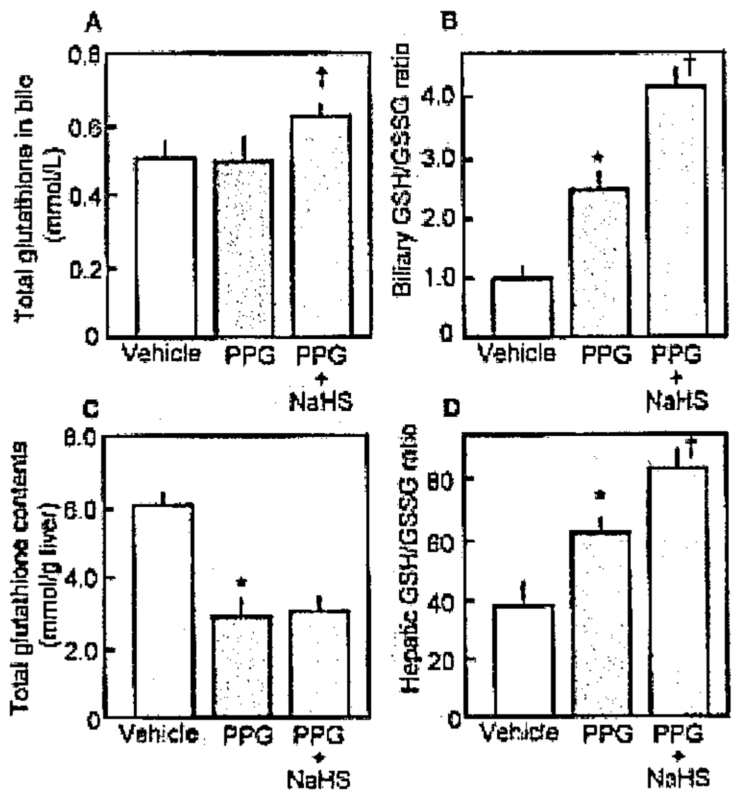

Fig. 4 Effects of the CSE blockade by PPG and supplementation of 30 mmol/L NaHS on biliary excretion and hepatic contents of glutathione in perfused rat livers. PPG at 1.5 mg/kg was administered in vivo intraperitoneally at 4 hrs prior to the isolation of the perfused liver. GSH and GSSG; reduced and oxidized forms of glutathione, respectively. Data indicate mean+/-SE of 7-9 separate experiments in each group. *P<0.05 as compared with the vehicle-treated group. $^{+}$P<0.05 versus the PPG-treated group.

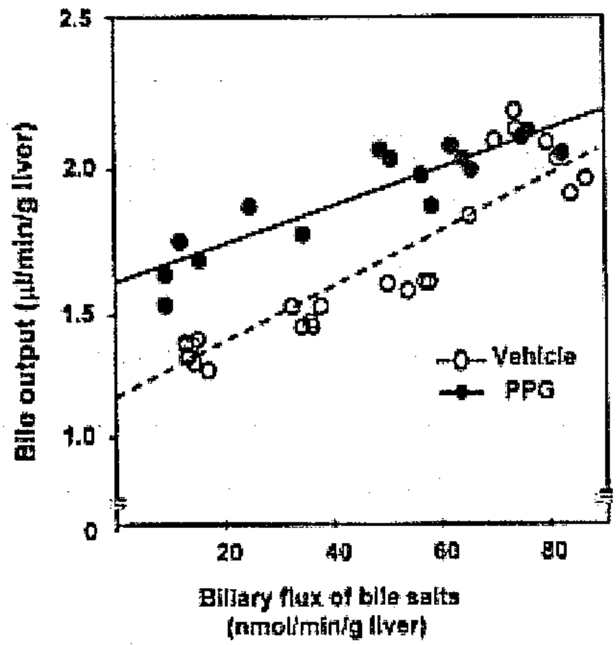

Fig.5 Alterations in bile salt-independent fraction of bile output by the blockade of CSE by PPG. PPG at 1.5 mg/kg was administered in vivo intraperitoneally at 4 hrs prior to the isolation of the perfused liver. Note the significant elevation ($P<0.05$) of the Y-intersection by the PPG treatment, and the difference in the basal bile output between the two groups becomes smaller with increased excretion of bile salts in bile.

Table 1 Effects of blockade of cystathionine γ-lyase by propagylglycyne (PPG) on basal bile output and biliary HCO_3^- excretion

Groups	Basal bile output (μl/min/g liver)	Biliary HCO_3^- concentration (mmol/L)
Vehicle (n=6)	1.73±0.09	27.9±1.2
PPG (n=6)	2.11±0.05 *	33.0±0.7 *

*P<0.05 as compared with the vehicle-treated control group.

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Research Communications:

Cadmium exposure alters metabolomics of sulfur-containing amino acids in rat testes

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Running Head: Hydrogen sulfide, methionine, cysteine, glutathione, cadmium

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Abstract

This study aimed to examine distribution of cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE), the H_2S-generating enzymes, and metabolomic alterations in sulfur-containing amino acids in rat testes exposed to stressors. Immunohistochemistry revealed distinct distribution of the two enzymes: CBS occurred mainly in Leydig cells and also detectable in germ cells, while CSE was evident in Sertoli cells and immature germ cells involving spermatogonia. The amounts of CSE and CBS in testes did not alter in response to administration of cadmium chloride, an anti-spermatogenic stressor leading to apoptosis. Metabolome analyses assisted by liquid chromatography equipped with mass spectrometry revealed marked alterations in sulfur-containing amino acid metabolism: amounts of methionine and cysteine were significantly elevated concurrently with a decrease in the ratio between S-adenosyl homocysteine and S-adenosyl methionine, suggesting expansion of remethylation cycle and acceleration of methyl donation. Despites a marked increase in cysteine, amounts of H_2S were unchanged, leading to a remarkable decline of the H_2S/cysteine ratio in the cadmium-treated rats. Under such circumstances, oxidized glutathione (GSSG) was significantly reduced, while reduced glutathione (GSH) was well maintained, and the GSH/GSSG ratio was consequently elevated. These results collectively showed that cadmium induces metabolomic remodeling of sulfur-containing amino acids even when the protein expression of CBS or CSE is not evident. Although detailed mechanisms for such a remodeling event remain unknown, our study suggests that metabolomic analyses serve as a powerful tool to pin-point a critical enzymatic reaction that regulates metabolic systems as a whole.

Key Words: cystathionine β-synthase, cystathionine γ-lyase, cysteine, methionine, H_2S, metabolome

Introduction

Testis is an organ characterized by active utilization of sulfur-containing amino acids. Metabolites derived from sulfur-containing amino acids have been shown to contribute to detoxification against noxious stressors as well as to maturation of testicular germ cells through multiple mechanisms. Cysteine metabolism plays a central role in such mechanisms (3)(7)(8)(13)(26) This amino acid serves as a substrate for synthesis of glutathione through reactions of glutamate ligase and glutathione synthase, and is also used to generate sulfate through aspartate transferase and sulfite oxidase. Another important substance generated upon cysteine metabolism in vivo is hydrogen sulfide (H_2S). This gaseous compound has recently been shown to account for a signaling molecule in neural and vascular systems (6)(9)(11)(12)(32). It is produced mainly by two types of pyridoxal-5 phosphate-dependent enzymes responsible for metabolism of L-cysteine, cystathionine γ-lyase (CSE, EC 4.4.1.1) and cystathionine β-synthase (CBS, EC 4.2.1.22) (1)(2)(14)(30). While the primary role of the two enzymes is to constitute the transsulfuration pathway that utilizes homocysteine to synthesize cysteine, both CSE and CBS are able to use cysteine as the substrate to generate H_2S (9)(12). Among the aforementioned amino acid derivatives, glutathione plays a central role in regulation of spermatogenesis. Male mice deficient in γ-glutamyl transpeptidase exhibit testicular atrophy concurrently with oligozoospermia (15). The fluid excreted from seminiferous tubules contains ample glutathione S-transferase that contributes to transport of testosterone into the fluid. The excretion of the protein is primary supported by Sertoli cells (20). Roles of methionine metabolism in maturation of testicular germ cells have been examined extensively: S-adenosyl methionine (SAM) decarboxylase constitutes a major pathway for biosynthesis of polyamines, which is essential for maturation of Sertoli cells and germ cells (26).

Although these lines of information suggest active utilization of remethylation and transsulfuration pathways in testes, the whole picture of metabolic remodeling in these biochemical pathways has not fully been investigated under stress conditions. Furthermore, the distribution of the enzymes responsible for metabolism of methionine and cysteine has been largely unknown in the testis at present. Furthermore, effects of exposure to stressors causing oligozoospermia on functional outcome of metabolic remodeling in the sulfur-containing amino acids and H_2S have not fully been examined. This study was designed to investigate distribution of CBS and CSE and to examine stress-induced metabolic responses of sulfur-containing amino acids and their derivatives in this organ.

Materials and Methods

Establishment of polyclonal antibodies against rat CBS and CSE.

To generate polyclonal antibodies against rat CSE, polypeptides for C-terminus of each enzyme were synthesized as immunoantigens and injected into rabbits. The peptide used for immunization to obtain the anti-CSE antibody was VYGGTNRYFRRVASE (1)(2). We also used a polyclonal antibody against CBS which was prepared in previous studies (12). The antiserum for CSE was purified by affinity purification using a commercially available kit (UltaLink Immunomobiization kit, PIERCE, Rockford, IL). For Western blot analyses, rat tissues were collected from testes and livers, and homogenized in lysing buffer (50 mmol/L Tris-HCl pH 7.6, 150 mmol/L NaCl, 1% NP40, 1 mmol/L phenylmethylsulfynyl fluoride, 5 μg/ml aprotinin, 5 μg pepstatin, 5 μg/ml leupepsin) and were subjected to Western blot analyses as described previously (5)(22).

Immunohistochemistry

Male Wistar rats were fasted overnight and were anesthetized with an intramuscular injection of 50mg/kg pentobarbital sodium. The testes of rats treated with an intraperitoneal injection of $CdCl_2$ at a dose of 20 μg/kg or with vehicle were removed and fixed for 4 hrs at 4 °C in periodate-lysine-paraformaldehyde solution as described previously (5)(22). The samples were washed sequentially for 4 hrs with PBS containing 10, 15 and 20% sucurose, and embedded in OCT compound and processed for preparing 4-mm slices to apply anti-CBS or –CSE antibody at a final concentration of 1 μg/ml at 4°C. After several washes with PBS, the sections were stained with a biotinylated anti-rabbit IgG for 1 hr (Vectastatin Elite ABC Lit, Vector Laboratories, Inc., Burlingame, CA). To inhibit endogenous peroxidase reactions, the samples were pretreated with 0.3% H_2O_2 in cold methanol for 30 min, and were subsequently incubated with avidin and HRP-conjugated biotin for 30 min. Finally, 0.1 mg/ml DAB tetrahydrochloride was applied to sections for 3 min. The sections were counterstained with methyl green after fixation with 20% formaldehyde for 20 min. In some experiments, the antibodies preabsorbed with an excess of antigens in advance were applied for immunohistochemistry as negative controls.

In separate sets of experiments, sections of testes were double stained by a method using DAB and nickel chloride according to our previous method (5)(22) to examine cell types expressing CBS or CSE. To this end, we applied the antibody against Ad4BP, an intranuclear DNA-binding protein expressed in steroidogenic cells (a gift from Professor Kenichiro Morohashi, National Institute of Molecular Biology, Okazaki). As described previously (20), this antibody allowed us to stain nuclei of Leydig cells and Sertoli cells. Leydig cells are located in the interstitial space out of seminiferous tubules, while Sertoli cells stand in the distal basement region of the tubules. Because of such anatomical topography of these cells, the Ad4BP staining led us to distinguish easily Sertoli cells from Leydig cells and also from testicular germ cells in the tubules (22). By this protocol, cells reacting only with the initial primary antibody were stained light brown, while those reacting with the second primary antibody was stained bright purple. When reacting with both primary antibodies simultaneously, cells were identified as those stained dark brown.

Sulfur-containing amino acid metabolome analyses in testes of $CdCl_2$-treated rats

Amounts of H_2S in tissues were determined by gas chromatography according to previous methods described elsewhere (11)(12). To determine amounts of metabolites in remethylation and transsulfuration pathways, high-performance liquid chromatography (HPLC) was used with three different detection systems. An approximate 1 gm of the testes was homogenized in 10 ml of 10 % trichloroacetic acid (TCA: Sigma, Inc. St. Louis, MO) containing 1 mM EDTA. The homogenate was then centrifused at 15000 rpm for 15 min at 4 °C, and that supernatant was collected and stored at -80 °C. Amounts of cysteine were determined by HPLC with fluorimetric detection and isocratic elution. The last step was derivatization with 7-fluorobenzene-2-oxy- 1,3-diazolic-4-ammonium sulfate (SBD-F: Wako, Inc. Tokyo, Japan) (0.3 g/L of 500 mM potassium borate, pH 11.5) at 60 °C for 60 min. The HPLC system (Shimazu, Kyoto, Japan) was used with a SIL-10Advp automatic sample injector and a RF-10AXL fluorescence detector. Chromatographic separation was performed on a ODS column (C18, 250 X 4.6 mm) using 0.1 M potassium dihydrogen phosphate as mobile phase at a flow rate of 1 ml/min and a column temperature of 30 °C. The fluorescence of the separated compounds was detected with a detector adjusted for excitation at 385 nm and emission at 515 nm. Amounts of the compounds were calculated with a calibration curve established by measurements of known concentrations of the standard compounds (19)(21). Contents of methionine, S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH), cystathionine, serine, and taurine were determined by a liquid chromatography assisted by double mass spectrometry (API 3000 LC-MS/MS) system. TCA-treated hepatic samples were precolumn-derivalizated with Waters AccQ-Fluor Reagent Kit (Waters, Milford, MA) to determine these compounds. To determine hepatic SAM levels, hepatic samples were added to 100-fold 100 mM ammonium acetate. Chromatographic separation was performed on a Atlantis column (dc18, 2.1 X 150 mm) using 5mM ammonium acetate, acetonitrile as mobile phase at a flow rate of 1 ml/min and a column temperature of 30 °C.

Statistical analyses

Differences in mean values among groups were examined by Fisher's multiple comparison analyses combined with ANOVA. $P < 0.05$ was considered statistically significant.

Results

Immunohistochemical detection of CBS and CSE in rat testes

The specificity of the polyclonal antibody against CBS used in the current study was characterized using rat liver lysates by Western blot analysis. As seen in Figure 1A, a major band was observed at 63kDa in the liver, suggesting the specificity to use this antibody for immunodetection. The CBS expression was unchanged in the liver of the cadmium-treated rats. When using the lysate derived from testes, CBS was undetectable irrespective of the presence or absence of the cadmium exposure, suggesting a paucity of the protein expression as a whole organ. The anti-CSE antibody used in the current study was identical to that used in our study (4), and Western blot analyses for the liver revealed the expression of CSE, as recognized at 40 kDa in the intact liver was unchanged irrespective of the stimulus such as cadmium. We thus hypothesized that these enzymes could be expressed locally in particular cell types and attempted to examine the cellular localization immunohistochemically. Figures 1B-1D illustrated representative pictures showing localization of CBS in the control and $CdCl_2$-treated rat testes. As seen in low-power images (Panel B), CBS occurred mainly in cells in the interstitial space and in the basement membrane of seminiferous tubules. We then examined if CBS is localized in Leydig cells and Sertoli cells. To distinguish these cells from others, immunostaining with Ad4BP, a nuclear transcriptional factor for steroidogenic cells, was conducted with CBS staining using double immunohistochemistry. As seen in Panel C, Leydig cells in the interstitial space (arrows) expressed this enzyme abundantly. The cells expressing CBS notably in the basement membrane of the tubules were Sertoli cells as characterized by their shape with cell processes protruding toward the central region of tubules (Panel C). At the same time, immature germ cells adjacent to Sertoli cells express the enzyme modestly. On the other hand, mature germ cells observed in the central region of tubules express the enzyme little, if any (Panel B). Such distribution patterns and immunoreactivities were unchanged in response to exposure to $CdCl_2$, so far as judged at 12 hrs after the administration (Panel D). These results suggest that testicular CBS is expressed in cell type-specific manners, resulting in failure of detection by Western blot analyses.

As seen in Figure 1A and also in the article of this forum (4), the anti-rat CSE antisera were purified through an affinity column and turned out to be usable for specific detection of the antigen at 40 kDa. Using the same antibody, distribution of CSE was examined in testes. As indicated in Figure 2A, CSE was abundantly expressed in vascular walls in the interstitial space of testes as well as in Sertoli cells which constitute the basement membrane of the tubules. The localization of CSE was also notable in immature germ cells occurring in the marginal regions of the tubules (Figure 2B). Furthermore, the enzyme expression in the individual cells appeared to be condensed in nuclei, while the expression in cytoplasm of these cells was modest relatively. Likewise that of the CBS expression, the response of the CSE expression was not altered by exposure to $CdCl_2$. (data not shown). Collectively, these results suggest that both CBS and CSE did not alter greatly in response to the heavy metal stressor so far as judged from the protein expression.

Analyses of sulfur-containing amino acid metabolism and H_2S generation

Table 1 illustrates metabolomic profiles of alterations in contents of sulfur-containing amino acids and their derivatives in testes of $CdCl_2$-treated rats. Data were collected at 12 hrs after the administration of the reagent. As seen, administration of $CdCl_2$ induced a significant elevation of contents of methionine, S-adenosylhomocysteine (SAH), serine and cysteine. By contrast, contents of cystathionine and oxidized form of glutathione (GSSG) were significantly decreased. On the other hand, terminal products of transsulfuration pathway such as hydrogen sulfide (H_2S) and taurine were unchanged in response to the exposure to $CdCl_2$. Homocysteine, a product standing at the intersection between remethylation and transsulfuration pathways, was undetectable in testicular samples so far as determined by the present analyses for measurements. Observation that testicular contents of cysteine were significantly elevated by the $CdCl_2$ exposure led us to examine if this event coincided with alterations in contents of sulfur-containing compounds in the plasma component. As seen in Table 2, the administration of $CdCl_2$ significantly decreases plasma contents of homocysteine and cysteine, while inducing no notable changes in glutathione. Interpretation of these events will be discussed later in Discussion.

Since contents of metabolites could be altered as a function of those of their upstream substrates, we analyzed the ratio values of the contents such as SAH/SAM and GSH/GSSG to estimate alterations in methyl donation and redox regulation of glutathione, respectively. In addition, since H_2S is synthesized through the reactions of CBS and/or CSE which consume cysteine as the common substrate, the ratio between H_2S and cysteine was calculated and compared between the control and cadmium-treated groups. As summarized in Figure 3, total amounts of the substrates for remethylation cycle in the cadmium-treated group became 2.5-fold greater than those of the control group. Under these circumstances, the SAH/SAM values were significantly elevated, suggesting acceleration of methyl donation in the cadmium-exposed testes. The cadmium exposure also turned out to elicit marked changes in the transsulfuration pathway. As indicated in a marked reduction of the H_2S/cysteine ratio, amounts of the gas generated in the tissue appeared to be far smaller than those expected from the elevation of cysteine contents. Another important change occurring at the transsulfuration processes is a modest decrease in total glutathione (GSH+2GSSG) which coincided with a marked elevation of the GSH/GSSG ratio. Consequently, in the testes exposed to $CdCl_2$, H_2S generation is relatively downregulated while GSH appears to be well preserved. These results suggest that remodeling of sulfur-containing amino acids elicited by $CdCl_2$ involves preservation of the anti-oxidative capacity of glutathione.

Discussion

This study first demonstrated distribution patterns of CSE and CBS in testes of rats. In this organ, these enzymes occurred in distinct topographic patterns. According to the classic concept for cysteine metabolism, CSE and CBS cooperatively execute biotransformation of sulfur-containing amino acids such as methionine into cysteine. In this context, Sertoli cells appear to play an important role in the amino acid metabolism, since both enzymes are colocalized with abundant expression. This fact is reasonable in that the cells are known to require ample amounts of glutathione to maintain their capacity to nurse germ cells for spermatogenesis (15)(17)(22). Anatomical distribution of these enzymes revealed in the current study led us to suggest putative pathways for methionine metabolism; because of its biochemical feature as an essential amino acid supplied through circulation from nutritional resources, methionine could access Sertoli cells directly or be captured primarily by Leydig cells to be converted to cystathionine and is then secondarily transferred to Sertoli cells where the both enzymes are colocalized.

Although such an intercellular pathways for amino acid transfer has not been investigated in the current study, roles of Leydig cells as a putative gateway to facilitate methionine metabolism is likely to be of great physiologic relevance. Previous studies revealed that testicular germ cells abundantly express anti-oxidative enzymes such as glutathione peroxidase and thioredoxin reductase (15)(23)(26). Considering that nutritional support via blood stream in the interstitial space plays a crucial role in maintenance of spermatogenesis, it is not unreasonable to speculate that both Leydig cells and Sertoli cells cooperatively execute the delivery of cysteine and H_2S as reductants to strengthen anti-oxidative capacity of the germ cells for a quality-control of spermatogenesis.

To further clarify mechanisms for metabolic remodeling of sulfur-containing amino acid derivatives, we quantitatively determined the metabolites using metabolomic approaches. Metabolomic information that cover the whole products of remethylation and transsulfuration pathways led us to speculate roles of CBS as a critical enzymatic step causing the cadmium-induced changes in the metabolites; first, the exposure to $CdCl_2$ significantly expanded amounts of substrates for the remethylation cycle (Σ remethylation cycle) and appeared to facilitate methyl donation, as judged from the SAH/SAM ratio. Of importance is a reduction of the tissue cystathionine contents, suggesting the inhibition of the CBS activity. Despite such a decrease in the substrate for CSE, the cysteine contents were significantly elevated.. Considering that cystine could access the intracellular space through cystine transporter (10), the results suggest that the cadmium exposure could increase the testicular contents of cysteine through active uptake of the substrates from circulation rather than through upregulation of intratesticular delivery of the upstream substrate from the remethylation pathway. During these processes, maintenance of testicular H_2S concentration could help the entry of cysteine through the transporter (12).

Since downregulation of the enzyme per se secondaliry greatly alters the expression of diverse genes (24), it is difficult to understand entire mechanisms for alterations in metabolites in the current experimental model (24). However, so far as judged from aforementioned results, metabolome analyses given by the current study led us to hypothesize that CBS could serve as a putative target for the $CdCl_2$-induced remodeling of the methionine metabolism in the testes. Such a hypothesis is in good agreement with another observation that the ratio of H_2S/cysteine was significantly reduced upon the exposure. Recent studies suggest multiple mechanisms by which CBS alters its catalytic activities: such mechanisms involve proteolytic cleavage of the enzyme by cytokines (33) or modulation of the activity by binding of adenosine ligands to its CBS domain, suggesting the enzyme senses cellular energetics (25). Another possible effector of the CBS activity is CO derived from heme oxygenase (HO), the gaseous monoxide that regulate neurovascular function (16)(18)(28). We have recently revealed that Leydig cells serve as a sensor for detecting exposure to heavy metal stressors such as cadmium and mediate downregulation of spermatogenesis under stress conditions (22). To sense the stressors, the cells induce heme oxygenase-1 and increase CO as an alert signal for triggering germ cell apoptosis (22). While molecular mechanisms by which CO tranduces signals involve soluble guanylate cyclase or MAPK, we recently collected evidence for the ability of the gas to inhibit the activity of CBS (Hoshikawa, et al. unpublished observation), being in agreement with previous experiments using the purified CBS enzyme in vitro (31). If CO-derived from Leydig cells could inhibit CBS in the same cells, biotransformation of methionine into cysteine and/or glutathione could largely be compromised to trigger germ cell apoptosis. Although further investigation is obviously necessary to specify which mechanisms could actually contribute to metabolic remodeling of the cadmium-exposed testes, the current results showed potential usefulness of metabolimic approaches to pin-point putative molecular candidates that play a critical role in metabolic regulation in vivo.

Acknowledgments

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Abbreviations:

CBS: cystathionine β-synthase
CSE: cystathionine g-lyase
GSH: reduced form of glutathione
GSSG: oxidized form of glutathione
HPLC: high performance liquid chromatography
PBS: phosphate buffered saline
SAH: S-adenosyl homocysteine
SAM: S-adenosyl methionine

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