Glycoconj J (2010) 27:181–187
DOI 10.1007/s10719-009-9250-0

Development and application of a system for seminolipid metabolism using mouse seminiferous tubules

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Received: 24 March 2009 / Revised: 6 June 2009 / Accepted: 11 June 2009 / Published online: 7 July 2009

Abstract A convenient tool for studying metabolism of seminolipid in testis was developed by using mouse isolated seminiferous tubules prepared by collagenase treatment. Because more than 99% of [^{35}S]sulfate-incorporation was distributed in seminolipid, its metabolism in seminiferous tubules can be analyzed without disturbance of the other sulfolipids in this assay system. Furthermore, the contents of seminolipid and its precursor, galactosylalkylacylglycerol, which were determined by liquid chromatography-electrospray ionization mass spectrometry, did not change within a few hours, indicating that the incorporations of [^{35}S] sulfate into seminolipid solely reflects the turnover rate of this sulfolipid. As an initial application of this system, we characterized heat-susceptibility of the seminolipid turnover rate in mouse seminiferous tubules. Severe heating (44°C for 10 min) of the isolated seminiferous tubules suppressed the ^{35}S-incorporation into seminolipid to 47% of heating at scrotal temperature (32°C for 70 min). In contrast, pretreatment of the testis *in vivo* under the same condition (44°C for 10 min) did not decrease the seminolipid turnover rate in the isolated seminiferous tubules. In addition, the activity of galactocerebroside sulfotransferase decreased in the temperature-dependent manner in seminiferous tubules as well as crude tubular homogenates, where the activity is significantly more stable in the former than the latter. The newly developed system could provide useful basic data for further analyses of seminolipid metabolism in the testis.

Keywords Galactocerebroside sulfotransferase · Heat-susceptibility · Isolated seminiferous tubule · Metabolism · Mouse · Seminolipid

We dedicate this article to the memory of Prof. Ishizuka (deceased).
Abbreviations for lipids follow those of the IUPAC-IUB Joint Commission on Biochemical Nomenclature [1] and the symbols for sulfoglycolipids follow the system of Ishizuka [2].

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Abbreviations

CST	galactocerebroside sulfotransferase
GalCer	galactosylceramide, galactocerebroside
GalEAG	galactosylalkylacylglycerol
HSP	heat shock protein
LDH	lactate dehydrogenase
LC-ESIMS	liquid chromatography-electrospray ionization mass spectrometry
Lyso-SM4g	lyso seminolipid
PAPS	3′-phosphoadenylyl sulfate
SM4g	seminolipid, GalEAG I^3-sulfate
SM4s	galactosyl sulfatide, GalCer I^3-sulfate

Introduction

Seminolipid (SM4g) is a unique sulfoglycoglycerolipid having a structure, galactosylalkylacylglycerol I^3-sulfate.

This sulfolipid is widely distributed in mammalian sperm membrane as the principal glycolipid component [2]. Its higher expression on sperm membrane has prompted investigations for sperm-egg interaction in mammalian fertilization [3–5]. Recent studies using ceramide galactosyltransferase-deficient [6] or CST-deficient [7] mice suggested that SM4g plays essential roles in spermatogenesis. These authors reported that lack of SM4g arrested the spermatogenesis before the first meiotic division and resulted in male sterility.

In most mammals, including humans, temperature is maintained lower in the testes than that in the abdominal tissues. Testicular or scrotal heating has a harmful effect on the spermatogenesis and fertility due to degeneration of cells in the seminiferous epithelium where the pachytene primary spermatocytes and round spermatids as the most sensitive cells; ultrastructural and functional changes in the epididymis; a decrease in sperm output and impaired sperm quality, etc. However, the mechanism which induces such effects is still unclear [8].

In the present study, we developed a new assay system for studying metabolism of seminolipid using isolated seminiferous tubules from adult mice. As an initial application of this system, we characterized heat-susceptibility of the seminolipid turnover rate in mouse seminiferous tubules.

Materials and methods

Materials

Male, 8-week-old ddY mice (ca. 35 g body weight) were purchased from Sankyo Labo Service, Tokyo, Japan. The present study was carried out in accordance with the Teikyo University Guide for the Care and Use of Laboratory Animals, accredited by the Japanese Ministry of Education, Culture, Sports, Science and Technology. Every effort was taken to minimize any pain or discomfort of animals used in experiments.

Reference gangliosides and sulfoglycolipids were prepared as previously described [9]. 3′-Phosphoadenylyl sulfate (PAPS), bovine brain galactocerebroside (GalCer), collagenase (type I from *Clostridium histolyticum*), 4-nitrocatechol, and *p*-nitorocatechol sulfate (2-hydroxy-5-nitrophenyl sulfate) were purchased from Sigma Aldrich Japan, Tokyo, Japan. Carrier-free $H_2{}^{35}SO_4$ and [^{35}S]PAPS (59.2 GBq/mmol) were obtained from Dupont NEN Research Products, Wilmington, DE, USA. Sulfate-free modified Krebs-Henseleit medium was made by replacing $NaHCO_3$ and $MgSO_4$ with triethanolamine-HCl buffer and $MgCl_2$ respectively [10].

Preparation of seminiferous tubules from mouse

Seminiferous tubules were isolated from mouse testes according to the methods of Romrell [11] and Rommerts [12] with slight modifications. Briefly, testes (0.1–0.12 g/one testis) were removed from mice sacrificed with cervical dislocation. The seminiferous tubules were gently expressed through a small incision of the tunica albuginea. The aggregate of seminiferous tubules was digested with collagenase (150 U/one testis in 2 ml of sulfate-free modified Krebs-Henseleit medium) at 32°C (scrotal temperature) for 20 min with vigorous shaking. After the reaction was terminated by adding 2 volumes of the ice-cold medium, the dispersed seminiferous tubules were allowed to sediment for 2 min and the supernatant was discarded. The isolated tubules were washed twice and resuspended in a fresh medium (2 ml/one testis, corresponding to 2.5–4.5 mg protein). This preparation was immediately used for heat stress treatments, followed by extraction of lipids, [^{35}S] sulfate incorporation experiment and enzyme assay as below.

Liquid chromatography-electrospray ionization mass spectrometry (LC-ESIMS) of testicular and seminiferous tubular lipids

Partial purification and determination of SM4g and galactosylalkylacylglycerol (GalEAG) from mouse testis and seminiferous tubule was carried out by LC-ESIMS as described [13].

Incorporation of [^{35}S]sulfate into tubular SM4g

The seminiferous tubule suspension (up to 0.25 mg protein) was transferred to a 15 ml polypropylene tube with cap, and incubated with the sulfate-free Krebs-Henseleit medium (final vol., 2 ml) containing 370 kBq of carrier-free $H_2{}^{35}SO_4$ and 5 mM glucose at 32°C with gentle shaking under O_2 gas phase. After indicated time, incubations were stopped by chilling in ice water and the tubules were spun down by short centrifugation. The supernatants were discarded by aspiration. The extraction procedures for total lipids of pelleted tubules using mixtures of $CHCl_3/CH_3OH/H_2O$ were similar to those described previously [14–16]. Because SM4g is alkali-labile, the saponification step was omitted. After partition in the Folch system [17, 18], the organic phase was washed twice with the theoretical upper phase to remove excess [^{35}S]sulfate and radioactivity of the final organic phase was measured in a liquid scintillation counter. The overall recovery of [^{35}S]SM4g in this assay system was 99.7± 3.0% (mean ± SD, n=6). For identification of the [^{35}S] sulfolipids, an aliquot of the tubular lipid was separated by

high-performance TLC (Merck, Darmstadt, Germany) in the solvent system, $CHCl_3/CH_3OH/CH_3COCH_3/CH_3COOH/H_2O$ (8:2:4:2:1, *v*/*v*), and analyzed using BAS-1500 Bioimaging Analyzer (Fuji firm, Tokyo, Japan). Dried tubular residue after the lipid extraction was solubilized by aqueous 1 M NaOH with heating prior to protein assay. The protein was quantitated by a modified method [19] of Bradford [20] using bovine serum albumin as the standard.

Heat treatment of mouse testis

Mice were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight) and their scrotal regions were submerged in a water bath at 44°C for 10 min [21]. After 1 h-rest period at ambient temperature, the seminiferous tubules were prepared and the [^{35}S]sulfate incorporation experiment was performed as mentioned above. Control mice were also anesthetized but the heat treatment was not carried out.

Enzyme assay

Galactocerebroside sulfotransferase (CST) activity was determined according to Tadano and Ishizuka [22] with a slight modification [23]. Galactosylceramide (galactocerebroside, GalCer) was used as the acceptor substrate instead of GalEAG due to the following reasons: CST is completely responsible for the biosynthesis of SM4g and galactosyl sulfatide (SM4s) *in vivo* [7], and GalCer is commercially available. Homogenates of the seminiferous tubules were prepared by sonication for 30 s in ice water using Handy Sonic UR-20P (Tomy Seiko, Tokyo) and the supernatant of centrifugation at 13,000× *g* for 15 min was used for the enzyme assay. The reaction mixture contained 140 μM GalCer, 0.4% Triton X-100, 40 μM [^{35}S] PAPS (0.6 kBq/nmol), 100 mM imidazole-HCl (pH 6.8), 40 mM $CaCl_2$, 4 mM ATP, and tubular proteins (up to 75 μg) in a total volume of 200 μl. The mixture was incubated at 32°C for 90 min and the reaction terminated by chilling in ice water followed by addition of 4 ml of $CHCl_3/CH_3OH$ (2:1, *v*/*v*). After partition in the Folch system and twice washing, radioactivity of the final organic phase was measured. The recovery of [^{35}S]SM4s from the lower phase was 95.2±1.8% (mean ± SD, n=6) [10].

For the determination of arylsulfatase A activity, the whole homogenates of the isolated tubules was centrifuged at 600× *g* for 15 min to remove cell debris. The cytosolic fractions were then obtained by centrifugation at 108,000× *g* for 60 min [24].

The activity of lactate dehydrogenase (LDH) leaked into incubation medium was taken as the indices of the stability of tubular cells [25]. Prior to and after incubation for indicated time at 0 or 32, 37, 44°C, the tubules were precipitated by centrifugation (10,000× *g* for 15 min) and the supernatants were used for LDH assay. Total tubular LDH activity was determined after lysing the tubules in 0.1% Triton X-100 (*v*/*v*). For all figures, means and standard deviations were calculated. Statistical comparison of two means was performed using unpaired Student's *t*-test.

Results

Assay system for biosynthesis of seminiferous tubular SM4g

Almost all radioactivity (99%) incorporated into the seminiferous tubular sulfolipids was distributed in SM4g fraction (Fig. 1a, lane 3). A faint band comigrating with SM4s with hydroxy fatty acid was observed, but the radioactivity was negligible (ca. 1% of the total [^{35}S] incorporation). The [^{35}S]sulfate incorporation into SM4g increased linearly up to about 0.2 mg of tubular protein (Fig. 1b) and 120 min of incubation time at 32°C (Fig. 1c).

SM4g and its precursor, GalEAG, of seminiferous tubules and pooled whole testes from mice were quantitated by LC-ESIMS (Table 1). The concentrations of both lipids were higher in seminiferous tubules than in whole testes, showing that collagenase treatment of the testes removed interstitial cells which express neither SM4g nor GalEAG. Heat treatment, at 44°C for 10 min followed by rest at 32°C for 2 h, of the seminiferous tubules did not affect the concentrations of SM4g and GalEAG, indicating that the contents of SM4g and its precursor do not change within a few hours with or without heat treatment. LDH-leakage assay showed that only 1.5% (at 32°C for 70 min) to 2.7% (at 37°C for 70 min) of total LDH activity in the tubules was released into the medium by the heat treatment. These results could confirm that the cell membrane of seminiferous tubules is stable in our experimental conditions.

Effect of heat on [^{35}S]sulfate incorporation into seminiferous tubular SM4g

The seminiferous tubules were pretreated at three temperature conditions for totally 70 min *in vitro*, and then [^{35}S] sulfate incorporation into SM4g was determined (Fig. 2). In comparison with freshly prepared untreated tubules, preheating decreased SM4g turnover rates to 67–30%. When compared with the treatment at scrotal temperature (32°C for 70 min), the treatment at abdominal temperature (37°C for 70 min) did not affect significantly, whereas that at severe condition (44°C for 10 min) suppressed the

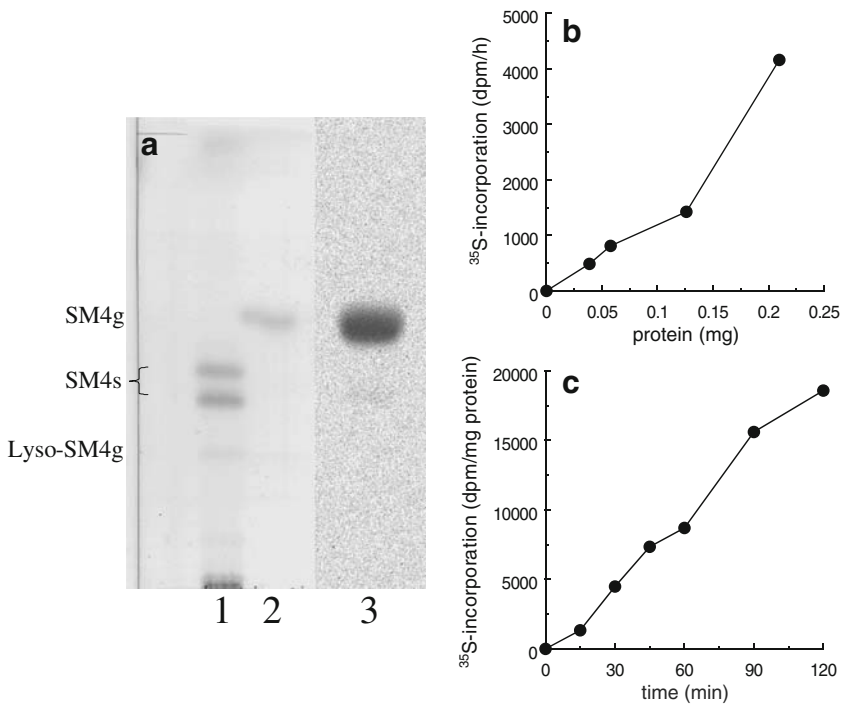

Fig. 1 TLC of ^{35}S-labeled seminiferous tubular sulfolipids, and protein and time dependencies of the incorporation of [^{35}S] sulfate into SM4g. **a** [^{35}S]Sulfolipids from seminiferous tubules (lane 3) were separated on TLC with the solvent system $CHCl_3/CH_3OH/CH_3COCH_3/CH_3COOH/H_2O$ (8:2:4:2:1, *v*/*v*) and analyzed using BAS-1500 Bioimaging Analyzer. The reference acidic glycolipids were obtained from rat brain (lane 1) and boar testes (lane 2) and stained with orcinol-sulfuric acid reagent. Protein (**b**) and time (**c**) dependencies were determined at 32°C (scrotal temperature) for 60 min and with about 0.16 mg protein, respectively. Each point is expressed as the means of four determinations

^{35}S-incorporation to 47%. In contrast to the *in vitro* severe heat pretreatment, *in vivo*-treatment of testes at 44°C for 10 min had no significant effect on the [^{35}S]sulfate incorporation into tubular SM4g (without treatment: 3,153±356 dpm·mg^{-1}·h^{-1}, with treatment: 3,263±1,104 dpm·mg^{-1}·h^{-1} (n=6)).

Heat stabilities of CST in seminiferous tubules and crude CST prepared from tubular homogenates

Seminiferous tubules or crude CST preparations from tubular homogenates were preheated at three temperature conditions *in vitro*, and then their CST activity was determined (Fig. 3). CST activities decreased in similar temperature-dependent manners in both preparations, where the activity in the seminiferous tubules was more stable than that in the crude CST preparation. In contrast to the CST, arylsulfatase A (a catabolic enzyme for SM4g) activities in seminiferous tubules were not affected by the heat pretreatments (data not shown), suggesting that the decreases in [^{35}S]sulfate incorporation into tubular SM4g after heat treatments (Fig. 2) is not due to activation of SM4g biodegradation but to suppression of SM4g biosynthesis.

Heat stability of crude CST prepared from mouse kidney homogenates

Crude CST preparations from mouse kidney homogenates were preheated *in vitro*, and their CST activity was

Table 1 Concentrations of seminolipid (SM4g) and galactosylalkylacylglycerol (GalEAG) in isolated seminiferous tubules from mice. Data for pooled whole testes were indicated for comparison

	nmol/mg protein[a] (mean ± SD, n=6)			
	SM4g		GalEAG	
	untreated[b]	heated[c]	untreated[b]	heated[c]
seminiferous tubule	20.63±2.07	21.25±2.25[d]	0.74±0.13	0.75±0.12[d]
pooled whole testes	15.80	–	0.48	–

[a] Valules were determined by liquid chromatography-electrospray ionization mass spectrometry

[b] Freshly prepared seminiferous tubules (untreated control)

[c] Seminiferous tubules were heated at 44°C for 10 min followed by rest (32°C, 2 h) *in vitro*

[d] Not significantly different from the data of untreated control

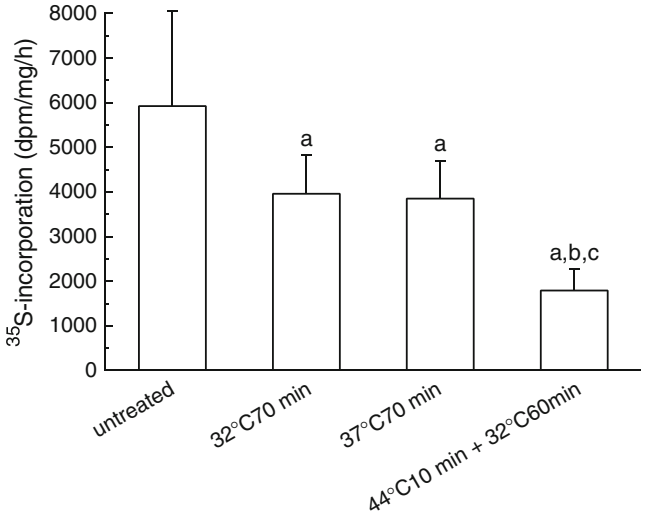

Fig. 2 Effect of heat treatments on [^{35}S]sulfate incorporation into seminiferous tubular SM4g. Isolated seminiferous tubules from mice were pretreated at three temperature conditions (32°C, scrotal; 37°C, abdominal; 44°C, severe) for a total of 70 min (32°C 70 min, etc.) *in vitro*. When the severe temperature was chosen, tubules were heated at 44°C for 10 min followed by rest (32°C for 60 min). After pretreatment, the tubules were incubated in a carrier-free $H_2{}^{35}SO_4$ containing medium at 32°C for 1 h and [^{35}S]sulfate incorporation into SM4g was determined as described in Materials and Methods. *Error bars* indicate SD (n=8) of total incorporation (dpm/mg protein). Significantly different (p<0.05): *a*, from untreated; *b*, from 32°C for 70 min; *c*, from 37°C for 70 min

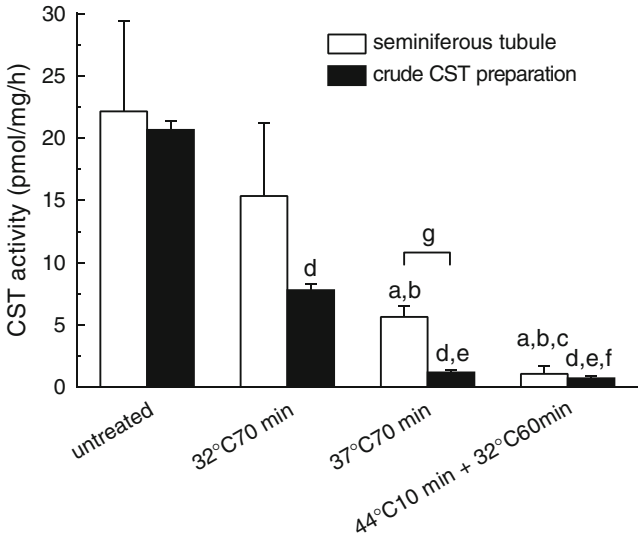

Fig. 3 Heat stabilities of CST in seminiferous tubules and crude CST prepared from seminiferous tubular homogenates. Seminiferous tubules (*open columns*) or crude CST preparations from seminiferous tubular homogenates (*closed columns*) were preheated *in vitro* as indicated, and then their CST activity was measured at 32°C using GalCer as the acceptor and [^{35}S]PAPS as the donor described in Materials and Methods. *Error bars* indicate SD (n=4). Heat treatment abbreviations are the same as Fig. 2. Significantly different (p<0.05): *a* and *d*, from untreated; *b* and *e*, from 32°C for 70 min; c and f, from 37°C for 70 min (*a–c*, for open; *d–f*, for closed columns). Significant differences (p< 0.05) between open and closed columns are marked with *g*

determined (Fig. 4). In contrast to the seminiferous CST (Fig. 3, closed columns), heat treatment at 32°C for 70 min did not decrease renal CST activity. At 37°C for 70 min, renal CST activity declined to 26.8% of untreated control, where the degree of suppression was less prominent as compared to seminiferous CST (5.8%) (Fig. 3).

Discussion

The present study showed that the isolated mouse seminiferous tubules are convenient tool for studying the metabolism of SM4g in the testis. This system was not only helpful to process a large number of experiments simultaneously but also allowed pretreatment of mice *in vivo* with heat stress, etc. Recently, we also reported that isolated rat renal tubule should be a useful metabolic system for clarification of proximal tubular sulfoglycolipids [10, 26]. Because more than 99% of ^{35}S-radioactivity incorporated into the total sulfolipids was distributed in SM4g (Fig. 1a), the SM4g metabolism in seminiferous tubules can be analyzed without disturbance of the other sulfolipids. The contents of SM4g and its precursor, GalEAG, did not change within a few hours under our assay condition (Table 1), indicating that the incorporation of [^{35}S]sulfate into SM4g solely reflects the turnover rate of this sulfolipid.

Heat-susceptibility of the SM4g turnover rate in seminiferous tubules substantially differed with heating conditions (heated in isolated seminiferous tubule *in vitro* or in testis *in vivo*). Severe heating (44°C for 10 min) of the

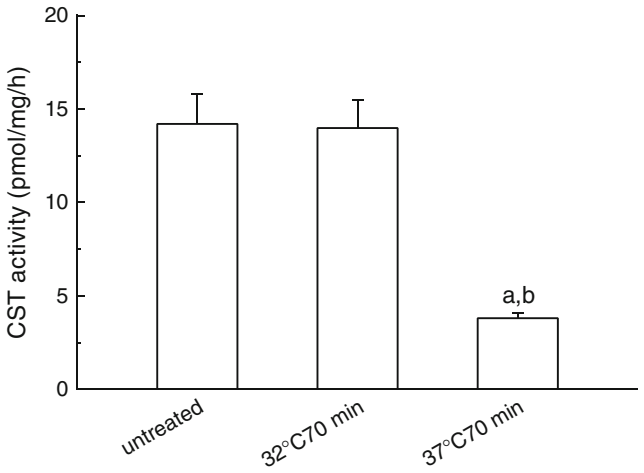

Fig. 4 Heat stability of crude CST prepared from mouse kidney homogenates. Crude CST preparations from mouse kidney homogenates were preheated *in vitro* as indicated, and then their CST activity was measured at 32°C by using GalCer as the acceptor and [^{35}S]PAPS as the donor described in Materials and Methods. *Error bars* indicate SD (n=4). Heat treatment abbreviations are same as Fig. 2. Significantly different (p<0.05): *a*, from untreated; *b*, from 32°C for 70 min

isolated seminiferous tubules suppressed the ^{35}S-incorporation into SM4g to 47% of that at scrotal temperature (32°C for 70 min) (Fig. 2). In contrast, the same severe heat pretreatment of testis *in vivo* did not decrease the SM4g turnover rate in the isolated seminiferous tubules, suggesting that in a living mouse certain factors, such as gonadotrophic hormones, nutrients and oxygen from testicular blood flow and also heat shock proteins (HSPs) or molecular chaperons in spermatocytes, may protect the SM4g biosynthesis in testis from heat stress.

CST activities in both preparations, seminiferous tubules and crude tubular homogenates, decreased in the similar temperature-dependent manner, where the former was significantly more stable than the latter (Fig. 3). In the crude tubular homogenates, it was suggested that disruption of an intracellular compartment, where CST and its protective HSPs might coexist, may cause segregation of CST from HSPs, resulting in higher susceptibility of CST to heat treatment. Furthermore, in contrast to the testis of a living mouse, the isolated mouse seminiferous tubules suffer little effects from any substances mentioned above in the testicular blood flow and interstitial cells. Thus, it seems likely that factors such as HSPs expressing constitutively and/or specifically in spermatogenic cells (see below) might partly protect CST activity in the isolated seminiferous tubules from the heat stress.

The testes in most mammals are maintained 5–7°C below body temperature by their location outside the body cavity in the scrotum and by a counter-current heat-exchange process that cools the blood entering the testis [27]. Spermatogenesis is disrupted readily by modest increases in temperature [8, 28], implying that, in spermatogenic cells, HSPs have specific functions that differ from those in other cell types [29]. The 70 kDa HSPs are molecular chaperones that assist a wide range of folding processes, including the folding and assembly of newly synthesized proteins, refolding of misfolded and aggregated proteins [30]. One of the HSP70 family, HSP70-2, is regulated developmentally and expressed specially in spermatogenic cells [31–33]. This protein synthesis occurs immediately after transcription in leptotene–zygotene spermatocytes [31, 34]. Interestingly, it was reported that the SM4g also expressed on the cell surface of primary spermatocytes from the end of the leptoene stage or the zygotene stage and later [2, 6, 35]. Furthermore, a sulfolipid binding protein of 68 kDa, referred to as SLIP1, which was affinity purified from rat testis homogenates using a SM4g column [36], was believed to be homologues to HSP70-2 [29, 37, 38]. These reports could suggest that HSP70-2 might also contribute to the protection of CST activity of spermatocytes from heat stress. In addition to the specification of HSP that protect CST from heat stress, further studies including morphological and quantitative examinations on the relationship between spermatogenesis and SM4g expression should be necessary.

In contrast to the seminiferous tubular CST activity, renal CST appears to be more heat-stable (Fig. 4). Because DNA sequences of all the isolated CST cDNA clones from various mouse tissues were identical in the coding region [39], the amino acid sequences of CST in testis and kidney are the same. Eckhardt *et al.* reported that *N*-glycosylation of Asn-312 and, to a lesser extent, Asn-66 of mouse CST, is necessary to form a fully active enzyme [40]. In addition, Yusa *et al.* indicated that recombinant mouse chondroitin 4-sulfotoransferase-1, in which three of the four potential *N*-glycosylation sites were deleted, was much more labile at 37°C than the control protein [41]. Collectively, the differences in heat-susceptibility between the testicular and renal CST may be partly due to variations in their *N*-linked oligosaccharide chains modified posttranslationally. However, it still remains possible that some factors, such as proteases, derived from the isolated seminiferous tubules damaged their CST activities.

Whereas the detailed investigation of heat-susceptibility of seminiferous tubular CST is beyond our present subject, further studies should be performed in future. Our newly developed system could provide useful basic data for the further analyses of seminolipid metabolism.

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