ANTIOXIDANT EFFECTS OF THE SYNTHETIC THYRONAMINE ANALOGUE IN EXPERIMENTAL CEREBRAL ISCHEMIA

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The oxidative stress associated with ischemic stroke is a major factor damaging the nervous tissue. Thyroid hormones have a significant effect on the body's redox status, however, the impact of their derivatives, thyronamines, considered as potential neuroprotectors, on the characteristics of lipid peroxidation (LP) is not clearly understood. The study was aimed to assess the impact of the T0AM thyronamine synthetic analogue on the main LP indicators in the model of acute cerebral ischemia. Permanent ligation of the right common carotid artery was performed to simulate acute cerebral ischemia in white rats. The animals were divided into two groups: the control group receiving no treatment and the experimental group, to which the T0AM thyronamine synthetic analogue was intraperitoneally administrated (75 mg/kg of the rat's body weight). After 24 h the rat was decapitated, and the cerebral cortex tissue was extracted for biochemical analysis. The following LP indicators were determined by spectrophotometry: malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GPx). When administering the T0AM thyronamine synthetic analogue, a significant (2-fold) decrease in MDA levels was observed in the ischemic hemisphere ($p = 0.022$), along with the 2.49-fold increase in the GPx activity in the brain tissue ($p = 0.004$) of the intact hemisphere and the 2.65-fold increase in its activity ($p = 0.021$) in the ischemic hemisphere, as well as the 1.23-fold increase in SOD activity in the ischemic hemisphere ($p = 0.042$). The T0AM thyronamine synthetic analogue has a great potential in terms of activation of the antioxidant protection mechanisms in the cerebral cortex of white laboratory rats under conditions of acute hemispheric ischemia.

Keywords: thyronamines, antioxidants, neuroprotection, ischemic stroke, oxidative stress, lipid peroxidation

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АНТИОКСИДАНТНЫЕ ЭФФЕКТЫ СИНТЕТИЧЕСКОГО АНАЛОГА ТИРОНАМИНА ПРИ ЭКСПЕРИМЕНТАЛЬНОЙ ИШЕМИИ ГОЛОВНОГО МОЗГА

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Окислительный стресс при ишемическом инсульте — один из основных факторов, повреждающих нервную ткань. Тиреоидные гормоны оказывают существенное влияние на редокс-статус организма, однако влияние их производных, тиронаминов, рассматриваемых в качестве потенциальных нейропротекторов, на показатели перекисного окисления липидов (ПОЛ) изучено недостаточно. Целью исследования было изучить влияние синтетического аналога тиронамина T0AM на основные показатели ПОЛ в модели острой ишемии головного мозга. Для моделирования острой ишемии головного мозга у белых крыс выполняли необратимую перевязку правой общей сонной артерии. Животные были разделены на две группы — контрольную без лечения и экспериментальную, в которой интраперитонеально вводили синтетический аналог тиронамина T0AM (75 мг/кг массы тела крысы). Спустя сутки крысу подвергали декапитации, и ткань коры больших полушарий головного мозга извлекали для биохимического анализа. Из показателей ПОЛ определяли малоновый диальдегид (МДА), супероксиддисмутазу (СОД), глутатионпероксидазу (ГПО) спектрофотометрически. На фоне введения синтетического аналога тиронамина T0AM наблюдали статистически значимое снижение содержания МДА в ишемизированном полушарии в 2 раза ($p = 0,022$), повышение активности ГПО в ткани головного мозга в 2,49 раза ($p = 0,004$) для интактного и в 2,65 раза ($p = 0,021$) — для ишемизированного полушарий и увеличение активности СОД в ишемизированном полушарии в 1,23 раза ($p = 0,042$). Синтетический аналог тиронамина T0AM обладает значительным потенциалом в отношении активации механизмов антиоксидантной защиты в коре головного мозга белых лабораторных крыс в условиях острой полушарной ишемии.

Ключевые слова: тиронамины, антиоксиданты, нейропротекция, ишемический инсульт, окислительный стресс, перекисное окисление липидов

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Stroke is one of the leading causes of mortality all over the world and the major cause of permanent disability that exerts a heavy economic burden on the entire society. The development of ischemia is associated with the rapid death of millions of neurons within seconds. Unfortunately, today there are still no effective neuroprotective agents capable of mitigating this process [1].

Oxidative stress (OS) accompanied by the release of reactive oxygen species (ROS) is one of the main mechanisms underlying such damage. The brain is particularly sensitive to oxidative damage, since it contains large amounts of polyunsaturated fatty acids representing one of the prime targets for ROS. In the rodent experiment, high levels of lipid peroxidation (LP), the products of which activate phospholipase A2 ensuring cleavage of the cell membrane phospholipids with the release of pro-inflammatory mediators, are observed in the ischemic stroke (IS) focus 24 h after the permanent middle cerebral artery (MCA) occlusion. Low levels of antioxidants with subsequent antioxidant buildup are reported in the same time period [2, 3]. The DNA bases are also to the great extent susceptible to the damaging effects of oxidants. Consequently, mutations and deletions occur in both primary and secondary structure of both nuclear and mitochondrial DNA, and the latter is more vulnerable, since it is located closer to the original source of ROS and has a lower repair capacity compared to the nuclear DNA. The LP products can behave as triggers of the p53 signaling pathway, causing changes in the membrane structure and the loss of mitochondrial DNA function [4]. Disruption of redox homeostasis is associated with damage to the nervous system that can result in the autoimmune and neurodegenerative diseases. In terms of OS intensity assessment, such indicators, as malondialdehyde (MDA) being one of the OS biomarkers, endogenous antioxidant superoxide dismutase (SOD), and glutathione peroxidase (GPx) that catalyzes the reduced glutathione oxidation, are of special interest.

The contribution of thyroid hormones (TH) to maintaining the redox status is ambiguous. There are literature data suggesting that hyperthyroidism results in the increased ROS production, while hypothyroidism leads to the decrease in ROS production, thereby reducing the antioxidant activity [5, 6]. The neuronal mitochondria represent one of the targets for both TH and their derivatives (particularly thyronamines), and morphofunctional alterations in the functioning of these organoids associated with the TH deficiency are observed. Upregulation of the genes, responsible for mitochondrial palmitate beta-oxidation affected by triiodothyronine resulting in the increase of adenosine triphosphate (ATP) essential for normal function of the ion pumps, was demonstrated in the astrocyte culture [7, 8]. Considering the central role of astrocytes in protecting neurons in ischemic brain damage, the authors concluded that reduction in the lesion size in experimental transient ischemia resulted specifically from normalization of energy exchange in astroglia ensured by T3.

However, this is just one of the possible mechanisms underlying neuroprotection ensured by TH. The description of the role of their derivatives, thyronamines, in protection of neurons, is usually limited to the hypothermic effect reported in the literature. We have assumed that thyronamines, specifically T0AM, also can contribute to the nervous tissue antioxidant protection. The study was aimed to assess the concentrations of products actively reacting with thiobarbituric acid (TBA-AP), SOD, and GPx in the brain tissue of laboratory rats after experimental acute ischemia against the background of administration of the T0AM thyronamine synthetic analogue (SA-T0AM) as a proposed neuroprotector.

METHODS

The authors synthesized the T0AM thyronamine synthetic analogue, 4-[4-(2-aminoethoxy)benzyl]aniline hydrochloride, by the method reported in the literature [9]. The structure of the resulting compound was confirmed by the ^{1}H and ^{13}C NMR spectroscopy.

A total of 40 male and female animals with the body weight of 190–210 g from the vivarium of the Gusak Institute of Emergency and Reconstructive Surgery were selected for the experiment. Permanent ligation of the right common carotid artery (CCA) in white non-linear laboratory rats was selected as a model of acute cerebral ischemia. According to the published studies, this model causes small cortical infarcts [10]. Surgery was performed under general anaesthesia (Calypsol, 100 mg/kg of the rat's weight). Dimethyl sulfoxide (DMSO) was used as a solvent for the SA-T0AM. In the study focused on assessing biological effects of various solvents, intraperitoneal administration of DMSO to rats in a dose of 5 mL/kg for a month was considered to be relatively safe [11]. The animals were divided into two experimental groups, 20 rats per group. In the first group (Control), the right CCA ligation surgery was performed; 0.5 mL of the DMSO solution + 0.5 mL of the 0.9% NaCl solution were administered intraperitoneally 10 min after the ligation. In the second group (Experiment), the animals were administered 0.5 mL of the DMSO solution + 0.5 mL of the 0.9% NaCl solution + SA-T0AM in a dose of 75 mg/kg of the rat's weight after surgery. The optimal dose of 75 mg/kg was selected based on the maximum hypothermia induction at zero mortality, in accordance with the previously reported study [12]. Given the maximum OS activity observed within minutes after the ischemia induction [13], the agent was administered 10 min after the CCA ligation. The animals were subjected to decapitation and extraction of the brain 24 h after the experiment. The cerebral cortex tissue (tissues of the intact hemisphere and the hemisphere affected by ischemia were used separately) was use to determine the LP indicators.

The weighted portion of the fresh rat cerebral cortex tissue was homogenized for at least 10 min in the glass homogenizer with the 50 mM Tris buffer containing 1 mM of EDTA and 0.25 M of sucrose, pH 7.4, in a ratio of 1:3. The as-prepared homogenate was frozen at a minimum of –70 °C for 24 h. After thawing is was centrifuged for 30 min at 4000 rpm, then the centrifugate was 6-fold diluted (50 µL of supernatant + 250 µL of 5 mM potassium phosphate buffer containing 1×10^{-4} M of EDTA, pH 7.8).

Protein was quantified by the photometric Lowry protein assay at λ = 750 nm in a 5 mm cell. The assay results are required to convert the SOD, GPx, and TBA-AP content in the animal brain homogenate (per 1 mg of protein). The method to determine SOD activity in the brain tissue homogenate is based on the enzyme capability of inhibiting epinephrine autoxidation to adrenochrome at pH 10.2. The oxidation kinetics was measured by spectrophotometry at λ = 480 nm in a 10 mm cell using the Eppendorf EPAC 6140 biochemical analyzer (Eppendorf AG; Germany). The TBA-AP concentration was determined based on the reaction of MDA with 2-thiobarbituric acid (TBA) yielding the colored "trimethine complex", the concentration of which was determined by photometry at λ = 532 nm in a 10 mm cell. Extinction was measured in the Eppendorf EPAC 6140 biochemical analyzer. The GPx activity was estimated based on the changes in the amount of reduced glutathione (GSH) before and after incubation with the model substrate (tert-butyl hydroperoxide) based on the reaction with Ellman's reagent (5,5-dithiobis-2-nitrobenzoic acid). Absorbance was measured

Table. The antioxidant protection indicators in the brain tissue of model rats with acute hemispheric ischemia (SOD, GPx and TBA-AP levels are provided per 1 mg of protein)

Groups	Control		Experiment		*p*
Indicator	*n*	Median	*n*	Median	
SOD, U, intact hemisphere	16	60	16	63	0.75
SOD, U, ischemic hemisphere	18	59	18	72.5	0.042
GPx, μmol/min*mg, intact hemisphere	16	278.5	18	693	0.004
GPx, μmol/min*mg, ischemic hemisphere	16	304	18	805	0.021
TBA-AP, μmol, intact hemisphere	20	0.44	20	1.5	< 0.001
TBA-AP, μmol, ischemic hemisphere	20	0.7	20	0.34	0.022

in a cell with the pathlength of 10 mm at 412 nm using the Eppendorf EPAC 6140 biochemical analyzer.

Statistical processing of the data obtained was performed in the R software package (R Core Team, 2018). According to the Shapiro–Wilk test results, the distribution of the TBA-AP ($W = 0.79$, $p = 0.01$), GPx ($W = 0.860$, $p = 0.016$), and SOD ($W = 0.89$, $p = 0.41$) parameters in the hemisphere affected with ischemia was non-normal. The nonparametric Mann–Whitney U test was used to reveal the differences between samples.

RESULTS

The results of biochemical tests characterizing the activity of antioxidant systems in the brain tissues of laboratory animals in the model of acute cerebral ischemia are provided in the Table and the Figure.

Comparison of TBA-AP levels in the intact hemisphere showed that these were 3.4 times higher in rats receiving SA-T0AM than in control animals ($p < 0.001$). However, the

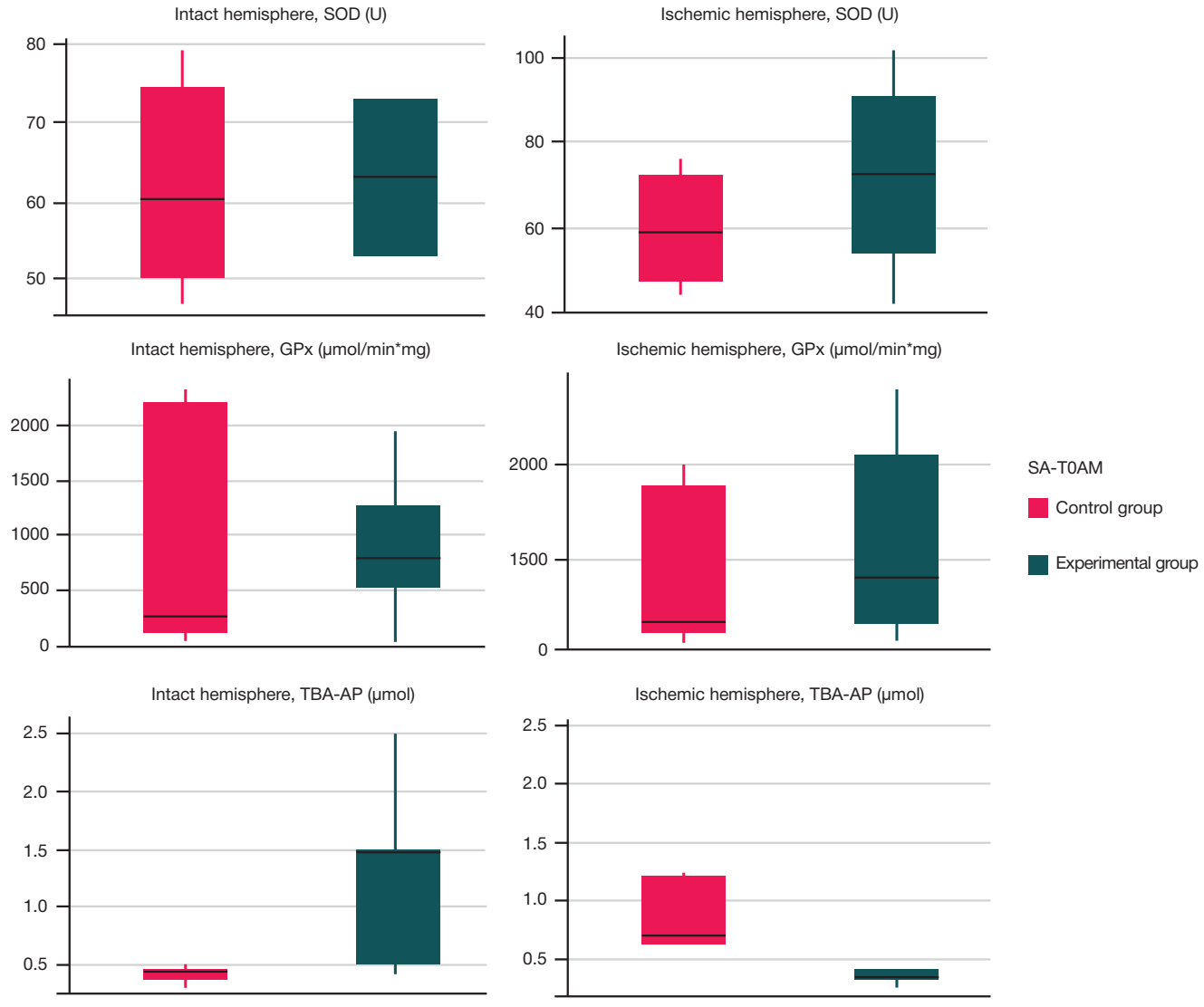

Fig. The oxidative stress marker activity in the brain of model rats with acute hemispheric ischemia. TBA-AP — concentration of products actively reacting with thiobarbituric acid; SOD — superoxide dismutase; GPx — glutathione peroxidase

levels of TBA-AP in the hemisphere affected by ischemia in the rats administered the studied potential neuroprotector were approximately twice lower than in control animals with ischemia receiving no therapy ($p = 0.022$).

Comparison of GPx activity in rats of the control and experimental groups showed that the SA-T0AM administration resulted in the 2.49- and 2.65-fold increase in activity of this enzyme, respectively, in the cortical tissue of both intact hemisphere ($p = 0.040$) and the hemisphere affected by ischemia ($p = 0.021$).

No significant differences in SOD activity in the intact hemisphere cortical tissue between the control and experimental rats were revealed ($p = 0.750$). However, the SOD activity in the hemisphere affected by ischemia in the animals receiving SA-T0AM turned out to be 1.23 times higher than that in control rats ($p = 0.042$).

DISCUSSION

Changes in MDA levels associated with acute ischemia

The increase in the TBA-AP levels observed in the homogenates of the rat brain hemispheres suggests activation of the OS processes in ischemia. MDA is a stable and toxic LP product. The increase in the levels of MDA, the main TBA-AP component, results in disturbance of permeability and subsequent cell membrane disruption, the release of lysosomal enzymes, and activation of the processes underlying lysis of the cellular structures.

The researchers have shown a significant increase in blood levels of MDA in patients with IS, without any correlation with the disease outcome; the other study revealed the relationship between the serum MDA levels and the functional disease outcome after 3 months, and, therefore, suggested to use MDA as a biopredictor [2, 3]. A number of authors confirm that there is a significant positive correlation between the MDA levels and the functional outcome of stroke a week after the patient admission to the unit; one of the studies has shown that the MDA levels determined at admission and 7 days later can be used to predict the patient's functional disability 6 months later based on the mRS scale [14, 15]. A significant correlation between the MDA levels and the stroke severity based on the NIHSS scale has been also revealed. Huge amounts of free radicals accumulate in the penumbra (as indicated by MDA accumulation) due to insufficient oxygen uptake. The extent of damage depends on the activity of the antioxidant protection mechanisms. In case of severe stroke, antioxidants fail to ensure binding of free radicals due to large amounts of damaged tissues. The antioxidant enzymes are induced enzymes, therefore, their transcription and synthesis take time. Thus, the increase in LP due to insufficient stimulation of the antioxidant protection mechanisms takes place in the early phase of stroke, which reflects the stroke volume, and therefore, the stroke severity; MDA serves as a sensitive marker of this process. The decrease in the levels of TBA-AP in the rat brain tissue homogenate against the background of CA-T0AM administration suggests the decrease in the OS degree in this group of animals [16].

Changes in GPx activity associated with acute ischemia

It is believed that GPx has a protective function; it ensures protection against brain damage. The increase in the infarct size and the apoptosis enhancement are observed in the glutathione peroxidase-1 (GPX1)-knockout mice in the experiment involving MCA occlusion with subsequent reperfusion [17, 18]. The increase in caspase-3 activity enhanced with OS was observed in these animals, which also testified in favor of the fact that ROS sensitive to GPX1 play an important role in regulation of apoptosis. The data confirm that GPX1 can effectively interact with both major neuronal death signaling pathways and the mechanisms of post-ischemic inflammation. This allows some authors to consider GPX1 as a promising tool for therapeutic intervention in the processes related to prevention or regulation of post–ischemic brain damage [19].

The GPx overexpression protective effect on the cerebral neurons of rats with focal ischemia was demonstrated. In the experiment involving 62 animals, the MCA occlusion was simulated, and the viral vectors expressing either GPx1/lacZ (experimental group), or lacZ only (control) were introduced in the striata (ischemic foci) by stereotactic injection. It was found that when the vectors were injected 12 h before surgery, the survival rate of neurons was 36% higher compared to that reported for the control group. When the vector was injected 2 h and 5 h after surgery, the survival rate of neurons was 26% and 25% higher, respectively, compared to controls. The fact that the ischemia severity was the same in both groups was confirmed by morphological data. The authors used immunofluorescence staining to demonstrate that GPx overexpression prevented the release of cytochrome from the neuronal mitochondria and limited the nitrogen-mediated damage to these organoids, suppressed Bax and caspase-3 expression, and activated Bcl-2 expression, which suggested GPx involvement in inhibition of the endogenous apoptosis pathway. Endogenous GPx is synthesized in the neurons, and transfer of the gene with the vector enhances GPx production. Astrocytes protect the neurons against OS due to glutathione they contain, and GPx overexpression also contributes to glutathione transformation into an oxidized form after the reaction with ROS. GPx is capable of directly inhibiting some phases of the apoptosis pathway without reduction of the total ROS levels. For example, the increase in Bcl-2 production against the background of GPx overexpression can inhibit the cytochrome c release. The cytosolic cytochrome forms a large part of the apoptosome of vertebrates, which also contains procaspase-9. The caspase-9 activation induces activation of caspase-3 triggering biochemical disruption of the cells. The data suggest that GPx prevents apoptosis at the stage of cytochrome c release, as evidenced by upregulation of Bcl-2 and downregulation of Bax. Injection of the vector carrying the GPx gene 4–6 h after the development of ischemia can prevent the second phase of caspase activation, thereby reducing the neuronal death rate in the ischemic focus. The researchers determine the therapeutic window of 9–11 h for this method of the GPx gene delivery to the disease site [20].

Thus, the increase in GPx activity represents an endogenous protective mechanism ensuring survival of neurons in stroke, and the SA-T0AM administration considerably increases the activity of this enzyme.

Changes in SOD activity associated with acute ischemia

SOD belongs to the major antioxidant enzymes. Minor SOD activation in the experimental hemisphere homogenates after occlusion is typical for ischemic tissues and suggests adaptive rearrangement of the antioxidant protection patterns in response to disturbances of the oxygen delivery to cells. In general, the literature data on the SOD activity in IS are controversial. Thus, a significant decrease in blood levels of SOD was reported in individuals with IS affecting large (not

small!) blood vessels [21], the same results were obtained when assessing SOD in blood serum of 41 patients with acute IS [22]. However, other researchers, in contrast, point to the rapid increase in plasma SOD levels in patients admitted to the unit and explain this phenomenon by the significant increase in the body's levels of free radicals [23]. Thus, today, the data on the SOD activity alterations associated with cerebral ischemia are controversial and require further investigation.

At the molecular level, the increase in the cytosolic cytochrome c levels together with DNA fragmentation was observed in the SOD2 knockout animals. The rats with SOD1 overexpression, in contrast, had low cytosolic cytochrome c levels. The release of cytochrome c results in the ROS production enhancement due to the respiratory chain inhibition. It is believed that ROS also initiate the release of cytochrome c from mitochondria. Thus, a "vicious circle" of cytochrome c release from the neuronal mitochondria in ischemia is formed, eventually resulting in the apoptotic cascade activation [20].

Our data suggest SOD activation in response to the SA-T0AM administration, which confirms the influence of this potential neuroprotector on the antioxidant protection enhancement in the brain tissue in ischemia.

Influence of thyronamines on the antioxidant protection characteristics

The T1AM and T0AM thyronamines are capable of binding to the TAAR1 receptor (Trace Amine-Associated Receptor 1) in the dose-dependent manner, which is accompanied by production of cyclic adenosine monophosphate (cAMP). However, it is currently difficult to credibly claim that TAAR1 is the only endogenous receptor, through which biogenic amines realize their effects. Thus, the increase in cAMP production at the cellular level is inconsistent with the development of hypothermia and the cardiac function decrease. Therefore, either TAAR1 activation is not G-protein-coupled in certain tissues, or thyronamines also can interact with other forms of TAAR [24]. Perhaps, the effects of thyronamines are also mediated by interaction with the receptors other than TAAR. Some authors note intracellular T1AM accumulation, suggesting the existence of intracellular targets for this TH derivative [25]. It is interesting that T0AM sometimes affects O_2 consumption to the greater extent, than T1AM, despite the fact that this thyronamine is less effective for in vivo hypothermia induction [9].

Thyronamines are natural decarboxylated TH derivatives. The *in vivo* administration of thyronamines often causes the effects opposite to that caused by TH, including the decrease in body temperature. Since it is well-known that the mitochondrial energy transduction apparatus is a potential target for TH and their derivatives, an *in vitro* study of the impact of T0AM and T1AM on the O_2 consumption rate and H_2O_2 release by the rat mitochondria was conducted in 2012. The study involved animals with hypothyroidism due to their low levels of endogenous thyronamines. The authors have found that incubation of mitochondrial preparations with thyronamines causes the decrease in the respiratory chain complex III activity, and endogenous T1AM can significantly reduce the O_2 consumption, thereby probably slowing down the rate at which electrons move through the respiratory chain, and enhance production of ROS by the liver mitochondria in rats with hypothyroidism. Furthermore, T1AM is oxidized by monoamine oxidases of the mitochondrial outer membrane due to O_2 that is subsequently reduced to H_2O_2 [26, 27].

The impact of thyronamine on the GPx enzyme activity in the brain is poorly understood and requires further investigation. Activation of the free-radical oxidation processes, the increase in the levels of ROS, as indirectly evidenced by the increase in the TBA-AP levels and SOD activity in our experiment, trigger the redox signaling processes. It is believed that the Nrf2-Keap1-ARE system is the main system responsible for activation of adaptive mechanisms in the cells under the conditions of OS. The Nrf2 nuclear factor is a transcription factor regulating a number of the antioxidant protection genes that act synergistically, ensuring binding of ROS via a cascade of enzymatic reactions. The Nrf2 target genes are involved in neutralization of free radicals, detoxification of xenobiotics, and maintaining the redox potential. Nrf2 is usually found in the cytoplasm and is bound to the Keap1 protein. The OS changes the position of the sulfhydryl groups in the Nrf2-Keap1 complex, causing dissociation and the Nrf2 transfer to the cell nucleus, where Nrf2 binds to the antioxidant response element (ARE) located in the promoter regions of a number of genes encoding the enzymes involved in the glutathione synthesis and metabolism (glutamate-cysteine ligase, glutathione S-transferase, GPx, glutathione reductase) and other enzymes involved in antioxidant protection (SOD, catalase) [28]. This is how activation of transcription of these genes is triggered; this mechanism can explain, why the TBA-AP levels are higher in the intact hemisphere, to the less extent affected by OS, than in the ischemic hemisphere. It has been shown in the animal model that Nrf2 activation can save the penumbra tissue, but not the tissue in the stroke core, while preventive treatment improves the functional outcome within a month. The animals having deletions in the Nrf2 gene become sensitive to the stress factors and susceptible to cerebral ischemia and other neurological disorders [29]. The increase in GPx activity in response to the increase in TBA-AP levels in the ischemic hemisphere homogenates before and especially after administration of SA-T0AM can be also caused by activation of the Nrf2-Keap1-ARE redox signaling [30].

In other words, the impact of TH and their derivatives (specifically thyronamines) on the redox status of the body and certain body tissues has been confirmed by a number of authors, however, the nature of this impact is extremely controversial and requires further investigation.

CONCLUSIONS

According to our findings, the TBA-AP levels in the white rat's ischemic hemisphere decrease in response to administration of the T0AM synthetic analogue (75 mg/kg of the rat's body weight, intraperitoneally). At the same time, the increase in SOD activity in the ischemic hemisphere along with the significant increase in GPx activity in the tissues of both brain hemispheres is observed in rats of the experimental group. This suggests that SA-T0AM used as a neuroprotector has a great potential in terms of activation of the antioxidant protection mechanisms in the cerebral cortex of white laboratory rats with acute hemispheric ischemia.

In the future we plan to continue the search for the most promising thyronamine synthetic analogues (water-soluble forms with the more prominent hypothermic effect that are more convenient to use in clinical settings), determine their biological properties in the model of focal cerebral ischemia, and identify the signaling pathways, through which their neuroprotective effects are realized.

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