Momohydroxylated bile acids, including tauroliocholate (TLC) and its 3-sulphate (TLC-S), have been shown to increase \([\text{Ca}^{2+}]_i\) in cytosol of rat hepatocytes [1, 2]. These bile acids mobilize \(\text{Ca}^{2+}\) from the internal pool which is sensitive to inositol trisphosphate (IP\(_3\)). However, bile-acid mediated \(\text{Ca}^{2+}\) release is independent of IP\(_3\) production. Nicotinic acid adenine dinucleotide phosphate (NAADP) is a nucleotide which can to release calcium from specific type of intracellular store defined as endo-lysosomal system or acidic store. The aim of this study was to examine influence of NED-19 (antagonist of NAADP) on TLC-S-induced change of calcium content in cytosol of and endoplasmic reticulum of isolated mice hepatocytes in order to elucidate the role of acidic store in bile-acid mediated \(\text{Ca}^{2+}\) release. Isolated hepatocytes of mice were loaded with fluo-4 (2.5 \(\mu\)M). Fluorescent images were obtained using Leica SP2 MP dual two-photon confocal microscope. Isolated hepatocytes were permeabilized in suspension with saponine (0.1 mg/mL). Next the permeabilized suspension of hepatocytes was loaded with Mag-Fura-2 AM (5 \(\mu\)M). Measurement of \(\text{Ca}^{2+}\) content in store of permeabilized cells was conducted using spectrofluorimetric method. We confirmed that TLC-S (50, 100 and 200 \(\mu\)M) elicited cytosolic \(\text{Ca}^{2+}\)-signals, which were not inhibited by the IP\(_3\)-receptors (IP\(_3\)Rs) antagonist 2-APB (100 \(\mu\)M). After application of TLC-S thapsigargin could release only 47.94 ± 3.05 %. Previous addition of NED-19 (100 nM) decreased fraction of calcium that is released by TLC-S and equals 33.25 ± 2.15 % of the total calcium. In this case, the following use of thapsigargin mobilized only 21.75 ± 10.68 %. Thus, previous application of NED-19 significantly (\(n = 6\); \(P \leq 0.01\)) reduced the proportion of calcium released by TLC-S 2-fold. It was observed that the rate of TLC-S-induced decrease of calcium content in the intracellular store was 1.8 times slower than after application of NED-19 (\(n = 6\); \(P \leq 0.05\)) Previous application of NED-19 increased the rate of thapsigargin-evoked calcium content reduction by a factor of 2.5 (\(n = 6\); \(P \leq 0.01\)). We suggest the impact of acid store in TLC-S-elicited cytosolic \(\text{Ca}^{2+}\)-signals in mice hepatocytes. Thus, the mechanism of TLC-S-induced calcium release is also NAADP-mediated.

**Keywords:** hepatocytes, tauroliocholate 3-sulphate, \(\text{Ca}^{2+}\), nicotinic acid adenine dinucleotide phosphate.
INTRODUCTION

Bile salts are synthesized from cholesterol in liver and represent the main driving force of the bile flow. Bile is crucial for intestinal absorption of fats and fat-soluble vitamins, as well as the elimination of excess cholesterol and waste products from body [3]. Previous work has shown that application of bile acids can cause the increase in the levels of cytosolic \([\text{Ca}^{2+}]\) in hepatocytes [1, 4].

Specifically, bile acids activate calcium entry into the cells and cause depletion of internal calcium store [5]. Other effects, not linked to calcium signaling, have also been observed, including the increase in intracellular Na\(^+\) concentration [6] and depolarization of inner mitochondrial membrane. [7].

In acinar pancreatic cells, it was also shown that bile acids can release calcium from both ER and acidic stores in secretory granular areas. In both stores TLC-S interacts with both the IP\(_3\)Rs and the RyRs. TLC-S opens the RyRs through activation of NAADP [8]. In hepatocytes, it is still unclear if NAADP-sensitive acidic store is involved in TLC-S-induced Ca\(^{2+}\)-signals. Therefore, the main purpose of this study was to examine such possibility.

MATERIALS AND METHODS

Isolation of hepatocytes. CD-1 male mice were humanely sacrificed in compliance with the provisions of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 1985) and in accordance with International Convention for the Protection of Animals. The protocol for hepatocyte isolation was as described in [9]. Isolated liver was perfused with buffer I without Ca\(^{2+}\): 140 mM NaCl; 4.7 mM KCl; 10 mM HEPES; 10 mM D-glucose; 100 \(\mu\)M EGTA; pH 7.4; the rate of perfusion was 5 mL/min at 37 °C. Next the liver was perfused with buffer I in the presence of 1.3 mM CaCl\(_2\) and collagenase I (Worthington) for 10 min at 37 °C. Dissociated hepatocytes were centrifuged at 50 \(\times\) g for 1 min and then transferred into buffer I containing 1 mM MgCl\(_2\) and 1.3 mM CaCl\(_2\), pH 7.4.

Fluorescent \([\text{Ca}^{2+}]\) measurement. After isolation, the cells were loaded with low affinity Ca\(^{2+}\)-sensitive dye fluo-4 (2.5 \(\mu\)M) for 30-45 minutes at 36.5 °C. Cells were attached to poly-L-lysine-coated coverslips in flow chamber. All experiments were performed at room temperature.

Fluorescent images were obtained using Leica SP2 MP dual two-photon confocal microscope with a \(\times\) 63 1.2 NA objective. For fluo-4 excitation and emission wavelengths were 488 nm (argon ion laser, 3 % power) and 510–590 nm, respectively. Fluorescent images were collected with frequency of 0.6–1.0 frame/second. Fluorescence signals were plotted as \(F/F_0\), with \(F\) as fluorescence during the experiment and \(F_0\) as the initial level of fluorescence.

Measurement of \([\text{Ca}^{2+}]\) content in store of permeabilized cells. Suspension of permeabilized hepatocytes (2 \(\times\) 10\(^6\)) was used to load with fluorescent dye Mag-Fura-2 AM (5 \(\mu\)M). The dye was washed out before permeabilization. Isolated hepatocytes were permeabilized with saponine (0.1 mg/mL) in intracellular solution for 10 min. Cells were later washed with an intracellular solution based on K-HEPES, containing 20 mM NaCl; 127 mM KCl; 1.13 mM MgCl\(_2\); 0.05 mM CaCl\(_2\); 0.1 mM EGTA; 10.0 mM HEPES (KOH); 5 \(\mu\)g/mL oligomycine; 1 \(\mu\)g/mL rotenone; 2.0 mM ATP; pH 7.0. 2 mL of cell suspension were transferred to the spectrofluorometer cuvette. The fluorescence of Mag-Fura-2 AM was monitored using excitation wavelength 340-380 nm with emission at 500 nm.
Cellular calcium content that was mobilized by 10 µM ionomycin was accepted as 100 % and represents the total amount of Ca\(^{2+}\) within the internal pool.

Reagents used in this study include fluo-4/Mag-Fura-2 AM (Invitrogen), thapsigargin (Calbiochem), collagenase (Worthington). All the other chemicals were purchased from Sigma.

RESULTS AND DISCUSSION

TLC-S-induced Responses in the Intact Hepatocytes. We have shown that TLC-S (50, 100 and 200 µM) elicited cytosolic Ca\(^{2+}\)-signals, consistent with the previous findings described early [1, 2]. A typical trace with repeated application of different concentration of TLC-S is shown in Fig.1. A and B. TLC-S (200 µM) induced calcium elevation in the cytosol of intact hepatocytes comparably half the size of 10 µM ATP effect (Fig.1, B). After TLC-S-elicited Ca\(^{2+}\)-signal takes place hepatocytes can answer to ATP but this signal has smaller amplitude yet longer plateau phase (Fig.1, B).

The liver expresses 2 principal intracellular, calcium-release channels: the inositol 1,4,5-trisphosphate receptor (IP\(_3\)R) (types 1 and 2) [10,11] and the ryanodine receptor type 1 (RyR), detected as a truncated but functional channel-protein [12]. Previous results of Mandi et al. [13] reported about NAADP-sensitive store in liver microsome fraction. Additionally, Zhang’s group has shown that NAADP-sensitive Ca\(^{2+}\) release channel is present in the lysosome of native liver cells [14]. We have also observed NAADP-evoked Ca\(^{2+}\)-release in permeabilized hepatocytes [15].

So, we investigated whether inhibitors of these channels influence the TLC-S-elicited Ca\(^{2+}\) release. We tested 2-aminoethyldiphenyl borate (2-APB) as the inhibitor of the IP\(_3\)Rs. It was revealed that TLC-S-induced Ca\(^{2+}\)-signals were not inhibited by the IP\(_3\)Rs antagonist 2-APB (100 µM) (Fig. 1, C).

Monitoring of TLC-S action on the Ca\(^{2+}\) storage organelles in suspension of permeabilized hepatocytes. In suspensions of murine hepatocytes, TLC-S (100 µM) mobilizes 66.10 ± 8.87 % of the total stored calcium released by ionomycin (10 µM). In this experiment, after exposure to TLC-S thapsigargin can release 47.94 ± 13.05 % of the total stored calcium. A typical trace showing the effect of bile acid on Mag-Fura-2 (5 µM) (F/F\(_0\)) in intracellular store of hepatocytes is shown on Fig.2, A.

NAADP is the most potent Ca\(^{2+}\)-mobilizing agent identified to date that acts in various cell types across phyla. It was shown to selectively target the lysosome-related organelles rich in Ca\(^{2+}\) and H\(^+\) and therefore called acidic Ca\(^{2+}\)-stores. In hepatocytes they are presented as endo-lysosomal system of the cell [16]. There are many hypotheses about the mechanisms of NAADP action. Much evidence suggests that NAADP induces small yet localized cytoplasmic Ca\(^{2+}\)-signals subsequently amplified into regenerative global Ca\(^{2+}\)-signals by recruitment of endoplasmic reticulum via calcium-induced calcium release (CICR) [17]. The actual data collected on the NAADP-receptors remain disputable. The potential NAADP-sensitive Ca\(^{2+}\)-channels candidates include TRPML1, TRPM2, TPCs and even RyRs [16, 17]. In order to investigate the effects of NAADP in the cell, there was synthesized the selective antagonist of NAADP – NED-19. This small molecule is cell-permeable and fluorescent derivative of tryptophan. NED-19 is a powerful noncompetitive inhibitor of NAADP-binding process. It is also able to label the NAADP-receptors in intact cells and effectively block the NAADP-induced Ca\(^{2+}\)-release. Thus, NED-19 is commonly used for studies of NAADP-mediated events [18]. We have found previously that NAADP triggered changes in stored Ca\(^{2+}\) were completely abolished by NED-19 as antagonist of NAADP in permeabilized rat hepatocytes [15].
Fig. 1. TLC-S-induced increase of Ca$^{2+}$ level in the cytosol of intact hepatocytes loaded with fluo-4: (A) TLC-S-mediated [Ca$^{2+}$] elevation is dependent on the concentration of TLC-S applied (30–50 µM); (B) TLC-S (200 µM) triggers [Ca$^{2+}$] rise about half the size of 10 µM ATP-evoked elevation; after TLC-S-elicited Ca$^{2+}$-signals hepatocytes answer to ATP but this signal has smaller amplitude yet longer plateau phase; (C) 2-APB does not block [Ca$^{2+}$], elevation induced by TLC-S.
After application of NED-19 (100 nM) fraction of calcium that was released by TLC-S decreases and made up only 33.25 ± 2.15 % of the total calcium released by ionomycin. In this case, the following use of thapsigargin mobilizes only 21.75 ± 10.68 % of the stored calcium in suspension of mice hepatocytes (Fig. 2, B). Thus, the previous application NED-19 significantly \((n = 6; P ≤ 0.01)\) reduced the proportion of calcium released by TLC-S 2-fold. We also calculated the velocity of calcium store emptying by TLC-S and thapsigargin in control and after previous application of NED-19. It was established that the rate of TLC-S-induced reduction of calcium level in the intracellular stores was 2-fold slower than after application of NED-19 \((n = 6; P ≤ 0.05)\). The same results were observed on thapsigargin-elicited calcium content decrease in endoplasmic reticulum – previous
application of NED-19 increased its rate by 2.5 fold (n = 6; P ≤ 0.01). We speculate that the rise in velocity of TLC-S- and thapsigargin-induced calcium release from endoplasmic reticulum after previous application of NED-19 is caused by destruction of the contact sites between NAADP-sensitive acidic stores and endoplasmic reticulum. We assume that the acidic Ca\(^{2+}\)-store is important for refilling endoplasmic reticulum with calcium.

**CONCLUSION**

We suggest that acidic Ca\(^{2+}\)-stores are involved in TLC-S-induced cytosolic Ca\(^{2+}\)-signals due to reduction of calcium level in the endoplasmatic reticulum of mice hepatocytes. Thus, the mechanism of TLC-S-induced Ca\(^{2+}\)-release is also NAADP-mediated.

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ВПЛИВ 3-СУЛЬФО-ТАУРОЛІТОХОЛЕВОЇ КИСЛОТИ НА ВМІСТ ЦИТОЗОЛЬНОГО ТА ДЕПОНОВАНОГО КАЛЬЦІЮ В ІЗОЛЬОВАНИХ ГЕПАТОЦИТАХ МИШЕЙ

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Моногідроксильовані жовчні кислоти, такі як тауролітохолева кислота (TLC) і її трисульфат (TLC-S), підвищують цитозольний кальцій у суспензії гепатоцитів [1, 2]. Вважають, що таке вивільнення відбувається за рахунок ІФ₃-чутливого депо, але без утворення ІФ₃. Нікотинацитденідинкулеотидфосфат (НААДФ) здатний вивільнювати Са²⁺ із органоїдів ендопліозомальної системи, яку зараховують до кислотного депо клітин через кислий вміст. Метою роботи було дослідити вплив NED-19 (антагоніста НААДФ) на TLC-S-індуковані зміни вмісту Са²⁺ в ізольованих гепатоцитах миші для того, щоб з'ясувати роль кислотного депо у TLC-S-індукуваному вивільненні Са²⁺. Ізольовані гепатоцити миші навантажували 2,5 мкмоль/л fluo-4. Зміни рівня концентрації кальцію в цитозолі реєстрували за допомогою скануючого двофотонного мікроскопа Leica SP2 MP. З метою пермебілізації гепатоцитів клітини обробляли сапоніном (0,1 мг/мл) у суспензії та навантажували magfura-2. Зміни депонованого кальцію реєстрували спектрофлуориметричним методом. Нами встановлено, що TLC-S у діапазоні концентрацій 50, 100 і 200 мкмоль/л здатний вивільнити короткочасне підвищення кальцію в цитозолі ізольованих гепатоцитів миші. Попередня аплікація 2-АРВ (100 мкмоль/л) не запобігала TLC-S-індукованим Са²⁺-сигналам. У суспензії ізольованих гепатоцитів миші TLC-S вивільняє 66,10 ± 8,87 % депонованого кальцію від усієї його кількості, яку здатний вивільнити іономіцин. У цьому експерименті після дії TLC-S, тапсигаргін ще може вивільнити 47,94 ± 13,05 % депонованого кальцію. Після застосування NED-19 (100 нмоль/л) частка депонованого кальцію після подальшого додавання TLC-S зменшується і становить лише 33,25 ± 2,15 %. При цьому подальше застосування тапсигаргіну мобілізує тільки 21,75 ± 10,68 % депонованого кальцію суспензії гепатоцитів миші. Отже, попередня аплікація NED-19 статистично достовірно (n = 6; P ≤ 0,01) зменшує частку депонованого кальцію після подальшого додавання TLC-S у 2 рази. Встановлено, що швидкість TLC-S-вивільнення кальцій з депо збільшується у 2 рази після подання NED-19 (n = 6; P ≤ 0,05). Також і швидкість тапсигаргін-індукуваного вивільнення кальцію з депо зростає у 2,5 рази після реінкубування TLC-S з NED-19 (n = 6; P ≤ 0,01). Ми припускаємо, що у реалізації впливу TLC-S на вміст кальцію в гепатоцитах миші, окрім тапсигаргін-чутливого, залучена ще й кислотна депозитарна система. Отже, механізм дії TLC-S-індукувані Са²⁺-сигнали досліджувалися клітин є НААДФ-опосередкованим.

Ключові слова: гепатоцити, 3-сульфо-тауролітохолева кислота, Са²⁺, нікотинацитденідинкулеотидфосфат.

ISSN 1996-4536 (print) • ISSN 2311-0783 (on-line) • Біологічні Студії / Studia Biologica • 2015 • Том 9/№1 • С. 49–56
ВЛИЯНИЕ 3-СУЛЬФО-ТАУРОЛИТОХОЛЕВОЙ КИСЛОТЫ НА СОДЕРЖАНИЕ ЦИТОЗОЛЬНОГО И ДЕПОНИРОВАННОГО КАЛЬЦИЯ В ИЗОЛИРОВАННЫХ ГЕПАТОЦИТАХ МЫШЕЙ

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Моногидроксилированные желчные кислоты, такие как тауролитохолевая кислота (TLC) и ее трисульфат (TLC-S), повышают цитозольный кальций в суспензии гепатоцитов [1, 2]. Полагают, что такое высвобождение происходит за счет ИФ₃-чувствительного депо, но без образования ИФ₉. Никотинацидадениндинуклеотидфосфат (НААДФ) способен высвобождать Ca²⁺ из органоидов эндоплазматической системы, которую причисляют к кислотному депо клеток из-за кислого содержимого. Целью работы было исследовать влияние NED-19 (антагониста НААДФ) на TLC-S-индуктированные изменения кальция в изолированных гепатоцитах мышей, чтобы выяснить роль кислотного депо в TLC-S-индуктированном высвобождении Ca²⁺. Изолированные гепатоциты мышей нагружали 2,5 мкмоль/л fluo-4. Регистрировали изменения концентрации кальция в цитозоле с использованием сканирующего двухфотонного микроскопа Leica SP2 MP. С целью пермемабилизации гепатоцитов клетки обрабатывали сапонином (0,1 мг/мл) в суспензии и нагружали mag-fura-2. Изменения депонированного кальция регистрировали спектрофлуориметрическим методом. Нами установлено, что TLC-S в диапазоне концентраций 50, 100 и 200 мкмоль/л способен вызывать кратковременное повышение кальция в цитозоле изолированных гепатоцитов мышей. Предыдущая аппликация 2-АРВ (100 мкмоль/л) не предотвратила TLC-S-индуцированного Ca²⁺-сигнала. В суспензии изолированных гепатоцитов мышей TLC-S вызывает 66,10 ± 8,87% депонированного кальция от всего, что способен высвободить иономицин. В этом эксперименте, после воздействия TLC-S, тапсигаргин еще способен высвободить 47,94 ± 13,05% депонированного кальция. После применения NED-19 (100 нмоль/л) доля кальция, высвобождаемая TLC-S уменьшается и составляет лишь 33,25 ± 2,15 %. При этом последующее применение тапсигаргина мобилизует только 21,75 ± 10,68 % депонированного кальция в суспензии гепатоцитов мышей. Итак, предыдущая аппликация NED-19 статистически достоверно (n = 6; P ≤ 0,01) уменьшает долю депонированного кальция после следующего добавления TLC-S в 2 раза. Установлено, что скорость TLC-S-вызванного освобождения кальция с депо увеличивается в 2 раза после предыдущей инкубации с NED-19 (n = 6; P ≤ 0,05). Также скорость тапсигаргин-индуктированного освобождения кальция с депо возрастает в 2,5 раза (n = 6; P ≤ 0,01). Мы предполагаем, что в реализации влияния TLC-S на содержание кальция в гепатоцитах мышей, кроме тапсигаргин-чувствительного, привлечено еще и кислотное депо, которое представлено эндоплазматическими элементами. Итак, механизм действия TLC-S на кальциевый гомеостаз исследуемых клеток является НААДФ-опосредованным.

Ключевые слова: гепатоциты, 3-сульфо-тауролитохолевая кислота, Ca²⁺, никотинацидадениндинуклеотидфосфат.

Одержано: 15.10.2014