Restraint stress in lactating mice alters the levels of sulfur-containing amino acids in milk

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ABSTRACT. It is well known that maternal stress during the gestation and lactation periods induces abnormal behavior in the offspring and causes a lowering of the offspring's body weight. Various causes of maternal stress during the lactation period, relating to, for example, maternal nutritional status and reduced maternal care, have been considered. However, little is known about the effects on milk of maternal stress during the lactation period. The current study aimed to determine whether free amino acids, with special reference to sulfur-containing amino acids in milk, are altered by restraint stress in lactating mice. The dams in the stress group were restrained for 30 min at postnatal days 2, 4, 6, 8, 10 and 12. Restraint stress caused a reduction in the body weight of lactating mice. The concentration of taurine and cystathionine in milk was significantly higher in the stress group, though stress did not alter their concentration in maternal plasma. The ratio of taurine concentration in milk to its concentration in maternal plasma was significantly higher in the stress group, suggesting that stress promoted taurine transportation into milk. Furthermore, taurine concentration in milk was positively correlated with corticosterone levels in plasma. In conclusion, restraint stress in lactating mice caused the changes in the metabolism and in the transportation of sulfur-containing amino acids and resulted in higher taurine concentration in milk. Taurine concentration in milk could also be a good parameter for determining stress status in dams.

KEY WORDS: lactation period, maternal stress, milk, sulfur-containing amino acids

Adverse experiences during early developmental stages, including the prenatal period and the postnatal period, alter the normal development of children [10, 15]. For instance, maternal stress during the gestation period has been shown to elevate the risk of attention deficit hyperactivity disorder [21] and autism [8]. Moreover, Gao *et al*. (2011) showed that maternal stress during the lactation period in mice caused maternal malnutrition that resulted in a decrease in the body weight gain of the offspring, and that its effect continued for 15 weeks after their birth [7]. Furthermore, it has been reported that restraint stress [6] and chronic social stress [17] in lactating mice decreased dams' care for their offspring, and also that decreased maternal care resulted in decreased body weight of offspring [17]. In short, maternal stress during the lactation period affects the growth and behavior of both dams and offspring via the changes in, among other things, the maternal nutritional status and maternal behavior. Although milk is surely one of the most important factors in terms of dams and their offspring, there are few papers focusing on the effects on milk status of maternal stress in lactating animals. In the present study, we examined how maternal restraint stress during the lactation period affects the composition of milk, with special reference to free amino acids because milk free amino acids rather than milk protein are rapidly available for offspring.

Taurine (2-aminoethanesulfonic acid), one of the sulfur-containing amino acids, is classified as a β-amino acid. It has been reported that intraventricular injection of taurine stimulated growth hormone secretion in rats [11] and that taurine administration resulted in anxiolytic-like effects in mice [4] and rats [13]. Taurine thus has several effects on the growth and behavior of rodent animals. In addition, taurine exists in large quantity in rodent milk, especially in the colostrum [25], and it is reported that taurine is the most abundant amino acid in the milk of mice [20]. This large amount of taurine in the milk seems to derive from: 1) maternal blood via taurine transporters; and 2) synthesized taurine in the mammary glands [2]. It has been reported that cysteine

dioxygenase (CDO) and cysteine sulfinic acid decarboxylase (CSAD) are expressed in lactating mammary glands [27].

In the present study, we focused on the relationships between stressful conditions and the levels of taurine and other sulfur-containing amino acids that are related to taurine synthesis. We chose to load restraint stress because the restraint stress is often used as the model of psychological stress and it has been shown that restraint stress affects not only lactating animals but also their offspring [7]. The present study examined the effects of restraint (psychological) stress in lactating mice on the transportation and synthesis of taurine and other sulfur-containing amino acids related to taurine metabolism.

MATERIALS AND METHODS

Animals

Pregnant ICR mice (at pregnancy day 13) were purchased from Japan SLC (Hamamatsu, Japan). They were housed individually in a cage (W 12 × D 30 × H 14 cm) suitable for breeding and kept at a constant room temperature of 23 ± 1°C and humidity of 60%. They were maintained under a 12-hr light/dark cycle (lights on at 08:00, lights off at 20:00). Six days after the arrival of the dams, all of them delivered between 9 and 18 offspring. At postnatal day 2 (P2), the sex of the offspring was checked, and the number of offspring was standardized (n=8/dam) in order to make the nutritional conditions the same among the dams. The dams were separated into two groups: 1) control group (n=8); and 2) stress treatment group (n=8). Those in the stress treatment group were subjected to restraint stress during the lactation period as described below. Water and a standard diet for laboratory rodents (MF, Oriental Yeast, Tokyo, Japan) were available *ad libitum* for dams and offspring throughout this experiment. The present study was performed in accordance with the Guidelines for Animal Experiments in the Faculty of Agriculture and in the Graduate Course of Kyushu University and conformed to Law No. 105 and Notification No. 6 of the Japanese government.

Maternal stress procedure

The dams in the stress treatment group were subjected to a daily stress session starting at 9.30 a.m. and lasting for 30 min. They were subjected to this treatment 6 times in total (once every two days) between P2 and P12 (i.e. at P2, 4, 6, 8, 10 and 12). They were wrapped in wire mesh (20 × 20 cm) and the mesh was folded so that they were not able to move freely in their own cages with their offspring. The control pregnant dams were left undisturbed in their own cages with their offspring and were gently handled only when the cages were cleaned once a week.

Milking

Milk samples were collected on P12 from the maternal mice from each group (control: n=8; stress: n=8), and were analyzed for the level of free amino acids. Maternal mice were separated from their offspring 6 hr before milking in order to collect enough milk. After 6 hr, the maternal mice were anesthetized with isoflurane and were injected subcutaneously with 0.1 m*l* (1 unit) of oxytocin (ZENOAQ, Fukushima, Japan) to promote the secretion of milk. Twenty min after the injection, they were anesthetized with isoflurane again and milked for 10–15 min using a KN-591 milking machine for mice and rats (Natsume Seisakusho Co., Ltd., Tokyo, Japan).

Analysis of sulfur-containing amino acids

The concentration of sulfur-containing amino acids in the liver, maternal plasma, mammary gland and milk were analyzed by HPLC. Plasma samples were filtrated through an ultrafiltration tube (Millipore, Bedford, MA, U.S.A.). Milk was also filtrated through an ultrafiltration tube. The liver and mammary gland samples were homogenized in ice-cold 0.2 M perchloric acid solution containing 0.01 mM ethylenediaminetetraacetic acid disodium salt dihydrate and left for deproteinization in ice. After 30 min, the mixtures were centrifuged at 20,000 × g for 15 min at 4°C and filtrated through a 0.20 μm filter. The pH of the filtrate was adjusted to approximately 7.0 with 1 M sodium hydroxide. Each 10 μl sample from the maternal plasma and milk and each 20 μl sample from the liver and mammary gland was dried under reduced pressure. The dried residues were dissolved in 10 μl of 1 M sodium acetate-methanol-triethylamine (2:2:1) and re-dried under reduced pressure, then dissolved in 20 μl of methanol-distilled water-triethylamine-phenylisothiocyanate (7:1:1:1) (derivatization solution). Twenty min after the phenylisothiocyanate had finished reacting with the amino groups at room temperature, the samples were dried again under reduced pressure and then dissolved in 200 μl of buffer A [70 mM sodium acetate (pH 6.45 with 10% acetic acid)-acetonitrile (975:25)]. The same methods were used on standard solutions that were prepared by diluting a commercially available L-amino acid solution (type ANII, type B, L-asparagine, L-glutamine and L-tryptophan; Wako, Osaka, Japan) with an HCl solution. A Waters HPLC system (Pico-tag free amino acid analysis column (3.9 × 300 mm), Alliance 2,690 separation module, 2,487 dual-wavelength UV detector, and Empower 2 chromatography manager; Waters, Milford, MA, U.S.A.) was applied to the samples. The samples were then equilibrated with buffer A and eluted with a linear gradient of buffer B [water-acetonitrile-methanol (40:45:15)] (0, 3, 6, 9, 40 and 100%) at a flow rate of 1 m*l*/min at 46°C. Absorbance at 254 nm was applied to determine the concentration of the free amino acids. Triethylamine and sodium acetate trihydrate were purchased from Wako (Osaka, Japan), while the other drugs, for which no manufacturer is noted, were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). The concentration of free amino acids in the plasma and milk samples was expressed as pmol/μl, and that in the liver and mammary gland was expressed as pmol/mg wet tissue.

Analysis of corticosterone

Maternal plasma samples collected on P12 were analyzed for total corticosterone concentration, using a corticosterone enzyme
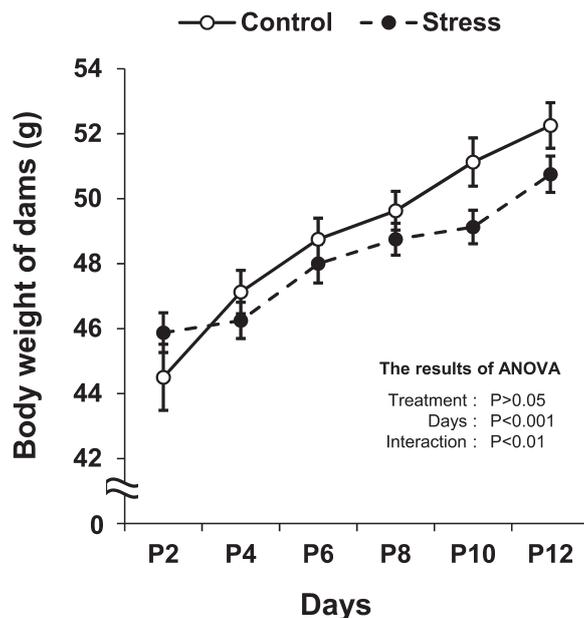
Fig. 1. The effects of restraint stress during the lactation period on the body weight of dams. Values are expressed as means ± SEM; n=8 in the control group and the stress group. P: postnatal age (day).

immunoassay kit (Cayman Chemical, Ann Arbor, MI, U.S.A.) according to the manufacturer's protocol, except for the use of Steroid Displacement Reagent (2.5%, Enzo Life Science, Farmingdale, NY, U.S.A.) in the plasma dilution step. The concentration of corticosterone was expressed as ng/ml.

Statistical analysis

All data were expressed as means ± SEM. The results concerning the free amino acids, the concentration ratio (concentration in milk/concentration in maternal plasma) of serine, and the cystathionine and taurine and plasma corticosterone were analyzed by t-test. The body weight of the dams was analyzed by a two-way repeated-measures ANOVA. The results concerning the concentration of serine, cystathionine and taurine in the maternal plasma and milk were analyzed by two-way ANOVA, but those concerning methionine and cysteine + cystine were not analyzed by a two-way ANOVA because they were not detected in the milk. When the interactions were significant in a two-way ANOVA, a post-hoc test (Tukey-Kramer test) was carried out. Differences were considered significant at $P<0.05$. All analyses were performed with Stat View (version 5, SAS Institute, Cary, NC, U.S.A., 1998). Outlying data were eliminated by applying Thompson's test criterion for outlying observations ($P<0.01$).

RESULTS

Corticosterone levels in maternal plasma

Corticosterone levels in the maternal plasma significantly increased (control=51 ± 18, stress=326 ± 16 ng/ml) as a result of restraint stress during the lactation period ($P<0.001$).

Body weight of dams

The effects of restraint stress in lactating mice on the body weight of dams are shown in Fig. 1. A significant ($P<0.01$) interaction between stress treatment and days was observed in the body weight of dams from P2 to P12, implying that maternal body weight gain was gradually retarded as a result of the restraint stress.

Sulfur-containing amino acid concentrations

The effects of restraint stress in lactating mice on the concentration of methionine, serine, cystathionine, cysteine + cystine and taurine in the liver, maternal plasma, mammary gland and milk are shown in Table 1. Maternal stress significantly ($P<0.01$) enhanced the concentration of cystathionine and taurine in milk, but not in the liver, maternal plasma or mammary gland. The concentration of methionine, serine and cysteine + cystine was not altered by maternal stress during the lactation period in any sample obtained. Figure 2 shows the results concerning the concentration of serine, cystathionine and taurine in maternal plasma and milk. Serine was not decreased by maternal stress treatment, but the concentration of serine in milk was significantly ($P<0.001$) lower than that in maternal plasma (Fig. 2A). Cystathionine was significantly ($P<0.01$) increased by maternal stress, and the concentration of cystathionine in the milk was significantly ($P<0.001$) lower than that in the maternal plasma (Fig. 2B). A

Table 1. The effects of restraint stress in lactating mice on the concentrations of methionine, serine, cystathionine, cysteine + cystine and taurine in the liver, maternal plasma, mammary gland and milk

	Methionine	Serine	Cystathionine	Cysteine + Cystine	Taurine
Liver					
Control	429 ± 27	250 ± 22	61.2 ± 7.2	20.7 ± 3.6	6,321 ± 332
Stress	439 ± 18	326 ± 36	43.3 ± 5.1	42.8 ± 10.0	8,542 ± 1,369
Maternal plasma					
Control	57.5 ± 6.1	124 ± 16	335 ± 19	1.32 ± 0.24	156 ± 10
Stress	49.3 ± 2.5	104 ± 5.9	323 ± 7.8	1.29 ± 0.26	140 ± 8.5
Mammary gland					
Control	124 ± 5.3	500 ± 14	7.07 ± 0.48	267 ± 23	1,846 ± 100
Stress	139 ± 11	504 ± 38	6.19 ± 0.56	301 ± 39	1,737 ± 126
Milk					
Control	ND	29.6 ± 2.8	193 ± 9.3	ND	212 ± 12
Stress	ND	23.3 ± 1.0	273 ± 7.8 [a]	ND	299 ± 18 [a]

The number of samples used for analysis was 8 for each group. Values for the concentrations of each amino acid in maternal plasma and milk are expressed as mean ± SEM in pmol/μl sample volume, and those for the concentrations of each amino acid in the liver and mammary gland are expressed as mean ± SEM in pmol/mg wet tissue. a) Significantly different ($P<0.05$) compared with control group. ND, not detectable.
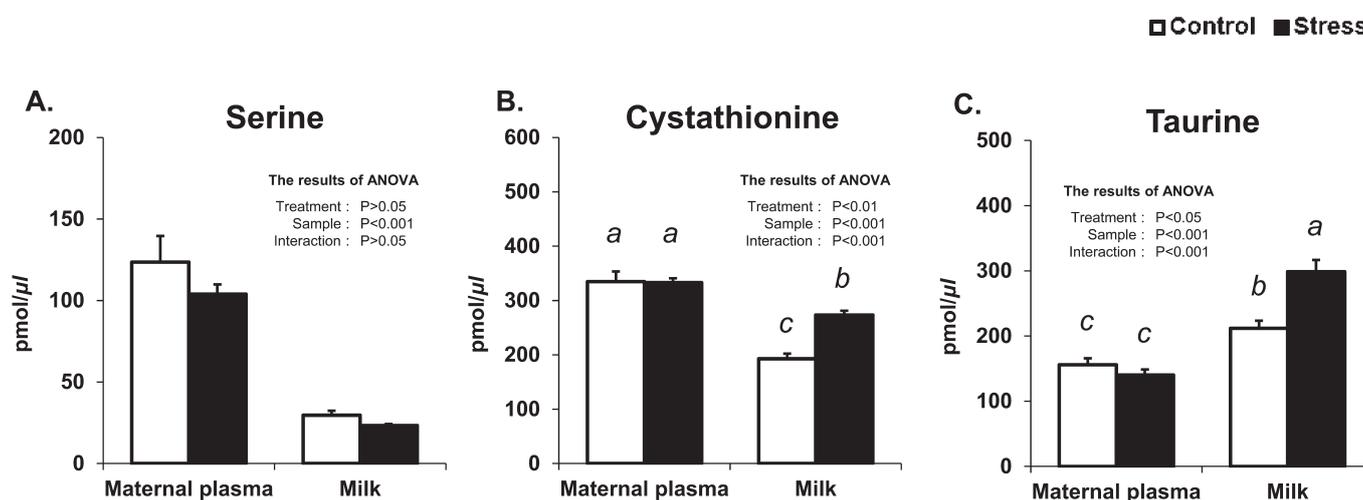

Fig. 2. The effects of restraint stress in lactating mice on the concentration of serine, cystathionine and taurine in maternal plasma and milk collected at P12. The values for the concentrations for each amino acid are expressed as means ± SEM in pmol/μl; n=8 in the control group and the stress group. Different letters denote significant difference ($P<0.05$).

significant ($P<0.001$) interaction suggests that the concentration of cystathionine in milk was lower than that in plasma, but that stress enhanced the lowered concentration of cystathionine in milk. Taurine was significantly ($P<0.05$) increased by the maternal stress treatment, and the concentration of taurine in milk was significantly ($P<0.001$) higher than that in maternal plasma (Fig. 2C). A significant ($P<0.001$) interaction implies that the enhanced taurine in milk was further increased by stress. Furthermore, the concentration of taurine in milk was positively correlated with the concentration of cystathionine in milk (Fig. 3) and also positively correlated with the corticosterone levels in maternal plasma (Fig. 4).

The concentration ratio (concentration in milk/concentration in maternal plasma) of serine, cystathionine and taurine was calculated. The results showed that maternal stress treatment did not alter the ratio of serine or cystathionine, but the ratio of taurine was significantly ($P<0.001$) increased (Table 2). Furthermore, the ratio of taurine was positively correlated with the corticosterone levels in maternal plasma (Fig. 5).

DISCUSSION

Few studies have demonstrated the effects of restraint stress in lactating mice on the concentration of free amino acids in milk. The present study examined the effects of restraint stress in lactating mice on growth and on the concentration of free amino acids, especially sulfur-containing amino acids, in the maternal liver, plasma, mammary glands and milk.

The corticosterone level was increased by restraint stress treatment during the lactation period. Our observation was comparable
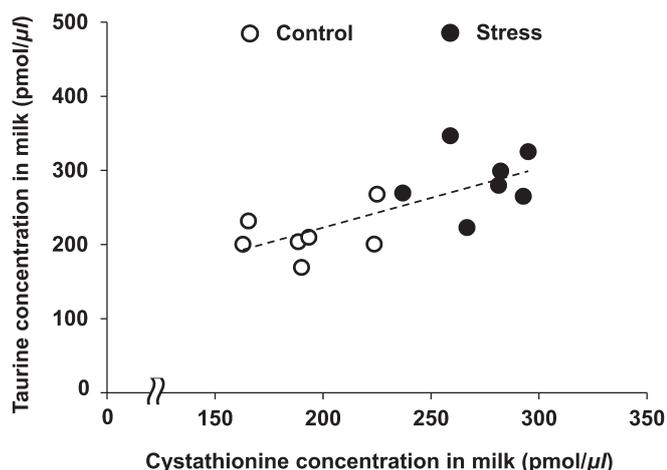
Fig. 3. Correlation between cystathionine concentration in milk and taurine concentration in milk collected at P12 (pmol/μl). r=0.72 (P<0.01).
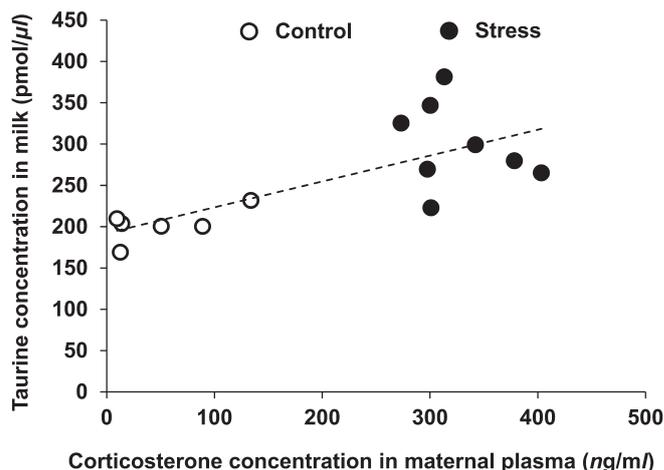

Fig. 4. Correlation between the concentration of taurine in milk collected at P12 (pmol/μl) and the concentration of corticosterone in maternal plasma (ng/ml). r=0.74 (P<0.01).
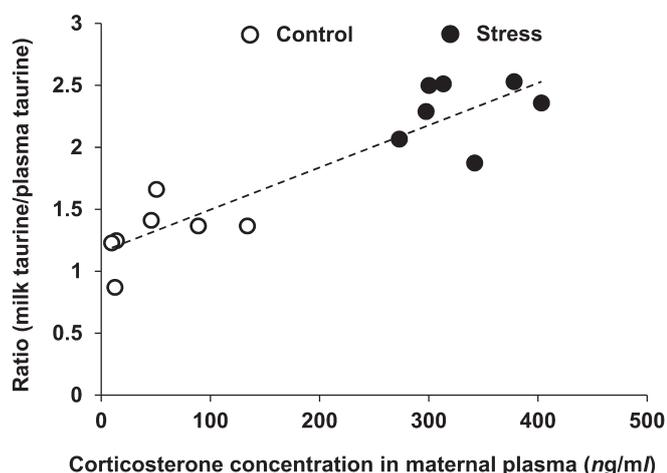

Fig. 5. Correlation between the concentration ratio (milk taurine/ plasma taurine) and the concentration of corticosterone in plasma (ng/ml). r=0.91 (P<0.001).

Table 2. The effects of restraint stress in lactating mice on the concentration ratio (concentration in milk/concentration in maternal plasma) of serine, cystathionine, and taurine

	Serine	Cystathionine	Taurine
Control	0.25 ± 0.02	0.67 ± 0.09	1.31 ± 0.09
Stress	0.23 ± 0.01	0.84 ± 0.02	2.30 ± 0.10[a]

The number of samples used for analysis was 8 for each group.
a) Significantly different (P<0.05) compared with control group.

with previous studies where plasma corticosterone levels were increased in mice and rats treated with restraint stress [9, 18, 19, 26]. In addition, the body weight of maternal mice during the stress treatment was decreased by restraint stress as the days passed. This result was in accordance with previous studies [12, 14, 22] and seems to be due to the decrease in food intake of maternal mice during the stress treatment. In the present study, the food intake of maternal mice during the stress treatment was not measured, but previous studies have reported that the restraint stress decreased the food intake in rodent animals [12, 14, 22]. Thus, maternal stress during the lactation period decreased the food intake of dams, and its decrease caused the decline in their body weight that was found in the present study. These results suggest that the dams in the stress treatment group were in a state of stress during the lactation period.

In the present study, methionine, cysteine and cystine were not detected in the dams' milk. In addition, the concentration of serine and cystathionine in the milk was significantly lower than it was in the maternal plasma, although the concentration of taurine in the milk was significantly higher than it was in the maternal plasma. Methionine, serine, cystathionine, cysteine and cystine are grouped into α-amino acids, which can be divided into L-type amino acids and D-type amino acids, but taurine is not grouped in this way. Nagaoka *et al*. (2009) reported that the free amino acids in mouse milk were converted into keto acids, ammonia, and hydrogen peroxide by high activity of L-amino acid oxidase in the mammary glands [16]. Due to the effect of L-amino acid oxidase, L-amino acids were catabolized a great deal in the mammary glands, with the result that the amino acids, except for taurine, seemed to be decreased in milk or to have disappeared from it altogether. The concentrations of taurine and cystathionine in the stress group were higher than they were in the control group. The concentration of taurine in the milk was
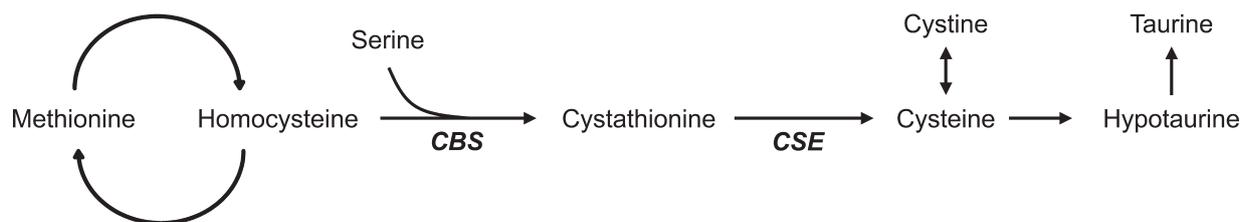
Fig. 6. Metabolic pathway of sulfur-containing amino acids. Cystathionine is synthesized from serine and homocysteine by cystathionine β-synthase (CBS) and metabolized to cysteine by cystathionine γ-lyase (CSE). Taurine is synthesized from cysteine through hypotaurine.

positively correlated with that of corticosterone, which is a good parameter for stress, in the maternal plasma (Fig. 4). This result strongly suggests that the concentration of taurine in the milk was increased by the restraint stress during the lactation periods. It has been reported that the concentration of taurine in rat milk was remarkably high and that it declined as the days passed [24]. In addition, the concentration of corticosterone in maternal plasma has been found to be higher during the lactation period with the dams in the same state with chronic stress [5]. Thus, these previous reports also suggest that the concentration of taurine in milk became higher as a result of higher corticosterone levels in the maternal plasma. Taurine is contained in large amounts in milk, especially in the colostrum [25]. It has been reported that the mRNA of amino acid transporters is expressed in the mammary gland [2, 28] and that their transporters transfer free amino acids from maternal blood to milk. The large amount of taurine in the milk seems to derive from 1) maternal blood via taurine transporters, and 2) the mammary glands. Smith *et al.* (1992) has isolated a cDNA clone (designated rB16a) encoding a taurine transporter from a rat brain [23], and it has been reported that rB16a was expressed in the mammary gland during the gestation and lactation periods [2]. Restraint stress in lactating mice seemed to increase the transportation of taurine from maternal blood to milk in the mammary gland. We examined the relationships between taurine concentration ratio (milk taurine/plasma taurine) and the concentration of corticosterone in plasma as shown in Fig. 5. The increased concentration ratio of taurine in the present study supports this hypothesis. On the other hand, the concentration ratio of cystathionine was not altered by maternal stress during the lactation period. Thus, the increased cystathionine concentration in the milk did not come from increased cystathionine transportation in the mammary gland. The metabolic pathway of sulfur-containing amino acids is shown in Fig. 6. Cystathionine is metabolized from serine and homocysteine by the catabolic action of cystathionine β-synthase (CBS), and metabolized to cysteine by cystathionine γ-lyase (CSE) [1, 29]. In the liver, it has been reported that the gene expression of CBS was down regulated by corticosterone [30]. Although little is known about the relationship between stress and the expression of CBS and CSE, there is a possibility that the expression of these enzymes was increased in the mammary gland by the stress, which resulted in increased cystathionine in the milk. In addition, the positive correlation between the taurine and cystathionine concentrations in milk presents the possibility that CSE is present in milk and promotes the metabolism of cystathionine in milk because the concentrations of cystathionine and taurine in the mammary gland were not changed by stress, which means that stress would not affect the metabolism of sulfur-containing amino acids in the mammary glands. However, the possibility that CSE and CBS are activated in the milk was remained. Further studies are needed to investigate the molecular mechanism whereby stress increased taurine transportation in the mammary gland and the means by which cystathionine was increased in the milk.

In conclusion, restraint stress in lactating mice significantly increased the concentration of cystathionine and taurine in the milk, although their concentrations in the liver, plasma and mammary glands were not altered. Taurine concentration in the milk seemed to be increased as a result of the increased taurine transportation from blood to milk. Although there is a possibility that the changes in the taurine concentration of the milk affect the offspring to some extent, we could not discuss the effects of restraint stress in lactating mice on the growth and behavior of their offspring only with increased taurine concentration in milk. This was because the restraint stress in the present study possibly caused not only taurine increment in milk but also decrement of milk produced, because it has been reported that the gene expression of prolactin in hypothalamus and the level of prolactin in plasma were decreased by stress [3, 7] and prolactin is known to promote milk synthesis. Thus, further studies are needed to investigate the effects of the stress-caused increment of taurine in milk on the growth and behavior of offspring. However, we suggest the possibility that taurine concentration in milk could be a good parameter for the stress status of dam mice because it was positively correlated with the concentration of corticosterone in the maternal plasma. The present study may contribute to elucidating the effects of maternal stress in lactating mice on the sulfur-containing amino acid levels of milk.

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