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INFLUENCE OF TAUROLITHOCHOLATE 3-SULPHATE ON ACTIVITY OF Na⁺, K⁺-ATPASE, Ca²⁺-ATPASE AND BASAL Mg²⁺-ATPASE IN RAT LIVER SUBCELLULAR FRACTION

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It is known that the monohydroxylated bile acids such as lithocholate and tauro-lithocholate markedly inhibit bile secretion. The mechanism of this inhibition has not been verified yet. In our work we have studied the effect of taurolithocholate 3-sulphate on the activity of Ca^{2+}-ATPase, Na^+/K^+-ATPase and basal Mg^{2+}-ATPase to clarify the role of effect of transport-ion pumps on taurolithocholate 3-sulphate's effect on hepatocytes. The activity of ATPases was estimated on postmitochondrial subcellular fractions of rat liver. It was found that taurolithocholate 3-sulphate (50 µM) decreased the activity of Ca^{2+}-ATPases by 1.84 fold ($P \leq 0.05$; n=5) and Na^+/K^+-ATPase by 3.67 fold ($P \leq 0.05$; n=5) in subcellular fractions of rat liver. At the same time taurolithocholate 3-sulphate increased the activity of basal Mg^{2+}-ATPase by 1.67 fold ($P \leq 0.05$; n=5). It was concluded that inhibition of bile secretion under the influence of taurolithocholate 3-sulphate observed is the result of decreased Ca^{2+}-ATPases and Na^+/K^+-ATPase activity, accompanied by increasing the concentrations of calcium and sodium in the cytosol. However, it has been suggested that activation of the basal Mg^{2+}-ATP-ase under the influence of taurolithocholate 3-sulphate may indicate a role of endo-lysosomal system, so-called acid store, in the implementation of the release of calcium. Thus transport-ion pumps (Ca^{2+}-ATPase, Na^+/K^+-ATPase and basal Mg^{2+}-ATPase) are involved in taurolithocholate 3-sulphate's effect on hepatocytes of rats.

Keywords: bile acid, taurolithocholate 3-sulphate, hepatocytes, liver, calcium, Ca^{2+}-ATPase, Na^+/K^+-ATPase, basal Mg^{2+}-ATPase.

Bile acids (BAs) are not only digestive surfactants, but also important cell signaling molecules, which stimulate several signaling pathways to regulate some important biological processes. BAs are amphipathic molecules synthesized from cholesterol in the liver. They are physiological detergents that play important roles in facilitating hepatobiliary secretion of endobiotic and xenobiotic metabolites [15]. In the intestines, BAs help intestinal absorption of dietary fats, fat-soluble vitamins, and other nutritions [11]. Over the past decade, BAs change beyond digestive surfactants to signaling molecules in a wide range of biological functions, including glucose and lipid metabolism, energy homeostasis, and the modulation of immune response [11, 18, 28] metabolism associated with liver injury, metabolic disorders, cardiovascular diseases and digestive system diseases such as inflammation bowel disease and colorectal cancer [12, 14, 16, 23]. The formation of bile is a vital function, and its impairment by drugs or infectious, autoimmune, metabolic, or genetic disorders results in the syndrome commonly known as cholestasis [7]. The secretion of bile normally depends on the function of a number of membrane transport systems in hepatocytes and bile-duct epithelial cells (cholangiocytes) and on the structural and functional integrity of the bile-secretory apparatus [7].

The monohydroxylated bile acids, including taurolithocholate (TLC) and taurolithocholate 3-sulphate (TLC-S), inhibit the secretion of bile from rat liver. This effect has been attributed to their ability to raise $[Ca^{2+}]_i$, as demonstrated in populations of rat hepatocytes [13]. BAs acti-

vate calcium entry into the cells and cause depletion of internal calcium store [20]. These BAs mobilize Ca^{2+} from the internal pool which is sensitive to inositol trisphosphate (IP_3). However, BAs-mediated Ca^{2+} release is independent of IP_3 production. The mechanism of TLC-S-induced Ca^{2+}-release was supposed mediated by another Ca^{2+}-release messenger such as nicotinic acid adenine dinucleotide phosphate (NAADP) [9]. NAADP is thought to target acidic store to release Ca^{2+}. It was previously proposed that the acidic Ca^{2+}-stores are involved in TLC-S-induced cytosolic Ca^{2+}-signals [9]. Besides it was found that NAADP decreased basal Mg^{2+}-ATPase activity of subcellular fraction rat liver [29] and it was assumed that this effect is also associated to acidic sore. Thus we supposed that basal Mg^{2+}-ATPase activity might be involved in TLC-S-induced toxic effect on cells such as apoptosis in hepatocytes [27]. It was demonstrated that BAs also inhibited Ca^{2+}-pump of endoplasmatic reticulum (SERCA) in pancreatic cells [18]. Thus role of Ca^{2+}-ATPases and basal Mg^{2+}-ATPase in the effect of TLC-S has not been estimated in hepatocytes yet. Other effects, not linked to calcium signaling, have also been observed, including the increase in intracellular Na^+ concentration [30]. Previously it was shown that TLC-S caused hyperosmolarity of hepatocytes [26] so it is possible to assume that Na^+/K^+–ATPase activity is also involved in TLC-S's effect. This is due to endosomal acidification and activation of NADPH oxidase isoforms. It has been shown that in mammalian cells extracellular messengers, such as angiotensin II [6], can activate an NADPH oxidase, and this might lead to increases in NAADP from NAADPH. NADPH oxidase may also serve to increase NADP levels that can then be converted to NAADP. NAADP is a nucleotide which can to release calcium from endo-lysosomal system or acidic store [6]. It was shown that NED-19 (antagonist of NAADP) decreased the fraction of Ca^{2+} released by TLC-S applying [9]. TLC-S-induced hepatocyte shrinkage was strongly blunted in presence of bafilomycin A1. Thus, the main purpose of this work was to estimate effect of TLC-S on activity of ATPases (Ca^{2+}-ATPase, Na^+/K^+–ATPase and basal Mg^{2+}-ATPase) in rat liver subcellular fraction.

MATERIALS AND METHODS

Isolation of a subcellular fraction. Experiments were conducted on male and female nonlinear white rats (0.18–0.2 kg). All procedures with animals were carried at in accordance with the "International Convention for working with animals" under approval of the Bioethics Committee of Biologycal Faculty (Ivan Franko National University of Lviv) (Protocol N 11/15 dated by 10.12.2015). After ether anaesthesia rats were decapitated. Isolated rat liver was perfused briefly with homogenizing medium (buffer solution) containing (mmol/L): sucrose – 250.0; EDTA – 1.0; Tris-HCl – 10.0 (pH 7.4 t=37°C). Then, chilled tissue was crushed by passing through the press. Next, buffer solution was added to the minced liver. Minced tissue with added buffer solution (in a ratio of 1:8) was homogenized using a Potter-Elvehjam teflon-glass homogenizer at a speed of 300 rev/min. Liver subcellular fractions obtained by differential centrifugation, conducting a series of successive centrifuging the mixture of organelles and membrane fragments obtained after homohenization of tissue. At the first step homogenates were centrifuged for 10 min at 3000 g (centrifuge PC-6) for the deposition of intact cells and nuclei, mitochondrial fraction precipitated for 10 minutes on a 6500 g and a temperature 0–2°C. The resulting postmitochondrial supernatant was used in the experiment as "subcellular fractions of rat liver".

Measurement of the ATPase activity. ATPase activity was determined by the measuring of orthophosphate content released after ATP hydrolysis. At the beginning of the experiment 200 μL of subcellular fraction were transferred to a standard incubation medium which contained (mmol/L) NaCI – 50.0; KCI – 100.0; Tris-HCI – 20.0; $MgCI_2$ – 3.0; $CaCI_2$ – 0.01; NaN_3 – 1; ATP – 3.0; pH 7.4 at 37°C. The reaction was started by adding 3 mM ATP (Sigma). Samples were incubated for 15 min at 37°C with moderate shaking in a water bath. Before the end of incubation

С. Бичкова

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0.4 ml of medium was taken off for the determination of protein content by the method of Lowry [21]. We used TLC-S (Sigma) at concentration 50 μmol/L for estimating their effect on ATPase activity. Reaction was stopped by adding 5 ml of 10% trichloroacetic acid to samples which were incubated during 10 min for full protein denaturation and then centrifugated during 10 min at 1600 g. Obtained supernatant was used to determine the content of inorganic phosphorus by the spectrophotometric method of Fiske–Subbarow.

The total ATPase activity of subcellular fraction was calculated as a difference between amounts of inorganic phosphorus in the media with different composition (with adding TLC-S and without TLC-S in control example). ATPase activity was expressed as micromoles of inorganic phosphorus equivalent to 1 mg of protein per 1 h. Specific Na^+/K^+-ATPase activity was calculated as difference between inorganic phosphorus contents in the media with or without ouabain (Sigma) at concentration 1 mmol/L. For the determination of Ca^{2+}/Mg^{2+}-ATPase activity, we quantified the difference between the total Ca^{2+}/Mg^{2+}- and Na^+/K^+-ATPase activities. Specific basal Mg^{2+}-ATPase activity was determined in incubational medium contained 1 mmol/L EGTA (Sigma) and lacked ouabain. In all experiments incubational medium without adding tissue was used as a control for background enzymatic ATP hydrolysis.

Data analysis. A significance of differences between experimental groups was calculated using Wilcoxon-Mann-Whitney test. P<0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

Influence of TLC-S on Na^+/K^+–ATPase activity in postmitochondrial subcellular fractions of rat liver. The basolateral plasma membrane contains the Na^+/K^+–ATPase that maintains the physiologic extracellular and intracellular ion gradients (more sodium outside the cell than inside; more potassium inside than outside). In addition, Na^+/K^+–ATPase, together with a potassium channel, helps to generate a transmembrane electrical potential of approximately –35 mV. These chemical and electrical potentials are used for the maintenance of intracellular ion and pH homeostasis. They provide the driving forces for proton extrusion by a mechanism of sodium–hydrogen exchange and for bicarbonate entry by a mechanism of sodium–bicarbonate symport, as well as for the electrogenic sodium-dependent uptake of conjugated bile salts (or BAs). BAs are the most abundant solutes in bile. Their transport from plasma into hepatocytes is predominantly mediated by the sodium-taurocholate co-transporter (NTCP) [7]. Thus no doubt that sodium transport and BAs transport has reversible correlation. It is known that TLC increases in the level of sodium in the cell [30].

It is possible to suppose that TLC-S might effect on activity of Na^+/K^+-ATPase in hepatocytes. Experiments were conducted on postmitochondrial subcellular fractions of rat liver to verify our assumption. Control samples showed activity of Na^+/K^+-ATPase about (4.32±0.79) μmol P_i/ mg protein per hour, at TLC-S (50 μM) presence in incubational medium it was equal (1.18±0.50). It was found that TLC-S decreased activity of Na^+/K^+-ATPase by 3.67 fold (P≤0.05; n=5) (See Figure). Thus, increasing in the level of sodium is accompanied with decreasing of activity of Na^+/K^+-ATPase at presence of TLC-S in incubational medium. This effect is thought to be the reason of inhibition BAs secretion by TLC-S. Thus our assumption that TLC-S might effect on activity of Na^+/K^+-ATPase was confirmed. It allows us to discuss the possible mechanism of this.

As shown by Becker and co-authors [5], TLC-S and other hydrophobic bile salts such as glycochenodeoxycholate, taurochenodeoxycholate induce hepatocyte shrinkage in perfused rat liver. Basing on our finding it is possible to assume that the mechanism of the shrinkage involve decreasing of Na^+/K^+-ATPase activity as well. Tauroursodeoxycholate (TUDC) which

is frequently used in the treatment of cholestatic liver disease reversed hyperosmolarity and triggered re-insertion of the hyperosmotically retrieved Ntcp into the membrane [26]. Apart from its choleretic effects, TUDC can protect hepatocytes from bile acid-induced apoptosis [27].

Thus our results point out another possible way to cholestatic treatment due to prevention the decreasing the Na^+/K^+-ATPase activity.

Influence of TLC-S on Ca^{2+}-ATPase activity in postmitochondrial subcellular fractions of rat liver. It is also known that TLC, as well as TLC-S, mobilizes Ca^{2+} from an intracellular pool [9, 13].Thus the main effect of TLC-S is connected to increasing calcium in hepatocytes and depletion calcium store. The TLC-S-induced cytosolic Ca^{2+} increase is known to be toxic for cells. It is probably involved in the toxicity of TLC-S. TLC-S induced apoptosis of hepatocytes due to activation of caspase-3 activity with approximately 50% [17]. The role of Ca^{2+}-pumps in this TLC-S-induced toxic effects in hepatocytes is unknown. We supposed that Ca^{2+}-ATPases are involved in action of TLC-S.

It was found that Ca^{2+}-ATPases activity in control samples was 9.70 ± 2.81 μmol P_i/ mg protein per hour and it was equal about (5.25 ± 1.44) μmol P_i/ mg protein per hour in samples incubated with adding TLC-S. We found that TLC-S (50 μM) decreased activity of Ca^{2+}-ATPases by 1.84 fold ($P\leq0.05$; n=5) in subcellular fraction of rat liver (See Figure). The influx of extracellular Ca^{2+} is balanced by Ca^{2+} removal from the cytosol by both the plasma membrane and internal store Ca^{2+}-ATPases. Common Ca^{2+}-ATPase activity of subcellular fraction consists of SERCA and Ca^{2+}-pump of plasmatic membrane (PMCA). We found that TLC-S decreased the common Ca^{2+}-ATPase activity in liver of rats. Our results are agreed with results of Kim and co-authors [18] demonstrated that bile acids cause the inhibition of SERCA, resulting in Ca^{2+} release from endoplasmatic reticulum stores in pancreatic cells.

In subcellular fraction TLC-S might cause direct and nondirect action on activity of Ca^{2+}-ATPases due to released calcium. We supposed that a contact sites between acidic organelles and the endoplasmatic reticulum exist in rat liver cells [10], which is unbroken in subcellular fraction [8]. However inhibitor effect of TLC-S probably reflects direct action which caused depletion of Ca^{2+}-store. It is important to known a type of Ca^{2+}-store realizing TLC-S-induced Ca^{2+} release. We supposed that acidic sore is also involved as well as endoplasmatic reticulum in TLC-S-induced effects. To test this assumption we estimate effect of TLC-S on basal Mg^{2+}-ATPase activity.

Influence of TLC-S on basal Mg^{2+}-ATPase activity in postmitochondrial subcellular fractions of rat liver. It should to note that activity of basal Mg^{2+}-ATPase is coupled to H^+-translocation in plasma membrane [2; 22] as well as in endosomal fraction [25]. Also in hepatocytes Mg^{2+}-ATPase is considered as markers of canalicular memebrane [4]. Previously it was found that basal Mg^{2+}-ATPase activity increased by ryanodine [3], IP_3 [1], bafilomicine A1 [8], but decreased by NAADP [29]. Thus basal Mg^{2+}-ATPase activity of subcellular fraction was thought to be associated to endo-lysosomal store (acidic sore).

Basal Mg^{2+}-ATPase activity was also estimated in rat liver subcellular fraction with TLC-S adding. It was found that basal Mg^{2+}-ATPase activity in control samples was about 15.42 ± 6.14 μmol P_i/ mg protein per hour and it was equal 25.76 ± 8.34 μmol P_i/ mg protein per hour in samples incubated with adding TLC-S. It was found that TLC-S in concentration 50 μM decreased activity of basal Mg^{2+}-ATPase increased by 1.67 fold ($P\leq0.05$; n=5) (See Figure). It is supposed that acidic store is also involved in TLC-S-induced effect.

It was found that TLC-S suppressed the activity of Ca^{2+}-ATPases and Na^+/K^+-ATPase, but increased the activity of basal Mg^{2+}-ATPase in subcellular fractions of rat liver. It is supposed that the main reasons inhibition of bile secretion by taurolithocholate 3-sulphate are inhibition of

Ca^{2+}-ATPases and Na^{+}/K^{+}-ATPase, thus increasing the concentration of calcium and sodium in the cytosol is observed. Besides, it has been suggested that activation of the basal Mg^{2+}-ATPase under the influence of taurolithocholate 3-sulphate, may indicate about a role of endo-lysosomal system, the so-called acid store, in the implementation of the release of calcium.
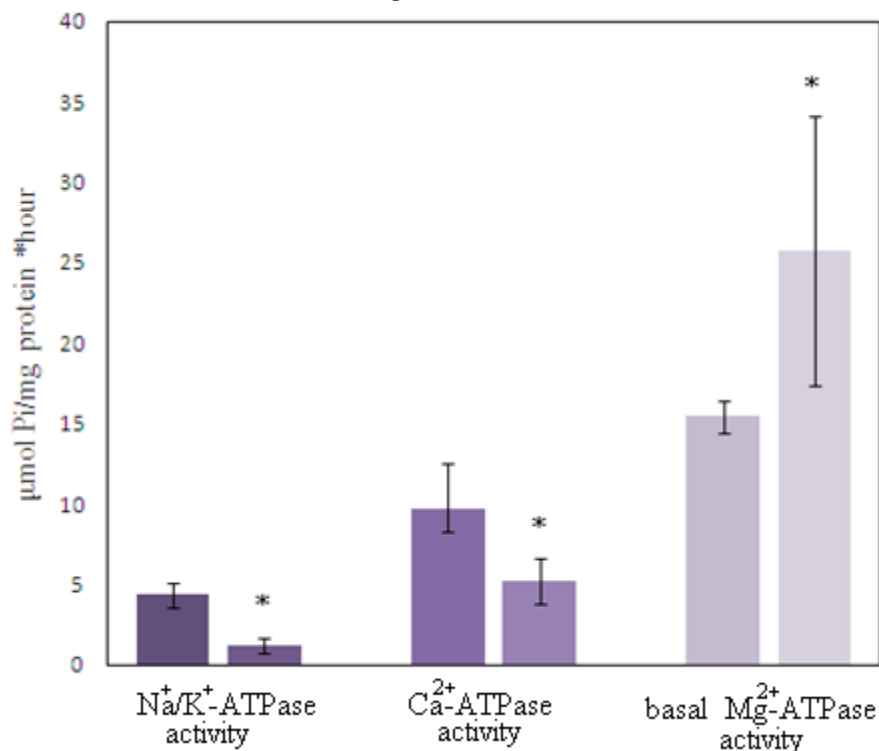
Influence taurolithocholate 3-sulphate on activity of ATPase in rat liver postmitochondrial fraction (M±m): *– P≤0.05; n=5 (*every first column is ATPase activity in control and every second column shows activity of ATPase at adding taurolithocholate 3-sulphate*).

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С. Бичкова

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ВПЛИВ 3-СУЛЬФО-ТАУРОЛІТОХОЛЕВОЇ КИСЛОТИ НА АКТИВНІСТЬ Na+, K+-АТФ-АЗИ, БАЗАЛЬНОЇ Mg²⁺-АТФ-АЗИ І Ca²⁺-АТФ-АЗ СУБКЛІТИННОЇ ФРАКЦІЇ ПЕЧІНКИ ЩУРІВ

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Відомо, що моногідроксильовані жовчні кислоти, такі як літохолева і таурохолева, суттєво пригнічують секрецію жовчі. Механізм цього явища ще не до кінця з'ясований. Встановлено, що за дії тауролітохолевої кислоти, а також її сульфату, спостерігається підвищення концентрації кальцію в цитозолі внаслідок вивільнення його з внутрішньоклітинних депо [9, 13]. Окрім того, спостерігається зростання рівня натрію в клітині [30]. Ми припустили, що ці процеси реалізуються за участю йон-транспортувальних помп. Для того, щоб перевірити наше припущення, ми вивчали вплив 3-сульфо-тауролітохолевої кислоти на активність Ca²⁺-АТФ-ази, Na+, K+-АТФ-ази та базальної Mg²⁺-АТФ-ази. Досліди проводили на постмітохондріальній субклітинній фракції печінки щурів, що унеможливлює вплив тауролітохолевої кислоти 3-сульфату на рецептори плазматичної мембрани гепатоцитів. АТФ-азну активність оцінювали за вмістом неорганічного фосфору. Встановлено, що 3-сульфо-тауролітохолева кислота пригнічувала активність Ca²⁺-АТФ-аз та Na+, K+-АТФ-ази, але підвищувала активність базальної Mg²⁺-АТФ-ази субклітинної фракції печінки щурів. Зроблено висновок, що пригнічення секреції під впливом 3-сульфо-тауролітохолевої кислоти спостерігається через інгібування роботи Na+, K+-АТФ-ази та Ca²⁺-АТФ-аз, внаслідок чого зростає концентрація кальцію та натрію в цитозолі. Поряд із тим, висловлено припущення, що активування базальної Mg²⁺-АТФ-ази під впливом 3-сульфо-тауролітохолевої кислоти може свідчити про роль ендолізосомальної системи, так званого кислого депо, у реалізації вивільнення кальцію.

Ключові слова: жовчні кислоти, 3-сульфо-тауролітохолева кислота, гепатоцити, печінка, кальцій, Ca²⁺-АТФ-аза, Na+, K+ -АТФ-аза, базальна Mg²⁺-АТФ-аза.

С. Бичкова
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201

ВЛИЯНИЕ 3-СУЛЬФО-ТАУРОЛИТОХОЛЕВОЙ КИСЛОТЫ НА АКТИВНОСТЬ Na+, K+-АТФ-АЗЫ, БАЗАЛЬНОЙ Mg2+-АТФ-АЗЫ И Ca2+-АТФ-АЗ СУБКЛЕТОЧНОЙ ФРАКЦИИ ПЕЧЕНИ КРЫС

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Известно, что моногидроксилированные желчные кислоты, такие как литохолевая и таурохолевая, существенно подавляют секрецию желчи. Механизм этого явления еще не до конца выяснен. Установлено, что при действии тауролитохолевой кислоты, а также ее сульфата, наблюдается повышение концентрации кальция в цитозоле вследствие высвобождения его из внутриклеточных депо [9, 13]. Кроме того, наблюдается рост уровня натрия в клетке [30]. Мы предположили, что эти процессы реализуются с участием ион-транспортных насосов. Для того чтобы проверить наше предположение, мы изучали влияние 3-сульфо-тауролитохолевой кислоты на активность Ca^{2+}-АТФ-азы, Na^+, K^+-АТФ-азы и базальной Mg^{2+}-АТФ-азы. Опыты проводили на постмитохондриальной субклеточной фракции печени крыс. АТФ-азную активность оценивали по содержанию неорганического фосфора. Установлено, что 3-сульфо-тауролитохолевая кислота подавляла активность Ca^{2+}-АТФ-аз и Na^+, K^+-АТФ-азы, но повышала активность базальной Mg^{2+}-АТФ-азы субклеточной фракции печени крыс. Сделан вывод, что подавление секреции под влиянием 3-сульфо-тауролитохолевой кислоты наблюдается путем ингибирования работы Na^+, K^+-АТФ-азы и Ca^{2+}-АТФ-аз, вследствие чего возрастает концентрация кальция и натрия в цитозоле. Вместе с тем, высказано предположение, что активирование базальной Mg^{2+}-АТФ-азы под влиянием 3-сульфо-тауролитохолевой кислоты может свидетельствовать о роли эндолизосомальной системы, так называемого кислого депо, в реализации высвобождения кальция.

Ключевые слова: желчные кислоты, 3-сульфо-тауролитохолевая кислота, гепатоциты, печень, кальций, Ca^{2+}-АТФ-аза, Na^+, K^+-АТФ-аза, базальная Mg^{2+}-АТФ-аза.