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研究課題名（和文）ホモカルノシンを骨格筋において増加させる新規手法およびその生理機能の探索

研究課題名（英文）Exploration of new methods to increase homocarnosine in skeletal muscle and their physiological functions

研究代表者

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研究成果の概要（和文）：本研究では、骨格筋の内因性ホモカルノシン合成を誘導するためには、食餌で摂取したGABAが有効であることを明らかにした。さらに、GABA分解阻害剤は血中GABA濃度と骨格筋中のホモカルノシン濃度を効率的に増加させることを見出した。ホモカルノシンとそのファミリーは、骨格筋における幹細胞であるサテライト細胞の細胞死への感受性を低下させることを発見し、ホモカルノシンが筋再生に促進的な効果を有することを示唆した。
本研究は、健全な骨格筋を維持し、サルコペニアを予防するために、筋再生におけるホモカルノシンとカルノシンの新たな生理的役割を研究するための強力な情報となるものである。

研究成果の学術的意義や社会的意義
This study provides the new strategy to induce endogenous homocarnosine synthesis in the muscles and new mechanisms in regulating muscle regeneration. The findings will lead to the development of functional foods for building healthy muscles and preventing sarcopenia in both young and aging people.

研究成果の概要（英文）：This study showed that dietary GABA is a great strategy to induce endogenous homocarnosine synthesis in skeletal muscles. However, dietary GABA is highly fed into a degradation pathway rather than fed into the homocarnosine synthesis. Thus, high doses of GABA intake is required to increase muscular homocarnosine levels. To solve the problem, inhibitors of GABA degradation efficiently increased blood GABA levels and muscular homocarnosine levels. The findings suggest that dietary homocarnosine with GABA or GABA-degrading inhibitors may be a new method to enhance the efficiency of increasing imidazole peptides in the muscles. we found that homocarnosine and its analogs decrease satellite cell susceptibility to early cell death upon activation, suggesting positive effects of homocarnosine on muscle regeneration. This study provides a strong foundation for the research on new roles of homocarnosine and carnosine in muscle regeneration to build up healthy muscles and to prevent sarcopenia.

研究分野：食品科学関連

キーワード：homocarnosine　GABA　skeletal muscle　muscle regeneration　satellite cell　sarcopenia　carnosine

１．研究開始当初の背景

(1)　Carnosine (ß-alanine-histidine) is a dipeptide present in high concentrations in skeletal muscle. Over 100 years since carnosine was discovered, it has been extensively studied and well reported to have a function in improving exercise performance. Moreover, recent research has suggested that carnosine may play a role in preventing sarcopenia in aging people. Based on those reported muscle beneficial functions, therefore, researchers in various fields have been trying to establish efficient methods for increasing carnosine in skeletal muscle instead of the current conventional method of an oral administration of carnosine or ß-alanine, which has low efficiency due to blood instability and side effects.

(2)　Therefore, in this study, we have proposed a peptide named homocarnosine (γ-aminobutyric acid (GABA) -histidine), which has a structure similar to that of carnosine, as an alternative functional peptide of carnosine. In general, homocarnosine is highly present in the brain, but present in very low concentrations in skeletal muscle. In my previous study, I have found that vitamin B6 could induce an increase in homocarnosine and its precursor GABA levels in heart muscle. Taken together, these findings suggest that intake of food factors such as GABA or vitamin B6 may possibly induce endogenous synthesis of homocarnosine in skeletal muscle.

２．研究の目的

Therefore, the main objectives of this study were 1) to determine food factors that can induce endogenous synthesis of homocarnosine in skeletal muscle and 2) to determine if homocarnosine has any beneficial functions in skeletal muscle.

３．研究の方法

(1)　Inducing endogenous homocarnosine synthesis in skeletal muscle by GABA intake

In this experiment, we gave a basal diet mixed with 0 (control), 5 (0.5%), 20 (2%), or 50 (5%) g GABA/kg diet to male ICR mice (5 weeks old) for 6 weeks. Then, we measured imidazole peptides (carnosine, anserine, and homocarnosine), GABA, and ß-alanine content in skeletal muscle, blood, heart, liver, kidney, and brain by HPLC and UPLC-MS/MS methods.

(2)　Increasing circulating GABA levels by inhibiting the GABA degradation pathway

In general, dietary GABA is highly degraded in liver by the degrading enzyme, GABA transaminase (GABA-T). Thus, in order to increase GABA levels in blood, the GABA-degrading enzyme inhibitor vigabatrin (Sabril®) at a dose of 250 mg/kg body weight was administered daily by subcutaneous injections for 2 weeks to mice receiving the GABA diets.

(3)　Mass production of homocarnosine

In this experiment, the method of Fmoc-based solid-phase homocarnosine synthesis was selected and established. The detailed method is shown in Fig. 1.

(4)　Muscle regeneration

In order to examine muscle regeneration, firstly muscle injury or damage is induced via intramuscular injection of 10 μM cardiotoxin (CTX; Latoxan) on tibialis anterior (TA) and/or gastrocnemius (GAS) muscles. At 0, 0.5, 2, 5, or 10 days post injury, muscles were harvested and subjected to further analysis to evaluate effects of an ability of muscle regeneration.

(5)　Satellite cell (SC) isolation and culture

Single myofibers isolation and culture was performed. Briefly, extensor digitorum longus (EDL) muscles were harvested and digested using collagenase type I. Isolated myofibers were cultured in plating medium at 37°C for 0, 24, or 48 hr. Then, fixed fibers were co-immunostained for Pax7 and MyoD. Without culturing (0 hr), SCs are in quiescent sate and stained positive with $Pax7^+$. Following 24 and 48 hr of culture, SCs will be activated and enter cell cycle for proliferation (stained positive with $MyoD^+Pax7^+$), commitment to differentiation (stained positive with $MyoD^+$), or self-renew (stained positive with $Pax7^+$). The effects of food factors on SC functions were evaluated by observing the number of $Pax7^+$,

MyoD⁺Pax7⁺, or MyoD⁺ cells under a fluorescent microscope.

Fig. 1

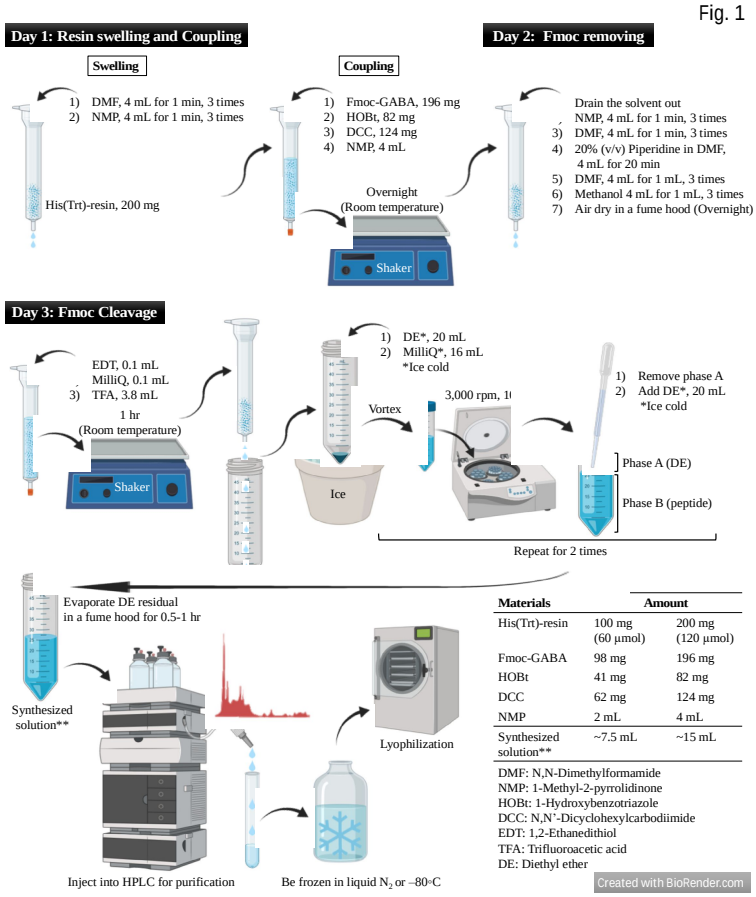

Fig. 1. Fmoc-based solid-phase homocarnosine synthesis

4．研究成果

(1) Dietary GABA induces endogenous synthesis of homocarnosine in skeletal muscle

We successfully demonstrated that dietary GABA is a great strategy to increase homocarnosine levels in skeletal muscle. As shown in Fig. 2, skeletal muscle homocarnosine levels were significantly increased in the 2% and 5% GABA intake groups (10-fold and 53-fold, respectively). However, GABA intake had no effect on the levels of homocarnosine analogs (carnosine and anserine) and ß-alanine. In addition, we found that vigabatrin (GABA-T inhibitor) administration to mice receiving 2% GABA intake for 2 weeks led to GABA-T inhibition in the liver and an increase in circulating GABA levels with a tendency increase in skeletal muscle homocarnosine levels.

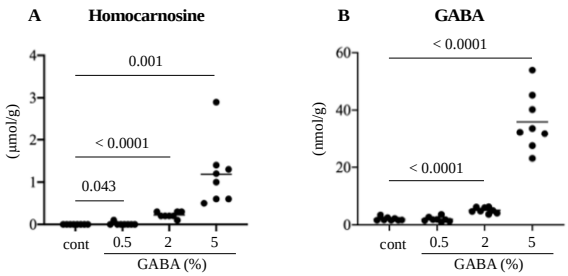

Fig. 2 Dietary GABA increased homocarnosine levels in skeletal muscle

Our study is the first to show that in addition to a typical muscle peptide carnosine, the new muscle peptide homocarnosine can be induced into the muscle by supplying its substrate GABA in tissues. As GABA availability is tightly regulated by GABA-T via GABA degradation, inhibitors of GABA or ß-alanine degradation could be novel potential interventions for increasing skeletal muscle imidazole dipeptides. The details of this research result are described in the published paper[1)].

(2) Mass production of homocarnosine

In this part, we successfully established an in-house method for synthesizing homocarnosine based on Fmoc-based solid-phase synthesis method as the protocol shown in Fig. 1. The representative peaks in a purification step by HPLC are shown in Fig. 3. In Fig. 3B, the synthesized solution (phase B) contained both contaminated histidine (P1) and synthesized homocarnosine (P2). The purified homocarnosine peak is shown in Fig. 3D. The efficiency of this synthesis method is > 95%; 100 mg resin gives homocarnosine 10-15 mg.

(3) Homocarnosine maintains satellite cell viability upon activation on mouse myofibers

In this study, we examined the effects of homocarnosine on satellite cells (SCs), the muscle stem cells playing a crucial role in muscle regeneration. As shown in Fig. 4, after 24 h, the number of activated SCs (control-24 hr) was significantly decreased as compared with those on immediately fixed myofibers (quiescent SCs, control-0 hr). The treatment with 25 mM homocarnosine and its analogs (carnosine and anserine) prevented this decrease in SC number. These results indicate that homocarnosine and its analogs decreases SC susceptibility to early cell death upon activation. We believe that the results of this study provide a strong foundation for the research on the role of homocarnosine and carnosine in muscle regeneration.

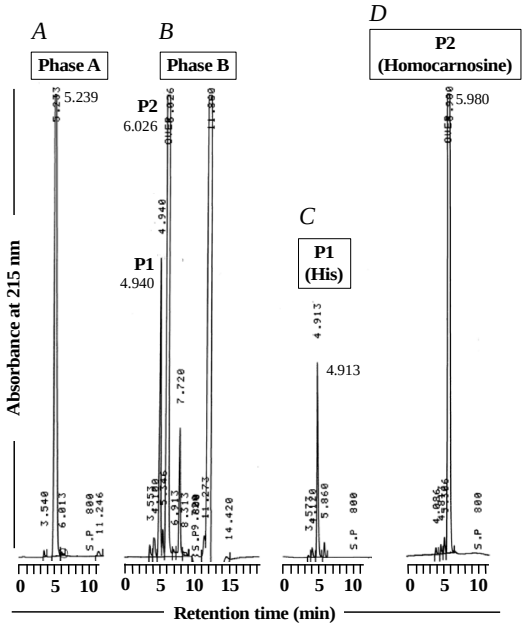

Fig. 3 Purification of synthesized homocarnosine from HPLC analysis

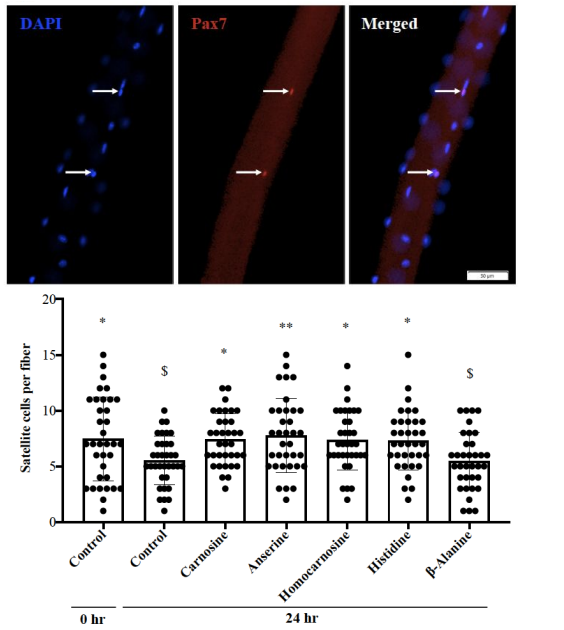

Fig. 4 Homocarnosine prevents cell death of SCs on myofibers after 24 h of activation

(4) Dietary GABA increasing homocarnosine in skeletal muscle and impacts muscle regeneration

In this experiment, we have collaborated with Professor Thomas Hawke at McMaster University, Canada. As we found that dietary GABA increased homocarnosine levels in skeletal muscle, we hypothesized that this increased homocarnosine by dietary GABA may enhance muscle regeneration ability. Since GABA has been reported to reverse diabetes, in this study we extended our interest into diabetic mice as well. We gave GABA in drinking water to both wild type (WT) and diabetic (Akita) mice for 6 weeks. We expected that mice receiving GABA water would have a better muscle regeneration ability. After 5 days of muscle injury, GABA water had no effects on regenerating myofiber size in WT mice. Surprisingly, diabetic mice receiving GABA water had smaller regenerating myofiber size, as compared with both WT and diabetic mice receiving drinking water. We further found that diabetic mice receiving GABA water had higher regenerating area (Fig. 5 A) with higher macrophage levels in injured area (Fig. 5B), suggesting that this group was still in the early phase of muscle reaeration. This implies that GABA possibly retarded the muscle regeneration process in diabetic mice. This finding is against our hypothesis that oral GABA administration may enhance muscle regeneration. Currently, ongoing experiments have been carried out to clarify why and how GABA intake specifically worsens muscle regeneration in diabetes conditions.

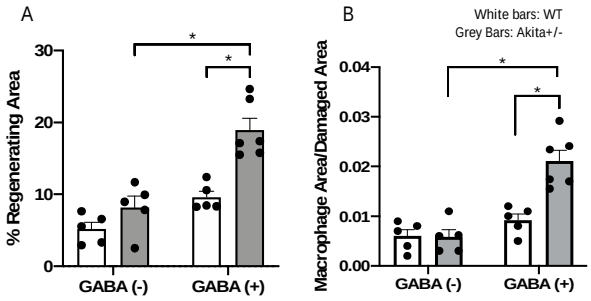

Fig. 5 GABA water retards muscle regeneration in diabetic mice

It can be speculated that GABA, which has a strong antioxidant effect, may suppress inflammatory process at the early phase of muscle regeneration where inflammation is important for inducing macrophage infiltration into the injured area in order to clear up damage cells. Therefore, if the clearance of damage tissues is slowed down, the muscle regeneration cannot be started and the process will be retarded. If this hypothesis is true,

our finding will provide new information about roles of antioxidants in muscle regeneration. Drinking much antioxidants right after exercise, where muscles undergo injury, may not be a good practice.

(5) Involvement of homocarnosine analogs during muscle regeneration process

Before we go to examine the effects of dietary homocarnosine on muscle regeneration, we checked if there is any mobilization or changes of homocarnosine analogs (carnosine and anserine) in response to muscle trauma. After the muscle injury by CTX injection, carnosine and anserine in the skeletal muscles were rapidly degraded within 12 h, along with an up-regulation of its degrading enzyme (carnosinase, CN1) (Fig. 6), while levels of amino acids released from muscle protein were not changed until 48 h. This suggests that imidazole peptides may be involved in energy metabolism in the acute phase during skeletal muscle injury, preventing muscle from breaking down its own muscle protein. This suggests a possible physiological function that protects against excessive degradation of skeletal muscle proteins in sarcopenia.

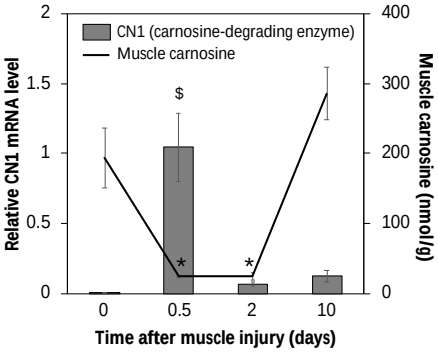

Fig. 6 Mobilization of muscle carnosine and its degrading enzyme during muscle injury/regeneration

(6) Dietary homocarnosine increases circulating homocarnosine levels with higher stability in blood

We found that oral administration of homocarnosine increased homocarnosine levels in blood up to 15 μM (Fig. 7A), while oral administration of carnosine increased carnosine levels in blood only 4 μM (Fig. 7B). The result indicates that homocarnosine has higher stability in blood than carnosine (almost 4-fold), suggesting that oral intake of homocarnosine is a great efficient method for increasing imidazole peptides in skeletal muscle. Currently, levels of homocarnosine in skeletal muscle and effects on muscle regeneration are under investigation.

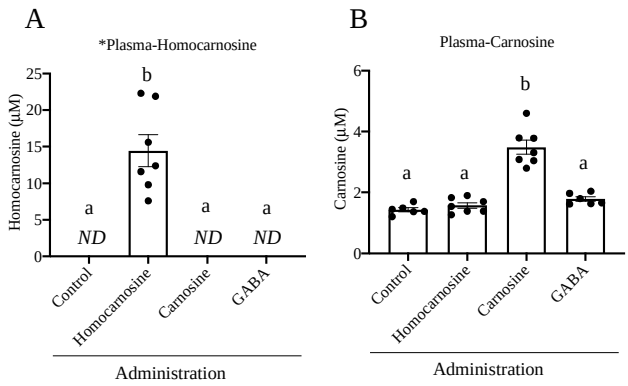

Fig. 7 Dietary homocarnosine increases homocarnosine levels in blood

In conclusion, this study is the first to demonstrate the new method for increasing homocarnosine as a new carnosine-liked peptide in skeletal muscle. This study also revealed novel roles of homocarnosine and carnosine in muscle regeneration and satellite cell functions, which are essential factors in preventing sarcopenia. We discovered that the muscle dipeptides, carnosine, anserine, and homocarnosine, may be frontline providers of energy used in the acute phase during skeletal muscle injury. This may explain the reason why our bodies store these peptide at high concentrations in our muscle. Without these peptides, the muscle may have to breakdown its own protein to release free amino acids for using during muscle injury. This study provides a strong foundation for the research on new roles of imidazole peptides in muscle functions and sarcopenia prevention.

(7) Unexpected discovery leads to new research

Additionally, this study showed that dietary GABA at a high dose (5%) exerted antiobesity-like effects by suppressing food intake and preventing weight gain in lean mice[2]. This finding led to a new study on GABA metabolism in appetite control for antiobesity drug development.

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５．主な発表論文等

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〔学会発表〕　計3件（うち招待講演　0件／うち国際学会　2件）

１．発表者名 Thanutchaporn Kumrungsee, Kai Onishi, Takumi Komaru, Noriyuki Yanaka
２．発表標題 The potential roles of vitamin B6 in satellite cell functions
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４．発表年 2020年

１．発表者名 Kumrungsee T, Onishi K, Komaru T, Yanaka N, Kato N.
２．発表標題 Vitamin B6 Regulates Muscle Satellite Cell Function: A Novel Possible Role of Vitamin B6 in Muscle Regeneration.
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２．発表標題 Vitamin B6 deficiency impairs imidazole homeostasis and energy metabolism in rat heart
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〔図書〕　計0件

〔産業財産権〕

〔その他〕

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６．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考

７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関