THE DELAYED EFFECT OF ROTENONE ON THE RELATIVE CONTENT OF BRAIN ISATIN-BINDING PROTEINS OF RATS WITH EXPERIMENTAL PARKINSONISM

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Isatin (indoldione-2,3) is an endogenous biological regulator found in the brain, peripheral tissues, and biological fluids of humans and animals. Its biological activity is realized via isatin-binding proteins, many of which were identified during proteomic profiling of the brain of mice and rats. A number of these proteins are related to the development of neurodegenerative diseases. Previously, using a model of experimental Parkinsonism induced by a seven-day course of rotenone injections, we have observed behavioral disturbances, as well as changes in the profile and relative content of brain isatin-binding proteins. In this study, we have investigated behavioral responses and the relative content of brain isatin-binding proteins in rats with rotenone-induced Parkinsonism 5 days after the last administration of this neurotoxin. Despite the elimination of rotenone, animals exhibited motor and coordination impairments. Proteomic profiling of isatin-binding proteins revealed changes in the relative content of 120 proteins (the relative content of 83 proteins increased and that of 37 proteins decreased). Comparison of isatin-binding proteins characterized by the changes in the relative content observed in the brain right after the last injection of rotenone (n=16) and 5 days later (n=11) revealed only two common proteins (glyceraldehyde-3-phosphate dehydrogenase and subunit B of V-type proton ATPase). However, most of these proteins are associated with neurodegeneration, including Parkinson's and Alzheimer's diseases.

Key words: Parkinsonism; neurotoxin rotenone; neurodegeneration; isatin-binding proteins; neuroprotectors; isatin; brain; proteomic profiling

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INTRODUCTION

Isatin (indoldione-2,3) is an endogenous regulator found in the body of mammals and humans and possessing a wide spectrum of biological activity [1–4]. A number of isatin-binding proteins play an important role in the development of neurodegenerative pathology (Alzheimer's and Parkinson's diseases) [3, 5]. Studies performed using toxin based models of Parkinsonism induced by administration of the neurotoxins MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) to mice and rotenone to rats showed that isatin had a neuroprotective effect and a significant impact on the profile of isatin-binding proteins [5–8]. The Parkinsonian syndrome (PS), caused by both neurotoxins, is characterized by significant weight loss in animals, their death, oligokinesia, rigidity, and postural instability. However, in contrast to MPTP-induced PS, in the case of rotenone-induced PS, the pool of isatin-binding proteins common to control and PS rats significantly exceeded the pool of proteins common for both control mice and mice with MPTP-induced PS. In other words, rotenone administration insignificantly changed the pattern of isatin-binding proteins [9]. Comparison of the profiles of isatin-binding proteins specific to rats with PS induced by each of these neurotoxins revealed a complete absence of proteins common to these two models, thus indicating differences in the molecular mechanisms of the action of rotenone and MPTP. A study of the quantitative parameters of isatin-binding proteins in the case of the rotenone model of Parkinsonism showed that, despite the absence of changes in the spectrum of these proteins compared to the control, the rotenone administration affected their relative content [10]. An increase in the relative content of 65 proteins and a decrease in the relative content of 21 proteins were detected.

The aim of this study was to compare the physiological reactions and quantitative changes in the proteomic profile of isatin-binding proteins in the brain of rats immediately after a course of rotenone administration and 5 days after the last administration of this neurotoxin.

MATERIALS AND METHODS

Reagents

The following reagents were used in the study: Tris (hydroxymethyl)aminomethane, ammonium bicarbonate, dithiothreitol, guanidine hydrochloride, urea, sodium chloride, Triton X-100, 4-vinylpyridine, Coomassie brilliant blue G-250 (Merck, USA); formic acid, sodium hydroxide (Acros Organics, USA), acetonitrile (Fisher Chemical, UK);

isopropanol, trifluoroacetic acid (Fluka, USA); Tris-(2-carboxyethyl)phosphine (Pierce, USA); modified trypsin (mass spectrometry grade, Promega, USA). Other reagents of the highest purity available were from local suppliers.

Experimental Animals

The study was performed on outbred albino rats obtained from the Stolbovaya nursery of (the branch of the Scientific Center for Biomedical Technologies, Russia). The animals were kept under standard vivarium conditions with free access to food and water under a twelve-hour light regime.

Modeling of Experimental Parkinsonism in Rats

Modeling of PS by means of systemic administration of rotenone was carried out as described in [11]. Rotenone solution in Miglyol 840 was administered intraperitoneally to rats at a daily dose of 2.75 mg/kg for 7 days. Rotenone preparation for injections to rats was described earlier [9]. Control animals received intraperitoneal injections of saline daily (during 7 days) in an equivalent volume of 0.2 ml per 100 g of animal body weight.

Behavioral Tests

Behavioral tests in animals with experimental PS were evaluated on day 12 of the experiment (5 days after the last administration of rotenone). These included "Open Field" and "Rotating Rod" tests, performed in details as described previously [9].

Statistical data processing was carried out using the Statistica v. 10.0 program. The normality of distribution was checked using the Shapiro-Wilk test, followed by assessment of equality of variances using Levene's test. Since there was no normal distribution in the experimental group, further processing was carried out using the nonparametric Mann-Whitney statistics method. Fisher's exact test was used to evaluate categorical data in small groups. The results in the tables are presented as mean ± error of the mean (Mean ± SEM). Differences between groups were considered as significant at $p<0.05$. Differences at $0.05<p<0.1$ were considered a statistical trend.

Preparation of Lysates of Brain Homogenates

The animals were decapitated under light ether anesthesia using a guillotine 5 days after the last administration of rotenone and behavioral testings. Brain tissue samples (cerebral hemispheres) were homogenized using a Heidolph SilentCrusher homogenizer (50,000 rpm) in 0.05 M potassium phosphate buffer (pH 7.4) to obtain a final protein concentration of 30 mg/ml. To assess relative quantitative changes in the content of brain proteins in animals of different experimental groups, the same amount of total protein was used during sample preparation; it was controlled using the Bradford method [12]. After incubation in the presence of 3% Triton X-100 (4°C, 1 h), the lysates were diluted 3 times with the same buffer and centrifuged for 30 min at 16,000 *g* to obtain a cleared supernatant.

Samples for mass spectrometric analysis (protein extraction, alkylation and trypsinolysis) were prepared as described previously [10].

The Mass Spectrometric Analysis

The mass spectrometric analysis was carried out using the equipment of the Center of Collective Use "Human Proteome" (IBMC) — the Ultimate 3000 RSLCnano highly efficient liquid-liquid separation system for peptides (Thermo Scientific, USA) operated in the nanoflow mode of the Q-Exactive HFX mass spectrometric detector (Thermo Scientific) as described previously [9].

Bioinformatics data processing was carried out according to [9].

Each protein presented in the tables was identified in at least three independent experiments.

RESULTS AND DISCUSSION

Delayed Effects of Rotenone on Behavioral Responses in Rats

Assessment of the level of oligokinesia in rats 5 days after the last administration of rotenone in the "Open Field" test showed the persistence of motor disorders. For example, horizontal and vertical activity and the number of peeks into holes remained reduced by 53%, 41%, and 61.4% ($p<0.05$), respectively, as compared to the control group. The delayed effect of rotenone also manifested itself in motor deficits: the duration of retention of animals on a rotating rod was reduced by 51% as compared to the control group (Table 1).

Delayed Effects of Rotenone on the Relative Abundance of Rat Brain Proteins

Results of proteomic analysis have shown that 5 days after the course of rotenone administration, differences remained in the relative content of proteins belonging to different functional groups in the brain of PS rats as compared to the control group. Comparing the proteomic data on the effect of rotenone immediately after the last administration and 5 days later, one can note that after 5 days the changes in the relative content (versus control) of only six proteins persisted (Fig. 1). These included alpha-synuclein, glyceraldehyde-3-phosphate dehydrogenase, amino acid transporter, subunits B and D1 of the V-type proton ATPase, 4 subunit 1 beta subcomplex of NADH dehydrogenase of the electron transport chain respiratory chain (Table S1,

Table 1. The delayed effects of the course of rotenone administration on motor activity and motor coordination of rats

Groups of animals and the number of animals in each group	Motor activity, units		Peeks into holes, units	Duration of retention of animals on a rotating rod, s
	Horizontal activity (number of movements)	Vertical activity (number of stands)		
Control, n=10	14.67±2.42	9.67±2.22	7.56±1.76	177.75±2.25
Rotenone, n=12	6.92±0.95	5.67±0.54	2.92±0.56	87.17±12.43
p	<0.01	<0.075	<0.05	<0.001

Rotenone was administered for 7 days [9], and motor activity and motor coordination were analyzed 5 days after the last rotenone administration. Data represent mean ± SEM.

Table 2. Functional distribution of brain proteins quantitatively changed during the development of rotenone-induced PS

Function	Total number of proteins		Total number of proteins after treatment with rotenone			
			↑		↓	
	7 days	12 days	7 days	12 days	7 days	12 days
Proteins/enzymes involved in energy generation and carbohydrate metabolism	19	19	17	7	2	12
Proteins involved in cytoskeleton formation and exocytosis	23	33	17	26	6	7
Proteins involved in signal transduction and regulation of enzyme activity	24	34	17	27	7	7
Antioxidant and protective proteins/enzymes	7	15	4	10	3	5
Protein regulators of gene expression, cell division and differentiation	7	3	4	3	3	0
Enzymes involved in metabolism of proteins, amino acids and other nitrogenous compounds	2	14	2	9	0	5
Enzymes involved in lipid metabolism	4	2	4	1	0	1
Total number	86	120	65	83	21	37

The "↑" sign indicates an increase in the amount of protein, and the "↓" sign indicates a decrease.

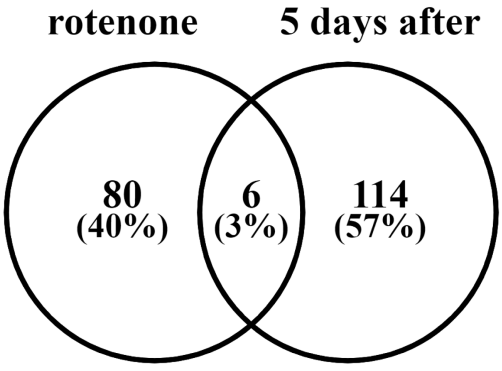

Figure 1. A Venn diagram comparing relative content of brain proteins (versus control) immediately after the course of rotenone administration and 5 days later.

Supplementary Materials, and Supplementary Materials to the article [10]). However, the quantitative values of this change at different stages of the experiment differed significantly. For example, immediately after the last administration of rotenone, the relative content of alpha-synuclein demonstrated a 5.6-fold increase over the control values, while 5 days later the alpha-synuclein level was only 1.4 times higher than in the control. For glyceraldehyde-3-phosphate dehydrogenase, the increase in the relative content at these time intervals was 1.6 and 0.7 times, respectively, for the NADH dehydrogenase subunit it was 2 and 1.3 times, and for the amino acid transporter and for B and D1 subunits of V-type proton ATPase the increase was 9.2 and 0.5 times and 21.1 and 1.3 times, respectively (cf. Table S1 of the Supplementary Materials [10] and this article). In other words, the number of proteins initially affected by the rotenone administration gradually returned to control. At the same time, there differences in the relative content of other proteins occurred. Table 2 shows data on changes in the relative content of proteins of different functional groups immediately after the last injection of rotenone and after 5 days. Interestingly, although these are different proteins, the number of proteins from certain functional groups was basically the same. In both cases, the most pronounced changes induced by rotenone were found in the functional groups of enzymes responsible for energy generation and carbohydrate metabolism,

cytoskeletal and exocytosis proteins, and proteins involved in signal transduction and regulation of enzyme activity. Less pronounced changes have been found in the relative content of antioxidant and protective proteins, regulators of gene expression, cell division and differentiation, enzymes of metabolism of proteins and other nitrogenous compounds, and enzymes of lipid metabolism (Table 2). In most cases, the relative content of the proteins increased both after the last rotenone injection and 5 days later. The only exception was the group of enzymes of energy generation and carbohydrate metabolism. Among 19 proteins in this group with altered relative content after the last rotenone injection, 17 proteins demonstrated an increase and 2 proteins demonstrated a decrease versus control. Five days later, among 19 proteins the relative content of 7 proteins increased and 12 proteins decreased versus control.

A comparative analysis of isatin-binding proteins characterized by altered relative content right after the last injection of rotenone (n=16) and 5 days later (n=11), revealed only two common proteins: glyceraldehyde-3-phosphate dehydrogenase and V-type proton ATPase subunit B (Fig. 2, Tables 3, 4). Immediately after the last rotenone administration, the relative content of isatin-binding proteins changed more significantly and mainly towards an increase. 5 days after the changes were less pronounced; in 7 out of 16 proteins, the relative content increased no more than 2 times, while the relative content of others slightly decreased.

It should be noted that most of detected isatin-binding proteins with altered relative content found at both stages of the experiment, are associated with neurodegeneration, including Parkinson's and Alzheimer's diseases [13–53].

Table 3. Isatin-binding proteins with significantly altered relative content in the brain of rats immediately 5 days after the end of a course of rotenone administration to animals as compared to control: delayed effect

##	Uniprot accession number	Uniprot gene name	Uniprot protein name	Function	Localization	Difference from control		References
						Fold change	-Log(P-value)	
1	A0A8I5Y1E2	*Add1*	Alpha-adducin	2	PM, M, C	2.2	1.1	[13, 14]
2	A0A8I6GDI3	*Actn1*	Alpha-actinin-1	2	PM, M, C	1.7	1.2	[15, 16]
3	P31000	*Vim*	Vimentin	2	C, PM, M, N	1.6	1.8	[17]
4	Q6AY84	*Scrn1*	Secernin-1	2	C	1.4	1.8	[18, 19]
5	Q00981	*Uchl1*	Ubiquitin carboxyl-terminal hydrolase isozyme L1	6	C, ER, N, Mch, PM	1.4	1.7	[20, 21]
6	P05065	*Aldoa*	Fructose-bisphosphate aldolase A	1	C	1.3	1.8	[22, 23]
7	P62815	*Atp6v1b2*	V-type proton ATPase subunit B, brain isoform	2	M, PM, Ve, S	1.3	2.2	[24]
8	P60203	*Plp1*	Myelin proteolipid protein	3	PM, M	0.7	3.0	[25]
9	Q5XIF6	*Tuba4a*	Tubulin alpha-4A chain	2	C	0.5	1.9	[26–28]
10	B4F7C2	*Tubb4a*	Tubulin beta chain	2	C	0.7	1.7	[29, 30]
11	P07323	*Eno2*	Gamma-enolase	1	C, PM	0.7	3.3	[31]
12	P04797	*Gapdh*	Glyceraldehyde-3-phosphate dehydrogenase	1	C, N	0.7	2.7	[32, 33]
13	P48500	*Tpi1*	Triosephosphate isomerase	1	C	0.6	3.4	[34]
14	P07335	*Ckb*	Creatine kinase B-type	1	C, PM, Mch	0.6	4.6	[35, 36]
15	P00507	*Got2*	Aspartate aminotransferase, mitochondrial	6	Mch, PM	0.4	3.9	[37]
16	Q5M7A7	*Cnrip1*	CB1 cannabinoid receptor-interacting protein 1	3	C, PM	0.7	2.3	[38–41]

Here and in Table 4 proteins names are the same as in the Uniprot database. Number in the column "Function" designate the following functional groups of proteins: *1*. Proteins/enzymes involved in energy generation and carbohydrate metabolism. *2*. Proteins involved in cytoskeleton formation and exocytosis. *3*. Proteins involved in signal transduction and regulation of enzyme activity. *4*. Antioxidant and protective proteins/enzymes. *5*. Protein regulators of gene expression, cell division and differentiation. *6*. Enzymes involved in metabolism of proteins, amino acids, and other nitrogenous compounds. *7*. Enzymes involved in lipid metabolism. Localization: C – cytoplasm, N – nucleus, M – membranes, PM – plasma membrane, ER – endoplasmic reticulum, G – Golgi complex, Mch – mitochondria, L – lysosomes, Mic – microsomes, E – endosomes, Ve – vesicles, S – synapse.

Table 4. Isatin-binding proteins with significantly altered relative content in the brain of rats immediately after the end of a course of rotenone administration to animals (compared to control)[a]

##	Uniprot accession number	Uniprot gene name	Uniprot protein name	Function	Localization	Difference from control		References
						Fold change	-Log(P-value)	
1	P04797	*Gapdh*	Glyceraldehyde-3-phosphate dehydrogenase	1	C, N	1.6	2.4	[32, 33]
2	P07943	*Akr1b1*	Aldo-keto reductase family 1 member B1	7	C	2.0	3.4	—
3	P19527	*Nefl*	Neurofilament light polypeptide	2	C, S	0.5	3.1	[42]
4	P38983	*Rpsa*	40S ribosomal protein SA	5	C, N, PM	2.8	4.0	[43]
5	P62744	*Ap2s1*	AP-2 complex subunit sigma	2	M, PM	2.4	3.4	[44, 45]
6	P62815	*Atp6v1b2*	V-type proton ATPase subunit B, brain isoform	2	M, PM, Ve, S	1.7	4.4	[24]
7	Q63198	*Cntn1*	Contactin-1	3	PM	3.7	3.0	[46]
8	Q6P0K8	*Jup*	Junction plakoglobin	2	C, M	10.5	3.0	[47, 48]
9	A0A8I6A1Y1	*Ogdh*	Oxoglutarate dehydrogenase (succinyl-transferring)	1	Mch, N	0.7	3.4	[49, 50]
10	A0A8I6A7U6	*Sfpq*	Splicing factor proline and glutamine rich	5	C, N	0.3	4.9	[51, 52]
11	A0A8I6A304	*Basp1*	Brain abundant, membrane attached signal protein 1	3	C, N	18.3	5.1	[53]

[a]Modified from [10] and supplemented.

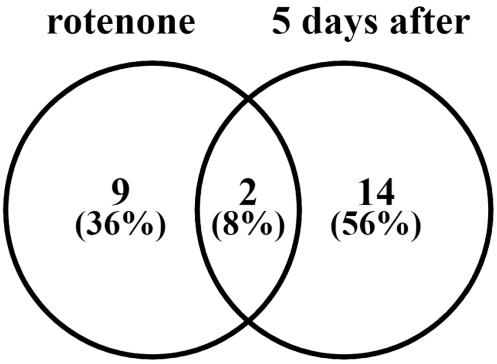

Figure 2. A Venn diagram comparing relative content of brain isatin-binding proteins (versus control) immediately after the course of rotenone administration and 5 days later.

Among all the isatin-binding proteins with altered relative content induced by rotenone and neuroprotectors, the most pronounced changes have been found in the case of plakoglobin and the BASP1 protein, which belongs to the group of acid-soluble brain proteins. Treatment of rats with rotenone increased their relative content in the brain by 10 and 20 times, respectively.

Plakoglobin, also known as gamma-catenin, is a cytoplasmic component of desmosomes homologous to beta-catenin. In addition to the formation of desmosomes, plakoglobin is also involved in the formation of adhesive intercellular contacts associated with actin microfilaments. Mutations in the *Jup* gene, which encodes plakoglobin, cause cardiomyopathies. In addition, it is known that proteins of the catenin family are involved in the regulation of the microenvironment of neuronal progenitor cells, proliferation and differentiation of cerebral cortex cells [48].

BASP1 (brain acid-soluble protein 1), which belongs to the group of acid-soluble proteins in the brain, along with the protein GAP-43 (growth-associated protein-43), regulates the maintenance of the presynaptic vesicle cycle and the release of neurotransmitters. Post-translational modifications and functions of this protein, its involvement in the processes of axon growth, regeneration and plasticity are now being actively studied in the context of neurodegenerative diseases [53].

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COMPLIANCE WITH ETHICAL STANDARDS

The experiments were carried out in compliance with generally accepted norms of humane treatment of laboratory animals. The work was carried out in accordance with the Order of the Ministry of Health of the Russian Federation No. 199n of April 1, 2016 "On Approval of the Rules for Good Laboratory Practice" and the Directive 2010/63/EU of the European Parliament and of the Council of the European Union of September 22, 2010 on the protection of animals used for scientific purposes.

CONFLICT OF INTERESTS

The authors declare no conflicts of interest.

Supplementary materials are available in the electronic version at the journal site (pbmc.ibmc.msk.ru).

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ОТСРОЧЕННОЕ ДЕЙСТВИЕ РОТЕНОНА НА ОТНОСИТЕЛЬНОЕ СОДЕРЖАНИЕ ИЗАТИН-СВЯЗЫВАЮЩИХ БЕЛКОВ МОЗГА У КРЫС С ЭКСПЕРИМЕНТАЛЬНЫМ ПАРКИНСОНИЗМОМ

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Изатин (индолдион-2,3) — эндогенный биологический регулятор, обнаруженный в мозге, периферических тканях и биологических жидкостях человека и животных. Его биологическую активность опосредуют изатин-связывающие белки, многие из которых были идентифицированы в ходе протеомного профилирования препаратов мозга мышей и крыс. Ряд этих белков имеет отношение к развитию нейродегенеративных заболеваний. Ранее на модели экспериментального паркинсонизма, индуцированного семидневным введением пестицида ротенона, были обнаружены выраженные нарушения поведенческих реакций, а также изменения профиля и относительного содержания изатин-связывающих белков мозга. В данной работе мы исследовали поведенческие реакции и относительное содержание изатин-связывающих белков мозга крыс с индуцированным ротеноном экспериментальным паркинсонизмом через 5 дней после завершения курсового введения этого нейротоксина. Несмотря на отмену введения ротенона, у животных сохранялись нарушения двигательной активности и координации движений. По результатам протеомного анализа выявлены изменения в относительном содержании 120 белков мозга (относительное содержание 83 белков увеличивалось, а 37 белков снижалось). Сравнительный анализ изатин-связывающих белков, относительное содержание которых в мозге менялось после последней инъекции ротенона (n=16) и через 5 дней (n=11), выявил совпадение только двух (глицеральдегид-3-фосфатдегидрогеназы и субъединицы B протонной АТРазы V-типа). При этом большинство обнаруженных белков ассоциировано с нейродегенерацией, включая болезни Паркинсона и Альцгеймера.

Полный текст статьи на русском языке доступен на сайте журнала (http://pbmc.ibmc.msk.ru).

Ключевые слова: паркинсонизм; нейротоксин ротенон; нейродегенерация; изатин-связывающие белки; нейропротекторы; изатин; мозг; протеомное профилирование

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