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ФІЗІОЛОГІЯ ЛЮДИНИ І ТВАРИН

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THE EFFECTS OF AMMONIA AND GLUTAMINE ON MITOCHONDRIAL RESPIRATION OF RAT PANCREATIC ACINAR CELLS

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During glutamine catabolism is produced ammonia, which can be toxic to cells. In hepatic encephalopathy neuron mitochondria ammonia causes the formation of free radicals, the opening of the mitochondrial permeability transition pore, oxidative phosphorylation disruption and swelling. It is still unknown whether the utilization of glutamine in the mitochondria of acinar cells of the pancreas produces toxic concentrations of ammonia. The experiments were performed on male Wistar rats weighing 250–300 g. Pancreatic acini were isolated using collagenase. Cells were incubated for 30 min with glucose (10 mM) in the control and additionally NH_4Cl (5 mM) or glutamine (2 mM) in the experiment. Acetylcholine (10 μM) or cholecystokinin (0.1 nM) was used to stimulate secretion. Respiration rate of isolated rat pancreatic acini was measured using a Clark electrode. Maximum respiration rate was stimulated by addition to the FCCP. Statistical significance (P) of difference between the groups was determined with two-way repeated-measures ANOVA followed by a Holm-Bonferroni corrected post-hoc t tests. The secretagogues acetylcholine and cholecystokinin did not affect basal and FCCP-stimulated respiratory rate. The basal respiratory rate of pancreatic acinar cells decreased with NH_4Cl compared to the basal respiratory rate with glucose oxidation, and this decrease was observed both at normal condition and under the action of secretagogues. Glutamine did not affect basal respiratory rate. During glutamine oxidation, the maximum respiratory rate increased compared to the control, regardless of the effect of acetylcholine or cholecystokinin. NH_4Cl reduced the maximum rate of FCCP-stimulated respiration in rest or upon stimulation with secretagogues compared to glucose control. Therefore, NH_4Cl causes a negative effect mitochondrial respiration regardless of secretory stimulation with acetylcholine or cholecystokinin. The toxic amount of ammonia required for inhibition of mitochondrial respiration is apparently not formed due to glutamine oxidation even when stimulated by acinar cells by secretagogues.

Keywords: pancreatic acini, ammonia, glutamine, acetylcholine, cholecystokinin, respiration, mitochondria

Introduction

Central physiological roles of pancreatic acinar cells are synthesis, transport, storage and secretion of digestive enzymes. The energy required for these processes is generated in the mitochondria. Mitochondria transform chemical energy from substrate oxidation into an electrochemical proton gradient across their inner membrane (ΔΨm) [18]. Bile acids, ethanol and non-oxidative ethanol metabolites damage the exocrine pancreas via calcium (Ca^{2+}) toxicity and mitochondrial injury [21].The abnormal Ca^{2+} signal promotes acinar cell necrosis by mitochondria depolarization and lowering ATP levels. Supraphysiologic concentrations of cholecystokinin

(CCK) and its analogues use in models of acute pancreatitis [17]. Experiments on isolated pancreatic mitochondria showed that Ca^{2+} directly depolarizes mitochondria by opening the mitochondrial permeability transition pore (MPTP). CCK-induced mitochondrial depolarization significantly reduces ATP levels in acinar cells and leads to necrosis [26].

A case of acute pancreatitis and hyperammonemia without liver damage in a patient with a deficiency of the urea cycle enzyme, ornithine carbamoyltransferase, has been described [20]. Ammonia causes the formation of free radicals, the disclosure of MPTP, oxidative phosphorylation and swelling of the neuron's mitochondria of hepatic encephalopathy patients [32]. Ammonia affects the basal and stimulated secretion of pancreatic acinar cells [13].

Glutamine plays a pleiotropic role in cellular functions. Glutamine as a "nitrogen shuttle" helps to protect the body against the toxic effects of high circulating levels of ammonia [11]. Glutamine is catabolized to glutamate and then to α-ketoglutarate by deamination via glutaminase and glutamate dehydrogenase. Decrease of glutamine serum levels was shown in pancreatitis patients during the metabolic stress [3, 11]. Intravenous administration of glutamine increased the concentration and total amount of glutamate secreted by acinar cells of the pancreas into pancreatic juice. Simultaneously with these processes, a large amount of ammonia is produced, which can be toxic to cells [4].

Pancreas uses large amount of absorbed glutamine for the synthesis of digestive enzymes [28]. The effect of glutamine on the functioning of the pancreatic acinar cells are controversial. Tissue cultures have a high requirement for glutamine. In 1955 it was discovered that glutamine deficiency in the incubation medium leads to cell death [8]. There is a difference in the degree of CCK-induced necrosis of pancreatic acinar cells between studies that used [10, 31] or did not use glutamine in the incubation medium [7]. Also, it has been reported that early administration of alanyl-glutamine dipeptide supplements (20 g per day or 0.40 g/kg per day) reduced morbidity and mortality in patients with severe acute pancreatitis [9, 33]. But in contrast, another study did not show a significant effect of enteral glutamine supplements (0.57 g/kg per day) on the development of infected necrosis and in-hospital mortality in patients with severe acute pancreatitis [5].

It is still unknown whether the utilization of glutamine in the mitochondria of pancreatic acinar cells can form toxic concentrations of ammonia and as this process depends on [Ca^{2+}]. The aim of our study was to determine how the oxidative capacity of mitochondria changes under the influence of ammonia and oxidation of glutamine under normal conditions and by stimulating the secretion of acinar cells in the pancreas of rats.

Materials and Methods

Experiments were carried out on 5 male Wistar rats weighing 250–300 g. The animals were kept at a constant room temperature with a 12-hour light cycle, with free access to water and standard food (D-Mix, Ukraine).

Protection of Vertebrate Animals used for Experimental and other Scientific Purposes' (Council of Europe No 123, Strasbourg 1985). Experimental protocols were approved by the Animal Care and Use Committee of Ivan Franko National University of Lviv.

Suspension of isolated acinar pancreatic cells was obtained using collagenase (type IV, 0.2 mg/ml), as previously reported [2]. The cells were calculated using a hemocytometer. Cell viability after isolation was> 93 %, as assessed by trypan blue test. All chemicals were purchased from Sigma-Aldrich unless otherwise noted.

The basic extracellular solution contained (mM): NaCl – 140.0, KCl – 4.7, $CaCl_2$ – 1.3, $MgCl_2$ – 1.0, HEPES – 10.0, glutamine – 2.0, sodium pyruvate – 2.0, glucose – 10.0; BSA – 2.5 mg/ml, soybean trypsin inhibitor – 0,1 mg/ml and the addition of basic amino acids MEM;

pH 7.4. The cells incubation medium for the respiration study contained (mM): NaCl – 140.0, KCl – 4.7, $CaCl_2$ – 1.3, $MgCl_2$ – 1.0, HEPES – 10.0, glucose -10.0; BSA – 2.5 mg/ml, soybean trypsin inhibitor – 0.1 mg/ml, and additionally glutamine – 2.0 or NH_4Cl – 5.0 (mM) in some experiments.

The rate of oxygen consumption was measured using a Clark oxygen electrode (biological oxygen monitor YSI 5300, USA) in a closed glass respiration chamber (volume of 1.6 ml at 37 °C). Before measuring respiration rate the suspension of isolated pancreatic acinar cells were preincubated for 30 minutes at 37 °C in the medium only with glucose, glutamine and glucose or NH_4Cl and glucose or additionally with secretagogues. Protonophore FCCP in increasing concentrations (0.5–2.0 μM) was added to the cell suspension directly into the polarographic chamber, to achieve the maximum frequency of uncontrolled respiration, as described previously [22]. Respiration rate was first normalized by cell number and scaled relative to the mean basal respiration rate in the control.

Results are presented as means ± SEM. Statistical analysis was performed using Origin Pro 2018. Significance of difference between the groups was determined with two-way repeated-measures ANOVA followed by a Holm-Bonferroni corrected post-hoc t tests in case of significant interaction between the factors. $P<0.05$ values were considered statistically significant.

Results and Discussion

It is known that high viability pancreatic acinar cells respond to the stimulation of secretagogues by increasing respiratory rate [23]. To test the functional ability of cells to respond to stimulation by secretagogues, acetylcholine (Ach) (10 μM) was injected into the polarographic chamber. Basal respiration rate was assessed for 4 minutes. Adding ACh to the polarographic chamber intensified pancreatic acinar cells respiration rate during 1 min after administration ACh, by ~ 16 %, compared with basal respiration rate.

The next step was to evaluate the effects of ammonia and glutamine on mitochondrial respiration. In the control, the acinar cells of the pancreas were incubated in the basic extracellular medium with glucose (10 mM). In the experiments, NH_4Cl (5 mM) or glutamine (2 mM) was added to the incubation medium with glucose. ACh (10 μM) or CCK (0.1 nM) was added to the solution for secretion stimulation. The cells were incubated for 30 min at 37 °C. After that, the basal respiration rate was registered. Maximal respiration rate was stimulated by adding to the cell FCCP at a concentration of 0.5; 1; 1.5; 2.0 μM.

ACh ad CCK did not affect basal and FCCP-stimulated respiratory rate (Fig. A). It is likely that the incubation time was too long for the stimulating effect of secretagogues to persist. The peak of secretion by acinar cells of the pancreas occurs in the first 5 min at optimal (average) concentrations of secretagogues [27]. There is evidence that ACh and CCK intensified maximal respiration rate during 15 min of incubation, but pyruvate was necessarily present in the medium [1].

The basal respiration rate of acinar cells under the influence of NH_4Cl decreased by 13.1–20.2 % ($p<0.05$) compared with the respiration rate for glucose oxidation, and this decrease was observed both at normal condition or under the action of secretagogues. Glutamine did not affect basal respiratory rate (Fig. A).

During glutamine oxidation, the maximum respiratory rate increased by 7.6–40.4 % ($p<0.05$) compared to the control, regardless of the effect of ACh or CCK, which is similar to the results of our previous studies [22]. NH_4Cl reduced the maximum rate of FCCP-stimulated respiration under the influence of control, with the addition of ACh or CCK by 24.8–31.0 % ($p<0.05$) compared with the control of glucose oxidation (Fig. B).

Mechanism of ammonia toxicity largely studied on brain tissues, because hyperammonemia is directly linked to a spectrum of neuropathology conditions. Hyperammonemia inhibits the

activity of mitochondrial dehydrogenases, which causes the collapse of the membrane potential of mitochondria and increase of ROS levels in isolated mitochondria of the liver and brain [25, 30]. Ammonia in pathological concentration (2 mM) is a potent inhibitor of the mitochondrial complex of brain α-ketoglutarate dehydrogenase. At toxic concentrations (10–20 mM), ammonia inhibits cerebral mitochondrial NAD (+) – and NADP (+) – bound isocitratedehydrogenase and NAD (+) - bound malatedehydrogenase and hepatic mitochondrial NAD (+) – bound isocitratedehydrogenase [16]. Under the influence of ammonia was shown decrease in the level of intermediate products of the cycle citric acid cycle (CAC), citrate, α-ketoglutarate and malate, in the mitochondria of the liver [32]. Instead, low concentrations of ammonium chloride (> 1 mM) stimulate the production of glucose from glutamine in mitochondria or isolated cells of the liver [15]. Ammonia also induces morphological abnormalities of mitochondria, the opening of MPTP, leading to mitochondrial swelling and cell death by apoptosis or necrosis [32].

Hyperammonemia is usually defined as plasma ammonia levels above 80 μM in newborns and above 45 μM in adults [19, 29]. In experiments with isolated mitochondria of the brain and liver, ammonia was used in pathologically significant concentrations of 5–10 mM [25]. In our study, we studied the effect of NH_4Cl at a concentration of 5 mM. Ammonia homeostasis is a multi-organ process involving the liver, brain, kidneys and muscles, as well as the gastrointestinal tract. Under normal conditions, ammonia from the gut is efficiently processed by the liver through two main metabolic pathways: the urea cycle (also known as the ornithine cycle) and glutamine synthetase, which converts glutamate to glutamine [30]. Hyperammonemia mainly occurs in hepatic encephalopathy and genetic defects of the urea cycle or other pathways of intermediate metabolism [6]. The effectiveness of the mechanisms of "detoxification" of ammonia is impaired in hepatocellular dysfunction in cirrhosis. Even in healthy patients, a sharp increase in ammonia concentration is mostly due to renal processes. Violations of potassium and acid homeostasis, lead to overproduction of ammonia by the kidneys. To support potassium balance, renal glutaminase generates an ammonium ion from glutamine that donates a proton to be exchanged across the membrane for the potassium ion. The end result is recovered potassium, acidified urine, and ammonia, a by-product that diffuses into the serum [30]. In patients with liver cirrhosis ammonia affects the endocrine pancreas causing an imbalance of insulin and glucagon, and as a consequence, amino acids in the plasma [24]. Hyperammonemia was associated with infected necrotic acute pancreatitis in patient with late-onset ornithine carbamoyltransferase deficiency [20].

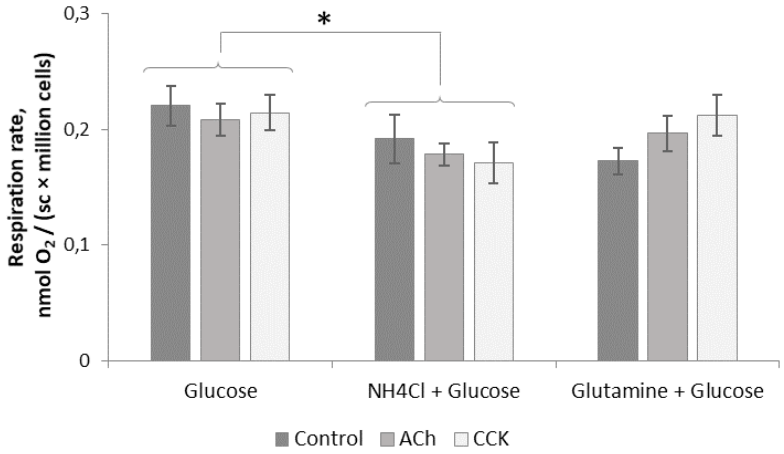
Our results confirmed that ammonia in pathological concentrations adversely affects the respiratory processes of the mitochondria of the pancreas. In our study, ammonia reduced the adaptive capacity of mitochondria, regardless of the influence of secretagogues. This can be explained by the fact that the pathological effect of ammonia is mediated by an increase in the level of cytoplasmic [Ca^{2+}] [12, 14]. Mitochondrial accumulation of Ca^{2+} impairs mitochondrial respiration, decreases ATP synthesis and increases the formation of free radicals, which lead to greater oxidative stress. An additional increase in cytoplasmic [Ca^{2+}] under the action of ACh or CCK did not increase the toxic effects of ammonia.

Glutamine is a multifunctional amino acid. In addition to protein syntesis, glutamine is an anaplerotic substrate for CAC. Mitochondrial glutamine is catabolized to glutamate by the amidohydrolase enzymes, which catalyze the conversion of glutamine to glutamate by releasing ammonium ions. Then mitochondrial glutamate is converted to α-ketoglutarate by glutamatedehydrogenase 1 or several mitochondrial aminotransferases. Mitochondrial α-ketoglutarate can participate in CAC by supporting the oxidative phosphorylation pathway or the reductive carboxylation pathway. During oxidative phosphorylation, glutamine metabolites are involved in the

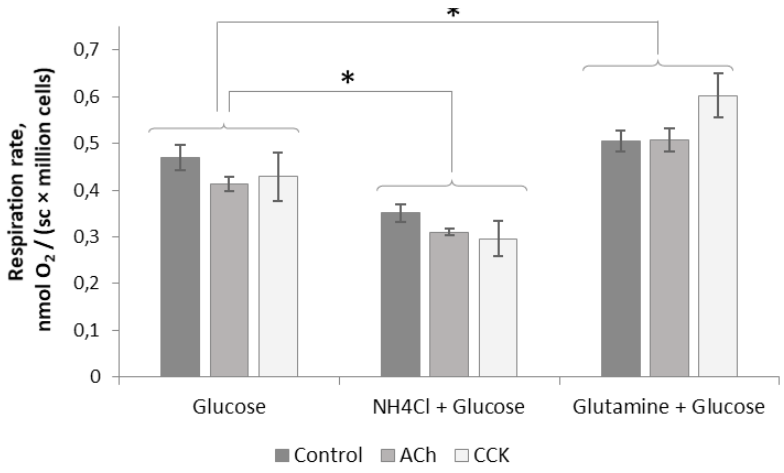
generation of electron donors, such as NADH or $FADH_2$, and the synthesis of GDF and ATP. The active metabolism of glutamine in the mitochondria of brain astrocytes by phosphate-activated glutaminase leads to the hyperproduction of ammonia [30]. But, as our study shows, glutamine catabolism in the pancreas promotes to increased adaptability of mitochondria, even under increased [Ca^{2+}].

Based on the results of our study, incubation of isolated acinar cells of the pancreas with NH_4Cl causes a negative effect on mitochondria regardless of stimulation with ACh or CCK. The toxic amount of ammonia required for such adverse effects is apparently not formed due to glutamine oxidation even when stimulated by acinar cells by secretagogues.

A)



B)



The effects of oxidative substrates and NH_4Cl on basal **(A)** and maximal uncoupled respiration rate **(B)** of isolated pancreatic acini; cells were pre-incubated in basic extracellular solution (30 min) with substrates and secretagogues; FCCP were added into the respiratory chamber; [glucose] = 10 mM, [glutamine] = 2 mM, [NH_4Cl] = 5 mM, [Ach] = 10 μM, [CCK] = 0.1 nM; two-way repeated-measures ANOVA and post-hoc Holm-Bonferroni corrected t-test: * – $p<0.05$, # – significant comparing with control (no ACh or CCK), $p<0.05$; M±S.E.M., n=5

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ВПЛИВ АМІАКУ І ГЛУТАМІНУ НА ДИХАННЯ МІТОХОНДРІЙ АЦИНАРНИХ КЛІТИН ПІДШЛУНКОВОЇ ЗАЛОЗИ ЩУРІВ

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За катаболізму глутаміну утворюється аміак, який може бути токсичним для клітин. За печінкової енцефалопатії у мітохондріях нейронів аміак спричиняє утворення вільних радикалів, розкриття мітохондріальної пори транзієнтної проникності, порушення окисного фосфорилювання та набряк. Досі невідомо, чи за утилізації глутаміну в мітохондріях ацинарних клітин підшлункової залози утворюються токсичні концентрації аміаку. Досліди виконували на щурах самцях лінії Вістар масою 250–300 г. Панкреатичні ацинуси ізолювали з використанням колагенази. Клітини інкубували упродовж 30 хв з глюкозою (10 ммоль/л) у контролі й додатково вносили NH_4Cl (5 ммоль/л) або глутамін (2 ммоль/л) – у досліді. Для стимуляції секреції використовували ацетилхолін (10 мкмоль/л) чи холецистокінін (0,1 нмоль/л). Дихання ізольованих панкреатичних ацинусів щурів вимірювали за допомогою електрода Кларка. Максимальну швидкість дихання стимулювали додаванням у FCCP. Статистичну вірогідність (P) різниці між середніми арифметичними оцінювали парним t-тестом або двофакторним дисперсійним аналізом із повторами та post-hoc тестом Холм-Бонферроні. Секретагоги ацетилхолін і холецистокінін не впливали на базальну та FCCP-стимульовану швидкістю дихання. Базальна швидкість дихання ацинарних клітин підшлункової залози за впливу NH_4Cl знизилася порівняно з базальною швидкістю дихання за окиснення глюкози, причому це зниження спостерігалось як у стані спокою, так і за дії секретагогів. Глутамін не впливав на базальну швидкість дихання. За окиснення глутаміну максимальна швидкість дихання зросла порівняно з контролем, незалежно від впливу ацетилхоліну чи холецистокініну. NH_4Cl знижував максимальну швидкість FCCP-стимульованого дихання як у спокої, так і за стимуляції секретагогами порівняно з контролем за окиснення глюкози. Отже, NH_4Cl негативно вливає на дихання мітохондрій незалежно від стимуляції ацетилхоліном або холецистокініном. Токсична кількість аміаку, необхідна для пригнічення дихання мітохондрій, очевидно, не утворюється через окиснення глутаміну навіть за стимулювання ацинарних клітин секретагогами.

Ключові слова: панкреатичні ацинуси, аміак, глутамін, ацетилхолін, холецистокінін, дихання, мітохондрії