increasingly recognized and findings suggest involvement of adaptation changes in gene expression patterns with the influence on cellular metabolism; to the very fundamental and ubiquitous housekeeping metabolism (Li et al., 2008). Reversibility of such changes is the platform for future anti-addiction treatments.

The induction of energy metabolism related enzymes due to ibogaine, previously triggered *in vivo* on rat model, was repeated on yeast *S. cerevisiae*, which is an accepted model for studies of primary metabolic pathways of higher eukaryotes (Ma, 2001; Menacho-Marquez and Murguia, 2007). A group of catabolism related enzymes was found to be induced after 5 h of exposure to ibogaine in a concentration of 1 mg/l (Fig. 1, Table 1); in higher proportion of induction and after shorter exposure compared to our previous study, assuming presently used concentration to be representative of brain tissue concentration in our previous experiment (Paškulin et al., 2006; Mash et al., 2000; Kontrimaviciute et al., 2006).

Ibogaine is known to affect numerous receptors and enzymes that are lacking in yeast cell. Therefore it is proved that enhanced expression of energy metabolism related enzymes is not mediated through receptor bindings, as previously described in the literature and it is not linked to cell differentiation or organization in tissue.

In search for a cause of enzyme induction, ATP pool and reactive oxygen species levels were investigated. ATP level falls to the minimum at 30 min of ibogaine's presence and then gradually returns towards control.

Table 1
List of *Saccharomyces cerevisiae* identified proteins whose expressions were stimulated by 1 mg/l ibogaine.

Spot	enzyme	Swiss-Prot accession number	Fold ibogaine/control	Theor. M_r(Da)/pI	Matched peptides	Mascot score
1	Glyceraldehyde-3-phosphate dehydrogenase 3	P00359	6.3	35747/6.46	16	381
2	Phosphoglycerate kinase	P00540	4.6	44738/7.11	19	492
3	Enolase 2 (2-phosphopyruvate dehydratase 2)	P00925	3.8	46914/5.67	22	933
4	Alcohol dehydeogenase 1	P00330	3.2	36823/6.26	29	694
5	Superoxide dismutase (Cu–Zn)	P00445	2.2	15855/5.62	7	125

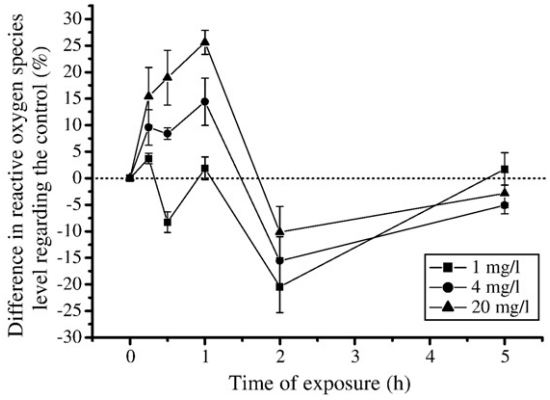

Fig. 3. Measuring of reactive oxygen species level during exposure of cells to different concentrations of ibogaine. Results are expressed as difference in reactive oxygen species level regarding the control and were measured in duplicate from two independent cultures for each concentration. The bars represent the averages ± S.E.

Table 2
Comparison of relative contents of low and high abundant protein fractions.

	Treated Σ normalized volumes (%)	Control Σ normalized volumes (%)	Ratio (treated/control)	Ratio (corrected)
High abundance proteins (normalized volume ≥0.5%)	36.398	50.103	0.726	1
Low abundance proteins (normalized volume <0.5%)	63.602	49.897	1.275	1.756

Restoration of energy status is due to induced energy metabolism related enzymes being translated. Besides, some immediate alosteric feed-back stimulation of regulatory glycolytic enzyme activity due to low ADP/ATP ratio could be recognized, indicated by immediate elevation of reactive oxygen species as byproducts of energy metabolism. Latter fall of reactive oxygen species level compared to control is due to efficient endogenous antioxidative systems with quick onset, as shown by the induction of Cu–Zn SOD (Fig. 1). This shows a typical exercise rebound effect — reactive oxygen species load provocation exerts protective role by "alarming and awakening" of antioxidative defence (Jamnik et al., 2006; Hakkiwell and Gutteridge, 2007).

It was shown that the induction of energy metabolism related enzymes is not an event per se, but compensation to a transient dose dependent fall in ATP level in the first hours of exposure to ibogaine (Fig. 2A). Whether this fall is a consequence of lower ATP production or results from higher ATP consumption is answered by immediate increase in intracellular reactive oxygen species levels after the ibogaine (Fig. 3). Since reactive oxygen species are mainly products of ATP synthesis in mitochondria, results negate decreased production. Rather, they suggest enhanced consumption of ATP pool, which is insufficiently buffered by an immediate increase of production due to alosteric feed-back modulation of glycolytic enzymes. Only after sufficient translation of additional quantity of enzymes, levels of ATP approach control values.

Induction i.e. bigger amount of enzymes being the cause of elevated specific activity is additionally confirmed by rebound effect, when eliminating the ibogaine from milieu brings ATP levels of samples, previously grown in the presence of ibogaine, above the control ones (Fig. 2B).

In which processes the consumption of ATP is increased remains unclear. We have excluded ibogaine's toxicity to cells and consequent energy cost of repair (data not shown). Neither, energy is consumed for metabolism of ibogaine itself, since yeast does not have cytochromes P450 that are known to be responsible for degradation of ibogaine in human (Walker, 1998; Maciulaitis et al., 2008).

Enhanced synthesis of numerous low abundance proteins with relative shift to low abundance fraction (Table 2) was observed. This is by itself an anabolic process that requires energy, which additionally suggests that enlarged energy demands are the primary trigger for induction of enzymes.

What is the exact mechanism and purpose of this wide non-specific activation of transcription, translation and consequent metabolic changes remain unclear, but metabolic turnover acceleration with even further energy demands is suspected. Shifts in quantities of energy related enzymes with subsequent elevated energy availability affect all metabolic processes inside and outside of the cells of any type and functional state; directly by fuelling ATP dependent reactions and indirectly by facilitating the synthesis of functional units. This could facilitate different healing processes, including restoration of physiological homeostasis in functionally remodeled cells after the development of tolerance to drugs of abuse, termed detoxification.

It should be pointed out here that substance-related disorders are not just a matter of neuronal circuits, being tuned on a drug seeking, craving-reward cycles, but are also a matter of a single cell, being habituated to the presence of drug of abuse and missing it, when it is gone. Interference with energy supply might be the crucial meeting point of these diverse adaptations to different types of drugs. Briefly, ibogaine has the opposite effect on energy metabolism than most of the drugs of abuse, which after chronic use downcast cellular energy status (Ryman and Walsh, 1952; Sadava et al., 1997; Sharma et al., 2003). On the contrary, after acute deprivation the ibogaine's induction of enzymes supplies additional energy; the effect once triggered, not being dependent to the presence of the drug and thereafter lasting for a prolonged period of time (Fig. 2B).

The proposed mechanism of action extends indications of ibogaine for medical use beyond syndrome of addiction, since induced catabolism enzymes with accelerated metabolism turnover facilitates detoxification and renewal of tissues after numerous pathological conditions like reconvalescence after infectious diseases, recovery after trauma, general exhaustion of chronic systemic diseases, cancer cachexia, depression etc. Ibogaine could be an adjuvant, non-specific therapy in synergism with disease targeting drug.

Besides, rebound effect of elevated energy availability after washing ibogaine from culture represents human individual after treatment, elevated in mood, strength and will, being capable of exerting resistance to their addiction for a prolonged period of time. These life changing, mind opening properties are exactly what the ibogaine medical subculture votes for.

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