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Molecular imaging of enhanced Na^+ expression in the liver of total sleep deprived rats by TOF-SIMS

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ABSTRACT

Sleep disorder is associated with metabolic disturbances, which was related to oxidative stress and subsequently sodium overload. Since liver plays important roles in metabolic regulation, present study is aimed to determine whether hepatic sodium, together with oxidative stress, would significantly alter after total sleep deprivation (TSD). Sodium ion was investigated by time-of-flight secondary ion mass spectrometry (TOF-SIMS). Parameter for oxidative stress was examined by heat shock protein-25 (HSP-25) immunohistochemistry. TOF-SIMS spectrum indicated that hepatic Na^+/K^+ ratio counting as 82.41 ± 9.5 was obtained in normal rats. Sodium ions were distributed in hepatocytes with several aggregations. However, following TSD, the intensity for Na^+/K^+ ratio was relatively increased (101.94 ± 6.9) and signals for sodium image were strongly expressed throughout hepatocytes without spatial localization. Quantitative analysis revealed that HSP-25 staining intensity is 1.78 ± 0.27 in TSD rats, which was significantly higher than that of normal ones (0.68 ± 0.15). HSP-25 augmentation suggests that hepatocytes suffer from oxidative stress following TSD. Concerning oxidative stress induced sodium overload would impair metabolic function; enhanced hepatic sodium expression after TSD may be a major cause of TSD relevant metabolic diseases.

1. Introduction

Sleep is a vital activity of daily living as it restores and refreshes our metabolic functions [1]. Chronic sleep deprivation would increase the risk of numerous metabolic diseases such as hypertension, atherosclerosis, insulin resistance, as well as stress-induced interruption of homeostatic function, which consequently lead to energy expenditure [2,3]. Our previous studies also demonstrated that total sleep deprivation (TSD) would predispose the liver to oxidative injury and depress the intracellular metabolic pathway of nodose neurons, which could cause clinical changes of metabolic and cardiovascular responses [4,5]. It is reported that increased energy expenditure would elevate the oxidative stress and subsequently increase in intracellular sodium levels [6,7]. Enhanced cytosolic sodium has been suggested to play an important role in the development of osmotic stress that effectively lead to membrane rupture and cell death [8].

However, as far as can be ascertained, no study concerning the effects of sleep deprivation on sodium expression has been reported. Furthermore, the corresponding changes of oxidative status following TSD have not been clearly explored. Since liver plays an important role in maintenance of metabolic function and is highly susceptible to chain reaction of oxidative stress [9], the present study is aimed to determine the hepatic sodium expression in TSD rats by the use of time-of-flight secondary ion mass spectrometry (TOF-SIMS). In addition, in order to clearly clarify the relationship between sodium expression and the extent of oxidative stress, heat shock protein 25 (HSP-25, the "stress" protein that has been documented to be induced by a variety of environmental stresses [10]) immunohistochemistry was concomitantly processed in the current study.

2. Experimental

2.1. Treatments and experimental animals

Adult male Wistar rats ($n = 36$, weighing 200–250 g) obtained from the Laboratory Animal Center of the National Taiwan

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University were used in this study. The experimental animals were divided equally into two groups. Rats in the first group were subjected to TSD for 5 days (TSD group), while those in the second group were housed in the TSD apparatus but were permitted to sleep (normal group). TSD was performed by the disc-on-water (DOW) method as described in our previous studies [4,5]. Sleep deprivation depends on the rat's aversion to water, since rats rarely entered the water spontaneously. As sleep deprivation begins, rats in the TSD group placing on the disc had to keep awake and walk against the direction of disc rotation to avoid being forced into the water. For normal group, rats were allowed to sleep wherein no disc movement was initiated. All experimental animals were exposed to an automatically regulated light:dark cycle of 12:12 at a constant temperature (25 ± 1 °C). Food and water were made available through grids placed on top of the chambers. In the care and handling of all experimental animals, the Guide for the Care and Use of Laboratory Animals (1985) as stated in the United States NIH guidelines (NIH publication No. 86-23) were followed.

2.2. Perfusion and tissue preparation

For TOF-SIMS analysis and HSP-25 immunohistochemistry, rats were deeply anesthetized and perfused transcardially with saline followed by 4% paraformaldehyde in 0.01 M phosphate buffer saline, pH 7.4. The liver was then removed and immersed in 30% sucrose buffer for cryoprotection at 4 °C. Serial 15 μm thick sections of the tissue were cut transversely with a cryostat and were alternately placed into two wells of a cell culture plate. Sections collected in the first well were attached to silica wafers and processed for TOF-SIMS analysis, while those in the second well were processed for HSP-25 immunohistochemistry.

2.3. TOF-SIMS analysis

TOF-SIMS analysis was carried out on a TOF-SIMS IV instrument (ION-TOF GmbH, Münster, Germany). The primary ion source was $^{69}Ga^+$ (pulsing current 2.0 pA) operated at 10 kV post-acceleration. An analysis area of 100×100 μm^2 with image data acquisition time of 200 s was measured. Charge compensation by applying low-energy electrons (~30 eV) from a pulsed flood gun was used during the measurements. The best resolution obtained was $m/\Delta m = 8071$. Spectrometric data was processed using inbuilt IonSpec and Ion image software. Positive ion mass spectra were averaged from an image (128×128 pixels) which was stored in four dimensional formats (x, y, ion counts, and drift time). Mass calibration of ion spectrum was achieved by using a set of mass peaks like m/z 15 (CH_3^+), 27 (C_2H_3), 41 ($C_3H_5^+$) and 55 ($C_4H_7^+$). As normal cells are intended to reach osmotic equilibrium by simultaneously maintaining intracellular lower sodium and higher potassium concentration, the "Corr. Area" of Na^+ intensity was normalized to K^+ one (Na^+/K^+) in which this ratio was used as an indicator for evaluating the physiological significance of sodium intensity.

2.4. HSP-25 immunohistochemistry

For HSP-25 immunohistochemistry, tissue sections were firstly treated with blocking medium to block non-specific binding. After that, sections were incubated in mouse monoclonal anti-HSP-25 antiserum (Chemicon, MAB3842, Temecula, CA, USA) at a dilution of 1:100 with blocking medium for 48 h at 4 °C. Following this, sections were further incubated with goat-anti-mouse biotinylated secondary antibody (Vector Laboratories, Burlingame, CA, USA) at room temperature. Immuno-reaction product was revealed by standard avidin–biotin complex procedure with 3,3′-diaminobenzidine as a substrate of peroxidase.

2.5. Quantitative image analysis

The general approach for quantitative image analysis was similar to that used in our previous studies [4,5]. HSP-25 staining intensity was quantified with a computer-based image analysis system and Image-Pro Plus software. True optical density (OD) of immuno-reaction was determined by subtracting the background staining (densitometric readings taken from vascular lumen space) from total OD, so that each measurement was made unbiasedly to correct for background.

3. Results and discussion

3.1. TOF-SIMS mass spectra

Fig. 1 shows the TOF-SIMS positive ion mass spectra in the m/z of 23 and Na^+/K^+ ratio that reflects sodium ion intensity in the liver of both normal and TSD rats. The positive ion spectrum revealed that the intensity for major peak of hepatic sodium was counted to be 2.97×10^5 in normal rats (Fig. 1A). However, following 5 days of TSD, the spectrum for hepatic sodium ion intensity was slightly increased to 3.43×10^5 (Fig. 1B). It is noteworthy that after using the "Corr. Area" for intensity of Na^+ and K^+ peaks, the calculated Na^+/K^+ ratio also revealed a higher expression in TSD (101.94 ± 6.9) than in normal group (82.41 ± 9.5) (Fig. 1C), suggested that the intensity change of sodium ion detected in current paradigm is a distinct effect. It is indicated that keeping Na^+/K^+ homeostasis is of particular importance for exerting normal physiological functions [11]. Impairment of Na^+/K^+ regulation would cause osmotic imbalance that disrupt plasma membrane integrity and subsequently interrupt cellular activities [12]. Previous studies have indicated that intracellular sodium accumulation could alter Na^+/K^+ translocase or other ion transport systems [12,13]. Impaired ion equilibrium would result to osmotic overload and cytosolic acidification that ultimately leads to metabolic dysfunctions [14]. Since liver acts as an essential part in numerous metabolic and detoxifying pathways, alteration in hepatic sodium expression would undoubtedly initiate numerous metabolic diseases. Although the detailed mechanism for pathogen-

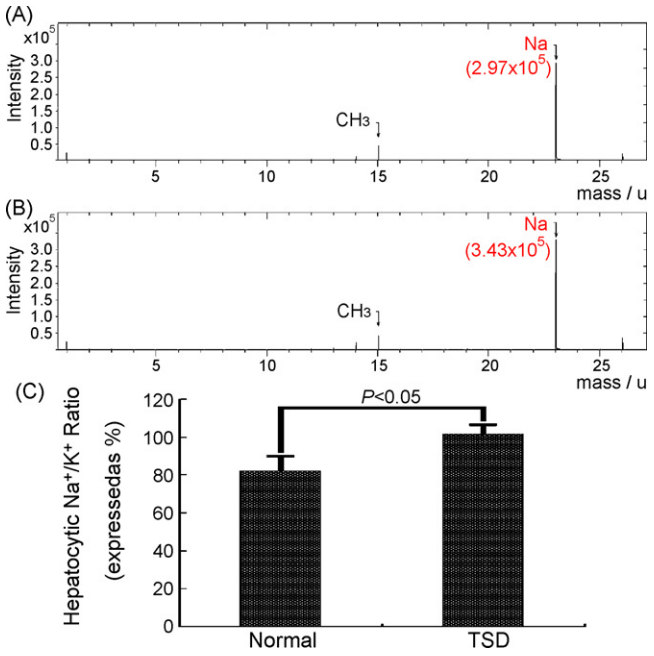

Fig. 1. TOF-SIMS positive ion spectrum (A, B) and histogram (C) showing the sodium ion intensity and Na^+/K^+ ratio in the liver of normal (A) and TSD (B) rats.

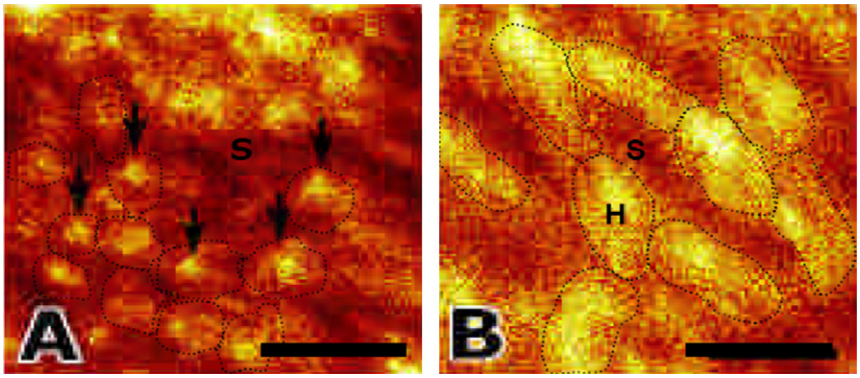

Fig. 2. TOF-SIMS positive ion image showing sodium ion expression in the liver of normal (A) and TSD (B) rats. The profile of each hepatocyte was outlined by dashed lines. Note the sodium mediated hepatocytic swelling was clearly identified in TSD group (B). H: hepatocyte; S: sinusoid; scale bar = 35 μm.

esis of TSD relevant disorders is not fully understood, our present study thus provided the first insight that enhanced hepatic sodium expression may play an important role in developing of TSD induced metabolic dysfunctions.

3.2. TOF-SIMS molecular image

TOF-SIMS positive ion image has revealed that in normal rats, sodium ions were mainly distributed in hepatocytes with apparent intracellular aggregations (Fig. 2A, arrows). The relatively round aggregations seem to locate in central portion of each hepatocyte with no obvious intensity discrepancy. However, in animals subjected to TSD, the sodium ions were strongly expressed throughout the whole hepatocytes without significant intracellular distribution (Fig. 2B). The swelling profile of each hepatocyte is clearly visible, suggested that hepatocytes would suffer from osmotic stress directly resulted from sodium overload. It is reported that TSD would cause oxidative stress that followed by energy expenditure [1–5]. Depletion of energy would lower intracellular pH and trigger the influx of sodium [13,14]. In the present study, we have provided the first molecular imaging evidence that augmented sodium expression following TSD is positively correlated with sodium mediated osmotic swelling of hepatocytes. Although the changes in hepatocytic volume following sodium overload may be crucial in triggering TSD relevant metabolic dysfunction, derangement of cytoskeletal structure due to energy depletion might also limit mechanical efficiency involving in cellular volume regulation and consequently lead to metabolic injury [15].

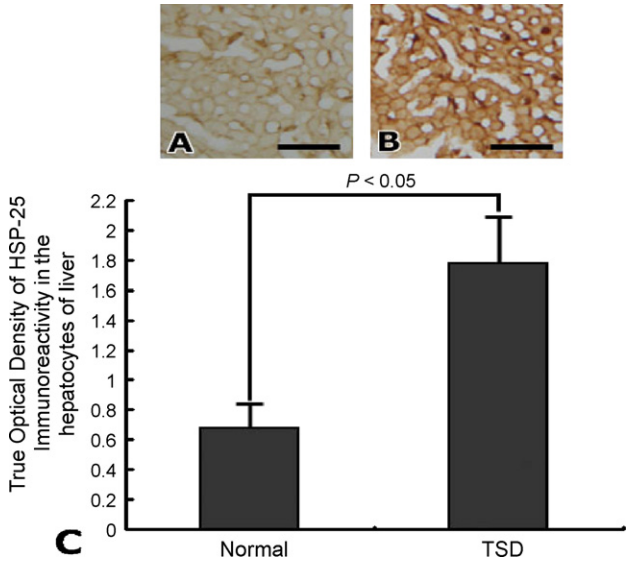

Fig. 3. Light photomicrographs (A, B) and histograms (C) showing quantitative HSP-25 immunoreactivity in hepatocytes of normal (A) and TSD (B) rats. Scale bar = 50 μm.

3.3. HSP-25 immunoreactivity and image analysis

In normal rats, only mild level of HSP-25 immunoreactivity was detected in hepatocytes (Fig. 3A). The HSP-25 staining was distributed diffusely throughout the parenchyma without any regional enrichment (Fig. 3A). However, following TSD, HSP-25 immuno-staining was remarkably increased (Fig. 3B). Up-regulated staining was homogeneously labeled in hepatic cytoplasm with no preferential localization (Fig. 3B). Quantitative image analysis revealed that the true OD of HSP-25 was estimated to be 0.68 ± 0.15 and 1.78 ± 0.27 in normal and TSD rats, respectively (Fig. 3C). Significant increase in HSP-25 true OD suggested that hepatocytes were suffered from severe oxidative stress following TSD. The enhancement of "stress protein" coincides well with the elevated sodium expression, which indicates that oxidative stress and subsequently sodium overload may be a key factor contributing to the development of TSD relevant metabolic damage.

4. Conclusions

The present study is the first report employing TOF-SIMS analysis to provide molecular imaging evidence that sodium ions would relatively up-regulate in the liver following TSD. The pathological significance of enhanced sodium expression is reflected by elevated HSP-25 immuno-expression, a morphologically reliable marker for oxidative stress. To the extent of our knowledge, the present study has addressed for the first time that *in vivo* hepatic sodium expression, together with profile of hepatocytes, could be clearly and simultaneously analyzed in tissue sections after TSD. Since sodium overload is an important upstream signal for metabolic disturbance, designing agents that effectively prevent sodium overload could thus represents an attractive target for strategies aimed at preventing TSD related metabolic damage.

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