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# MAXIMAL OXIDATIVE CAPACITY OF NK/LY LYMPHOMA CELLS UPON GLUCOSE, PYRUVATE AND GLUTAMINE OXIDATION

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Respiration and oxidative phosphorylation of cancer cells are substantially determined by energy substrates and oxygen availability, as well as by intrinsic mechanisms of metabolism regulation. The aim of the study was to establish the maximal mitochondrial capacity of intact NK/Ly cells to oxidize important exogenous energy substrates – glucose, pyruvate and glutamine. After 15 min of incubation, cells were placed into the respiratory chamber. Oxygen consumption rate was determined using YSI 5300 Biological Oxygen Monitor with Clark oxygen electrode at 37°C. To assess the maximal oxidative capacity of cells the respiration was stimulated with different concentrations of uncoupling agent cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP, 0.25–2 μM). The maximal stimulation of respiration was achieved with 0.5 or 1 μM FCCP, depending on the oxidative substrate. Glucose or pyruvate significantly and substantially elevated the respiration stimulated with 0.5–1.5 μM FCCP or 0.5–2 μM FCCP, respectively. Respiration rate and maximal oxidative capacity did not change significantly upon glutamine oxidation compared to control. The obtained data suggest that glucose and pyruvate might be an energy fuel for mitochondria of NK/Ly cells, whereas glutamine may be used as a precursor for intensive synthetic processes.

*Keywords:* lymphoma, mitochondria, glucose, pyruvate, glutamine, maximal oxidative capacity.

Cancer cells are characterized by a specific choice of substrates for intensive catabolic and synthetic processes. Most cancer cells even in aerobic conditions utilize glycolytic pathway as a main energy source, which is generally named “Warburg effect”. This leads to excessive lactate production and acidification of cancer cells. From other hand, glycolisis has much less energy efficiency (two ATP molecules per one glucose molecule) compared to complete glucose cleavage to $CO_2$ and $H_2O$ (30–32 ATP molecules per one glucose molecule). Thus, even minor changes in mitochondrial respiration or in coupling of respiration and oxidative phosphorylation might significantly shift metabolism of cancer cells. However, “Warburg effect” is not characteristic of all cancer cell lines [12]. Thus, understanding of tumor cells aerobic oxidative processes might prove to be important in future cancer therapy.

NK/Ly lymphoma is an experimental model of murine malignant tumor. This cell line is convenient for investigation of metabolism peculiarities of tumor cells due to its short growth period (~ 24–26 days), large amount of cells (100–150 millions/ml; 6–10 ml of ascitic fluid drained usually), and intoxication emergence only on a terminal stage of development. This model is used also for anti-tumor drugs testing [15, 21, 22], including nanoparticle carriers evaluation in chemotherapy [5]. Energy metabolism of this tumor line, substrate transport and utilization were not studied in detail yet.

Ensuring mitochondrial substrate oxidation process is critical to maintain the required level of ATP synthesis. Uptake of di- and tricarboxylic Krebs cycle substrates and their derivatives,

alongside with glucose and aminoacids, may play an important role in an activation of mitochondrial catabolic processes. Analysis of substrate-dependent respiration provides important insight into energetic processes in tumor cell metabolism [12, 16, 33]. Evaluation of maximal oxidative capacity of mitochondria *in situ*, i.e. mitochondria are preserved in their natural milieu, is a promising approach to study functional state of these organelles in norm and in pathological states [6, 30]. Horbay et al. [14] studied permeabilized NK/Ly cells and showed marked increase of respiration rate in presence of α-ketoglutarate and succinate. In that study authors used digitonin-treated plasma membrane permeabilization technique, to facilitate transport of energy substrates into mitochondria. Today no natural mechanisms of Krebs cycle substrates plasma membrane transport are known for intact NK/Ly cells. In our previous study [17], it was established that methyl ester of α-ketoglutarate apparently freely penetrates the plasma membrane causing an increase in the maximal FCCP-stimulated respiration rate of NK/Ly cells, whereas non-methylated α-ketoglutarate as well as succinate did not influence the mitochondrial oxygen consumption.

The aim of the study was to establish the maximal mitochondrial capacity of intact NK/Ly cells to oxidize important exogenous energy substrates – glucose, pyruvate and glutamine. To assess the maximal oxidative capacity, an uncoupling agent carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) was used, which effectively dissipate the mitochondrial membrane and stimulate maximal activity of respiratory chain [13, 24–26].

## Materials and methods

Experiments were conducted on white wild-type male mice (20–30 g). All manipulations with animals were done in accordance with European Convention for the Protection of Animals (1998) and Law of Ukraine "On Protection of Animals from Cruel Handling" (2014). Animals were kept in standard vivarium conditions at constant temperature on mixed ration. Ascites tumor cells were passaged by intreperitoneal inoculation of $10–15\times10^6$ cells to mice. Ascites was drained from the abdominal cavity of anaesthetized mice with sterile syringe on 7–10 day after inoculation. Cells were counted in haemocytometer. The plasma membrane integrity was assessed with trypan blue staining (> 99% trypan-negative cells). Cell suspension was stored in closed test-tube at room temperature.

NK/Ly cells were incubated for 15 min at 37°C in basic extracellular medium (control) comprising of (in mM): NaCl – 140.0, KCl – 4.7, $CaCl_2$ – 1.3, $MgCl_2$ – 1.0, HEPES–10.0; pH 7,4. The medium was supplemented with glucose (10 mM), pyruvate (2 mM) or glutamine (2 mM). Cells (~10 millions) were then introduced into the respiratory chamber. Respiration rate was determined using YSI 5300 Biological Oxygen Monitor with Clark oxygen electrode at 37°C. To reveal the maximal oxidative capacity of cells the respiration was stimulated with different concentration of FCCP (0.25, 0.5, 1, 1.5 and 2 μM in respiratory chamber).

The cell suspension was continuously stirred with propeller stirrer [26]. Respiration rate calculation was based on oxygen solubility in basic medium (200 nmol $O_2$ in 1 ml) [26]. Each experiment was repeated by six times with groups of cells, obtained from different animals (n=6). The statistical calculations were performed using Microsoft Office Excel. Data are presented as M±m. Statistical significance of difference between two samples (P) was determined by paired t-test [9].

## Results and discussion

The intensity of substrate oxidation in FCCP-uncoupled mitochondria of intact NK/Ly cells was investigated. To allow the sufficient substrate accumulation within cells, they were incubated for 15 minutes at 37°C with glucose, glutamine or pyruvate. The cell suspension was then placed into respiration chamber and basal respiration was registered for ~2 minutes. Afterwards

FCCP was added in increasing concentrations since 0.25 to 2 μM to reach the maximal respiratory capacity of mitochondria (Fig. 1).

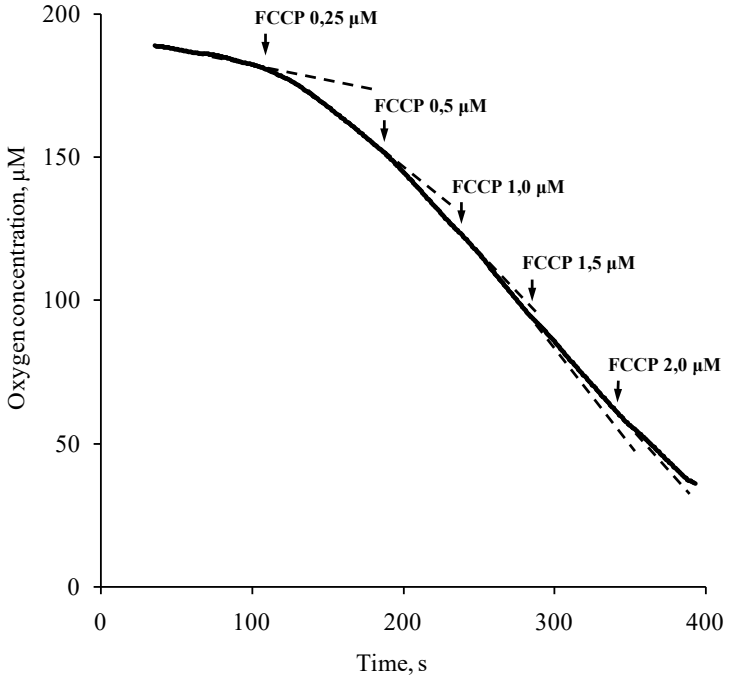


Fig. 1. Original recording of NK/Ly cells respiration stimulated with FCCP. Glucose (10 мМ) was used as oxidative substrate. For better estimation of the respiratory rates, dashed lines were applied to extrapolate cell respiration tracks.

Basal respiration rate of NK/Ly cells did not change upon incubation with oxidative substrates. The maximal oxidative capacity was observed with 0.5 μM FCCP in control and with 1 μM FCCP when exogenous substrates were added. At higher FCCP concentrations (1.5 and 2 μM) respiration rate gradually decreased. FCCP-stimulated respiration was markedly higher when glucose or pyruvate was present in the medium. Significant differences comparing to control were observed with 0.5–2 μM FCCP (Fig. 2, A and B, $P \leq 0.05$, n=6). Maximal oxidative capacity upon pyruvate or glucose oxidation was 0.078±0.009 and 0.079±0.008 nmol $O_2$/(s$10^6$ cells) respectively, being ~55% higher than control value. It is interesting that glutamine did not influence the uncoupled respiration rate of NK/Ly cells compared to control (Fig. 2, B).

Thus, FCCP-stimulated respiration rate significantly increased upon glucose oxidation. This could be explained by extensive needs in glucose in tumor cells. It is well-established that glucose is transported by transmembrane protein carriers of GLUT family, found in most mammalian cell types [3] including many B-cell lymphomas [31]. There is no data for glucose transport in NK/Ly cells, but the most common molecular glucose transporters in cancer cells are GLUT1 and GLUT3 [1, 19, 23]. Considering the well-known Warburg effect, where cancer cells tend to produce lactate even in aerobic conditions, the high capacity of NK/Ly cells to metabolize glucose completely is surprising. In addition, the end-product of glycolysis, pyruvate, is also strongly stimulating the uncoupled respiration rate. The pyruvate MCT transporter [23] is present in plasma membrane of many tumor cells [1, 28, 29]. Activity of this transporter correlates with intensity of tumor growth and Warburg effect [10]. It is also quite possible that the liquid ascitic

NK/Ly tumor is not very hypoxic [20] and, thus, oxidative processes play important role in energetic supply alongside with glycolysis.

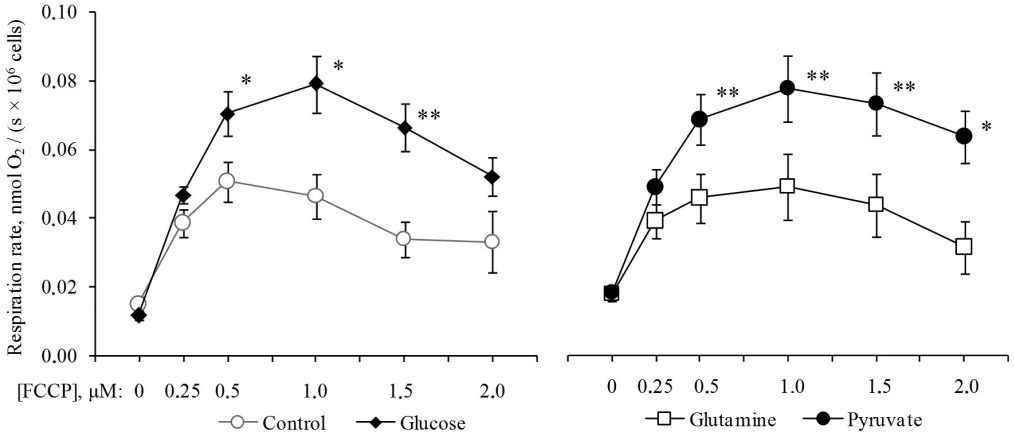


Fig. 2. Dependence of FCCP-uncoupled NK/Ly cells respiration on oxidative substrate: glucose (10 mM), pyruvate (2 mM) or glutamine (2 mM); no exogenous substrate was present in control; M±m, n=6; * – P≤0.05; ** – P≤0.01 comparing to control.

Glutamine transport across the plasma membrane is carried out by many types of proteins including SLC1,6,7, and 38 transporters; $Na^+$-cotransporters B0AT1, SNAT1,2,3,5 and 7; antiporters ASCT2, LAT1 and 2 [27]. Glutamine did not increase FCCP-stimulated respiration rate, indicating either low plasma membrane permeability for glutamine (low expression/activity of transporters [27]) or low extent of glutamine oxidation in mitochondria of NK/Ly cells. Targeting glutamine transporters, expressed in wide range of cancer cell types, could be an effective anticancer therapeutic mean, at least because glutamine blood levels are decreased in tumor bearers [4, 32]. Quite possibly this amino-acid is transported into NK/Ly cells and utilized for non-oxidative processes. Glutamine is known not only as energy source for cancer cells [2], but also as an element of acid resistance [18]. When glutamine is present, tumors synthesize proteins and carbohydrates more actively by utilizing some amounts of Krebs cycle metabolites for synthetic purposes, reducing the rate of cellular respiration [7, 8]. It is then reasonable to assume that inability of glutamine to support high rate of uncoupled mitochondrial respiration is due to anaplerotic shift of metabolic pathways of NK/Ly lymphoma cells.

Therefore, mitochondria of NK/Ly tumor cells do possess great latent respiratory potential and can oxidize glucose and pyruvate in conditions of high cellular energy demand e.g. when mitochondrial membrane potential decreases. Whether NK/Ly cells utilize their oxidative phosphorylation capacity, or merely depend on glycolysis *in vivo* is a question for further consideration.

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## МАКСИМАЛЬНА ОКИСНА ЗДАТНІСТЬ КЛІТИН ЛІМФОМИ NK/LY ЗА ОКИСНЕННЯ ГЛЮКОЗИ, ПІРУВАТУ І ГЛУТАМІНУ

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У ракових клітинах процеси дихання й окисного фосфорилювання суттєво детермінуються наявністю енергетичних субстратів і кисню, а також властивими їм механізмами регуляції метаболізму. Метою роботи було встановити максимальну мітохондріальну здатність інтактних клітин NK/Ly окиснювати найбільш важливі екзогенні енергетичні субстрати – глюкозу, піруват і глутамін. Після 15-хвилинної інкубації 10 млн пухлинних клітин вносили у полярографічну комірку. Швидкість споживання кисню визначали полярографічним методом за допомогою установки, зібраної на базі електрода Кларка за температури 37°С. Максимальну окисну

здатність мітохондрій клітин лімфоми NK/Ly досліджували за стимуляції дихання клітин різними концентраціями протонофора ціанід-4-(трифлуорометокси)феніл-гідразону (FCCP, 0,25–2 мкмоль/л). Встановлено, що максимальну стимуляцію дихання викликали концентрації FCCP 0,5 і 1 мкмоль/л, залежно від субстрату окиснення. Швидкість дихання і максимальна окисна здатність за окиснення глутаміну достовірно не відрізнялися від контролю. У процесі окиснення пірувату і глюкози швидкість стимульованого FCCP дихання достовірно та суттєво зростала за концентрацій протонофора 0,5–1,5 мкмоль/л, а у процесі окиснення пірувату – і за 2 мкмоль/л. Отримані результати свідчать про те, що глюкоза і піруват можуть бути енергетичним паливом для мітохондрій клітин NK/Ly, на відміну від глутаміну, який може використовуватися для інтенсивних синтетичних процесів.

*Ключові слова:* лімфома, мітохондрія, глюкоза, піруват, глутамін, максимальна окисна здатність.

## МАКСИМАЛЬНАЯ ОКИСЛИТЕЛЬНАЯ СПОСОБНОСТЬ КЛЕТОК ЛИМФОМЫ NK/LY ПРИ ОКИСЛЕНИИ ГЛЮКОЗЫ, ПИРУВАТА И ГЛУТАМИНА

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Дыхание и окислительное фосфорилирование в раковых клетках существенно определяются наличием энергетических субстратов и кислорода, а также присущими им механизмами регуляции метаболизма. Целью работы было установить максимальную митохондриальную способность интактных клеток NK/Ly окислять наиболее важные экзогенные энергетические субстраты – глюкозу, пируват и глутамин. После 15-минутной инкубации 10 млн клеток вносили в полярографическую ячейку. Расход кислорода определяли полярографическим методом с помощью установки, собранной на базе электрода Кларка при температуре 37°C. Максимальную окислительную способность митохондрий лимфомных клеток NK/Ly исследовали, стимулируя использование кислорода в клетках различными концентрациями протонофора цианид-4-(трифлуорометокси)фенилгидразона (FCCP, 0,25–2 мкмоль/л). Установлено, что максимальную стимуляцию дыхания вызывали концентрации FCCP 0,5 и 1 мкмоль/л, в зависимости от субстрата окисления. В процессе окисления пирувата и глюкозы скорость стимулированного FCCP дыхания достоверно и существенно возрастала при концентрациях протонофора 0,5–1,5 мкмоль/л, а в процессе окисления пирувата – и при 2 мкмоль/л. Скорость дыхания и максимальная окислительная способность при окислении глутамина достоверно не отличались от контроля. Полученные результаты свидетельствуют о том, что глюкоза и пируват могут быть энергетическим топливом для митохондрий клеток NK/Ly, в отличие от глутамина, который предположительно может использоваться как субстрат для синтетических процессов.

*Ключевые слова*: лимфома, митохондрии, глюкоза, пируват, глутамин, максимальная окислительная способность.