

# Activation of Cx-43 Hemichannels Induces the Generation of $Ca^{2+}$ -Oscillations in White Adipocytes and Stimulates Lipolysis.

Maria Turovskaya

Institute of Cell Biophysics RAS: Institut biofiziki kletki RAN

Egor Turovsky (✉ [turovsky.84@mail.ru](mailto:turovsky.84@mail.ru))

Institute of cell biophysics RAS <https://orcid.org/0000-0002-2528-8798>

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## Research Article

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# Abstract

With fluorescence microscopy, it was revealed that a decrease in the concentration of extracellular calcium ( $[Ca^{2+}]_{ex}$ ) results in two types of  $Ca^{2+}$ -responses in white adipocytes:  $Ca^{2+}$ -oscillations and transient  $Ca^{2+}$ -signals. Activation of connexin hemichannels is involved in the mechanism of generation of  $Ca^{2+}$ -oscillations, since the blockers of connexin hemichannels - carbenoxolone, octanol and proadifen, as well as Cx43 gene knock-down lead to complete suppression of these signals. TIRF microscopy confirmed activation of Cx-43 in response to the reduction of  $[Ca^{2+}]_{ex}$ . In response to the activation of Cx-43, the secretion of ATP-containing vesicles from adipocytes occurs. And this ATP release is suppressed in adipocytes along with the Cx43 gene knock-down and is inhibited by Bafilomycin A1, a vacuolar ATPase inhibitor. At the level of intracellular signaling, the generation of  $Ca^{2+}$ -oscillations in white adipocytes in response to a decrease in  $[Ca^{2+}]_{ex}$  takes place due to the mobilization of  $Ca^{2+}$  ions from the thapsigargin-sensitive  $Ca^{2+}$ -pool in the endoplasmic reticulum via IP3R as a result of the activation of P2Y1 purinergic receptors and the phosphoinositide signaling pathway. Such paracrine activation of white adipose tissue in response to the opening of Cx43 hemichannels leads to local signal propagation and regulation of gene expression. At 24 hours after activation of Cx-43 and generation of  $Ca^{2+}$ -oscillations in white adipocytes, there is a change in the expression of key genes involved in the regulation of lipolysis, which is accompanied by a decrease in the number of adipocytes that contained lipid droplets. Meanwhile, inhibition or knock-down of Cx-43 leads to inhibition of lipolysis and accumulation of lipid droplets.

In this study, we elucidate and research the mechanism of generation of  $Ca^{2+}$ -oscillations in white adipocytes in response to decreased concentration of  $Ca^{2+}$  ions in the external environment and show the correlation between periodic  $Ca^{2+}$ -modes and lipolysis/lipogenesis balance regulation.

## Introduction

There are 2 main types of adipose tissue, white and brown adipose tissue. White adipose tissue (WAT) stores energy as triglycerides in lipid droplets, whereas brown adipose tissue determines adaptive thermogenesis and dissipates energy as heat. White adipose tissue dysfunction leads to upon excess caloric intake, resulting in obesity and an enhanced predisposition to many pathophysiological changes, including diabetes, cardiovascular disease and cancer [1]. White adipocytes form fat depots in different parts of the organism and therefore adipose tissue is a distributed organ under the control of central and peripheral signals. Adipocytes are also a major cell type within the bone marrow and their number increases under various pathological conditions, including radiotherapy [2]. However, there is also a third type of adipose tissue - "beige" or "brite" adipocyte, which occurs as inclusions in white fat depots and is characterized by a distinct and different origin and molecular identity from classical brown adipocytes [3, 4]. WAT includes different types of cells: adipocytes, pre-adipocytes, macrophages, endothelial cells, and mesenchymal stem cells. Up to 50 percent of white adipose tissue is adipocytes [5].

Under normal conditions, with a positive caloric balance, FFA supplied with blood is stored both by hypertrophy of adipocytes and due to proliferation and differentiation of preadipocytes into adipocytes [6, 7]. It is believed that impairment of these processes and/or remodeling of the extracellular matrix, as well as impaired angiogenesis, can lead to pathological hypertrophy and dysfunction of adipocytes [8].

Lipolysis stimulated by norepinephrine is provided by the activation of  $\beta$ -adrenergic receptors, adenylate cyclase and the synthesis of cAMP, as well as key lipases HSL and ATGL, and phosphorylation of perilipin. Inhibition of this process is provided as a result of activation of the  $\alpha$ 2-adrenergic receptors, heterotrimeric Gi protein,  $\alpha$ i-subunit, inhibiting adenylate cyclase and cAMP synthesis [9]. The  $\beta\gamma$  subunits of Gi proteins also play an important role in the processes of signal transduction from the receptor to targets [10]. Moreover, WAT is an active secretory organ [11], which secretes a number of biologically active substances - adipokines in response to various stimuli, including stressful ones. The mechanisms of secretion in the cells of most tissues are closely related with changes in the concentration of cytosolic  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_i$ ). In this case, electrically non-excitable cells are characterized by the formation of connections with the connexin hemichannels and their activity is also regulated by changing in the concentration of  $\text{Ca}^{2+}$  ions [12]. In obesity and type II diabetes, the concentration of free fatty acids circulating increases. In obesity, it has been shown for the brain that saturated fatty acids activate the gap junction, which leads to the activation of microglia, the release of pro-inflammatory factors, ATP and glutamate into the extracellular space. As a result, it causes neuronal damage and secondary inflammation of glial cells, accompanied by a disturbance of the feeding pattern [12, 13, 14]. For adipose tissue, similar effects of connexin activation in obesity can be expected. In addition, the effects of activating hemichannels in adipose tissue are poorly understood.

Vertebrate connexins (Cx) are presented by 20 isoforms of proteins that have 4 transmembrane domains and differ in the length of the cytoplasmic domain. In the membranes of contacting cells, connexins form connexon channels and provide the distribution between these cells of calcium and potassium ions, water, molecules up to 1-1.2 kDa - ATP, small RNA, glutamate, pro-apoptotic molecules [15, 16].

Connexins which do not form contacts with a nearby cell but open into the extracellular space are named uncoupled "free" hemichannels and are also used for the transport of molecules [17]. The permeability of the connexons is regulated by the membrane potential and their opening occurs in response to depolarization, as well as conformational changes in the connexin molecule. This change depends on the concentration of  $\text{Ca}^{2+}$  ions and the channel opens when the concentration of extracellular  $\text{Ca}^{2+}$  decreases [11]. Another manner of regulating the connexin channels permeability is post-translational modification achieved by phosphorylation of PKB/Akt, MAPK, PKC, PKA, GSK-3, glycosylation, acetylation, nitrosylation, or ubiquitination [18, 19].

Cx43, among other connexins, is distinguished by the ability to form both connexons and uncoupled "free" hemichannels. In addition, Cx43 regulates cellular metabolism, and the gene expression of this connexin affects the expression pattern of other genes [20, 21]. Cx43 is also expressed on the inner mitochondrial membrane and its activity is required for the inhibition of MPT-megachannels and

suppression of the apoptotic process in brain cells [22]. The activity of Cx43 in cardiomyocytes is necessary for the transport of potassium ions from the cytosol [23], the efficient function of complex I and oxidative phosphorylation [24], stimulation of ATP-dependent K<sup>+</sup>-channels, and activation of hypoxic preconditioning mechanisms [25].

Connexins play an important role in the cell differentiation, adhesion and apoptosis; they do regulatory functions during embryonic development, synaptic transmission, immune responses, and carcinogenesis [26]. Connexins are associated with the pathogenesis of types I and II diabetes [27]. Cx36 form gap junctions between pancreatic beta cells required to maintain basic insulin secretion and glucose-induced insulin release. Animal studies have shown that loss of Cx36 is associated with the loss of pulsatile insulin release, increase in basal insulin output, and reduced glucose-induced insulin release – an defects characteristic of diabetes [28, 29]. Cx36 is coded for by GJD2 gene, which is located on the 14q region of chromosome 15, a susceptibility locus for type II diabetes, and the diabetic syndrome [30]. Cx43 is widely found in the human anatomy, and more than 80 different mutations in the gene encoding Cx43 (GJA1) are associated with the pleiotropic developmental disorder known as oculodentodigital dysplasia (ODDD), которая характеризуется craniofacial malformations, enamel hypoplasia, syndactyly, ocular deficits [31]. Cx36 form the gap junctions between pancreatic beta cells required to maintain basic insulin secretion and glucose-induced insulin release. Animal studies have shown that loss of Cx36 is associated with the loss of pulsatile insulin release, increase in basal insulin output, and reduced glucose-induced insulin release - defects characteristic of diabetes [28, 29]. Cx36 is coded for by GJD2 gene, which is located on the 14q region of chromosome 15, a susceptibility locus for type II diabetes, and the diabetic syndrome [30]. Cx43 is widely found in the human anatomy, and more than 80 different mutations in the gene encoding Cx43 (GJA1) are associated with the pleiotropic developmental disorder known as oculodentodigital dysplasia (ODDD), characterized by craniofacial malformations, enamel hypoplasia, syndactyly, ocular deficits [31]. Many tissues are protected from these abnormalities because other isoforms of connexin are expressed in them. However, in white adipocytes, Cx43 is the main connexin and therefore patients with ODDD are characterized by abnormalities in the physiology of white or brown adipose tissue. It was also found that Cx43 play a role in adipose-derived stromal cell differentiation into adipocytes, as well as adipose beiging in rodents and humans [32, 33].

Panexins have a similar structure and function, which are presented by 3 isoforms in vertebrates [34]. Pannexins can also form channels that open into the extracellular space and their permeability does not depend on the concentration of extracellular Ca<sup>2+</sup> ions [35], and their ability to form full-fledged intercellular channels remains unconfirmed [36, 37]. It is known that pannexins are involved in the mechanism of synaptic ATP release and suppression of hyperexcitation of neuronal networks through the activation of adenosine receptors on the presynaptic membrane and suppression of glutamate secretion [38]. The role of pannexins and their signaling in adipose tissue is currently poorly understood.

It should be said that an increase in [Ca<sup>2+</sup>]<sub>i</sub>, as a secondary messenger, mediates the action of almost all neurotransmitters and hormones. However, the role of Ca<sup>2+</sup> ions in the regulation of the balance between lipolysis and lipogenesis in white adipose tissue has not been sufficiently studied, including the role of

Ca<sup>2+</sup> + oscillations. Therefore, it is of interest to study the intracellular mechanism of Ca<sup>2+</sup> + oscillation generation caused by the activation of Cx-43 connexin channels and their role in the regulation of lipolysis/lipogenesis in white adipocytes.

## Materials And Methods

Isolation of preadipocytes. Cell cultures were prepared as described in detail previously [39].

All studies were approved by the Animal Ethic committee of the Institute of cell biophysics. NMRI mice (aged 3–5 weeks) were decapitated after a brief (45–60 sec) anesthesia with carbon dioxide before sacrifice. Mice were subjected to cervical dislocation and disinfected with 70% ethanol prior to dissection. All operations were performed in a sterile environment on ice. White adipose tissue was removed from the epididymal fat depot and placed in a Petri dish with cold DMEM-medium. Scissor-minced white adipose tissue was transferred into a tube containing sterile DMEM with 7 mg type II collagenase (Sigma-Aldrich, USA) and 4% bovine serum albumin (BSA, free from fatty acids). Then the tissue was incubated for 18 min at 37°C. To stop the enzymatic reaction, the tube was chilled on ice for 20 min with intermittent shaking followed by filtration through 250 µm filter and centrifugation at 1000 g for 10 min. The pellet was then resuspended in cold DMEM medium, filtered through 50 µm filters and centrifuged at 1000 g for 10 min. Finally, the pellet was resuspended in cultural medium containing: DMEM, 10% fetal bovine serum (FBS; Gibco), 4 mM L-glutamine, 4 nM insulin, 0.004% gentamicin and 25 µg/ml sodium ascorbate (Sigma-Aldrich, USA). The obtained suspension contained preadipocytes, since mature adipocytes carry vesicles of fat and do not precipitate under the given conditions.

Cultures of white adipocytes. 100 µl of culture medium containing  $3 \times 10^4$  preadipocytes were placed on round coverglasses (25 mm in diameter), which were then transferred into 35 mm Petri dishes. 6 hours after adhesion of the cells to the glass, additional culture medium was added to the Petri dishes. On the third day the medium in the dishes was replaced with a fresh portion of medium, which included 10 nM cytosine arabinoside (Sigma-Aldrich, USA) to suppress the proliferation of fibroblasts, and incubation in CO<sub>2</sub> atmosphere was continued for 8 hours. After that the medium was replaced with fresh culture medium. On the ninth day of culture, the cells form a monolayer and become differentiated.

Transfection with small interfering RNA (siRNA). When cell confluence reached at 40% (5 days *in vitro*), cells were transfected with siRNA against mouse Gja1 (Cx-43) (Thermo Fisher Scientific, USA) using lipofectamine RNAiMax (Invitrogen, USA) according to the manufacturer's instructions. After incubating white fat cells with siRNA-reagent mixtures in Opti-MEM (Gibco, USA) containing 50 pM of siRNA Gja1 were added for 6 hours. Then cultural medium changed and cells were incubated for an additional 48 hours. The efficiency of knockdown was at least 85–90% as confirmed by RT-PCR. Эксперименты проводили на 9 DIV.

The measurement of cytosolic calcium concentration. The measurement of cytosolic [Ca<sup>2+</sup>] was performed by fluorescent microscopy using Fura-2AM (Molecular probes, USA), a ratiometric fluorescent

calcium indicator. Cells were loaded with the probe dissolved in Hanks balanced salt solution (HBSS), containing 10 mM HEPES, pH 7.4, at a final concentration of 5  $\mu$ M at 37<sup>0</sup>C for 40 min with subsequent 15 min washout. The coverslip containing the cells loaded with Fura-2 was then mounted in the experimental chamber. During the experiment we used a perfusion system, which enables complete replacement of the cell bathing solution within 30 seconds. We used an Axiovert 200M based imaging system (Carl Zeiss, Germany) equipped with HBO100 mercury lamp, AxioCam HSm CCD camera and MAC5000 high speed excitation filter wheel. Fura-2 fluorescence was excited at two wavelengths using band-pass filters BP 340/30 and BP 387/15; fluorescence was registered in the wavelength range of 465–555 nm. Excitation light intensity was lowered using 25 and 5% neutral density filters in order to prevent phototoxicity. Image frames were acquired at 3 seconds intervals with a Plan Neofluar 10 $\times$ /0.3 objective. The time lapse image sequences were analyzed using ImageJ 1.44 (NIH Image, Bethesda, MD). Graphs were plotted using OriginPro 8.0 software Microcal Software Inc., Northampton, MA). Statistical analysis was performed using the same software. Results are presented as means  $\pm$  standard error (SE) or as representative calcium signal of the cells.

Assessment of hemichannel open probability by dye loading. Connexin hemichannels are permeable to the fluorescent dye carboxyfluorescein (376 Da) and in an open state can act as conduits of carboxyfluorescein transport across the membrane in accord with the concentration gradient of the dye [40, 41]. Carboxyfluorescein (100  $\mu$ M) was added to the cell incubation media for 10 min, resulting in background connexin-mediated dye loading, followed by application of the experimental stimulus. Then the cells were washed for 5 min, and the degree of intracellular carboxyfluorescein accumulation (dye loading) was assessed by measuring the intensity of carboxyfluorescein fluorescence in individual cells. Images of carboxyfluorescein fluorescence were taken using an inverted confocal microscope Leica TCS SP5 (Leica, Germany) before and after addition of the dye, after the application of the Ca<sup>2+</sup>-free stimulus in the presence of the dye in the media and after the washout of carboxyfluorescein. Using ImageJ software, regions of interest were drawn around the cell bodies of white adipocytes and the mean pixel intensity for all the cells in the field of view was calculated. Background fluorescence was subtracted.

Quinacrine staining. To visualize ATP-containing vesicles, adipocytes were stained with 5  $\mu$ M quinacrine in Hanks solution containing 10 mM HEPES at 37<sup>0</sup>C for 15 min. Quinacrine, a derivative of acridine, is a weak base that binds ATP with high affinity [42, 43]. After the incubation the cells were washed 5 times with Hanks solution and used to visualize vesicles using TIRF microscopy.

Total internal reflection fluorescence (TIRF) microscopy. To visualize and investigate the dynamics of the release from adipocytes of ATP-containing vesicles stained with quinacrine, TIRF microscopy was used. An inverted TIRF microscope (IX71, Olympus, Japan) equipped with an immersion oil lens with a high numerical aperture (60 $\times$ /1.65 NA) and a cooled high-resolution camera (Hamamatsu, Japan) was used for this. Series of images were obtained and analyzed using the Olympus Cell software (Olympus). The quinacrine fluorescence was excited using an argon laser at a wavelength of 488 nm, emission was recorded at a wavelength range 500–530 nm. To assess changes in the fluorescence intensity, an area of interest (ROI) was selected and fluorescent granules containing ATP were detected. A decrease in the

fluorescence intensity in the region of interest testified to the secretion of vesicles into the extracellular space. Experiments were performed at 37°C.

**Total RNA isolation.** Total RNA was isolated from the primary culture of white adipocytes using the Mag Jet RNA reagent kit (Thermo Scientific, USA) according to the manufacturer's instructions. The quality of the RNA was assessed by electrophoresis in 2% agarose gel in TBE buffer in the presence of ethidium bromide (1 µg/mL). The RNA concentration was measured using a NanoDrop 1000c spectrophotometer (Thermo Scientific, USA). cDNA was synthesized using the RevertAid H Minus First Strand kit according to the protocol recommended by the manufacturer (Thermo Scientific). Single-stranded cDNA preparations were used as a template for real-time PCR analysis.

**Real-time polymerase chain reaction (RT-qPCR).** Each PCR was performed in a 25 µL mixture composed of 5 µL of qPCRmix-HS SYBR (Evrogen, Moscow, Russia), 1 µL (0.2 µM) of the primer solution, 17 µL water (RNase-free), 1 µL cDNA. Dtlite Real-Time PCR System (DNA-technology, Moscow, Russia) was used for amplification. Amplification process consisted of the initial 5 min denaturation at 95°C, 40 cycles of 30 s denaturation at 95°C, 20 s annealing at 60–62°C, and 20 s extension step at 72°C. The final extension was performed for 10 min at 72°C. The sequences of the used primers are presented in Table 1. All the sequences were designed with FAST PCR 5.4 and NCBI Primer-BLAST software. The data were analyzed with Dtlite software (DNA-technology, Moscow, Russia). The expression of the studied genes was normalized to gene encoding Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Data were analyzed using Livak's method [44].

Table 1  
Primer sequences for real-time polymerase chain reaction (RT-PCR).

<b>Gapdh</b>	<b>Forward 5'-ccacggcaagttcaacggcac-3'</b> <b>Reverse 5'-gatgatgaccctttggccccacc-3'</b>
Lipe	Forward 5'-gagcactacaaacgcaacgagaca-3' Reverse 5'-aaattcagccccacgcaactct-3'
Sitr1	Forward 5'-ctttcagaaccaccaaagcgga-3' Reverse 5'-acagaaaccccagctccagtca-3'
Sitr3	Forward 5'-acctttgtaacagctacatgcacgggt-3' Reverse 5'-ccatcacatcagcccatatgtcttc-3'
Igf2	Forward 5'-cctcctggagacatactgtgccac-3' Reverse 5'-tgtctccaggtgtcatattggaagaa-3'
Gja1	Forward 5'- cttcaatggctgctcctcacca - 3' Reverse 5'- gctcgctggcttgctgtgt - 3'

Lipid droplets assessment. Adipogenic differentiation of the cells was histologically assessed by staining with Oil Red. Samples were fixed with 3.7% buffered formalin for 1 hour. Then the cells were washed from the formalin solution. Then the cells were stained with Oil Red solution (3 mg/ml Oil Red in 60% isopropanol; Sigma-Aldrich, Germany) for 2 hours and washed three times with PBS. Then the samples were dried in a thermostat at 37°C. Samples were imaged using an Axiovert 200M based imaging system (Carl Zeiss, Germany). ImageJ software was used in order to analyze images.

Statistical analysis. All presented data were obtained from at least three cell cultures from 2–3 different passages. n – number of the experiments. All values are given as mean  $\pm$  standard error (SE). The differences between the columns were estimated with paired t-test. Two-way or One-way analysis of variance (ANOVA) followed by the post-hoc Tukey-Kramer test was used for multiple group comparisons. The statistical tests were performed with GraphPad Prism 5 software.

## Results

### **Ca<sup>2+</sup>-responses of white adipocytes to Ca<sup>2+</sup>-free medium application.**

When the complete medium is replaced with calcium-free medium supplemented with 0.5 mM EGTA (Ca<sup>2+</sup>-free), different Ca<sup>2+</sup>-releases are observed in mature white adipocytes (9 days in vitro, DIV). Directly after stimulation, Ca<sup>2+</sup>-oscillations occur in  $45 \pm 11\%$  of cells, Ca<sup>2+</sup>-oscillations are observed in  $23 \pm 8\%$  of cells after a lag period of  $5.2 \pm 3.5$  minutes, and transient Ca<sup>2+</sup>-signals are recorded in  $32 \pm 11\%$  of adipocytes (Fig. 1A). Ca<sup>2+</sup>-oscillations in adipocytes occur without changing the basal level [Ca<sup>2+</sup>]<sub>i</sub> (Fig. 1A, black and green curves), and after transient signals, a new basal level of [Ca<sup>2+</sup>]<sub>i</sub> is established (Fig. 1A, red curve). When the Ca-free medium is replaced with a medium containing 1.2 mM Ca<sup>2+</sup>, no changes in [Ca<sup>2+</sup>]<sub>i</sub> are found in adipocytes (Fig. 1A).

Figure 1B shows the representative calcium signals of white adipocytes in response to repeated addition of Ca<sup>2+</sup>-free buffer. After the first short-term (~ 160 seconds) stimulation of adipocytes by Ca<sup>2+</sup>-free medium, the complete medium (containing 1.2 mM Ca<sup>2+</sup>) was washed, and consequently, Ca<sup>2+</sup>-oscillations were suppressed. To recover of calcium signaling system of white adipocytes repeated application of Ca<sup>2+</sup>-free medium was performed after 30 minutes pause in the registration of [Ca<sup>2+</sup>]<sub>i</sub>. Repeated application of Ca<sup>2+</sup>-free medium induces Ca<sup>2+</sup>-release in  $87 \pm 21\%$  of adipocytes from the population of cells that responded to the first stimulation. Diminution in the number of cells in response to the repeated stimulation is most likely associated with irreversible desensitization of the calcium-transport systems. Moreover, each of adipocytes is still able to respond to Ca<sup>2+</sup>-free buffer exclusively in the form of Ca<sup>2+</sup>-response that occurs during the first short-term stimulation, i.e., adipocytes, which responded to the first stimulation with impulse rise in [Ca<sup>2+</sup>]<sub>i</sub>, react with the same type of signal to the repeated stimulation (Fig. 1D, black curve), but never with oscillations. Such a feature of white adipocytes may be due to an individual set of receptors and expression of calcium-transport systems, which is a



good property for inhibitory analysis and establishing the mechanisms underlying the sensitivity of white fat adipocytes to changes in the concentration of extracellular calcium.

Therefore, the replacement of the extracellular medium of cultured white adipocytes with calcium-free medium induces generation of two types of Ca<sup>2+</sup>-signals, transient Ca<sup>2+</sup>-responses and Ca<sup>2+</sup>-oscillations, which are suppressed when the concentration of extracellular Ca<sup>2+</sup> is restored.

### **Activation of Cx-43 connexin hemichannels promotes generation of Ca<sup>2+</sup>-oscillations in response to low extracellular Ca<sup>2+</sup>-level but this has no effect on Ca<sup>2+</sup>-transients**

It is known that connexin hemichannels can be activated by a decrease in the concentration of extracellular Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>ex</sub>), and Cx-43 type is most abundantly expressed in white fat adipocytes [32]. Connexin hemichannels blockers, such as carbenoxolone (CBX, 100 μM) and octanol (1 mM), completely suppress Ca<sup>2+</sup> oscillations in all white adipocytes directly after 30-min preincubation (Fig. 2A), and their signals to addition of Ca<sup>2+</sup>-free medium convert into a single Ca<sup>2+</sup> transient. Incubation of cells with proadifen (100 μM) (Fig. 2D) elicited a similar effect. In the population of white adipocytes that respond to the first addition of Ca<sup>2+</sup>-free medium, in the form of transient signals, connexin hemichannels blockers had no effects on the cells' responses after the second addition of Ca<sup>2+</sup>-free medium (Fig. 2B). In general, no decreases in the amplitude of Ca<sup>2+</sup> transients were found (Fig. 2D). Along with connexin hemichannels, pannexin 1 hemichannels (Pannexin-1) are also expressed in white adipocytes and perform several important physiological functions [45]. Incubation of white adipocytes with a pannexin blocker, probenecid (PROB, 1 mM), had no impact on generation of Ca<sup>2+</sup>-oscillations (Fig. 2A) and the amplitude of Ca<sup>2+</sup>-transients (Fig. 2D). After addition of Ca<sup>2+</sup>-free medium, the highly selective peptide blocker Pannexin-1 (<sup>10</sup>Panx, 100 μM) did not influence the Ca<sup>2+</sup>-signals of white adipocytes either (Fig. 2D).

Interestingly, cellular Cx43 gene knock-down using Gja1 siRNA not only to completely inhibits Ca<sup>2+</sup>-free medium-induced Ca<sup>2+</sup>-oscillations (Fig. 2C), but also provokes a statistically significant decrease in the amplitude of Ca<sup>2+</sup>-transients (Fig. 2D). Herewith, cellular Cx43 knock-down does not change the density of a cell culture, cell morphology (not shown) and their response to physiological stimuli (Fig. 2C – NE); the response of white adipocytes is a high-amplitude increase in [Ca<sup>2+</sup>]<sub>i</sub> after application of 1 μM of noradrenaline, which is typical for normal cell cultures of mature white adipocytes [46].

It is known that connexin hemichannels in an opened state are permeable to carboxyfluorescein [41]. In the presence of carboxyfluorescein in the incubation media, application of Ca<sup>2+</sup>-free to white adipocytes facilitated intracellular accumulation of the dye (Fig. 3A, B), indicating increased open probability of membrane channels. Ca<sup>2+</sup>-free stimulation-induced dye accumulation was prevented by CBX (Fig. 3A, B) or peptide blockers of Cx43 – Gap-26 (рис. 3B) and by Cx43 gene knock-down using Gja1 siRNA (Fig. 3A, B). Preincubation of white adipocytes with the blocker of Panexin-1 – <sup>10</sup>Panx (100 μM) did not affect intracellular accumulation of the dye (Fig. 3B), which confirms that pannexins are not involved in the response of white adipocytes to a decrease in the concentration of external calcium.

Thus, a decline in the concentration of extracellular  $\text{Ca}^{2+}$  induces generation of  $\text{Ca}^{2+}$ -oscillations in white adipocytes due to the activation and opening of connexin, mainly Cx43 but not pannexin hemichannels. However, the blockers of connexin and pannexin hemichannels did not affect the generation and amplitude of  $\text{Ca}^{2+}$ -transients, which probably occur due to the activation of other signaling pathways.

### **Decrease in extracellular $\text{Ca}^{2+}$ ions induces vesicular ATP secretion by white adipocytes.**

A fairly large range of active molecules, including ATP, can be secreted through hemichannels [16]. The cells were loaded with fluorescent probe quinacrine to visualize ATP-containing vesicles and to research the dynamics of their secretion in white adipocytes during a decrease of  $[\text{Ca}^{2+}]_{\text{ex}}$  and opening of Cx-43. Figure 4A shows a single white adipocyte loaded with quinacrine prior to stimulation with  $\text{Ca}^{2+}$ -free medium, and the image obtained by TIRF-microscopy shows a great number of ATP-containing vesicles. After a 5-minute exposure to  $\text{Ca}^{2+}$ -free medium, almost complete secretion of ATP-containing vesicles takes place (Fig. 3A,  $\text{Ca}^{2+}$ -free). Time analysis of the dynamics of the secretion shows that the secretion of most ATP-containing vesicles occurs within the first 20–30 seconds after addition of  $\text{Ca}^{2+}$ -free medium (Fig. 3B). In other non-excitabile cells, for example, astrocytes, it was shown that ATP secretion is a  $\text{Ca}^{2+}$ -dependent process [47] and incubation with Tetanus toxin (TeNT, 50 ng/mL), an inhibitor of  $\text{Ca}^{2+}$ -dependent vesicular fusion, leads to the development of a lag phase followed by secretion of ATP-containing vesicles after addition of  $\text{Ca}^{2+}$ -free medium (Fig. 3B, +TeNT). Furthermore, the number of secreted vesicles usually decreases (Fig. 3E). This experiment shows that  $\text{Ca}^{2+}$ -ions are needed for secretion of ATP-containing vesicles, and taking into account that after addition of  $\text{Ca}^{2+}$ -free medium,  $\text{Ca}^{2+}$ -ions are absent in the external environment, then, probably, adipocytes use accumulated  $\text{Ca}^{2+}$ . In fact, incubation of white adipocytes with  $\text{Ca}^{2+}$ -chelator, BAPTA-AM, for an hour changes the form of  $\text{Ca}^{2+}$ -free medium-induced  $\text{Ca}^{2+}$ -oscillations. As a result, either the interval between oscillations becomes longer (Fig. 3C, black curve), or the amplitude of  $\text{Ca}^{2+}$ -oscillations become smaller and their frequencies increase (Fig. 3C, red curve). Also, at the level of secretion of ATP-containing vesicles, incubation with BAPTA-AM, commonly, significantly reduces the number of secreted vesicles (Fig. 3E). A decrease in the number of secreted vesicles in  $\text{Ca}^{2+}$ -free medium was also observed upon incubated with Bafilomycin A1 (BafA) a vacuolar ATPase inhibitor (Fig. 3E), or cellular knock-down of Cx43, when ATP secretion is completely suppressed in white adipocytes (Fig. 3D, E).

As a result, activation of Cx43 hemichannels occurs in response to a decrease in  $[\text{Ca}^{2+}]_{\text{ex}}$ , thereby leading to vesicular secretion of ATP-containing vesicles, a process dependent on the intracellular concentration of  $\text{Ca}^{2+}$ -ions.

### **Activation of Cx-43 in response to a decrease in $[\text{Ca}^{2+}]_{\text{ex}}$ contributes to phosphoinositide signaling pathway and activation of G-proteins.**

Application of ATP (10  $\mu\text{M}$ ) to the cell culture of white adipocytes contributes to generation of predominantly  $\text{Ca}^{2+}$ -oscillations that occur without any change in the basal level of  $[\text{Ca}^{2+}]_{\text{i}}$  in  $22 \pm 16\%$  of adipocytes (Fig. 5A, designation 2), or with an increase in the basal level of  $[\text{Ca}^{2+}]_{\text{i}}$  in  $47 \pm 11\%$  of

adipocytes (Fig. 5A, designation 3).  $\text{Ca}^{2+}$ -free medium-induced  $\text{Ca}^{2+}$ -oscillations are rapidly suppressed with application of apyrase (apyrase, 35 U/ml, Fig. 5B), an ATP hydrolyzing enzyme. At the same time, in one population of white adipocytes, the basal  $[\text{Ca}^{2+}]_i$  level returned to that observed at rest, while in another population, no similar effect is found.

To generate  $\text{Ca}^{2+}$ -signals, cells can use both the input of  $\text{Ca}^{2+}$ -ions from outside and mobilization from intracellular calcium stores. Depletion of calcium from the endoplasmic reticulum (EPR) of the cells incubated with thapsigargin (TG, 10  $\mu\text{M}$ ), the SERCA inhibitor, leads to disappearance of both  $\text{Ca}^{2+}$ -oscillations and  $\text{Ca}^{2+}$ -transients of white adipocytes following a decrease in  $[\text{Ca}^{2+}]_{\text{ex}}$  (Fig. 5C). U73122 (10  $\mu\text{M}$ ), a phospholipase C (PLC) inhibitor, completely inhibits  $\text{Ca}^{2+}$ -release from white adipocytes after repeated addition of  $\text{Ca}^{2+}$ -free buffer (Fig. 5D). Similarly, inhibition of the IP3R by Xestospongin C (XeC, 1  $\mu\text{M}$ , Fig. 5E) prevents generation of  $\text{Ca}^{2+}$ -oscillations and transients in all adipocytes in response to the first addition of  $\text{Ca}^{2+}$ -free buffer. However, the ryanodine receptor as an inhibitor of ryanodine receptors ryanodine (Rya, 10  $\mu\text{M}$ , Fig. 5F) has no effect on generation of  $\text{Ca}^{2+}$ -signals from adipocytes.

MRS-2179 (30  $\mu\text{M}$ , Fig. 5G), the P2Y1 receptor antagonist, suppressed completely  $\text{Ca}^{2+}$ -oscillations of white adipocytes, but the signals in response to addition of  $\text{Ca}^{2+}$ -free medium were in the form of single fast  $\text{Ca}^{2+}$ -impulses. Furthermore, suramin (5  $\mu\text{M}$ , Fig. 5H), an uncoupler of G-proteins [48], inhibits completely  $\text{Ca}^{2+}$ -release from adipocytes suggesting that G-proteins participate in activation of  $\text{Ca}^{2+}$ -transients in response to a decrease in  $[\text{Ca}^{2+}]_{\text{ex}}$ ; this issue requires further study.

The  $\text{Ca}^{2+}$ -oscillations (red curves) and  $\text{Ca}^{2+}$ -transients (black curves) of single cells are presented. Between the first and second applications of  $\text{Ca}^{2+}$ -free, there was a 30 minutes pause in the  $\text{Ca}^{2+}$ -dynamics registration. During recording pause, an inhibitor was added.

Thus, the signaling pathway involved in generation of  $\text{Ca}^{2+}$ -oscillations by white adipocytes in response to a decrease in extracellular  $\text{Ca}^{2+}$  includes mobilization of  $\text{Ca}^{2+}$ -ions from the thapsigargin -sensitive  $\text{Ca}^{2+}$ -pool of endoplasmic reticulum with participation of phospholipase C and IP3R activation. In this case, paracrine activation of P2Y1 takes place in response to the opening of Cx43 hemichannels and secretion of ATP by adipocytes which responded to addition of  $\text{Ca}^{2+}$ -free medium that leads to local signal propagation throughout the cells of white adipose tissue.

### **A decrease in $[\text{Ca}^{2+}]_{\text{ex}}$ stimulates lipolysis and correlates with generation of $\text{Ca}^{2+}$ -oscillations in white adipocytes.**

It is known that  $\text{Ca}^{2+}$ -oscillations can regulate many physiological processes both in excitable and electrically unexcitable tissues [49]. Replacement of the extracellular medium with a calcium-free medium results in generation of  $\text{Ca}^{2+}$ -oscillations, on average, in  $29 \pm 14\%$  of cells, which last for  $52 \pm 5$  minutes and tend to decrease the amplitude of  $\text{Ca}^{2+}$ -signals (Fig. 6A, red curve). At the same time,  $47 \pm 9\%$  of white adipocytes show transient  $\text{Ca}^{2+}$ -signals in the presence of  $\text{Ca}^{2+}$ -free solution and no  $\text{Ca}^{2+}$ -oscillations occur in this cell population for 60 minutes of recording, and increased basal  $[\text{Ca}^{2+}]_i$  level returned to the basal level observed at rest by the end of the cell response during the experiment (Fig. 6A,

blue curve). The use of Ca<sup>2+</sup>-free solution and application of 100 μM of CBX (Fig. 6B, black curve) or Cx43 knockout (Cx-43-KD, Fig. 6B, green curve) to adipocytes generated Ca<sup>2+</sup>-transients exclusively in 54 ± 18% and 21 ± 7% of cells, respectively, the amplitudes of which were usually lower as compared to control (Fig. 6A, blue curve). Having recorded adipocyte Ca<sup>2+</sup>-dynamics after addition of Ca<sup>2+</sup>-free solution, the cell cultures were put into the CO<sub>2</sub> incubator again for 24 hours. Total RNA was then isolated from one part of the cells and used for PCR analysis, while the other part of the cells was fixed and loaded with a probe that stained lipid inclusions (OilRed, Fig. 6D).

The expression of *Gja1*, a gene encoding Cx-43, does not significantly change after the use of Ca<sup>2+</sup>-free solution, in combination with the connexin blocker CBX (Fig. 6A), while the cell knockdown of *Gja1* leads to almost complete suppression of expression of this gene. 24 hours after application to Ca<sup>2+</sup>-free solution in white adipocytes we observed a rise in the expression level of *Lipe*, *Sirt1*, *Sirt3* and *Atgl* genes encoding hormone-sensitive lipase, sirtuins 1 and 3, triglyceride lipase, by a factor of 8.6, 3.1, 3.3 and 3.6, respectively (Fig. 6C). Simultaneously, there is a 54% suppression of the expression of the gene *Igf2* encoding insulin-like growth factor-2 (Fig. 6C). Data on changes in gene expression coincide with those on diminution in the number of adipocytes loaded with lipid droplets (Fig. 6D, +Ca<sup>2+</sup>-free) as compared to the control (Fig. 6D, Control) (adipocytes on the 9 DIV, without Ca<sup>2+</sup>-free); this may indicate an activation of lipolysis. The addition of Ca<sup>2+</sup>-free solution for 60 min in the presence of CBX (100 μM) blocker leads to a significant increase in the expression level of 2 out of 5 genes studied, such as *Igf2* (by 3,2 times) and *Atgl* (by 2,9 times) (Fig. 6C). The use of Ca<sup>2+</sup>-free solution in combination with CBX leads to smaller morphological changes in the white adipocytes (Fig. 6D, CBX + Ca<sup>2+</sup>-free) as compared to those observed when only Ca<sup>2+</sup>-free solution is used: the number of adipocytes loaded with lipid droplets begins to decline when compared to the control (Fig. 6D, Control), but increase when Ca<sup>2+</sup>-free solution is added (Fig. 6D, +Ca<sup>2+</sup>-free). A knockdown of the *Gja1* gene suppresses not only Ca<sup>2+</sup>-oscillations in white adipocytes in response to application of Ca<sup>2+</sup>-free medium, but also contributes to a decrease in the expression of the *Lipe* (на 19%), *Sirt1* (by 31%) genes and a 5.8-fold increase in *Igf2* expression (Fig. 6C); this exactly correlates with the removal of the effect Ca<sup>2+</sup>-free medium has on intracellular accumulation of lipid droplets (Fig. 6D, Cx-43-KD + Ca<sup>2+</sup>-free); OilRed stained adipocyte culture is similar in morphology to the control (Fig. 6D, Control).

Thus, 24 h after Cx-43 activation and generation of Ca<sup>2+</sup>-oscillations in white adipocytes we observed a change in the expression of key genes involved in the regulation of lipolysis accompanied by a diminution in the number of adipocytes loaded with lipid droplets. It should be noted that inhibition or Cx-43 knockdown not only suppresses Ca<sup>2+</sup>-oscillations, but also tends to decrease the level of genes encoding key enzymes of lipolysis.

## Discussion

The physiological effects of connexins depend on both the type of connexin hemichannel and the type of tissue in which it is expressed, and, probably, the type of exposure. For example, Cx32 and Cx26 form connexon hemichannels that bind hepatocytes and are responsible for regulation of blood glucose and

hepatocyte glycogenolysis [50, 51]. On the other hand, neointima formation following balloon catheter injury is significantly reduced in heterozygous Cx43 knockout mice, suggesting a correlation between neointima formation and high levels of Cx43 during the inflammatory response to injury [52], i.e. participation of this hemichannel in the inflammatory response.

It has been found that disturbances in brain energy metabolism during hypoxia/ischemia lead to increased expression of Cx43 and inhibition of this mechanism can be destructive to cells [53, 54], most likely, through disconnected astrocytic network. Also, reoxygenation after ischemia leads to a change in the level of phosphorylation and expression of Cx43 in astrocytes. [55]. On the one hand, connexins' blockers exert neuroprotective effect during brain ischemia and [56], on the other hand, Cx43 activity may contribute to the development of the effect of hypoxic preconditioning [57]. Cx43 is highly expressed both in the mesenchymal fraction of the stem cells and in resident adipose-derived stem cells. The expression level of this connexin is increased during differentiation of adipocytes [32, 58], that distinguishes them significantly from the astrocytes, suggesting other physiological functions of the connexin. Thus, our work established correlation between the activation of  $Ca^{2+}$ -oscillations, enhanced expression of the genes encoding lipolysis proteins and diminution in the number of lipid droplets in the cytosol of adipocytes, in which Cx-43 was activated for 60 minutes. This can be defined as a positive effect.

As shown in our experiments using TIRF microscopy, in response to the activation of Cx-43, ATP-containing vesicles are secreted by white adipocytes. There is evidence from brain cells that an increase in the level of expression of Cx43 and Panx1, which occurs during ischemia, enhances the ATP release into the extracellular space and thus ATP acts as an "alarm signal". In adipose tissue, ATP secretions can strictly different functions. For example, a Chang and Cuatrecasas investigations showed that pre-exposure of white adipocytes with ATP inhibits subsequent stimulation of glucose uptake by insulin. Later studies convincingly prove that extracellular ATP elicit an increase in the cAMP level and causes an increase in  $[Ca^{2+}]_i$  through the activation of P2-purinoreceptors, which leads to the activation of protein kinase A and increased lipolysis and an a decrease in the leptin production by white adipocytes. ATP rapidly suppresses leptin secretion when insulin is added. The analogue of ATP, BzATP, leads to a similar effect, which also enhances the lipolytic activity of adipocytes. In addition, it is known that ATP is co-localized with norepinephrine in the sympathetic nerve terminals and released simultaneously in response to neuronal activity [59] and acts as a co-transmitter for norepinephrine. Leptin production by white adipocytes was decreased in P2Y1 receptor knockout mice [60]. At the same time, with metabolic syndrome in humans, the expression level of P2X7 receptors in white adipose tissue significantly increases, the activation of which mediates the release of inflammatory cytokines [61], and activation of these receptors under the action of ATP may contribute to the pathogenesis of the disease. On the other hand, for the activation of P2X7-receptors, high ATP concentrations are required [62], which are not observed normally and are unlikely to be realized under the conditions of our experiments.

At the level of intracellular signaling, it has been shown that, in addition to the mobilization of  $Ca^{2+}$  ions from intracellular stores, ATP can regulate the amount and voltage dependence of voltage-gated  $K^{+}$  currents in brown adipocytes [63], and increases membrane conductance in single rat adipocytes [64].

It has been shown earlier in our experiments that in response to acetylcholine [65], noradrenaline [66], or calmodulin inhibition [67], the generation of short-term Ca<sup>2+</sup>-signals and Ca<sup>2+</sup>-oscillations, which have different mechanisms of generation and maintenance [68], occurs in white adipocytes. Such differences in Ca<sup>2+</sup>-signaling should undoubtedly lead to different physiological effects in white adipocytes. Furthermore, in our previous studies, our findings do not suggest that connexin channels contribute to generation and maintenance of Ca<sup>2+</sup>-oscillations irrespective of high expression of this protein in adipose tissue. However, there is evidence that Cx-43 is involved in oscillatory Ca<sup>2+</sup>-responses of adipocytes during absorption of microparticles and other effects. Thus, Cx43 activation in adipose-derived stromal/stem cells has been found to be involved in regulation of Ca<sup>2+</sup>-oscillations that occur with involvement of NOS and internalization of quantum dots [69, 70], while the role of these oscillations in regulation of differentiation or the development of these cells is yet to be understood.

The mechanisms of calcium oscillations can be divided into two large classes that depend on the receptor type, IP3R or RyR, used for mobilization of Ca<sup>2+</sup> from the intracellular structures [80, 81]. Our data show, that in white adipocytes, Ca<sup>2+</sup>-oscillations activated by Cx43 in response to decreased extracellular Ca<sup>2+</sup> occur exclusively due to activation of PLC and IP3R, although the functional activity of RyR in adipocytes [68]. At the same time, Ca<sup>2+</sup> transients in response to addition of Ca<sup>2+</sup>-free solution were also suppressed by PLC, IP3R, and G-protein inhibitors, but were not influenced by the inhibitors of connexin and pannexin hemichannels, probably, due to other mechanisms of action. Nevertheless, these Ca<sup>2+</sup>-transients may also play an important role in regulation of lipolysis in adipocytes, as there is convincing evidence that activation of IP3Rs and PPARs promotes conversion of human white fat cells into brite adipocyte [82]. Cx43 expression can be regulated by renin and angiotensin II levels via activation of the extracellular signal-regulated kinase and NF-κB pathways [26], and these signaling systems are well designed in white adipose tissue, and closely linked to generation of Ca<sup>2+</sup>-oscillations and transients [83].

There is evidence of both the protective effect of connexin hemichannels and their negative effects in obesity. It has been shown that injections of the hemichannel inhibitor INI-0602 after 4 weeks of application leads to a decrease in the weight of mice kept on a high-calorie diet. It was found that inhibition of the gap junction hemichannel pathway affects the initial phases of nutritional disorders, i.e. prevents the initial stage of obesity development and does not affect the mice locomotor activity. However, in this in vivo study model, hemichannel inhibition was performed in brain cells, and the effects were observed in the body as a whole. At the same time, there is convincing evidence that connexins play a key role not only in adipogenesis, but also in lipid metabolism of adipocytes [84]. It has been found that an increase in the expression level or activation of Cx43 in white adipose tissue is an effective approach for combat lipid accumulation in obesity and other metabolic diseases [85]. It has been shown that GJIC inhibition by 18 α-glycerrhetic acid or adipocyte-specific Cx43 gene knockout reduced white adipose tissue beiging and found Cx43 to be important in maintaining mitochondrial integrity in brown fat [85, 86], which is also observed in our studies, when the knockdown of Cx43 led to the accumulation of lipid droplets at the control level or even higher.

Due to calcium signaling of the cells, it has been shown that an increase in  $[Ca^{2+}]_i$  level after treatment of cells with capsaicin is due to activation of Cx43 and this causes lipolysis in the visceral depot of the white adipose tissue [84]. Cx43 inhibition in white adipocytes leads to activation of autophagy, enhancing ROS production, i.e. the activity of this connexin can play a protective role for adipose tissue against damage induced by oxidative stress [87, 6]. The level of Cx43 expression is higher in brown adipose tissue than in white adipose tissue [33] and Cx43 plays an important role during cold stress, contributing to beiging of white adipose tissue via cAMP transport between adipocytes [85]. In brown adipocytes, like in white adipocytes, stimulation of  $\beta$ 3-adrenoreceptors increases ROS production, and inhibition of Cx43 enhances this process [88] that may cause oxidative stress.

In the regulation of lipolysis and lipogenesis of adipose cells, the following enzymes are crucial – hormone-sensitive lipase, sirtuins, insulin-like growth factor-2, triglyceride lipase, etc. [89], so in this work, we decided to investigate the expression of genes that encode these proteins in light of the generation of  $Ca^{2+}$  oscillations by white adipocytes through the activation of Cx-43. 24 hours after the activation of Cx-43 and  $Ca^{2+}$ -oscillations in a calcium-free medium, first there is an increase in the expression level of the *Lipe* gene encoding HSL. HSL is known to be rate-limiting for diacylglycerol and cholesterol ester hydrolysis in adipose tissue and essential for complete hormone-stimulated lipolysis [90]. In mice with HSL knock-out we observed diminution in genes encoding lipogenic enzymes, lipid related proteins, and insulin signaling proteins [90]. Also in HSL<sup>-/-</sup>-mice detected numerous morphological changes in white adipocytes, among them heterogeneity of cell size, appearance of hypertrophied adipocytes population, and a large number of undifferentiated preadipocytes [91, 92]. It is interesting that HSL<sup>-/-</sup>-mice do not show signs of obesity and to some extent are able to resist weight gain with a high-calorie diet [91, 93]) and are characterized by a decrease in circulating FFA in the blood, but accumulation of an increased amount of DAG in various tissues [91, 94]. Consequently, the absence of HSL results in impaired white adipocytes differentiation or impaired mechanisms of accumulation of lipids in the form of DAG [90]. Lipolysis of the lipid inclusions accumulated by adipocytes occurs also due to activation of triglyceride lipase (ATGL) [95], and the expression level of the gene, encoding it, also significantly arises after activation of Cx-43. ATGL is responsible for the initial stage of lipolysis and the cleavage of TAG into diacylglycerol (DAG), while HSL has already been responsible for the hydrolysis of DAG [96]. In our experiments, Cx-43 knock-down promotes the removal of the effect of connexin activation in response to the expression of *Lipe* and *Atgl* genes, which correlated with a rise in the number of lipid droplets in white adipocytes.

In addition to the lipases mentioned above, sirtuins also play important role in WAT physiology. Sirtuins (silent information regulators, sirts) via modification of histones, transcription factors and co-regulators, control expression of genes involved in the organism's responses to various stresses [97]. Sirtuins are associated with cell survival, apoptosis, inflammation, glucose and lipid homeostasis [98]. Sirt1 is located mainly in the nucleus and is responsible for the modification of transcription factors, cofactors, histones, and DNA repair [99]. Overexpression of Cx43 improves renal function in db/db spontaneous diabetic model mice through increased Sirt1 expression, decreased HIF-1 $\alpha$  expression, and reduced extracellular

matrix components. In the kidney, the probable coupling mechanism of Cx43 and Sirt1 may exert a protective effect through up-regulation of SIRT1 expression and enhance SIRT1-dependent deacetylation of HIF-1 $\alpha$  to reduce HIF-1 $\alpha$  activity, which eventually ameliorated renal epithelial to mesenchymal transition and diabetic renal tubulointerstitial fibrosis [100]. It was shown that Cx43 and SIRT1 are co-localized in the cytoplasm in NRK-52E cells, and the overexpression of Cx43 leads to an increase in Sirt1 expression, at the same time, the knockdown of Cx43 in white adipocytes, as our studies have shown, coincides with a decrease in Sirt1 [100]. It was found that in humans, obesity leads to a decrease in the level of Sirt1 in adipose tissue and its level is restored with a decrease in body weight [101]. Peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) is considered to be the main transcription factor responsible for promoting adipogenesis. Sirt1, through interaction with two PPAR $\gamma$  corepressors, the nuclear receptor corepressor (N-CoR) and silencing mediator of retinoid and thyroid hormone receptors (SMRT), can suppress adipogenesis [102]. Overexpression of Sirt1 inhibits adipogenesis in the 3T3-L1 cells [103, 104], as well as determines the differentiation of mesenchymal stem cells into myogenic cells, but not into preadipocytes, through interaction with the Wnt signaling pathway [105]. In our experiments, there is an increase in Sirt1 expression upon activation of Cx43, which, on the one hand, can prevent the accumulation of lipid inclusions, which is recorded using OilRed, and, on the other hand, can promote adipocyte differentiation. Sirt1 activation also promotes the phosphorylation of AMPK and inhibits the synthesis of fatty acids and an increase in the number of lipid inclusions in adipose cells in response to high glucose levels [106]. In our experiments suppression of Sirt1 gene expression after application of Ca<sup>2+</sup>-free medium with CBX or to Cx-43-KD-adipocytes can abolished the anti-lipolytic effect of Cx43 activation through this signaling pathway.

Sirt3 is expressed in mitochondria and is involved in the regulation of the activity of mitochondrial enzymes responsible for glycolysis, fatty acid (FA) oxidation, ketone body synthesis, the catabolism of amino acids, as well as proteins that regulate apoptosis and oxidative stress [107]. Sirt3 is required for the activation of the bioenergetic functions of mitochondria in the early stages of adipocyte differentiation. Silencing of Sirt3 decreases the protein level of forkhead box O3a (FoxO3a) transcription factor and subsequently downregulates the expression of several antioxidant enzymes and increases oxidative stress in mesenchymal stem cells after adipogenic induction. Therefore, Sirt3 depletion diminishes the ability of mesenchymal stem cells to undergo adipogenic differentiation and leads to adipocyte dysfunction [108]. Sirt1, by interference with the nuclear factor  $\kappa$ B (NF- $\kappa$ B) signaling pathway, represses inflammatory gene expression in adipocytes and macrophages infiltrating adipose tissue [109, 110].

Another important factor, Insulin-like growth factor-2 (IGF2) is a growth-promoting polypeptide that shares a high degree of structural homology with insulin, a widely expressed peptide hormone in cell division [111]. IGF2 is a key factor regulating cell proliferation, growth, migration, differentiation, survival, lipid metabolism [112]. IGF2 is synthesized primarily by the liver, but it is also produced locally by many tissues, where it acts in an autocrine or paracrine manner [113].



It is known that the level of IGF2 protein in the blood serum is considerably higher in patients with metabolic syndrome, and the level of expression of the gene encoding IGF2 is also increased in obese mice on a high-calorie diet [114]. IGF2 promotes the proliferation and differentiation of preadipocytes line 3T3-L1 and enhances accumulation of lipid inclusions by these cells [115]. Overexpression of IGF2 contributes to formation and accumulation of lipid inclusions by hepatocytes in adult mice [116, 117]. Interestingly, that in our experiments, the activation of Cx43 after application of Ca<sup>2+</sup>-free medium leads to the suppression of the expression of Igf2 gene encoding insulin-like growth factor-2 (IGF2), which can promote lipolysis. It is known that insulin markedly increases the rate of synthesis and accumulation of triglyceride by 3T3-L1 adipocytes [115]. At the same time, the use of Ca<sup>2+</sup>-free medium in combination with CBX promotes a significant increase in the expression of Igf2, so does Cx43 knock-down, which correlates with the inhibition of lipolysis and the appearance of a large number of lipid droplets.

## Conclusions

Thus, in differentiated white adipocytes, the reduced concentration of [Ca<sup>2+</sup>] results in the generation of two types of Ca<sup>2+</sup>-signals, such as Ca<sup>2+</sup>-oscillations and Ca<sup>2+</sup>-transients. Ca<sup>2+</sup>-oscillations occur due to the activation of Cx-43 hemichannels and vesicular ATP secretion, leading to paracrine activation of most white adipocytes in vitro. The generator of these Ca<sup>2+</sup>-oscillations is mobilization of Ca<sup>2+</sup>-ions from the thapsigargin-sensitive endoplasmic reticulum pool via IP3R, which also involves the activation of P2Y1 purinoreceptors and G-proteins. Long-term Ca<sup>2+</sup>-oscillations in adipocytes change the expression level of genes involved in the regulation of lipogenesis/lipolysis and make a shift in the balance in favor of activation of lipolysis and diminution in the number of the lipid droplets.

## Declarations

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**Author contributions** MVT performed all experiments, contributed to the experimental design and data analysis, prepared figures, participated in the discussion of results, and edited the manuscript. EAT conceived the study, designed the experiments, analyzed and interpreted the data, wrote the manuscript, contributed reagents and materials.

**Availability of data and material:** Data will be made available on reasonable request

**Compliance with ethical standards.**

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