学 位 論 文 の 要 旨

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学　位　論　文　名　Pathophysiological Significance of *Stim1* Mutation in Sympathetic Response to Stress and Cardiovascular Phenotypes in SHRSP/Izm: In vivo Evaluation by Creation of a Novel Gene Knock-in Rat Using CRISPR/Cas9

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論 文 内 容 の 要 旨

INTRODUCTION

The stroke-prone spontaneously hypertensive rat (SHRSP) is one of the best disease models for essential hypertension. Previous studies have indicated that SHRSP shows sympathetic hyper-responsiveness to various forms of stress. Stress sensitivity is an important physiological factor associated with the pathogenesis of hypertension and an increased risk of cardiovascular events in humans. Therefore, identification of genes responsible for the exaggerate sympathetic response to stress in SHRSP would provide an important clue to uncover the pathophysiological basis of essential hypertension in humans as well as in rodents.

We identified a quantitative trait locus (QTL) for the exaggerated sympathetic response to stress in SHRSP in chromosome (chr) 1, which was confirmed in reciprocal congenic strains constructed between SHRSP and a normotensive rat strain, WKY. Recently, we further identified the stromal interaction molecule 1 (*Stim1*) as a promising candidate gene within the QTL since SHRSP had a premature stop codon (p.Arg640X) in this gene that resulted in expression of a truncated form of STIM1 ($STIM1^{SHRSP}$) lacking 46 amino acids at the C-terminal end.

STIM1 is an endoplasmic reticulum (ER)-resident transmembrane protein and an essential component in store-operated Ca^{2+} entry (SOCE) which plays a pivotal role in maintenance of intracellular Ca^{2+} homeostasis in various cells. Defective SOCE activity due to abnormal STIM1 function has been implicated in the pathogenesis of diseases such as cardiac hypertrophy, type 2 diabetes, immunodeficiency, and autoimmunity.

In an *in vitro* study, we showed that the STIM1^{SHRSP} caused a decrease in SOCE activity in primary culture of cerebral astrocytes which is an abundant cell type in brain and participate in regulation of sympathetic activity. To clarify whether the impaired SOCE would be causally related to the exaggerated sympathetic response in SHRSP *in vivo*, in the present study, we created *Stim1* knock-in SHRSP (SHRSP- *Stim1*^{em1Izm}; abbreviated as KI SHRSP hereafter), in which the mutated *Stim1* was substituted for the wild-type *Stim1* by the gene-editing technology using CRISPR/Cas9.

MATERIALS AND METHODS

Stim1 knock-in SHRSP

SHRSP/Izm was provided by the Disease Model Cooperative Research Association (Kyoto, Japan). The CRISPR/Cas9-mediated knock-in of the wild-type *Stim1* into SHRSP/Izm was performed in Kyoto University as described previously (Mahal Z et al. *Hypertens Res* 2019; 42: 610-617). A single-stranded oligodeoxynucleotide used for a nucleotide substitution in the *Stim1* was as follows;

5'-ATAGCCTTCTTGCCAGCCAAGTGGGGAATTCGTGTGTTTCGGCTACCCTGCAGGGC TCGGCTGTCCCCAACTGGAGATGGCCATCTCCAGTTGGGGACAGCCGAGCCCTGCAG GGTAGCCGA(Arg)AACACACGAATTCCCCACTTGGCTGGCAAGAAGGCTAT-3'.

Potential off-target sites in the rat genome (rn5) were identified using the CRISPR design tool (crispr.mit.edu), which were sequenced in KI SHRSP to confirm no changes in off-target sites. STIM1 expression was examined by western blotting using brainstem lysates obtained from SHRSP and KI SHRSP. All experiments with animals in this study were approved by the Ethics Committee for Animal Experimentation of Shimane University.

Culture of cerebral astrocytes and fluorescent Ca^{2+} imaging for evaluation of SOCE

This experiment was performed as previously described (Ohara H, Nabika T. 2016 *Biochem Biophys Res Commun* 2016; 476: 406-11). Briefly, astrocytes obtained from neonatal rats (1-3 days old) were loaded with 4 μM Fluo-8 AM (an intracellular Ca^{2+} indicator) and observed using a confocal fluorescent microscope, FV1000-D.

Evaluation of stress response

Blood pressure (BP) and heart rate (HR) were measured using the tail-cuff method (BP-98A, Softron) at 12, 16 and 20 weeks of age. To evaluate sympathetic stress response, the rats were imposed to restraint stress (by placing a rat in a stainless-steel holder adjusted to the rat's body size) and cold stress (by placing a rat in a cage kept at 4 °C) as previously described (Ferdaus MZ et al. *PLOS ONE* 2014; 9: e95091). BP and HR changes under restraint and cold stress were monitored using the telemetry system (HD-S10, Data Science). Urine was collected under the cold stress for 6h and urinary norepinephrine (NE) excretion was measured to evaluate

sympathetic nerve activity.

RESULTS AND DISCUSSION

Stim1-specific genome-editing in KI SHRSP and expression of wild-type STIM1 in the brainstem as well as cultured astrocytes from KI SHRSP were confirmed by sequencing and western blotting, respectively. In addition, Ca^{2+} imaging showed that SOCE in astrocytes from KI SHRSP was significantly greater than that from SHRSP. Taken together, the results indicated functional recovery of STIM1 in KI SHRSP *in vivo* without potential off-target mutations.

BP measured by the tail-cuff method did not significantly differ between SHRSP and KI SHRSP at all examined ages, indicating that increased SOCE in SHRSP did not affect baseline BP. On the other hand, HR of KI SHRSP at 16 and 20 weeks of age was significantly lower than that of age-matched SHRSP. Regarding stress response, increase in urinary NE excretion under cold stress and BP elevation under cold/restraint stress did not significantly differ between SHRSP and KI SHRSP, while HR elevation under restraint stress in KI SHRSP was significantly lower than that in SHRSP.

In summary, the present results indicated that the rescue of the natural nonsense mutation in *Stim1* of SHRSP did not reduce the exaggerated sympathetic response to stress, while we unexpectedly found inter-strain differences in HR measured by two different methods (tail-cuff and telemetry under restraint stress). The tail-cuff measurement requires restriction of active movement of rats using holders, thus the two methods are partly in common in terms of 'restriction'. The observation on HR might therefore suggested that KI SHRSP was resistant not to physical stress (cold stress) but to mental stress (restraint stress). STIM1 is known to play an important role in cardiac pacemaking through regulation of Ca^{2+} dynamics in the sinoatrial cells. It may be worth exploring effects of the truncated STIM1 on the heart function in future studies.

CONCLUSION

We could not indicate that *Stim1* was the gene responsible for the exaggerated sympathetic response in SHRSP. Nevertheless, HR response under "restraint conditions" would be an intriguing cardiovascular phenotype of KI SHRSP in future investigation.