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Effect of Xenon on the Phosphorylation of Glycogen Synthase Kinase 3β and Antioxidant Enzymes in Rat Brain

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RELEVANCE The increase in the number of severe brain injuries due to stroke and traumatic brain injury determines the need to study and develop effective strategies for neuroprotection. The article highlights new mechanisms of the neuroprotective action of the inhalation anesthetic xenon based on the data of our own experimental studies.

AIM OF STUDY To assess the effect of anesthesia with xenon at a concentration of 0.5 MAC (minimum alveolar concentration) on the phosphorylation of glycogen synthase kinase 3β (GSK-3β) and the content of antioxidant defense enzymes in the rat brain.

MATERIAL AND METHODS The effect of inhalation anesthesia with xenon on the phosphorylation of the GSK-3β enzyme in comparison with lithium chloride, as well as on the content of heme oxygenase, catalase, and Mn-superoxide dismutase in rat brain homogenates was studied by immunoblotting.

RESULTS The use of xenon at a concentration of 0.5 MAA causes an almost twofold increase in the content of the phosphorylated form of the GSK-3β enzyme in comparison with the control ($p<0.05$) and significantly increases the pool of antioxidant defense enzymes: heme oxygenase by 50% ($p <0.05$) and Mn-superoxide dismutase by 60% ($p<0.05$).

CONCLUSION The conducted experimental study revealed new molecular mechanisms of action of the inhalation anesthetic xenon. The effect of xenon on the pool of enzymes involved in the protection of the brain from oxidative distress was found. The data obtained indicate the prospects for using xenon and require further research in this direction.

The use of xenon at a concentration of 50 vol.% (0.5 MAA) for 30 minutes does not affect the content of the glycogen synthase-3β enzyme, at the same time causing an almost twofold increase in its phosphorylated form, the glycogen synthase-3β enzyme, and is accompanied by a significant increase the content of heme oxygenase, Mn-superoxide dismutase and a slight increase in the content of catalase in rat brain homogenates. Thus, the results of the study suggest that one of the possible mechanisms of the neuroprotective effect of xenon is the phosphorylation of glycogen synthase-3β, which prevents the opening of the mitochondrial pore, inhibiting the death of mitochondria-mediated apoptosis of neurons and increasing the level of antioxidant protection in them.

Keywords: xenon, neuroprotection, glycogen synthase kinase-3β (GSK-3β), heme oxygenase, Mn-superoxide dismutase

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i / p - intraperitoneally
GSK-3β - glycogen synthase kinase-3β
ABB - acid-base balance
MAC - minimum alveolar concentration
r. u. - relative units

INTRODUCTION

Cardiovascular diseases are the leading cause of death worldwide, and ischemic stroke is second only to coronary artery disease. In the Russian Federation alone, more than 450 thousand people experience it annually [1]. Qualified medical care properly provided helps reduce the number of deaths, but disability among stroke survivors ranks first among the causes of primary disability [2, 3].

Another equally significant medical and social problem is the increase in the number of craniocerebral injuries, which are considered the most common cause of disability and mortality among young people of working age [4–6]. In the Russian Federation, about 600 thousand cases of traumatic brain injury are annually registered, of which more than 50 thousand people die, and about 240 thousand are disabled [7]. Thus, the development of new methods of neuroprotection in severe brain damage of various origins is one of the most important tasks in the treatment of critical conditions. However, the methods of neuroprotection existing today are not effective enough, which is confirmed by clinical trials [8, 9].

According to a large number of experimental studies, the inhalation anesthetic xenon has a pronounced neuroprotective effect [10-14]. Xenon is an inert gas that is found in the atmosphere in insignificant amounts - $8.7 \cdot 10^{-6}$ vol.% [15].

The anesthetic properties of xenon were reported after its use in animal experiments in 1946 [16], and in 1951 Cullen and Gross used it as an anesthetic in human surgeries [17]. In 2000, thanks to the work of domestic researchers, permission was obtained for the clinical use of xenon in Russia, while in Western Europe it began to be used for general anesthesia only in 2005 [18].

In addition to the anesthetic effect, xenon was found to have neuroprotective properties [19–21], and the mechanisms of neuroprotection were explained by blockade of N-methyl-d-aspartate (NMDA-receptors) receptors [22]. NMDA receptors provide important functions such as synaptic plasticity, memory formation, mood mediation, reward motivation, brain development, and neuronal survival [23–25]. Hyperactivity of NMDA receptors under pathological conditions can lead to neuronal death as a result of excitotoxicity [26]. High doses of glutamate cause hyperactivation of NMDA receptors and β-amino-3-hydroxy-5-methyl-isoxazole propionic acid receptors (AMPA receptors), which leads to excess calcium influx into neurons [27, 28].

The mechanisms by which excessive calcium influx leads to apoptosis are complex and not well understood. Recent studies have shown the importance of mitochondrial dysfunction, excess production of reactive oxygen species, and activation of the calpain cascade [29]. As for the mechanisms of realization of the neuroprotective properties of xenon, they cannot be explained only by the blockade of NMDA receptors and a decrease in glutamate excitotoxicity. According to recent studies, the implementation of the pathological cascade of glutamate excitotoxicity occurs in the first minutes and hours after brain damage. Apoptosis of neurons and glia in the penumbra zone occurs in the first 24–72 hours after injury, and therefore it seems so important to study the molecular mechanisms of neuroprotection by xenon during this period [30].

Experimental data have shown that glycogen synthase kinase 3β (GSK-3β) can claim the role of a key enzyme providing neuroprotection. Phosphorylation of this enzyme prevents the induction of the mitochondrial pore, as a result, apoptosis of neurons decreases and inflammation in the damaged area is limited [31, 32].

The inert gas xenon belongs to the class of inhaled gaseous anesthetics, which molecular mechanisms of the protective properties remain largely unknown.

The aim of the study was to evaluate the effect of xenon anesthesia on the phosphorylation of GSK-3β and the content of antioxidant defense enzymes in the rat brain.

MATERIAL AND METHODS

THE FIRST STAGE OF EXPERIMENTAL STUDIES (STUDY OF THE INFLUENCE OF INHALATION ANESTHESIA BY XENONE ON PHOSPHORYLATION OF GSK-3ß IN COMPARISON WITH THE EFFECT OF LITHIUM CHLORIDE)

1. Laboratory animal and experimental conditions.

Male Sprague Dawley rat. Animal weight: 450-500 g.

The experiment was carried out in accordance with the accepted national and international standards:

- European Convention for the Protection of Vertebrate Animals used for Experimental or Other Scientific Purposes (Strasbourg, March 18, 1986);

- Directive 2010/63/EU of the European Parliament and of the Council of the European Union on the protection of animals used for scientific purposes;

- GOST 33215-2014 Guidelines for the maintenance and care of laboratory animals. Rules for equipping premises and organizing procedures;

- GOST 33216-2014 Guidelines for the maintenance and care of laboratory animals. Rules for the maintenance and care of laboratory rodents and rabbits.

2. Anesthesia.

6% solution of chloral hydrate in 0.9% sodium chloride solution. Chloral hydrate 300 mg / kg intraperitoneally (i/p) at the end of the experiment as a component of euthanasia before the collection of internal organs.

Animal preparation and procedures.

The animal was placed in a transparent induction chamber of the Combi-Vet anesthesia machine, connected with an adapter to a KNP-01 xenon anesthesia device and an oxygen compressor. During the experiment, spontaneous breathing was maintained.

3. Groups of animals.

Group I (n = 4) - "xenon". For 30 minutes, a gas mixture with a gas flow was fed into the induction chamber of the anesthesia machine, where the rat was located: 95% oxygen - 0.5 L/min, 100% xenon - 0.5 L/min. The concentration of gases in the mixture at the inlet to the chamber: oxygen - 40–45%, xenon - 50–55%. The used gas mixture was passively removed through the xenon adsorber to the outside.

Group II (n = 4) - "lithium". Thirty minutes before placing the animal into the chamber, a 4.2% solution of lithium chloride was injected into the anesthesia chamber in a dose of 50 mg/kg i/p. Then, for 30 minutes, an oxygen-air mixture (50% oxygen) - 1.0 L/ min was fed into the induction chamber of the anesthesia machine with the rat. The spent gas mixture was passively removed from the chamber to the outside.

Group III (n = 4) - "control". For 30 minutes, a gas mixture with a gas flow was fed into the induction chamber of the anesthesia machine, where the rat was located: oxygen-air mixture (50% oxygen) - 1.0 L/ min. The used gas mixture was passively removed to the outside.

4. Euthanasia and organ retrieval.

Five minutes after the injection of chloral hydrate and reaching the surgical stage of anesthesia, the animal underwent thoracotomy. A blood sample was taken from the left ventricle for the analysis of the acid-base balance of the blood (ABB) (in some animals). Then, craniotomy was performed and the brain was removed from the cranium. The entire organs were wrapped in foil, labeled, and frozen in liquid nitrogen. Storage and transportation of samples to the laboratory was carried out in a thermos with liquid nitrogen.

THE SECOND STAGE OF EXPERIMENTAL STUDIES (STUDY OF THE INFLUENCE OF INHALATION ANESTHESIA BY XENON ON ENZYMES OF THE ANTIOXIDANT SYSTEM OF THE CELL)

1. Groups of animals.

Group I (n = 4) - "xenon". For 30 minutes, a gas mixture with a gas flow was fed into the induction chamber of the anesthesia machine, where the rat was located: 95% oxygen - 0.5 L/min, 100% xenon - 0.5 L/min. The concentration of gases in the mixture at the inlet to the chamber: oxygen - 40–45%, xenon - 50–55%. The used gas mixture was passively removed through the xenon adsorber to the outside.

Group II (n = 4) - "control". For 30 minutes, a gas mixture with a gas flow was fed into the induction chamber of the anesthesia machine, where the rat was located: oxygen-air mixture (50% oxygen) - 1.0 L/min. The used gas mixture was passively removed to the outside.

2. Euthanasia and organ retrieval.

Five minutes after i/p injection of chloral hydrate and reaching the surgical stage of anesthesia, the animal underwent thoracotomy. A blood sample was taken from the left ventricle for ABB analysis. Then, craniotomy was performed and the brain was removed from the cranium. Excess fluid from the surface of organs was removed with blotting paper. The entire organs were wrapped in foil, labeled, and frozen in liquid nitrogen. Storage and transportation of samples to the laboratory was carried out in a thermos with liquid nitrogen.

THE THIRD STAGE OF EXPERIMENTAL STUDIES (STUDY OF THE GSK-3ß ENZYME, HEMOXYGENASE, Mn-SUPEROXIDE DISMUTASE AND CATALASE BY IMMUNOBLOTTING)

1. Protein concentration measurement.

Protein concentration measurement in tissue homogenates in all experiments was performed according to the method based on the colorimetric reaction of bicinchoninic acid with proteins. The solution of the following composition was used: sodium salt of bicinchoninic acid (Sigma Chemical Co., USA), sodium tartrate (Sigma Chemical Co., USA), 0.95% sodium bicarbonate (reagent A) and a 4% mixture of copper sulfate with water in 1:5 ratio (reagent B), which was prepared immediately before measuring the protein concentration by mixing the starting reagents A and B in a 50:1 ratio. To an aliquot of 50 µl of the analyzed sample 1 ml of a solution was added, mixed and incubated for 30 minutes at 37 °C, after which the optical density of the solution was determined at 562 nm in an acrylic cuvette using a Hitachi-557 spectrophotometer (Hitachi Ltd., Japan).

The protein concentration in the analyzed sample was measured by the calibration curve using the SigmaPlot 2000 software. A commercial preparation of bovine serum albumin (Fermentas) with a concentration of 2 mg/ml was used as a standard for constructing the calibration curve.

2. Western blotting.

The homogenate was dissolved in a buffer containing 0.125 M Tris-HCl (pH 6.8), 4% sodium dodecyl sulfate (Sigma Chemical Co., USA), 20% glycerol, 0.005% bromophenol blue (Sigma Chemical Co., USA) and 10% 2β–mercaptoethanol (Merck, Germany). Samples were boiled for 2 minutes in a water bath and loaded onto a 15% Tris-glycine polyacrylamide gel at a concentration of 50 mg protein per well. Electrophoresis was performed at a constant current of 10 mA in the concentration mode and 15 mA in the separation mode. At the end of electrophoresis, proteins were transferred to a PVDF membrane (Amersham Pharmacia Biotech, UK). Membranes were blocked in 5% defatted milk in 0.1% phosphate buffer, and then incubated with primary antibodies against GSK3β (mouse monoclonal anti-total-GSK3β 1: 1000, Cell Signaling, USA), phosphorylated form of GSK3β (mouse monoclonal anti-P-GSK3β 1: 1000, Cell Signaling, USA), heme oxygenase, Mn-superoxide dismutase, and catalase. Then the membranes were incubated with secondary antibodies conjugated with horseradish peroxidase (Calbiochem, USA) in a dilution of 1:10000. Detection of bound antibodies was performed using a chemiluminescent substrate of horseradish peroxidase ECL (Enhanced chemiluminescence system, Amersham Pharmacia Biotech, UK). Chemiluminescence was detected on photographic film. The scanned images were analyzed using the ImageJ software (NIH, Bethesda, MD, USA). The signal intensity of the P-GSK3β protein was normalized to the intensity of the total GSK3β protein for each band. The quantitative content of the phosphorylated form of GSK-3β, heme oxygenase, Mn-superoxide dismutase, and catalase was expressed in relative units (r.u.)

The data were statistically processed using the Statistica 7.0 software using the Mann – Whitney U-test. Differences at the $p < 0.05$ level were considered significant.

RESULTS AND DISCUSSION

EFFECT OF INHALATION ANESTHESIA WITH XENONE AND LITHIUM CHLORIDE ON THE CONTENT OF GSK-3ß IN RAT BRAIN HOMOGENATES

Experiments have shown that in all studied series (control, xenon, lithium), the total content of GSK-3β did not change, remaining at the level of 80–90 rel. units of chemiluminescence. Lithium chloride (the most studied inhibitor of the GSK-3β enzyme) in a dose of 50 mg / kg i.p. was used as a positive control.

The research results showed that all changes in the content of the discussed enzyme (GSK-3β) occurred due to the process of phosphorylation/dephosphorylation.

In the "Xenon" group, where xenon was used for inhalation anesthesia in a concentration of 50 vol% (0.5 minimum alveolar concentration, MAC), there was an almost two-fold increase in the content of the phosphorylated form of the GSK-3β enzyme compared to the control group ($p < 0.05$).

In the "Lithium" group, where lithium chloride (LiCl 4.2%) was used as a positive control in a dose of 50 mg / kg i.p., almost two-fold increase in the content of the phosphorylated form of the GSK-3β enzyme was observed in comparison with the control ($p < 0.05$) (Fig. 1).

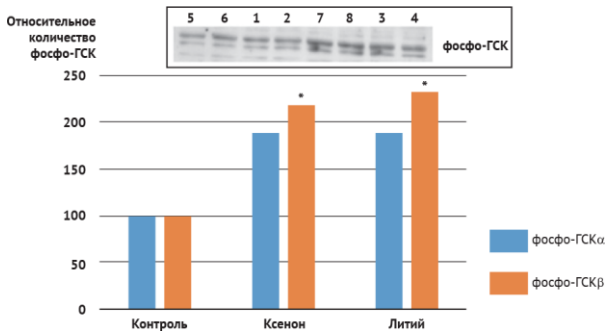

Fig. 1. Immunoblot calculation data for the phosphorylated form of the GSK-3β enzyme in rat brain homogenates exposed to xenon anesthesia for 30 minutes

Notes: group “Control” — intraperitoneally chloral hydrate at a dose of 300 mg/kg body weight; group “Xenon” — xenon in a concentration of about 0.5 minimum alveolar concentrations (MAC); group “Lithium” — lithium chloride (LiCl 4.2%) at a dose of 50 mg/kg intraperitoneally

The results of our study showed that the use of xenon in a concentration of 0.5 MAC does not affect the content of the GSK-3β enzyme in rat brain homogenates, but is accompanied by an almost two-fold statistically significant increase, comparable to the results of exposure to lithium chloride, the content of its phosphorylated (inactivated) form compared with control data ($p < 0.05$).

EFFECT OF INHALATION ANESTHESIA WITH XENONE ON THE CONTENT OF HEMOXYGENASE IN RAT BRAIN HOMOGENATES

The results of the study showed that inhalation anesthesia with xenon for 30 minutes - 50 vol.% (0.5 MAC) was accompanied by a statistically significant 50% increase ($p < 0.05$) in the content of heme oxygenase in rat brain homogenates (Fig. 2).

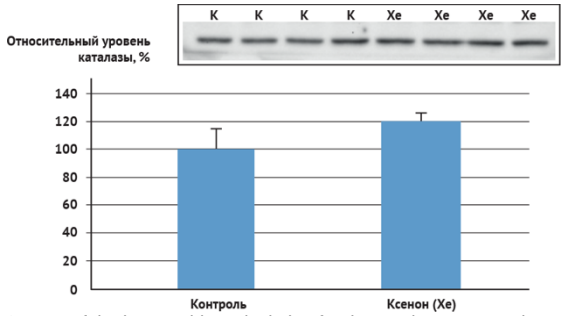

Fig. 3. Data of the immunoblot calculation for the catalase enzyme in rat brain homogenates

Notes: group “Control” — intraperitoneal chloral hydrate at a dose of 300 mg/kg body weight, group “Xenon (Xe)” — xenon at a concentration of about 0.5 minimum alveolar concentrations (MAC)

EFFECT OF INHALATION ANESTHESIA WITH XENONE ON THE CONTENT OF Mn-SUPEROXIDE DISMUTASE IN RAT BRAIN HOMOGENATES

The results of the study showed that inhalation anesthesia with xenon for 30 minutes - 50 vol.% (0.5 MAC) was accompanied by a statistically significant increase by 60% ($p < 0.05$) in the content of Mn-superoxide dismutase in rat brain homogenates (Fig. 4).

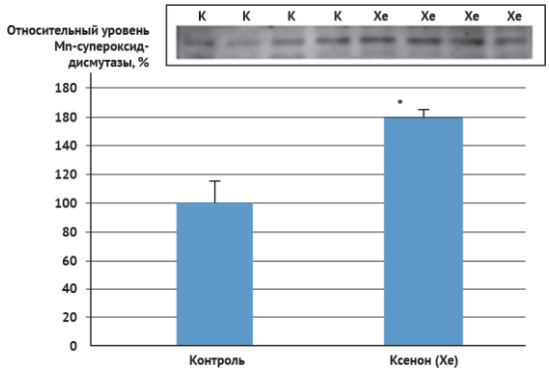

Fig. 4. Immunoblot calculation data for the Mn-superoxide dismutase enzyme in rat brain homogenates

Notes: group C — “Control” — intraperitoneally chloral hydrate at a dose of 300 mg/kg of body weight, group “Xenon” (Xe) — xenon at a concentration of about 0.5 minimum alveolar concentrations (MAC)

Glycogen synthase kinase-3, GSK-3 is an enzyme responsible for the phosphorylation of serine and threonine residues in various proteins. The existence of GSK-3 was first discovered when studying glycogen metabolism [33]. Later it was found that the GSK-3β isoenzyme regulates immune and migration processes and is a key enzyme that protects cells from ischemia / reperfusion. Phosphorylation of this enzyme prevents mitochondrial pore induction and protects post-mitotic cells (neurons, cardiomyocytes, kidney cells) from ischemia / reperfusion [34].

It is known that halogenated inhalation anesthetics have a protective effect on the brain due to the effect of anesthetic preconditioning [35, 36].

In our previous studies, it was shown that preconditioning with sevoflurane in a concentration of 1.5-2 MAC leads to a statistically significant two-fold increase in the content of the phosphorylated form of GSK-3β (phospho-GSK-3β) ($p < 0.05$) and protects neurons in the rat brain (reduces the death of neurons in the C1 field of the hippocampus by 45% ($p = 0.007$) during ischemia / reperfusion [37, 38].

A recent study showed that the use of lithium chloride in the postresuscitation period causes a statistically significant increase in the phosphorylation of GSK-3β in the rat brain by 180% ($p < 0.05$) and reduces the death of neurons in the C1 field of the hippocampus by 37% ($p = 0.01$), and in the C3 / C4 field - by 12% ($p < 0.05$) [39].

Today, it is known that Nrf2 (nuclear factor erythroid 2-related factor 2) is a master regulator of the level of antioxidant defense enzymes (heme oxygenase, superoxide dismutase, glutathione peroxidase, catalase, etc.). In this regard, recent studies on the effect of GSK-3β on Nrf2 and antioxidant defense of cells are also of great interest [40, 41]. The results of these studies showed that phosphorylation of GSK-3β causes a statistically significant increase in the level of the transcription factor Nrf2 by 50% ($p < 0.05$) in neuronal cells and by 75% ($p < 0.05$) in hepatocytes.

The results of our study found that the use of xenon in a concentration of 0.5 MAC causes an almost two-fold statistically significant increase in the content of the phosphorylated form of the GSK-3β enzyme in comparison with the control ($p < 0.05$) and statistically significantly increases the pool of antioxidant defense enzymes: heme oxygenase - by 50% ($p < 0.05$) and Mn-superoxide dismutase - by 60% ($p < 0.05$).

The results of the study suggest that one of the possible mechanisms of the neuroprotective action of xenon is the phosphorylation of GSK-3β, which prevents the opening of the mitochondrial pore, inhibition of mitochondrial death-mediated apoptosis of neurons and an increase in the level of antioxidant protection in them (Fig. 5).

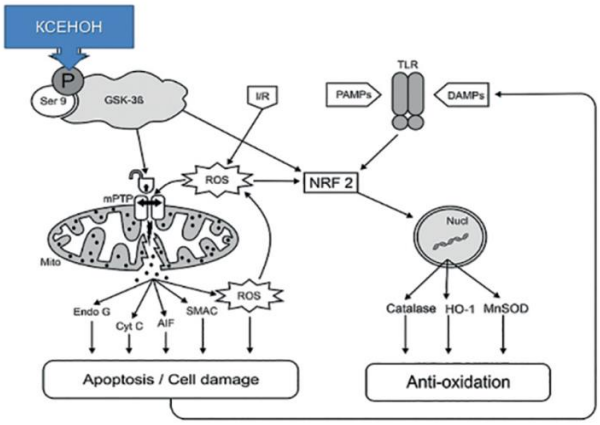

Fig. 5. Xenon phosphorylates (inactivates) GSK-3β — a key enzyme in the implementation of mechanisms of cellular damage and systemic inflammatory response. Phosphorylation of GSC-3β prevents the opening of the mitochondrial pore and the release of apoptosis factors (AIF, Cyt C, Endo G, SMAC) into the cytosol. Phosphorylation of GSK-3β leads to an increase in the level of the nuclear transcription factor Nrf2 in cells, the action of which is accompanied by an increase in the level of antioxidant enzymes of the cell (HO-1, MnSOD, catalase).
Notes: AIF — apoptosis-inducing factor; Cat — catalase; ROS — reactive oxygen species; Cyt C — cytochrome C; DAMP — damage-associated molecular pattern; Endo G — endonuclease G; I/R — ischemia-reperfusion; Mito — mitochondria; MnSOD, mitochondrial Mn superoxide dismutase; mPTP — time of non-specific mitochondrial permeability; HO-1 — heme oxygenase; SMAC — apoptotic protein; PAMP — pathogen-associated molecular pattern; Ser 9 — Serine 9 amino acid residue; TLR — toll-like receptor

CONCLUSION

The conducted experimental study revealed new molecular mechanisms of action of the inhalation anesthetic xenon, which are realized through the phosphorylation of glycogen synthase-3β and an increase in the pool of enzymes involved in the antioxidant protection of the brain. The data obtained in this experiment indicate that xenon is promising and require further research in this direction.

FINDINGS

The use of xenon at a concentration of 50 vol.% (0.5 MAC) for 30 minutes does not affect the content of the glycogen synthase-3β enzyme, at the same time causing an almost two-fold increase in its phosphorylated form, the glycogen synthase-3β enzyme, and is accompanied by a significant increase the content of heme oxygenase, Mn-superoxide dismutase and a slight increase in the content of catalase in rat brain homogenates. Thus, the results of the study suggest that one of the possible mechanisms of the neuroprotective effect of xenon is the phosphorylation of glycogen synthase-3β, which prevents the opening of the mitochondrial pore, inhibiting the death of mitochondria-mediated apoptosis of neurons and increasing the level of antioxidant protection.

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