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The role of adipose tissue cell elements in the regulation of the nitroxidergic system and possible ways of pharmacological modulation

I. F. Bielenichev*[1,A,C,F], S. O. Maslennikov[1,C,D,E], N. V. Dobrelia[2,B], O. S. Khromov[2,B], M. L. Holovakha[1,A], V. P. Ryzhenko[1,B], O. O. Brek[3,B]

[1]Zaporizhzhia State Medical and Pharmaceutical University, Ukraine, [2]State Institution “Institute of Pharmacology and Toxicology of the National Academy of Medical Sciences of Ukraine”, Kyiv, [3]Kharkiv National Medical University, Ukraine

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***E-mail:** i.belenichev1914@gmail.com

Endothelial dysfunction is characterized by a decrease in the bioavailability of the vasodilator – nitric oxide (NO), and an increase in the level of vasoconstrictor substances. This imbalance leads to vasoconstriction, leukocyte attachment and inflammatory reactions in the vascular wall, atherosclerosis and thrombosis.

The aim: to evaluate the role of adipose tissue elements in the regulation of parameters of the nitroxidergic system under hypoxia conditions.

Materials and methods. The studies were carried out on 30 adult white male Wistar rats. All animals were randomly assigned and divided into groups: a control group (15 rats), type 2 diabetes mellitus (T2DM) was induced in the animals of the second group (15 rats). Isolated fragments of the popliteal arteries (PA) and intrapulmonary artery (IPA) were cleared of perivascular adipose tissue (PVAT-) or left uncleaned (PVAT+) and cut into rings. The simulation of acute hypoxia with further study of medical agents were performed.

Results. The PA and IPA with PVAT responded to acute hypoxia with vasoconstriction – an increase in the amplitude of contraction in the first and second phases, and after removing PVAT, they responded with a decrease in the maximum amplitude of contraction by 3.4 times in the 1st phase and an increase in amplitude by 1.8 times in the 2nd phase. Perfusion with Angiolin reduced 2nd phase of HV of the PA and IPA. Adding a combination of Thiotriazoline and L-arginine (1:4) to a solution for perfusion of fragments of arteries of animals with T2DM, causes a significant increase in constrictor reactions in both the 1st and 2nd phases of HV, regardless of presence of perivascular adipose tissue.

Conclusions. The presence of PVAT affects the HV of arteries, both in normal and in T2DM. The possibilities of ways of pharmacological modulation of the nitroxidergic system depending on the state of PVAT were determined.

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Роль клітинних елементів жирової тканини в регуляції нітроксидергічної системи та можливі шляхи фармакологічної модуляції

І. Ф. Бєленічев, С. О. Масленніков, Н. В. Добреля, О. С. Хромов, М. Л. Головаха, В. П. Риженко, О. О. Брек

Ендотеліальна дисфункція характеризується зниженням біодоступності вазодилататора оксиду азоту (NO) і підвищенням рівня вазоконстрикторних речовин. Цей дисбаланс призводить до звуження судин, адгезії лейкоцитів, запальних реакцій у судинній стінці, атеросклерозу та тромбозу.

Мета роботи – оцінити роль елементів жирової тканини в регуляції параметрів нітроксидергічної системи в умовах гіпоксії.

Матеріали і методи. Дослідження здійснили на 30 дорослих білих щурах-самцях лінії Вістар. Усіх тварин рандомно поділили на групи: у першу (контрольну) включили 15 щурів; тваринам другої групи (n = 15) індукували цукровий діабет 2 типу (ЦД2). Ізольовані фрагменти підколінних артерій (ПА) і внутрішньолегеневої артерії (ВЛА) очищали від периваскулярної жирової тканини (ПВЖТ-) або залишали неочищеними (ПВЖТ+) і нарізали на кільця. Моделювали гостру гіпоксію, вивчали ефекти лікарських засобів.

Результати. ПА та ВЛА із ПВЖТ+ на гостру гіпоксію реагували вазоконстрикцією – збільшенням амплітуди скорочення в першій і другій фазі, а після видалення ПВЖТ – зниженням максимальної амплітуди скорочення в 3,4 раза в першій фазі, збільшенням амплітуди в 1,8 раза в другій фазі. Перфузія тіотріазоліном майже не впливала на гіпоксичну вазоконстрикцію ПА та ВЛА в ПВАТ+ і знижувала амплітуду в другій фазі гіпоксичної вазоконстрикції в ПВАТ- у контрольних тварин. Перфузія ангіоліном знижувала другу фазу гіпоксичної вазоконстрикції ПА та ВЛА, особливо у ПВЖТ+. Додавання комбінації тіотріазоліну та L-аргініну (1:4) до розчину для перфузії фрагментів артерій тварин із ЦД2 спричиняє достовірне посилення

констрикторних реакцій і в першій, і в другій фазах гіпоксичної вазоконстрикції незалежно від наявності периваскулярної жирової тканини.

Висновки. Наявність периваскулярної жирової тканини впливає на гіпоксичну вазоконстрикцію артерій у нормі та при цукровому діабеті 2 типу. Визначені можливі шляхи фармакологічної модуляції нітроксидергічної системи залежно від стану периваскулярної жирової тканини.

Сучасні медичні технології. 2024. Т. 16, № 2(61). С. 122-131

Few evidence suggests that COVID-19 infection can act as a causative agent or as a trigger for the development of reactive arthritis even in patients who did not have antibodies of rheumatological disorders that is why the treating physician or rheumatologist should have a high index of suspicion while treating any post infectious COVID-19 patient with arthralgia. The exact mechanism through which SARS-CoV-2 might cause arthritis is not fully understood, and mechanistic data are still lacking. The most common hypothesis is the existence of molecular mimicry between SARS-CoV-2 viral epitopes and the synovial membrane causing local inflammation, but other theories have proposed a role for the presence of circulating immune complexes or localization of the virus directly in joint tissue [1]. Molecular mimicry triggers humoral and cellular autoreactivity in the host [2]. Primary SARS-CoV-2 infection induces systemic inflammation that can affect the musculoskeletal system allowing direct viral infection [1].

Coronavirus infection is associated with a pronounced inflammatory process, including the so-called “cytokine storm”, which can lead to cell membranes and cellular organelles damage by reactive oxygen species (ROS), free radicals and peroxidation products [3,4,5]. Such pathobiochemical processes ultimately lead to dysfunction of cells and their death by apoptosis and even necrosis. In addition, endothelial dysfunction (ED) develops against the background of SARS-CoV-2 infection [4]. Endothelial cells produce and release several mediators, including vasodilators such as nitric oxide (NO) and prostacyclin, vasoconstrictors such as endothelin-1 and prostaglandin F2α (PGF2α), and substances involved in coagulation, fibrinolysis, and inflammatory and immunological reactions, such as ROS and growth factors that promote cell growth. Various structural and functional disorders of the endothelium, for example, changes in the ability to produce and release these substances, or disruption of the endothelium barrier function can lead to ED development [5,6]. It is known that post-COVID complications associated with arthropathy are known to be more severe in patients with diabetes mellitus that may be associated with a more intensive development of ED against the background of microcirculatory disorders.

ED is characterized by a decrease in the bioavailability of the vasodilator – NO, and an increase in the level of vasoconstrictor substances (ROS, thromboxanes, lipoperoxides, prostaglandins F2α). This imbalance leads to vasoconstriction, leukocyte attachment, inflammatory reactions in the vascular wall, atherosclerosis and thrombosis [6,7,8]. It is known that the formation of ED in type 2 diabetes mellitus (T2DM) occurs against the background of a decrease in the bioavailability of NO. Other factors that may contribute to ED in T2DM include decreased production of other dilating factors, increased production of vasoconstrictors (ROS, lipid peroxidation products of fatty acids and phospholipids, cytotoxic metabolites of NO), and decreased sensitivity of vascular smooth muscle to vasodilators [6,7,9]. Oxidative stress not only leads to a decrease in the bioavailability of nitric oxide (NO) (due to its conversion to peroxynitrite), but also contributes to a decrease in the level of reduced thiols (glutathione, cysteine, etc.), which act as a carrier and protector of nitrogen monoxide [6,7].

Advanced glycation end products in T2DM increase oxidative stress and reduce the endothelial response to the action of vasodilators. Hyperglycemia also contributes to an imbalance between vasodilators and vasoconstrictors as it increases endothelin-1 secretion in vitro and decreases NO production in the aorta of diabetic rats and human coronary microvessels. IL-1β and IL-6, pro-inflammatory signaling molecules, also play an important role in the indirect mechanisms of ED formation, enhancing free radical processes and reducing endothelial nitric oxide synthase isoenzyme (eNOS) expression [6,7,10].

It is known that the progression of T2DM has differentially affects endothelial function and vascular contractility in many vessels, including the intrapulmonary artery, the microvasculature of the joints, the popliteal artery, the artery of the ligament of the femoral head, etc. The formation of ED in the popliteal artery and intrapulmonary artery is associated with oxidative stress, inflammation, disorders in nitrooxydergic system and lipid metabolism [6,9,11,12]. Impaired oxygenation and trophism due to dysfunction of the microvasculature leads to the development of aseptic inflammation aggravated by proinflammatory mediators, that manifests with a clear clinical picture of arthropathy. Understanding the mechanisms of popliteal and intrapulmonary arteries ED in T2DM can lead to significant advances in both preventive and therapeutic purposes in the treatment of diabetic patients with joint diseases, as well as the treatment of post-COVID arthropathy and pulmonary diseases in patients with T2DM.

The above is a theoretical basis for the use of Thiotriazoline in the complex therapy of SARS-CoV-2, which has established immunomodulatory, anti-inflammatory, antioxidant, anti-ischemic, cardio- and endothelial-protective, antiaggregant and hepatoprotective activities. The effectiveness of Thiotriazoline for the above types of activity has been proven both at the preclinical and clinical stages of research and is confirmed by more than 20 years of history of its use in health care systems of different countries [14]. Our studies showed that the administration of Thiotriazoline led to an objective improvement in general clinical indicators in patients with post-COVID syndrome – complaints of palpitations disappeared, blood pressure was stabilized (without additional correction with antihypertensive drugs, unlike patients who received only basic therapy), weakness and fatigue were decreased, and blood oxygen saturation increased to 98–99 % in 93.4 % of patients treated with Thiotriazoline.

However, there is currently no data on the endothelioprotective effect of Thiotriazoline in T2DM [14]. We have obtained data

on the endothelioprotective effect of the combination of L-arginine and Thiotriazoline (4:1) and the new original drug – Angiolin, a structural analogue of Thiotriazoline ((S)-2,6-diaminohexanoic acid 3-methyl-1,2,4-triazolyl-5-thioacetate). All these facts are very attractive from the point of view of the prospects for further research of adipose tissue cellular elements influence on the development of vascular ED of the knee joint and the possibility of its drug correction in patients with T2DM [14].

Aim

The purpose of the study was to evaluate the role of adipose tissue elements in the regulation of parameters of the nitroxidergic system under hypoxia conditions.

Materials and methods

Laboratory animals used in experiments. The studies were carried out on 30 adult white male Wistar rats weighing 190 ± 15 g, which were kept on a standard vivarium diet consisting of dry briquetted feed (vivarium of the Institute of Pharmacology and Toxicology of the Academy of Medical Sciences of Ukraine). All manipulations with animals were carried out in accordance with the Law of Ukraine No. 3447-IV "On the Protection of Animals from Cruelty" and the European Convention for the Protection of Vertebrate Animals Used for Experimental Research and Other Scientific Purposes. All animals were randomly assigned and divided into two groups: a control group (15 rats), T2DM was induced in the animals of the second group (high-fat diet + streptozotocin, 15 rats). The experimental research protocols and their results were approved by the decision of the Bioethics Commission of the Zaporizhzhia State Medical University (protocol No. 33 of March 2, 2021).

Modeling diabetes mellitus. The development of T2DM was induced by keeping rats on a high-fat diet (6 % vegetable fats, 10 % animal fats) for 21 days and a single intraperitoneal injection of streptozotocin (STZ) at a dose of 40 mg/kg [15]. STZ was dissolved in citrate buffer, pH = 4.5. Rats of the first and second groups were intraperitoneally injected with citrate buffer used for STZ dilution. Animals were accepted into the experiment 11 days after STZ administration. The experimental study was carried out 3 months after the formation of the diabetes mellitus model.

Determination of blood glucose in rat. The concentration of glucose in the blood plasma was measured immediately before the start of the experiments and according to the experimental protocols using a Bionime glucose meter (BIONIME Rightest GM 300, Switzerland).

Glucose tolerance test. An oral glucose tolerance test was performed in rats of all groups on the 32nd day of the experiment using the standard protocol [15]. Before the test, the rats were fasted for 6 hours. Glucose solution at a rate of 2 g/kg of animal weight was administered per os using a probe. Blood was collected from the tail vein on an empty stomach and 30, 60, 90 and 120 min after glucose administration.

Insulin resistance test. An insulin resistance test was performed in rats of all groups on the 31st day of the experiment using the standard protocol [13,15]. Before the test, the rats were fasted for 6 hours. Blood was taken from the tail vein before insulin injection and 30, 60, 90 and 120 minutes after it. Insulin was injected intraperitoneally at a dose of 0.175 IU/kg.

Animal euthanasia. Euthanasia of animals was carried out by overdose of anesthesia (chloralose and urethane in a ratio of 1:10, 800 mg per 100 g of body weight; i. v.). The capsule of the knee joint with paraarticular soft tissues and lungs with intrapulmonary artery were removed from the animals.

Registration and calculation of contractile activity of selected arteries. Samples of isolated rat tissues were stored in a cooled Krebs solution of the following composition (mmol): 132 NaCl, 4.7 KCl, 1.4 NaH_2PO_4, 1.0 $MgCl_2$, 1.8 $CaCl_2$, 25 $NaHCO_3$, 6.5 glucose, pH 7.4 maintained with a gas mixture of 5 % CO_2 / 95 % air. Isolated fragments of the popliteal arteries (PA) and intrapulmonary artery (IPA) were cleared of perivascular adipose and connective tissue (PVAT-) or left uncleaned (PVAT+) and cut into rings (up to 1 mm). The rings were placed in a flow chamber (2–4 ml) with Krebs solution (36 °C) and stretched on metal hooks with a preload of up to 0.5 g (5 mN). The contractile activity of arterial rings was recorded in isometric mode using strain gauges (FTK-0.1, Ukraine), a LabTrax 4-CDA adapter (WPI, USA) and DataTrax 2 (WPI, USA). The studies were carried out on fragments of vessels after stabilization of their contractile activity for 40–60 minutes, and the maximum response under the influence of 60 mmol KCl in Krebs solution was taken as 100 % in subsequent calculations of the contraction amplitude (%, KCl). The reactions of the studied arteries rings were carried out according to the next experimental protocols: measured of the amplitude of the rings contraction under the influence of phenylephrine (PE, 10^{-6} mol) and the level of endothelium-dependent relaxation of PE-contracted rings (%, PE) under the influence of acetylcholine (ACh, 10^{-6} mol); measured the amplitude of the phase hypoxic reaction of the vessels rings, previously contracted by phenylephrine (%, PE), under conditions of reducing the oxygen concentration in Krebs solution by blowing a gas mixture of CO_2 – 5 % and N_2 – 95 % for 50–60 minutes. The studies have been carried out on the vessel fragments after stabilization of their contractile activity for 40–60 minutes, and the maximum response under the influence of phenylephrine (PE, 3×10^{-6} mol/l) in Krebs solution was taken as 100 % in subsequent calculations of the contraction amplitude (%, PE). The responses of the studied artery rings were documented according to the following experimental protocols: amplitudes of contraction of the rings under influence of phenylephrine and levels of endothelium-dependent relaxation of the rings contracted by phenylephrine under influence of acetylcholine (ACh, 10^{-6} mol/l) were measured; as well as amplitudes of the phasic hypoxic reaction of the vessel rings, previously narrowed with phenylephrine (%, PE) were measured under conditions of decreased oxygen concentration in Krebs solution owing to blowing a gas mixture of 5 % CO_2 and 95 % N_2 for 50–60 minutes. As known, hypoxic vasoconstriction of isolated vessels has biphasic nature and consists of an initial transitory constrictor response – the first phase, followed by a slow long-term constriction – the second phase.

The transitory (1st) phase is observed in studies on isolated arteries of the pulmonary circulation (including deendothelial vessels) and can be characterized as acute because it develops in a few seconds after hypoxia onset and lasts 2 to 6 minutes,

according to the different data. The long-term (2nd) phase of hypoxic pulmonary vasoconstriction begins in a few minutes after hypoxia onset and can last for several hours or even days in chronic hypoxia. It is believed that the second phase is physiologically more important compared to the transient phase and is responsible for maintaining blood flow changes in response to decrease in oxygen partial pressure throughout the entire period of hypoxia [16,17,18].

Simulating acute hypoxia. The prepared artery (both PA and IPA) rings were sequentially kept in an oxygenated Krebs solution (bubbling with a gas mixture of O_2 – 95 % and CO_2 – 5 %) in the presence of PE (3×10^{-6} mol/l), and then washed for 50–60 min with a Krebs solution with a low oxygen content (bubbling with a gas mixture of CO_2 – 5 % and N_2 – 95 %).

Method of the studied pharmacological agents' administration. The vessels fragments with preserved and removed PVAT were stored in a cooled Krebs solution with the addition of Thiotriazoline (TTZ, 10^{-5} mol/l), Thiotriazoline with L-arginine (1:4, TTZ + Arg, 10^{-5} mol/l per Thiotriazoline) or Angiolin (ANG, 10^{-5} mol/l). Subsequently (in the chamber), perfusion of the rings obtained from the arteries was carried out with Krebs solution containing the indicated substances. Substances of Thiotriazoline, L-arginine and Angiolin, obtained at the State Scientific Institution "Institute for Single Crystals" of National Academy of Sciences of Ukraine (Kharkiv) with the participation of Research and Production Association "Pharmatron" (Zaporizhzhia) were used in the work.

At RPA "Pharmatron", the feasibility of combining Thiotriazoline and L-arginine was theoretically substantiated, their physicochemical, chemical and pharmacological compatibility were determined, quantum chemical calculations were carried out, and the optimal ratios of the active components in the drug combination were established experimentally on animals under the conditions of the corresponding model pathology, leading to potentiation of the pharmacological effect and ED50 and LD50 were also experimentally determined. The fine chemical structure of Angiolin has been established using modern physicochemical methods (IR, NMR spectroscopy, mass spectrometry, X-ray diffraction analysis, gas-liquid chromatography, high-performance liquid chromatography); quantum chemical calculations were carried out; chemical and physicochemical properties have been established; Factory production regulations have been developed, as well as quality control methods (QCM) in accordance with modern requirements.

A full range of preclinical studies was carried out for Angiolin as an endothelial-, cardio- and neuroprotective drug, in accordance with the requirements of the Center for Drug Evaluation and Research of the Ministry of Health of Ukraine. Dosage forms for parenteral use and tablets have been developed for Angiolin. Angiolin passed phase 1 of clinical trials with permission from the Center for Drug Evaluation and Research of the Ministry of Health of Ukraine.

Histochemical methods. Both IPA and PA rings were fixed in Carnoy's fluid and then, according to the standard procedure, poured into Paraplastome-X100 blocks, from which serial frontal 14-micron histological sections were prepared. To determine nitrotyrosine, histological sections were isolated from paraplast and rehydrated, washed three times for 5 minutes with 0.05 M phosphate buffer (pH = 7.4) and incubated for 30 minutes with 2N hydrochloric acid (T = 37 °C). The sections were incubated for 30 minutes (T = 37 °C) in a blocking solution consisting of 0.3 % Triton X-100, 5 % BSA (bovine serum albumin) without fatty acids in 0.05 M phosphate buffer (pH = 7.2). Then, washing was carried out twice with 0.05 M phosphate buffer (pH = 7.2) for 5minutes each one. Sections were incubated overnight in a humidified chamber at 4 °C with antibodies against 3-nitrotyrosine (Mouse monoclonal [HM.11] to Nitrotyrosine (ab7048)) diluted to a concentration of 1:100 in blocking solution. Then they were washed twice with 0.05 M phosphate buffer (pH = 7.2) for 5 minutes each. Secondary antibodies to the rabbit IgG fragment conjugated to fluorescent dye (FITC) (FITC Anti-Nitrotyrosine antibody (ab27647)) were diluted to a concentration of 1:200 in blocking solution and incubated with tissues for 1 hour. Sections were treated with blocking solution and then washed with 0.05 M phosphate buffer (pH = 7.2) with 0.3 % Triton X-100. For 3-nitrotyrosine, negative controls were prepared by adding 0.5 M dithionite in 0.1 N NaOH. Positive controls were prepared by adding 0.1 M sodium nitrite mixed at a 1:1 concentration with 0.1 N HCl.

After a final 4-fold wash with 0.05 M phosphate buffer (pH = 7.2), the sections were placed in a mixture of glycerol-phosphate buffer (9:1). The immunofluorescence reaction was studied on an AxioImager-M2 microscope (Carl Zeiss, Germany), equipped with an AxioCam-HRm camera (Carl Zeiss, Germany), using a 38HE high-emissivity filter (lex = 470/40 nm, lem = 525/50 nm) (Carl Zeiss, Germany). Quantitative analysis of the immunofluorescence reaction was carried out using the AxioVision-4.8.2 digital image analysis system (Carl Zeiss, Germany): the relative content of nitrotyrosine in the structure was determined, $mUif/mm^2$.

Real time polymerase chain reaction. The method of Polymerase Chain Reaction with reverse transcription in Real Time (qRT-PCR) was used to assess the state of expression of inducible nitric oxide synthase isoenzyme (iNOS) matrix ribonucleic acid (mRNA) and eNOS mRNA. The molecular genetic research included several stages. Isolation of total RNA from rat blood was carried out using the TrizolRNAPrep100 kit, which contains the following reagents: Trizol reagent and Extra Gene E. RNA is isolated according to the recruitment protocol. For reverse transcription (DNA synthesis), the "Reagent kit for reverse transcription (RT-1)" was used. The preparation and performance of the reaction were carried out following the kit protocol. Amplification CFX96 TM Real-Time PCR Detection Systems and a set of reagents for performing qRT- PCR in the presence of SYBRGreenR-402 were used to determine the expression level of the studied genes. The final reaction of the mixture for amplification included SYBRGreen dye, SynTaq DNA polymerase with antibodies inhibiting the enzyme activity, 0.2 µl of forward and reverse specific primers, dNTP-deoxynucleoside triphosphates, 1 µl of a template (cDNA). The reaction mixture was brought to a total volume of 25 µl by adding deionized H_2O. Specific primer pairs (5'–3') for the analysis of the studied and reference genes were selected using the PrimerBlast software (www.ncbi.nlm.nih.gov/tools/primer-blast). Amplification took place under the following con-

Table 1. The effect of Thiotriazoline (TTZ, 10^{-5} mol/l) on the contraction phases of hypoxic vasoconstriction of purified (PVAT-) and unpurified (PVAT+) arteries of rats

Study group	n	Hypoxic vasoconstriction phase (amplitude, % PE)	
		Phase 1	Phase 2
Control (PVAT+)	11	72.4 ± 5.1	29.3 ± 0.8
Control (PVAT+) + TTZ	6	50.4 ± 4.2	33.0 ± 3.0
		p < 0.05	p > 0.05
Control (PVAT-)	6	21.4 ± 2.5	52.8 ± 4.4
Control (PVAT-) + TTZ	6	17.6 ± 5.2	7.4 ± 0.9
		p > 0.05	p < 0.001
T2DM (PVAT+)	21	50.4 ± 4.3	1.1 ± 0.1
T2DM (PVAT+) + TTZ	6	56.6 ± 5.3	32.6 ± 4.2
		p > 0.05	p < 0.001
T2DM (PVAT-)	11	6.8 ± 0.6	60.2 ± 5.9
T2DM (PVAT-) + TTZ	5	43.0 ± 4.2	23.6 ± 2.7
		p < 0.001	p < 0.01

Table 2. The effect of Angiolin (ANG, 10^{-5} mol/l) on the contraction phases of hypoxic vasoconstriction of purified (PVAT-) and unpurified (PVAT+) arteries of rats

Study group	n	Hypoxic vasoconstriction phase (amplitude, % PE)	
		Phase 1	Phase 2
Control (PVAT+)	11	72.4 ± 5,1	29.3 ± 0.8
Control (PVAT+) + ANG	6	51.8 ± 3.6	6.1 ± 5.1
		p < 0.05	p < 0.001
Control (PVAT-)	6	21.4 ± 2.5	52.8 ± 4.4
Control (PVAT-) + ANG	6	58.5 ± 6.7	19.0 ± 3.9
		p < 0.001	p < 0.001
T2DM (PVAT+)	21	50.4 ± 4.3	1.1 ± 0.1
T2DM (PVAT+) + ANG	6	38.8 ± 5.4	16.2 ± 3.0
		p > 0.05	p < 0.001
T2DM (PVAT-)	11	6.8 ± 0.6	60.2 ± 5.9
T2DM (PVAT-) + ANG	5	22.1 ± 3.9	38.9 ± 6.2
		p < 0.001	p < 0.001

ditions: initiated denaturation at 95 °C – 10 min; then 50 cycles: denaturation -95 °C, 15 sec., primer annealing – 58–63 °C, 30 sec., elongation 72 °C, 30 sec.

The registration of the fluorescence intensity took place automatically at the end of the elongation stage of each cycle along the SybrGreen channel. The actin beta (Actb) gene was used as a reference gene to determine the relative value of the change in the expression level of the studied genes. The expression levels of the target genes were quantified relative to the expression of the housekeeping gene using the comparative Ct (2−ΔΔCt) method. The Ct values were converted to relative expression values using a formula that compares the target gene's Ct value to the housekeeping gene's Ct value. The relative expression values were then converted to Log2 values using the formula Log2 (relative expression).

Expression level calculation. After the Cq values are measured, different methods can be used to determine the expression level of the target gene in the test sample relative to the calibrator sample.

Analysis and statistical processing of received data. The research results were processed using the statistical package of the licensed program Statistica 13.0 TIBCO Software Inc. (StatSoft Inc., No. JPZ804I382130ARCN10-J), as well as SPSS16.0, Microsoft Excel 2010. The actual material was processed by methods of variation statistics. A test for normality of the Shapiro-Wilk distribution was performed. To make it easier to understand, in any case, the data are presented as the mean ± mean accuracy (M ± m), in the nonparametric case – the median, first and third quartiles (Med, Q25 and Q75). To compare dependent samples, the Wilcoxon test was used. Multiple comparisons were made using the Kruskal–Wallis ANOVA test. Differences were considered statistically significant if the p value was less than 0.05.

Results

As studies have shown, the popliteal artery and intrapulmonary artery with PVAT (control PVAT+) responded to acute hypoxia with vasoconstriction – an increase in the amplitude of contraction in the first and second phases. When PVAT was removed, both arteries (control PVAT-) responded to acute hypoxia with a decrease in the maximum amplitude of contraction by 3.4 times in the 1st phase and an increase in amplitude by 1.8 times in the 2nd phase of hypoxic vasoconstriction (HV). Addition of Thiotriazoline (TTZ) to a perfusion solution containing segments of the PA and IPA with preserved perivascular adipose tissue (PVAT+) in rats of the control group had a moderate effect on vascular contractility in response to acute hypoxia: the amplitude of contraction in the 1st phase decreased slightly, remaining unchanged in the 2nd phase of HV *(Table 1)*.

Addition of TTZ to the perfusate containing the studied arteries without PVAT (PVAT-), the contraction amplitude in the 1st phase of the HV remained unchanged, and the amplitude of the 2nd phase decreased by 7 times ($p < 0.001$). T2DM leads to a significant suppression of both the first and second phases of hypoxic vasoconstriction. The popliteal artery and intrapulmonary artery obtained from animals with experimental type 2 diabetes, in the presence of PVAT, responded to hypoxia with a decrease in vasoconstriction (a decrease in amplitude by 1.5 times in the 1st and 26.7 times in the 2nd contraction phase), which indicates a violation of the mechanisms of vascular tone regulation. The addition of TTZ to a perfusion solution containing PVAT+ vessels from rats with T2DM did not affect the amplitude of the 1st phase of vasoconstriction in response to acute hypoxia compared to the control (PVAT+ without T2DM) but increased the amplitude by

32 times in the 2nd phase compared to T2DM (PVAT+), bringing it to the values of the control group PVAT+.

Removal of PVAT from the PA and the IPA in T2DM further suppresses the maximum amplitude of arterial contraction in response to hypoxia in the 1st phase (10.7 times compared to the control PVAT+ and 3.14 times compared to the control PVAT- and 1.4 times compared to T2DM PVAT+), but at the same time there is an increase in the contraction amplitude in the 2nd phase of HV (54.7 times compared to T2DM PVAT+ and 2 times compared to control PVAT+). The addition of TTZ to the perfusate containing the study vessels from rats with T2DM (PVAT-) led to a significant increase of contraction in phase 1 response to hypoxia in 6.3 times compared to the T2DM(PVAT-) group and in 2 times compared to control group (PVAT-), but at the same time reduced the amplitude of HV in phase 2 (by 2.5 and 2.3 times, respectively) compared with these groups, bringing it to the level of control values (PVAT+).

The addition of Angiolin to a perfusion solution containing a segment of both the popliteal artery and the intrapulmonary artery with PVAT+ did not have a significant effect on the amplitude of HV in the 1st phase and some of its suppression in the 2nd phase *(Table 2)*. In the vascular preparations of the control group with PVAT-, the addition of Angiolin to the perfusion solution promotes an increase in the 1st phase of HV, increasing the contractile response by 2.7 times ($p < 0.001$), but significantly inhibits the 2nd phase of hypoxic vasoconstriction 52.8 ± 4.4 to -19.0 ± 3.9 % ($p < 0.001$).

The addition of ANG to the perfusion solution containing arterial segments of PVAT- obtained from animals with T2DM led to an increase in amplitude by 14.7 times in the 2nd phase of HV compared with the indicators of T2DM PVAT+ ($p < 0.001$) and bringing it closer to similar to the values of the control group (PVVT +). The addition of ANG to the perfusate with PVAT+ vessels from rats with T2DM did not have a significant effect on the amplitude of the 1st phase of HV. This indicator was lower than that in the PVAT+ control group. In case of PVAT- in arterial segments of rats with T2DM, the contraction in the 1st phase of HV when ANG was added to the perfusion solution increased by 3.25 times ($p < 0.001$) compared with the T2DM group PVAT- and was at the level of control values (PVAT-), and the contraction in the 2nd phase decreased by 1.5 times ($p < 0.001$) compared with the values of the T2DM group (PVAT-), but at the same time reached the control values (PVAT+).

The addition of a combination of Thiotriazoline and L-arginine (TTZ/Arg) to the perfusion solution containing vessels led to a significant increase in the constrictor response, regardless of the presence or absence of PVAT *(Table 3)*.

In the segments of the studied arteries of rats in the control group (PVAT+), it was revealed that the contraction amplitude of the 1st phase of hypoxic vasoconstriction doubled, and the amplitude of the 2nd phase increased more than 4 times. After removal of perivascular adipose tissue from the PA and IPA segments of rats in the control group, it was found that the contraction amplitude of the 1st phase of hypoxic vasoconstriction increased almost 12 times, and the amplitude of the 2nd phase constriction increased by 3 times. This direction of reactions was also observed when conducting experiments on vascular segments of rats with experimental T2DM, although the magnitude

Table 3. The effect of Thiotriazoline and L-arginine (1:4) (TTZ/Arg, 10^{-5} mol/l in terms of TTZ)) on the contraction phases of hypoxic vasoconstriction of purified (PVAT-) and unpurified (PVAT+) arteries of rats

Study group	n	Hypoxic vasoconstriction phase (amplitude, % PE)	
		Phase 1	Phase 2
Control (PVAT+)	11	72.4 ± 5.1	29.3 ± 0.8
Control (PVAT+) + TTZ/Arg	6	150.9 ± 13.0	125.7 ± 8.7
		p < 0.001	p < 0.001
Control (PVAT-)	6	21.4 ± 2.5	52.8 ± 4.4
Control (PVAT-) + TTZ/Arg	6	256.5 ± 15.1	169.7 ± 11.3
		p < 0.001	p < 0.001
T2DM (PVAT+)	21	50.4 ± 4.3	1.1 ± 0.1
T2DM (PVAT+) + TTZ/Arg	5	95.6 ± 11.2	61.1 ± 13.8
		p < 0.001	p < 0.001
T2DM (PVAT-)	11	6.8 ± 0.6	60.2 ± 5.9
T2DM (PVAT-) + TTZ/Arg	5	94.0 ± 14.0	88.9 ± 11.6
		p < 0.001	p < 0.05

Table 4. Nitrotyrosine content in samples of lung and paraarticular tissues of experimental animals

Study group	Indicator of the relative content of nitrotyrosine in the structure, mUif/mm^2
Control (PVAT+)	12.9 ± 0.75
Control (PVAT-)	13.8 ± 0.55
T2DM (PVAT+)	32.7 ± 1.5
T2DM (PVAT-)	41.8 ± 1.7
Control (PVAT+) +TTZ	12.8 ± 0.82
Control (PVAT-) +TTZ	12.8 ± 1.22
T2DM (PVAT+) +TTZ	28.7 ± 1.2
T2DM2 (PVAT-)+TTZ	30.0 ± 2.12*
Control (PVAT+) + ANG	12.0 ± 0.6
Control (PVAT-)+ANG	11.7 ± 0.7
T2DM (PVAT+) +ANG	26.2 ± 1.2*
T2DM (PVAT-)+ANG	28.8 ± 1.4*
Control (PVAT+) + TTZ/ARG	12.8 ± 0.7
Control (PVAT-) + TTZ/ARG	12.8 ± 0.3
T2DM (PVAT+) + TTZ/ARG	30.0 ± 1.00
T2DM (PVAT-) + TTZ/ARG	30.0 ± 1.20*

***:** $p < 0.05$ relative to the corresponding T2DM group.

of the changes in the reactions were somewhat smaller. Thus, the contraction amplitude in the 1st phase of HV in the presence of perivascular adipose tissue (PVAT+) increased by 1.9 times ($p < 0.001$), and in the absence of perivascular adipose tissue (PVAT-) by 13.8 times ($p < 0.001$) and these indicators were not statistically different in magnitude from the phasic reaction of rats in the control group (PVAT+ control) (72.4 ± 5.1). An increase in the magnitude of constriction was also observed in the 2nd phase of HV both in the presence of PVAT and without it.

Immunohistochemical studies revealed that the content of a marker of nitrosative stress – nitrotyrosine in the studied samples of lung tissue and PA animals is within the physiological norm *(Table 4)*, regardless of the presence or absence of PVAT. Modeling of T2DM leads to an increase in nitrotyrosine content in tissue samples with PVAT by 153 %, in tissues without PVAT – by 203 %.

The introduction of the studied drugs into the perfusate with vessel's samples from control animals did not lead to a significant change in nitrotyrosine values.

The introduction of TTZ into the perfusate with vascular samples led to a decrease in nitrotyrosine values compared with the corresponding diabetes groups in PVAT+ by 12.2 %, and in PVAT- by 28.2 %. When Angiolin was added to the perfusate, the concentration of nitrotyrosine decreased in PVAT+ by 19.8 % and in PVAT- by 31.1 %. The combination of Thiotriazoline with L-arginine reduced the concentration of nitrotyrosine in the PVAT+ group by 8.2 % and in the PVAT- group by 28.2 % *(Table 5)*.

PCR in samples of the PA and IPA with PVAT+ in rats with T2DM revealed an increase in the expression of iNOS mRNA by 5 times and a decrease in the expression of eNOS mRNA by 28 times. Removal of PVAT from the vessels in rats with T2DM led to more pronounced changes in the expression of NOS isoforms – the expression of iNOS mRNA increased in this group by 7.25 times, and the expression of eNOS mRNA decreased by 37 times.

The administration of TTZ into the perfusate did not affect the expression of NOS in control animals, regardless of the presence of PVAT. The administration of TTZ into the perfusate led to an increase in the expression of eNOS mRNA by 54.3 % in rats with T2DM and PVAT+ and by 104 % in the group PVAT-. TTZ decreased iNOS mRNA expression by 33 % in the vessels of rats with T2DM, regardless of the presence of PVAT. Angiolin slightly increased the expression of eNOS mRNA in the control group with PVAT+ (1.3 times), without affecting this indicator in the control group PVAT-, and had no effect on iNOS expression in the control groups. Angiolin increased the expression of eNOS mRNA in rats with T2DM by 8 times, regardless of the presence of PVAT. Angiolin reduced the expression of iNOS mRNA in the vessels of T2DM rats with PVAT+ by 76 % and in the T2DM PVAT- group by 71 % *(Table 6)*.

The combination of TTZ/Arg had a slight effect on the expression of eNOS mRNA in rats of the control group (1.2–1.3 times), regardless of the presence of PVAT, without affecting the expression of iNOS mRNA. The addition of this combination to the perfusate with PVAT led to an increase in the expression of eNOS mRNA by 51 times, to a level exceeding the control value by 1.8 times and decreased the expression of iNOS mRNA by 59 %. The TTZ/Arg combination increased the expression of eNOS mRNA in rats with T2DM in the absence of PVAT by 35 times, while decreasing the expression of iNOS mRNA by 54 %.

Table 5. eNOS mRNA and iNOS mRNA expression in lung and paraarticular tissues PVAT+ of experimental animals (relative unit)

Study group	eNOS mRNA, r. u.	iNOS mRNA, r. u.
	control 1.0000 ± 0.0132	control 1.0000 ± 0.013
T2DM	0.0350 ± 0.0001	5.1600 ± 0.0440
Control + TTZ	1.0750 ± 0.0120*	1.1800 ± 0.010*
T2DM + TTZ	0.0540 ± 0.0013*	3.4500 ± 0.0100*
Control + ANG	1.3100 ± 0.0260*	1.0000 ± 0.0023*
T2DM + ANG	0.2870 ± 0.0001*	1.2140 ± 0.0110*
Control + TTZ/ARG	1.2110 ± 0.0160*	1.0000 ± 0.0060*
T2DM + TTZ/ARG	1.8800 ± 0.0160*	2.1200 ± 0.0105*

***:** $p < 0.05$ in relation to the T2DM group.

Table 6. eNOS mRNA and iNOS mRNA expression in lung and paraarticular tissues PVAT- of experimental animals (relative unit)

Study groups	eNOS mRNA, r. u.	iNOS mRNA, r. u.
	control 1.0000 ± 0.0120	control 1.0000 ± 0.0220
T2DM	0.0217 ± 0.0001	7.2560 ± 0.0770
Control + TTZ	0.9970 ± 0.0010*	1.0500 ± 0.0120*
T2DM + TTZ	0.0430 ± 0.0001*	4.7700 ± 0.0154*
Control + ANG	1.1100 ± 0.0117*	0.9810 ± 0.0001*
T2DM + ANG	0.1780 ± 0.0001*	2.1000 ± 0.0010*
Control + TTZ/ARG	1.3300 ± 0.0110*	1.0000 ± 0.0010*
T2DM + TTZ/ARG	0.7720 ± 0.0120*	3.2890 ± 0.0120*

***:** $p < 0.05$ in relation to the T2DM group.

Discussion

It is known that PVAT has significant endocrine and paracrine functions, such as the release of bioactive adipokines, cytokines and chemokines, and is involved in non-endothelial NO synthesis [5,8,9,11]. AMP-activated protein kinase (AMPK) has been shown to regulate adipocyte metabolism and vascular function. Activation of AMPK inhibits the production of adipokines released by PVAT and prevents endothelial dysfunction by increasing NO bioavailability [17,19]. PVAT of different vessels expresses eNOS differently. Thus, in PVAT of the abdominal aorta, NO production caused by eNOS was lower than in PVAT of the thoracic aorta, indicating the susceptibility of the abdominal aorta to vascular damage. Considering its topographic location near the vascular wall, PVAT may play a decisive role in the mechanisms of atherosclerosis and endothelial dysfunction development [9,12]. The lack of PVAT is considered a risk factor for the development of vas-

cular atherosclerosis. In pathological metabolic disorders, such as dyslipidemia, obesity and diabetes, PVAT loses its protective functions and turns into a source of formation of ROS, cytotoxic NO derivatives, pro-inflammatory cytokines, pro-apoptotic factors, molecules that promote fibrin expression – factors that cause endothelial dysfunction and inflammatory cell infiltration, which contributes to the development of atherosclerosis.

Thus, in T2DM, increased expression of MCP-1 (monocyte chemoattractant protein-1) and TNF-α was found in PVAT of the abdominal aorta, but decreased expression of adiponectin. Under these same conditions, extensive inflammation, lymphangiogenesis and fibrosis, increased expression of VEGF-C, VEGF-D and overexpression of angiopoietin-2 are observed [10,17].

There is also increased expression of Cav-1 protein from PVAT, which may inhibit endothelial NO production and induce vasocontraction. It has been established that lipid peroxidation products in T2DM promote the expression of the adiponectin gene in PVAT through a peroxisome proliferator-activated receptor γ (PPARγ)-dependent mechanism. Adiponectin reconnects with eNOS to improve redox state. In addition, PPARγ deficiency in PVAT increases atherosclerosis and leads to vascular and systemic inflammation. A growing number of studies have focused on glucose metabolism in endothelial cells. T2DM inhibits the activation of glycolysis in endothelial cells under hypoxic conditions. Adiponectin is involved in the regulation of a related process. High glucose levels lead to vascular resistance to adiponectin and contribute to diabetic endothelial dysfunction. High glucose levels may reduce the expression of adiponectin by stromal cells in epicardial adipose tissue, which induces an inflammatory paracrine process in endothelial cells [12,17].

PVAT is a source of DPP-4 (dipeptidyl peptidase-4), the inhibition of which leads to a decrease in oxidative stress in the vascular wall. Pioglitazone, an insulin sensitizer, reduced PVAT damage in oxidative stress responses in T2DM. In addition, pioglitazone dramatically reduced the expression of vascular cell adhesion molecule-1 (VCAM-1) and matrix metalloprotein-9 (MMP-9), as well as MMP-9 activity in the aortic wall environment, and markedly reduced the accumulation of macrophages and lipids in atheroma plaques. Treatment with pioglitazone increased serum adiponectin levels, which enhanced endothelial-mediated vasodilation and increased the number and function of endothelial progenitor cells, thereby reducing the formation of endothelial dysfunction. It has been reported that glucagon-like peptide-1 (GLP-1)-based therapeutics can positively influence autophagy in PVAT, thereby improving obesity-induced endothelial dysfunction. For example, tenegliptin can reduce the expression of the major subunit of NADPH oxidase, Nox-4, and a macrophage marker in perivascular adipocytes of normoglycemic ApoE−/− mice [10,11,13]. Sodium glucose cotransporter 2 (SGLT2) inhibitors were found to suppress PVAT inflammation and attenuate atherogenesis in an in vivo mouse model. Empagliflozin improved RNA expression of inflammatory factors in PVAT, attenuated diabetes-induced endothelial dysfunction, and reduced atherosclerotic lesion area in the aortic arch of diabetic ApoE-/- mice [8,12]. Empagliflozin suppressed PDGF-B expression in PVAT macrophages, thereby attenuating neointimal hyperplasia following diabetic vascular injury in HFD-fed mice [8,10].

Antioxidants and modulators of the nitroxidergic system are of great interest. The use of Thiotriazoline in our experiments showed its protective effect on blood vessels in T2DM. Short-term intensive perfusion with Thiotriazoline attenuates PVAT inflammation by inhibiting ROS/NO-dependent mechanisms of IL-1β expression, as well as increasing the bioavailability of NO, thereby significantly inhibiting the formation of endothelial dysfunction.

Thiotriazoline is a trap for ROS and NO prevents the development of imbalance of the thiosulfide system during overproduction of ROS, providing functions such as cell signal transmission through the receptor-ion form complex, preserving the activity of proteins, enzymes, transcription factors and the integrity of cell membranes [14]. Thiotriazoline prevents irreversible inactivation of the transcription factor NF-kappa B, protecting sensitive cysteine residues – Cys 252, Cys 154 and Cys 61 in its DNA-binding domains – from excess ROS. In addition, Thiotriazoline may participate in the restoration of these groups upon reversible inactivation, taking on the role of Redox Factor-1. By inhibiting the oxidative inactivation of the transcription factor NF-kappa B in the presence of excess ROS, Thiotriazoline may enhance the activation of the expression of redox-sensitive genes, which are necessary to protect cells from the toxic effects of oxidative stress [14]. Thanks to these properties, Thiotriazoline normalizes the nitroxidergic system in the PA and IPA in rats with T2DM, eliminates pathological processes in PVAT in T2DM and preserves the mechanisms of hypoxic vasoconstriction of the vessels in the presence of PVAT.

Angiolin exhibits specific endothelial protective properties, with the existing evidence-based preclinical base [14]. Angiolin can maintain the density of endothelial cells of muscular vessels and capillaries, increase the density of proliferating endothelial cells, increase the expression of VEGF, eNOS, and reduce iNOS during experimental ischemia and hypoxia [8,14]. Angiolin is also able to exhibit mitoprotective effects by preserving mitochondrial ultrastructure, increasing the expression of intramitochondrial HSP70, normalizing the thiol-disulfide system and increasing the level of reduced glutathione in the cytosol and mitochondria of ischemic brain and heart [6,8]. Having a similar mechanism of endothelial protective action, Angiolin affects endothelium-dependent and endothelium-independent mechanisms of vascular contraction and relaxation. Apparently, under physiological conditions in the absence of PVAT, Angiolin increased NO production by increasing the expression of eNOS mRNA (both in the control group with PVAT+ and, especially, in the group with T2DM, regardless of PVAT+ and a decrease in nitrosative stress) and reduced the 2nd phase of HV in studied vessels in the control groups, especially in PVAT+. In case of T2DM, ANG increased the 1st phase of HV in the PA and IPA with PVAT- and increased the 2nd (longer) phase of HV in the arteries with PVAT+ due to the normalization of fine metabolic pathways (possibly glutathione metabolism, energy metabolism, reduction of pyruvate aldehyde) and normalization of the nitroxidergic system and inhibition of nitrosative stress. In terms of the influence of these indicators, Angiolin was the leader among the studied drugs.

The pharmacological effect of the combination of Thiotriazoline and L-arginine (4:1) is due to a positive effect on the

synthesis, transport and bioavailability of NO and physiological functions of this molecular messenger. NO is an unstable, short-lived radical, and for its stabilization and subsequent transport, mechanisms such as the formation of stable S-nitrosol complexes with thiocontaining low-molecular-weight compounds are provided. Under conditions of thiol compounds deficiency (oxidative stress, ischemia, intoxication, hypertension, etc.), NO transport is disrupted, because it is attacked by ROS such as superoxide radical and hydroxyl radical with transformation into a cytotoxic product – peroxynitrite [14].

The combination of L-arginine and Thiotriazoline increases the level of reduced thiols, particularly glutathione, through Thiotriazoline activation of glutathione reductase and direct reduction of the oxidized thiol group. In addition, the combination of L-arginine and Thiotriazoline, due to the antioxidantive properties of Thiotriazoline, prevents the oxidative modification of NO by oxygen radicals. Thiotriazoline can act as a transport molecule for NO, forming nitrosothiols. Another component of the combination, L-arginine, has a direct stimulating effect on NO synthase activity and NO production. Therefore, the combination of L-arginine and Thiotriazoline has unique properties to exert a protective effect in relation to the synthesis and transport of NO, its bioavailability, which underlies the mechanism of such properties as cardioprotective, anti-ischemic, hepatoprotective, fetoprotective [14].

Short-term perfusion of the popliteal and intrapulmonary arteries with a drug combination leads to an increase in the constrictor properties of the vessel in response to hypoxia both under T2DM conditions and, especially, under physiological normal conditions (control). Moreover, this effect did not depend on the presence or absence of PVAT. At the same time, the combination had a slight effect on the expression of eNOS mRNA in control rats, regardless of the presence of PVAT, and increased the expression of eNOS mRNA (especially in PVAT+) and decreased the expression of iNOS mRNA equally in the presence / absence of PVAT in T2DM. The constrictor properties of the combination and a less pronounced effect on nitrosative stress appear to be related to the deficiency of the antioxidant system in the PA in T2DM, NO formed from arginine can be converted into peroxynitrite. The amount of Thiotriazoline in the combination is not sufficient to prevent this conversion and maintain NO bioavailability.

Conclusions

1. The popliteal and intrapulmonary arteries with PVAT, in response to acute hypoxia, responded with vasoconstriction – an increase in the amplitude of contraction in the first and second phase, in case PVAT-, it responded with a decrease in the maximum amplitude of contraction in the 1st phase and an increase in the amplitude in the 2nd phase. When modeling T2DM, it leads to a significant suppression of both phases of hypoxic vasoconstriction, especially, in PVAT-.

2. In the samples of the studied arteries of rats with T2DM, in response to hypoxia, a significant increase in the content of nitrotyrosine and impaired expression of NOS isoforms was observed – an increase in the expression of iNOS mRNA and a decrease in the expression of eNOS mRNA. Removal of PVAT from the vessels of rats with T2DM led to more pronounced changes in the nitrogen monoxide system.

3. Perfusion with the modulators of the NO system of different mechanisms of action – Thiotriazoline and Angiolin at a concentration of 10^{-5} M and a combination of Thiotriazoline and L-arginine (1:4) to varying degrees of severity influenced hypoxic vasoconstriction of arteries depending on the presence of PVAT in normal conditions and in case with T2DM.

4. Thiotriazoline and Angiolin and the combination of Thiotriazoline and L-arginine (1:4) had different effects on the expression of iNOS mRNA and eNOS mRNA and the concentration of nitrotyrosine in the arteries of rats, depending on the presence of PVAT in normal conditions and in T2DM.

5. The obtained results provide an experimental substantiation of the prospects for further studies of the regulatory function of PVAT.

Information about the authors:
Bielenichev I. F., MD, PhD, DSc, Professor, Head of the Department of Pharmacology and Medical Formulation with Course of Normal Physiology, Zaporizhzhia State Medical and Pharmaceutical University, Ukraine.
ORCID ID: 0000-0003-1273-5314
Maslennikov S. O., MD, PhD, Associate Professor of the Department of Traumatology and Orthopedics, Zaporizhzhia State Medical and Pharmaceutical University, Ukraine.
ORCID ID: 0000-0002-7505-8587
Dobrelia N. V., PhD, Senior Researcher, State Institution "Institute Pharmacology and Toxicology of National Academy of Medical Science of Ukraine", Kyiv.
ORCID ID: 0000-0001-7067-9213
Khromov O. S., MD, PhD, DSc, Leading Researcher in "Institute Pharmacology and Toxicology of National Academy of Medical Science of Ukraine", Kyiv.
ORCID ID: 0000-0003-1876-6031
Holovakha M. L., MD, PhD, DSc, Professor, Head of the Department of Traumatology and Orthopedics, Zaporizhzhia State Medical and Pharmaceutical University, Ukraine
ORCID ID: 0000-0003-2835-9333
Ryzhenko V. P., PhD, Associate Professor of the Department of Medical and Pharmaceutical Informatics and Advanced Technologies, Zaporizhzhia State Medical and Pharmaceutical University, Ukraine.
ORCID ID: 0000-0003-3466-7148
Brek O. O., MD, PhD, Assistant Professor of the Department of Surgery No. 4, Kharkiv National Medical University, Ukraine.
ORCID ID: 0009-0007-5327-4610

Відомості про авторів:
Бєленічев І. Ф., д-р мед. наук, професор, зав. каф. фармакології та медичної рецептури з курсом нормальної фізіології, Запорізький державний медико-фармацевтичний університет, Україна.
Масленніков С. О., PhD, доцент каф. травматології та ортопедії, Запорізький державний медико-фармацевтичний університет, Україна.
Добреля Н. В., канд. біол. наук, старший науковий співробітник, ДУ «Інститут фармакології і токсикології НАМН України», м. Київ.
Хромов О. С., канд. мед. наук, провідний науковий співробітник, ДУ «Інститут фармакології і токсикології НАМН України», м. Київ.
Головаха М. Л., д-р мед. наук, професор, зав. каф. травматології та ортопедії, Запорізький державний медико-фармацевтичний університет, Україна.
Риженко В. П., канд. біол. наук, доцент каф. медичної та фармацевтичної інформатики та перспективних технологій, Запорізький державний медико-фармацевтичний університет, Україна.
Брек О. О., канд. мед. наук, доцент каф. хірургії № 4, Харківський національний медичний університет, Україна.

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