Title

Glucagon-like peptide-1 is involved in the thermic effects of dietary proteins in male rodents

Authors

[1]Keita Ochiai, [2]Asuka Muto, [3]Bong Soo Seok, [1]Yuta Doi, [2]Yusaku Iwasaki, [3]Yuko Okamatsu-Ogura, [4]Daniel J. Drucker, [1,5]Tohru Hira

Affiliations

[1]Laboratory of Nutritional Biochemistry, Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan

[2]Laboratory of Animal Science, Graduate School of Life and Environmental Sciences, Kyoto Prefectural University, 1-5 Hangi-cho, Shimogamo, Sakyo-ku, Kyoto 606-8522, Japan

[3]Laboratory of Biochemistry, Faculty of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

[4]Lunenfeld Tanenbaum Research Institute, Mt. Sinai Hospital, 600 University Avenue TCP5-1004 Mailbox 39, Toronto Ontario M5G 1X5, Canada

[5]Laboratory of Nutritional Biochemistry, Research Faculty of Agriculture, Hokkaido University, Sapporo 060-8589, Japan

Corresponding author and request for reprints

Tohru Hira

Research Faculty of Agriculture, Hokkaido University

Kita-9, Nishi-9, Kita-ku, Sapporo 060-8589, Japan

Tel: +81-11-706-2504, Fax: +81-11-706-2504

E-mail: hira@agr.hokudai.ac.jp

ORCID ID: https://orcid.org/0000-0002-0354-1617

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Abstract

Protein intake potently increases body temperature and energy expenditure, but the underlying mechanism thereof remains incompletely understood. Simultaneously, protein intake potently stimulates glucagon-like peptide-1 (GLP-1) secretion. Here, we examined the involvement of GLP-1 in the thermic effects of dietary proteins in rodents by measuring rectal temperature and energy expenditure and modulating GLP-1 signaling. Rectal temperature of rats or mice fasted for 4 or 5 h were measured using a thermocouple thermometer before and after an oral administration of nutrients. Oxygen consumption after oral protein administration was also measured in rats. Rectal temperature measurements in rats confirmed an increase in core body temperature after refeeding, and the thermic effect of the oral administration of protein was greater than that of a representative carbohydrate or lipid. Among the five dietary proteins examined (casein, whey, rice, egg, and soy), soy protein had the highest thermic effect. The thermic effect of soy protein was also demonstrated by increased oxygen consumption. Studies using a nonselective β-adrenergic receptor antagonist and thermal camera suggested that brown adipose tissue did not contribute to soy protein-induced increase in rectal temperature. Furthermore, the thermic effect of soy protein was completely abolished by antagonism and knockout of GLP-1 receptor, yet potentiated via augmentation of intact GLP-1 levels through inhibition of dipeptidyl peptidase-4 activity. These results indicate that GLP-1 signaling is essential for the thermic effects of dietary proteins in rats and mice, and extend the metabolic actions of GLP-1 ensuing from nutrient ingestion to encompass the thermic response to ingested protein.

23 **Abbreviations**

24 AA	amino acid
25 BIIE	BIIE 0246
26 BAT	brown adipose tissue
27 βAR	β-adrenergic receptor
28 CCK	cholecystokinin
29 CCK-A	cholecystokinin-A
30 Dvz	devazepide
31 DIT	diet-induced thermogenesis
32 DPP-4	dipeptidyl peptidase-4
33 EE	energy expenditure
34 Ex9	exendin (9-39)
35 GLP-1	glucagon-like peptide 1
36 GLP-1R	glucagon-like peptide 1 receptor
37 *Glp1r* KO	glucagon-like peptide 1 receptor knockout
38 GIP	glucose-dependent insulinotropic polypeptide
39 iBAT	interscapular brown adipose tissue
40 PYY	peptide-YY
41 SNS	sympathetic nervous system
42 VO_2	oxygen consumption
43 WT	wild-type

Introduction

The postprandial rise in body temperature, termed the thermic effect of food or diet-induced thermogenesis (DIT), is induced by increased energy expenditure (EE) after ingestion of a meal. This is, together with basal metabolic rate and physical activity EE, one of the components of daily EE (1). Although DIT accounts for only approximately 10% of daily EE, its decline is thought to positively tilt the energy balance and lead to body fat accumulation in the long term (2). DIT is typically divided into two components: obligatory and facultative thermogenesis. Obligatory thermogenesis refers to the obligatory response, including digestion, absorption, and storage of ingested nutrients, whereas facultative thermogenesis refers to the additional responses to obligatory thermogenesis and dissipate excess energy as heat (3).

The magnitude of DIT, especially obligatory thermogenesis, depends not only on the food's energy content but also on its component; proteins 20–30%, carbohydrates 5–10%, lipids 0–3%, and mixed meals about 10% of energy intake (4–6). Although it has been speculated that the potent thermic effect of proteins is due to the consumption of a large amount of energy for digestion, absorption, and metabolism (7), the underlying mechanisms are not fully understood.

Glucagon-like peptide-1 (GLP-1) is a gut hormone secreted from the enteroendocrine L-cells in response to nutrient ingestion (8,9). GLP-1 has various postprandial physiological effects, such as inducing satiety (10,11), suppressing gastric emptying (12), and stimulating glucose-dependent insulin secretion, which is known as the incretin effect (13,14). Because of its beneficial incretin and extrapancreatic effects, GLP-1 receptor (GLP-1R) agonists and dipeptidyl peptidase-4 (DPP-4) inhibitors are widely used for the therapy of type 2 diabetes (15,16).

68 Several previous studies have examined the effect of GLP-1 on thermogenesis.
69 Intravenous (iv) administration of GLP-1 elicited dose-dependent increases in EE and
70 core body temperature in rats (17), and iv infusion of GLP-1 also increased EE in humans
71 (18). Furthermore, intracerebroventricular administration of GLP-1 or GLP-1R agonists
72 promoted thermogenesis in interscapular brown adipose tissue (iBAT) via activation of
73 the sympathetic nervous system (SNS) (19,20). However, the contribution of endogenous
74 (gut-derived) GLP-1 to postprandial thermogenesis remains unclear. Furthermore, recent
75 studies with brown adipose tissue (BAT)-positive or BAT-negative subjects have
76 demonstrated that the contribution of BAT to the DIT response to an energy-balanced diet
77 is only partial (~30%) (21), suggesting a major role of additional primary mechanisms in
78 postprandial thermogenesis, independent of BAT activation.

79 We have recently demonstrated that dietary proteins stimulate GLP-1 secretion
80 more potently than carbohydrates and lipids in rats (22,23); the mechanism may be due
81 to the large contribution of peptides produced by the digestion of dietary proteins (such
82 as whey protein), which simultaneously promote GLP-1 secretion while inhibiting DPP-
83 4 activity. Based on our previous findings that protein intake, a potent inducer of
84 postprandial thermogenesis, strongly promotes GLP-1 secretion, we hypothesized that
85 GLP-1 is involved in the thermic effect of dietary protein.

86 In the present study, to examine this hypothesis, we first examined whether
87 dietary proteins have a potent thermic effect in comparison with carbohydrates and lipids
88 in rats by measuring rectal temperature, a simple and common method for measuring core
89 body temperature. We then investigated the involvement of various gut hormones,
90 including GLP-1, in protein-induced thermogenesis by employing several gut hormone
91 receptor antagonists, GLP-1R knockout (*Glp1r* KO) mice, and separately, treatment with

92 a DPP-4 inhibitor.

93

94 **Materials and Methods**

95 *Animals and diet*

96 Male Sprague-Dawley rats (6 weeks old) from Japan SLC (Hamamatsu, Japan)
97 and male C57BL/6J mice (8 weeks old) from The Jackson Laboratory Japan, Inc.
98 (Yokohama, Japan) were purchased. *Glp1r* KO C57BL/6J mice were generated and
99 provided by Dr. D. J. Drucker at the Lunenfeld Tanenbaum Research Institute (24). The
100 animals were individually housed in a temperature- and humidity-controlled room (22 ±
101 2°C, 55 ± 5%), maintained on a 12 h light-dark cycle (8:00-20:00 light period). Rats had
102 free access to water and were fed AIN-93G diet (25). Mice were available in standard
103 chow (CE-2, CLEA Japan Inc., Tokyo, Japan) and water *ad libitum*. All animal
104 experiments were performed after an acclimatization period of 4-7 days and when animals
105 were sufficiently habituated to the handling and measurement environment. This study
106 was approved by the Hokkaido University Animal Committee and the Institutional
107 Animal Experiment Committee of Kyoto Prefectural University and was carried out in
108 accordance with their Institutional Regulations for Animal Experiments.

109

110 *Materials*

111 Soy protein isolate (soy protein) was purchased from MP Biomedicals (92%
112 protein; 905456; Solon, OH, USA). Whey protein isolate (whey protein, > 90% protein;
113 WPI8855) was obtained from Fonterra. Purified rice endosperm protein (rice protein;
114 78.5% protein) was kindly provided by Kameda Seika Industry Co., Ltd. (Niigata, Japan).
115 Dextrin (TK-16; degree of polymerization: 5.5) was kindly provided by Matsutani

116 Chemical Industry Co. Ltd. (Hyogo, Japan). Soybean oil was kindly provided by J-Oil
117 Mils Inc. (Tokyo, Japan). Propranolol hydrochloride, a nonselective β-adrenergic receptor
118 (βAR) antagonist, was purchased from LKT Laboratories, Inc. (Minneapolis, MN, USA).
119 Exendin (9-39) (Ex9; analytical grade, purity > 95%), a GLP-1R antagonist, and GIP (7-
120 30)-NH_2 (analytical grade, purity > 95%), a glucose-dependent insulinotropic polypeptide
121 (GIP) receptor antagonist, were purchased from Thermo Fisher Scientific Inc.
122 Devazepide (Dvz), a cholecystokinin-A (CCK-A) receptor antagonist, was donated by
123 ML Laboratories (Liverpool, UK), and BIIE 0246 (BIIE), a peptide-YY (PYY) receptor
124 (Y2R) antagonist, was purchased from Tocris Bioscience (Ellisville, MO, USA). Unless
125 otherwise specified, all other reagents were purchased from FUJIFILM Wako Pure
126 Chemical Corporation (Osaka, Japan).
127
128 *Measurement of rectal temperature in rats*
129 The rectal temperatures of rats fasted for 4 or 5 h were measured using a rectal
130 probe (RET-2 rectal probe for rats, Physitemp, Clifton, NJ, USA) connected to a
131 thermocouple thermometer (Delta OHM HD 2128.2 T-type, Caselle di Selvazzano, Italy)
132 before (0 h) and after the oral administration of nutrients. The probe was pre-warmed by
133 exposing it to Vaseline at approximately 34°C maintained by a heating block. The probe
134 was gently inserted into the anus (3 cm). During the measurements, the rats were briefly
135 (within 20 s) and lightly restrained in a cotton glove. Unless otherwise specified, rectal
136 temperature was measured during an awake state and under normal housing conditions
137 (22 ± 2°C).
138
139 *Measurement of rectal temperature in wild-type and Glp1r KO mice*

140 Wild-type (WT; C57BL/6J) and *Glp1r*$^{-/-}$ mice were fasted for 5 h (9:00-14:00).
141 Basal rectal temperature was measured using a thermocouple probe (RET-3 rectal probe
142 for mice, Physitemp, Clifton, NJ, USA) connected to a microprobe thermometer (BAT-
143 12, Physitemp). Following the oral administration of soy or whey protein solution (2 g/kg,
144 20 mL/kg) or saline (control, 20 mL/kg), rectal temperature was measured at 1, 2, and 3
145 h after administration. Rectal temperature was measured in conscious mice and normal
146 housing conditions (22 ± 2°C).
147
148 *Postprandial rectal temperature measurement in rats*
149 The rectal temperatures of the rats (0 h) were measured after fasting for 5 h
150 (9:00–14:00). Then, the fasting group continued fasting, and the refeeding group had *ad*
151 *libitum* access to the chow (AIN-93G). Food intake and rectal temperature were measured
152 at 1, 2, and 3 h after refeeding.
153
154 *Rectal temperature measurements in rats under oral gavage of macronutrients*
155 After fasting for 4 h (9:00-13:00), basal rectal temperatures (0 h) were measured.
156 Then rats received oral gavage (15 mL/kg body weight) of test liquids (isocaloric protein,
157 carbohydrate, and lipid) using a 6 Fr feeding tube (Atom Medical, Tokyo, Japan). As a
158 representative of proteins and carbohydrates, casein sodium and dextrin (both at 2 g/kg =
159 33·5 kJ (8 kcal)/kg) were dissolved in water, respectively. As a representative of lipids,
160 soybean oil (0.89 g/kg = 33·5 kJ (8 kcal)/kg) was suspended in a 1.5% carboxymethyl
161 cellulose solution. Rectal temperatures were measured at 0.5, 1, 2, and 3 h after oral
162 administration.
163

164 *Rectal temperature measurements in rats under oral gavage of various proteins*

165 Soy protein, casein, whey protein, rice protein, and egg albumin were each
166 dissolved in water (2 g/15 mL/kg) as test solutions for oral administration. In a separate
167 experiment, the rats received different oral protein doses (1 or 2 g/kg). The control group
168 received the vehicle (water, 15 mL/kg). The experimental procedure was the same as that
169 described above.

170

171 *Nonselective βAR antagonist treatment in rats*

172 Rats fasted for 4 h received an intraperitoneal injection (1 mL/kg) of propranolol
173 solution (dissolved in saline and dosed at 3 mg/kg (26)) as a nonselective βAR antagonist
174 or vehicle (saline) immediately after oral administration (15 mL/kg) of soy protein
175 solution (2 g/kg) or vehicle (water). In a separate experiment, rats fasted for 4 h received
176 a subcutaneous injection (1 mL/kg) of L-norepinephrine hydrochloride (norepinephrine;
177 Sigma-Aldrich, St. Louis, MO, USA; dissolved in saline and dosed at 0.3 mg/kg) as a
178 BAT activator (27,28). Immediately after the subcutaneous administration, the rats
179 received an intraperitoneal injection (1 mL/kg) of propranolol (3 mg/kg) or vehicle
180 (saline). The rectal temperature was measured as described above.

181

182 *Measurement of skin surface temperature on iBAT of rats by infrared thermography*

183 The interscapular area of the rats was shaved (20) under isoflurane anesthesia
184 the day before the test. Infrared videos were captured with a thermal camera (FLIR ONE
185 PRO) on freely moving rats to minimize stress (28,29). After fasting for 3-4 h (9:00 to
186 12:00-13:00), infrared videos of rats were taken for 3 min with a mode that displays the
187 warmest point in view. The maximum skin surface temperature (highest temperature

188 measured in one 3-min session) of the interscapular area—that is, between the shoulder
189 blades where the iBAT is located underneath—was determined using FLIR ONE Version
190 4.4.0 and defined as the baseline temperature. From 13:00 onwards, rats were
191 subcutaneously injected (1 mL/kg) with saline or norepinephrine (dissolved in saline and
192 dosed at 1 mg/kg) to confirm that the iBAT temperature change was accurately measured.
193 Skin temperatures were measured for 1 min at 0.5, 1, 2, and 3 h after administration, and
194 the maximum iBAT surface temperature in one 1-min session was determined as
195 described above.

196 In a separate experiment, rats were fasted for 4 h, after which they received an
197 oral gavage (15 mL/kg) of the vehicle (water) or soy protein solution (2 g/kg), and the
198 interscapular skin temperatures were measured as described above.

199

200 *Measurement of tail skin surface temperature of rats by infrared thermography*

201 Infrared images were taken with a thermal camera (FLIR ONE PRO) on freely
202 moving rats to minimize stress. After fasting for 3-4 h (9:00 to 12:00-13:00), infrared
203 images of skin temperatures were taken in a room at an ambient temperature of 22°C. The
204 maximum surface temperature of the hairless tail was determined using FLIR ONE
205 Version 4.4.0 and defined as the baseline tail skin surface temperature. Then, at 13:00,
206 one group of rats was placed in a room at 18°C, while the other group was placed in a
207 room at 22°C. Skin temperature was measured after 0.5 h, and the maximum tail surface
208 temperature was determined as described above.

209 In a separate experiment, rats were fasted for 4 h and received an oral gavage (15
210 mL/kg) of vehicle (water) or soy protein solution (2 g/kg). Skin temperatures under
211 normal housing conditions (22 ± 2°C) were measured at 0.5, 1, 2, and 3 h after oral

212 administration, and the maximum tail skin surface temperature was determined as
213 described above.
214
215 *Various gut hormone receptor antagonist treatments in rats*
216 Rectal temperature was measured in rats treated with various gut hormone
217 receptor antagonists. First, after fasting for 4 h (9:00-13:00), the basal rectal temperatures
218 (0 h) were measured. The rats then received an oral gavage (15 mL/kg) of vehicle (water)
219 or soy protein solution (2 g/kg). Immediately after oral administration, the rats received
220 an intraperitoneal injection (1 mL/kg) of an antagonist solution or vehicle. Ex9, a GLP-
221 1R antagonist, was dissolved in saline and administered at 30 nmol/kg (100 μg/kg)
222 (30,31). GIP (7-30)-NH_2, a GIP receptor antagonist, was dissolved in saline containing
223 7% DMSO and dosed at 6.9 nmol/kg (20 μg/kg) (32). Dvz, a CCK-A receptor antagonist,
224 was dissolved in saline containing 5% DMSO and 5% Tween 80 and dosed at 222
225 μmol/kg (0.5 mg/kg) (33,34). BIIE, a PYY receptor (Y2R) antagonist, was dissolved in
226 saline containing 7% DMSO and administered at 653 nmol/kg (0.62 mg/kg) (35). Rectal
227 temperatures were measured at 0.5, 1, 2, and 3 h after oral administration. All experiments
228 were conducted separately.
229
230 *Measurements of GLP-1 concentrations in portal vein plasma of rats*
231 After fasting for 4 h (9:00-13:00), basal rectal temperatures (0 h) were measured.
232 The rats received an oral gavage (15 mL/kg) of vehicle (water) or soy protein solution (2
233 g/kg). Immediately after oral administration, the rats received an intraperitoneal injection
234 (1 mL/kg) of Ex9 solution (dissolved in saline and dosed at 30 nmol/kg) or its vehicle
235 (saline). Rectal temperatures were measured at 0.5 and 1 h after oral administration in the

236 awake state. Then, the blood samples were collected from the portal vein under sodium
237 pentobarbital anesthesia (50 mg/kg). Immediately after the procedure, rats were
238 euthanized by exsanguination. The sampling syringe contained heparin (final
239 concentration, 50 IU/mL), aprotinin (final concentration, 500 KIU/mL), and DPP-4
240 inhibitor (final concentration, 50 μM; Millipore, MA, U.S.A.). Plasma was collected after
241 centrifugation (2300 × *g*, 10 min at 4°C) and stored at −80°C until analysis.
242 Active GLP-1 concentrations in plasma were measured using an ELISA kit
243 (Catalog No. 27700, RRID: AB_2892225, Immuno-Biological Laboratories Co., Ltd.).
244
245 *Measurement of oxygen consumption and physical activity*
246 Oxygen consumption (VO_2) was measured (36,37) using an O_2 metabolism
247 measuring system (model MM102R; Muromachikikai, Tokyo, Japan) in a transparent
248 chamber (230 mm (width) × 320 mm (depth) × 250 mm (height)) with an airflow of 2.4-
249 2.5 L/min. The VO_2 of each rat was measured for 1 min at 3-min intervals from 4 h before
250 to 3 h after oral administration of the test solutions. Cumulative VO_2 over 0.5 h was
251 calculated and expressed as mL O_2 per 0.75 power of kg body weight (17,38). Each rat
252 was acclimatized to the chamber overnight before measurements were started. After
253 fasting for 4 h (9:00-13:00), the rats received an oral gavage (15 mL/kg) of the vehicle
254 (water) or soy protein suspension (2 g/kg). The rats had free access to water during
255 measurements. Simultaneously, the physical activity of each rat was continuously
256 recorded using a video recording system (digital video camera NV-GS50K and DVD
257 video recorder DMR-E90H; Panasonic, Osaka, Japan) and assessed later. The video
258 records were checked for locomotion, rearing, grooming, and drinking. If each of these
259 behaviors was observed during a 3-min period, each was scored as one count of physical

260 activity. The cumulative physical activity for 0.5 h was calculated, giving a maximum
261 possible score over 0.5 h of 40 counts.

262 The difference in thermogenesis between pre- and post-administration [ΔVO_2
263 (thermogenesis)] was calculated as the DIT based on previous reports (36,37). In brief,
264 using the correlation between the total VO_2 and physical activity from the data of fasted
265 rats, the VO_2 dependent on physical activity [VO_2 (activity)] was calculated. Activity-
266 independent VO_2 pre- and post-administration was obtained as VO_2 (activity-
267 independent) = VO_2 – VO_2 (activity). Finally, ΔVO_2 (thermogenesis) was calculated by
268 subtracting pre-administration VO_2 (activity-independent) from post-administration VO_2
269 (activity-independent).

270 In a separate experiment, the rats received an oral gavage (15 mL/kg) of soy
271 protein suspended in water (2 g/kg). Immediately after oral administration, the rats
272 received an intraperitoneal injection (1 mL/kg) of Ex9 (30 nmol/kg) or its vehicle (saline).
273 VO_2 and physical activity were measured and ΔVO_2 (thermogenesis) was estimated as
274 described above.

275

276 *Measurements of GLP-1 concentrations in portal vein plasma in WT and Glp1r$^{-/-}$ mice*

277 C57BL/6J and *Glp1r* KO mice were fasted for 5 h (9:00-14:00). Blood was
278 collected from the portal vein under isoflurane anesthesia 1 h after oral administration of
279 soy protein solution (2 g/20 mL/kg) or saline (20 mL/kg). The sampling syringe contained
280 heparin, aprotinin, and the DPP-4 inhibitor, vildagliptin (final concentrations, 50 IU/mL,
281 500 KIU/mL, and 10 μM, respectively). Plasma was collected after centrifugation and
282 stored at –80°C. Total GLP-1 was measured using an ELISA kit (Catalog No. EZGLP1T-
283 36K, RRID: AB_2813786, Millipore).

284

285 *Measurements of GLP-1 and insulin concentrations in tail vein plasma of rats treated*
286 *with or without DPP-4 inhibitor*

287 Sitagliptin phosphate (sitagliptin; Sigma-Aldrich, St. Louis, MO, USA) was used
288 as an orally effective DPP-4 inhibitor. First, after fasting for 2 h (9:00-11:00), sitagliptin
289 (50 mg/kg dissolved in water) or water (5 mL/kg) was orally administered at –2 h using
290 a 6Fr feeding tube. Soy protein solution (2 g/kg) or water (control) was administered
291 orally (15 mL/kg) at 0 h. Blood samples were collected from the tail vein at –2, –1, 0, 0.5,
292 1, 2, and 3 h and immediately transferred to chilled tubes containing heparin (final
293 concentration, 50 IU/mL), aprotinin (final concentration, 500 KIU/mL), and DPP-4
294 inhibitor (final concentration, 50 μM). Plasma was collected and stored as described
295 above.

296 Active GLP-1 concentrations in plasma were measured using the ELISA kit
297 described above. Plasma insulin concentrations were measured using a Rat Insulin ELISA
298 kit (U-E type, AKRIN-130, RRID: AB_2933972, Shibayagi Company Limited). Since
299 plasma DPP-4 activity was 20-50% suppressed at 1-4 h after sitagliptin treatment in our
300 previous study (22), the test solutions were orally administered 2 h after oral sitagliptin
301 treatment.

302

303 *Rectal temperature measurements in rats treated with or without DPP-4 inhibitor and*
304 *oral gavage of nutrients*

305 After fasting for 2 h (9:00-11:00), rectal temperature was measured, and
306 sitagliptin (50 mg/kg dissolved in water) or water (5 mL/kg) was administered orally (–2
307 h). Rectal temperatures were measured 1 and 2 h after oral administration (–1 and 0 h).

308 The test solutions (water or isocaloric protein, carbohydrate, and lipid, as described
309 above) were orally administered (15 mL/kg) at 0 h, and rectal temperatures were
310 measured at 0.5, 1, 2, and 3 h after the oral administration of nutrients.
311
312 *Statistical analyses*
313 Data are expressed as mean ± SEM. Statistical analyses were performed using
314 JMP Pro version.16.1.0 software (SAS Institute, Inc., Cary, NC, USA). Statistical
315 significance was assessed using one-way or two-way ANOVA, or a mixed model with
316 unstructured covariance. Statistical differences between mean values were determined by
317 Dunnett's test, Student's *t*-test, paired *t*-test, Tukey Kramer's test, and Bonferroni's test,
318 as appropriate, as described in the figure legends. In all analyses, $p < 0.05$ was considered
319 statistically significant.
320
321 **Results**
322 *Postprandial changes in rectal temperature of rats fed the AIN-93G diet*
323 We first examined the feeding-induced changes in rectal temperature. Rectal
324 temperatures at 0 h (basal state) were 36.08 ± 0.10°C in the fasting group and 35.86 ±
325 0.05°C in the refeeding group; therefore, results are presented as changes from the basal
326 state (ΔRectal temperature). Rats consumed the AIN-93G diet (0-1 h, 1.77 ± 0.21 g; 1-2
327 h, 0.80 ± 0.22 g; 2-3 h, 0.63 ± 0.28 g), and displayed significantly increased rectal
328 temperature (~0.4 °C), and the elevation was maintained for up to 3 h (Fig. 1A). In
329 contrast, in the fasting group, the rectal temperature did not change. The AUC (ΔAUC)
330 at 0-3 h was significantly higher in the refeeding group (Fig. 1B).
331

332 *Effects of oral administration of macronutrients on the rectal temperature in rats*

333 We next compared rectal temperature changes in response to oral administration
334 of various nutrients. Among the isocaloric nutrients (casein, dextrin, and soybean oil),
335 only casein significantly increased the rectal temperature at all time points (Fig. 1C), and
336 its ΔAUC was significantly higher than that of dextrin and soybean oil (Fig. 1D).

337

338 *Effects of various proteins on the rectal temperature in rats*

339 All five dietary proteins examined (casein, whey, rice, egg, and soy) increased
340 rectal temperature by approximately 0.3-0.4°C at 0.5 h after oral administration (Fig. 2A).
341 Subsequently, the elevated rectal temperature gradually decreased in rice protein-
342 administered rats, while the elevated rectal temperature was maintained in whey protein-
343 treated rats until 3 h after oral administration. For casein, egg albumin, and soy protein,
344 the rectal temperature peaked at 1 h after oral administration; thereafter, the temperature
345 gradually decreased after casein and egg albumin administration yet was maintained for
346 ~3 hrs after gavage with soy protein. As shown by the ΔAUC (Fig. 2B), soy protein had
347 the highest thermic effect.

348 In a separate experiment, soy protein at 1 g/kg and 2 g/kg increased rectal
349 temperature and its ΔAUC in a dose-dependent manner (Fig. 2C, D). Dose-dependent
350 responses were also observed for the other four proteins (Supplementary Fig. 1) (39).
351 Because of its potent effect on elevating the rectal temperature, we used a 2 g/kg dose of
352 soy protein in further experiments.

353

354 *Effect of nonselective βAR antagonism on the soy protein-induced rectal temperature*
355 *increase in rats*

356 To examine the involvement of BAT in soy protein-induced increase in rectal
357 temperature, we used the nonselective βAR antagonist, propranolol. Soy protein
358 increased the rectal temperature in the presence or absence of propranolol (Fig. 3A, B);
359 propranolol treatment itself did not affect rectal temperature (Fig. 3C, D). Intraperitoneal
360 propranolol treatment adequately suppressed the norepinephrine-induced increase in
361 rectal temperature (Supplementary Fig. 2) (39).
362 We further investigated the involvement of BAT using an infrared camera to
363 measure skin surface temperature in iBAT. The iBAT surface temperature significantly
364 increased (~1.0 °C), peaking at 1 h after subcutaneous administration of norepinephrine
365 (Supplementary Fig. 3) (39), indicating that our experimental method was compatible
366 with a previously reported method (28). On the other hand, oral administration of soy
367 protein did not increase iBAT surface temperature (Fig. 3E, Supplementary Fig. 4) (39).
368
369 *Effect of oral administration of soy protein on the tail skin surface temperature of rats*
370 In rats, a cold environment causes vasoconstriction of the tail skin, preventing
371 heat loss and maintaining a constant body temperature; sympathetic nerve-ending
372 noradrenaline activity is partly involved in this response (40,41). In the present study,
373 when rats were moved from the 22°C room to the 18°C room for 0.5 h, the tail skin surface
374 temperature was decreased by 5.13 ± 0.70°C (Supplementary Fig. 5) (39), likely
375 reflecting the vasoconstriction of the tail skin. We next examined whether the soy protein-
376 induced increase in rectal temperature consequently occurred through the suppression of
377 heat loss associated with vasoconstriction. As shown in Fig. 3F and Supplementary Fig.
378 6 (39), the tail skin surface temperature remained in the range of 27.5-29.5°C and did not
379 decrease from the baseline level (27.47 ± 0.37°C) after oral administration of soy protein.

380

381 *Involvement of gut hormones in the soy protein-induced increase in rat rectal temperature*

382 To assess the possible involvement of gut hormones in soy protein-induced
383 increase in rectal temperature, we used various gut hormone receptor antagonists. Rats
384 receiving oral administration of soy protein and an intraperitoneal injection of saline
385 significantly increased rectal temperature at 0.5-2 h and its ΔAUC during 0-3 h; however,
386 this effect of soy protein was completely abolished by treatment with Ex9, a GLP-1R
387 antagonist (Fig. 4A, B). Ex9 treatment itself did not affect rectal temperature. In contrast,
388 administration of antagonists directed against GIP, CCK-A, or PYY receptor (GIP (7-30)-
389 NH_2, Dvz, or BIIE) did not attenuate soy protein-induced increase in rectal temperature
390 (Fig. 4C-H); in addition, these antagonist treatments did not independently impact rectal
391 temperature, as in the control/vehicle group.

392

393 *Effect of soy protein on GLP-1 secretion in rats*

394 Similar to the results shown in Fig. 4, Ex9 intraperitoneal treatment counteracted
395 the increase in rectal temperature at 0.5 and 1 h and its ΔAUC during 0-1 h after oral
396 administration of soy protein (Fig. 5A, B). Consistent with a role for GLP-1R signaling,
397 active GLP-1 concentrations in the portal plasma were increased at 1 h after oral
398 administration of soy protein with or without Ex9 treatment ($p < 0.01$, two-way ANOVA;
399 Fig. 5C).

400

401 *Effects of oral administration of soy protein on VO_2 and involvement of GLP-1 signaling*
402 *in rats*

403 To investigate whether soy protein increases EE, VO_2 and physical activity were

404 measured. Although the difference was not significant, oral administration of soy protein
405 tended to suppress physical activity (Supplementary Fig. 7A) (39). As shown in
406 Supplementary Fig. 7B (39), VO_2 increased with increased physical activity, giving a
407 regression line with a positive correlation in fasted rats ($y = 9.5703x + 445.45$, $n = 80$, R^2
408 $= 0.83$, $p < 0.01$). Using this regression line, the calculated ΔVO_2 (thermogenesis), as
409 described above, was significantly higher at 1, 2, and 3 h after oral administration of soy
410 protein compared to that in the control group (Fig. 6 A), and a significant effect of soy
411 protein was detected by the mixed model analysis ($p < 0.05$). In addition, the AUC for
412 changes in VO_2 (thermogenesis) during 0-3 h in the soy protein group was significantly
413 higher than in the control group (Fig. 6B).

414 Next, we examined the involvement of GLP-1 signaling in soy protein-induced
415 increases in EE. Physical activity did not differ significantly with or without Ex9
416 treatment (Supplementary Fig. 7C) (39). ΔVO_2 (thermogenesis) was calculated based on
417 the regression line with a positive correlation in fasted rats ($y = 9.8341x + 412.85$, $n = 77$,
418 $R^2 = 0.54$, $p < 0.01$; Supplementary Fig. 7D) (39); Ex9 treatment diminished the soy
419 protein-induced VO_2 increase ($p < 0.05$ for the effect of Ex9 by mixed model; Fig. 6C).
420 Although the difference was not significant ($p = 0.12$, paired *t*-test, Fig. 6D), the ΔAUC
421 was lower in the Ex9-treated group.

422

423 *Effects of oral administration of soy protein on the rectal temperature in Glp1r KO mice*

424 To further explore the involvement of GLP-1 signaling in the rectal temperature-
425 increasing effect of soy protein, we employed *Glp1r* KO mice. In WT mice (Fig. 7A, C),
426 oral administration of soy protein at 2 g/kg significantly increased rectal temperature at 1
427 and 2 h, and its ΔAUC was significantly higher than that of the control group. In contrast,

428 oral administration of soy protein failed to increase the rectal temperature at any time
429 point in *Glp1r* KO mice (Fig. 7B), and its ΔAUC did not differ from that of the control
430 group (Fig. 7D). Total GLP-1 concentrations in the portal plasma were significantly
431 higher 1 h after oral administration of soy protein in both WT and *Glp1r* KO mice (Fig.
432 7E, F).

433 To examine whether GLP-1 signaling is also involved in rectal temperature
434 increases induced by other proteins, we performed similar experiments using whey
435 protein. Oral administration of whey protein at 2 g/kg did not increase the rectal
436 temperature at any time point in *Glp1r* KO mice (Supplementary Fig. 8B, D) (39), while
437 it increased rectal temperature at 1 and 2 h in WT mice (Supplementary Fig. 8A, C) (39).
438

439 *Effect of DPP-4 inhibitor treatment on the effects of soy protein in rats*

440 The results from Ex9-treated rats and *Glp1r* KO mice prompted us to explore the
441 possibility that increases in endogenous GLP-1 would potentiate the soy protein-induced
442 increase in rectal temperature. First, we examined the effects of sitagliptin, an orally
443 administered DPP-4 inhibitor, on plasma GLP-1 concentrations. In rats without sitagliptin
444 treatment, plasma GLP-1 concentrations were significantly increased at 0.5, 1, and 2 h
445 after oral administration of soy protein, compared to the basal (0 h) value (Fig. 8A).

446 When rats were pretreated with sitagliptin, plasma GLP-1 levels were elevated
447 before the nutrient challenge (Fig. 8A), consistent with a reduction in plasma DPP-4
448 activity as demonstrated in our previous study (22). After oral administration of soy
449 protein, plasma GLP-1 concentrations were significantly increased at 0.5 h compared
450 with the basal value (0 h); GLP-1 concentrations at –1-3 h, except at 2 h, in sitagliptin-
451 pretreated soy protein groups were significantly higher than in the sitagliptin-untreated

452 soy protein group. Oral administration of soy protein did not stimulate insulin secretion,
453 with or without sitagliptin treatment (Supplementary Fig. 9) (39).

454 We next examined the effects of sitagliptin treatment on the soy protein-induced
455 increase in rectal temperature. Rats pretreated with sitagliptin had higher rectal
456 temperature responses to soy protein than rats without sitagliptin treatment ($p < 0.05$ for
457 the effect of sitagliptin by mixed model, Fig. 8B). In addition, the AUC for changes in
458 rectal temperature during 0-3 h in the sitagliptin treatment group was higher than in the
459 sitagliptin untreated group (Fig. 8C). There were no significant differences in rectal
460 temperature before soy protein administration with or without sitagliptin treatment.

461 We further investigated the effects of sitagliptin on rectal temperature changes
462 in response to carbohydrates (dextrin) and lipids (soybean oil). Rectal temperature
463 significantly increased under sitagliptin treatment at 0.5 h after oral dextrin administration
464 compared to basal values (Fig. 8D); however, there were no significant differences
465 between the groups (with or without sitagliptin treatment) at any time point and in the
466 ΔAUC during 0-3 h (Fig. 8E). Furthermore, soybean oil did not increase the rectal
467 temperature with or without sitagliptin treatment (Fig. 8F, G). Nor did sitagliptin
468 treatment itself affect rectal temperature (Fig. 8H, I).

469

470 **Discussion**

471 Although dietary proteins induce potent thermic effects (4–6), the underlying
472 mechanisms have not been clarified. In the present study, we examined whether a gut
473 hormone, GLP-1, whose secretion is strongly stimulated by protein intake, or other
474 representative gut hormones (GIP, CCK, and PYY) are involved in the thermic effects of
475 dietary proteins. Notably, we found that oral administration of soy protein (2 g/kg)

476 increased rectal temperature and EE via GLP-1 signaling. These results propose a novel
477 physiological role of GLP-1 and contribute to a better understanding of the potent thermic
478 effects of dietary proteins.

479 Consistent with a previous study (42), refeeding significantly increased rectal
480 temperatures in rats (Fig. 1A, B); the AIN-93G diet contains 0.2 g casein/g (25), and rats
481 (250-320 g) consumed about 3.2 g diet (= 2-2.56 g casein/kg BW) in 3 h. Almost
482 equivalent doses (2 g/kg) of casein, but not dextrin or soybean oil, increased the rectal
483 temperature (Fig. 1C, D), confirming the potent thermic effect of protein, similar to
484 previous studies (4–6). Although rectal temperature fluctuations differed (Fig. 1A, C),
485 these results suggested that casein had a major contribution to the thermic effect of AIN-
486 93G ingestion.

487 Among the five dietary proteins examined (casein, whey, rice, egg, and soy), soy
488 protein had the highest thermic effect (Fig. 2A, B). In a previous human study, Acheson
489 et al. (43) found a greater thermic effect after the consumption of a meal containing whey
490 (50% protein) than after casein and soy protein meals (50% protein, respectively). In
491 contrast, Alfenas et al. (44) observed no significant difference in DIT after breakfast meals
492 containing casein, whey, or soy protein. It should be noted that these previous studies
493 employed protein-containing mixed meals rather than a single administration of protein.
494 Further studies are warranted to elucidate the reasons for the differences in the thermic
495 effects among dietary proteins. However, to our knowledge, the present study is the first
496 to compare the thermic effects of a single administration of as many as five different
497 dietary proteins in rats.

498 Previous reports in rodents and humans have suggested that activation of the
499 SNS-BAT axis is higher in a high-carbohydrate diet and lower in a high-protein and high-

500 lipid diet (45–48). Consistent with these reports, propranolol, a nonselective βAR
501 antagonist, treatment did not affect soy protein-induced increase in rectal temperature
502 (Fig. 3A, B). Alternatively, secretin and bile acids have been reported to increase BAT
503 thermogenesis independently of the βAR signaling pathway (49,50). However, soy
504 protein did not increase the iBAT temperature (Fig. 3E). A recent study (51) showed no
505 difference in the DIT after high-protein diet ingestion in humans with high vs. low BAT
506 activity. This report further supports the likelihood that BAT is not meaningfully involved
507 in the soy protein-induced increase in rectal temperature. Another paper (52) reported that
508 habitual animal protein intake was positively associated, whereas plant protein intake was
509 negatively correlated with cold-induced BAT activity in humans. Thus, dietary proteins
510 may chronically affect adaptive changes in BAT activity.

511 The tail skin surface temperature did not decrease after oral administration of
512 soy protein (Fig. 3F), suggesting that the soy protein-induced increase in rectal
513 temperature was not due to the suppression of heat loss associated with vasoconstriction
514 but due to thermogenesis.

515 Oral soy protein increased active GLP-1 concentrations in the portal plasma of
516 rats treated with or without the GLP-1R antagonist, Ex9 (Fig. 5C), and treatment with
517 Ex9 completely counteracted the thermic effect of soy protein (Fig. 4A, B, and 5A, B),
518 implicating the involvement of GLP-1 signaling. These results, including Fig. 3F, are
519 partly supported by a previous finding that iv GLP-1 increases body temperature in rats
520 without causing tail skin vasoconstriction (17). On the other hand, GIP, CCK-A, and PYY
521 receptor antagonism did not affect soy protein-induced increases in rectal temperature
522 (Fig. 4C-H). A previous study demonstrated negative correlations between postprandial
523 GIP levels and thermogenic efficiency (53), and GIP secretion is stimulated preferentially

524 by dietary carbohydrates and lipids (54,55). These findings are consistent with the potent
525 thermic effects of dietary proteins independent of GIP. CCK reportedly activates BAT
526 thermogenesis (56,57); however, soy protein did not increase BAT activation (Fig. 3A-E),
527 and the results using a CCK receptor antagonist (Fig. 4E, F) suggest that CCK signaling
528 was not involved in soy protein-induced increase in rectal temperature. Although some
529 reports link PYY to DIT (53,58), the present study suggests that PYY is not involved in
530 the thermic effect of soy protein (Fig. 4G, H). This heterogeneity in the data may reflect
531 differences in experimental design, including the use of mixed meals and species
532 differences between rats and humans.

533 Oral administration of soy protein increased EE, as demonstrated by the
534 measurement of O_2 consumption (Fig. 6A, B), but was not associated with increased
535 physical activity (Supplementary Fig. 7A) (39). Consistent with rectal temperature
536 measurement experiments, soy protein-induced increase in EE [ΔVO_2 (thermogenesis)]
537 was counteracted by Ex9 treatment (Fig. 6C, D) (39). These results further support the
538 notion that soy protein increases DIT via GLP-1 signaling. In humans, iv administration
539 of GLP-1 increased EE, which was likely mediated by insulin (18). However, in the
540 current studies, oral soy protein did not promote insulin secretion (Supplementary Fig. 9)
541 (39), suggesting that soy protein increases DIT independently of insulin signaling.
542 Furthermore, it has been shown that central GLP-1R activation by intracerebroventricular
543 administration of GLP-1 or GLP-1R agonist increased EE (17,59), probably via
544 subsequent activation of the SNS-BAT axis (19,20). However, gut-derived GLP-1 is
545 unlikely to directly activate the hypothalamic GLP-1R because of its rapid inactivation
546 by DPP-4 (60). Although intestinal GLP-1 might act on vagal afferent nerves, which then
547 triggers activation of the SNS-BAT axis, the lack of contribution of BAT to the thermic

548 effect of oral soy protein (Fig. 3A-E) suggests a difference in the underlying mechanisms.

549 The involvement of GLP-1 signaling in soy protein-induced thermogenesis was
550 further demonstrated in experiments employing *Glp1r* KO mice (Fig. 7). Notably, the oral
551 whey protein-induced increase in rectal temperature observed in WT mice was
552 completely abolished in *Glp1r* KO mice (Supplementary Fig. 8) (39). Taken together,
553 these results collectively suggest that GLP-1 signaling is essential for the full thermic
554 effects of dietary proteins.

555 A previous study reported an increase in EE and a relative reduction in body
556 weight gain in high-fat diet-fed mice lacking the DPP-4 gene (61). Similar to our recent
557 study (22), sitagliptin pretreatment effectively increased plasma GLP-1 concentrations
558 before and after the oral administration of soy protein (Fig. 8A). Sitagliptin pretreatment
559 also enhanced soy protein-induced increases in rectal temperature (Fig. 8B, C).
560 Conversely, compared to soy protein, the effect of sitagliptin treatment was limited,
561 transient, and not statistically significant when dextrin was orally administered (Fig. 8D,
562 E). Moreover, oral soybean oil and sitagliptin treatment did not further increase rectal
563 temperature (Fig. 8F-I). These results indicate that GLP-1 is important for the increase in
564 body temperature and EE ensuing from protein, but not carbohydrates or lipids ingestion.

565 A limitation of the current study is that the tissue(s) contributing to soy-induced
566 and GLP-1R-dependent DIT was not identified. Some previous studies have
567 demonstrated that iv administration of an amino acid (AA) mixture increased rectal
568 temperature in awake rats (62), and increased EE in rats was greater with oral
569 administration than with iv administration of the AA mixture (63). In addition, an *in vitro*
570 previous study demonstrated the thermogenic effect of GLP-1R agonist in muscle cells
571 (64). Accordingly, GLP-1 may enhance body temperature and EE by increasing the

572 availability of AAs and their metabolism, for example, in intestinal epithelial cells, liver,
573 and skeletal muscle. It will be important to interrogate these possibilities and elucidate
574 the mechanism(s), such as how and where GLP-1 signaling in one or more sites augments
575 thermogenesis.

576 In conclusion, we confirmed that dietary proteins have potent thermic effects
577 compared to carbohydrates and lipids in rats, as assessed by measuring rectal temperature.
578 Soy protein had an especially potent thermic effect and increased EE. Importantly, we
579 found that GLP-1 signaling mediates the thermic effects of dietary proteins. These
580 findings provide novel insights into the underlying mechanisms of the thermic effects of
581 dietary proteins, which have long remained ambiguous, and new directions for studies
582 linking macronutrient ingestion to DIT, physiological processes with potential
583 translational relevance.

584

585 **Author Contributions**

586 K.O. and T.H. conceived and designed the study; K.O., A.M., Y.D., and B.S.S.
587 performed the experiments; D.J.D. provided KO mice; K.O., A.M., B.S.S., Y.I., Y.O.O.,
588 and T.H. analyzed the data; K.O. and T.H. wrote, and K.O., Y.I., Y.O.O., D.J.D., and T.H
589 revised the manuscript, and all the authors contributed and approved the final draft.

590

591 **Data Availability**

592 Some or all datasets generated during and/or analyzed during the current study
593 are not publicly available but are available from the corresponding author on reasonable
594 request.

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Figure legends

Fig. 1. Feeding and protein administration increase the rectal temperature in rats. (A) Changes in rectal temperature (ΔRectal temperature, n = 6) after refeeding or continued fasting in 5 h-fasted rats and (B) its AUC (ΔAUC). (C) Changes in rectal temperature after oral administration of isocaloric casein (2 g/kg), dextrin (2 g/kg), or soybean oil (0.89 g/kg) solution in 4 h-fasted rats and (D) its AUC (n = 6). Values are expressed as the mean ± SEM. In (A, C), plots with asterisks (*) show significant differences compared with 0 h values within each treatment ($^{*}p < 0.05$ and $^{**}p < 0.01$, Dunnett's test). # symbols and different letters indicate significant differences between the treatments (A and B, crossover design, $^{\#\#}p < 0.01$, paired t-test; C and D, crossover design, $p < 0.05$, Bonferroni's test).

Fig. 2. Among dietary proteins, rectal temperature is increased in a dose-dependent manner and to the greatest extent by soy protein. (A) Changes in rectal temperatures after oral administration of different dietary proteins (2 g/15 mL/kg) in 4 h-fasted rats and (B) its AUC (n = 5-6). (C) Changes in rectal temperatures after oral administration of different soy protein doses (1 or 2 g/kg) or water (control, 15 mL/kg) in 4 h-fasted rats and (D) its AUC (n = 8-9). Values are expressed as the mean ± SEM. Different letters indicate $p < 0.05$ between treatments by Tukey's test. The p values of the mixed model with unstructured covariance are shown in each panel (A, C). In (C), plots with asterisks (*) show significant differences compared with 0 h values within each treatment ($^{**}p < 0.01$, Dunnett's test).

Fig. 3. BAT activation and heat loss from the tail skin surface are not involved in the

thermic effect of soy protein. (A-D) Propranolol (3 mg/kg) or its vehicle (saline) was intraperitoneally injected at a dose of 1 mL/kg immediately after oral administration of (A, B) soy protein (2 g/kg) or (C, D) water (control, 15 mL/kg) in 4 h-fasted rats. (A, C) Changes in rectal temperatures and (B, D) its AUC (n = 5). (E) iBAT or (F) tail skin surface temperatures of 4 h-fasted rats before and after oral administration of water (control, 15 mL/kg) or 2 g/kg soy protein (E, n = 5-6; F, n = 6). Values are expressed as the mean ± SEM. Plots with asterisks (*) show significant differences compared with 0 h (baseline) values within each treatment ($^{*}p < 0.05$ and $^{**}p < 0.01$, Dunnett's test), and comparisons between treatments were performed using Student's t-test. The p values of the mixed model with unstructured covariance are shown in each panel (A, C, E, F).

Fig. 4. GLP-1 receptor antagonist abolishes soy protein-induced increase in rectal temperature. Various receptor antagonists (A, B, 30 nmol/kg Ex9; C, D, 6.9 nmol/kg GIP (7-30)-NH_2; E, F, 222 μmol/kg Dvz; G, H, 653 nmol/kg BIIE) or their vehicle was intraperitoneally injected at a dose of 1 mL/kg immediately after oral administration of soy protein (2 g/kg) or water (control, 15 mL/kg) in 4 h-fasted rats. (A, C, E, G) Changes in rectal temperatures and (B, D, F, H) its AUC (n = 6-7, respectively). Values are expressed as the mean ± SEM. Plots with asterisks (*) show significant differences compared with 0 h values within each treatment ($^{*}p < 0.05$ and $^{**}p < 0.01$, Dunnett's test). Different letters indicate $p < 0.05$ between treatments by Tukey's test. The p values of two-way ANOVA are shown in each panel (B, D, F, H).

Fig. 5. Oral soy protein stimulates GLP-1 secretion with or without Ex9 treatment. Ex9 (30 nmol/kg) or its vehicle (saline) was intraperitoneally injected at a dose of 1 mL/kg

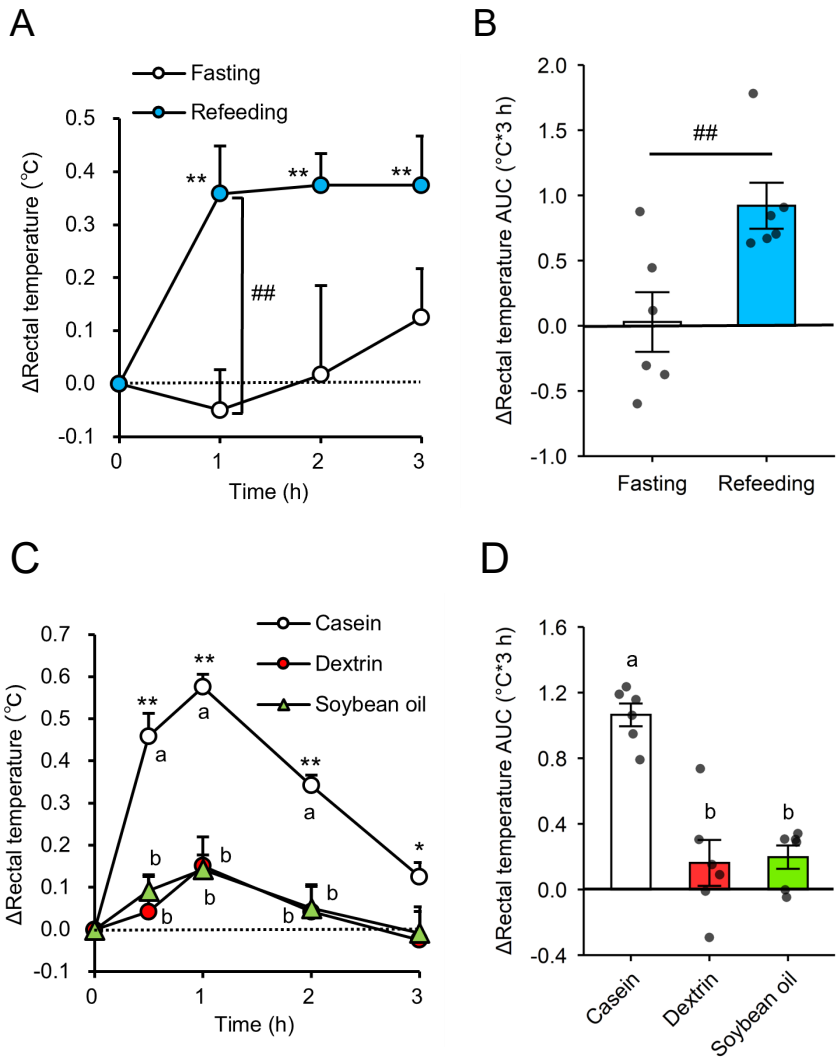
immediately after oral administration of soy protein (2 g/kg) or water (control, 15 mL/kg) in 4 h-fasted rats. (A) Changes in rectal temperatures and (B) its AUC (n = 6-7). (C) Active GLP-1 concentrations in portal vein plasma 1 h after oral administration of test solutions. Values are expressed as the mean ± SEM. Plots with asterisks (*) show significant differences compared with 0 h values within each treatment ($^{**}p < 0.01$, Dunnett's test). Different letters indicate $p < 0.05$ between treatments by Tukey's test. The p values of two-way ANOVA are shown in each panel (B, C).

Fig. 6. Oral soy protein increases oxygen consumption (VO_2) via GLP-1 signaling. (A, B) Soy protein (2 g/kg) or water (control, 15 mL/kg) was orally administered in 4 h-fasted rats. (C, D) Ex9 (30 nmol/kg) or saline (1 mL/kg) was intraperitoneally injected immediately after oral administration of soy protein (2 g/kg) in 4 h-fasted rats. (A, C) The changes of thermogenesis from pre-administration [ΔVO_2 (thermogenesis)] and (B, D) its AUC during 0-3 h post-administration are presented. Values are expressed as the mean ± SEM. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ by paired t-tests between treatments (crossover design). The p values of the mixed model with unstructured covariance are shown in each panel (A, C).

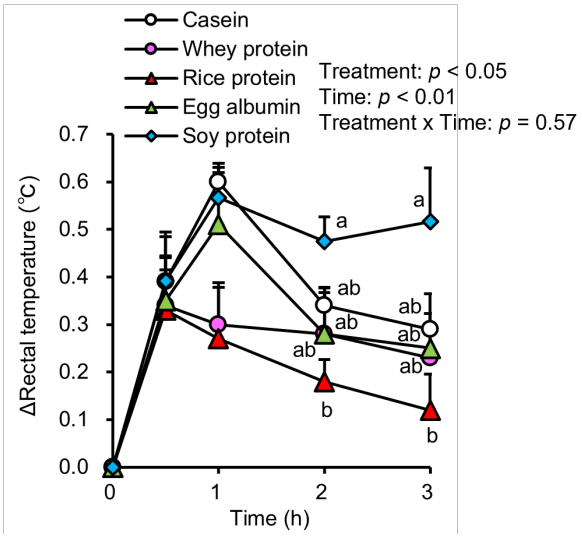
Fig. 7. The canonical GLP-1 receptor is essential for the increasing effect of soy protein on rectal temperature. Changes in rectal temperatures after oral administration of soy protein (2 g/kg) or saline (control, 20 mL/kg) and its AUC in (A, C) wild-type (WT) C57BL/6J mice (n = 8) and (B, D) *Glp1r* KO mice (n = 12, crossover design) fasted 5 h. Total GLP-1 concentrations in portal vein plasma 1 h after oral administration of test solutions in (E) WT mice (n =6) and (F) *Glp1r* KO mice (n = 5). Values are expressed as

the mean ± SEM. In (A, B), plots with asterisks (*) show significant differences compared with 0 h values within each treatment ($^{*}p < 0.05$ and $^{**}p < 0.01$, Dunnett's test), and comparisons between treatments were performed using (A) Student's *t*-test ($^{\#\#}p < 0.01$) or (B) paired *t*-test. $^{*}p < 0.05$ and $^{**}p < 0.01$ by (C, E, F) Student's *t*-test or (D) paired *t*-test.

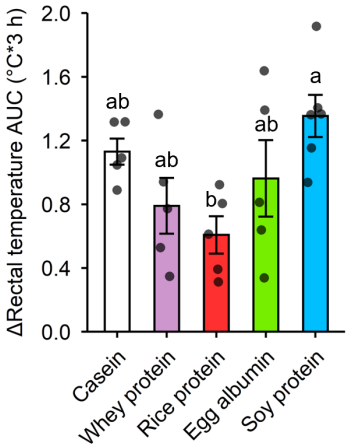
Fig. 8. DPP-4 inhibition enhances the rectal temperature-elevating effect of soy protein. Sitagliptin (50 mg/kg) or water (5 mL/kg) was administered orally at 2 h before the oral administration of soy protein (2 g/kg) or water (control, 15 mL/kg) in rats fasted for 2 h. (A) Active GLP-1 concentrations in tail vein plasma (n = 6-7). Changes in rectal temperatures and its AUC during 0-3 h in rats orally given (B, C) soy protein (2 g/kg), (D, E) dextrin (2 g/kg), (F, G) soybean oil (0.89 g/kg), or (H, I) water (15 mL/kg) with or without 50 mg/kg sitagliptin treatment (B-E, n = 7; F-I, n = 6). Values are expressed as the mean ± SEM. In (A, B, D, F, H), plots with asterisks (*) show significant differences compared with 0 h values within each treatment ($^{*}p < 0.05$ and $^{**}p < 0.01$, Dunnett's test), and different letters or symbols indicate $p < 0.05$ between treatments by Tukey's test or Student's *t*-test. In (C, E, G, I), $^{*}p < 0.05$ by Student's *t*-test. The p values of the mixed model with unstructured covariance are shown in each panel (B, D, F, H).


A
Fasting
Refeeding
ΔRectal temperature (°C)
Time (h)
**
##
B
ΔRectal temperature AUC (°C*3 h)
##
Fasting
Refeeding
C
Casein
Dextrin
Soybean oil
ΔRectal temperature (°C)
Time (h)
D
ΔRectal temperature AUC (°C*3 h)
Casein
Dextrin
Soybean oil

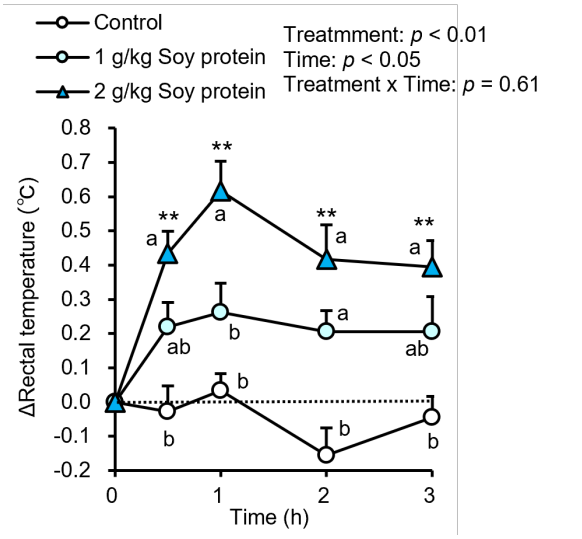
A


Casein
Whey protein
Rice protein
Egg albumin
Soy protein
Treatment: p < 0.05
Time: p < 0.01
Treatment x Time: p = 0.57
ΔRectal temperature (°C)
Time (h)

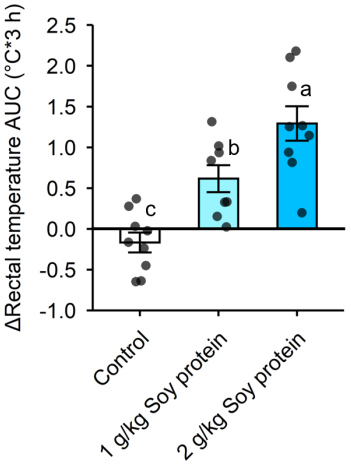
B

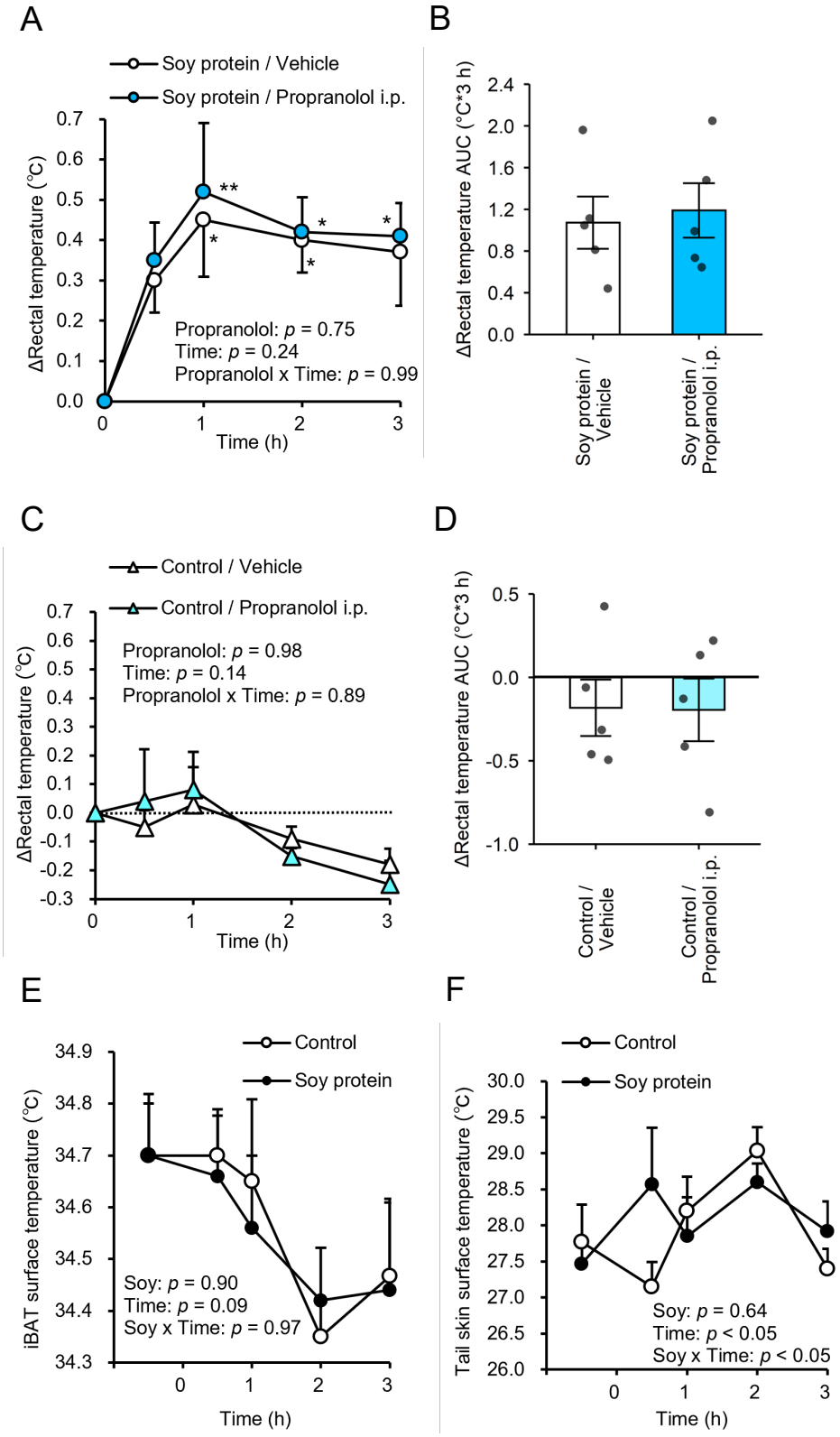

ΔRectal temperature AUC (°C*3 h)
Casein
Whey protein
Rice protein
Egg albumin
Soy protein

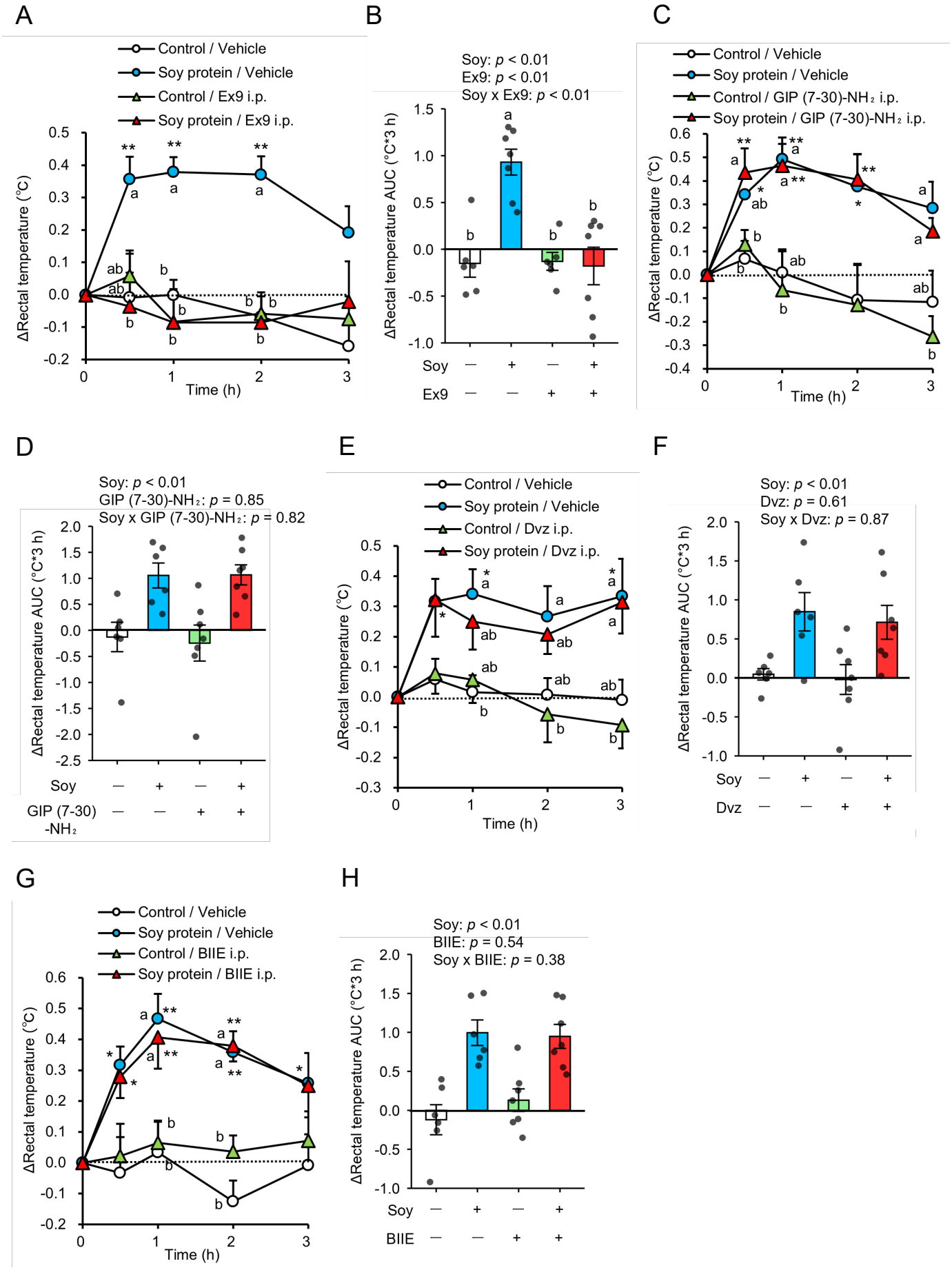
C

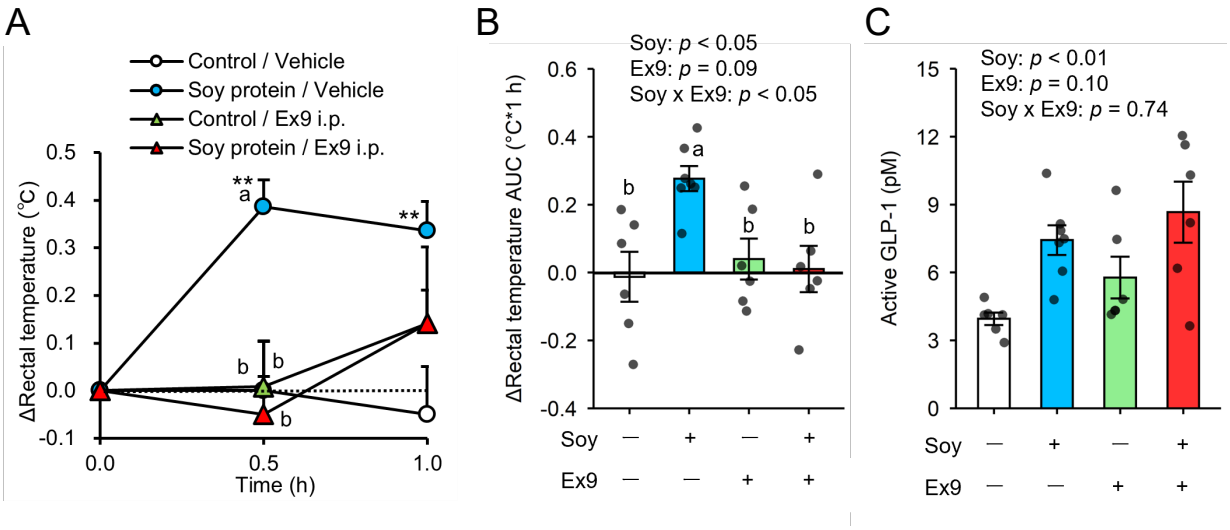

Control
1 g/kg Soy protein
2 g/kg Soy protein
Treatmment: p < 0.01
Time: p < 0.05
Treatment x Time: p = 0.61
ΔRectal temperature (°C)
Time (h)

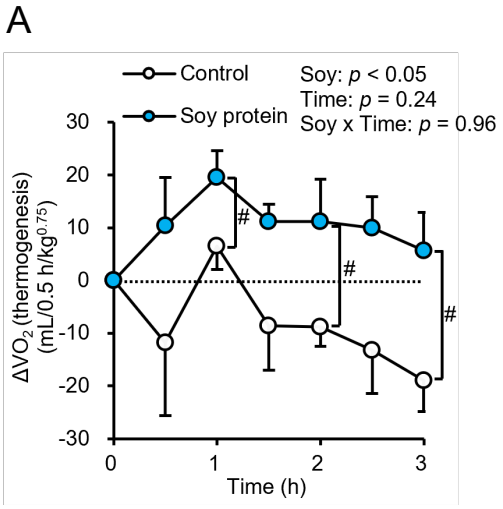
D

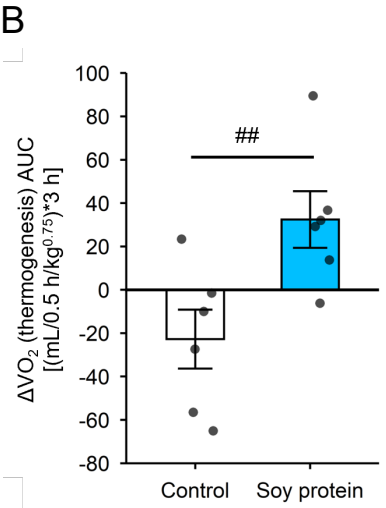

ΔRectal temperature AUC (°C*3 h)
Control
1 g/kg Soy protein
2 g/kg Soy protein

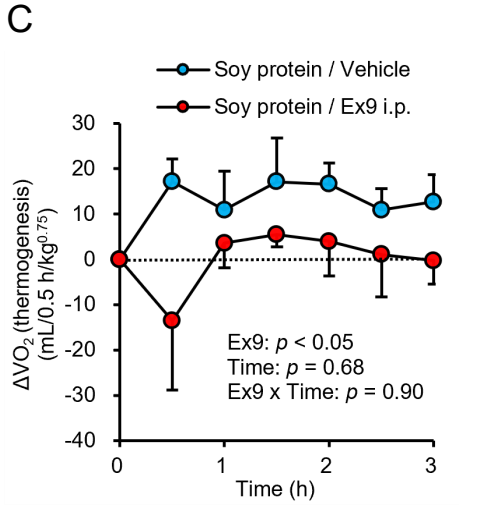

A
Soy protein / Vehicle
Soy protein / Propranolol i.p.
ΔRectal temperature (°C)
Time (h)
Propranolol: p = 0.75
Time: p = 0.24
Propranolol x Time: p = 0.99
B
ΔRectal temperature AUC (°C*3 h)
Soy protein / Vehicle
Soy protein / Propranolol i.p.
C
Control / Vehicle
Control / Propranolol i.p.
ΔRectal temperature (°C)
Time (h)
Propranolol: p = 0.98
Time: p = 0.14
Propranolol x Time: p = 0.89
D
ΔRectal temperature AUC (°C*3 h)
Control / Vehicle
Control / Propranolol i.p.
E
Control
Soy protein
iBAT surface temperature (°C)
Time (h)
Soy: p = 0.90
Time: p = 0.09
Soy x Time: p = 0.97
F
Control
Soy protein
Tail skin surface temperature (°C)
Time (h)
Soy: p = 0.64
Time: p < 0.05
Soy x Time: p < 0.05

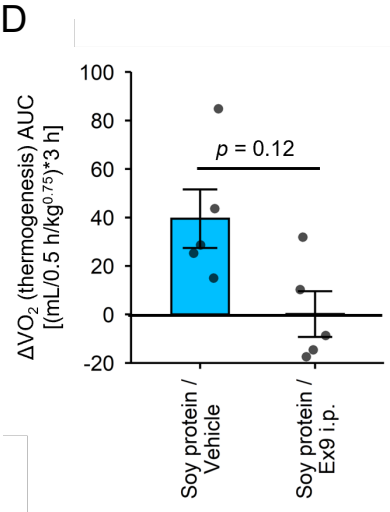

A
Control / Vehicle
Soy protein / Vehicle
Control / Ex9 i.p.
Soy protein / Ex9 i.p.
ΔRectal temperature (°C)
Time (h)
B
Soy: p < 0.01
Ex9: p < 0.01
Soy x Ex9: p < 0.01
ΔRectal temperature AUC (°C*3 h)
Soy
Ex9
C
Control / Vehicle
Soy protein / Vehicle
Control / GIP (7-30)-NH₂ i.p.
Soy protein / GIP (7-30)-NH₂ i.p.
ΔRectal temperature (°C)
Time (h)
D
Soy: p < 0.01
GIP (7-30)-NH₂: p = 0.85
Soy x GIP (7-30)-NH₂: p = 0.82
ΔRectal temperature AUC (°C*3 h)
Soy
GIP (7-30) -NH₂
E
Control / Vehicle
Soy protein / Vehicle
Control / Dvz i.p.
Soy protein / Dvz i.p.
ΔRectal temperature (°C)
Time (h)
F
Soy: p < 0.01
Dvz: p = 0.61
Soy x Dvz: p = 0.87
ΔRectal temperature AUC (°C*3 h)
Soy
Dvz
G
Control / Vehicle
Soy protein / Vehicle
Control / BIIE i.p.
Soy protein / BIIE i.p.
ΔRectal temperature (°C)
Time (h)
H
Soy: p < 0.01
BIIE: p = 0.54
Soy x BIIE: p = 0.38
ΔRectal temperature AUC (°C*3 h)
Soy
BIIE

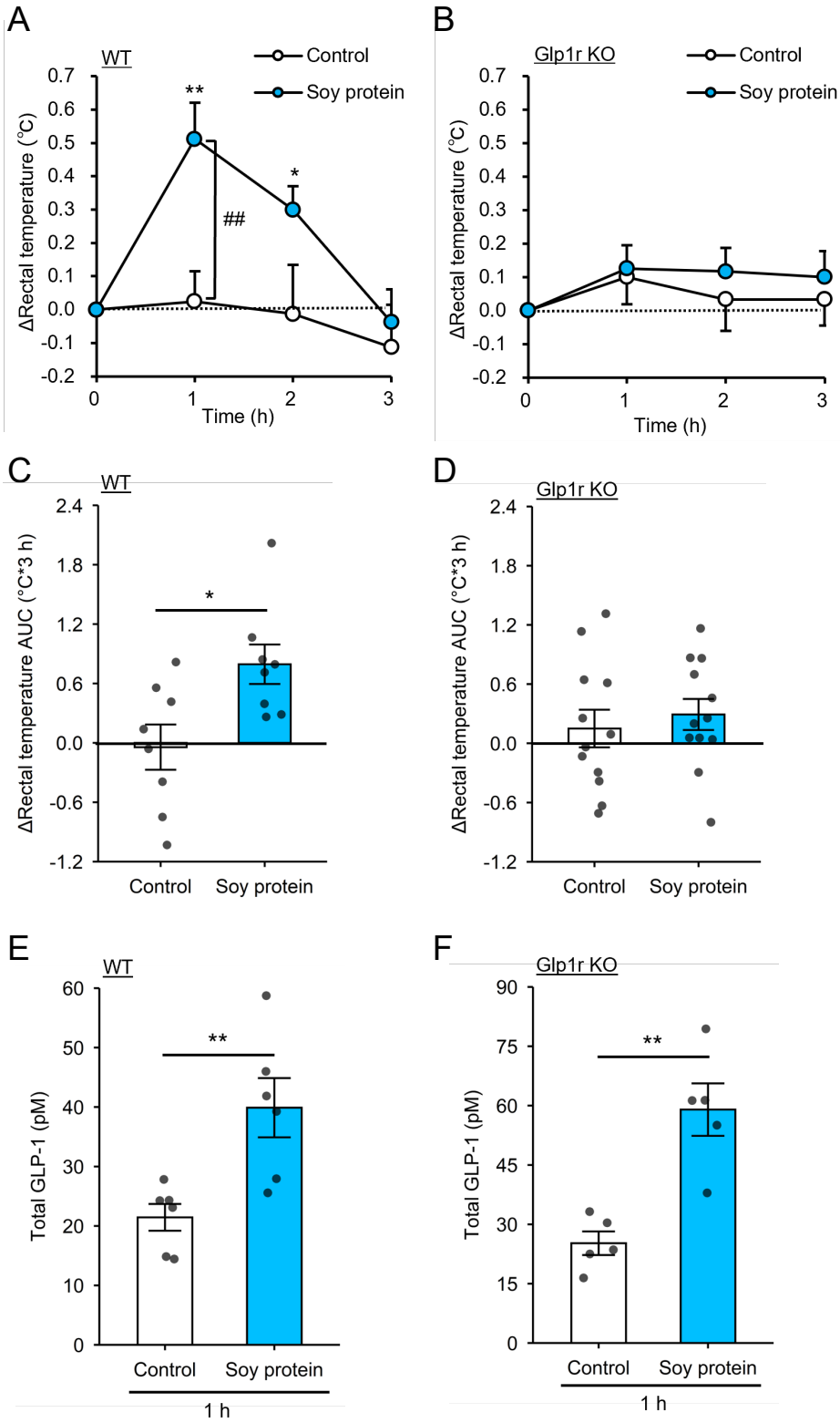

A
Control / Vehicle
Soy protein / Vehicle
Control / Ex9 i.p.
Soy protein / Ex9 i.p.
ΔRectal temperature (°C)
Time (h)
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
0.0
0.5
1.0
**
a
**
b
b
b
B
Soy: p < 0.05
Ex9: p = 0.09
Soy x Ex9: p < 0.05
ΔRectal temperature AUC (°C*1 h)
0.6
0.4
0.2
0.0
-0.2
-0.4
b
a
b
b
Soy
Ex9
C
Soy: p < 0.01
Ex9: p = 0.10
Soy x Ex9: p = 0.74
Active GLP-1 (pM)
15
12
9
6
3
0
Soy
Ex9

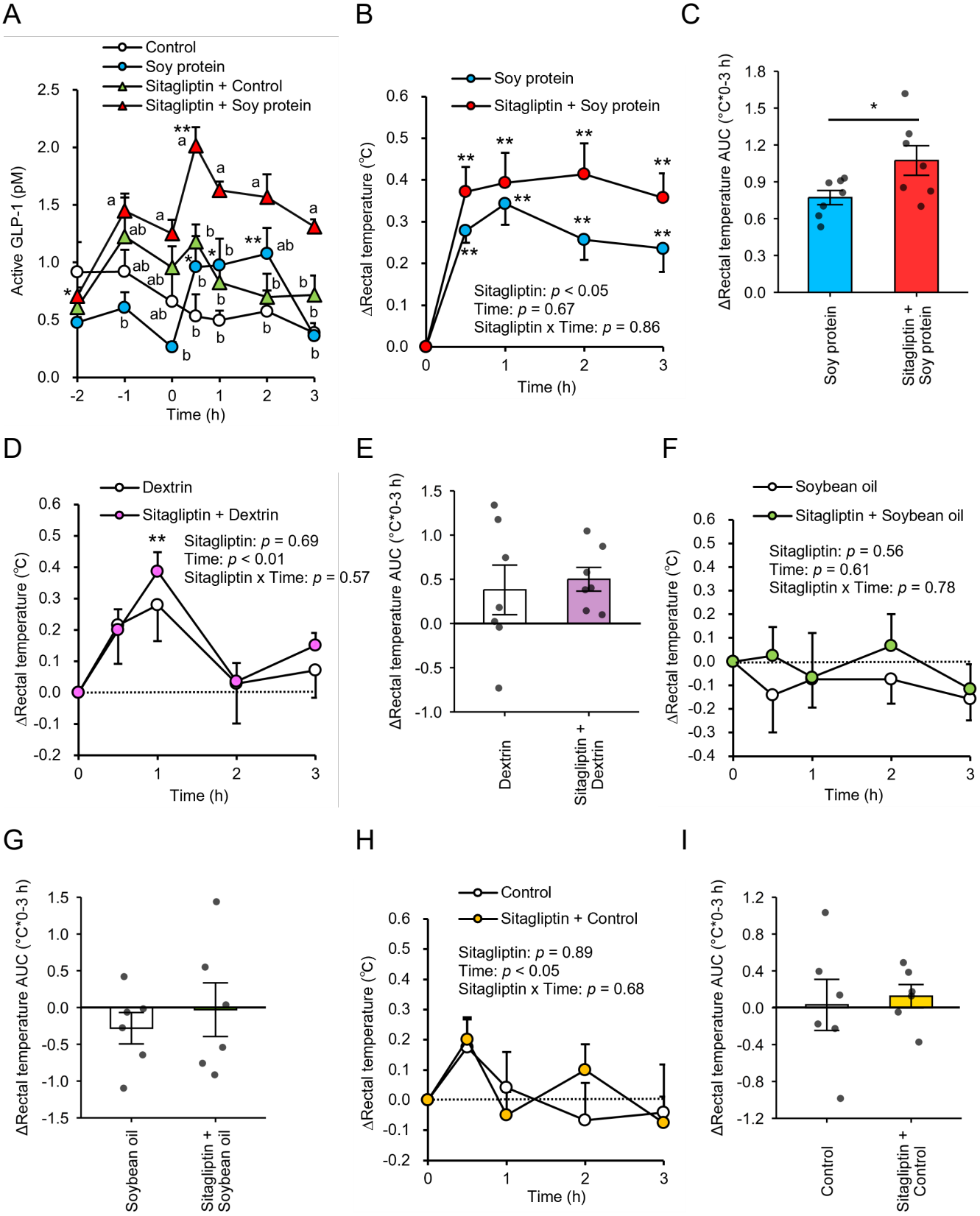

A
Control
Soy protein
Soy: p < 0.05
Time: p = 0.24
Soy x Time: p = 0.96
ΔVO2 (thermogenesis) (mL/0.5 h/kg0.75)
Time (h)
#
#
#


B
ΔVO2 (thermogenesis) AUC [(mL/0.5 h/kg0.75)*3 h]
##
Control
Soy protein


C
Soy protein / Vehicle
Soy protein / Ex9 i.p.
ΔVO2 (thermogenesis) (mL/0.5 h/kg0.75)
Ex9: p < 0.05
Time: p = 0.68
Ex9 x Time: p = 0.90
Time (h)


D
ΔVO2 (thermogenesis) AUC [(mL/0.5 h/kg0.75)*3 h]
p = 0.12
Soy protein / Vehicle
Soy protein / Ex9 i.p.


A
WT
Control
Soy protein
ΔRectal temperature (°C)
Time (h)
**
*
##
B
Glp1r KO
Control
Soy protein
ΔRectal temperature (°C)
Time (h)
C
WT
ΔRectal temperature AUC (°C*3 h)
*
Control
Soy protein
D
Glp1r KO
ΔRectal temperature AUC (°C*3 h)
Control
Soy protein
E
WT
Total GLP-1 (pM)
**
Control
Soy protein
1 h
F
Glp1r KO
Total GLP-1 (pM)
**
Control
Soy protein
1 h


A
Control
Soy protein
Sitagliptin + Control
Sitagliptin + Soy protein
Active GLP-1 (pM)
Time (h)
B
Soy protein
Sitagliptin + Soy protein
ΔRectal temperature (°C)
Sitagliptin: p < 0.05
Time: p = 0.67
Sitagliptin x Time: p = 0.86
Time (h)
C
ΔRectal temperature AUC (°C*0-3 h)
Soy protein
Sitagliptin + Soy protein
D
Dextrin
Sitagliptin + Dextrin
ΔRectal temperature (°C)
Sitagliptin: p = 0.69
Time: p < 0.01
Sitagliptin x Time: p = 0.57
Time (h)
E
ΔRectal temperature AUC (°C*0-3 h)
Dextrin
Sitagliptin + Dextrin
F
Soybean oil
Sitagliptin + Soybean oil
ΔRectal temperature (°C)
Sitagliptin: p = 0.56
Time: p = 0.61
Sitagliptin x Time: p = 0.78
Time (h)
G
ΔRectal temperature AUC (°C*0-3 h)
Soybean oil
Sitagliptin + Soybean oil
H
Control
Sitagliptin + Control
ΔRectal temperature (°C)
Sitagliptin: p = 0.89
Time: p < 0.05
Sitagliptin x Time: p = 0.68
Time (h)
I
ΔRectal temperature AUC (°C*0-3 h)
Control
Sitagliptin + Control