

# Trehalose increases jejunum cytoplasmic lipid droplets and suppresses adipocyte hypertrophy

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## Research

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# Abstract

**Background:** Trehalose is a functional disaccharide that has anti-metabolic activities such as suppression of adipocyte hypertrophy in mice and alleviation of impaired glucose tolerance in humans. Trehalase hydrolyzes trehalose in the small intestine into two glucose molecules. In this study, we investigated whether trehalose can suppress adipocyte hypertrophy in mice in the presence or absence of trehalase.

**Methods:** Trehalase knockout (KO) mice and wild-type (WT) mice were fed a high fat diet (HFD) and administered water with 0.3% (w/v) or without trehalose for 8 weeks. At the end of the experimental period, mesenteric adipose tissues and the small intestine were collected and the adipocyte size and proportion of cytoplasmic lipid droplets (CLDs, %) in jejunum epithelium were measured by image analysis.

**Results:** Trehalose treatment was associated with suppressed adipocyte hypertrophy in both trehalase KO and WT mice. The rate of CLDs in the jejunal epithelium was increased in both trehalase KO and WT mice given water containing trehalose relative to untreated control mice. Since there was a negative correlation between jejunal epithelial lipid droplet volume and mesenteric adipocyte size, together with these results, trehalose treatment would suppress adipocyte hypertrophy. Because of jejunal epithelium containing lipid droplets fallen into the intestinal lumen, triglyceride (TG) levels in feces tended to be higher in the KO/HFD/Tre group than in the KO/HFD/Water group. Whereas feces from trehalose-treated trehalase KO and WT mice tended to have more free fatty acids (FFA) than the untreated groups. Chylomicron-TG tended to be decreased in both trehalose-treated trehalase KO and WT mice. In vitro, addition of trehalose to differentiated Caco-2 cells increased intracytoplasmic lipid droplets and decreased secretion of the chylomicron marker ApoB48.

**Conclusions:** The suppression of adipocyte hypertrophy in the presence and absence of trehalase indicates that trehalose mediates effects prior to being hydrolyzed into glucose. In both trehalase KO and WT mice, trehalose treatment increased the rate of CLDs in jejunal epithelium, reduced chylomicron migration from the intestinal epithelium to the periphery, and suppressed adipocyte hypertrophy. Thus, trehalose ingestion could prevent metabolic syndrome by trapping fat droplets in the intestinal epithelium and suppressing rapid increases in chylomicrons.

## Background

We have continuously been reported that trehalose suppresses adipocyte hypertrophy and mitigates insulin resistance [1–3]. We also demonstrated that trehalose induces white adipose tissue (WAT) browning with suppressing white adipocyte hypertrophy, increasing body temperature, and reducing blood glucose levels even under normal dietary conditions [4]. Trehalose has an inhibitory effect on adipocyte hypertrophy that is not seen for other saccharides. In the upper small intestine, trehalose is hydrolyzed into two molecules of glucose by trehalase, which have different activity among individuals.

Mizote et al. [5] reported that ingestion of 10 g/day trehalose improved glucose tolerance in human subjects for 12 weeks. These results suggested that trehalose could mediate its effects prior to being hydrolyzed by trehalase.

Oral administration of 1 g/kg trehalose to mice for 5 consecutive days was previously shown to be associated with a significant decrease in the total number of Peyer's patch (PP) lymphocytes and suppression of spontaneous release of interleukin-6 (IL-6) [6]. Furthermore, Kikusato et al. [7] reported that juvenile chicks fed a diet supplemented with 0.5% trehalose for 18 days showed reduced expression of intestinal inflammatory genes such as interferon- $\gamma$  and tumor necrosis factor-like ligand 1A. Thus, we assumed that trehalose had some effect on small intestine and trehalose-mediated suppression of adipocyte hypertrophy might be triggered in the small intestine.

We previously generated trehalase-deficient mice using a gene-targeting procedure [8] and deposited these mice with RIKEN. An oral trehalose tolerance test revealed that these trehalase-deficient mice exhibited no changes in blood glucose levels. These mice are thus useful tools for analyzing the mechanisms of trehalose action. Here we used trehalase KO mice and WT mice to investigate whether trehalose can suppress adipocyte hypertrophy and elicit changes in intestinal function in the presence or absence of trehalase. We hypothesized that trehalase KO mice would show a stronger effect of trehalose compared to WT mice.

## Materials And Methods

### Animals

Ten-weeks-old female trehalase KO mice (RBRC00857, background strain C57BL/6J) and WT mice were obtained from the RIKEN BioResource Research Center (Tsukuba, Japan) and fed a standard diet (CE-2; CLEA Japan, Inc.) and water ad libitum for two weeks. The mice were kept in a temperature-controlled room with a 12-hour light cycle. This study was approved by the Laboratory Animal Care Committee of the Hayashibara Co., Ltd. (Okayama, Japan) and all experiments involving animals were conducted in accordance with the Guidelines for Care and Use of Laboratory Animals of the Hayashibara Co., Ltd.

### Test substance

TREHA™ (Hayashibara Co., Ltd.) containing > 98.0% trehalose dihydrate was used as the source of trehalose.

### Study design

The experimental protocol is shown in Fig. 1. A total of 40 mice were acclimated for 2 weeks and then the 12-weeks-old mice were randomly divided into 5 groups and matched for average body weight. Two groups of trehalase KO mice and two groups of WT mice were fed a high fat diet (HFD32, CREA Co., Ltd., Japan) and then given drinking water ad libitum that either lacked (control) or contained 0.3% (w/v) trehalose (KO/HFD/Water, KO/HFD/Tre, WT/HFD/Water, WT/HFD/Tre, n = 8 for each group). As the

experimental control, another group of trehalase KO mice were given a normal diet and water ad libitum (KO/CE-2/Water, n = 8). Four mice were grouped per cage with each cage containing animals from a single experimental group. Food and water were replaced every other day and food intake was monitored; body weights were recorded weekly throughout the experimental period. After 8 weeks of treatment, the animals were euthanized under pentobarbital anesthesia. The adipose tissue was weighed, and blood samples were collected from the abdominal vena cava for measurement of serum lipids and chylomicrons (CM). Serum TG and non-esterified fatty acids (NEFA) were measured using Triglyceride E-test kits and NEFA C-test kits (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), respectively. CM was measured by high sensitivity gel filtration HPLC (Skylight-biotech, Akita, Japan).

## Calculation of energy intake

The energy densities of the HFD, a normal diet, and the drinking water containing 0.3% (w/v) trehalose were 20.9 kJ, 14.2 kJ, and 0.05 kJ per gram, respectively. Based on these data, the mean energy intake per mouse in each group was calculated using the following formulas:

Energy intake (kJ/mouse/day):

Mice consuming HFD and 0.3% (w/v) trehalose:

Food intake (g) × 20.9 (kJ) + water intake (g) × 0.05 (kJ)

Mice consuming HFD and water:

Food intake (g) × 20.9 (kJ) + water intake (g) × 0 (kJ)

Mice consuming normal diet and water:

Food intake (g) × 14.2 (kJ) + water intake (g) × 0 (kJ)

## Histological analysis of adipocyte size

Mesenteric adipose tissue samples were fixed in 10% (v/v) buffered formalin and embedded in paraffin. The sections were deparaffinized with xylene, stained with hematoxylin and eosin, and then examined by light microscopy. Photographs of 5 random areas per section in the respective adipose tissue were taken at 200 × magnification. More than 200 adipocyte sizes were measured by image analysis software (cellSens, Olympus Corporation, Tokyo, Japan).

## Rates of CLDs in jejunal epithelium

Sections of intestine were stained with hematoxylin and eosin, and then examined using light microscopy. Jejunal cytoplasmic vacuoles were demonstrated to contain neutral lipids by Oil Red O staining of frozen sections. Photographs of 5 random areas in the respective jejunal section were taken at 400x magnification. The proportion of area containing lipid (%) was measured in more than 20 intestinal villi/mouse by image analysis software (cellSens).

## Cell culture

We prepared Caco-2 cells, a human colon carcinoma cell line, according to the method described by Vidal et al. [9] and Morel et al. [10]. The cells were seeded at  $1.5 \times 10^4$  cells/well in a 24-well insert cup (0.4  $\mu$ m pore size, polyethylene terephthalate) treated with atelocollagen, and cultured for one week in Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal bovine serum (FCS) at 37 °C. Confluency was confirmed by measuring electric resistance before serum-free DMEM and DMEM containing 20% FCS DMEM were added to the apical and basal side, respectively. The cells were cultured for an additional week to promote differentiation into intestinal epithelial cells, which were then divided into groups according to the electrical resistance values before use in experiments.

## Micelle treatment of Caco-2 cells

As reported by Harnell et al. [11], micelles in the duodenum consist of 0.3 mM oleic acid, 0.025 mM cholesterol, 0.1 mM 2-monooleylglycerol, 1.0 mM taurocholic acid and 0.1 mM  $\alpha$ -lysophosphatidylcholine. Lipids at these concentrations were dissolved in ethanol in a glass test tube, dried with  $N_2$  gas, and stored at -80 °C until use. After adding serum-free DMEM medium to the lipids and sonicating for 20 minutes, they were mixed with the same amount of serum-free DMEM medium with or without trehalose (50 mM final concentration) and sonicated for 5 minutes. The medium on the apical side of the differentiated Caco-2 cells was removed, and 300  $\mu$ L/well of the micelle solution was added. After incubating for 24 hours, basal side culture medium was collected in a tube, mixed with polyoxyethylene (10) octylphenyl ether and EDTA at a final concentration of 1% and 5 mM, respectively, and protein inhibitors before storage at -80 °C until use. When assessing the amount of intracellular lipid accumulation, the content of micelle components was reduced by half to prevent cytotoxicity, and mixed with the fluorescently labeled fatty acid BODIPY™ FL C<sub>16</sub> (Thermo Fisher Scientific Inc., Waltham, MA, USA) at a final concentration of 10  $\mu$ M.

## Measurement of lipid droplet accumulation in Caco-2 cells

The cultured cells were fixed by treatment with 4% formaldehyde for 5 minutes. The membrane at the bottom of the insert cup was cut with a scalpel, transferred to a slide glass, and encapsulated with mounting medium (50% glycerol, 0.05%  $NaN_3$  in phosphate buffered saline). Then, the specimen was observed with a fluorescence microscope (Olympus BX53-FK, exposure time: 10 milliseconds), and the ratio of BODIPY™ FL C<sub>16</sub> that accumulated in areas containing lipid droplets was analyzed with Olympus cellSens.

## Evaluation of secreted ApoB48 by western blotting

Basal side culture medium was collected and mixed with  $\times 1.25$  sample buffer (2.08% sodium lauryl sulfate, 6.25% glycerol, 1.94% dithiothreitol in 0.073 M Tris-HCl buffer, pH 6.8) and denatured by boiling for 5 minutes. The protein was subjected to 5% SDS-PAGE and transferred to a PVDF membrane. The membrane was treated with blocking agent, and labeled with a mouse anti-human ApoB monoclonal antibody (7B8) (ab39560, Abcam plc, Cambridge, UK) as the primary antibody, and horseradish

peroxidase-labeled goat anti-mouse IgG polyclonal antibody (Nr.P0447, Dako, Agilent Technologies, Santa Clara, CA) as the secondary antibody, which was detected by ECL prime detection agent (GE Healthcare, Little Chalfont, UK). The resulting images were analyzed with Image J (version 1.52a).

## Statistics

Data are expressed as means  $\pm$  standard deviations. A power analysis (G\*Power 3.1.9.4, <http://www.gpower.hhu.de/>) showed that a sample size of 8 mice per group was suitable to detect a difference between 5 experimental groups ( $1-\beta = 0.95$ , effect size = 0.8,  $\alpha = 0.01$ ). In addition, the calculated p values were described. Statistically significant effects of trehalose on dispersion uniformity and normality were examined using Tukey-Kramer (Statcel, ver. 3). Non-parametric data were analyzed by the Steel-Dwass test (Statcel 3). A p-value less than 0.05 was considered significant.

## Results

### Continuous ingestion of trehalose had no effect on energy intake, body weight, and tissue weight

The energy intake values for trehalase KO and WT mice given water with or without trehalose during the experimental period were similar at  $59.8 \pm 4.7$ ,  $59.0 \pm 4.2$ ,  $58.2 \pm 4.5$ ,  $55.2 \pm 5.1$ , and  $56.0 \pm 3.0$  kJ per mouse per day in the KO/CE-2/Water, KO/HFD/Water, KO/HFD/Tre, WT/HFD/Water, and WT/HFD/Tre, groups respectively. Furthermore, the four HFD groups exhibited no significant differences in overall body weight, or weight of adipose tissue, serum NEFA. Liver weight was significantly lower in the WT/HFD/Water group compared to the KO/HFD/Water group. Serum TG was significantly higher in the KO/HFD/Tre group compared to the WT/HFD/Water group (Table 1).

Table 1. Energy intake, body, organ weights, serum lipids in mice after 8 weeks of trehalose intake.

		KO/CE-2/Water	KO/HFD/Water	KO/HFD/Tre	WT/HFD/Water	WT/HFD/Tre
	Body weight (g)	22.6 $\pm$ 1.5	31.3 $\pm$ 4.7*	30.4 $\pm$ 2.6*	27.3 $\pm$ 3.7*	26.8 $\pm$ 2.3*
	Energy intake (kJ)	59.8 $\pm$ 4.7	59.0 $\pm$ 4.2	58.2 $\pm$ 4.5	55.2 $\pm$ 5.1	56.0 $\pm$ 3.0
	mesenteric adipose	10.9 $\pm$ 3.2	18.3 $\pm$ 4.7*	18.6 $\pm$ 3.1*	17.9 $\pm$ 5.7*	15.0 $\pm$ 1.9
Adipose weight/ body wight (mg/g)	Perirenal adipose	7.4 $\pm$ 4.3	19.6 $\pm$ 4.1*	24.9 $\pm$ 13.3*	19.2 $\pm$ 8.0*	18.6 $\pm$ 3.5*
	Retroperitoneal adipose	15.1 $\pm$ 0.2	52.2 $\pm$ 18.5*	55.1 $\pm$ 12.4*	41.2 $\pm$ 20.3*	40.6 $\pm$ 14.1*
	Visceral adipose	33.5 $\pm$ 13.9	90.2 $\pm$ 22.4*	98.7 $\pm$ 12.7*	78.4 $\pm$ 29.9*	74.3 $\pm$ 16.0*
	Liver (g)	1.043 $\pm$ 0.132	1.414 $\pm$ 0.239*	1.382 $\pm$ 0.150*	1.224 $\pm$ 0.138 <sup>§</sup>	1.253 $\pm$ 0.145
Serum	Triglyceride (mg/dL)	54.2 $\pm$ 20.0	33.4 $\pm$ 6.7	42.5 $\pm$ 11.1 #	34.5 $\pm$ 5.1	37.2 $\pm$ 6.6
	Non-esterified fatty acid (mEq/L)	0.477 $\pm$ 0.140	0.522 $\pm$ 0.143	0.578 $\pm$ 0.186	0.534 $\pm$ 0.049	0.502 $\pm$ 0.060

Values are shown as means  $\pm$  SD for 7-8 mice per group. There were 8 mice in the KO/CE-2/Water and WT/HFD/Water groups and 7 mice in the other groups. Statistical analysis was performed using a Steel-Dwass test. \*Statistically significant ( $p < 0.05$ ) difference compared to the KO/CE-2/Water group; §Statistically significant ( $p < 0.05$ ) difference between the KO/HFD/Water and WT/HFD/Water group; #Statistically significant ( $p < 0.05$ ) difference between the KO/HFD/Tre and WT/HFD/Water group.

## **Trehalose suppressed adipocyte hypertrophy in mesenteric adipose tissues**

We examined the histology of the mesenteric adipose tissues and measured the adipocyte sizes from both trehalase KO and WT mice groups (with and without trehalose in the water) using cellSens imaging software. Histologically, mesenteric adipose tissues from both the trehalose-treated trehalase KO and WT groups comprised many small adipocytes (Fig. 2a). For trehalase KO mice, the size of mesenteric adipocytes from the HFD/Tre group ( $1,953 \pm 209 \mu\text{m}^2$ ) was significantly smaller than that for the HFD/Water group ( $2,809 \pm 541 \mu\text{m}^2$ ;  $p < 0.05$ ; Fig. 2b). The WT/HFD/Tre group ( $1,683 \pm 189 \mu\text{m}^2$ ) had significantly smaller mesenteric adipocytes than for the WT/HFD/Water group ( $2,515 \pm 717 \mu\text{m}^2$ ,  $p < 0.05$ ). Moreover, the suppressive effects of trehalose on adipocyte hypertrophy were nearly the same between the trehalase KO and WT mice.

## **Trehalose increased the proportion of CLDs in jejunum epithelium**

Histopathological examination of the entire intestine of the trehalase KO and WT mice showed that intracytoplasmic vacuole was most intense in the upper jejunum. In both the trehalase KO and WT mice, the number of intracytoplasmic vacuoles were markedly increased in the groups given trehalose (Fig. 3a). Vacuoles of the jejunal epithelium were demonstrated to contain neutral lipids by Oil Red O staining of frozen sections (Fig. 3b). Then, we determined intracytoplasmic vacuoles as CLDs.

We also measured the proportion of CLDs in the jejunum by image analysis. Trehalase KO mice given water containing trehalose tended to have an increased proportion of CLDs ( $41.6\% \pm 4.5\%$ ) compared to the water only group ( $34.1\% \pm 6.9\%$ ). Moreover, in WT mice, the proportion of CLDs in the trehalose group ( $44.0\% \pm 4.3\%$ ) was significantly higher than that of the water group ( $27.7\% \pm 5.9\%$ ) (Fig. 3c). Surprisingly, a negative correlation was observed between the proportion of CLDs (%) in the jejunum and the size of mesenteric adipocytes across the HFD groups (Fig. 4). Whereas the adipocytes in the water only group exhibited various sizes in both trehalase KO and WT mice, in the groups given water containing trehalose, most of animals had small adipocyte size and a high proportion of CLDs. Mice with small adipocytes had many CLDs in the jejunum.

## **Trehalose promoted detachment of lipid droplets from the intestinal epithelium to the intestinal lumen and the lipids**

## were excreted in the feces

Intestinal epithelium is reported to turn over every 3–4 days [12]. When we observed jejunum microvilli, the intestinal epithelium contained many lipid droplets that were detached in the intestinal lumen of mice fed HFD compared to mice fed a normal diet (Fig. 5a). We also measured TG and FFA excreted in feces. In the trehalase KO mice, the amount of TG excreted in the feces of the trehalose group was slightly increased compared to that for the water group (Fig. 5b). In both the trehalase KO and the WT mice, FFA excreted in feces of the trehalose groups tended to be increased relative to that for the water groups (Fig. 5c). Since the daily fecal weight for the groups fed a normal diet was 7-fold higher than that for the HFD group, the amount of lipid excretion in the normal group was apparently increased.

## Trehalose decreased serum CM-TG

We next measured serum CM-TG levels to examine whether the amount of CM-TG was decreased by jejunal lipid droplet trapping. In both the trehalase KO and WT mice, the amount of serum CM-TG tended to decrease in the trehalose group compared to the water group (Fig. 6).

## Inhibitory effect of ApoB48 secretion by trehalose in vitro

Caco-2 cells differentiated into intestinal epithelium cells were treated with lipid micelles for 24 hours, and the lipid droplet area accumulated in the cells was measured (Fig. 7a). In the absence of micelles, the lipid droplet area in the control group ( $0.52\% \pm 0.21\%$ ) and 50 mM trehalose-treated group ( $0.05\% \pm 0.02\%$ ) was similar. Upon micelle treatment, the area increased to  $7.46\% \pm 1.72\%$ . When cells were treated with both micelles and 50 mM trehalose, the lipid droplet area increased to  $22.51\% \pm 2.74\%$ , approximately three times that of the control group treated with micelles only (Fig. 7b). The differentiated Caco-2 cells were subsequently treated with the micelles for 24 hours, and the amount of ApoB48 secreted in the basal culture medium was measured (Fig. 7c, d). The micelle treatment increased the amount of ApoB48 secretion on the basal side, and this secretion was markedly suppressed in the presence of 50 mM trehalose.

## Discussion

In the present study, we first demonstrated that trehalose suppressed adipocyte hypertrophy in both WT mice and those with trehalase KO mice. We further showed a negative correlation between the size of mesenteric adipocytes and the proportion of jejunal CLDs. To our knowledge, this is the first study to assess the effects of trehalose on adipocyte hypertrophy and accumulation of jejunal CLDs in trehalase KO mice.

Comparing trehalase KO mice and WT mice fed HFD indicated that trehalose had nearly similar effects in terms of suppression of adipocyte hypertrophy and the jejunal CLD trap rate. Because trehalose is degraded by trehalase in the upper small intestine, the amount of trehalose that reaches the lower intestine is higher in the trehalase KO mice than the WT mice. However, the effects of trehalose on adipocyte size and jejunal lipid droplet trapping were nonetheless equivalent. The similarity in the effects

of trehalose on WT and trehalase KO mice suggested that the trehalose-mediated action occurs before trehalose is degraded by trehalase and thus these effects could be induced in sites such as the oral cavity, stomach, duodenum and upper jejunum.

D'Aquila et al. [13] reviewed the significance of jejunal CLDs. Dietary fat consumed as TG is efficiently digested into fatty acids (FA) in the gastrointestinal lumen and is absorbed by enterocytes. The digested products taken up by enterocytes are re-synthesized into TGs and packaged either in chylomicrons (CMs) for secretion or in CLDs for storage. Although the CLDs were thought to be an inactive reservoir of neutral lipids, they are now recognized as dynamic organelles that have functions beyond lipid metabolism [14, 15]. The synthesis of CLDs is thought to buffer enterocytes from FA toxicity and control the rate of synthesis and secretion of CMs [13]. Although all regions of the small intestines can take up and absorb digestive products of TG, the jejunum is the main site for TG metabolite uptake and absorption [16, 17].

When we examined whole intestine tissues histopathologically, the upper jejunum had the strongest intensity of CLD staining and this result was consistent with earlier reports [16, 17]. The finding of substantially more jejunal lipid droplets in the trehalose group compared to the water group was unexpected. Since the accumulation of CLDs suppresses the secretion of CM that migrates to the lymphatic system, according to the review by D'Aquila et al. [13], intestinal CLDs would not necessarily be detrimental. Moreover, this review also reported that the amount CLD increased after meals and decreased upon fasting due to enterocyte turnover such that malabsorption of fat that occurs during steatorrhea did not occur under normal physiological conditions. We also conducted histopathological evaluation of the intestine, liver and pancreas, but observed no abnormal changes associated with trehalose treatment. In particular, there were no differences in the number of intestinal villi and villi length in specific areas between the HFD groups. In contrast, there was a negative correlation between the size of mesenteric adipocytes and the proportion of jejunal CLD content. Trapping lipid droplets in the jejunum could be responsible for the induction of adipocyte hypertrophy suppression.

Xiao et al. [18] performed a single lipid challenge test in which glucose or water was consumed 5 hours after a high fat liquid meal challenge, and a duodenal biopsy was performed 1 hour later to compare the amount of CLDs. Their data indicate that oral glucose mobilizes TGs stored within enterocyte CLDs and provides a substrate for CM synthesis and secretion. At 1 hour after glucose ingestion, the amount of CM-TG was significantly higher and the number of CLDs in the jejunum was significantly lower compared to the water group. This result suggested that glucose suppressed CLD accumulation in the intestine and they were instead rapidly transferred to lymphatic vessels as CM. When glucose was given to mice fed a HFD in our study, we found no suppression of adipocyte hypertrophy [1]. This phenomenon was apparently different from the effect of trehalose on CLDs.

Soriguer et al. [19] reported that plasma levels of glucose, insulin, TG, CM, apoB48, apo A-IV levels and HOMA-IR were all significantly higher in morbidly obese patients with T2DM. But the jejunal wall TG concentration in these patients was markedly lower than in morbidly obese patients without T2DM. In this study, amounts of ApoB48, CM-TG, and HOMA-IR were negatively correlated with jejunal TG. As such,

the difference between diabetes and obesity may be due to the suppression of a transition from jejunal lipid droplet traps to CM. These findings were consistent with data for our study.

In terms of the inhibitory effects of trehalose on CM secretion, we measured the amount of APOB48 that passed under the basement membrane of Caco-2 cells. A decrease in the amount of APOB48 was observed in the presence of trehalose. Since APOB48 is a component protein of CM, CM secretion could likely be suppressed by trehalose.

Studies by DeBosch et al. [20, 21] indicated that trehalose inhibited glucose transport to induce hepatic autophagy and prevent hepatic steatosis in an SLC2A- and AMPK-dependent manner. The beneficial effects of hepatic AMPK activation in increasing fat oxidation and insulin sensitivity are well documented [22]. In our previous study, trehalose maintained high serum HMW-adiponectin levels in HFD-fed mice compared to the water group [2]. This result suggests that trehalose maintains high levels of HMW-adiponectin that in turn activates AMPK. Meanwhile, Auclair et al. [23] reported that intestinal factors regulate CLDs and showed that increased intestinal AMPK activity reduced lipolysis and decreased secretion of CM from CLDs. We will need to investigate whether there are differences in intestinal AMPK activity with trehalose treatment of mice in future studies.

In recent years, proteins in CLDs have been frequently analyzed to investigate the

relationship between CLDs and systemic lipid metabolism. D'Aquila et al. [24] analyzed the proteome of CLDs isolated from enterocytes harvested from the small intestine of mice following dietary fat challenge. They identified 181 proteins associated with the CLD fraction, 37 of which are related to lipid-related metabolic pathways. Moreover, using confocal and electron microscopy they confirmed that perilipin 3, apolipoprotein A- $\text{II}$  and acyl-CoA synthetase long-chain family member 5 localized on or around the CLD. However, which proteins actually suppress CM secretion from CLDs remains unclear.

The increase in the rate of jejunum CLDs associated with trehalose treatment compared to the water group may also be due to differences in CLD proteins. Future studies will help clarify what protein(s) is the causative factor of this increase in CLD content. In addition, the effect of lipolysis and lipophagy on CLDs as well as the effect of trehalose on these functions should be investigated.

## Conclusions

In this study we demonstrated that trehalose suppressed adipocyte hypertrophy in both WT and trehalase KO mice fed a HFD. We observed a negative correlation between the size of mesenteric adipocytes and the proportion of jejunal CLDs in both trehalase KO mice and WT mice. When trehalose was administered to mice fed a HFD, trehalose increased the accumulation of CLDs in the jejunum and suppressed the secretion of CM that migrates throughout the body via lymphatic vessels. Our results suggest that prevention of rapid migration of lipids into the blood and peripheral tissues may have a preventive effect on metabolic syndrome.

# Abbreviations

AMPK:AMP-activated protein kinase; ApoB48:Apolipoprotein B48; CLDs:cytoplasmic lipid droplets; CM:chylomicron; CM-TG:chylomicron-triglyceride; FFA:free fatty acids; HFD:high fat diet; HMW-adiponectin:High molecular weight-adiponectin; IL-6:interleukin-6; KO:knockout; KO/CE-2/Water:trehalase KO mice fed normal diet with water; KO/HFD/Tre:trehalase KO mice fed high fat diet with trehalose; KO/HFD/Water:trehalase KO mice fed high fat diet with water; PP:Peyer's patch; SLC2A:solute carrier 2A; TG:triglyceride; WAT:white adipose tissue; WT/HFD/Tre:wild type mice fed high fat diet with trehalose; WT/HFD/Water:wild type mice fed high fat diet with water; w/v:weight/volume; v/v:volume/volume

# Declarations

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## Availability of data and materials

All data generated and analyzed during this study are included in the manuscript.

## Author contributions

CA, NA, SA, CY, SE and HM designed the study. CA, NA, SA, CY, SM, AM and AS performed the experiments. Analysis and data mining were performed by CA, NA and SA. SA, CY, SM, AM and AS provided technical assistance. SA, NA, SE, TA, HM and CA interpreted the results. CA and SA wrote the manuscript, which was approved by HM, TA and US. All authors have read and approved the final manuscript.

## Ethics approval

All experiments involving animals were conducted in accordance with the Guidelines for Care and Use of Laboratory Animals of the Hayashibara Co., Ltd.

## Consent for publication

All authors support the submission to this journal.

## Competing interests

All authors are employees of Hayashibara Co. Ltd., and all funding for the study was sponsored by the company. The authors declare that there are no other conflicts of interest.

## Authors information (optional)

Not applicable.

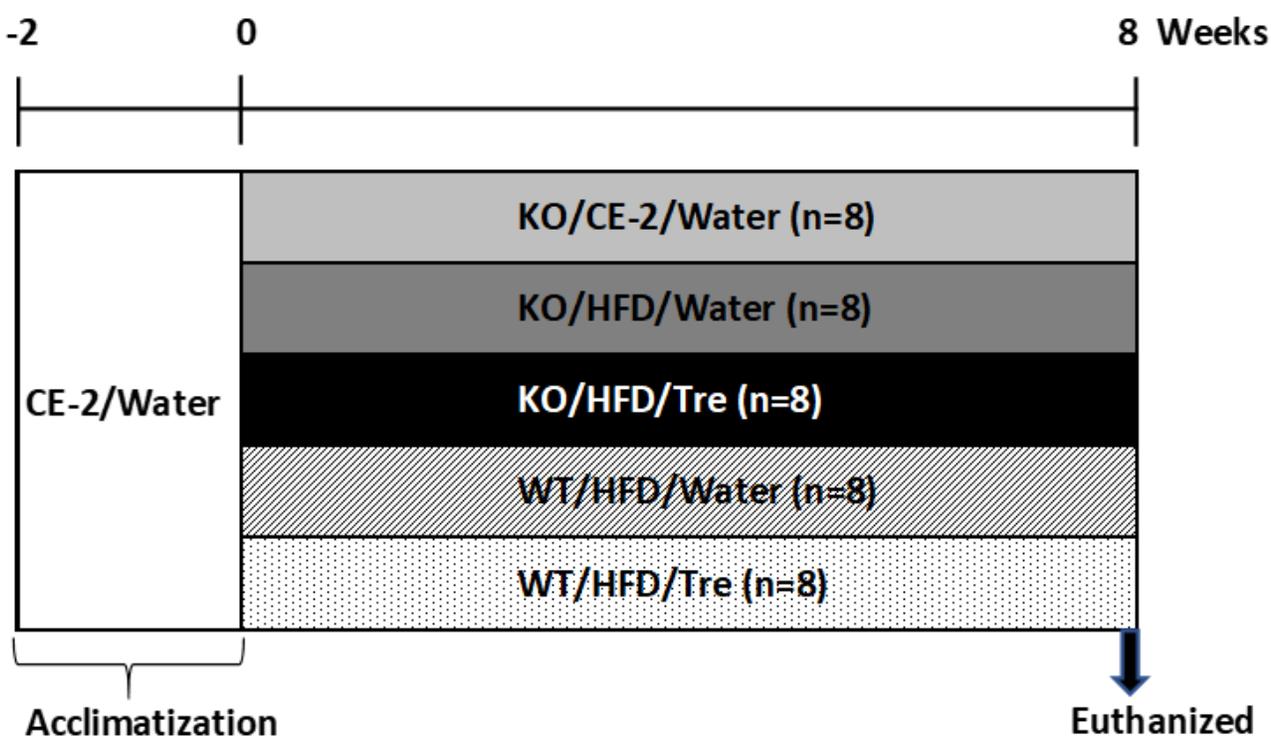
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## Figures



- Divided into 3 groups for trehalase KO or 2 groups for wild type that were matched for body weight
- Starting trehalose administration: 0

**Figure 1**

Experimental protocol. After 2 weeks of acclimatization, trehalase KO mice were divided into 3 groups and WT mice were divided into 2 groups matched by body weight and then administered with 0.3% (w/v) trehalose in the drinking water or water alone. The mice were euthanized after 8 weeks. Histological

examination of the intestine was carried out and measurement of adipocyte size and the rate of jejunal lipid accumulation was conducted by image analysis and serum lipid analysis, respectively.

Figure 2a

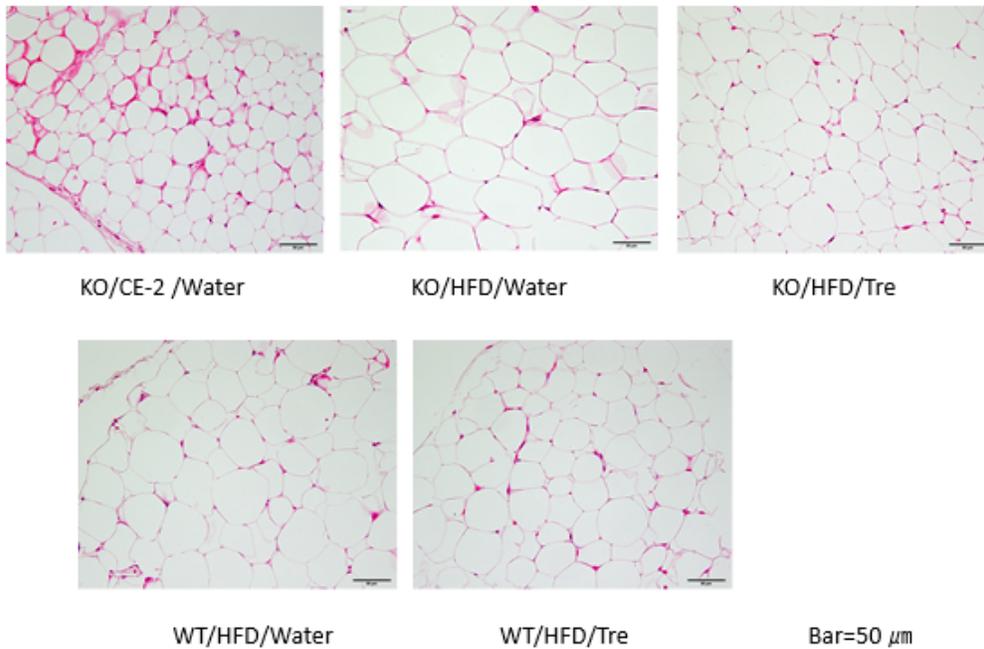


Figure 2b

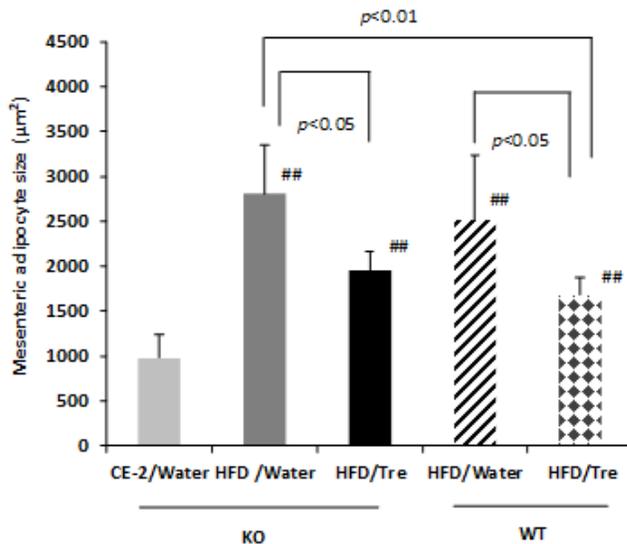


Figure 2

Effect of drinking water containing trehalose on the size of mesenteric adipocytes. The histology of the mesenteric adipose tissues was analyzed and the adipocyte size was measured using cellSens image software. Representative images (400x) of hematoxylin-eosin staining in sections of mesenteric adipose

tissue are shown to assess mesenteric adipocytes histologically (a) and to determine their size (b). Values are shown as means  $\pm$  standard deviation (n=7-8). Statistical analysis was performed with a Tukey-Kramer test. The values indicate that the statistical significances were  $p < 0.05$  and  $p < 0.01$ , respectively. Each group vs. CE-2/Water:  $##p < 0.01$ .

Figure 3a

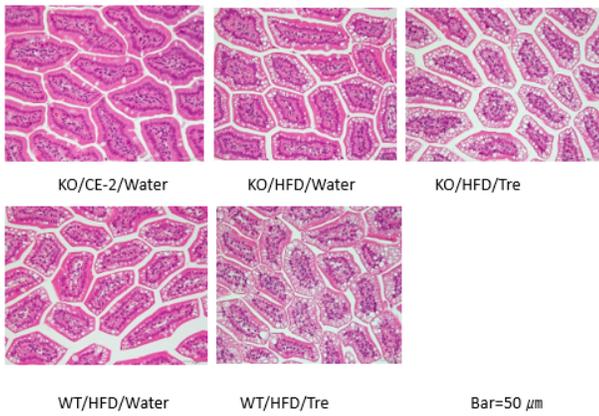


Figure 3b

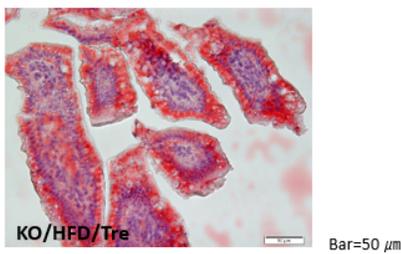


Figure 3c

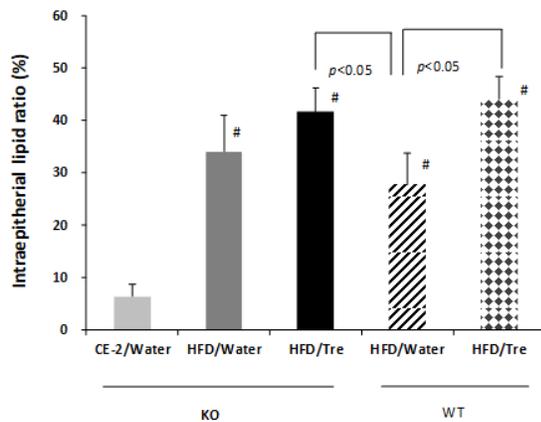
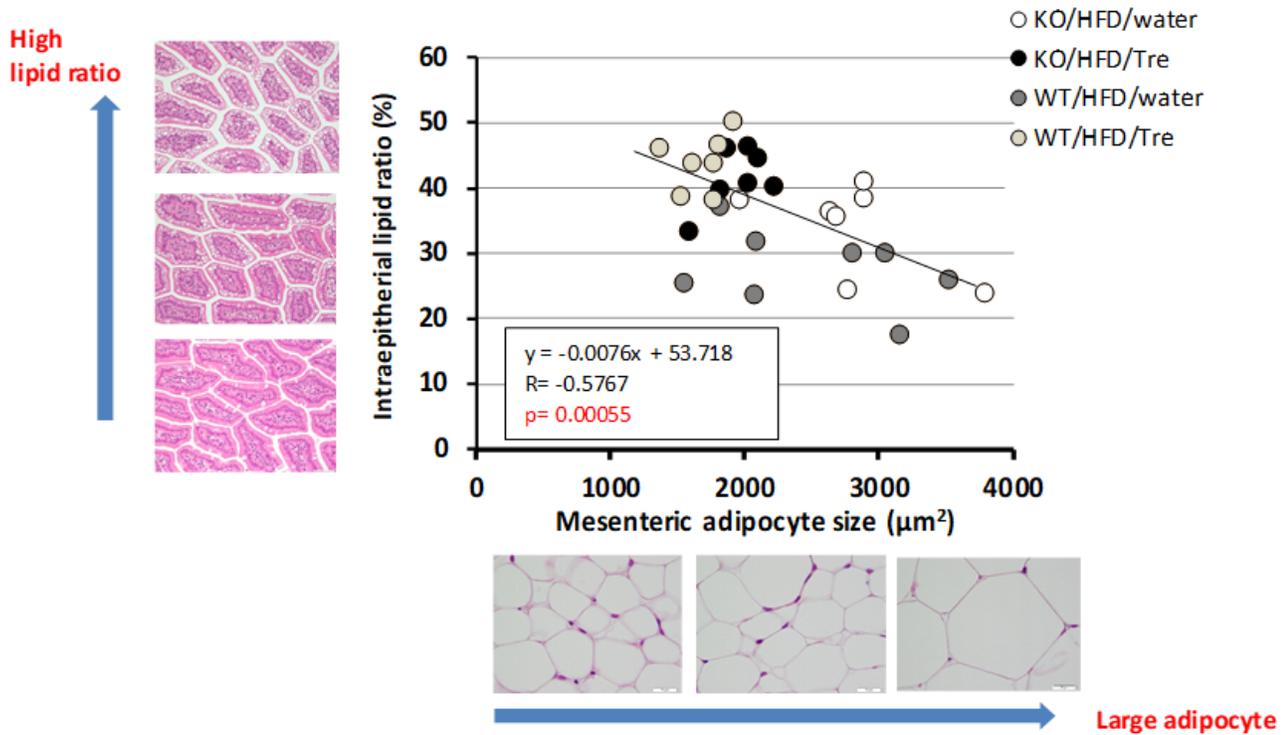


Figure 3

Effect of drinking water containing trehalose on intracytoplasmic lipid droplets in jejunal epithelium. Representative images (400x) of hematoxylin-eosin staining in sections of jejunum are shown (a) Oil Red O staining showing the presence of neutral lipids in jejunal intracytoplasmic vacuoles (b) intraepithelial lipid ratio (%) in the jejunal epithelium determined using the image software cellSens (c). Values are shown as means  $\pm$  standard deviation (n=7-8). Statistical analysis was performed using a Steel-Dwass test. The values indicate that the statistical significances were  $p < 0.05$ . Each group vs. CE-2/Water: # $p < 0.05$ .



**Figure 4**

Correlation between intraepithelial lipid ratio (%) and mesenteric adipocyte size. There was a negative correlation between mesenteric adipocyte size and jejunal intraepithelial lipid ratio (%) (n=7-8, each group: Pearson correlation coefficients  $r = -0.57$ ). The values indicate statistical significance ( $p < 0.01$ ).

Figure 5a

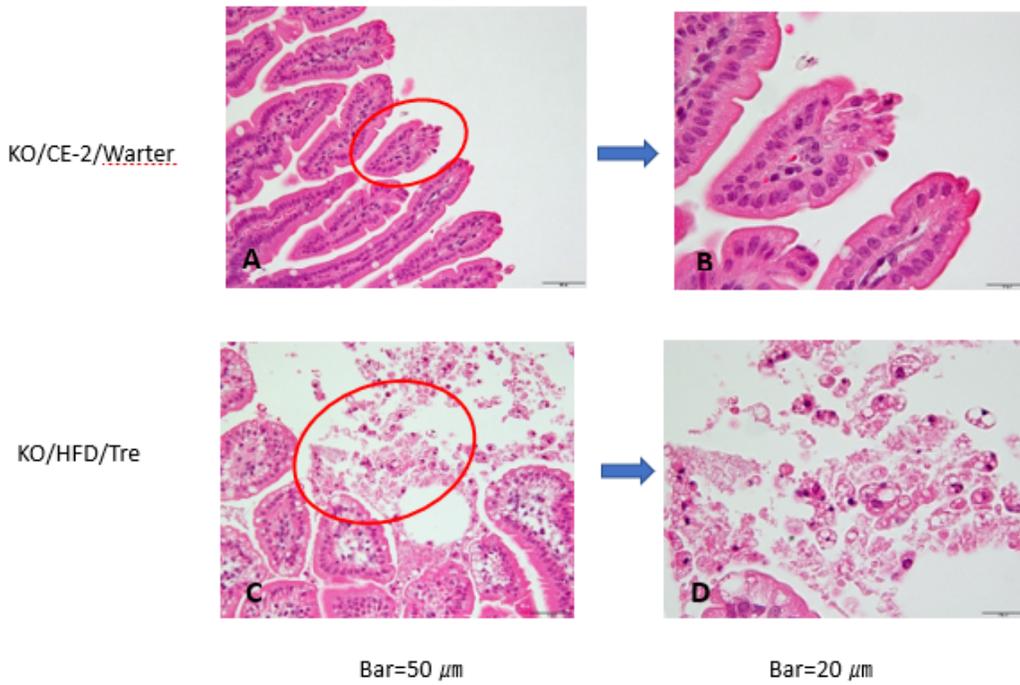


Figure 5b

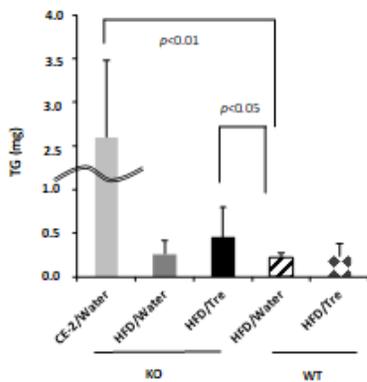


Figure 5c

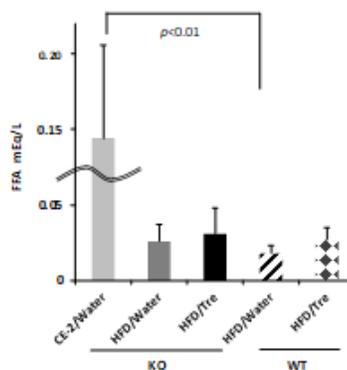
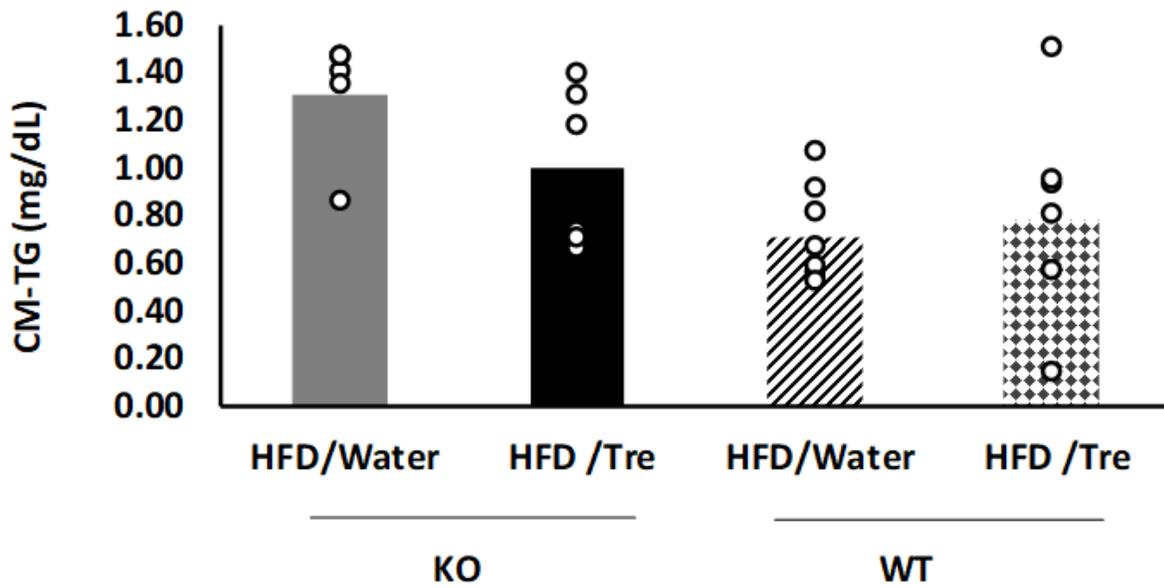


Figure 5

Effect of drinking water containing trehalose on fecal lipid excretion. Detached jejunal microvillus to the lumen of KO/CE-2/Water (a-A), and KO/HFD/Trehalose (a-C) and magnified images of jejunal microvillus (a-B, -D). The amount of TG (b) and FFA (c) excreted in feces. Values are shown as means  $\pm$  standard deviation (n=7-8). Statistical analysis was performed using Steel-Dwass test. The values indicate statistical significance (5b and 5c;  $p < 0.01$ , 5b;  $p < 0.05$ ).



**Figure 6**

Effect of drinking water containing trehalose on CM-TG. Serum CM-TG was measured by high sensitivity gel filtration HPLC. The individual scores were plotted (n=5-8). No statistical significance was observed.

Figure 7a

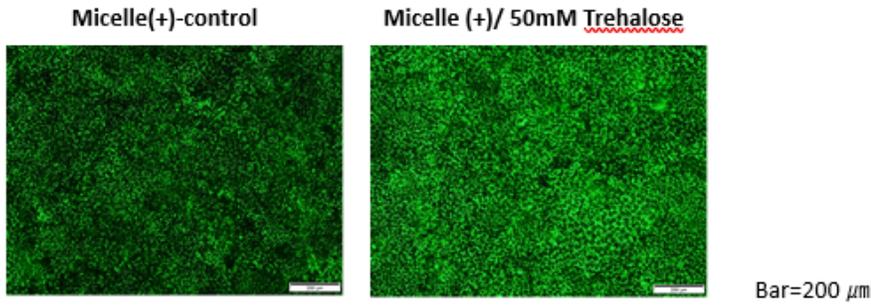


Figure 7b

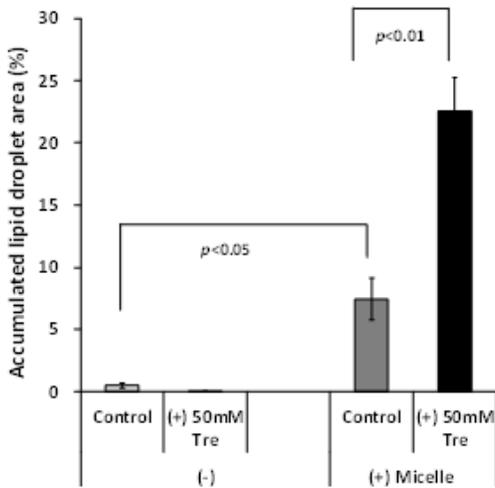


Figure 7d

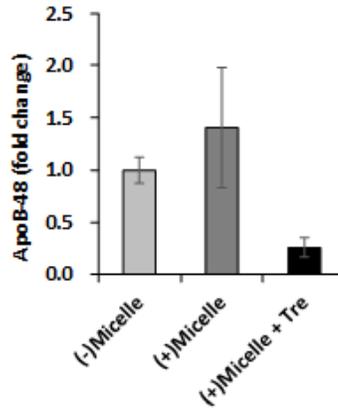


Figure 7c

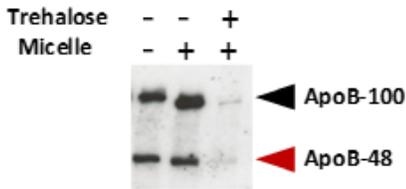


Figure 7

Effect of trehalose on lipid droplet accumulation and ApoB48 secretion in Caco-2 cells. Caco-2 cells were treated with or without micelles and 50mM trehalose. The cultured cells were fixed with formaldehyde, mounted and observed with a fluorescence microscope (a). The bright areas with BODIPY<sup>TM</sup> FL C16 were analyzed (b). Culture supernatants from the basal side were analyzed for ApoB48 by western blotting (c). The relative amount of ApoB48 relative to micelles (-) (d). Values are shown as means  $\pm$

standard deviation (n=3). The values indicate statistical significance (7b:  $p < 0.05$ ,  $p < 0.01$ , Tukey-Kramer test). No statistical significance was observed (7d: t-test).