

**Mechanisms of changes in energy metabolism
by allyl isothiocyanate via TRP channels**

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GENERAL INTRODUCTION

The transient receptor potential (TRP) channel family is composed of a wide variety of cation-permeable channels and shows a great diversity of activation mechanisms. TRPV1 and TRPA1 are cation channels belonging to the TRP channel family that are activated by high (>43°C) (1,2) and low (<18°C) (3,4) nociceptive temperatures, respectively; therefore, they are termed thermosensitive TRP channels. Interestingly, they are also activated by spicy or pungent compounds in foods.

Recent studies have indicated that activation of TRPV1 or TRPA1 is involved not only in nociception and thermosensation but also in thermoregulation and energy metabolism (5-10). It has been reported that intragastric administration of TRPV1 agonists (capsaicin and capsiate) enhances oxygen consumption and increases core body temperature (7,11,12). Intragastric administration of TRPA1 agonists (AITC and cinnamaldehyde) enhances thermogenesis in interscapular brown adipose tissue (IBAT) (6,13). Intravenous administration of both TRPV1 and TRPA1 agonists also enhances adrenalin secretion from the adrenal medulla through the central nervous system (14-18), and it enhances metabolisms in skeletal muscles (19,20). From these facts, it is expected that activation of TRPV1 or TRPA1 might affect thermogenesis and energy metabolism.

The relationship between TRP channels and energy metabolism has been well studied; however, there have been few reports on energy substrate utilization, i.e., whether carbohydrate or fat is preferentially metabolized by TRP channel activation. Recent studies suggest that TRPV1 dysfunction is associated with the development of diabetes and obesity (21), and several studies indicate a relationship between TRPV1 and blood glucose metabolism (22-25).

From these facts, the author hypothesized that we would be able to control energy metabolism by intake of foods via activation of TRP channels. To test this hypothesis, it is needed to elucidate how changes in energy metabolism would be happen by intake of foods that activate TRP channels and how is the mechanisms of changes in energy metabolism via TRP channels. Allyl isothiocyanate (AITC), a natural compound in plants belonging to the family *Cruciferae*, is known as the pungent ingredient in mustard, horseradish and wasabi. AITC activates TRPA1 (26), and the author focused AITC. The aim of this study is to elucidate the effects of intragastric administration of AITC on changes in substrate oxidation and its

mechanisms. The author investigated the effect of intragastric administration of AITC on changes in substrate oxidation by respiratory gas analysis. The relationship between TRP channels and substrate utilization by AITC was also investigated by using TRPA1 KO and TRPV1 KO mice. Furthermore, the author investigated that the effects of intragastric administration of AITC on the blood glucose levels in mice by using an intraperitoneal glucose tolerance test (IPGTT).

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CHAPTER 1

Intragastric administration of allyl isothiocyanate increases carbohydrate oxidation via TRPV1 but not TRPA1 in mice

Introduction

Allyl isothiocyanate (AITC), a natural compound in plants belonging to the family *Cruciferae*, is known as the pungent ingredient in mustard, horseradish and wasabi. Ingestion of spicy or pungent compounds in foods has traditionally been thought to enhance thermogenesis and energy expenditure. These effects of capsaicin, one of the spicy compounds in foods, have been well studied, however, it is unclear whether the similar pungent compound, AITC, affects thermogenesis and energy metabolism.

The transient receptor potential (TRP) channel family is composed of a wide variety of cation-permeable channels and shows a great diversity of activation mechanisms. TRPV1 and TRPA1 are cation channels belonging to the TRP channel family that are activated by high (>43°C) (1,2) and low (<18°C) (3,4) nociceptive temperatures, respectively; therefore, they are termed thermosensitive TRP channels. Interestingly, they are also activated by spicy or pungent compounds in foods. For example, TRPV1 is activated by ingredients of spicy foods such as hot pepper (capsaicin) (1), pepper (piperine) (5), and ginger (gingerols and shogaols) (6,7). TRPA1 is activated by ingredients of pungent foods such as cinnamon (cinnamaldehyde) (3), and garlic (allicin) (8). AITC has also been reported to activate TRPA1 (9).

Recent studies have indicated that activation of TRPV1 or TRPA1 is involved not only in nociception and thermosensation but also in thermoregulation and energy metabolism (10-15). It has been demonstrated that intragastric administration of TRPV1 agonists (capsaicin and capsiate) enhances oxygen consumption and increases core body temperature (12,16,17). Furthermore, studies of TRPV1 knockout (KO) mice and TRPV1 antagonists revealed that such effects were mediated by TRPV1 (15,16). Intragastric administration of TRPA1 agonists (AITC and cinnamaldehyde) enhances thermogenesis in interscapular brown adipose tissue (IBAT) (11,18). Intravenous administration of both TRPV1 and TRPA1 agonists also enhances adrenalin secretion from the adrenal medulla through the central nervous system (19-23), and it enhances metabolisms in skeletal muscles (24,25). From these facts, it is expected that

activation of TRPV1 or TRPA1 might affect thermogenesis and energy metabolism.

The relationship between TRP channels and energy metabolism has been well studied; however, there have been few reports on energy substrate utilization, i.e., whether carbohydrate or fat is preferentially metabolized by TRP channel activation. Recent studies suggest that TRPV1 dysfunction is associated with the development of diabetes and obesity (26), and several studies indicate a relationship between TRPV1 and blood glucose metabolism (27-30). If the author could reveal the relationship between TRP channel activation and the regulation of substrate utilization, we could control energy metabolism depending on the type of metabolic disorder by using TRP channel agonists. This kind of information is expected to be very useful for our modern lifestyle, with excessive energy intake and reduced physical activity.

In the present study, the author investigated the effect of intragastric administration of AITC, which is generally regarded as a TRPA1 agonist, on changes in substrate oxidation. The author also investigated the relationship between TRP channels and substrate utilization by AITC by using TRPA1 KO and TRPV1 KO mice. Together with examination of TRPV1-overexpressing cells, the author examined how AITC enhanced substrate utilization.

Materials and Methods

Animals

Male C57BL/6 mice (6–9 weeks old; SLC) were used. Mutant TRPV1-null mice and TRPA1-null mice were generously provided by Dr. D. Julius (University of California, San Francisco). The procedure of mutant mice was reported in previous literature (31,32). Mutant mice were back-crossed into the C57BL/6 genetic background. The mice were housed in a vivarium maintained at $23 \pm 2^\circ\text{C}$ under a 12:12-h light-dark cycle (lights on 0600–1800 h) with free access to a commercial standard laboratory chow (MF; Oriental Yeast, Tokyo, Japan) and drinking water. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Kyoto University and were in complete compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Respiratory gas analysis

The mice were kept individually in a chamber for 3 h to attain a constant respiratory exchange ratio (RER). A sample was intragastrically administered and the expired air was

analyzed. The oxidation of total fatty acids and carbohydrates was computed on the basis of oxygen consumption (V_{O_2}) and carbon dioxide production (V_{CO_2}). Gas analysis was performed using an open-circuit metabolic gas analysis system connected directly to a mass spectrometer (model Arco2000; ArcoSystem, Chiba, Japan). The gas analysis system has been described in detail elsewhere (33,34). Briefly, each metabolic chamber had a 72-cm² floor and was 6 cm in height. Room air was pumped through the chambers at a rate of 0.5 l/min. Expired air was dried in a cotton thin column and then directed to an O₂/CO₂ analyzer for mass spectrometry.

On the basis of the volume of CO₂ production per unit of time (l/min; V_{CO_2}) and V_{O_2} , total glucose and lipid oxidation were calculated using the stoichiometric equations of Frayn (35) as follows: total fatty acid oxidation = $1.67 V_{CO_2} - 1.67 V_{O_2}$ and carbohydrate oxidation = $4.55 V_{O_2} - 3.21 V_{CO_2}$.

Materials

Dulbecco's modified Eagle's medium (DMEM) and other cell culture reagents were obtained from Invitrogen (Carlsbad, CA, USA). Fura-2 acetoxymethyl ester (Fura-2 AM) was obtained from Dojindo Chemicals (Kumamoto, Japan). Doxycycline was obtained from BD Clontech (CA, USA). Capsaicin and capsazepine were obtained from Sigma (St. Louis, MO, USA). Allyl isothiocyanate was purchased from Nacalai Tesque (Kyoto, Japan). Cinnamaldehyde was obtained from Wako Chemicals (Tokyo, Japan). For intragastric administration, both AITC and cinnamaldehyde were diluted in saline containing 3% ethanol and 10% Tween 80.

Cell culture

The cDNA encoding rat TRPV1 was kindly provided from D. Julius. The entire coding regions of rat TRPV1 were subcloned into pcDNA5/FRT to yield pTRPV1FRT. To establish doxycycline-inducible TRPV1-expressing cells, Flp-In-T-Rex-293 cells (Invitrogen) were co-transfected with pTRPV1FRT and pOG44 (BD Clontech) by using lipofectamine 2000 (Invitrogen). At 24 h after transfection, transfected cells were selected for hygromycin B (400 µg/mL) resistance by using medium containing. After 14 days, hygromycin-resistant clones were collected and combined. TRPV1 expression was induced by incubation with doxycycline (1 µg/mL) for 12–24 h before experiments.

Cells were routinely maintained in DMEM supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO₂. They were passaged twice a

week at a ratio of 1:5 to maintain an exponentially growing state. Experiments were performed 2 days after each passage.

[Ca²⁺]_i measurement

[Ca²⁺]_i was measured with a Fura-2 AM imaging method. Cells on dishes were detached using Ca²⁺-free PBS containing 0.5 mM EDTA and then collected by centrifugation. The collected cells were washed with PBS and resuspended in Krebs solution [containing (in mM): 135 NaCl, 5 KCl, 2 CaCl₂, 2 MgCl₂, 10 HEPES, adjusted to pH 7.4 with NaOH] containing the cytoplasmic calcium indicator Fura-2 AM (2 mM) at 37°C for 45 min. After washing with nominal Ca²⁺-free Krebs solution containing 1 mM EGTA, the cells were stocked in Krebs solution or nominal Ca²⁺-free Krebs solution at 25°C for 5 min. The cells were suspended at a concentration of 3 × 10⁵ cells/mL in Krebs solution.

A cuvette containing Fura-2-loaded cells was placed in a fluorospectrophotometer (CAF-110; Jasco Inc., Tokyo, Japan). After incubation with stirring at 37°C for at least 1 min, the test compound was added. Time-dependent changes in fluorescence (excitation wavelength set at 340 or 380 nm and emission wavelength at 550 nm) were recorded and analyzed using PowerLab system MacLab/4e and Chart 4 (AD Instruments). The fluorescence ratio (340/380) was converted according to the equation published by Grynkiewicz et al (36), where R_{max} and R_{min} were determined using 0.4% Triton X-100 and 20 mM EDTA, respectively. The effective dissociation constant for Fura-2 at 37°C was 224 nM.

TRPV1 antagonist capsazepine was added with AITC. When inhibition by capsazepine is measured, 10 μM capsazepine was added to 10, 100 μM AITC and 0.1, 1 μM capsaicin. The test compounds were prepared in DMSO and add to the loading solution (final DMSO conc. 0.2%).

[Ca²⁺]_i measurement of DRG neuron

TRPA1-null mice (7–9 weeks old) and WT mice (C57/BL6) were deeply anesthetized with an intraperitoneal injection of pentobarbital sodium (30 mg/kg) and killed by decapitation. The DRG were removed and were minced in Hank's balanced salt solution. The cells were dissociated with collagenase (5.0 mg/mL) and dispase (5.0 mg/mL) for 1 h and separated on a percoll gradient (37). Cells were plated onto collagen-I-coated coverslips in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and supplemented with penicillin and streptomycin (Gibco) for 2 hours.

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Cells on the collagen-I-coated coverslips were loaded with Fura-2 AM (2 μ M) at 37 °C for 1 h. After washing with Krebs solution, the coverslip was put on a recording chamber (Warner Instruments, Inc., Hamden, CT, USA) mounted on the stage of an upright fluorescence microscope (BS50WI, Olympus). Cells in the chamber were perfused with Krebs solution. AITC was diluted to the indicated concentration just before the experiment. Ca²⁺ imaging was performed by using Fura-2 with a digital image analysis system (AQUACOSMOS; Hamamatsu Photonics, Hamamatsu). The Fura-2 fluorescence emission, which was caused by excitation at 340 and 380 nm, was measured at 510 nm (F340 and F380). The images of the cells were captured every 4 s. The signal ratio at F340 : F380 was converted into [Ca²⁺]_i levels using an in-vivo calibration with 1 μ m ionomycin (38).

Vagotomy procedure.

Mice were anaesthetized using pentobarbital sodium (50 mg/kg), and the stomach and lower esophagus were visualized following an upper midline laparotomy. The skin and abdominal wall were incised along the ventral midline, and the intestines were moved aside to allow access to the left lateral lobe of the liver and the stomach. The left lateral lobe of the liver was gently retracted, and a ligature was placed around the esophagus at its entrance to the stomach, which allowed access to the esophagus. The stomach was gently pulled down beneath the diaphragm to clearly expose both vagal trunks, which were then transected. All neural and connective tissue surrounding the esophagus was removed to ensure transection of all small vagal branches.

To confirm completeness of vagotomy, a food intake analysis test was performed based on the satiety effect of cholecystokinin-octapeptide (CCK-8) (Sigma) (39,40). Animals were deprived of food for 8 h and then received an intraperitoneal injection of 8 μ g/kg CCK per mouse. Food intake over a 30 min period was measured. Subdiaphragmatic vagotomy abolished the satiety effect of CCK-8 so the food intake in vagotomized mice was similar to saline-injected animals (vagotomy/saline 0.375 \pm 0.035 g vs. vagotomy/CCK 0.318 \pm 0.035 g food intake). Any vagotomized animal that decreased their food intake significantly was excluded from the study.

Data analysis

All values are presented as means \pm SEM. The effect of intragastric administration of AITC or cinnamaldehyde on RER, oxygen consumption, carbohydrate oxidation, and fat

oxidation were examined by two-way repeated-measures ANOVA with Bonferroni post hoc test (Prism 4.0; GraphPad Software, San Diego, CA). In TRPA1 or TRPV1 KO mice, the effects of intragastric administration of AITC on RER, oxygen consumption, carbohydrate oxidation, and fat oxidation were examined by two-way repeated-measures ANOVA followed by unpaired *t* test. The effects of intragastric administration of AITC on Average RER and cumulative oxygen consumption, carbohydrate oxidation, and fat oxidation for 2 hours were examined by one-way ANOVA (see Fig. 2). Tukey's test was used as a post hoc test. In TRPA1 or TRPV1 KO mice, the effects of intragastric administration of AITC on Average RER and cumulative oxygen consumption, carbohydrate oxidation, and fat oxidation for 2 hours (see Figs. 4, 9) were examined by using an unpaired *t*-test. The effect of intraperitoneal administration of AITC to mice or intragastric administration of AITC to vagotomized mice on RER and oxygen consumption, were examined by two-way repeated-measures ANOVA followed by unpaired *t* test.

Results

Effects of intragastric administration of AITC on changes in energy substrate utilization

The author measured RER after intragastric administration of AITC by indirect calorimetry. Mice were fasted for 3 hours before the experiment to avoid the effect of components in diet (MF) on its digestion and absorption on its metabolism. Intragastric administration of AITC dose-dependently elevated RER for 2 hours after administration compared to vehicle (Fig. 1). Significant differences were observed at a dose of 25 mg/kg but not at a dose of 5 mg/kg (data not shown). At 25 mg/kg dose, the peak RER elevation was observed at 30 min after administration; at 50 mg/kg dose, it was observed at 60 min after administration. AITC dose-dependently increased carbohydrate oxidation for 2 hours after administration compared to vehicle. In contrast, AITC dose-dependently decreased fat oxidation for 2 hours after administration. Oxygen consumption was slightly higher for 20–40 min after administration in the AITC-treated group than in the vehicle-treated group. However, there was no significant difference between each group in the cumulative total oxygen consumption for 2 hours after administration (Fig. 2). Since a dose of 25 mg/kg AITC was regarded as a sufficient dose to affect metabolism, it was used in further experiments.

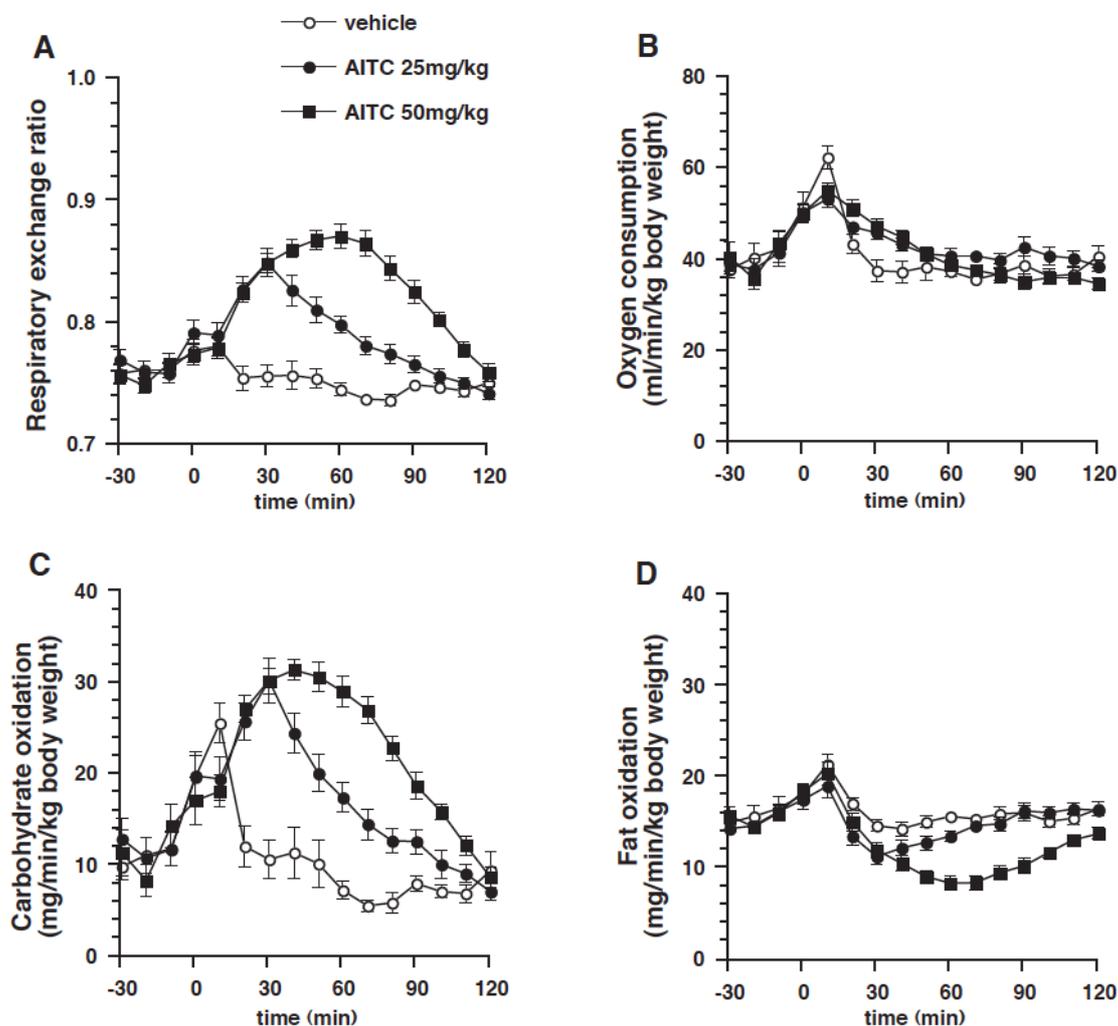


Fig. 1 (A) Changes in the respiratory exchange ratio of mice administered with AITC or vehicle (control). Values are expressed as means \pm SEM. $n = 15-16$ (vehicle vs. AITC 25 mg/kg: $P < 0.05$ at 20–70 min; vehicle vs. AITC 50 mg/kg: $P < 0.05$ at 20–100 min; two-way repeated-measures ANOVA, followed by Bonferroni's post hoc test).

(B) Changes in oxygen consumption of mice administered with AITC or vehicle (control). Values are expressed as means \pm SEM. $n = 15-16$ (vehicle vs. AITC 50 mg/kg: $P < 0.05$ at 30 min; two-way repeated-measures ANOVA, followed by Bonferroni's post hoc test).

(C) Changes in carbohydrate oxidation of mice administered with AITC or vehicle (control). Values are expressed as means \pm SEM. $n = 15-16$ (vehicle vs. AITC 25 mg/kg: $P < 0.05$ at 20–70 min; vehicle vs. AITC 50 mg/kg: $P < 0.05$ at 20–100 min; two-way repeated-measures ANOVA, followed by Bonferroni's post hoc test).

(D) Changes in fat oxidation of mice administered with AITC or vehicle (control). Values are expressed as means \pm SEM. $n = 15-16$ (vehicle vs. AITC 25 mg/kg: $P < 0.05$ at 30 min; vehicle vs. AITC 50 mg/kg: $P < 0.05$ at 40–90 min; two-way repeated-measures ANOVA, followed by Bonferroni's post hoc test).

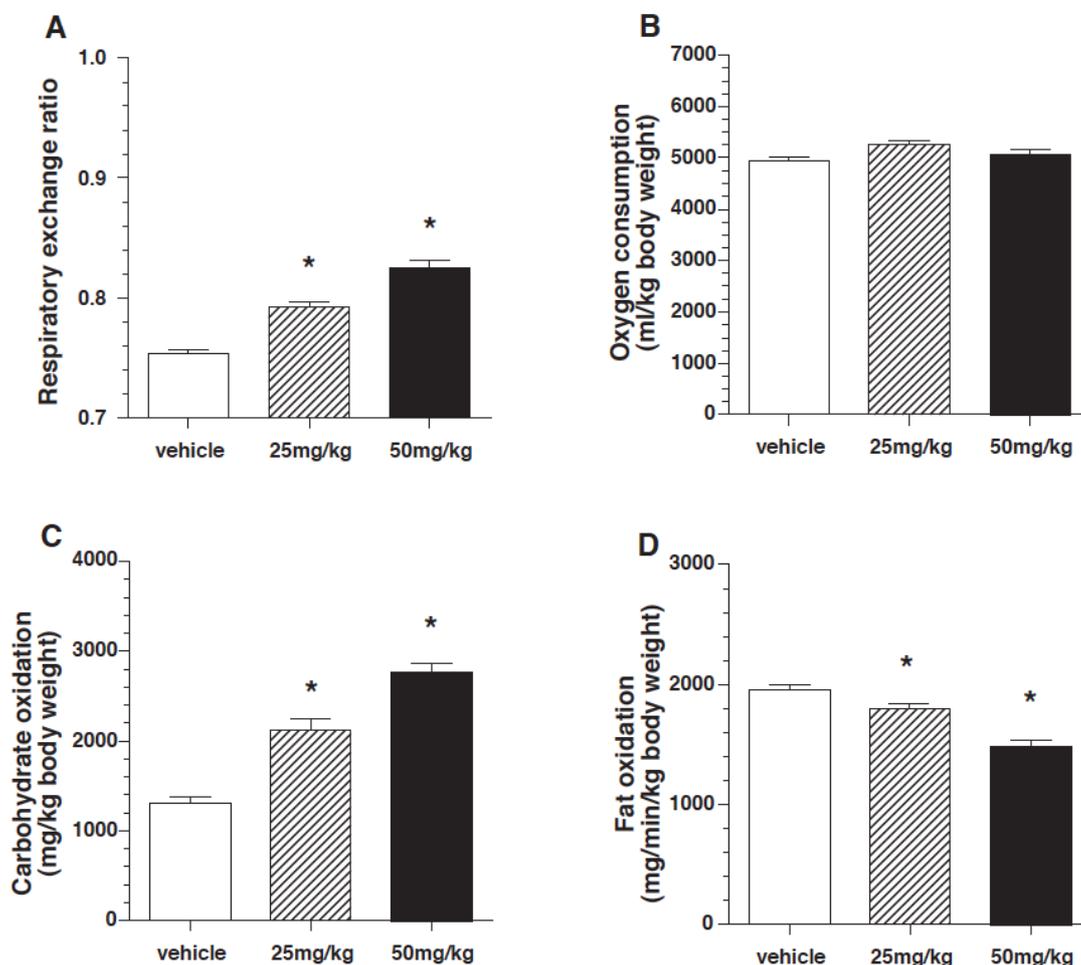


Fig. 2 (A) Average respiratory exchange ratio of mice for 2 hours after intragastric administration of AITC or vehicle (control). (B-D) Cumulative oxygen consumption, carbohydrate oxidation, and fat oxidation of mice for 2 hours after intragastric administration of AITC or vehicle (control). Values are expressed as means \pm SEM. $n = 15-16$; * $P < 0.05$ (Tukey's test).

Contribution of TRPA1 to changes in energy substrate utilization

AITC is generally regarded as a TRPA1 agonist. To examine the contribution of TRPA1 to changes in energy substrate utilization induced by AITC administration, The author administered AITC to TRPA1 KO mice. In TRPA1 KO mice, intragastric administration of AITC significantly elevated RER for 40–90 min after administration compared to vehicle administration (Fig. 3). This finding was similar to that observed in WT mice. AITC increased carbohydrate oxidation for 40–90 min after administration compared to vehicle. AITC also

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decreased fat oxidation, although two-way repeated-measures ANOVA showed no significant difference. However, there was a significant difference between each group in the cumulative total fat oxidation for 2 hours after administration (Fig. 4). Oxygen consumption was not affected for at least 2 hours after administration.

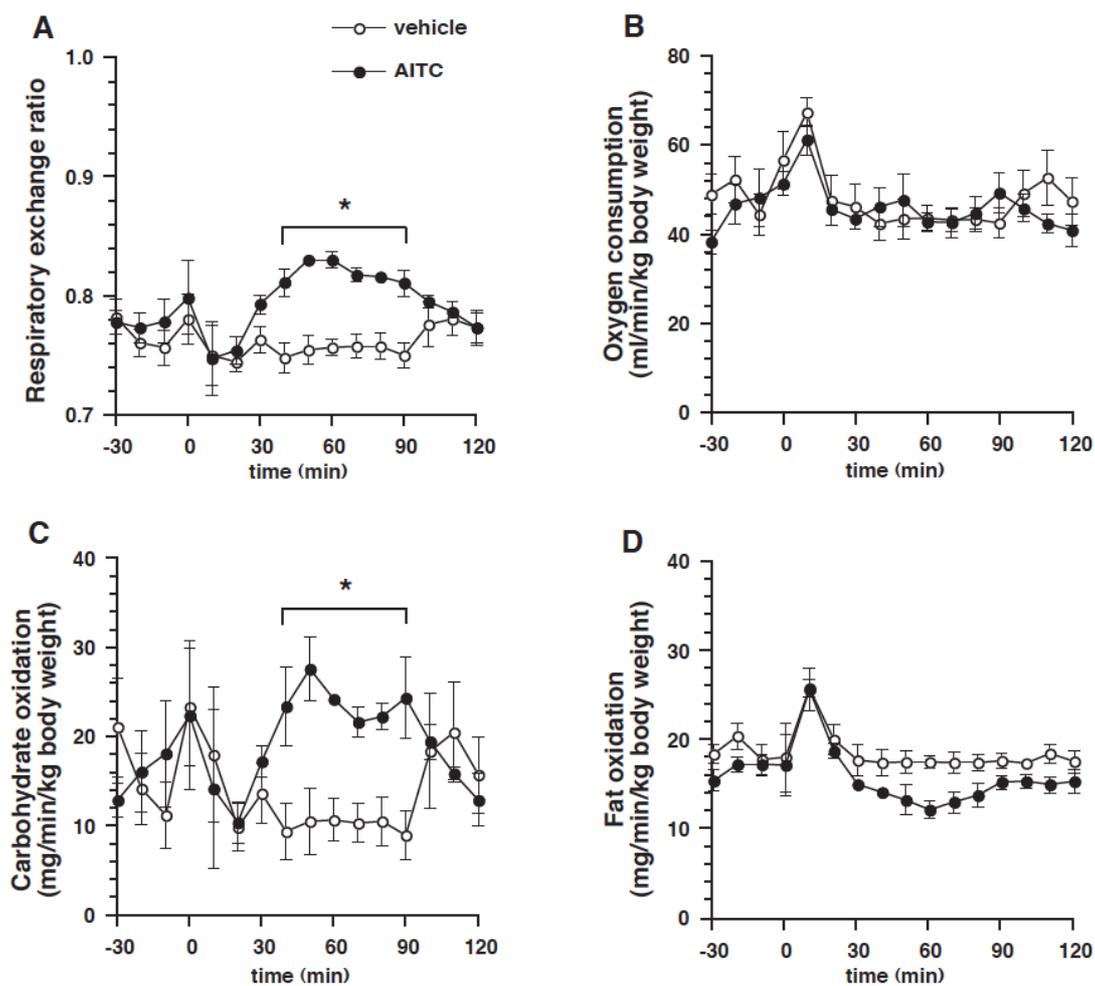


Fig. 3 Changes in the respiratory exchange ratio, oxygen consumption, carbohydrate oxidation, and fat oxidation of TRPA1 KO mice administered AITC or vehicle (control). Values are expressed as means \pm SEM. $n = 4$; * $P < 0.05$ (two-way repeated-measures ANOVA, followed by unpaired t test).

AITC Changes Energy Substrate Utilization via TRPV1

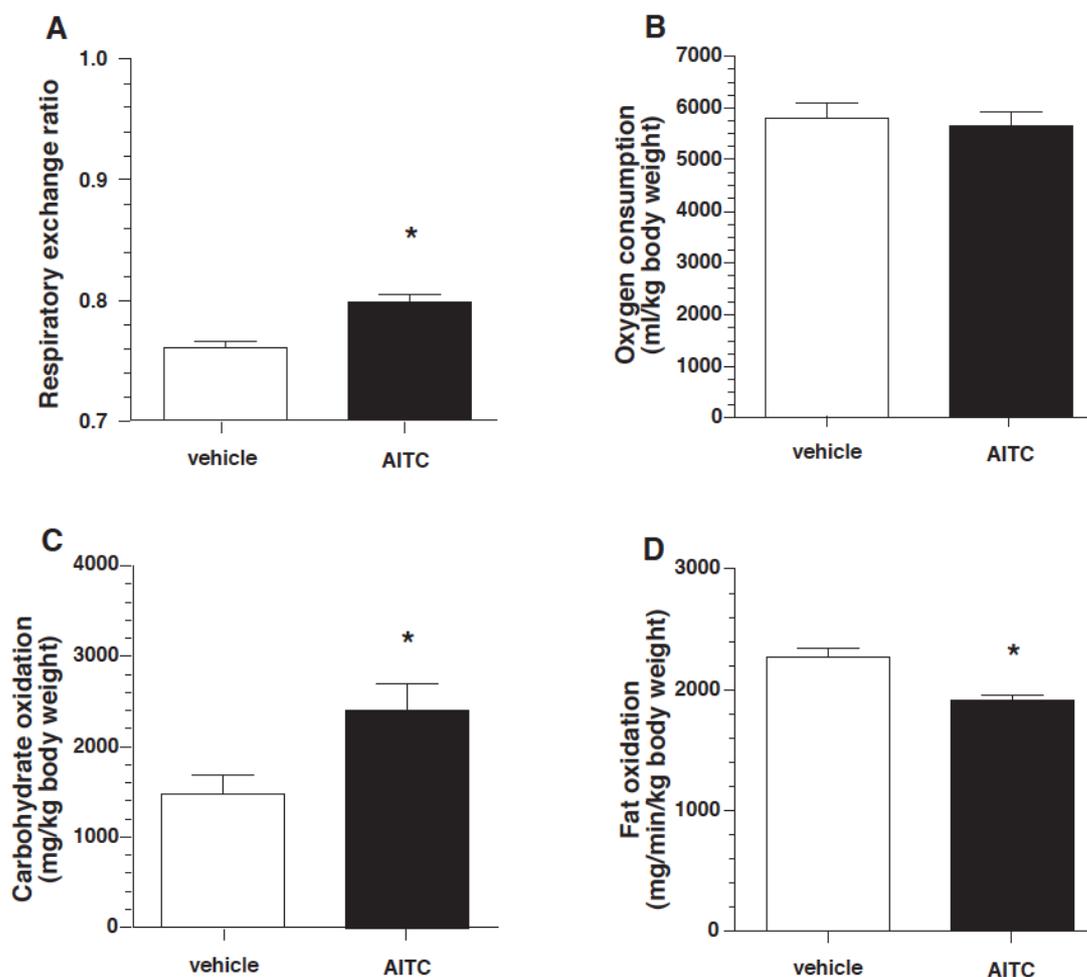


Fig. 4 (A) Average RER of TRPA1 KO mice administered with AITC or vehicle (control) for 2 hours. (B-D) Cumulative oxygen consumption, carbohydrate oxidation, and fat oxidation of TRPA1 KO mice administered with AITC or vehicle (control) for 2 hours. Values are expressed as means \pm SEM (n = 4). *P < 0.05 (unpaired t test).

Effects of intragastric administration of cinnamaldehyde on changes in energy substrate utilization

The author used another TRPA1 agonist, cinnamaldehyde to examine the contribution of TRPA1 to changes in energy substrate utilization. Intragastric administration of cinnamaldehyde did not affect RER, oxygen consumption, carbohydrate oxidation, and fat oxidation for at least 2 hours after administration at a dose below 100 mg/kg (Fig. 5).

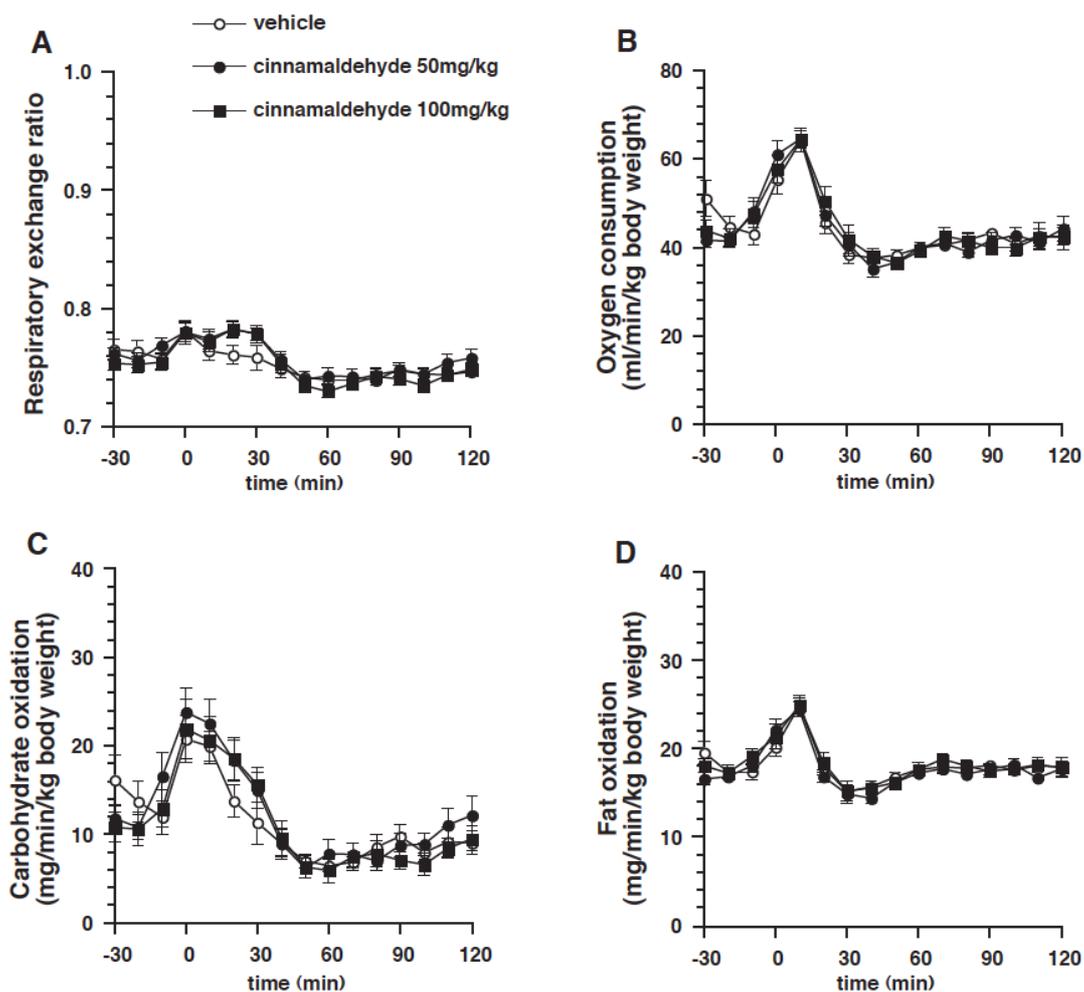


Fig. 5 Changes in the respiratory exchange ratio, oxygen consumption, carbohydrate oxidation, and fat oxidation of mice administered with cinnamaldehyde or vehicle (control). Values are expressed as means \pm SEM (n = 16). There is no significant difference between the groups.

Activation of TRPV1 by AITC

It has been reported that TRPA1 is co-expressed with TRPV1 in a subset of small- to medium- diameter peripheral sensory neurons (4,9), and that allicin, structurally similar to AITC, activates TRPV1 (41). Consequently, the author considered that TRPV1 might be involved in change in energy substrate utilization by AITC. To elucidate whether AITC directly activates TRPV1, the author examined whether AITC affected $[Ca^{2+}]_i$ in HEK 293 cells expressing TRPV1. To perform this experiment, the author established doxycycline-inducible cell lines expressing TRPV1. Expression of TRPV1 in these cells was confirmed by their responses to capsaicin. AITC at concentrations higher than 20 μ M caused a significant increase of $[Ca^{2+}]_i$ in cells expressing TRPV1. AITC had no effect on cells that were not induced to express TRPV1 by doxycycline. To determine whether AITC induced the increase of $[Ca^{2+}]_i$

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due to Ca^{2+} influx, the effects of extracellular Ca^{2+} ion depletion were examined. The AITC-induced increase of $[\text{Ca}^{2+}]_i$ was not observed in the absence of extracellular Ca^{2+} , indicating that AITC caused Ca^{2+} influx. The peaks of $[\text{Ca}^{2+}]_i$ change induced by various concentrations of AITC were plotted and analyzed. AITC induced Ca^{2+} influx in a dose-dependent manner (Fig. 6A). TRPV1 antagonist capsazepine significantly decreased Ca^{2+} influx induced by AITC (Fig. 6B).

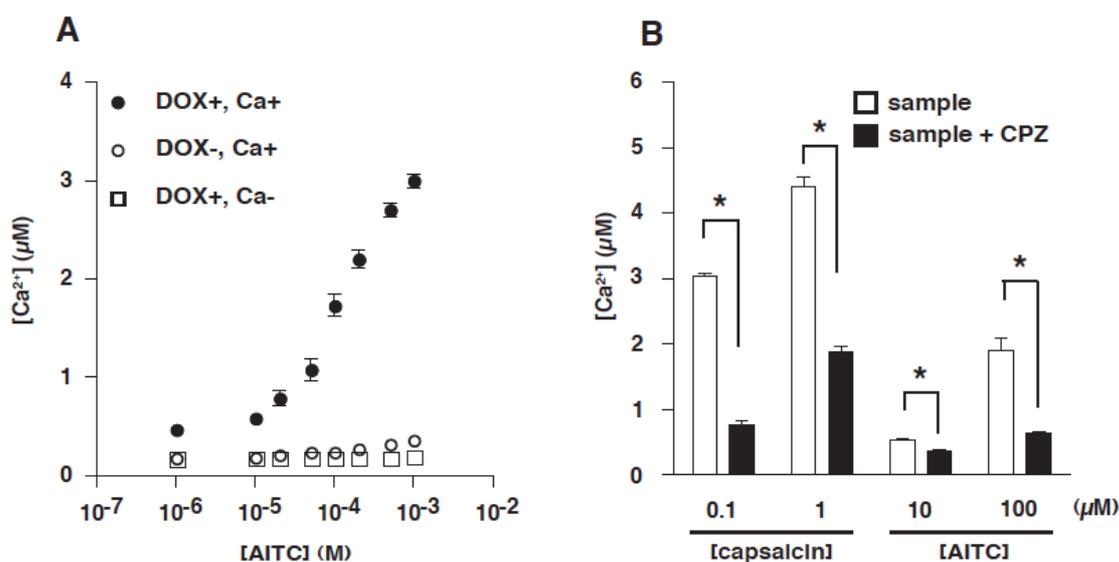


Fig. 6 (A) Dose responses for intracellular calcium ion concentration in TRPV1-expressing HEK293 cells. TRPV1 expression was induced (filled circles and empty squares) by doxycycline or not induced (empty circles). Intracellular calcium ion concentration in TRPV1-expressing HEK293 cells by AITC in presence (filled circles and empty circles) or in absence (empty squares) of extracellular calcium ions. Values are expressed as means \pm SEM (n = 3–5). **(B)** Calcium responses induced by AITC were inhibited by TRPV1 antagonist, capsazepine. White columns indicate TRPV1 activities by capsaicin (0.1, 1 μM) and AITC (10, 100 μM). Black columns indicate TRPV1 activities by these compounds with capsazepine (10 μM). *P < 0.05 (unpaired t test).

AITC activates capsaicin-sensitive DRG neurons from TRPA1KO mice

TRPV1 is expressed in a part of DRG neurons. To test whether AITC activates TRPV1-expressing but not TRPA1-expressing DRG neurons, the author examined $[\text{Ca}^{2+}]_i$ measurement of DRG neuron from TRPA1KO mice (Fig. 7A and B). The author used capsaicin to identify TRPV1-expressing sensory neurons. AITC gradually increased $[\text{Ca}^{2+}]_i$ in a subset of DRG neurons from TRPA1KO mice (29.8%, Fig. 7B). Most of the neurons that responded to AITC also responded to capsaicin (84.4% of neurons that responded to AITC, Fig. 7B). Considering that capsaicin is a TRPV1 agonist, these results suggest that AITC activates

AITC Changes Energy Substrate Utilization via TRPV1

TRPV1-expressing sensory neurons in vivo. In a subset of DRG neurons from WT mice, AITC rapidly increased $[Ca^{2+}]_i$ (Fig. 7C), however, these responses were not observed in DRG neurons from TRPA1KO mice (for 322 DRG neurons from TRPA1 KO mice). Therefore, it was considered that the rapid response to AITC was induced via TRPA1. Consequently, AITC could activate TRPV1-expressing sensory neurons not via TRPA1.

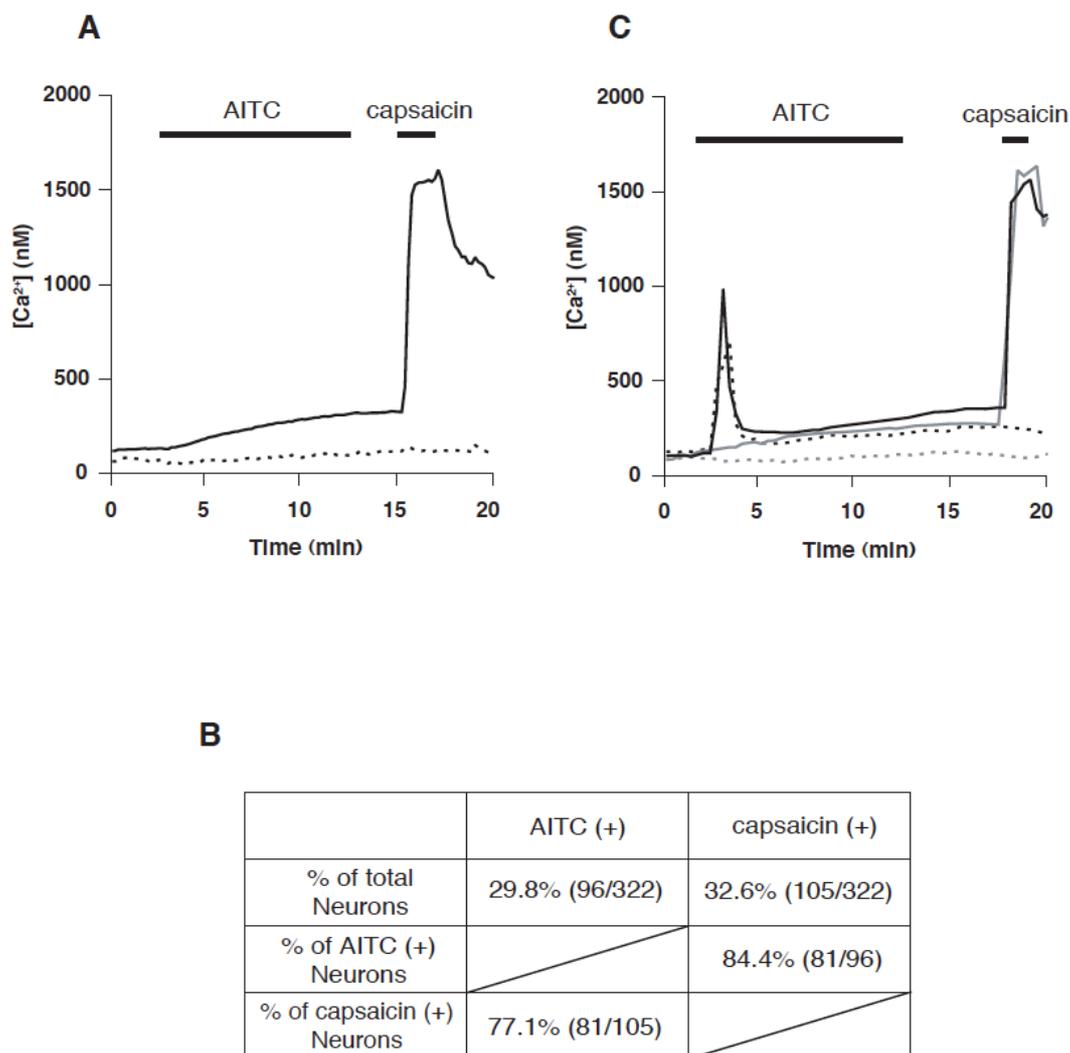


Fig. 7 (A) Typical intracellular calcium ion concentration responses of AITC (500 μ M) and capsaicin (1 μ M) on a single DRG neuron from TRPA1 KO mice. Represented traces are from a neuron that responded to AITC and capsaicin (solid line) and did not respond to AITC and capsaicin (dotted line).

(B) Tabulation of AITC and capsaicin response profiles in TRPA1 KO mice. Responses are listed as a percentage of total DRG neurons, AITC-responding DRG neurons and capsaicin-responding DRG neurons. Neurons were identified as 0.3 M KCl-responding cells.

(C) Typical intracellular calcium ion concentration responses of AITC (500 μ M) and capsaicin (1 μ M) on a single DRG neuron from WT mice. Represented traces are from a neuron that responded to AITC rapidly and capsaicin (black solid line), to AITC rapidly but not to capsaicin (black dotted line), not to AITC rapidly but to capsaicin (gray line) and not to AITC rapidly and not to capsaicin (dotted gray line).

Contribution of TRPV1 to changes in energy substrate utilization

To examine the contribution of TRPV1 to changes in energy substrate utilization induced by AITC administration, the author administered AITC to TRPV1 KO mice. In TRPV1 KO mice, intragastric administration of AITC did not elevate RER; this finding differed from that observed in WT mice (Fig. 8). RER seemed to slightly elevate in the AITC-treated group compared to that in the vehicle-treated group; however, there was no significant difference between the groups in the average RER for 2 hours after administration (Fig. 9). AITC slightly increased carbohydrate oxidation and decreased fat oxidation for 2 hours after administration compared to vehicle; however, there was no significant difference between the groups in the cumulative total carbohydrate oxidation and fat oxidation for 2 hours after administration. Oxygen consumption was not affected for at least 2 hours after administration.

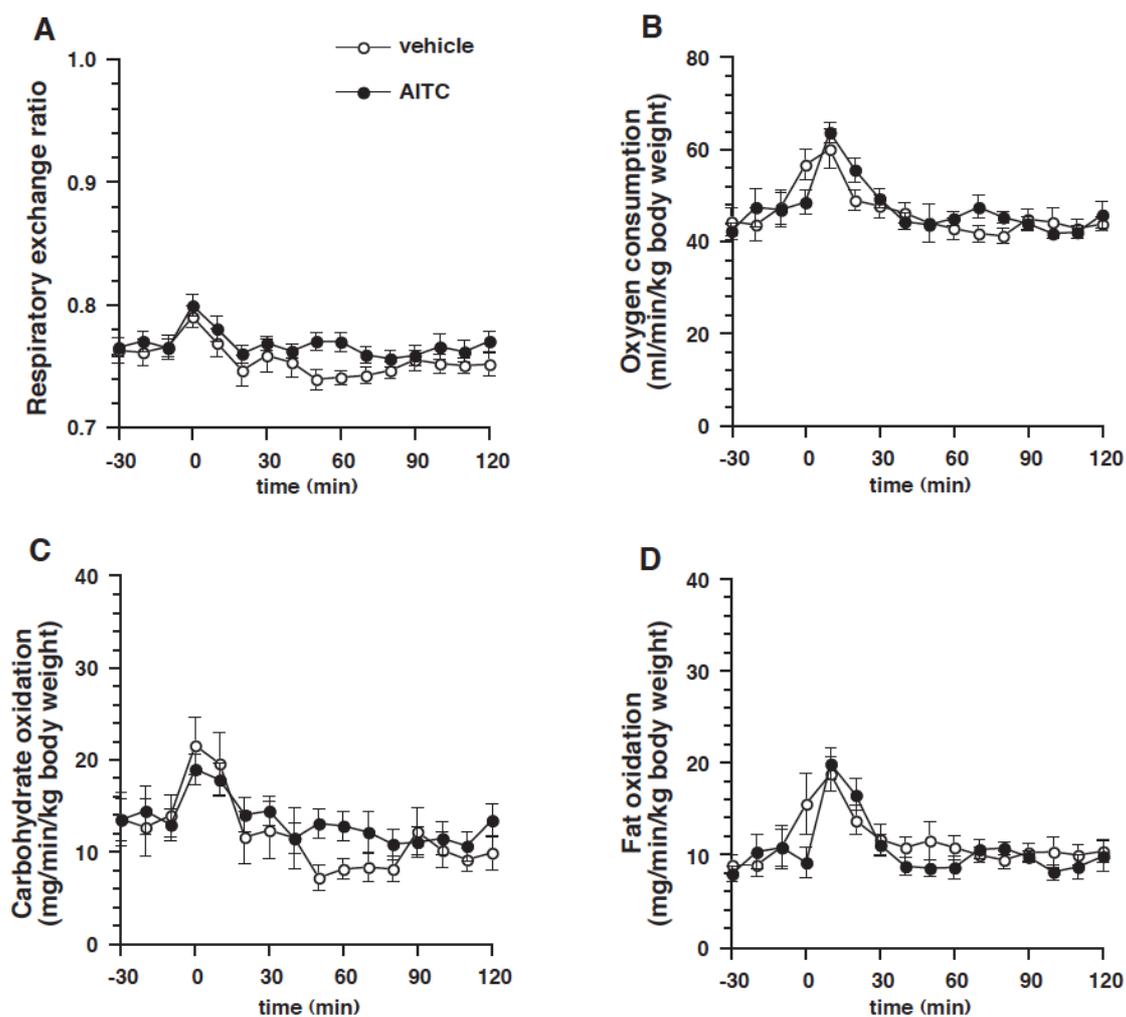


Fig. 8 Changes in the respiratory exchange ratio, oxygen consumption, carbohydrate oxidation, and fat oxidation of TRPV1 KO mice administered with AITC or vehicle (control). Values are expressed as means \pm SEM ($n = 11$). There is no significant difference between the groups.

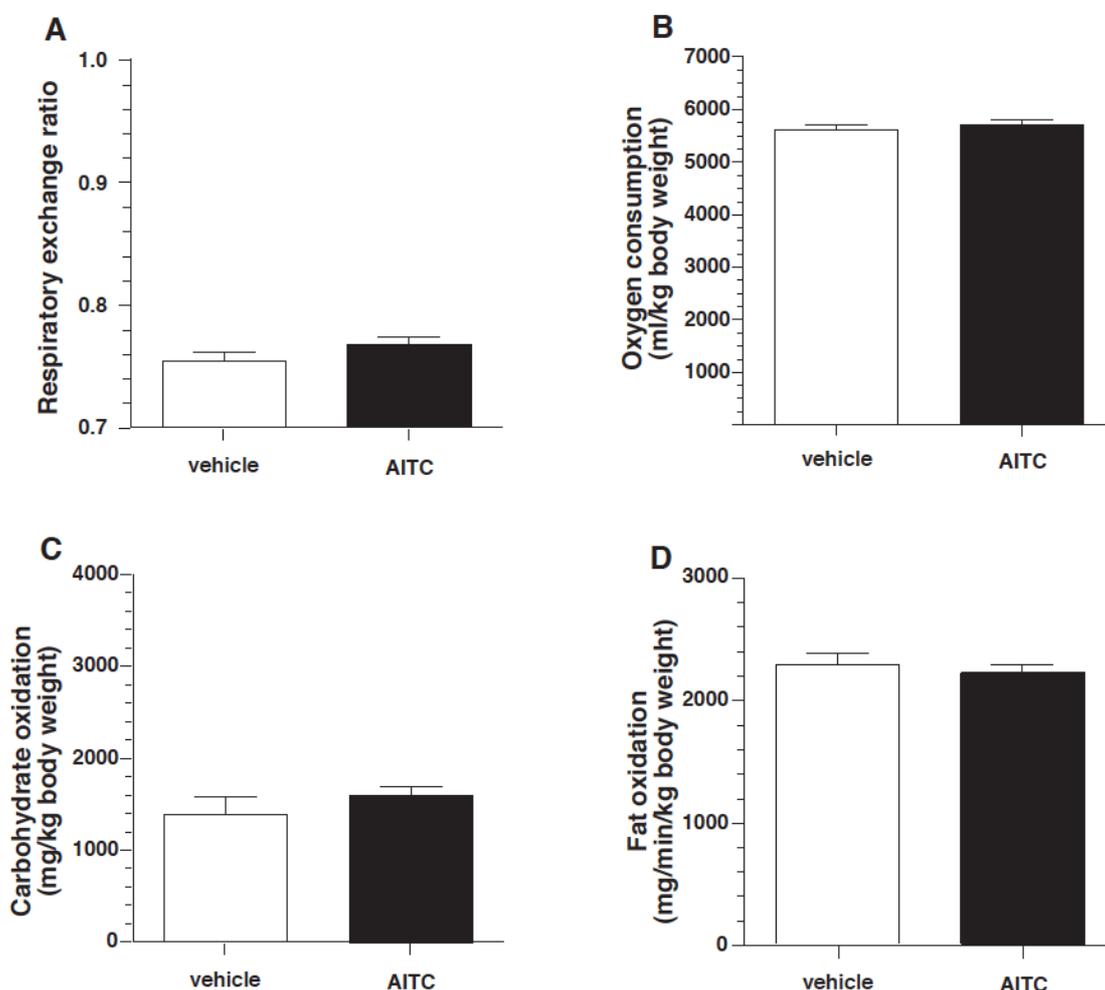


Fig. 9 (A) Average RER of TRPV1 KO mice administered with AITC or vehicle (control) for 2 hours. (B-D) Cumulative oxygen consumption, carbohydrate oxidation, and fat oxidation of TRPV1 KO mice administered with AITC or vehicle (control) for 2 hours. Values are expressed as means \pm SEM (n = 11). There is no significant difference between the groups.

Investigation of the site of action of AITC for changes in energy substrate utilization

To elucidate the site of action of AITC, the author administered AITC to mice intraperitoneally and used vagotomized mice. Intraperitoneal administration of AITC elevated RER for 20-100 min after administration compared to vehicle. However, oxygen consumption was decreased for 60 min after administration in the AITC-treated group than in the vehicle-treated group (Fig. 10). Carbohydrate oxidation was slightly increased and fat oxidation was decreased for 2 hours after administration of AITC in the AITC-treated group compared to that in the vehicle-treated group (data not shown).

In vagotomized mice, intragastric administration of AITC significantly elevated RER

for 30–40 min after administration compared to vehicle administration. Oxygen consumption was slightly higher around 30 min after administration in the AITC-treated group than in the vehicle-treated group (Fig. 11). However, there was no significant difference between each group in the cumulative total oxygen consumption for 2 hours after administration (data not shown). In sham-operated mice, intragastric administration of AITC significantly elevated RER for 20–90 min after administration compared to vehicle administration (Fig. 11). Oxygen consumption was slightly higher around 30 min after administration in the AITC-treated group than in the vehicle-treated group. However, there was no significant difference between each group in the cumulative total oxygen consumption for 2 hours after administration (data not shown).

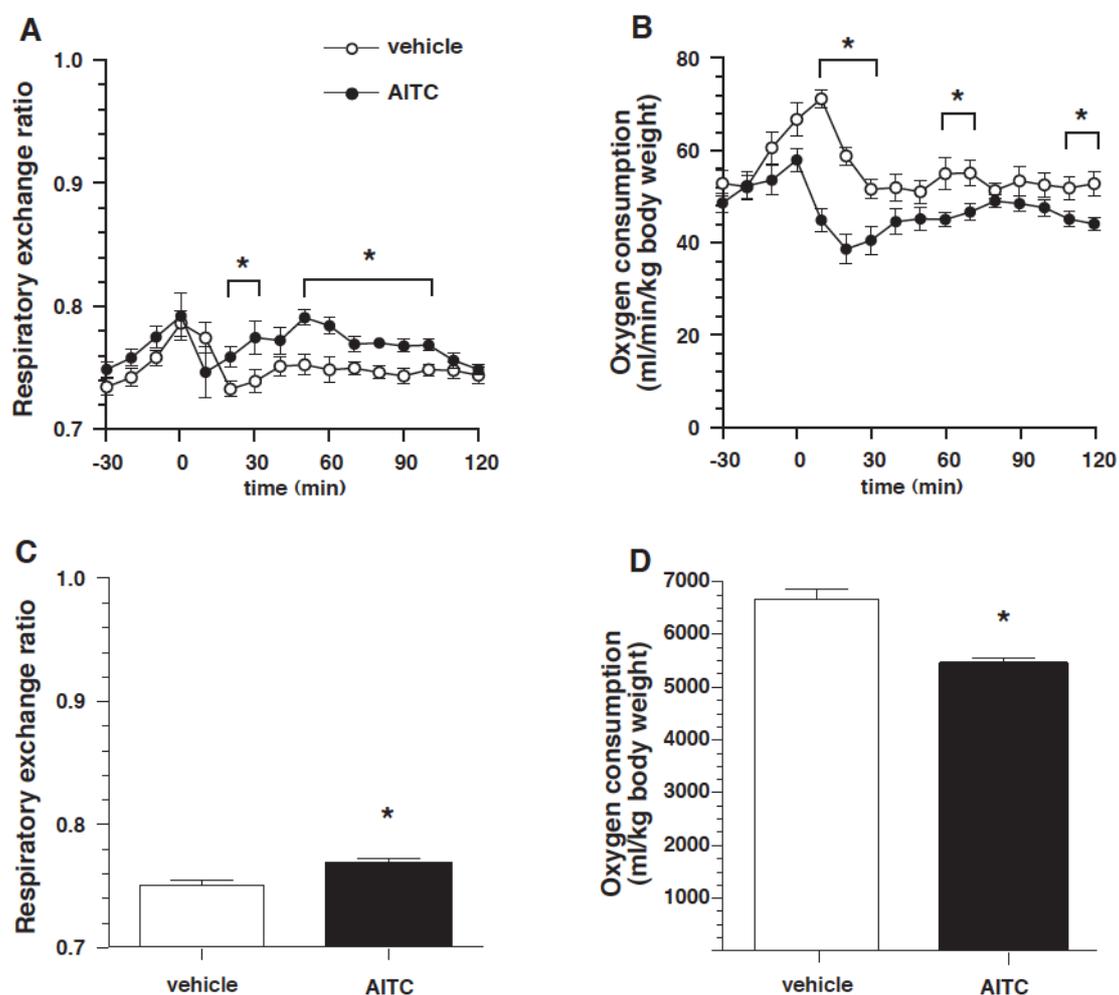


Fig. 10 (A, B) Changes in the respiratory exchange ratio and oxygen consumption of mice intraperitoneal administered with AITC (25 mg/kg) or vehicle (control). Values are expressed as means \pm SEM. $n = 10$; * $P < 0.05$ (two-way repeated-measures ANOVA, followed by unpaired t test). **(C)** Average respiratory exchange ratio of mice for 2 hours after intraperitoneal administration of AITC or vehicle (control). **(D)** Cumulative oxygen consumption of mice for 2 hours after intraperitoneal administration of AITC or vehicle (control). Values are expressed as means \pm SEM ($n = 10$). * $P < 0.05$ (unpaired t test).

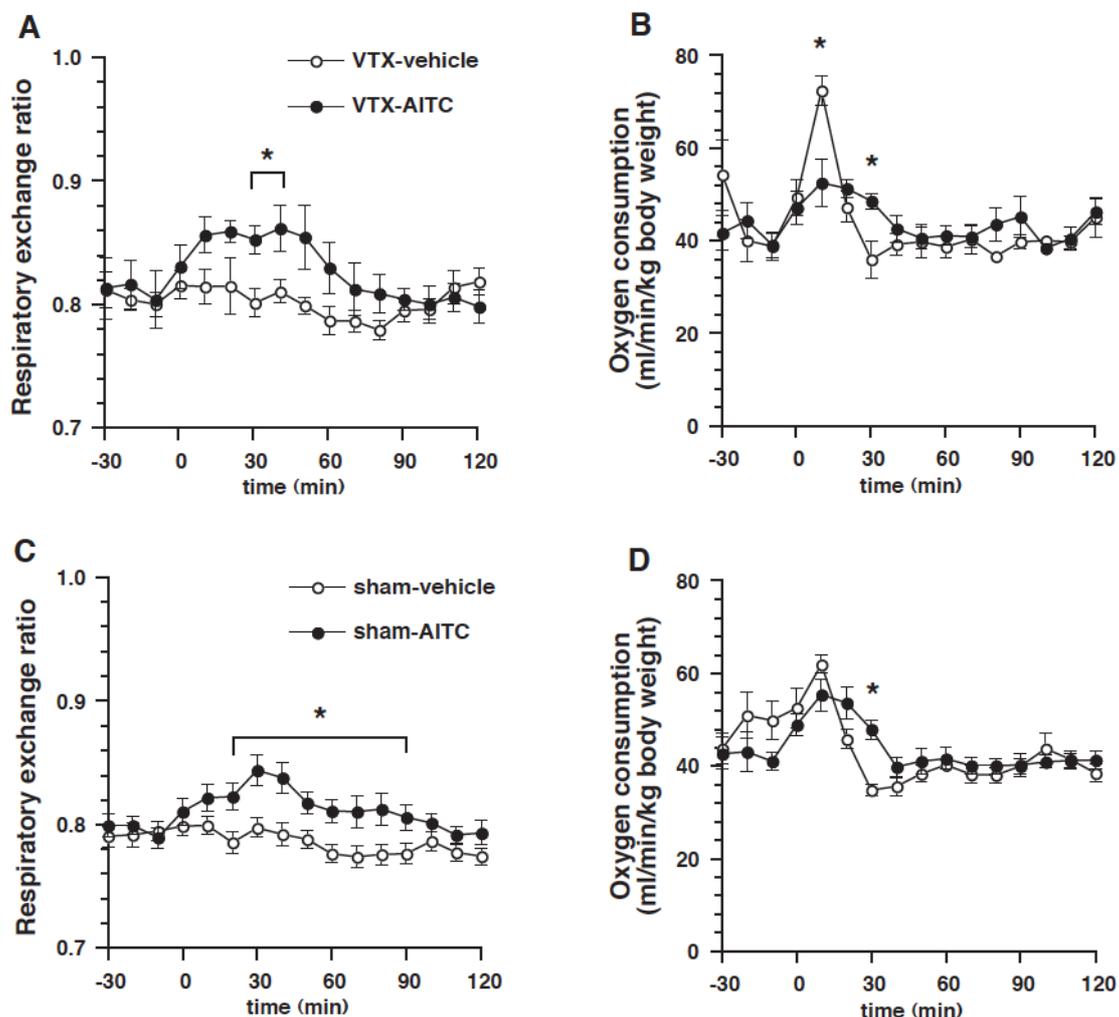


Fig. 11 (A, B) Changes in the respiratory exchange ratio and oxygen consumption of vagotomized mice administered with AITC (25 mg/kg) or vehicle (control). Values are expressed as means \pm SEM (n = 6).

(C, D) Changes in the respiratory exchange ratio and oxygen consumption of sham-operated mice administered with AITC or vehicle (control). Values are expressed as means \pm SEM (n = 10).

*P < 0.05 (two-way repeated-measures ANOVA, followed by unpaired t test).

Discussion

In the present study, the author observed that intragastric administration of AITC elevated RER, markedly increased carbohydrate oxidation, and decreased fat oxidation. Oxygen consumption was slightly increased for 30 min after AITC administration; however, there was no significant difference between the AITC-treated group and vehicle-treated groups in cumulative total oxygen consumption for 2 hours after administration. Therefore, AITC did not

considerably affect oxygen consumption.

Intraperitoneal administration of AITC elevated RER. However, oxygen consumption was decreased after AITC administration. It is considered that the dose of AITC was too high to administrate intraperitoneally and that activities of mice were suppressed by damages from AITC. In previous studies, the author showed that intragastric administration of AITC to anesthetized mice increased colon and IBAT temperatures and decreased tail temperature (11). The author also obtained similar results in the experiments of intravenous administration of AITC (unpublished data). These reports suggest the possibility that AITC acts on not only in the pre-absorptive state but also in the post-absorptive state through the bloodstream and affects carbohydrate oxidation.

In vagotomized mice, AITC also elevated RER similar to sham-operated mice. This result indicated that vagus nerves were not involved in an increase in carbohydrate oxidation by AITC. If AITC acts within gastrointestinal tracts, extrinsic nerves are involved in transmission of stimulation by AITC. Extrinsic nerves of gastrointestinal tract are vagus and spinal nerves and the literature contains reports of vagal and spinal afferent fibres expressing TRPA1 and V1 (42,43). In the present study, the data indicate that vagus nerves are not involved in changes whole body metabolisms by AITC. Together with intraperitoneal administration experiments, the action site of AITC might be beyond gastrointestinal tract or spinal nerves within gastrointestinal tract. It is important to elucidate the action site of AITC apparently and it needs further studies.

The mechanisms by which AITC causes an increase in carbohydrate oxidation remain unclear. At present, the author demonstrated that TRPV1 was involved in increase in carbohydrate oxidation by AITC. Since it has been reported that TRPV1 is expressed in primary sensory neurons and dorsal root ganglia (42-44), AITC might increase carbohydrate oxidation via TRPV1 expressed in the sensory nerve terminals of the gastrointestinal tract or throughout the body. In fact, capsaicin, a TRPV1 agonist, induced adrenaline secretion primarily through activation of the adrenal sympathetic nerve (21). Furthermore, it is reported that TRPV1 is involved in insulin secretion and glucose metabolism (27-30). It was assumed that adrenaline secretion through activation of the central nervous system was induced by AITC and that glucose uptake was increased via beta-adrenoreceptor activation by noradrenaline in peripheral tissues, skeletal muscles, and brown adipose tissues (45). Further studies are required to elucidate where and how carbohydrate is metabolized.

Intragastric administration of TRPV1 agonists, capsaicin and capsiate, did not

increase carbohydrate oxidation similar to AITC (15). However, intraperitoneal administration of capsaicin to anesthetized rats elevated RER (19). Capsaicin is absorbed from the gastrointestinal tract and is to a great extent metabolized in the liver before it reaches the general circulation (46,47). AITC is also absorbed from the gastrointestinal tract and intact AITC is detected in urine (48-50). Consequently, differences in stability and action site of these compounds may lead to differences in metabolic changes of carbohydrate oxidation.

It is possible that differences in binding sites of AITC and capsaicin in TRPV1 caused a differential effect on carbohydrate oxidation. It has been reported that TRPV1 is activated by multiple pathways and has multiple ligand-binding sites (18). For example, not one but three amino acids are involved in TRPV1 activation by capsaicin and other vanilloids, and that some other amino acids are involved in TRPV1 activation by proton, one of TRPV1 agonists (51-54). Since AITC is not a vanilloid, its binding site for TRPV1 activation may be different from that of capsaicin. Recent study suggested that a single N-terminal cysteine plays an important role in the activation of TRPV1 by allicin (41). AITC is structurally similar to allicin; therefore, TRPV1 activation by AITC may use the same binding site as TRPV1 activation by allicin. Further studies are needed to elucidate this point.

Intragastric administration of capsaicin at high doses (20 or 50 mg/kg) did not elicit an increase in carbohydrate oxidation similar to AITC (data not shown). Therefore, it is suspected that not only pain stress but also other mechanisms may be involved in the increase of carbohydrate oxidation by AITC.

Intragastric administration of cinnamaldehyde, a TRPA1 agonist, did not increase carbohydrate oxidation. The dose of cinnamaldehyde was determined from a previous *in vitro* study (3). In that study, the concentration for half-maximal activation of TRPA1 (EC_{50} value) by AITC was three times greater than cinnamaldehyde (22 and 61 mM, respectively). In this study, the author used cinnamaldehyde at the dose of three times greater than the effective dose of AITC to increase carbohydrate oxidation (760 $\mu\text{mol/kg}$ and 250 $\mu\text{mol/kg}$, respectively). If intragastric administration of AITC activated TRPA1 in the present experiment, the dose of cinnamaldehyde was thought to be sufficient to activate TRPA1. However, the author observed that cinnamaldehyde did not increase carbohydrate oxidation at that dose. Moreover, AITC increased carbohydrate oxidation in TRPA1 KO mice. Consequently, it is considered that TRPA1 is not involved in the increase in carbohydrate oxidation by AITC.

AITC did not increase carbohydrate oxidation in TRPV1 KO mice, suggesting that the increase in carbohydrate oxidation by AITC observed in this study is mediated by TRPV1.

Furthermore, the author demonstrated that AITC dose-dependently increased $[Ca^{2+}]_i$ in cells expressing TRPV1. An AITC-induced increase of $[Ca^{2+}]_i$ was not observed in the absence of extracellular Ca^{2+} , indicating that AITC caused Ca^{2+} influx. Since TRPV1 is a Ca^{2+} -highly permeable non-selective cation channel (1), it is considered that AITC increased $[Ca^{2+}]_i$ via activation of TRPV1. In previous studies, AITC did not activate TRPV1 (9,41). In the present study, the author used different experimental methods from those of previous studies, which may have resulted in obtaining different results. In comparison with the previously reported EC_{50} value (711 nM or 1.47 μ M) of representative TRPV1 agonist capsaicin (1,55), the EC_{50} (~200 μ M) of AITC was considerably high. At high doses, it is likely that AITC exerts a non-specific effect on HEK293 cells. However, AITC did not increase $[Ca^{2+}]_i$ in cells in which TRPV1 was not induced by doxycycline and addition of TRPV1 antagonist, capsazepine decreased Ca^{2+} influx by AITC; therefore, AITC increased $[Ca^{2+}]_i$ in cells via TRPV1.

AITC increased $[Ca^{2+}]_i$ in a subset of DRG neuron from TRPA1KO mice. There is no difference between the number of AITC responding neurons and capsaicin responding neurons in the DRG neurons (by chi-square tests). Most of the neurons that responded to AITC also responded to capsaicin. Together with HEK 293 cells data, the author considered that AITC could directly activate TRPV1 in vivo. However, some points have remained unclear (ex. differences between time course of $[Ca^{2+}]_i$ by AITC and capsaicin). To elucidate these points, more detailed studies are needed in future.

Previous study of Akopian reported that TG neurons from TRPA1 KO mice were not responded to AITC (56). In this study, the author used not TG neurons but DRG neurons. The application time of AITC for TG neurons was only two minutes, but that for DRG neurons, in this study, was more than ten minutes. In the present study, the response to AITC in DRG neurons from TRPA1 KO mice was slow compared to that of capsaicin. Considering these facts, the application time of AITC in the previous study might be too short to observe the response to AITC in neurons from TRPA1 KO mice. In fact, the author observed the rapid response to AITC (that was within two minutes) in neurons from WT mice and not in TRPA1 KO mice. Differences between the methodologies of previous reports and that of this study might explain these unexpected results.

It has been reported that TRPA1 is co-expressed with TRPV1 in a subset of small- to medium- diameter peripheral sensory neurons (4,9). In the present study, the author demonstrated that AITC activated TRPV1. From these reports and the present data, it was expected that cross-interaction between TRPA1 and TRPV1 (57) might be involved in the

changes in metabolisms by AITC. However, TRPA1 is not involved in the increase in carbohydrate oxidation by AITC because AITC increased carbohydrate oxidation in TRPA1 KO mice similar to WT mice. It is, therefore, unlikely that cross-interaction is involved in the changes in metabolisms by AITC in the present study.

In the present study, AITC directly activated TRPV1 in vitro experiments. However, it is possible that TRPV1 was indirectly activated by AITC in vivo. It is reported that bradykinin, ATP, prostaglandins, and trypsin or tryptase are inflammatory mediators involved in the activation of TRPV1 by protein kinase C-dependent phosphorylation (58-61). AITC might induce inflammation, resulting in the release of bradykinin, ATP, prostaglandins, and trypsin or tryptase, which might affect TRPV1 activation.

It is possible that the effects of AITC on the increase in carbohydrate oxidation were caused by metabolites. It has been reported that AITC was metabolized to some compounds after oral administration (48). The author showed that intraperitoneal administration of AITC also elevated RER, indicating that AITC could act without gastric digestion. Although it could not be denied completely that metabolites of AITC are involved in the increase in carbohydrate oxidation by AITC, the author considered that AITC is important to the increase in carbohydrate oxidation by AITC.

The author administered AITC to mice at high doses. Concentrations of AITC solution administered mice were 50 and 100 mM. It was too high to get from spicy meals. In previous studies, absorption and metabolisms of AITC was different between rats and mice (48,50), indicating that there might be species differences in effective concentration of AITC. So the author consider that more lower concentration or long term administration at low concentration might be effective in application for human. Further studies are required to consider application for human.

Recent studies have indicated the evidence to support the concept of TRP channels as targets in metabolism (10-15). In those reports, activation of TRPV1 and TRPA1 considered to reflex activation of sympathetic pathways or inactivation of parasympathetic pathways via the central nervous system (19-23). Other reports indicate a relationship between TRPV1 and blood glucose metabolism (27-30). TRPV1 and TRPA1 are also involved in nociception. It might be reasonable to consider that changes in metabolisms through the central nervous system are involved in responses to avoid painful stimuli. Consequently, it is expected that activation of TRPV1 or TRPA1 affect energy metabolism, and in future they would be important targets in the modulation of energy metabolisms.

In conclusion, the author demonstrated that intragastric administration of AITC increased carbohydrate oxidation in mice and that the effects were mediated by TRPV1 but not TRPA1. The author also showed that AITC increased $[Ca^{2+}]_i$ in cultured cells and in DRG neurons, and suggested that AITC might directly activate TRPV1.

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CHAPTER 2

Intragastric administration of allyl isothiocyanate reduces hyperglycemia in intraperitoneal glucose tolerance test (IPGTT) by enhancing blood glucose consumption in mice

Introduction

Controlling glucose metabolism is important to prevent the development of metabolic disorders such as obesity and type 2 diabetes. Recent studies revealed that postprandial dysmetabolism (i.e., hyperglycemia and hyperlipidemia) is related to the risk of developing obesity and cardiovascular disease (1-4). Postprandial hyperglycemia and hyperlipidaemia induce endothelial dysfunction associated with increased oxidative stress and vascular inflammation, and are involved in the pathogenesis of atherosclerosis. Therefore, it is important to control postprandial metabolic states and reduce postprandial metabolic abnormalities.

The transient receptor potential (TRP) channel family is composed of a wide variety of cation-permeable channels and shows great diversity in its mechanisms of activation. TRPV1 and TRPA1 are cation channels belonging to the TRP channel family, and are activated by high (5,6) and low (7,8) nociceptive temperatures, respectively. Interestingly, they are also activated by spicy or pungent compounds in foods, such as capsaicin, pipeline, cinnamaldehyde and allicin (5,7,9,10). Recent studies indicate that the activation of TRPV1 or TRPA1 is involved not only in nociception and thermosensation but also thermoregulation and energy metabolisms (11-15).

Allyl isothiocyanate (AITC) is a natural compound in plants belonging to the family *Cruciferae*, and is the pungent ingredient in mustard, horseradish and wasabi. AITC activates TRPA1 (16), and recent studies suggest that it could activate not only TRPA1 but also TRPV1 (17,18).

Research has suggested that TRPV1 is involved in the emergence of diabetes and obesity (19-22). Long-term treatment with TRPV1 antagonists reduces fasting glucose, triglyceride, and insulin levels in ob/ob mice (21). Moreover, dietary capsaicin, which is a TRPV1 agonist, is expected to improve not only obesity-induced inflammation but also

obesity-related metabolic disorders such as insulin resistance (20). The author previously demonstrated that intragastric administration of AITC increases carbohydrate oxidation via TRPV1 (18). Based on these facts, TRPV1 is considered to play a key role in glucose metabolism.

Approaches for suppressing postprandial hyperglycemia have been studied, but that research was generally aimed at preventing glucose absorption by inhibiting digestive enzymes (23-25). The author considered that the increase in carbohydrate oxidation by administering AITC might enhance glucose metabolism under excessively increased blood glucose conditions, such as during the postprandial period, followed by suppressing hyperglycemia. The approach for suppressing postprandial hyperglycemia by increasing the utilization of blood glucose has not been well studied, and it is expected to become a novel approach for suppressing postprandial hyperglycemia.

In the present study, the author investigated the effects of intragastric administration of AITC on the blood glucose levels in mice by using an intraperitoneal glucose tolerance test (IPGTT). To elucidate the relationship between carbohydrate oxidation and blood glucose utilization, respiratory gas analysis of the changes in $^{13}\text{CO}_2$ emission after administering ^{13}C -labeled glucose was performed. The author also investigated the relationship between TRP channels and changes in the blood glucose levels by using TRPA1 knockout (KO) mice and TRPV1 KO mice.

Materials and Methods

Animals

Male C57BL/6 mice (Japan SLC, Hamamatsu, Japan) were used. Mutant TRPV1-null mice and TRPA1-null mice were generously provided by Dr. D. Julius (University of California, San Francisco, CA). The procedure for generating mutant mice is reported in previous literature (26,27). Mutant mice were back-crossed into the C57BL/6 genetic background. The mice were housed in a standard cage and maintained at $23 \pm 2^\circ\text{C}$ under a 12:12-h light-dark cycle (lights on 0600–1800 h) with free access to a commercial standard laboratory chow (MF; Oriental Yeast, Tokyo, Japan) and drinking water. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Kyoto University and were in complete compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Materials

Allyl isothiocyanate was purchased from Nacalai Tesque (Kyoto, Japan). D-glucose was obtained from Wako pure chemical Industries (Osaka, Japan). ¹³C-labeled glucose was obtained from Isotec (Miamisburg, OH). For intragastric administration, AITC was diluted in saline containing 3% ethanol and 10% Tween 80.

Intraperitoneal glucose tolerance test

The mice (14-17 weeks old) were deprived of food overnight (about 13 h; 2300-1200 h) and had free access to water. They were administered glucose (2 g/kg body weight) intraperitoneally and then AITC (25 mg/kg body weight) or the vehicle intragastrically. Blood glucose levels were measured before and after AITC or vehicle was administered. For blood glucose measurements, blood samples were obtained from the tail vein and analyzed using a Glucocard Diameter (Arkray, Kyoto, Japan).

Respiratory gas analysis

The mice (14-17 weeks old) were kept individually in a chamber for 12 h to attain a constant respiratory exchange ratio. AITC or vehicle was administered and the expired air was then analyzed. The oxidation of total carbohydrate was computed on the basis of oxygen consumption (V_{O_2}) and carbon dioxide production (V_{CO_2}). Gas analysis was performed using an open-circuit metabolic gas analysis system connected directly to a mass spectrometer (model Arco2000; ArcoSystem, Chiba, Japan). The gas analysis system is described in detail elsewhere (28,29). Briefly, each metabolic chamber had a 72-cm² floor and was 6 cm in height. Room air was pumped through the chambers at a rate of 0.5 L/min. Expired air was dried in a cotton thin column and then directed to an O₂/CO₂ analyzer for mass spectrometry.

On the basis of the volume of CO₂ production per unit of time (l/min; V_{CO_2}) and V_{O_2} , total carbohydrate oxidation was calculated using the stoichiometric equations of Frayn (30) as follows: total carbohydrate oxidation = $4.55 V_{O_2} - 3.21 V_{CO_2}$.

Oxidation of exogenous carbohydrate in the IPGTT was assessed on the basis of the relative abundance of ¹³CO₂ (¹³CO₂-¹²CO₂ ratio) in the respiratory gas after intraperitoneal administration of ¹³C-labeled glucose. A solution containing 10% glucose and 0.02 mol/l of ¹³C-labeled glucose was administered intraperitoneally to the mice (0.01 ml/g of body mass) and then AITC or vehicle was intragastrically administered. The total amount of administered

¹³C-labeled glucose was 0.2 mmol/kg of body mass.

Data analysis

All values are presented as means ± SE. The effect of intragastrically administering AITC on blood glucose levels, the relative abundance of ¹³CO₂, carbohydrate oxidation and Vo₂ were examined by two-way repeated-measures ANOVA (Prism 5.0; GraphPad Software, San Diego, CA) followed by unpaired t-test (see Fig. 1, 3, 5). The effects of intragastric administration of AITC on the area under the curve (AUC) of blood glucose and average the relative abundance of ¹³CO₂ and cumulative carbohydrate oxidation and Vo₂ for 2 hours (see Figs. 2, 4, 6) were examined by using an unpaired t-test.

Results

Effects of intragastric administration of AITC on blood glucose levels

The blood glucose levels after intragastric administration of AITC in the IPGTT were measured. The dose of AITC was determined from the previous study, wherein it was considered to be sufficient to affect energy metabolism (18). The blood glucose levels increased after intraperitoneal glucose administration, however, intragastric administration of AITC reduced the increase in blood glucose level compared with vehicle administration for 15-60 min after administration (Fig. 1A). The AUC for the blood glucose, 2 h after administration, was lower in the AITC-treated group than in the vehicle-treated group (Fig. 2A).

Effects of intragastric administration of AITC on excess blood glucose utilization

The author considered that the increase in carbohydrate oxidation by administering AITC might have increased the utilization of excessive glucose in the blood, followed by a reduction in the increase in blood glucose level as seen by the IPGTT. To elucidate the relationship between the increase in carbohydrate oxidation and the reduction in the increase in blood glucose level, the author measured the ¹³CO₂-¹²CO₂ ratio in respiratory gas after intragastric administration of AITC with ¹³C-labeled glucose. In the IPGTT, an increase in blood glucose levels was derived from intraperitoneally administered glucose. Therefore, an increase in the utilization of intraperitoneally administered glucose means an increase in the utilization of excessive blood glucose.

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Intragastric administration of AITC elevated the $^{13}\text{CO}_2$ - $^{12}\text{CO}_2$ ratio for 10-50 min after the administration, compared to vehicle administration (Fig. 1B). AITC increased carbohydrate oxidation for 20-110 min after the administration, compared with vehicle (Fig. 1C). The average of the $^{13}\text{CO}_2$ - $^{12}\text{CO}_2$ ratio and the cumulative total carbohydrate oxidation for 2 h after administration were higher in the AITC-treated group than in the vehicle-treated group (Fig. 2B and C). V_{O_2} was not influenced for at least 2 h after administration (Fig. 1D and 2D).

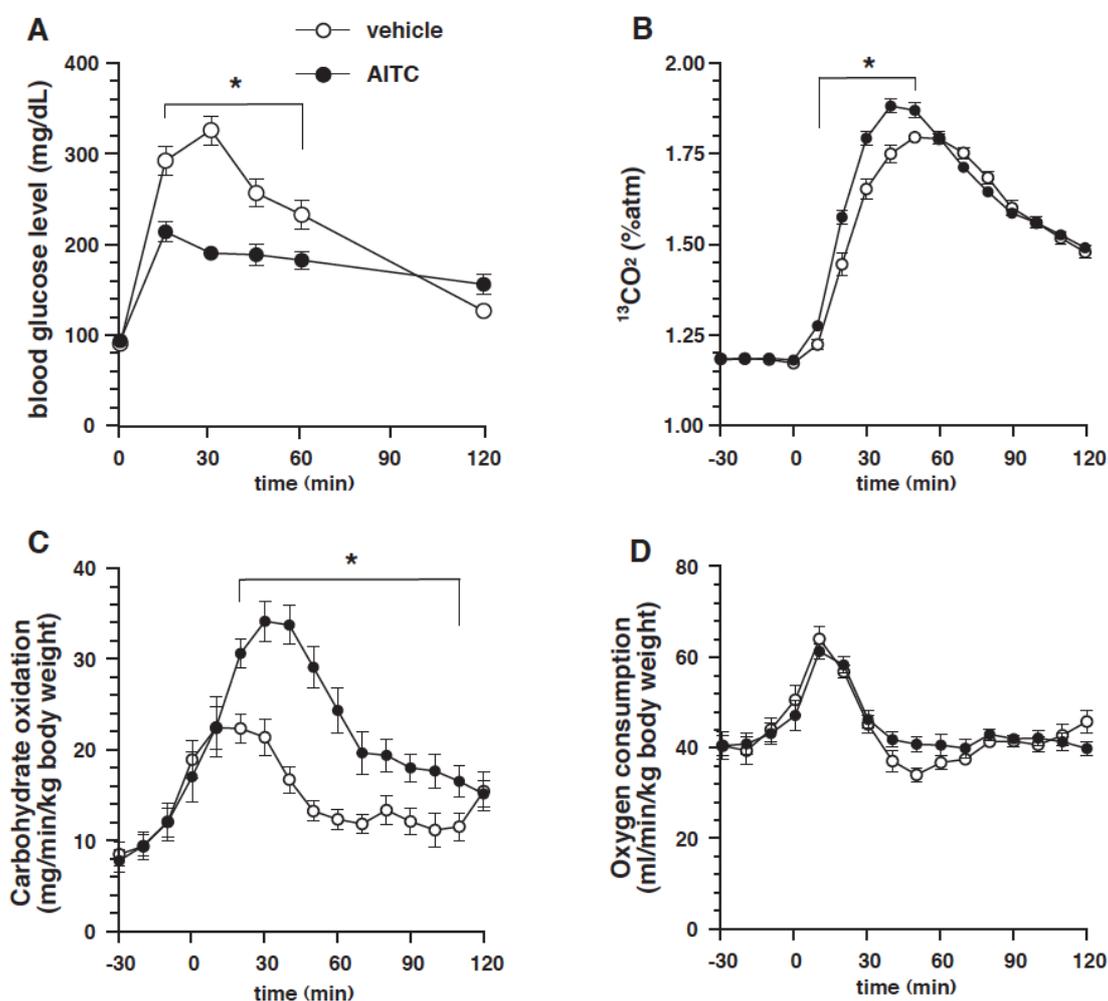


Fig. 1 (A) Changes in the blood glucose levels of mice administered with AITC or vehicle (control) in IPGTT. Values are expressed as means \pm SE. $n = 5$; $*P < 0.05$ (two-way repeated-measures ANOVA, followed by unpaired t-test).

(B-D) Changes in the relative abundance of $^{13}\text{CO}_2$, carbohydrate oxidation and $\dot{\text{V}}_{\text{O}_2}$ of mice administered AITC or vehicle (control) in IPGTT. Values are expressed as means \pm SE. $n = 10$; $*P < 0.05$ (two-way repeated-measures ANOVA, followed by unpaired t-test).

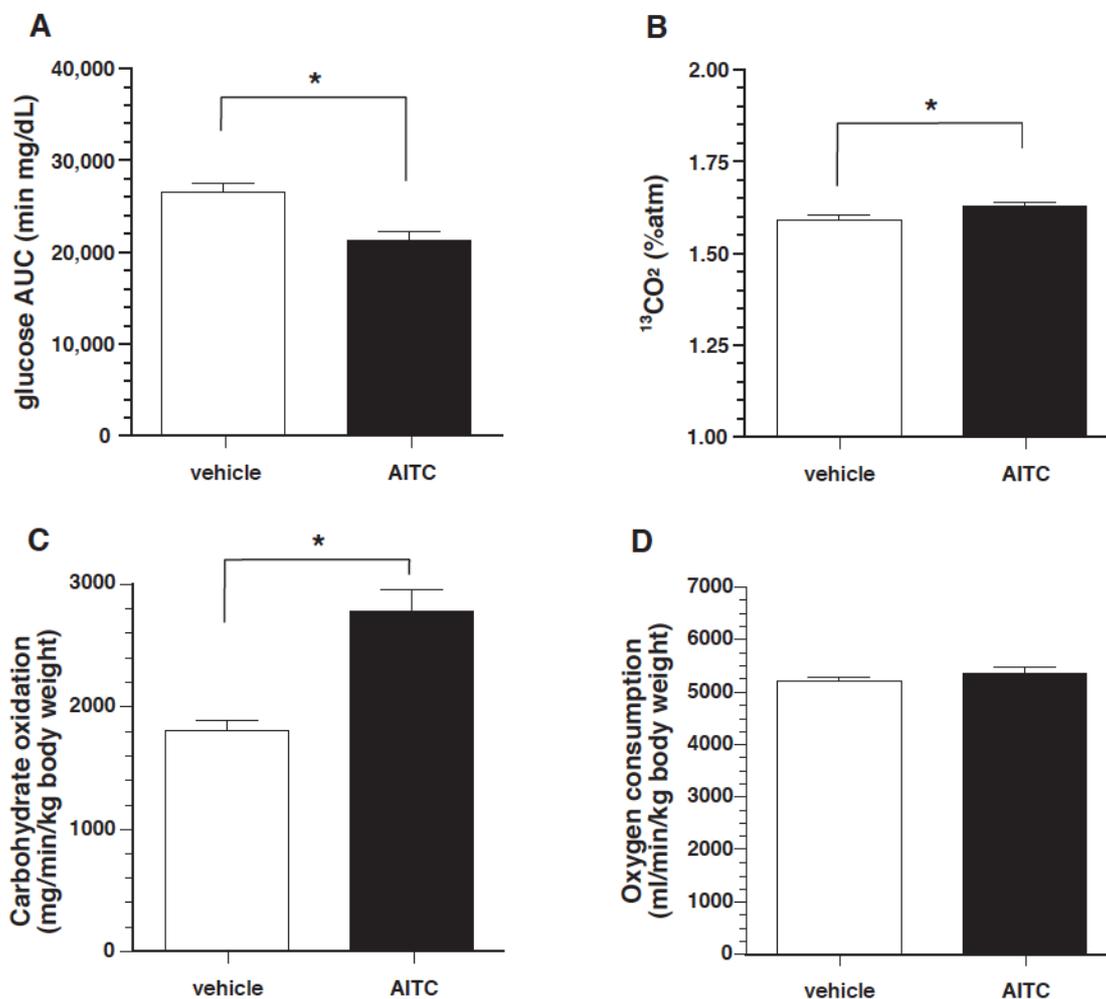


Fig. 2 (A) Area under the curve (AUC) of blood glucose levels of mice in IPGTT. Values are expressed as means ± SE. n = 5; *P < 0.05 (unpaired t test).

(B-D) Average the relative abundance of ¹³CO₂ and cumulative carbohydrate oxidation and $\dot{V}O_2$ of mice administered with AITC or vehicle (control) in IPGTT for 2 hours. Values are expressed as means ± SE (n = 10). *P < 0.05 (unpaired t test).

Contribution of TRPA1 in enhancing the utilization of excessive blood glucose

To examine the contribution of TRPA1 in reducing the increase in blood glucose level by administering AITC, TRPA1 KO mice were employed. In TRPA1 KO mice, intragastric administration of AITC reduced the increase in blood glucose level in the IPGTT compared with the vehicle administration for 15-60 min after administration (Fig. 3A). This finding was similar to that observed in wild type (WT) mice. The AUC for the blood glucose, 2 h after administration, was lower in the AITC-treated group than in the vehicle-treated group

(Fig. 4A).

In the respiratory gas analysis, intragastric administration of AITC elevated the $^{13}\text{CO}_2$ - $^{12}\text{CO}_2$ ratio at 20-30 and 60 min after the administration, compared to vehicle administration (Fig. 3B). AITC increased carbohydrate oxidation for 20-60 min after the administration compared with vehicle (Fig. 3C). The average of the $^{13}\text{CO}_2$ - $^{12}\text{CO}_2$ ratio and the cumulative total carbohydrate oxidation for 2 h after administration were higher in the AITC-treated group than in the vehicle-treated group (Fig. 4B and C). Vo_2 was not influenced for at least 2 h after administration (Fig.3D and 4D).

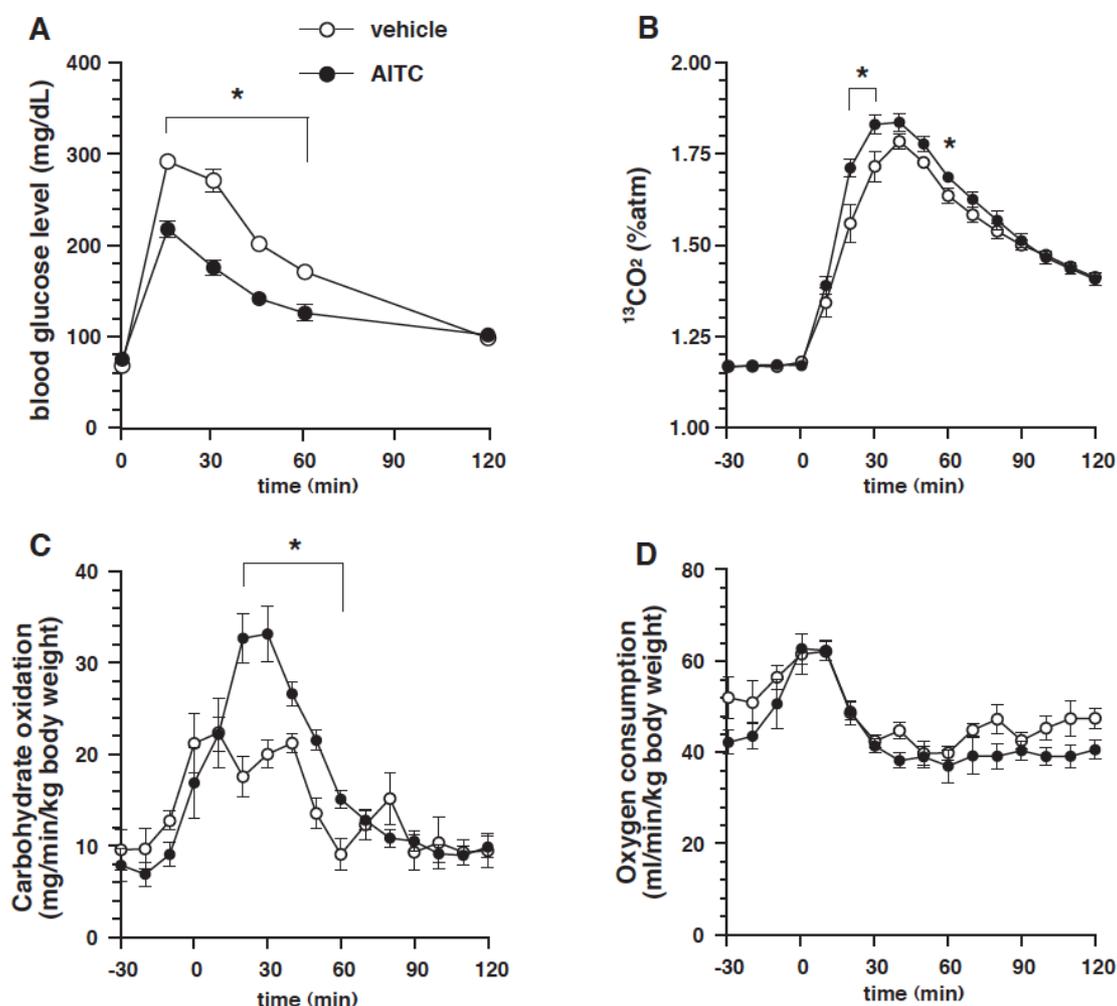


Fig. 3 (A) Changes in the blood glucose levels of TRPA1 KO mice administered with AITC or vehicle (control) in IPGTT. Values are expressed as means \pm SE. $n = 4-5$; $*P < 0.05$ (two-way repeated-measures ANOVA, followed by unpaired t-test).

(B-D) Changes in the relative abundance of $^{13}\text{CO}_2$, carbohydrate oxidation and Vo_2 of TRPA1 KO mice administered AITC or vehicle (control) in IPGTT. Values are expressed as means \pm SE. $n = 7$; $*P < 0.05$ (two-way repeated-measures ANOVA, followed by unpaired t-test).

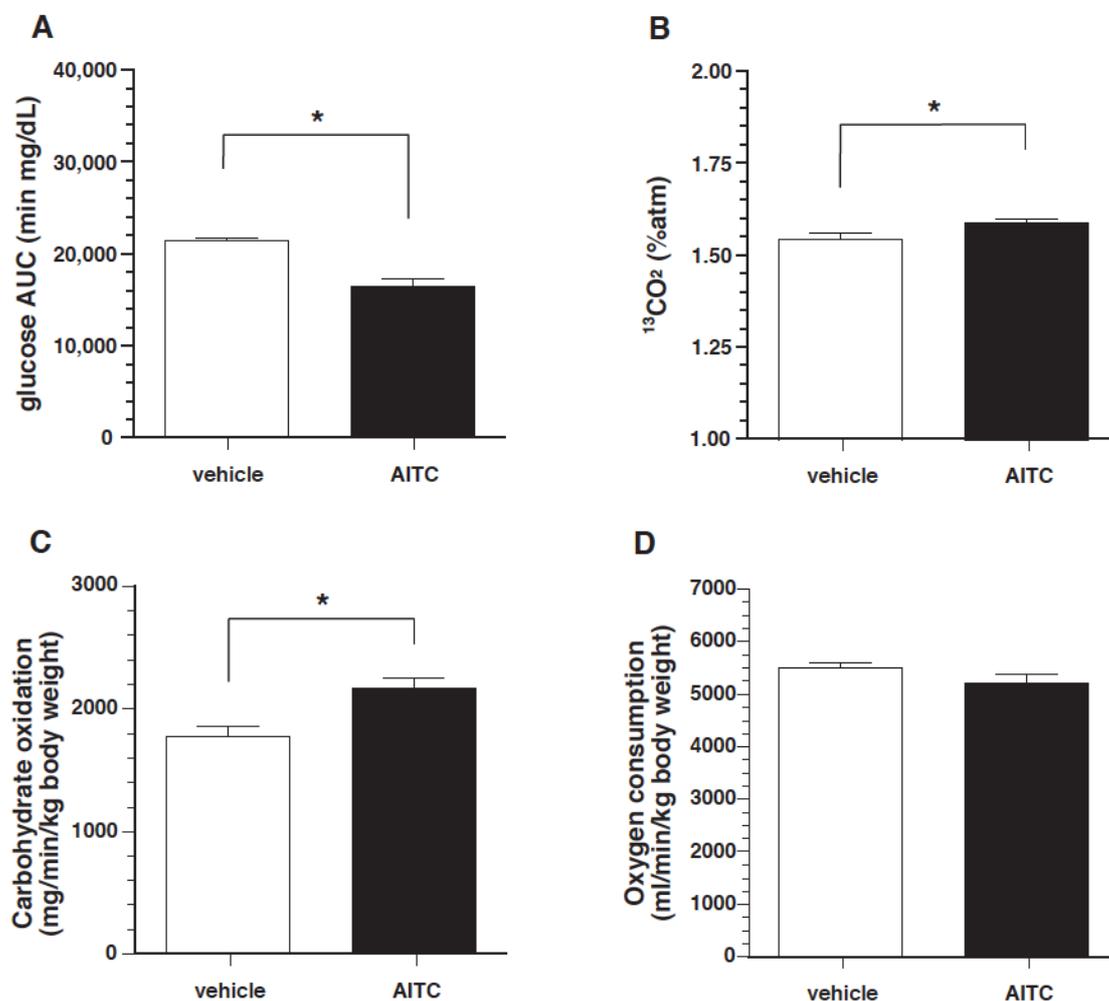


Fig. 4 (A) AUC of blood glucose levels of TRPA1 KO mice in IPGTT. Values are expressed as means ± SE. n = 4-5; *P < 0.05 (unpaired t test).

(B-D) Average the relative abundance of ¹³CO₂ and cumulative carbohydrate oxidation and $\dot{V}O_2$ of TRPA1 KO mice administered with AITC or vehicle (control) in IPGTT for 2 hours. Values are expressed as means ± SE (n = 7). *P < 0.05 (unpaired t test).

Contribution of TRPV1 in the enhancement of utilization of excessive glucose in blood

The author previously reported the involvement of TRPV1 in the increase in carbohydrate oxidation by AITC (18). To examine the contribution of TRPV1 in reducing the increase in blood glucose level by administering AITC, IPGTT on TRPV1 KO mice was performed. Intragastric administration of AITC did not reduce the increase in blood glucose level caused by the IPGTT. This finding differed from that observed for WT mice (Fig. 5A). One hundred twenty minutes after administration, the blood glucose levels of the AITC-treated group was higher than those of the vehicle-treated group. However, there was no significant

difference between each group in terms of the AUC for the blood glucose 2 h after administration (Fig. 5B).

Using respiratory gas analysis, the author found that there was no significant difference between each group in the $^{13}\text{CO}_2$ - $^{12}\text{CO}_2$ ratio (Fig. 5B). There was no significant difference between each group in the average of the $^{13}\text{CO}_2$ - $^{12}\text{CO}_2$ ratio for 2 h after administration (Fig. 6B). These findings differed from those observed in WT mice. AITC slightly increased carbohydrate oxidation for 2 h after administration compared to vehicle administration (Fig. 5C), however, there was no significant difference between each group in the cumulative total carbohydrate oxidation for 2 h after administration (Fig. 6C). V_{O_2} was not influenced for at least 2 h after administration (Fig. 5D and 6D).

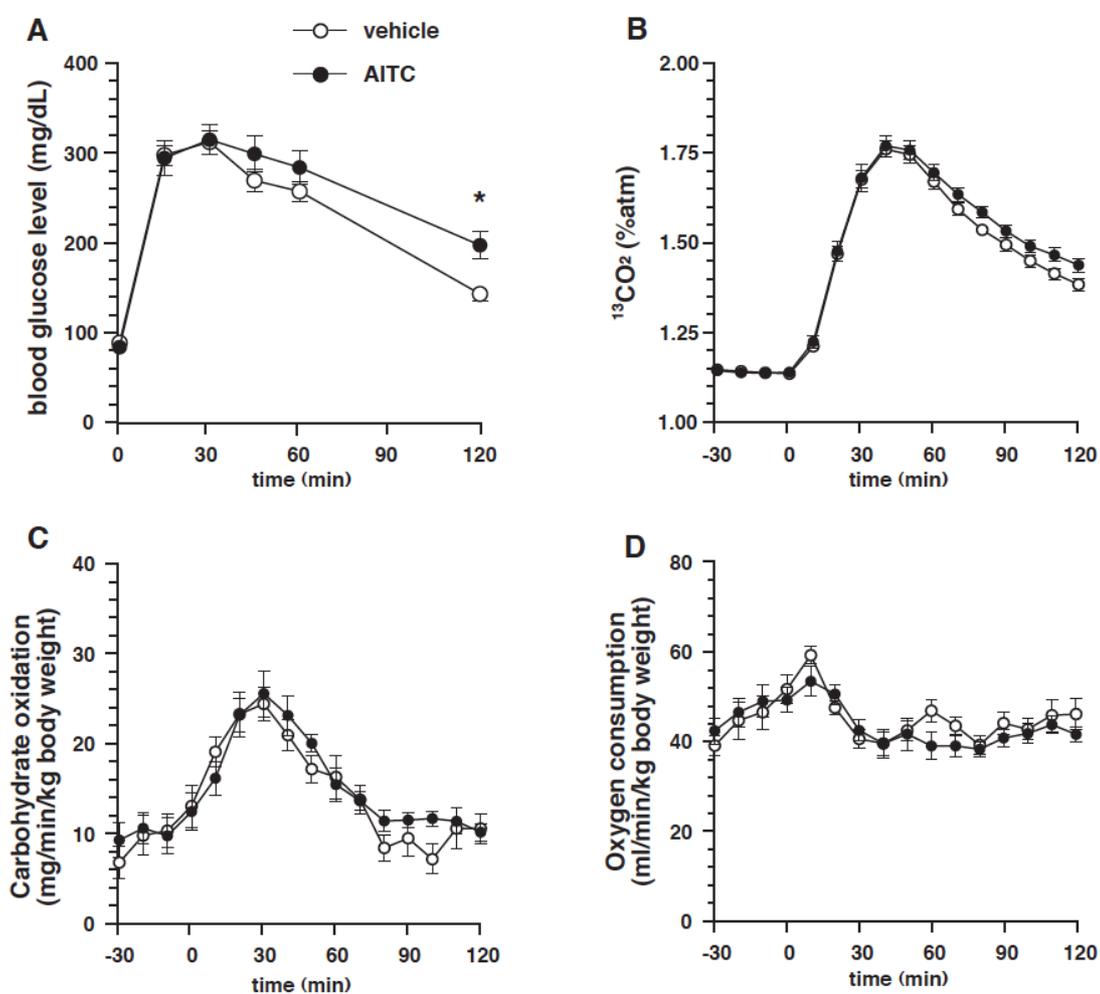


Fig. 5 (A) Changes in the blood glucose levels of TRPV1 KO mice administered with AITC or vehicle (control) in IPGTT. Values are expressed as means \pm SE. $n = 11$; * $P < 0.05$ (two-way repeated-measures ANOVA, followed by unpaired t-test).

(B-D) Changes in the relative abundance of $^{13}\text{CO}_2$, carbohydrate oxidation and $\dot{\text{V}}_{\text{O}_2}$ of TRPV1 KO mice administered AITC or vehicle (control) in IPGTT. Values are expressed as means \pm SE ($n = 11$). There is no significant difference between the groups.

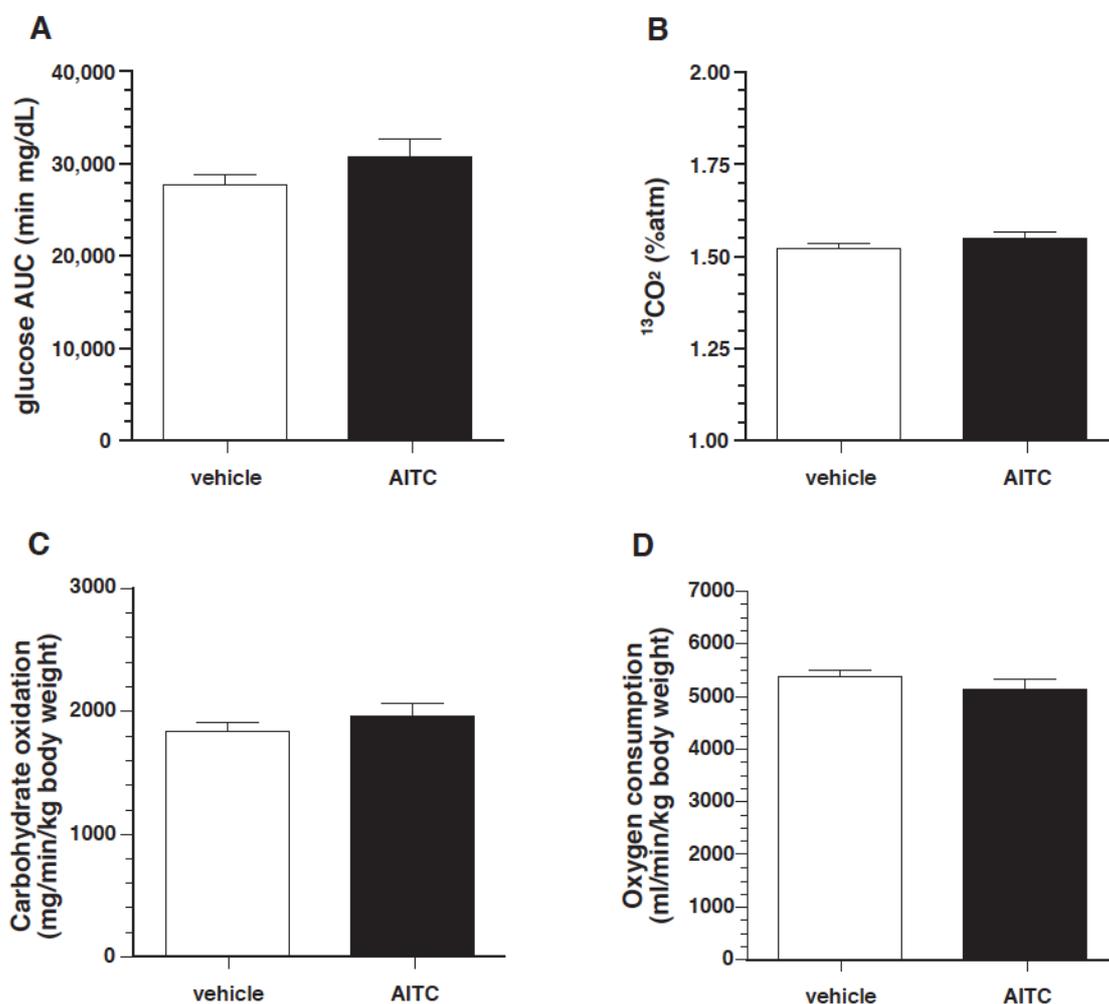


Fig. 6 (A) AUC of blood glucose levels of TRPV1 KO mice in IPGTT. Values are expressed as means ± SE (n = 11).

(B-D) Average the relative abundance of ¹³CO₂ and cumulative carbohydrate oxidation and $\dot{V}O_2$ of TRPV1 KO mice administered with AITC or vehicle (control) in IPGTT for 2 hours. Values are expressed as means ± SE (n = 11). There is no significant difference between the groups.

Discussion

In the present study, the author observed that intragastric administration of AITC reduced the increase in blood glucose level for 15-60 min after administration in the IPGTT. Respiratory gas analysis showed that intragastric administration of AITC increased the ¹³CO₂-¹²CO₂ ratio for 10-50 min after administration. This period was in accordance with the period of the reduction in the increase in blood glucose level by AITC. The increase in the ¹³CO₂-¹²CO₂ ratio meant an increase in the oxidation of intraperitoneally administered

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¹³C-labelled glucose. The increase in blood glucose level was because of the intraperitoneally administered ¹³C-labelled glucose. Therefore, these results indicate that intragastric administration of AITC reduces the increase in blood glucose level by increasing the utilization of excessive glucose in the blood.

AITC is a typical TRPA1 agonist (7,16). In TRPA1 KO mice, however, intragastric administration of AITC reduced the increase in blood glucose level and increased the ¹³CO₂-¹²CO₂ ratio in the IPGTT, which is similar to that observed in WT mice. These results agree with the previous results that TRPA1 is not involved in the increase in carbohydrate oxidation by administering AITC (18). Therefore, TRPA1 is considered to be not involved in reducing the increase in blood glucose level by intragastrically administering AITC.

In TRPV1 KO mice, intragastric administration of AITC did not reduce the increase in blood glucose level and did not increase the ¹³CO₂-¹²CO₂ ratio in the IPGTT. Therefore, TRPV1 is involved in reducing the increase in blood glucose level by intragastrically administering AITC.

The blood glucose levels at 2 h after administering AITC were higher than those of the control in WT mice. This phenomenon was also observed in TRPV1 KO mice but not in TRPA1 KO mice. Therefore, this elevation in blood glucose level was involved not in TRPV1 but in TRPA1. AITC might not only bring about a reduction in the increase in blood glucose level, which is involved in TRPV1, but also cause side effects such as increasing the blood glucose levels for 2 h after administration, which is involved in TRPA1. Further studies are needed to elucidate the mechanism about increased blood glucose levels.

The mechanisms by which AITC increases carbohydrate oxidation remain unclear. Previous studies reported that administering AITC induces adrenalin secretion (11) and that capsaicin, which is a typical TRPV1 agonist, induces adrenaline secretion primarily by activating the adrenal sympathetic nerve (14). Therefore, it was assumed that adrenaline secretion through the activation of the central nervous system was induced by AITC and that glucose uptake was increased via adrenoreceptor activation by adrenaline or noradrenaline in skeletal muscles and adipose tissues. It remains unclear where and how glucose was metabolized, for which further studies are required.

Recent studies suggested that TRPV1 KO mice have insulin resistance or obesity (19-22). Therefore, it is possible that impairments in glucose metabolism, like insulin resistance, cause differences in blood glucose level between WT mice and TRPV1 KO mice. However, the changes in blood glucose level of the vehicle treated-group in TRPV1 KO mice were similar to

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those in WT mice, which indicates that there was no difference in glucose tolerance ability between WT mice and TRPV1 KO mice. Therefore, the author believe that the differences in blood glucose level between WT mice and TRPV1 KO mice were not derived from chronic changes in glucose metabolism between each mouse and that TRPV1 might play a key role in the control of glucose metabolism.

My results showed that intragastric administration of AITC reduced the increase in blood glucose level after glucose loading by increasing the utilization of excessive blood glucose. The approach for suppressing postprandial hyperglycemia that entails increasing the utilization of blood glucose is expected to become a novel approach for suppressing postprandial hyperglycemia. However, it remains unclear how AITC reduced the increase in blood glucose level, for which further studies are required to consider applying this discovery in humans.

In conclusion, the author demonstrated that intragastric administration of AITC reduced the increase in blood glucose level in the IPGTT and that the reduction in the increase was derived from the increased utilization of excessive blood glucose. The author also showed that these effects were involved in not TRPA1 but TRPV1 and suggest that the activation of TRPV1 may be involved in controlling the blood glucose levels.

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SUMMARY

CHAPTER 1

The transient receptor potential (TRP) channel family is composed of a wide variety of cation-permeable channels activated polymodally by various stimuli and is implicated in a variety of cellular functions. Recent investigations have revealed that activation of TRP channels is involved not only in nociception and thermosensation but also in thermoregulation and energy metabolism. The author investigated the effect of intragastric administration of TRP channel agonists on changes in energy substrate utilization of mice. Intragastric administration of allyl isothiocyanate (AITC, a typical TRPA1 agonist) markedly increased carbohydrate oxidation but did not affect oxygen consumption. To examine whether TRP channels mediate this increase in carbohydrate oxidation, the author used TRPA1 and TRPV1 knockout (KO) mice. Intragastric administration of AITC increased carbohydrate oxidation in TRPA1 KO mice but not in TRPV1 KO mice. Furthermore, AITC dose-dependently increased $[Ca^{2+}]_i$ in cells expressing TRPV1. These findings suggest that AITC might activate TRPV1 and that AITC increased carbohydrate oxidation via TRPV1.

CHAPTER 2

The author investigated the effects of allyl isothiocyanate (AITC) on the blood glucose levels of mice using an intraperitoneal glucose tolerance test. The intragastric administration of 25 mg/kg body weight AITC reduced the increase in blood glucose level after 2 g/kg body weight glucose was given intraperitoneally, compared with that of control mice. To elucidate the mechanism responsible for the reduction, respiratory gas analysis employing ^{13}C -labeled glucose was performed. The intragastrically administering AITC increased $^{13}CO_2$ emission, compared to vehicle, after intraperitoneal administering ^{13}C -labeled glucose. This indicated that AITC increased the utilization of exogenously administered glucose, which was excessive glucose in the blood. To examine whether transient receptor potential (TRP) channels mediated this reduction in the blood glucose levels, we used TRPA1 and TRPV1 knockout (KO) mice. Intragastrically administering AITC reduced the increase in the blood glucose level in TRPA1 KO mice but not in TRPV1 KO mice. These findings suggest that dietary AITC

might reduce the increases in blood glucose levels by increasing the utilization of excessive glucose in the blood by activating TRPV1.

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