Increased *N*-Acetyltaurine in Serum and Urine After Endurance Exercise in Human

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Abbreviations

NAT	*N*-acetyltaurine
ACS2	Acetyl-CoA synthase 2
GM	Growth medium
DM	Differentiation medium

1 Introduction

Taurine (2-aminoethanesulfonic acid), which is the most abundant free amino acid-like compound found in mammalian tissues including liver and skeletal muscle (Awapara 1956; Jacobsen and Smith 1968; Huxtable 1980), has been previously reported to have many physiological and pharmacological actions (Pasantes et al. 1998; Huxtable 1992; Miyazaki and Matsuzaki 2014; Nakamura et al. 1993; Miyazaki 2010; Nieminen et al. 1988). The most established role of taurine is the conjugation with hydrophobic bile acids in the liver to increase hydrophilicity and facilitate excretion into the bile (Danielsson 1963; Sjovall 1959).

Recently, Shi et al. has reported that a novel metabolite *N*-acetyltaurine (NAT) that is *N*-acetylated form of taurine with acetate was increased in urine during alcohol catabolism (Shi et al. 2012). NAT is a highly hydrophilic and hygroscopic compound that has also found as a major compound in the viscid droplet of orb spider

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web (Vollrath et al. 1990; Higgins et al. 2001). The synthesis of NAT is depending on acetate level in the body, and acetate is produced from some metabolic sources including alcohol consumption (Buckley and Williamson 1977). Consumed alcohol is degraded to acetate via two-steps enzymatic metabolism in the liver. In addition, acetate is synthesized from acetyl-CoA by acetyl-CoA hydrolase in the liver and utilized as an alternative energy source via conversion to acetyl-CoA in the mitochondria of peripheral tissues by acetyl-CoA synthetase 2 (ACS2) under ketogenic conditions such as fasting and diabetes mellitus (Fukao et al. 2004; Luong et al. 2000; Sakakibara et al. 2009). Because the ketogenic condition would be induced during endurance exercise for continuous energy production in the skeletal muscle and heart, it is suggested that acetate is likely to be synthesized during endurance exercise. Therefore, there is a possibility that NAT biosynthesis might increase in result of *N*-acetylation of taurine with the elevated acetate or acetyl-group of AcCoA during the exercise in the skeletal muscles that abundantly contain taurine.

The present study purposed to determine the NAT in serum and urine after endurance exercises in humans, and in addition, to confirm the cellular production of NAT in skeletal muscle cell line following exposure to taurine and acetate, using HPLC-MS/MS analysis.

2 Methods

2.1 Serum NAT and Taurine Levels After Full-Marathon

Thirty-one healthy non-professional runners who participated in the 32nd Tsukuba Full-Marathon held in November 2012 were recruited as volunteers. Blood was collected from the participants at a day before, immediately after, and a day after the full-marathon race. Taurine and NAT in serum were measured by HPLC-MS/MS system. The study was carried out in accordance with the Declaration of Helsinki and was approved by the Human Subjects Committee of the University of Tsukuba. All subjects provided informed written consent.

2.2 NAT and Taurine Excretions in Urine After an Endurance Exercise

A healthy male (40 years of age) was loaded to a transient running. Experimental protocol was shown in Fig. 1. Before the exercise, urine was collected for 24 h between am11 on previous day and am11 on exercise day. At am11, blood was collected immediately before the exercise. Thereafter, the subject was loaded to run for an hour (running speed; approximately 9 km/h). Blood was also collected at immediately, 1, 2, 3, 4, and 24 h after the exercise. In addition, urine was

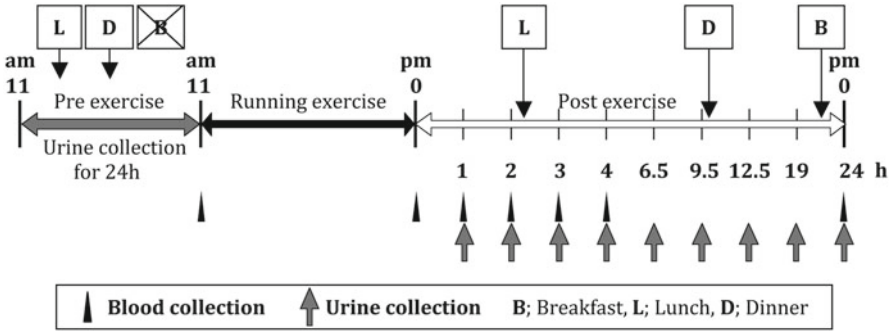

Fig. 1 Experimental protocol of the transient running exercise in a healthy subject. Urine was collected for 24 h before the exercise, and at 1, 2, 3, 4, 6.5, 9.5, 12.5, 19.5, and 24 h after the exercise. Blood was collected immediately before and after the exercise, and at 1, 2, 3, 4, and 24 h after the exercise. On the exercise day, subject did not take the breakfast, but took the lunch and dinner at 2.5 and 10 h after the exercise. Running speed was approximately 9 km/h

collected at 1, 2, 3, 4, 6.5, 9.5, 12.5, 19.5, and 24 h after the exercise. Furthermore, urinary volume in each collection was measured to calculate the amount of daily urinary excretion of taurine and NAT. Data of taurine and NAT in urine were expressed as the urinary excretion per hour. Taurine and NAT concentrations in serum and urine were quantified using HPLC-MS/MS system. Figure 1 Experimental protocol of the transient running exercise in a healthy subject. Urine was collected for 24 h before the exercise, and at 1, 2, 3, 4, 6.5, 9.5, 12.5, 19.5, and 24 h after the exercise. Blood was collected immediately before and after the exercise, and at 1, 2, 3, 4, and 24 h after the exercise. On the exercise day, subject did not take the breakfast, but took the lunch and dinner at 2.5 and 10 h after the exercise.

The study was carried out in accordance with the Declaration of Helsinki and was approved by the Human Subjects Committee of the Tokyo Medical University Ibaraki Medical Center.

2.3 NAT Production from Cultured Skeletal Muscle Cell Exposed to Acetate and Taurine

Mouse differentiable myoblast (C2C12) was purchased from ATCC (Manassas, VA). C2C12 cells were cultured with growth medium (GM; DMEM supplemented with 10 % fetal bovine serum) until confluent, and thereafter, the medium was switched to differentiation medium (DM; DMEM supplemented with 2 % horse serum) (Miyazaki et al. 2013). After differentiation, myotube was exposed to 20 mM taurine in GM for 24 h. Thereafter, the myotube was washed with PBS twice, and further exposed to 1 mM sodium acetate in GM for 24 h. After the incubations,

the culture medium and the myotube were collected to analyze NAT and taurine using HPLC-MS/MS system. Data are obtained from four independent experiments ($N=4$).

2.4 Quantification of Taurine and NAT

Taurine and NAT in serum, urine, culture cell, and culture medium were quantified by HPLC-LC/LC system according to the methods of Shi et al. (2012) and Johnson et al. (2011) with some modifications. In brief, NATs as standard and internal standard were synthesized from taurine (Wako Pure Chemical Industries, Osaka, Japan) and 2-aminoethane-d4 sulfonic acid (taurine-d4; C/D/N Isotopes Inc., Quebec, Canada), respectively, by reaction with acetic anhydride. Five microliter of serum and urine samples and 50 μL of cultured medium were mixed with 100 ng taurine-d4 and 1 ng NAT-d4 as internal standard in 50 μL of acetonitrile-water (19:1, v/v) in a microcentrifuge tube. In assay for culture medium, ten-times volumes of sample and acetonitrile-water were used. After centrifugation, the supernatant was evaporated to dryness at 80 °C under a nitrogen stream. The residue was redissolved in 60 μL of 0.1 % formic acid, and an aliquot (1 μL) was analyzed by HPLC-MS/MS system.

2.5 Statistic Analysis

Statistical significances were determined by unpaired Student's *t*-test or one-way ANOVA multiple comparison test. Data were expressed as the mean ± SEM. Differences were considered as statistically significant when the calculated *P* value was less than 0.05.

3 Results

3.1 NAT and Taurine Concentrations in Serum After Full-Marathon

NAT was detectable in the serum sample (3.2 ± 0.2 nM at before the full-marathon). Immediately after the marathon, serum NAT concentration was significantly increased (18.8 ± 2.4 nM, $P<0.01$ compared to the before). Taurine concentration in serum was also significantly increased immediately after the marathon race (30.9 ± 1.6 μM vs. 39.8 ± 1.8 μM before the marathon race, $P<0.01$). After 1 day, taurine and NAT concentrations in serum recovered to the levels before the marathon race (31.4 ± 1.1 μM and 3.1 ± 0.2 nM, in taurine and NAT, respectively).

3.2 NAT and Taurine Concentrations in Serum and Urine After a Transient Exercise

Figure 2 shows the changes over time of NAT and taurine concentrations in serum (**a**) and urine (**b**) before and after the transient running exercise in a subject. In serum, taurine concentration increased immediately after the exercise compared to that before the exercise, and further increased at 1 h later (Fig. 2a). Thereafter, serum taurine concentration decreased. Compared to before the exercise, NAT concentration in serum markedly increased immediately after the exercise. However, serum NAT concentration recovered to the normal level until 1 h later.

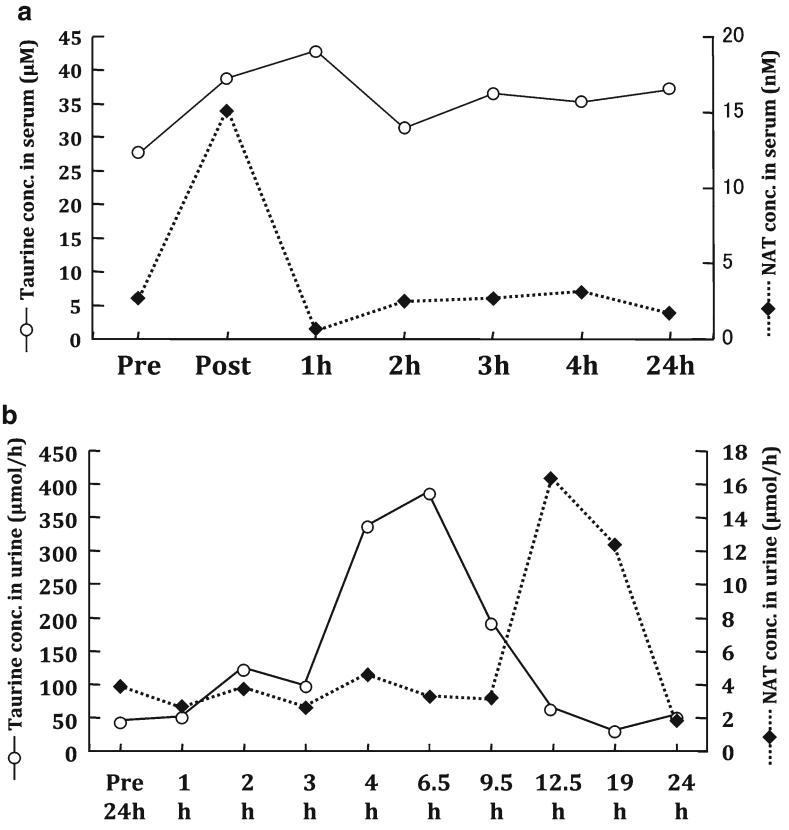

Fig. 2 Changes over time of taurine and NAT concentrations in serum and urine before and after a transient exercise. (**a**) Taurine and NAT concentrations in serum. (**b**) Taurine and NAT concentrations in urine. The concentrations in urine were expressed as the urinary excretion per unit time. *Pre* immediately before the exercise, *Post* immediately after the exercise, *Pre 24 h* 24 hours before the exercise, *conc*. concentration

Figure 2b demonstrates taurine and NAT excretions in urine expressed as its content per hour. In urine, there was little change in taurine concentration until 3 h later of the exercise, but markedly increased at 4 and 6.5 h later (Fig. 2b). Thereafter, the concentration gradually decreased, and returned to the level of before the exercise until 24 h later. NAT was also detectable in urine. Urinary NAT concentration unchanged until after 9.5 h of exercise. Thereafter, urinary NAT concentration dramatically increased between after 12.5 and 19 h of exercise, and returned to the level of before the exercise until 24 h later. Amount urinary excretions of both taurine and NAT increased after 24 h of the exercise compared to before 24 h (taurine; 0.97 mmol/day before exercise vs. 2.79 mmol/day after exercise, NAT; 91.4 μmol/day before exercise vs. 182.9 μmol/day after exercise).

3.3 Taurine and NAT Concentrations in Cell and Cultured Medium in the Myotube Exposed to Taurine and/or Acetate

Intracellular level of taurine was significantly increased by 20 mM taurine treatment for 24 h in both absent and present of acetate, while there was no significant difference between with and without acetate exposures regardless of taurine pretreatments (Fig. 3a). In cultured medium, taurine was undetectable in the conditions without taurine pretreatment (Fig. 3b). Taurine concentration in the cultured medium was detected after the taurine pretreatment, but there was no significant difference between with and without acetate exposures.

NAT could be detected in the both cell and cultured medium (Fig. 3c, d). Intracellular NAT level was significantly increased by 20 mM taurine pretreatment (Fig. 3c) in both with and without acetate exposures. In the taurine pretreated cells, intracellular NAT concentration was significantly higher in the 1 mM acetate exposure than in the absent of acetate. In the cultured medium, NAT was undetectable in the absent of taurine pretreatment (Fig. 3d). In the taurine pretreatment conditions, NAT concentration in the cultured medium was significantly higher in the acetate exposure than in the absent of acetate.

4 Discussion

In the present study, a novel metabolite NAT could be detected in human serum using HPLC-MS/MS system. Furthermore, the present study showed for first time that serum NAT concentration was significantly increased by the endurance exercise as full-marathon race, although blood level of taurine has been already known to be elevated after endurance exercises (Cuisinier et al. 2002; Ishikura et al. 2008). The significantly increased serum concentrations of both taurine and NAT returned to the level before the exercise by a day later.

Because Shi et al. has reported that urinary NAT concentration was increased during alcohol catabolism (Shi et al. 2012) to excrete the excess acetate into urine through *N*-acetylation of taurine, the resent study evaluated the effect of a transient endurance exercise on the urinary excretion of NAT. In the result, daily excretion of urinary NAT was significantly increased by the endurance exercise, suggesting that taurine might be acetylated with the elevated acetate during the exercise. The peak of urinary taurine excretion appeared between 4 and 6.5 h after the exercise. But, the peak of urinary NAT excretion was about 12 h later. There is a possibility that the urinary excretions of taurine and NAT might be influenced by taking meal, because

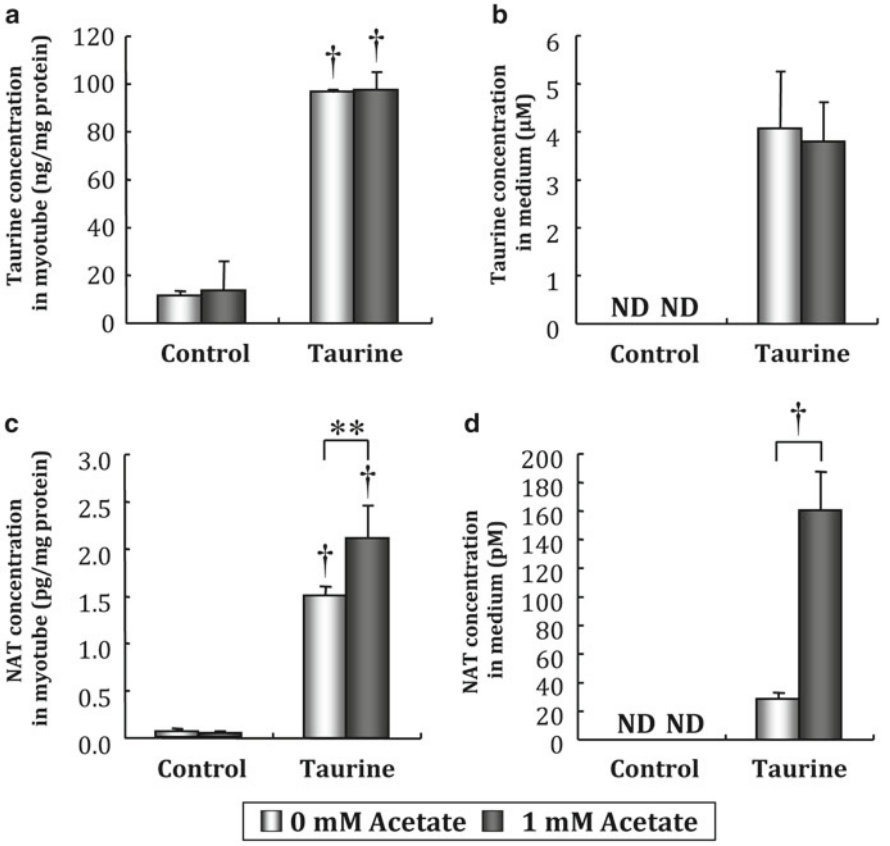

Fig. 3 Taurine and NAT concentrations in the myotube and cultured medium in the cultured skeletal muscle cell exposed to taurine and/or acetate. (**a**) Taurine concentration in the myotube, (**b**) Taurine concentration in the medium, (**c**) NAT concentration in the myotube, (**d**) NAT concentration in the medium. Control and Taurine show the 0 and 20 mM taurine in the culture medium, respectively, prior to the acetate exposure. Taurine and NAT concentrations in the myotube were expressed as per total protein content measured by the BCG method. Data are the mean ± SEM. *ND*; means "no detected". Symbols on the column without bar show the significant difference compared to the respective control. $^{**}P<0.01$, †$P<0.001$

the excretory peak of taurine was at after taking a lunch. However, the excretory peaks of taurine and NAT did not appear after taking a dinner and lunch, respectively, and the excretory peak of taurine to urine appeared after the peak of serum taurine after the exercise, and therefore, the changes of their excretions into urine is suggested to be influenced by the exercise rather than taking meals. It is unclear the reason why the timing of NAT excretion in urine was different with taurine, but there is a possibility of reaction time of acetylation with taurine and acetate.

Shi et al. described that *N*-acetylation of taurine is synthesized mainly in the liver and kidney during alcoholic catabolism (Shi et al. 2012). Because acetate produced in the liver would be used for acetyl-CoA synthesis as energy source through ACS2 in the skeletal muscle (Sakakibara et al. 2009), we hypothesized that *N*-acetylation of taurine might react in the skeletal muscle during endurance exercise. In the skeletal muscle cell culture experiment in the present study, NAT excretion from the myotube was significantly increased by exposing to taurine and acetate. Therefore, the increased NAT in serum after the endurance exercises might be derived from the skeletal muscle.

However, Shi et al. also mentioned that taurine would react directly with acetate in one-step enzymatic and ATP-independent pathway that does not require the conversion of acetate to acetyl-CoA, in the cytosol of kidney and liver during alcoholic catabolism (Shi et al. 2012). Indeed, acetyl-carnitine, a product after reaction with carnitine and excess acetyl-group of acetyl-CoA in β-oxidation (Liu et al. 2008), was unchanged in the both cell and medium by exposing to acetate (no data shown). Therefore, the production of NAT in the cultured muscle cell might be also due to the direct reaction of taurine with acetate, but not through acetyl-CoA. Furthermore, there is a possibility that NAT might be synthesized in other tissues including the kidney, and further studies are needed to clarify. Taurine has been reported to have a lot of biological, physiological, and pharmacological functions in various tissues including membrane stabilization (Pasantes et al. 1998), detoxification (Huxtable 1992; Miyazaki and Matsuzaki 2014), antioxidation (Nakamura et al. 1993; Miyazaki 2010), and osmoregulation (Nieminen et al. 1988). During the alcoholic catabolism, it is suggested that NAT might be produced in the kidney in order to facilitate the urinary excretion of excess acetate by higher hydrophilicity via reaction with taurine. On the other hand, the physiological role of the novel metabolite NAT has been unclear still now.

5 Conclusion

The present study confirmed (1): Serum NAT level significantly increased after the full-marathon race, (2): The increased NAT in serum recovered to the normal level after a day, (3): Urinary NAT excretion significantly increased after endurance running, (4): NAT production from the skeletal muscle cultured cell significantly increased following taurine and acetate treatments. These results indicate the significant increases of NAT in serum and urine after endurance exercises in humans, suggesting that taurine might be *N*-acetylated with excess acetate in the skeletal muscle during the exercise.

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