Effect of RSPU-189 Compound and Sulodexide on Placental Mitochondrial Respiration in Female Rats with Experimental Preeclampsia

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Abstract

We explored the effect of RSPU-189 compound, a derivative of GABA, on placental mitochondrial respiration in female rats with Experimental Preeclampsia (EP), induced by the replacement of drinking water with 1.8% NaCl solution from the first to the last day of gestation. We found that the respiratory control ratio (V3/V4 as proposed by Chance) in mitochondria isolated from the placenta of the control group of animals with EP significantly decreased as compared to the indices of the females with an uncomplicated pregnancy. This mainly resulted from a change in the rate of endogenous respiration, which was 3 times higher for the substrates of malate oxidation and 1.5 times higher for succinate. We also discovered that both RSPU-189 compound and the comparator drug sulodexide which the females with EP received orally once a day throughout gestation at doses of 15mg/kg and 30 dosage units, respectively, inhibit the development of mitochondrial dysfunction in placenta. This was displayed as diminished non-stimulated respiration and increased respiratory control ratio as compared to the indices of the control group females with EP.

Keywords: Experimental Preeclampsia; Placental Mitochondrial Dysfunction; Respiratory Control; Rate of Oxygen Consumption; GABA-Derivatives; Sulodexide

Introduction

Preeclampsia (PE) is a severe complication of pregnancy, the main cause of preterm delivery and fetal development disorders [1,2,3]. It is associated with hypertension, decreased uterine placental circulation, proteinuria, and edemas. Many authors claim that oxidative stress and mitochondrial dysfunction account for the development of PE [4,5,6,7,8,9]. Mitochondrial dysfunction may be caused by the damage of the enzymes of Electron Transport Chain (ETC) and enzymes of tricarboxylic acid cycle or mitochondrial DNA by Reactive Oxygen Species (ROS) [10]. Placental mitochondria can act as a source of ROS, which cause Lipid Peroxidation (LPO) or spontaneous protein oxidation. Since fetal development requires greater energy costs to transport nutrients and synthesize proteins, nucleic

acids, etc., respiration rate in mitochondria significantly increases as gestation progresses [11,12]. A higher rate of Oxygen Consumption (OCR) can result in electron leakage from the respiratory chain, which promotes formation of ROS and peroxynitrites as well as development of oxidative stress [13,14]. Affected by ROS, ATP antiporter is converted into a Permeability Transition Pore (PTP), a non-specific channel permeable to any low-molecular-weight substances, which results in impaired mitochondrial osmotic equilibrium, damage to their membranes and escape of pro-apoptotic proteins into the cytosol, which triggers a set of reactions causing placental cell apoptosis [12,15]. On the other hand, impaired mitochondrial performance can result from endothelial dysfunction of placental vessels. External factors such as tumor necrosis factor-alpha (TNF-alpha), interleukin-6 (inhibitors of the ETC complexes I and III) forming due to endothelial damage [16] and placental hypoxia can induce enhanced ROS production [12]. In any case, mitochondria are both produced and targeted by free radicals [12,13].

Therefore, a search for agents restraining mitochondrial dysfunction, which may be used to prevent and treat PE in pregnant women, is very promising. In this context, GABA derivatives are of particular interest as they have pregnancy- and endothelium-protective, and antihypoxic properties, as well as the ability to inhibit LPO processes, enhance the activity of antioxidant enzymes, and affect the functional condition of mitochondria of cardiomyocytes when they are damaged [17,18,19].

The purpose of this study was to investigate the effect of a GABA derivative, the compound, whose laboratory code is RSPU-189, on mitochondrial respiration in rats with Experimental Preeclampsia (EP).

Materials and Methods

Animals

The experiments were performed on pregnant white female outbred rats weighing 200-220g. The animals were

kept and cared for according to the National Standard of the Russian Federation GOST P-53434-2009, the international recommendations of the European Convention for the Protection of Vertebrate Animals Used for Experimental and other scientific purposes, 1986, the Order of the Ministry of Public Health of the Russian Federation No 750, the WHO recommendations on the experimental work involving animals. We also performed this study according to the Ethics Review (protocol № 176-2013 of May 8, 2013).

For mating, two female rats and one male rat were placed in a separate cage for 12 hours. The pregnancy was determined by means of a vaginal smear which showed the presence of sperm cells in female rats. Pregnant rats were housed in individual cages.

Experimental preeclampsia was modeled by replacing drinking water consumed by pregnant female rats with 1.8% NaCl solution throughout gestation [20].

Four groups were formed. Group 1 - positive control – intact animals (baseline) – pregnant females without EP (n=7); group 2 - negative control – females with EP (n=9), each receiving 0.3ml of saline solution; group 3 - experimental group (n=7) – females with EP receiving RSPU-189 compound at a dose of 15 mg/kg (0.3ml per animal); group 4 – experimental group (n=7) – females with EP to whom sulodexide, a comparator drug, was administered at a dose of 30 dosage units (0.3ml per animal). Saline solution, RSPU-189 compound and sulodexide were administered daily orally from the 1st to the 21st day of gestation. Both experimental groups receiving the agents under study used the same regimen which was applied in the negative control group.

The dosage of sulodexide and RSPU-189 compound was adjusted on the basis of literature findings and our earlier studies [18,19].

Measuring Arterial Pressure (AP)

Arterial pressure was measured on the 1st and 21st days of gestation on the tails of awake pregnant rats using the device for non-invasive measuring (Kent Scientific Corporation, Canada).

Measuring the Level of Protein in Urine

24-hour urine sample was collected in metabolic chambers (Nalgene, Italy). Protein detection employed the Pyragoll Red method (Olvex Diagnosticum, Russia) and spectrophotometry (PE-5400B spectrophotometer, Ekros, Russia).

Reagents

Sodium chloride (Reachim, Russia), N-Tris-Aminomethane (TRIS), (Sigma, USA) Saccharose (Sigma, USA) Mannitol(Sigma,USA), Ethylene diamine tetraacetate (EDTA) (Fluka Analytical, Czech Republic). Monobasic potassium phosphate (Sigma, USA), Potassium chloride (Sigma, USA), L-Glutamic acid potassium salt monohydrate (AppliChem GmbH, Germany), Potassium malate (Sigma, USA), Potassium succinate (Sigma, USA), Adenosine diphosphate (ADP) (Sigma, USA),

RSPU-189 compound (synthesized by the department of organic chemistry of Herzen State Pedagogical University, St Petersburg, Russia) and Vessel Due F (sulodexide) (Alfa Wassermann , Italy).

Isolation of Placental Mitochondria

Placenta was taken from narcotized animals (chloral hydrate, 400 mg/kg) on the 21st day. The removed placentas were weighed at a cool temperature and homogenized in a Potter Homogenizer (glass/teflon) in the medium for isolating mitochondria (300 mM saccharose, 220mM mannitol, 10mM EDTA, 100mM tris, pH – 7.4) in the ratio of 1:5. The homogenate was centrifuged at 600g for 15 minutes to sediment cell nuclei and debris. Sediment No 1 was removed, and the supernatant was centrifuged again at 5000g for 20 minutes. Supernatant 2 was removed, while sediment No 2 was resuspended and used as a mitochondrial fraction [1,21].

Mitochondrial Respiration Measurements

Mitochondrial respiration was measured using a Clark electrode which was connected to an Expert-01 Ekonix device (Econika, Russia) in a 1ml chamber; mitochondria were continuously stirred with a magnetic stir bar. Mitochondrial suspension containing 0.5 - 1 mg protein was added to the polarographic medium (300mM saccharose, 10mM KCl, 5 mM KH_2PO_4 , 1mM EDTA, 1.2 mM MgCl_2 , 5 tris-HCl pH - 7.4, T 33C). Oxygen consumption was registered either in the absence (condition 4 proposed by Chance) or in the presence of 0.02mM ADP (condition 3) and 1mM potassium succinate, or 0.5mM potassium malate/0.5mM potassium glutamate as substrates [22] and was expressed as nM O_2 /mg of protein/min. Respiratory control was calculated as the ratio of the rate of oxygen consumption in condition 3 to condition 4. Protein concentration was assessed using a commercial Pierce™ BCA Protein Assay Kit (Thermo Scientific, USA).

Statistical Analysis

The data were statistically processed using the methods of variation statistics. The rates of oxygen consumption were calculated using linear regression, then, the mean value and standard deviation were computed (Excel 2007). ANOVA test was used to assess the data validity.

Results

Arterial Pressure, Proteinuria, Weight of the pups

To assess the development of PE in the animals of all the groups under study we measured AP and the level of protein in the 24-hour urine sample.

AP did not actually change in the control females without PE on the 21st day of gestation whereas this indicator increased in the animals which received 1.8% sodium chloride solution (Table 1). There was an insignificant increase in AP in the females with EP which received RSPU-189 compound and sulodexide throughout gestation and it was significantly lower than the indicator in the animals of the control group with PE (Table 1).

The level of protein in the 24-hour urine sample in the females with an uncomplicated pregnancy increased by 20.5% by the 21st

day of gestation whereas it increased by 117.6% in the females with EP. There was a 28.3% and 58.9% rise in this indicator in the females with EP receiving RSPU-189 and sulodexide, respectively. These findings prove the development of EP in the animals of the control group and the protective action of the agents under study (Figure 1).

The newborns were weighed. The weight of the pups of the females without EP was 5.05 ± 0.51 g ($M \pm p$), while the weight of the pups of the females with EP was -4.17 ± 0.47 g which was 17.4% less ($p < 0.05$). The weight of the pups of the rats with EP receiving RSPU-189 compound or sulodexide throughout pregnancy was 4.87 ± 0.54 and 4.39 ± 0.64 ($p < 0.05$), respectively, which was 16.7% and 5.3% higher than in the pups of the control rats with EP.

Mitochondrial Respiration in Experimental Preeclampsia

The study of respiration of the mitochondria isolated from the placentas demonstrated that the overall rate of oxygen consumption in the animals with EP significantly increases as compared to that of the intact group. This increase gets even more pronounced when malate and glutamate are used as oxidation substrates (Figure 2).

The statistical analysis of the areas of polarogram graphs corresponding both to the respiration after exogenic substrates I and II of the respiratory chain complexes were added (V_2), and the respiration after ATP was depleted (V_4) made it possible to reveal that these indicators significantly increased in the mitochondria isolated from the placentas of the animals with EP as compared to those in the intact animal group (Figure 2). When malate/glutamate was used as an oxidation substrate, non-stimulated mitochondrial respiration was 3 times higher ($p < 0.05$), when succinate was used, it was 1.5 times higher ($p < 0.05$). On the other hand, OCR was not statistically different from the values obtained in the intact animal group after ATP was added (condition V_3) (Figure 2).

To assess mitochondrial dysfunction we calculated the respiratory control ratio (RCR) (the ratio of V_3 / V_4). In the group of animals with EP the RCR value was significantly lower when malate/glutamate and succinate were used (Figure 5).

Therefore, the preeclampsia modeled by replacing drinking water with 1.8% NaCl solution throughout gestation results in mitochondrial respiratory dysfunction.

Effect of RSPU-189 and Sulodexide on Mitochondrial Respiration in Experimental Preeclampsia

In females with EP receiving RSPU-189 compound and sulodexide, the oxygen consumption rate decreased, mainly due to the rates V_{basal} , V_2 , V_4 , which were not stimulated by ATP addition (Figure 3).

Thus, for the substrates of Complex I of the respiratory chain – malate/glutamate, the oxygen consumption rate, measured when ATP was not added, decreased ($p < 0.05$) (Figure 4), whereas RCR increased ($p < 0.05$) (Figure 5) as compared to the indicators of

Animal groups	Arterial pressure (mmHg) ($M \pm p$)		
	Before pregnancy	On the 21 st day of pregnancy	% of increase
Animals without PE (n=7)	118.9 $\pm$ 2.7	123.1 $\pm$ 6.4	3.5
EP +saline solution (n=9)	118.7 $\pm$ 8.7	148.0 $\pm$ 14.5*	24.3
EP +RSPU-189 (n=7)	117.1 $\pm$ 5.4	124.3 $\pm$ 5.8**	6.1
EP+sulodexide (n=7)	118.7 $\pm$ 17.1	129.0 $\pm$ 18.2**	8.6

*p < 0.05 – in relation to the indicator of the animals without EP; ** p < 0.05 in relation to the indicator of the animals of the control group with EP + saline solution

Animals without EP – pregnant females; EP + saline solution – females with EP receiving saline solution orally once a day in the amount of 0.3ml per animal throughout gestation; EP + RSPU-189 – females with EP receiving RSPU-189 at a dose of 15mg/kg in the amount of 0.3ml per animal throughout gestation; EP + sulodexide – females with EP receiving sulodexide at a dose of 30 dosage units in the amount of 0.3ml per animal throughout gestation.

Figure 1: Changes in the urine protein level of the pregnant females
*p < 0,05 – in relation to the indicator of the animals without EP; ** p < 0,05 in relation to the indicator of the animals of the control group with EP + saline solution Animals without EP – pregnant females; EP + saline solution – females with EP receiving saline solution orally once a day in the amount of 0.3ml per animal throughout gestation; EP + RSPU-189 – females with EP receiving RSPU-189 at a dose of 15mg/kg in the amount of 0.3ml per animal throughout gestation; EP + sulodexide – females with EP receiving sulodexide at a dose of 30 dosage units in the amount of 0.3ml per animal throughout gestation.

the control animal group with EP.

When succinate, a substrate of Complex II of the respiratory chain, was used, similar trends of changes in the mitochondrial functional condition were observed – decreased V_2 and V_4 , and increased RCR. In animals with EP which received RSPU-189 during gestation, non-ATP-stimulated respiration was 1.5 times lower, whereas when sulodexide was administered, it was 2.5 times lower ($p < 0.05$) as compared to the indicator in the control group of animals with EP.

Figure 2: Oxygen consumption rates (OCR) in placental mitochondria in rats with experimental preeclampsia

Baseline – mitochondria, isolated from the placentas of the pregnant females without EP; EP – mitochondria, isolated from the placentas of pregnant females with EP.

OCR was measured under basal conditions (T 33°C, 1 ml chamber, stirring) followed by the sequential addition of mitochondrial suspension (1 mg protein/ml) and twice ADP (20μM), using 0.5 mM malate and 0.5mM glutamate as sources of reducing equivalents for Complex I (A) or succinate as a Complex II substrate (B).

We started to register an oxygen decrease after a mitochondrial preparation (1mg protein/ml) was added into a 1ml chamber; it was continuously stirred at a temperature of 33°C, then ATP was added twice (20μM), the polarographic medium contained 0.5mM potassium malate/0.5mM potassium glutamate as a source of reducing equivalents for Complex I of the respiratory chain (A, C) or succinate as a Complex II substrate (B, D).

Mean values ± SE computed when the placentas of 4 allelically determined animals were investigated are presented. The arrows show the points when ATP was added.

C and D are individual parameters of V₂ (absence of ATP), V₃ (after ATP was added), and V₄ (after ATP depletion) which were calculated using the polarograms similar to A and B and expressed as nM O₂/min/mg protein. The graph provides the mean values M ± σ, n= 10 (placentas of allelically determined animals) # - p< 0,05 as compared to the females without EP

Figure 3: Effects of sulodexide and RSPU-189 on placental mitochondrial respiration in rats with experimental preeclampsia

EP – mitochondria isolated from the placentas of pregnant females with EP, EP+sul – mitochondria isolated from the placentas of pregnant females with EP receiving sulodexide, EP+RSPU-189 – mitochondria isolated from the placentas of pregnant females with EP receiving RSPU-189.

OCR was measured as described above, A – malate/glutamate, B – succinate.

Mean values computed when the placentas of 3 allelically determined animals were investigated are presented. The arrows show the points when mitochondrial suspension and ATP were added.

Figure 4: Effects of sulodexide and RSPU-189 on placental mitochondrial basal OCR in rats with experimental preeclampsia

The graph shows the median value of basal OCR indices alongside with the interquartile range and the limits of the range including 90% of values expressed as $\text{nM O}_2/\text{mg protein}/\text{min}$ for the condition V_4 (proposed by Chance), $n=10$ (placentas of allelically determined animals). The measurements were made when either malate/glutamate (A) or succinate (B) was used.

* - $p < 0.05$ as compared to the female group without EP

- $p < 0.05$ as compared to the female group with EP

Figure 5: Effects of sulodexide and RSPU-189 on placental mitochondrial respiratory control ratio (RCR) in rats with experimental preeclampsia

The graph shows the mean values $M \pm \sigma$, calculated as the ratio of the rates of oxygen consumption V_3 / V_4 (proposed by Chance), $n=10$ (placentas of allelically determined animals). The measurements were made when either malate/glutamate (A) or succinate (B) was used as a substrate for respiratory chain. The respiratory control ratio was computed as the ratio of condition 3 to condition 4 values using the oxygen consumption curve. $N=10$.

* - $p < 0.05$ as compared to the female group without EP

- $p < 0.05$ as compared to the female group with EP

Discussion

The replacement of drinking water with 1.8% NaCl solution in the females throughout gestation induces EP which is displayed by increased AP and proteinuria. The paper of Beausejour A, et al. [20] together with our earlier studies involving this model of EP demonstrated increased oxidative stress in the placenta, higher production of proinflammatory cytokines, Tumor Necrosis Factor- α (TNF- α), prostanooids, thromboxane, prostacyclin PGF 1 alpha, and decreased activity of antioxidant enzymes.

Numerous findings prove that the development of oxidative stress brings about the damage of mitochondrial proteins. As a result, placental mitochondria produce ROS and become

their target. The more severely a mitochondrion is damaged, the higher the production of ROS and the more likely are the pregnancy complications such as Intrauterine Growth Restriction (IUGR) and preeclampsia [11].

As the placenta plays a crucial role in the selective transport of ions, nutrients and immunoglobulins between the maternal bloodstream and that of the fetus and the majority of these processes are ATP-dependant, we can suggest that the proper functioning of the placenta directly relies on energy supply of cells and, therefore, on oxidative phosphorylation in mitochondria.

Under normal conditions, the rate of oxygen consumption in mitochondria is closely associated with the production and

utilization of ATP. As oxidative stress develops, all complexes of respiratory chain (I, II, III) start using consumed oxygen as an electron acceptor and produce superoxide anion and hydrogen peroxide regardless of ATP production. It brings about uncoupling and hypoenergetic conditions [16].

An increase in non-stimulated respiration in the group with EP discovered by us conforms with the findings of the study of mitochondria of parturient women with preeclampsia [11]. Increased OCR is a sign of electron escape from the respiratory chain and uncoupling of respiration and phosphorylation which is proved by low values of respiratory control. Placental hypoxia which develops as a result of vasoconstriction and the damaging effect of cytokines and TNF- α on respiratory chain are the most likely causes of mitochondrial dysfunction in this case [23]. Electron escape yields an increase in ROS production which causes further damage to the placenta, thus, a vicious cycle occurs [14,24].

Under normal conditions, formation of superoxide anion in mitochondria is an important signaling pathway which triggers the expression of transcription factors such as Ets-1, STAT-3, regulating angiogenesis and trophoblast invasion [10]. However, excess production of ROS damages DNA and proteins. As a result, the amount of STAT-3 reduces in preeclampsia which leads to decreased invasion and angiogenesis [25] as well as to impaired adaptation to oxidative stress [26].

RSPU-189 significantly improved the functional parameters of mitochondria as compared to the negative control group. It is probably due to its endothelium- protective and antihypoxic action, and the ability to inhibit LPO processes and activate antioxidant enzymes [18,27]. This compound had a more pronounced effect on respiratory Complex II. As a GABA derivative, it is likely to promote the stable functioning of succinate dehydrogenase which yields an increase in the rate of oxygen consumption by mitochondria when stimulated by ATP and coupling of respiration and oxidative phosphorylation in animals with EP receiving RSPU-189 during gestation.

Sulodexide had a similar effect on mitochondrial respiration. A decrease in non-stimulated respiration in mitochondria of the animals with EP receiving the agent indicates less likelihood of electron escape from the respiratory chain which is supposed to mitigate free radical processes in mitochondria. However, stimulated respiration in this group was also diminished, respiratory control did not restore to the values in healthy animals which indicates the presence of damage in the respiratory chain.

Sulodexide has an antioxidant and endothelium-protective action. Enhanced Superoxide Dismutase (SOD) and Glutathione Peroxidase (GP) gene expression and inhibition of oxidative stress in endothelial cells of the human umbilical vein in ischemia induced by oxygen and glucose deprivation were demonstrated to be its effects [28]. Moreover, sulodexide protects cells from apoptosis induced by ischemia, promotes their viability and prevents mitochondrial depolarization [29]. Its antioxidant action is proved by its ability to enhance SOD, catalase and GP

activity in the renal tissues of rats with experimental diabetes mellitus [30], reduce oxidative stress in intercellular matrix and protect cells from damage by free radicals [31]. Endothelium-protective action of sulodexide also involves the inhibition of endothelial glycocalyx destruction which prevents cell ageing. Clinical trials provide evidence that glycosaminoglycans play an essential role in restoring the proper functioning of endothelium [32]. Some findings show that sulodexide decreases the number of inflammatory cytokines, chemokines and colony-stimulating factors improving endothelial function [33].

Conclusion

Therefore, EP induced by the replacement of drinking water with 1.8% NaCl solution in rats during gestation is associated with mitochondrial respiratory dysfunction. RSPU-189 compound and sulodexide, its comparator drug, restrict the uncoupling of respiration and oxidative phosphorylation and enhance the functional activity of mitochondria in animals with EP.

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