DOI

UDC: (616.153.455.01:616.33-018.73-092:615.015.11]-085.37-092.9)

**ATB 340 (A MODULATOR OF SULFITE OXIDASE ACTIVITY)
REDUCES OXIDATIVE STRESS DURING HYPERGLYCEMIA AND
IN STRESS EXPOSED GASTRIC MUCOSA IN OLD RATS**

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Introduction. Sulfite oxidase (SO) is one of the mitochondrial enzymes involved in the synthesis of H₂S and multiple physiological processes, including oxidative stress. The dominant sulfide oxidation products vary in a tissue-specific manner, and SO/oxidative stress which is involved in gastric mucosa (GM) injury is unclear. Our studies have shown potent cytoprotective and anti-inflammatory effects of H₂S-rich compounds, including hybrid nonsteroidal anti-inflammatory drugs (H₂S-NSAIDs) but their effects on age-related metabolic dysfunction in the gastric mucosa (GM) exposed to long-term postprandial hyperglycemia with high-fructose diet (HFD) is still unknown.

Aim. To characterize the activity of sulfite oxidase (SO) and the role of oxidative stress in the GM of older rats exposed to HFD and assess the therapeutic effect of the recently synthesized H₂S donor, hybrid NSAID, H₂S-acetylsalicylic acid (H₂S-ASA, ATB 340) on the GM of adult vs old rats.

Material and methods. Adult (n=24) and old (n=43) rats were divided into control groups with a standard diet (SD) and experimental groups with a 28-day high fructose level diet (HFD, V. Kozar, 2008) with and without acute stress induction (Takagi, 1964). During 19-28 day, HFD groups consumed per os: a) placebo (1.0 ml of saline per os); b) NaHS, 100 µmol/kg/day; c) placebo and stress induction; d) NaHS, 100 µmol/kg/day and stress induction; e) ASA, 10 mg/kg/day and stress induction; f) conventional aspirin (ASA), 10 mg/kg/day and NaHS, 100 µmol/kg/day and stress induction; g) ATB-340, 17.5 mg/kg/day and stress induction. The integrity of the GM was analyzed based on the histological severity index, which was calculated with account for morphological studies of GM samples. The indicator of oxidative stress, malondialdehyde (MDA) and the activity of SO in the GM were assessed using the standard biochemical methods.

Results. We have observed a different SO expression in the GM of adult vs old rats with SD and HFD with and without WIS. The effect of HFD resulted in an increase in the SO activity in old rats by 15%, whereas in adults this value was 14%. MDA content changes were similar. In animals that received NaHS, the changes were similar, whereas the SO activity in old rats was 23% higher. Stress induction reduced the SO activity by 35% in old animals and by 22% in adults. The difference in the MDA content amounted to 41%. The effect of exogenous NaHS in old rats under stress was the highest when combined with ASA, while the MDA content was the highest when classical aspirin was used, which also coincided with similar changes in histological severity index.

Conclusion. Sulfite oxidase is the essential contributor to age-related oxidative stress in the GM under research. The use of ATB-340 (H_2S -aspirin) increases the H_2S content in the tissues, reduces symptoms of GM damage associated with a decrease in the SO activity and the content of MDA, which confirms alleviation of oxidative stress and has anti-radical and anti-oxidant effects.

Disclosures. No conflicts of interest, financial or otherwise, are declared by the authors.

Author contribution. Yaroslav Pavlovskiy, Maksym Lutsyk drafted manuscript; Antonina Yashchenko edited and revised manuscript; Natalia Zaichko, John Wallace, Oksana Zayachkivska approved final version of the manuscript.

Key words: Hydrogen Sulfide, Mucosal Defense, Gastric Mucosa, Aspirin, Non-Steroidal Anti-Inflammatory Drugs, Sulfite Oxidase, MDA, Fructose

АТВ 340, МОДУЛЯТОР АКТИВНОСТІ СУЛЬФІТ ОКСИДАЗИ, ЗМЕНШУЄ ОКИСНИЙ СТРЕС У СЛИЗОВІЙ ОБОЛОНЦІ ШЛУНКА СТАРИХ ЩУРІВ ЗА УМОВ ГІПЕРГЛІКЕМІЇ ТА СТРЕСУ

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Вступ. Сульфит-оксидаза (SO) є одним з мітохондріальних ферментів, що беруть участь у синтезі H_2S і численних фізіологічних процесах, включаючи окисний стрес. Продукти окиснення сульфідів мають тканинну специфічність, однак особливості їхнього пошкоджувального впливу на слизову оболонку шлунка (GM) не відомі. В наших дослідженнях показано потужний цитопротекторний та протизапальний ефект сполук, збагачених H_2S і гібридних нестероїдних протизапальних препаратів (H_2S -NSAIDs), але їх вплив на вікові особливості метаболічної дисфункції у СОШ на тлі довготривалої її постпрандіальної гіперглікемії за умов гіперкалорійної високо-вуглеводної дієти фруктозою (HFD) не вивчено.

Мета. Охарактеризувати активність сульфітоксидази (SO) та роль окисного стресу у СОШ старих щурів при HFD та оцінити терапевтичний ефект недавно синтезованого донора H_2S , гібрид NSAIDs, H_2S -ацетилсаліцилова кислота (H_2S -ASA, АТВ 340) на СОШ старих тварин порівняно із дорослими.

Матеріали і методи. Дорослих ($n = 24$) і старих ($n = 43$) щурів поділили на контрольні групи зі стандартною дієтою (SD) та експериментальні групи із дієтою з високим вмістом фруктози (HFD, V. Kozar, 2008) впродовж 28 днів, яким додатково індукували гострий стрес (WIS) (Takagi, 1964). Групи з HFD у 19-28 дні отримували per os: а) плацебо (1,0 мл фізіологічного розчину per os); б) $NaHS$, 100 мкмоль/кг/добу; в) плацебо та індукція стресу; г) $NaHS$, 100 мкмоль/кг/добу та індукція стресу; д) ASA, 10 мг/кг/добу та індукція стресу; е)

ASA, 10 мг/кг/добу, NaHS, 100 мкмоль/кг/добу та індукція стресу; є) АТВ-340, 17,5 мг/кг/добу та індукція стресу). Цілісність СОШ аналізували за гістологічним індексом ураження, що враховували враховуючи морфологічні дослідження зразків СОШ. Індикатор окисного стресу, малоновий диальдегід (MDA) та активність SO в GM оцінювали стандартними біохімічними методами.

Результати. Ми спостерігали різну експресію SO в GM у дорослих щурів у порівнянні зі старими щурами з SD і HFD без і з WIS. Вплив HFD призводив до збільшення активності SO у старих щурів на 15%, тоді як у дорослих на 14%. Зміни вмісту MDA були схожими. У тварин, яким вводили NaHS, зміни MDA мали подібну тенденцію, тоді як активність SO у старих щурів була вища на 23%. Індукція стресу зменшувала активність SO у старих тварин на 35%, тоді як у дорослих – на 22%, а відмінність у вмісті MDA сягала 41%. Дія екзогенного NaHS у старих щурів за умов стресу була найвищою у разі комбінації з ASA, тоді як вміст MDA був найвищий при застосуванні класичного аспірину, що співпадало з аналогічними змінами гістологічного індексу ураження.

Висновок. Сульфат оксидаза є важливим фактором вікового окисного стресу у GM, який був досліджений. Застосування АТВ 340 (H_2S -аспірину), що збільшує вміст H_2S в тканинах, зменшує прояви пошкоджень GM, пов'язаних зі зменшенням активності SO і вмісту MDA, що свідчить про облегшення проявів окисного стресу, виявляючи анти-радикальну та анти-оксидантну дію.

Ключові слова: Гідроген Сульфід, захист слизової оболонки, шлунок, аспірин, нестероїдні протизапальні засоби, сульфат оксидаза, окисний стрес, MDA, фруктоза

Introduction

The gastrointestinal (GI) tract shows a remarkable resilience to damage induced by the beverages and foods that we ingest, which can have a wide range of osmolarity, pH and temperature [20]. This resistance to tissue damage is collectively referred to as "mucosal defense" and many different chemical mediators participate in GM defense, inflammation and repair [9, 18]. A new classification of human diseases will become available soon, based on consensus in expert committees that decided to include new category „stress-related diseases" (SRD) [7]. It is widely accepted that SRD result from complex reciprocal interaction between epigenetic and genetic factors. One of the epigenetic factors is fructose-rich nutrition, which results in oxidative stress, however its prevention remains incompletely understood [3, 14]. There are age-related features in gastric mucosa (GM) defense, inflammation and repair against cytolytic agents, including prostaglandin/cyclooxygenase activities [11]. The issue of preventing and correcting the cyto-aggressive action of nonsteroidal anti-inflammatory drugs (NSAIDs), which widely used by elderly patients and most often causes GM damage to the elderly patients [1, 2,

6] and whatever age-related features in H_2S mucosal defence against oxidative stress is related to that, is open.

Important bioregulators of prostaglandin-independent mechanisms of cytoprotection of the GM is sulfur compounds. Recent extensive research into their effects confirmed as potent cyto- and vasoprotective effects, including against low-grade inflammation [17]. It has been shown that Sulfhydryl (SH) groups play prominent role in gastroprotection by depleting glutathione (GSH) and induction oxidative stress [11, 18]. Another important regulator is the gas transmitter H_2S (hydrogen sulfide), which is known for its vasodilator, antioxidant, antiapoptotic and pro-angiogenic properties, which provide cytoprotective and anti-inflammatory effects [16, 22]. A number of studies point out the unity of various ways of H_2S synthesis: enzymatic and non-enzymatic (the transformation of thiols and thiol-containing compounds) [5, 15, 23]. The four most important enzymes involved in formation of hydrogen sulfide are: cystathionine β -synthase – CBS, cystathionine γ -lyase – CSE, 3-mercaptopyruvate sulfurtransferase – 3-MST, which cooperates with cysteine aminotrans-

ferase – CAT [17]. It is known that these enzymes have specific tissue and cellular localization. We have shown that for the purpose of cytoprotection of the mucosal barrier of the digestive system (esophagus, stomach and intestines) and anti-inflammatory action, the synthesis of H_2S is carried out using CSE, as well as the maintenance of normal microflora in the cardiovascular system under physiological conditions [16, 23]. It has been found that in activated neutrophils, H_2S can be oxidized to thiosulfate (under the influence of sulfite oxidase (SO, EC 1.8.3.1) oxidizes sulfite to sulfate), and then converted to SO_2 (by enzymes thiosulfate sulfutransferase or thiosulfate reductase). SO is one of the enzymes involved in the utilization of H_2S in mitochondria too [5]. Both H_2S pathways enzymatic and non-enzymatic act as crucial rheostat regulators of the redox state, affected by active forms of oxygen levels in cells, and have both been shown to combine together and perform key roles in cellular and subcellular survival and progression under extreme factors actions. However, the full concept explaining the role of SO in GM cytoprotection in the aspect of age-related changes is still unknown. Metabolic dysfunction induced by long-term fructose influence is not finally clarified. Taking in account that of nonsteroidal anti-inflammatory drugs (NSAIDs), potent cytolysis' agents for GM, are among the most commonly used medications, and their use is increasing with aging populations worldwide despite of the significant risk for pro-oxidative gastrointestinal (GI) effects, bleeding and ulceration, sometimes leading to death [6, 10, 12].

Aim. To characterize the role of sulfite oxidase (SO) and activity of oxidative stress in GM in older rats with fructose exposed hyperglycemia and to assess the therapeutic effect of the recently synthesized H_2S donor – hybrid NSAID H_2S -acetylsalicylic (H_2S -ASA, ATB 340) adult vs old rat GM.

Materials and Methods

All experiments were carried out on adult and old rats (N=67, body weight 350 ± 40 g; n=5-6) in accordance with the norms of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (1986) and in accordance with the Committee on Bioethics of

Danylo Halytsky Lviv National Medical University (protocol № 6 from 29.03.2017). Animals were maintained under a constant 12 h light and dark cycle and an ambient temperature of $21-23^\circ C$ with $50 \pm 10\%$ relative humidity. All animals were kept in raised mesh-bottom cages to prevent coprophagy. Animal from the control group were allowed free access to tap water and kept on standard diet (SD), experimental groups were on 28-days hypercaloric high-fructose diet (HFD by V. Kozar, 2008), with unrestricted access to 40% solution of fructose *ad libitum* within drinking-water [8].

Acute stress was induced by model of Takagi et al. that involves short-term exposure to water-immersion stress [20]. The rats were placed in restraint cages and immersed vertically to the level of the xiphoid process in a water bath of 23° for 3.5 h. The initial and final body weights were recorded. Blood glucose concentrations were measured daily by glucometer (Achtung TD-4207, Germany) using a blood sample from the tail vein.

The animals were subdivided into control groups of adult rats (AR) and old rats (OR) with standard diet (SD) and vehicle (1.0 ml of physiological solution), and experimental groups receiving 28-days hypercaloric high-fructose diet, HFD V. Kozar, 2008) without and with acute stress (Takagi, 1964). Experimental groups were pre-treated 9-days *per os* by: 1) vehicle (adult); 2) vehicle (old); 3) NaHS, 100 μ mol/kg/day (adult); 4) NaHS, 100 μ mol/kg/day (old); 5) vehicle with stress induction (adult); 6) vehicle with stress induction (old); 7) NaHS, 100 μ mol/kg/day and induction of stress; 8) conventional aspirin (ASA), 10 mg/kg/day and induction of stress; 9) ASA, 10 mg/kg/day and NaHS, 100 μ mol/kg/day and stress induction; 10) ATB-340 -releasing, 17.5 mg/kg/day and induction of stress. The administration of NaHS and ATB-340 was performed in doses tested by J.L. Wallace, 2012-2017 [21, 22, 24].

The rat stomach was removed immediately after euthanasia and cut with scissors in the longitudinal direction from the gastroesophageal junction to the pylorus. The gastric mucosal surface was gently washed with phosphate-buffered (pH 7.4) saline. Samples of the GM were fixed in 10% formalin and embedded

in paraffin. Serial sections of 5-mm thickness were stained with hematoxylin/eosin. The sections were blindly evaluated by two individuals and their scores were averaged. The GM damage was estimated by histological scoring (HS), including three components: a). the state of the epithelial plate on the degree of alteration (0 – no changes; 1 – stratification of the stratum corneum; 2 – focal basophilia of keratin masses; 3 – cell mass desquamation, vacuolation of basal cells, vesicular nuclei; 4 – erosion); b). Condition of subepithelial structures according to stromal damage (0 – no changes; 1 – diffuse swelling of the submucosal basis; 2 – pronounced uneven swelling of the submucosal base and insignificant infiltration; 3 – severe swelling and disorganization of the submucosal basis; 4 – perivascular or subepithelial infiltrates, perivascular hemorrhages), c). Leukocyte infiltration of the epithelial layer (0 – no leukocyte infiltration; 1 – moderate leukocyte infiltration; 2 – average leukocyte infiltration; 3 – severe leukocyte infiltration; 4 – multiple leukocyte infiltrates).

Visualization was performed using a microscope (Swift Instruments International, Japan) and a digital video camera (Echo-Imager 5020200 Microscope Digital Eyepiece, China).

The activity of SO was determined in GM homogenates biochemically by the rate of oxidation of sulfate anion in the presence of potassium hexacyanoferrate by standard biochemical methods. GM was homogenized at 3000 rpm (Teflon-glass) in a medium of 1.15% potassium chloride (ratio 1:3). The homogenates were centrifuged for 30 min. at 600g, the aliquots of the post-native supernatant were taken in the Eppendorf microtubules and stored at -20°C until the research was carried out [23].

The intensity of the oxidative component of oxidative stress was evaluated by changes in the rate of generation of an unstable free radical of oxygen – superoxide anion (O_2^-), hydroxyl radical ($OH^\cdot$) and by changes in the content of terminal products of lipid peroxidation (malonic dialdehyde – MDA).

The level of MDA was determined by reaction with thiobarbituric acid (TBA) by the diagnostic set TBK-Agate (Biokont, RF). With a thiobarbituric acid the lipid peroxidation

products form a red stained complex, which is extracted with butanol, with a maximum light absorption at $\lambda = 535$ nm.

Statistical analysis of the results was carried out using programs «Excel» i «Statistica 7.0». The certainty of the changes was estimated by ANOVA Dunnett's test.

The relative difference between the physiological and biochemical parameters against control was calculated by formula:

$$\Delta(\%) = 100 \cdot (X_i - X_n) / X_n,$$

where X_i – value in the main group;
 X_n – value in the control group.

Results and discussion

Healthy adult rats on SD treated with vehicle exhibited normal gastric mucosa appearance, with histological scores of zero. The GM histological scores in old rats ranged between 1 and 2 (on a 0 to 12 scale) in HS. There was no detectable mucosal damage by HS of GM in adult rats under HFD vs old rats, however combination of WIS and HFD exposure caused sharply rise of HS (Fig. 1). The effects

on the GM in old rats were with irregular subepithelial exudates, submucosal vascular dilation and mild leukocyte infiltration in the gastric epithelium and submucosal clots (Fig. 2A). Influence of treatment with NaHS and ATB 340 (donors of H_2S synthesis) resulted in attenuated gastric injury induced by HFD, acute stress and ASA (Fig. 2B-2D).

We have observed a different expression of SO activity in GM in adult vs old rats with SD and

Fig.2. Administration of donor of hydrogen sulfide (H_2S) synthesis, NaHS (A, B, C) and ATB 340 (D) attenuates gastric injury/inflammation in HSD rats under stress induction and aspirin treatment.

HFD without and with WIS. In control groups that consumed SD, which we considered relative physiological norm, the SO activity in adult rats was 2,9 nmol/min·mg protein, in the old – 4,1 nmol/min·mg protein, the effect of HFD caused an increase in SO activity in old animals by 15%, and in adult by 14%, as compared to control (Fig. 3).

Changes in MDA content had the same tendency. The use of exogenous NaHS, which increases the H_2S content in the tissues of the body, reduced the activity of SO by 24% of adult rats, whereas in old rats it decreased by 23% compared to the groups without correction of the synthesis of H_2S . The obtained data allows us to interpret the effect of H_2S , on the background of HFD, as anti-oxidant and anti-radical. Investigation of changes of MDA levels in all experimental groups are represented on Fig. 4.

Regarding the importance to test age-related activity of H_2S enzyme SO against GM injury caused by long-term high fructose diet (HFD), we combined exposure to HFD with WIS. Stress induction showed age-related changes in the activity of SO. Older animals showed an increase in SO activity compared to adults by 18%, indicating better

adaptive-compensatory properties of young rats, while NaHS application increased SO activity by 7% and reduced MDA content by 16%. Distortion of the natural stereotype of the cytoprotective response of GM by the administration of ASA decreased the activity of SO by 20% and increased the content of MDA by 8%, while the combination of ASA and NaHS increased the activity of SO by 18% and reduced the MDA content by 7% compared to the groups without correction of H₂S synthesis. Our obtained results correlates with other scientific groups [2, 4].

To test effects of novel hybrid NSAIDs on changes of pro- and antioxidant balance and its impact on GI injury during aging which are still unknown, we used 7-days supplementation of conventional and H₂S-releasing NSAIDs during 21-28 days of HSD. Administration of the newest ATB 340, the H₂S-ASA hybrid drug, showed similar SO activity to the administration of ASA and NaHS, while the MDA content decreased by

27%. H₂S-ASA shows a potent antioxidant effect, which attenuates oxidative stress. Similar results were shown in Liu L, et al study [10]. The relative difference between experimental groups and control data are represented on Tab. 1.

Conclusions. Long-term postprandial hyperglycemia induced by HFD is one agent that declines mucosal defense in stomach. Sulfite oxidase is the essential contributor to age-related oxidative stress in GM during exposure of HFD. Supplementation of NaHS and ATB-340 (H₂S-aspirin), which increases the H₂S content in the tissues, reduces the symptoms of GM damage associated with a decrease in the activity of SO and the content of MDA, indicating the alleviation of oxidative stress, showing an anti-radical and anti-oxidant effects.

Disclosures. No conflicts of interest, financial or otherwise, are declared by the authors.

Author contribution. Yaroslav Pavlovskiy, Maksym Lutsyk drafted manuscript; Antonina Yashchenko edited and revised manuscript; Natalia Zaichko, John Wallace, Oksana Zayachkivska approved final version of the manuscript.

Groups		Sulfite oxidase (SO) nmol/min*mg protein	Malonic dialdehyde (MDA) µmol/l	Histological score (HS)
SD	Adult	2,92±0,24	2,58±0,26	0,0
	Old	4,14±0,78	3,7±0,51	2,0±0,71
HFD	Adult	3,33±0,28	2,97±0,21	0,5±0,55
	Old	4,79±0,22	4,22±0,15	3,33±1,03
	Adult + NaHS	2,54±0,17	2,9±0,42	1,83±0,98
	Old + NaHS	3,65±0,51	4,15±0,49	3,0±0,71
	Adult + stress	2,61±0,18	3,51±0,11	5,17±0,98
	Old + stress	3,1±0,69	5,95±0,43	6,4±0,89
	Old + NaHS	3,3±1,0	5,02±1,07	5,18±0,75
	Old + ASA	2,48±0,21	6,41±0,45	6,0±1,0
	Old + ASA + NaHS	3,64±0,6	5,5±0,83	4,6±0,89
	Old + ATB 340	3,45±0,99	4,33±0,77	4,17±1,47

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Стаття надійшла 12.11.2018

Після допрацювання 14.12.2018

Прийнята до друку 27.12.2018