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MOLECULAR CELL BIOLOGY

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Long-Chain Free Fatty Acids Influence Lipid Accumulation, Lysosome Activation and Glycolytic Shift in Various Cells In Vitro

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Abstract—Hydrophobic molecules may be toxic when present in excess. When dissolved in membranes, hydrophobic molecules disrupt membrane function. Studies on the effects of free fatty acids (FFA) on cultured cells contradict each other. Here we describe the effects of FFA on various human cells in culture. The addition of long-chain FFA (oleic, palmitic, linoleic, linolenic, etc.) to cultured cells led to lipid accumulation in hepatocytes and muscle cells, initiation of autophagy, and uncoupling of oxidative phosphorylation. Although treated cells increase their oxygen consumption, metabolic shifts in favor of glycolysis were observed. All these effects were expressed to varying degrees in different cells and with the addition of different FFAs. The mechanisms of these FFA effects are discussed, as well their practical implications.

Keywords: free fatty acids, metabolic reprogramming, cell culture, glycolysis, respiration, uncoupling

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INTRODUCTION

Lipids in general and free fatty acids (FFA) in particular have poor water solubility and are transported within the body by various carriers, such as chylomicrons, lipoproteins of various densities, albumin, and lipid-binding proteins within cells.

Before the discovery of penicillin, FFAs were considered promising antibiotics. The antimicrobial action of FFA against the causative agent of anthrax was discovered by Robert Koch in 1890 [1]. Study of the antimicrobial effects of fatty acids continues to this day, and it has been noted that it is practically impossible for microbes to develop resistance to FFA [2].

It is believed that the main antimicrobial effect of FFA is associated with membrane modification. There is both a direct detergent effect, and modification of the membrane, leading to impaired respiration and suppression of energy [3]. Endogenous FFAs are involved in the antimicrobial defense of the body [4] and are also widely used in creams to suppress fungal growth and treat acne.

The mechanisms of FFA action on human cells are extremely diverse. First, FFA is a high-energy substrate that undergoes beta-oxidation and "feeds" (with acetate) the Krebs cycle. Secondly, FFA become ready-made blocks for the synthesis of phospholipids and triglycerides. The human body is efficient, and often fatty acids supplied with food are incorporated into more complex lipids [5]. This can lead to modification of the properties of the membrane by altering their fatty acid composition.

FFA are part of lipids with high biological activity, they are diacylglycerides (secondary messengers) and lysophosphatidic acids (lipid hormones, the action of which is mediated by receptors) [6].

FFAs are ligands for peroxisome proliferator-activated receptors (PPARs), which in turn affect energy metabolism by regulating glucose metabolism and mitochondrial biogenesis through the induction of the transcription factors FOXO1 and PGC1-α. A number of nuclear receptors are known that interact with FFA: LXR (liver X receptor), FXR (farnesoid X receptor), RAR (retinoic acid receptor), VDR (vitamin D receptor), TR (thyroid hormone receptor), and HNF4a (Hepatocyte nuclear factor 4a). On the cell surface, there are G-protein coupled receptors that recognize FFA (GPR40 and GPR120) [7].

Until 2018, it was even believed that palmitate activates TLR4, but it turned out that this is not the case [8].

Fatty acids are the precursors of a huge family of prostanoids (prostaglandins, thromboxanes, leukotrienes, resolvins, etc.), which includes thousands of compounds studied to date that are key regulators (inducers and "finalizers") of inflammation [9]. The vast majority of anti-inflammatory drugs used today (aspirin, paracetamol, ibuprofen, indomethacin, etc.) are, in fact, inhibitors of the formation of prostanoids from FFA. One of the mechanisms of action of glucocorticoids (dexamethasone) is to increase the expression of annexins, which reduce the availability of phospholipids for phospholipases A2—enzymes that produce the raw materials for prostanoids.

The data on the effects of FFA on human cells are extremely contradictory. First, it should be noted that there are significant differences in the effect of FFA on cells of different differentiation [10–12]. It is possible that many contradictions are due to differences in experimental conditions and the transfer of data obtained in one model to another (for example, work on isolated mitochondria, on cells, and in vivo).

Fatty acids exhibit the properties of oxidative phosphorylation uncouplers. Endogenous uncouplers act as regulators of thermogenesis. It is this mechanism that allows animals and humans to survive in low temperatures and survive the winter in hibernation [13]. Furthermore, uncouplers can increase life expectancy and fight common pathologies [14].

There are only two options for energy production in human cells: glycolysis and oxidative phosphorylation. The ratio of these processes varies in different ways in different cells, depending on energy needs and other factors. During glycolysis, lactate accumulates in the cells. The attitude towards lactate has undergone a significant change in recent years. For about 100 years it was considered a waste byproduct, but now theories are being considered that lactate may be a key redox regulator of metabolism [15]. In 2020, an article was published in which lactate was called the "ugly duckling" of energy metabolism [16]. Indeed, it has recently been shown that suppression of the electron transport chain and the corresponding increase in lactate production stimulates hair stem cells and initiates the hair growth cycle [17–19].

The aim of our work was to comprehensively study the effect of long-chain FFAs on different types of cells in culture under standard conditions.

EXPERIMENTAL

Cells. The following cell cultures were used in this work: 977—human embryonic fibroblast strain (obtained from S.M. Terekhov, Medical Genetic Research Center of the Russian Academy of Medical Sciences); 977 hTERT—embryonic fibroblasts expressing the protein component of telomerase (obtained independently); 1608 hTERT—a line of adult human skin fibroblasts, immortalized by the introduction of the gene for the protein component of human telomerase (the original strain was obtained from S.M. Terekhov, immortalization was carried out by us [20]); IC-21—a continuous culture of mouse macrophages; Caki-1—a culture derived from clear cell human kidney cancer (retains a number of characteristics of the primary culture); HepG2—a culture of human hepatocellular carcinoma (retains a number of characteristics of hepatocytes); SK-N-SH—a culture of human neuroblastoma; L6—a rat skeletal myoblast culture; HaCat—human immortalized keratinocytes.

Cells (except IC-21) were cultured at 37°C in an atmosphere of 5% carbon dioxide in DMEM medium (PanEko, Russia) with a glucose content of 4.5 g/L and the addition of glutamine, gentamicin (40 μg/mL) and 10% embryonic bovine serum. IC-21 cells were cultured in RPMI-1640 medium with the same supplements.

Experiments to study the effect of FFA were carried out in complete medium with serum.

Reagents. The influence of long-chain FFAs on various parameters of the vital activity of cells in culture was determined. The following fatty acids were used in the analysis:

1. C16:0 saturated palmitic acid (Sigma-Aldrich P0500, USA);

2. Oleic acid, C18:1, *cis*, omega 9 (Sigma-Aldrich O1630);

3. Linolenic acid, C18:3, omega 3, *cis*, *cis*, *cis* 9,12,15 (Sigma-Aldrich L2376);

4. Linoleic acid, C18:2 omega 6, *cis*, *cis* 9, 12 (Sigma-Aldrich L8134);

5. Conjugated linoleic acid C18:2, a mixture of the *cis* and *trans* 9,11 and 10,12 isomers (Sigma-Aldrich O5507);

6. Elaidic acid (*trans*- isomer of oleic acid) (Sigma-Aldrich E4637);

7. A mixture of the listed fatty acids.

To convert FFA into a solution, complexes of FFA with serum albumin were prepared: a hot alcoholic solution of fatty acids was mixed with a warm (37°C) solution of bovine serum albumin (7–10%). After that, the pH of the solution was brought to 7.2–7.4 with 10% sodium hydroxide. The solution was filtered through a 0.2 μm filter, aliquoted, and stored at –20°C.

Lactate was determined using the Lactate-Glo™ Assay kit (Promega, United States) according to the manufacturer's recommendations. The added lactate dehydrogenase reacts with lactate to form NADH. In the presence of NADH, a special reductase converts proluciferin to luciferin, which is detected by luciferase. Measurements are carried out 2 days after the addition of FFA.

The concentration of glucose in the medium was determined with OneTouch Select Plus test strips 2 days after FFA addition. In the calculations, the difference between the initial and final glucose concentrations was normalized to the number of cells.

Oxygen consumption was determined using the ab197243 Extracellular O2 Consumption Reagent kit (AbCAM, UK). The procedure specified by the manufacturer was used. An oxygen-sensitive fluorescent dye was added to living cells, the diffusion of oxygen from the air was blocked with a special (from the kit) mineral oil. Oxygen causes quenching of the dye fluorescence, reducing the lifetime of the excited state, which was measured using a Spark multi-modal plate reader (Tecan, Switzerland) according to the manufacturer's recommendations.

Fat inclusions were detected in cells fixed with 4% formaldehyde and stained with a saturated solution of Sudan 4 dye. Cell morphology was determined using a Diaphot inverted phase contrast microscope (Nikon, Japan) and a Nikon D5000 camera.

The state of lysosomes was assessed using intravital microscopy of cells stained with acridine orange (1 μg/mL) directly in the growth medium [21], and photographed using an Olympus BX53 microscope.

Lysosome activation was determined using a BD LSR Fortessa flow cytometer. FFA was added one day before measurement. Cells were removed with trypsin. Cell suspension (5×10^5 cells/mL) was stained with acridine orange for 15 min. Green and red fluorescence was recorded in the FITC and PE-Cy5-5 channels, respectively.

Transcriptome analysis was performed using a HiSeq 2000 (Illumina, United States) as described previously [22]. A mixture of FFA (total concentration 500 μM) was added to 1608hTERT cells 24 h before the experiment.

RESULTS

Primary Analysis of FFA Effects

Toxic concentrations of FFA were determined in preliminary experiments. For this purpose, FFA at different concentrations was added to the growing cells. At a concentration of 500 μM, all the FFAs used inhibited cell growth to varying degrees. At the same time, conjugated linoleic acid had a cytotoxic effect on IC-21 and HepG2 cells, causing their death within 2 days. HaCat keratinocytes turned out to be the most resistant and IC-21 macrophages the least resistant to FFA.

The action of FFA led to specific changes in cell morphology. Granules located around the nucleus appeared and grew in the cytoplasm (Fig. 1d). Vital staining with acridine orange showed that lysosomes are the basis of this granular material (Figs. 1b, 1c, 1f, 1k, 1l). Lipid vesicles are also formed to varying degrees, which, when stained with acridine orange, look black (Fig. 1c).

By combining various FFAs, we developed a mixture that vaguely resembles the mixture of fatty acids in human blood plasma [23], which is slightly toxic for all the cells studied. This mixture consists of: 65% oleic, 22% linoleic, 10% palmitic and 3% linolenic acids and at a concentration of 250 μM (total concentration of FFA) does not inhibit the growth of all studied cells. At a concentration of 500 μM, the FFA mixture suppresses proliferation (single injection, the duration of the experiment is 3 days).

The degree of activation of lysosomes and the formation of lipid inclusions were different in different cells and under the action of different FFA. The activation of lysosomes (the first stage of autophagy) was observed starting with a FFA concentration of 125 μM. A quantitative study of the activation of lysosomes in 1608hTERT cells was performed using flow cytometry with in vivo staining with acridine orange (Fig. 2). It is known that the ratio of red and green signals of acridine orange reflects the level of autophagy determined in other tests [21].

The least activation was caused by oleic acid and a mixture based on oleic acid (1.10 and 1.11, respectively). The greatest activation was caused by elaidic acid (2.22). However, this result indicates the toxic effect of elaidic acid, since the intensity of cell signals along the green channel decreased, which may be associated with the loss of the barrier function of the membranes. Also, the signal through the red channel expanded unusually, which indicates an increase in cell heterogeneity. We probably recorded an intermediate variant of the onset of cell death under the influence of elaidic acid (a *trans*-isomer of oleic acid).

Of the remaining variants, the most active were linolenic (1.52) and linoleic (1.50) acid. Palmitic (1.39) and conjugated linoleic (1.37) acid were found to be intermediate.

An increase in side scatter signals means an increase in refractive surfaces in the cells, i.e. formation and growth of vacuoles (lysosomes and lipid inclusions). The small lateral scatter under the action of conjugated linoleic acid (as in the control) may reflect the fact that under the action of conjugated linoleic acid, cells shrink and lysosomes are arranged in a dense lump, as shown in Fig. 1l. The collection of lysosomes in one lump usually reflects a toxic effect, which ends in cell death.

The second (apart from lysosomes) noticeable morphological manifestation of FFA action was the appearance of lipid inclusions (Table 1) (Fig. 1c, 1h, 1i, 1j). First, it should be noted that palmitate (the only saturated FFA in the study) promotes the formation of lipid inclusions to a lesser extent than other FFAs. Cells treated with palmitate undergo insignificant morphological changes and are similar to control cells (Fig. 1k).

The formation of lipid inclusions is highly dependent on the cell type. This process is most pronounced in liver cells (Table 1) (Figs. 1h, 1i). Lipid inclusions became large, round, and captured a significant volume of the cytoplasm. Other cells, primarily kidney, muscle and nerve cells, also formed significant lipid accumulations, but their size was smaller (Fig. 1j). Epithelial cells of the HaCat epidermis had the least ability to accumulate lipid inclusions.

Unsaturated FFAs (oleic, linoleic and linolenic) contributed to the formation of fatty inclusions to a greater extent, the effect of the FFA mixture resembled the effect of oleic acid (the most represented in the FFA mixture), conjugated linoleic acid was clearly weaker. Oleic and elaidic FFA (*cis*- and *trans*-isomers) acted in approximately the same way (Table 1).

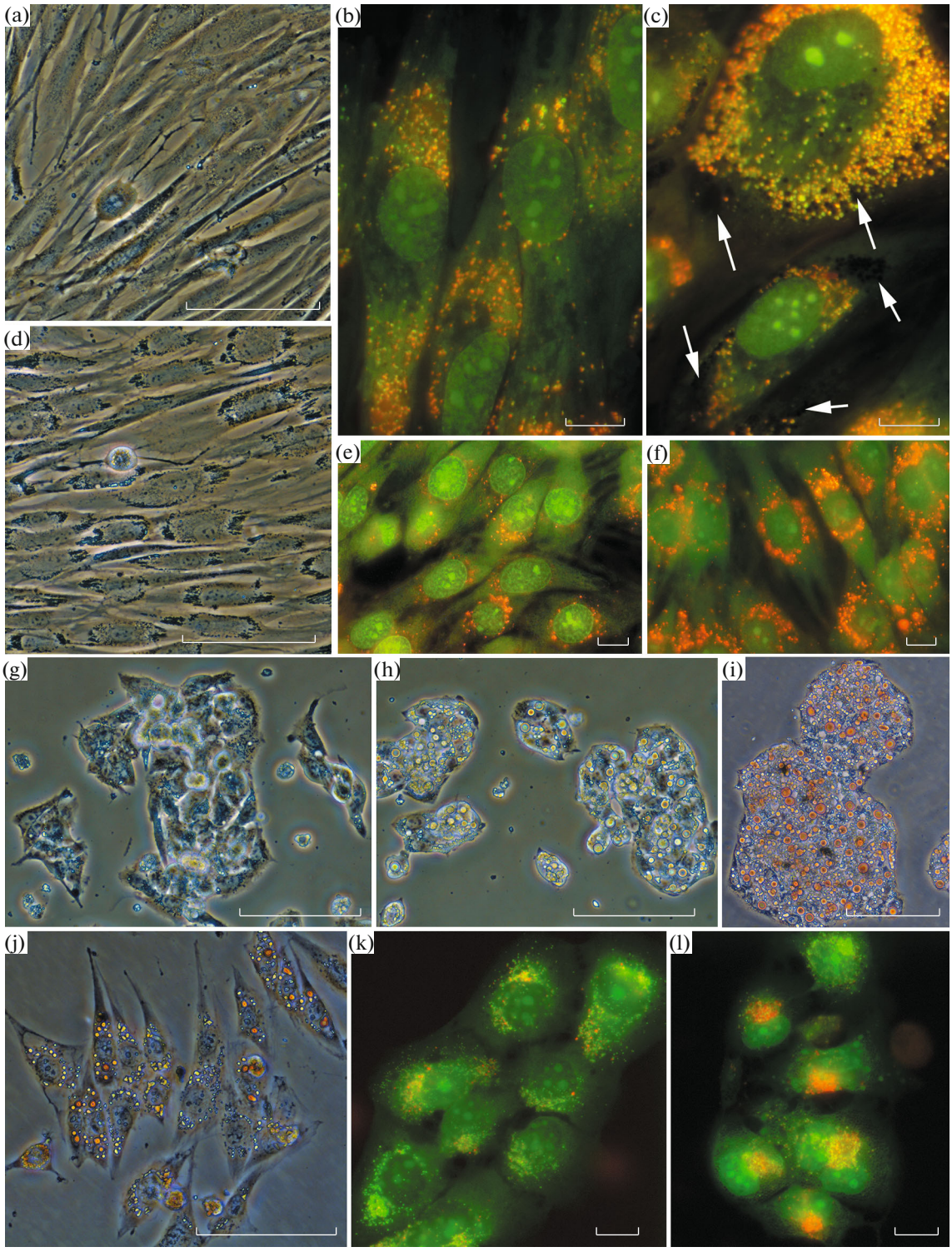

Fig. 1. Visible effects of adding FFA to cells. (a–d) 977 cells; (e–g) L6 cells; (h–j) HepG2 cells; (k, l) HaCat cells. (a, b, e, g) Controls. (d) Addition of linolenic acid. It can be seen that some kind of granular material has gathered around the cell nuclei, which strongly refracts light. A mitotic cell is visible. (c) Granular material is represented by activated lysosomes (red) and hydrophobic vacuoles (black, shown by white arrows), which does not include acridine orange. (f) The activation of lysosomes is clearly visible (red). (h) HepG2 cells after exposure to elaidic acid. Large round vacuoles are visible. (a) Staining with Sudan 4 reveals lipid inclusions. (j) L6 cells in the presence of conjugated linoleic acid, lipid inclusions are visible; (k) HaCat in the presence of palmitic acid; (l) HaCat in the presence of conjugated linoleic acid. (a, d, g, h, i, k) Phase contrast; (i, j) staining by Sudan 4; (b, c, e, f, k, l) staining with acridine orange. Scale segment 100 (a, d, g, h, i, k); 10 μm (b, c, e, f, k, l).

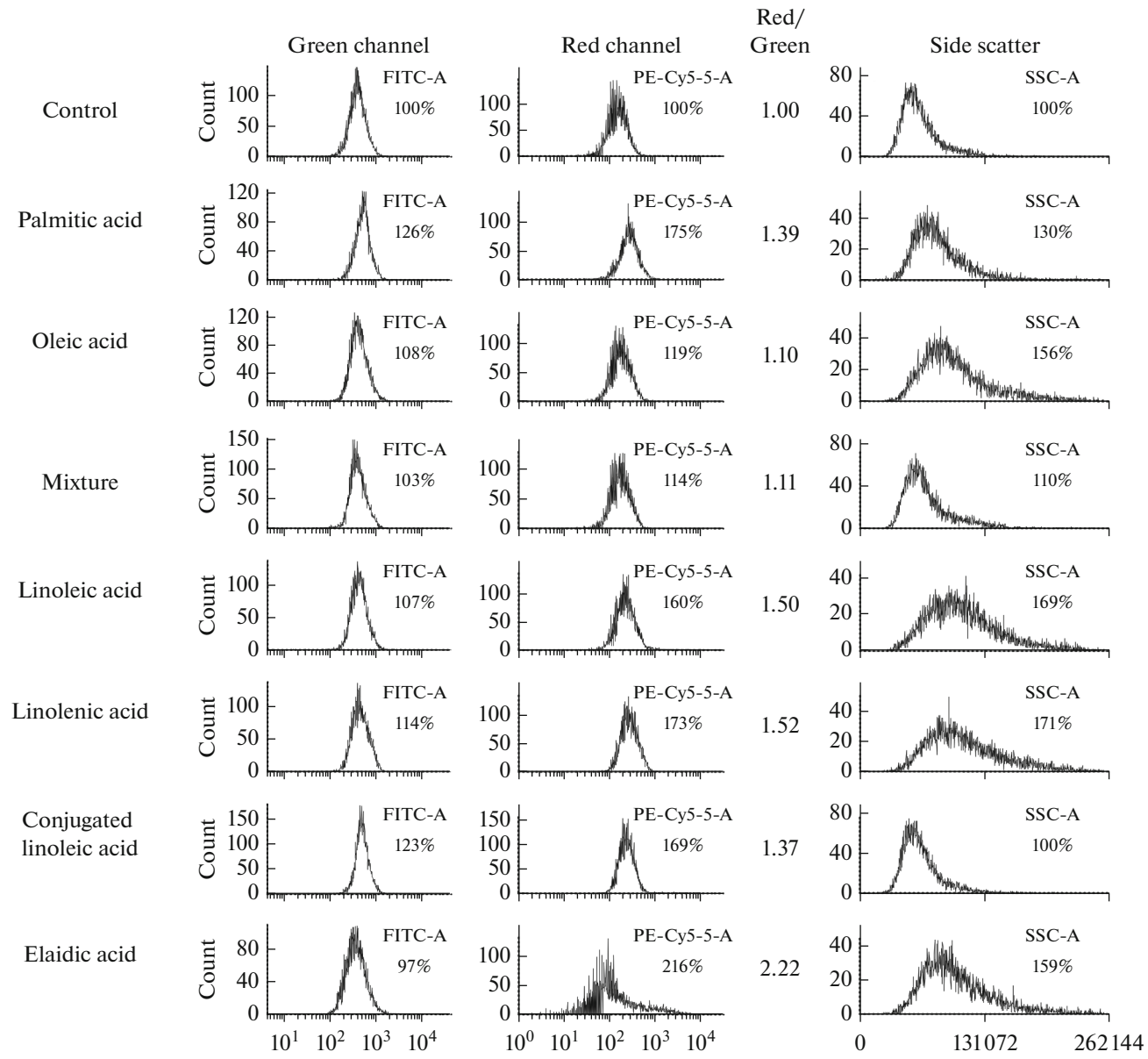

Fig. 2. Study of lysosome activation using acridine orange in 1608hTERT cells. FFA (500 μM) was added to the cells 24 h before the experiment. Distributions of green and red fluorescence signals, as well as side scattering (a measure of object granularity) are given. In all histograms, the abscissa shows the fluorescence intensity, and the ordinate shows the number of events (cells). The signals from the control cells are taken as 100%. Red/Green—the ratio of the average signals for the red and green channels—which reflects the level of autophagy.

Assessment of Glycolysis

When studying the effect of FFA, we were interested in changes in metabolism, primarily glycolysis. In the process of glycolysis, pyruvate is formed, which is subsequently either reduced to lactate (when cells strongly require glycolysis, often with a lack of oxygen), or pyruvate is oxidized by mitochondria [24, 25]. The resulting lactate is released into the environment, which leads to acidification. Sometimes glycolysis is assessed by extracellular acidification, such as the ab197244 Glycolysis Assay kit.

We observed changes in pH that occur during cell growth by changes in the color of the medium (Fig. 3). The greatest acidification occurs in the medium with palmitate (wells numbered 3). All the other FFAs and mixtures cause approximately the same weaker acidification effect.

Similar changes occurred when different FFAs and their mixtures were added to different cells. A stronger acidification effect was observed in fast-growing cells (HepG2, SK-N-SH, and L6), and was less noticeable in primary fibroblasts and macrophages. With a denser

Table 1. Accumulation of fatty inclusions in cells treated with FFA at a concentration of 500 μM for 3 days

Cells	Palmitic acid	Oleic acid	Linoleic acid	Linolenic acid	Conjugated linoleic acid	Elaidic acid	FFA mixture
IC-21	0	++	++	++	++	++	++
HaCat	+	++	++	+	+	++	++
L6	+	++	++	+	+	++	++
SK-N-SH	+	++	++	+++	++	++	++
977hTERT	+	++	+++	++	+	++	+
Caki-1	+	+++	+++	+++	+	+++	++
HepG2	++	++++	++++	*	++**	++++	++++

* Toxic effect, cell death.
** Signs of toxic effects, growth inhibition.

planting of cells (when they formed a monolayer and divided less), the acidification of the medium decreased.

The effect of acidification of the medium correlated with the accumulation of lactate (Table 2) and glucose consumption.

We did not observe the accumulation of lactate by HepG2 cells, apparently, due to the peculiarities of the metabolism of liver cells, in which the resulting lactate is converted into pyruvate (the Corey cycle).

Medium glucose was analyzed with OneTouch Select Plus test strips at baseline glucose concentrations of 1 and 4.5 g/L. In all cases, cells in the presence of FFA consumed more glucose than without FFA (Table 3, Fig. 4).

Thus, in the presence of FFA, the cells acidify the medium more, secrete more lactate and consume more glucose, which means stimulation of glycolysis by FFA.

Changes in Cellular Oxygen Consumption

It is known that FFAs cause uncoupling of oxidative phosphorylation in cells, which is accompanied by increased oxygen consumption. We decided to verify this by measuring the oxygen consumption of whole cells in a complete culture medium. Indeed, FFA caused increased respiration (Fig. 5).

Palmitic acid exhibited the greatest ability to stimulate respiration in almost all studied cells. Its effect

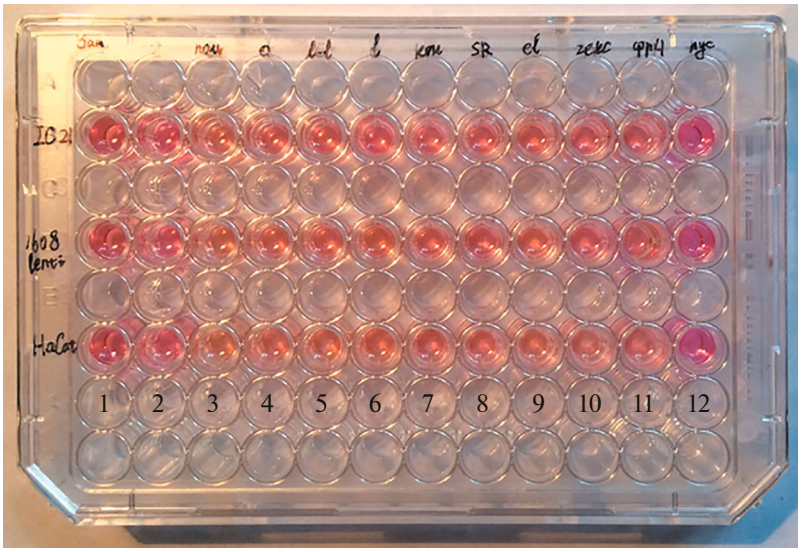

Fig. 3. Influence of FFA on the pH of the cultivation medium. A dye (phenol red) was added to the culture medium for HaCat cells (bottom row), 1608hTERT cells (middle row), and IC-21 cells (top row), the color of which changes with changes in pH. When acidified, the medium turns yellow; when alkalized, it becomes crimson. Into the wells (3–11) various fatty acids and mixtures thereof were added. The cells grew for 2 days. As a result, all these wells became more yellow. It should be noted that immediately after the addition of fatty acids, the color of all wells was the same. (1) Uncoupler Bam-15, (2) the control, (3) palmitate, (4) oleate, (5) linolenic acid, (6) linoleic acid, (7) conjugated linoleic acid, (8) a mixture of FFA, (9) elaidic acid, (10 and 11) mixtures of FFA, (12) wells without cells.

Table 2. Lactate accumulation (%) upon exposure to 500 μM FFA

FFA	977	IC-21	HepG2	HaCat	L6	SK-N-SH
Control	100	100	100	100	100	100
Palmitate	106	105	87	112	109	111
Linolenic	106	106	96	108	106	109
Linoleic	107	105	97	106	108	107
Oleic	105	103	94	106	107	108
Conjugated	100	99*	70*	104	102	102
Elaidic	104	105	96	105	107	106

* Observed toxic effects.

Table 3. Glucose consumption by 977 fibroblasts in the presence of various free fatty acids (500 μM)

Free fatty acid	Glucose, mm	Decrease in glucose concentration, mM	Specific consumption of glucose, %
Control	27.8	0.4	100
Palmitic	27.3	0.9	241
Oleic	27.5	0.7	192
Linoleic	27.8	0.4	114
Linolenic	27.1	1.1	322
Conjugated linoleic	27.7	0.5	170

was comparable to that of the uncoupler Bam-15 (10 μM) (Fig. 5c) [26].

Conjugated linoleic acid had the weakest uncoupling effect; in a number of cases (coinciding with the cytotoxic effect), it can concluded that it suppressed respiration (Figs. 5a, 5b).

Oleic acid had a weak effect. Intermediate effects were also observed: other FFAs, differed in different cells (Fig. 5c). In a number of experiments, individual FFAs did not stimulate respiration.

Thus, it has been shown that most FFAs stimulate oxygen consumption by cells.

Transcriptomic Analysis

A full-transcriptomic study of the effect of the FFA mixture on 1608hTERT cells was carried out. Computer processing of the results revealed a number of processes (cell division, development, changes in lipid metabolism), in which many differentially expressed genes are involved, but did not allow assessment of the change in the intensity of certain metabolic processes. Manual processing of the results made it possible to draw up the following table (Table 4).

From Table 4 it follows that the expression of all key glycolysis genes increased by about 1.5 times. The activity of the uncoupling protein 2 (UCP2) gene increased by an order of magnitude. Considering the short lifetime of UCP2 (about 30 min) [27], it can be assumed that the expression of this protein, leading to uncoupling, increases in response to FFA. It is known that uncouplers, including FFA, increase the expression of UCP2 [28].

DISCUSSION

First, we want to note that, unlike many scientists who have studied the effect of fatty acids on various cells, we did not observe a particularly pronounced toxic effect of palmitate.

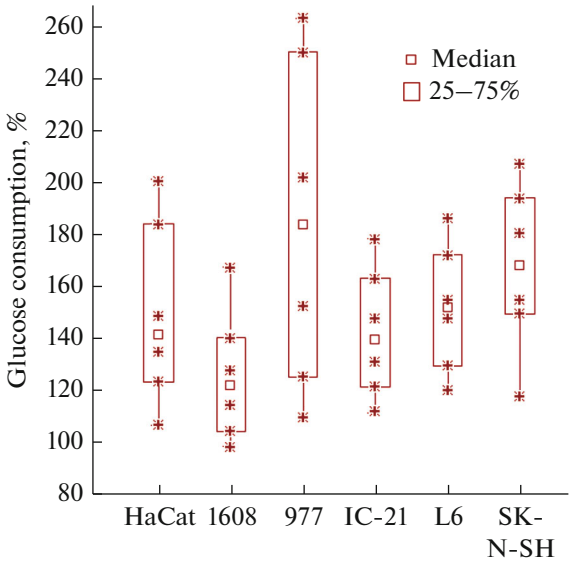

Fig. 4. Glucose uptake by cells in the presence of a FFA mixture.

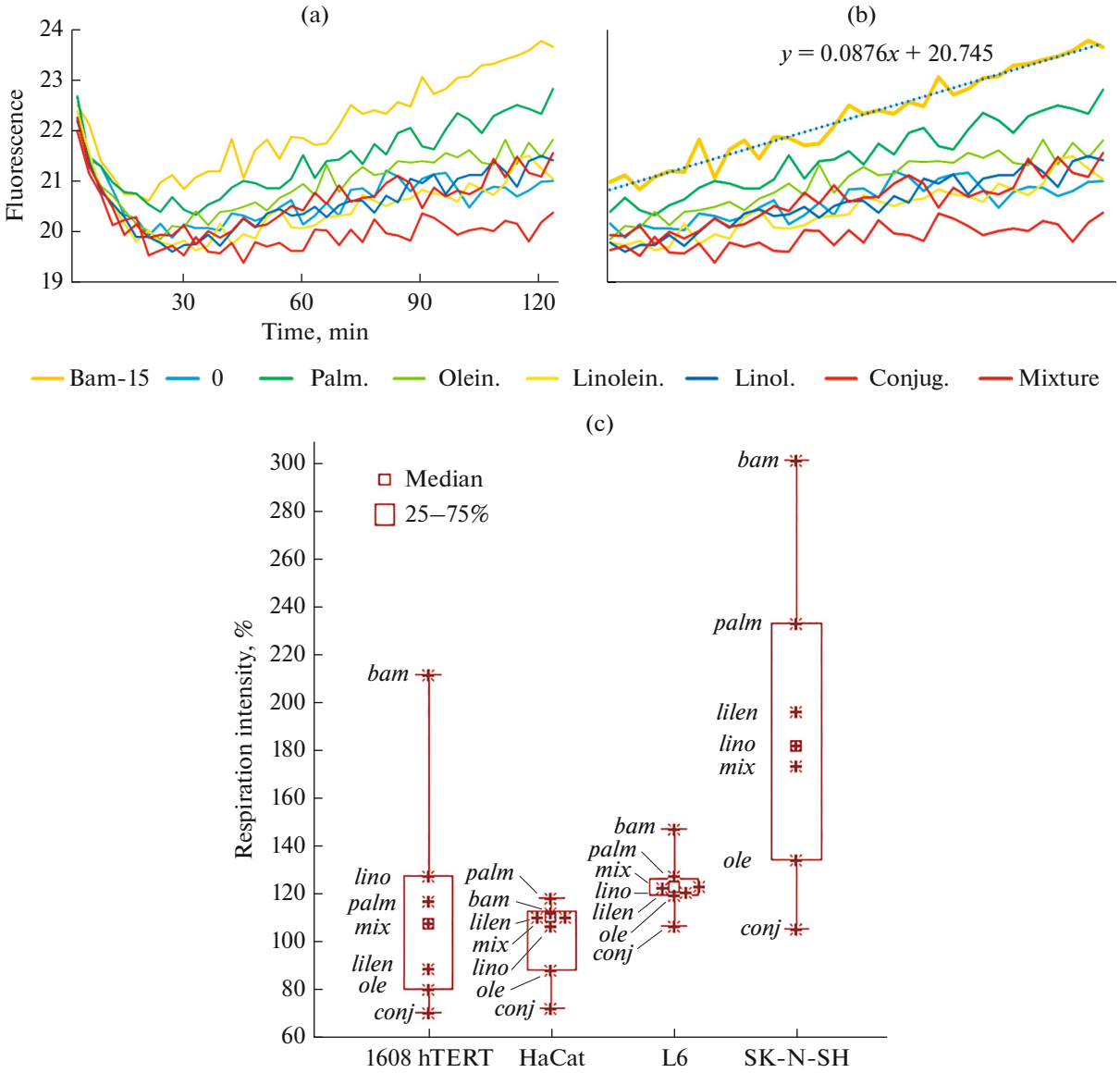

Fig. 5. Oxygen consumption by cells with the addition of FFA. (a) Initial curves of changes in the lifetime of the excited state of the dye, dependent on oxygen. The initial stage is characterized by the diffusion of oxygen from the mineral oil. Once equilibrium is established, the curve reflects the rate of respiration. (b) A method for assessing the rate of respiration. As a measure of respiration, the coefficient was chosen as the argument of the function after the corresponding trend. (c) Breathing rate. Individual FFAs are indicated: *bam*—Bam-15, *lino*—linoleic, *palm*—palmitic, *mix*—mixture, *lilen*—linolenic, *ole*—oleic, *conj*—conjugated linoleic acid. Statistical processing was carried out on the amount of FFA.

The lipotoxicity of palmitate has been shown many times on hepatocytes [29, 30], myoblasts [31–34], beta cells of the pancreas [35], and kidney cells [36]. When studying the toxic effect of palmitate at the time of addition, either 1% serum or albumin solution (2–5%) was used [11, 29–38].

In our experiments, on the contrary, the toxicity of palmitate was the lowest of all the studied FFAs. This is due to the conduction of experiments under conditions close to the conditions in vivo, in which a mixture of FFA is always present. We cultivated the cells in a complete standard medium, namely, in a medium supplemented with 10% blood serum.

Serum is known to almost always improve cell survival. The mechanisms of the protective action of serum can be different: inhibition of proteases, the presence of various growth factors, a decrease in the permeability of the plasma membrane, etc. That is why other authors still use at least 1% serum. Probably, in our case, palmitate was diluted with other serum FFAs. It is known that palmitate is very easily included in the composition of fatty acids of phospho-

Table 4. Changes in the transcription of key glycolysis and uncoupling genes in 1608hTERT cells upon addition of a mixture of free fatty acids

Process	Gene	Change in number of transcripts	Gene product	Product functions
Glycolysis	*HK1*	5560 → 8550	Hexokinase	First glycolysis reaction
	PFKP	2982 → 5496	Phosphofructokinase	Key stage of glycolysis
	PFKL	3818 → 5953		
	PKM2	62733 → 125288	Pyruvate kinase	ATP and pyruvate formation
	LDHA	41399 → 53613	Lactate dehydrogenase	Interconversion of lactate and pyruvate
	LDHB	4181 → 5771		
Uncoupling	*UCP1*	0 → 0	Uncoupling proteins	Uncoupling of oxidative phosphorylation
	UCP2	26 → 227		
	UCP3	32 → 18		

lipids and its concentration reaches high values (for example, 43% of all fatty acids in phospholipids [29]).

It is easy to assume that a phospholipid with two palmitate residues is extremely unusual for membranes; it changes their curvature and in this way disrupts their barrier functions, which leads to fatal consequences [33].

Dilution of saturated palmitate with unsaturated fatty acids normalizes the properties of the formed phospholipids and dramatically reduces toxicity. It is known that oleate "rescues" muscle cells from the toxic effect of palmitate [32, 33].

Our calculations show that the concentration of FFA in serum is determined by fatty acids bound to albumin and does not exceed 50 μM (10 times less than that used by us). In experiments without serum or with 1% serum, we observed a pronounced toxic effect of palmitate (data not shown). We believe that the known toxicity of palmitate manifests itself only in experiments carried out in the absence of other fatty acids, and therefore is an artifact, since it does not occur in vivo.

If one of the two sources of "energy supply" to cells is reduced (as a result of uncoupling of oxidative phosphorylation), then an alternative source (glycolysis) must increase in order to maintain a normal level of energy consumption. This is exactly what happens in our experiments. Similar results have been obtained quite recently when uncoupling with Bam-15 [26]. It is known that increased expression of UCP2 (in fact, an uncoupling protein) stimulates glycolytic enzymes and enhances glycolysis [39]. During the differentiation and maturation of immune cells, regular changes in metabolism occur, from glycolytic and oxidative [40, 41].

The behavior of cells in our experiments can be regarded as an attempt to maintain their homeostasis in the action of toxic factors in the external medium. FFAs added to cells in large excess are detached from their extracellular carrier (albumin) and enter the cells. At some point, there is a lack of intracellular FFA carriers in the cells. FFA begin to dissolve in membranes and change their properties (the action of FFA as antibiotics), and form agglomerates [3].

In the case of palmitate, FFA begins to intensively integrate into phospholipids [29], which also changes the properties of the membranes. To maintain viability, the cell needs to remove excess FFA.

(1) One of the options is to "burn" the FFA through beta-oxidation, through the Krebs cycle and the respiratory chain. However, under conditions of partial uncoupling, beta-oxidation is unable to lead to an increase in ATP production, since such an increase must also occur as a result of oxidative phosphorylation. The only way to maintain the decreased level of ATP is the production of ATP, which is not associated with the respiratory chain of mitochondria, i.e. glycolysis.

A well-known example of stimulating glycolysis is limiting the availability of oxygen. The oxidation of fatty acids can produce more ATP than the oxidation of glucose (for the same substrate weight), however, the oxygen consumption for obtaining a unit of ATP during beta-oxidation is higher due to the higher ratio of $FADH_2$/NADH. Glucose gives 5.19 kcal/L of oxygen, fatty acids—4.81 kcal/L of oxygen [42]. It is known that with an increase in physical activity, when the need for ATP increases, the metabolism of athletes switches from burning lipids to burning carbohydrates.

Perhaps the limitation of oxygen availability is relevant to our case of growing cells in atmospheric oxygen.

Cell biologists usually think in the following way. Since the concentration of oxygen in the air is always higher than in the body, cells in a thermostat with atmospheric air encounter an excess, not a lack of oxygen [43]. However, due to the low solubility of oxygen in the medium and, mainly, the high diffusion barrier (a several mm thick layer of medium), the rate of oxy-

gen consumption by cells at the bottom of the culture flask can exceed the entry rate [44]. It can be assumed that under our conditions (taking into account increased respiration due to uncoupling), cells may be in limited oxygen, therefore, the transition of energy metabolism towards glycolysis is natural.

(2) Another option to protect against excess FFA is to pack the FFA into triglycerides for further storage. If the corresponding genes are expressed in the cell, then this option is preferable [45]. In our experiments, all cells accumulated (but to varying degrees) lipids in the form of lipid bubbles – most significantly liver cells, then kidney and muscle cells. Epidermal cells and fibroblasts did this to a lesser extent.

(3) In response to the addition of FFA, the expression of UCP2 increases in cells (according to both our data and published data) [46–48]. The mechanism of uncoupling is activated, respiration is enhanced, and an enhanced combustion of any available substrate, including the end products of beta-oxidation of fatty acids, occurs, thereby reducing the concentration of FFA. It is assumed that this mechanism works in adipocytes and prevents the entry of FFA into free circulation [49].

(4) Utilization of FFA through autophagy. We found no experimental evidence for direct utilization of FFA by autophagy. It is known that lipophagy (a variant of autophagy) degrades the lipid vesicle material, but not the FFA [50]. In this case, there is no fusion of lysosomes with lipid vesicles. First, an autophagosome is formed on the basis of a lipid vesicle, and only then fusion with a lysosome occurs [51]. In this case, FFA are directly consumed during the growth of phagophores. The synthesis of phospholipids occurs in situ [52].

We can assume that due to low solubility, FFA begin to interact with each other, forming supramolecular complexes resembling micelles [3], which can no longer enter into reactions characteristic of FFA. Such complexes are probably recognized by the cell as garbage and enter the lysosomes.

The switch of energy metabolism from mitochondrial to glycolytic metabolism and vice versa takes place during development. Therefore, an artificial influence on this process (metabolic reprogramming) may be important in the treatment of certain disorders. For example, the effect of uncouplers rearranges metabolism, which can positively affect life expectancy and the course of many pathologies [14, 26].

In recent years, it has become obvious that the rearrangement of metabolism from oxidative to glycolytic functions as a regulator of the differentiation of cells of the immune system [53–55].

Classic activated macrophages (type M1) have glycolytic metabolism. They target microbes and support inflammation. The metabolism of alternative, or regulatory, macrophages (M2) is based on mitochondrial oxidation. These macrophages are observed in the microenvironment of tumors, and they reduce inflammation [56].

It is known that tumors create an immunosuppressive environment around themselves. It is possible that the basis for this is the locally enhanced production of lactate, which inhibits glycolysis and reprograms macrophages into the M2 regulatory type [57, 58]. The anti-inflammatory effects [58–61] and regenerating angiogenic effect of lactate have been described many times [62]. It is possible that epigenetic mechanisms are involved in lactate regulation [63–65].

The medical use of FFA can be summarized as follows. FFA is applied (injected) locally, after which the cells that received FFA become a source of lactate for a long time. As a result, local inflammation is reduced, and the attracted macrophages have a regulatory (regenerative) effect, trying to eliminate the area of "hypoxia" in the body (the appearance of lactate is a sign of hypoxia), promoting angiogenesis. Unlike the use of uncouplers, the action of FFA allows cells to be divided into two categories: cells in which a prolonged glycolytic shift has occurred (cells that are trying to get rid of excess FFA by uncoupling), and cells, which, responding to the production of lactate, reduce the level of inflammation (migrating cells of the immune system). Under the action of low molecular weight uncouplers, glycolytic shift can have a pro-inflammatory effect, since it will be applicable to most cells in the body.

The issue of the therapeutic use of FFAs is extremely difficult, since the toxic effects of FFAs have been described many times [66, 67], an increased concentration of FFAs in the blood plasma is considered as a poor prognostic sign accompanying obesity, type II diabetes mellitus, and atherosclerosis. This problem needs additional research.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare they have no conflict of interest. This study does not contain any research involving humans or animals as research objects.

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