present study might be a reason for the lack of severe hypoglycemic episodes.

Progression of retinopathy was observed in three patients. Aggressive reduction of BG levels in patients with long-term poor glycemic control has been reported to be a factor contributing to early retinopathy progression after glycemic control[29-31] and a slow reduction rate of HbA_{1c} levels (slower than -0.5% per month) is recommended for such patients[32]. The patient in the present study who withdrew because of marked retinopathy progression had a long-term poor glycemic state; longer insulin titration intervals than those used in the present study might be desirable for such patients. However, progression of retinopathy was reported in another two patients, whose baseline HbA_{1c} levels and reduction rates were not as high and not as aggressive, respectively (from 7.8 to 6.9% and from 7.2 to 6.3% during 6 months), indicating that the fast reduction rate of HbA_{1c} levels is only a definitive cause of early retinopathy worsening after aggressive glycemic control. Although causes for retinopathy progression in our patients are not clear, patients who undergo intensified glycemic control regimens should be followed up with careful and regular observation of retinopathy to prevent or to take an appropriate action against its worsening.

The present study was limited by low numbers of patients. Accordingly, the aforementioned results must be interpreted as those of a limited pilot study. A large number of participants is necessary to conclude that the regimen of the present study is a suitable method for the initiation of insulin therapy in Japanese T2D patients.

In summary, an addition of a once-daily pre-dinner injection of BIAsp 30 using a patient-directed insulin titration method on the basis of SMBG levels according to a predetermined algorithm can provide satisfactory reduction of HbA_{1c} levels and flat BG profiles from post-dinner to pre-breakfast in Japanese T2D patients with failure of OAD therapies. Lower post-breakfast BG excursions at baseline, shorter diabetes duration and younger age might warrant optimal glycemic control, including daytime postprandial BG profiles, with safety after this simple insulin initiating regimen.

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The effect of gastric inhibitory polypeptide on intestinal glucose absorption and intestinal motility in mice

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ABSTRACT

Gastric inhibitory polypeptide (GIP) is released from the small intestine upon meal ingestion and increases insulin secretion from pancreatic β cells. Although the GIP receptor is known to be expressed in small intestine, the effects of GIP in small intestine are not fully understood. This study was designed to clarify the effect of GIP on intestinal glucose absorption and intestinal motility. Intestinal glucose absorption in vivo was measured by single-pass perfusion method. Incorporation of [^{14}C]-glucose into everted jejunal rings in vitro was used to evaluate the effect of GIP on sodium-glucose co-transporter (SGLT). Motility of small intestine was measured by intestinal transit after oral administration of a non-absorbed marker. Intraperitoneal administration of GIP inhibited glucose absorption in wild-type mice in a concentration-dependent manner, showing maximum decrease at the dosage of 50 nmol/kg body weight. In glucagon-like-peptide-1 (GLP-1) receptor-deficient mice, GIP inhibited glucose absorption as in wild-type mice. In vitro examination of [^{14}C]-glucose uptake revealed that 100 nM GIP did not change SGLT-dependent glucose uptake in wild-type mice. After intraperitoneal administration of GIP (50 nmol/kg body weight), small intestinal transit was inhibited to 40% in both wild-type and GLP-1 receptor-deficient mice. Furthermore, a somatostatin receptor antagonist, cyclosomatostatin, reduced the inhibitory effect of GIP on both intestinal transit and glucose absorption in wild-type mice. These results demonstrate that exogenous GIP inhibits intestinal glucose absorption by reducing intestinal motility through a somatostatin-mediated pathway rather than through a GLP-1-mediated pathway.

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1. Introduction

Gastric inhibitory polypeptide (GIP), also called glucose-dependent insulinotropic polypeptide, is an incretin of 42-amino-acid polypeptide synthesized by K cells of the duodenum and small intestine [1]. We previously generated GIP receptor-deficient mice (GIPR$^{-/-}$ mice) and showed that GIPR$^{-/-}$ mice have higher blood glucose levels as well as impaired initial insulin response after oral glucose load [2]. Thus, early insulin secretion stimulated by GIP plays an important role in glucose tolerance after oral glucose load.

While GIP receptor mRNA was reported to be present in rat gut [3], the role of the GIP receptor in the gut has not been fully clarified. In this in vivo study, we investigated the effect of exogenous GIP on intestinal glucose absorption in mice using the intestinal perfusion method. We investigated the effect of exogenous GIP on SGLT-dependent glucose uptake in vitro by using the everted jejunal ring method. Because intestinal motility and absorption are positively related [4,5], we investigated the effect of exogenous GIP on gastrointestinal motility by non-absorbed marker method. Since SST secretion has been reported to be stimulated by GIP and to prolong intestinal motility, we also investigated the involvement of SST in the inhibitory effect of exogenous GIP on both intestinal transit and intestinal glucose absorption by using somatostatin receptor antagonist. Our results demonstrate that exogenous GIP inhibits intestinal glucose absorption by reducing intestinal motility through a somatostatin-mediated pathway rather than through a GLP-1-mediated pathway.

2. Materials and methods

2.1. Animals

Male C57/BL6J mice weighing 25–30 g (8–14 weeks old) were housed in a temperature (25 ± 2 °C)- and moisture (50%)-controlled room with a 12 h light/dark cycle (6:00 AM/6:00 PM). The mice were fed standard mouse chow (Oriental Yeast, Osaka) and tap water *ad libitium*, and used as wild-type mice.

Generation of GIPR$^{-/-}$ mice and GLP-1 receptor-deficient mice (GLP-1R$^{-/-}$ mice) was described previously [2,6]. GLP-1R$^{-/-}$ mice were kindly provided by Dr. Daniel J. Drucker [6]. Age-matched male GIPR$^{-/-}$ and GLP-1R$^{-/-}$ mice were used in the experiments. The Animal Care Committee of Kyoto University Graduate School of Medicine approved animal care and procedures.

2.2. Materials

Synthetic human GIP was purchased from Peptide Institute (Osaka, Japan). The somatostatin receptor antagonist, cyclo(7-aminoheptanoyl-PHE-$_D$-TRP-LYS-THR(BZL)) (cyclosomatostatin (CSS)) and somatostatin 28 (SST) were from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of reagent grade.

2.3. Perfusion experiment

Single-pass perfusion method [7] was used to measure the effect of exogenous GIP or SST on intestinal glucose absorption using C57/BL6J mice. Preperfusion was done for a 45 min equilibration period and the samples were discarded. Three 15 min samples were then collected. GIP or SST was administered intraperitoneally at 60 min after starting the preperfusion according to the protocol (Fig. 1A). The change of absorption was calculated as the glucose concentration of the first sample collected (Period 1) minus the glucose concentration of the last sample collected (Period 2), and expressed as per centimeter perfused bowel. Negative values indicate an inhibitory effect on absorption; positive values indicate an increased effect on absorption.

2.4. Glucose uptake in jejunum in vitro

Incorporation of D-glucose into everted jejunal rings was determined as described previously [8]. SGLT-dependent glucose uptake for 15 min was determined as the glucose uptake in the absence of phlorizin minus the glucose uptake in the presence of phlorizin.

2.5. Small intestinal transit after intraperitoneal administration of GIP

Transit through the stomach and small intestine was measured by administering a non-absorbed marker containing 10% charcoal suspension in 5% gum Arabic, as previously described [9]. The mice were given 0.2 ml of the suspension by gavage through a straight blunt-ended feeding needle. GIP (50 nmol/kg body weight) or SST (75 nmol/kg body weight) or vehicle (saline) was administered intraperitoneally 15 min prior to the administration of the non-absorbed marker. CSS (1 μg/kg body weight), or vehicle (saline) was intraperitoneally administered 10 min prior to GIP administration.

2.6. Plasma GIP and SST assays

Blood was collected from the tail vein before the intraperitoneal administration of GIP (50 nmol/kg body weight) and collected again 20 min after the administration. ELISA assay kit was used according to the manufacture's instruction for the determination of plasma total GIP concentration (Linco Research, St. Charles,
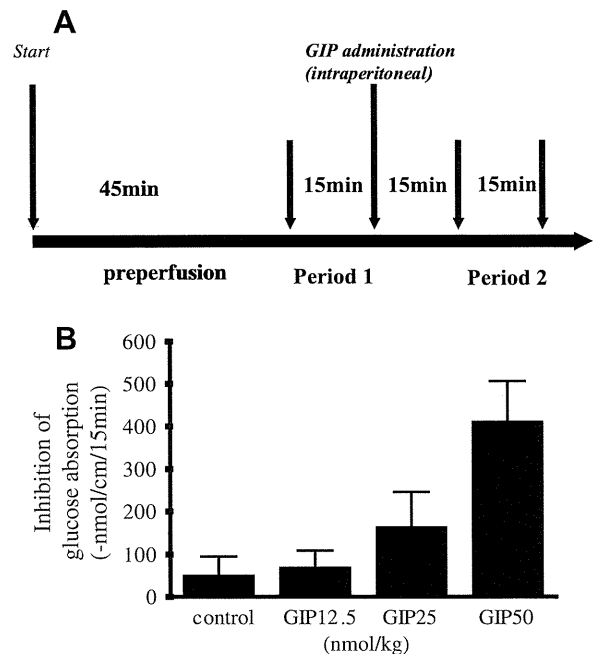

Fig. 1. (A) Diagram showing the sampling protocol of intestinal perfusion. The flow rate of the perfusion fluid was 2 ml/15 min. Perfusion began with an equilibration period of 45 min, which samples were discarded. The samples of Period 1 and Period 2 were then collected. GIP was administered intraperitoneally 60 min after the beginning of preperfusion. The change of absorption was calculated as the glucose concentration of the first samples collected (Period 1) minus the glucose concentration of the last samples collected (Period 2), and expressed as per centimeter perfused bowel. (B) Concentration-dependence of inhibition of glucose absorption by GIP in wild-type mice. Data are shown as means with SEM (*n* = 6 for each group, *P* < 0.05 by ANOVA).

MO) and SST concentration (Phoenix Pharmaceuticals INC., Belmont, CA), respectively.

2.7. Analysis

The results are given as mean ± standard error (SEM, *n* = number of mice). Statistical significance was determined using paired and unpaired Student's *t*-test and analysis of variance (ANOVA). *P* < 0.05 was considered significant.

3. Results

3.1. Perfusion experiment

Inhibition of glucose absorption was calculated by change in glucose concentration in effluent perfusate in wild-type mice (Fig. 1A). Spontaneous inhibition of glucose absorption of 49 ± 44 nmol/cm/15 min is shown in saline-administered controls (Fig. 1B). Inhibition of glucose absorption was enhanced to 67 ± 40, 163 ± 84, and 409 ± 96 nmol/cm/15 min when the amount of intraperitoneally-administered GIP was increased to 12.5, 25, and 50 nmol/kg body weight, respectively.

3.2. Glucose uptake by jejunum in vitro

We investigated glucose uptake by the jejunum *in vitro* using everted jejunal rings. In the presence of 100 nM GIP in the incubation medium, glucose uptake into jejunal rings in wild-type mice was similar to that in the presence of vehicle (control: 4.2 ± 0.9 μmol/g weight; GIP: 3.5 ± 0.9, *P* = NS; Fig. 2A). Additionally, glucose uptake into jejunal rings in GIPR$^{-/-}$ mice was similar
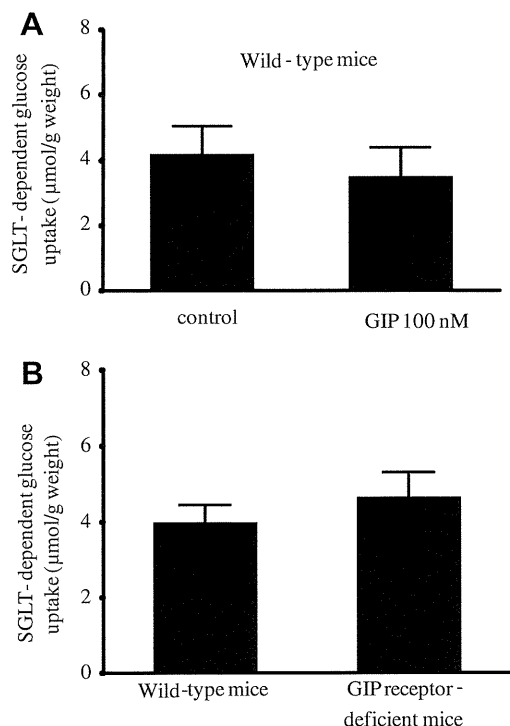
Fig. 2. Glucose uptake in the jejunum. (A) Glucose uptake in the jejunum in wild-type mice in the absence and in the presence of 100 nM GIP. (B) Glucose uptake in the jejunum in wild-type and GIPR$^{-/-}$ mice. SGLT-dependent glucose uptake was determined as the glucose uptake in the absence of 1 mM phlorizin minus the glucose uptake in the presence of 1 mM phlorizin. Data are shown as means with SEM (n = 8 for each group).

to that in wild-type mice (wild-type mice: 4.0 ± 0.5 μmol/g weight; GIPR$^{-/-}$ mice 4.6 ± 0.7, P = NS; Fig. 2B).

3.3. Small intestinal transit after intraperitoneal administration of GIP

Intestinal transit rate was measured by the length of small intestine traversed by the charcoal suspension. In wild-type mice,
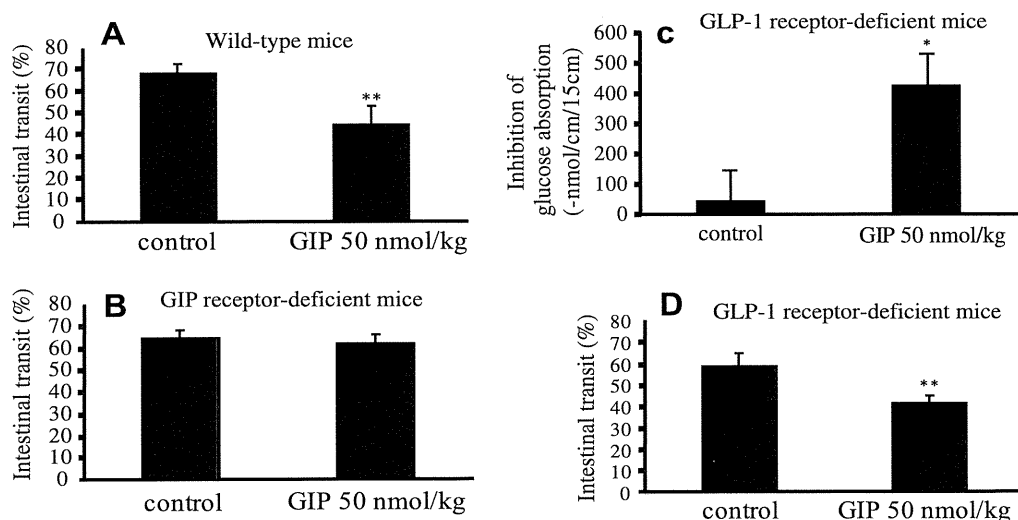
the intestinal transit rate in GIP-administered mice was significantly less than that in saline-administered control (45 ± 8% vs. 68 ± 4%, P < 0.01; Fig. 3A). On the other hand, in GIPR$^{-/-}$ mice, the intestinal transit rate was similar to that in saline-administered control and GIP-administered mice (65 ± 3% vs. 63 ± 4%; Fig. 3B).

3.4. Perfusion and intestinal transit in GLP-1 receptor-deficient mice

To determine whether GIP affects intestinal glucose absorption through GLP-1 signaling, inhibition of glucose absorption by GIP was measured in GLP-1R$^{-/-}$ mice. Inhibition of glucose absorption in GLP-1R$^{-/-}$ mice was 44 ± 100 nmol/cm/15 min in saline-administered control mice and 426 ± 104 nmol/cm/15 min in GIP-administered mice (50 nmol/kg body weight, P < 0.05, Fig. 3C). Thus, GIP significantly inhibited glucose absorption in GLP-1R$^{-/-}$ mice.

The intestinal transit rate was also evaluated in GLP-1R$^{-/-}$ mice, and was 59 ± 13% in saline-administered control and 42 ± 7% in GIP-administered mice, respectively. Thus, GIP significantly inhibited the intestinal transit rate in GLP-1R$^{-/-}$ mice (P < 0.01, Fig. 3D). Consequently, the genetic disruption of GLP-1 receptor did not affect GIP action on intestinal glucose absorption and intestinal transit.

3.5. Involvement of SST in the action of GIP

To determine whether the inhibitory effect of GIP on intestinal transit is due to release of SST, a somatostatin receptor antagonist, CSS (1 μg/kg body weight), was intraperitoneally administered 10 min prior to GIP administration in wild-type mice (Fig. 4A). In the presence of CSS, the intestinal transit rate in GIP-administered wild-type mice was significantly higher than that in the absence of CSS (60 ± 3% vs. 45 ± 8%; P < 0.01). Accordingly, CSS reduced the inhibitory effect of GIP on intestinal transit. Moreover, intraperitoneally-administered SST itself significantly inhibited the intestinal transit rate in wild-type mice compared to control (SST: 37 ± 5% vs. control: 68 ± 4%, P ⩽ 0.01).

In a perfusion experiment, to confirm that the inhibitory effect of GIP on intestinal glucose absorption is attributable to release of SST, CSS (1 μg/kg body weight) was intraperitoneally administered 10 min prior to GIP administration in wild-type mice (Fig. 4B). In

Fig. 3. Intestinal transit after oral administration of non-absorbed marker (10% charcoal suspension in 5% gum Arabic) in wild-type (A) and GIPR$^{-/-}$ (B) mice. Twenty minutes after administration of non-absorbed marker by gavage, the animals were killed and the entire gastrointestinal transit tract was removed. GIP (50 nmol/kg body weight) or saline was administered intraperitoneally 15 min prior to the administration of non-absorbed marker. Data are shown as means with SEM (n = 6 for each group). Statistical significance was determined using Student's t-test. **P < 0.01 compared with control. (C) Inhibition of glucose absorption in GLP-1R$^{-/-}$ mice with or without intraperitoneal GIP administration as indicated in the legends of Fig 1. (D) Intestinal transit after oral administration of non-absorbed marker in GLP-1R$^{-/-}$ mice with or without intraperitoneal GIP administration as indicated in the legends of Fig 3A. Data are shown as means with SEM (n = 6 for each group). Statistical significance was determined using Student's t-test. *P < 0.05 compared with control.
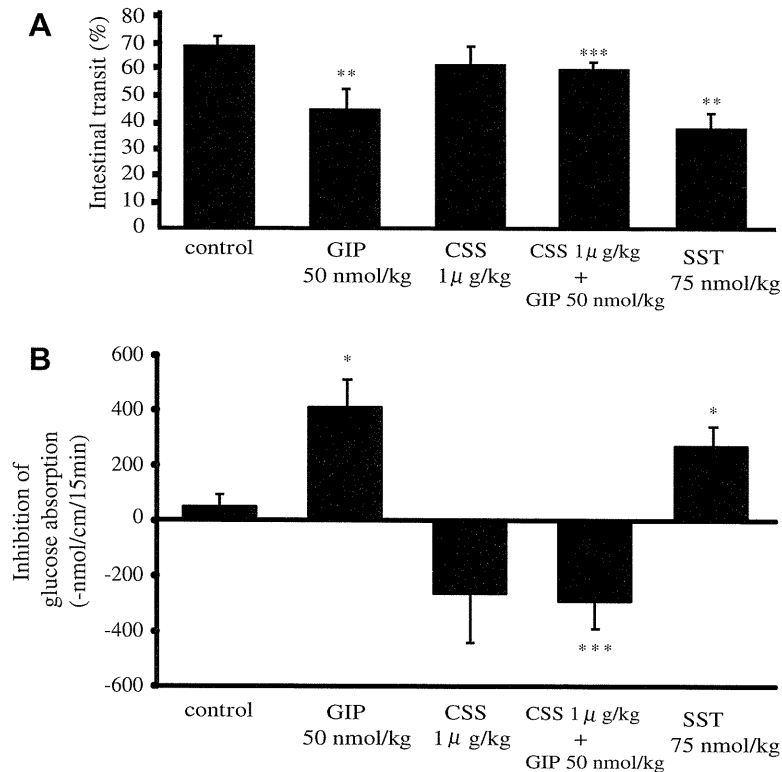
Fig. 4. (A) Intestinal transit after oral administration of non-absorbed marker in wild-type mice with or without pretreatment of CSS. The rate of transit was determined as indicated in the legend of Fig 3A. GIP or SST or saline was administered intraperitoneally 15 min prior to the administration of non-absorbed marker. CSS or saline was intraperitoneally administered 10 min prior to GIP administration. Data are shown as means with SEM (n = 6 for each group). Statistical significance was determined using Student's t-test. **P < 0.01 compared with control. ***P < 0.01 compared with GIP alone administered mice. (B) Inhibition of glucose absorption by GIP in wild-type mice with or without pretreatment of CSS, and inhibition of glucose absorption by SST. CSS or saline was intraperitoneally administered 10 min prior to GIP administration. Data are shown as means with SEM (n = 6 for each group). Statistical significance was determined using Student's t-test. *P < 0.05 compared with control. ***P < 0.01 compared with GIP alone administered mice.

the presence of CSS, the inhibition of glucose absorption in GIP-administered wild-type mice was significantly lower than that in the absence of CSS (410 ± 96 nmol/cm/15 min vs. −290 ± 99 nmol/cm/15 min; P < 0.01). Accordingly, CSS reduced the inhibitory effect of GIP on intestinal glucose absorption. Furthermore, inhibition of glucose absorption in wild-type mice was 49 ± 44 nmol/cm/15 min in saline-administered control mice and 278 ± 63 nmol/cm/15 min in SST-administered mice (75 nmol/kg body weight, P < 0.05).

In an experiment of glucose uptake in everted jejunal ring, 100 nM SST did not alter glucose uptake compared to control (control: 4.2 ± 0.9 μmol/g weight; SST: 4.2 ± 0.4, n = 8; P = NS).

3.6. Measurement of plasma GIP and SST levels

The plasma levels of total GIP and SST in mice were significantly enhanced 20 min after the intraperitoneal GIP-administration at a dosage of 50 nmol/kg body weight compared to the respective basal levels (GIP: 58 ± 5 pg/ml vs. 3400 ± 257 pg/ml, n = 8; P < 0.01; SST: 9.9 ± 0.5 ng/ml vs.11.9 ± 0.3 ng/ml, n = 8; P < 0.05).

4. Discussion

We investigated the inhibitory effect of exogenous GIP on glucose absorption in small intestine. GIP has been known as an important insulinotropic hormone released from duodenal K cells. However, there have been few reports on the effects of GIP on intestinal glucose absorption. In this study, GIP was found to inhibit glucose absorption in a concentration-dependent manner by the perfusion method.

Glucose absorption includes two steps in enterocytes, permeation through brush-border membrane and subsequently through basolateral membrane. Glucose and galactose cross the brush-border membrane by means of SGLT-1, which is a rate-limiting step of glucose absorption [10]. Recent in vitro study by Singh et al. found that exogenous GIP stimulates SGLT-dependent glucose absorption by using an Ussing chamber experiment [11]. In the experiment, intestine was fixed between two chambers, and short-circuit-current representing SGLT activity was measured. However, in our experiments using everted jejunal rings, which is another method to measure SGLT-dependent glucose absorption in vitro, the lack of effect of exogenous GIP on SGLT-dependent glucose uptake was shown, and genetic disruption of the GIP receptor was found not to affect SGLT-dependent glucose absorption. The reason why our results and theirs are different is unknown, but may be attributable to difference in method.

It is generally accepted that there is a positive relationship between intestinal motility and absorption [4,5]. It has been shown that increased intestinal motility, besides enhancing the functional surface area, facilitates diffusion of glucose to the transporters of the brush-border membrane by altering the unstirred water layer [12,13]. We investigated the effect of GIP on motility of small intestine by evaluating intestinal transit. In this study, GIP was found to inhibit intestinal transit compared to control in wild-type but not in GIPR$^{-/-}$ mice. Thus, the inhibitory effect of GIP on glucose absorption may be attributable, in part, to inhibition of intestinal motility.

GLP-1, another incretin hormone, is secreted from L cells found predominantly in ileal mucosa, and is known to be part of the "ileal brake" that acts as an inhibitor of upper gastrointestinal motility

[14]. In this study, GIP was found to inhibit intestinal transit in GLP-1R$^{-/-}$ mice as well as in wild-type mice, indicating that the inhibitory action of GIP on gastrointestinal transit is not mediated by GLP-1. Furthermore, glucose absorption was found to be inhibited significantly by GIP in GLP-1R$^{-/-}$ mice as well as in wild-type mice, suggesting that the primary mechanism of the inhibition of intestinal glucose absorption by GIP most likely does not involve the GLP-1-mediated pathway.

Recently, Miki et al. reported that GLP-1 inhibited gut motility while GIP did not [15]. In this study, however, GIP was found to inhibit intestinal transit. The inconsistency could be due to their use of a non-absorbed marker containing a high concentration (as much as 50%) of glucose to evaluate gut motility, whereas we used a non-absorbed marker without glucose. Intraduodenal infusion of hyperosmolar solution was reported to increase duodenal motility, which is mediated by activation of osmoreceptors in duodenum [16]. In our preliminary experiment on small intestinal transit using 10% charcoal suspension in 5% gum Arabic with 50% glucose, the intestinal transit rate was significantly greater than that when using glucose-free solution (88 ± 8% vs. 68 ± 4%, $P < 0.05$, unpublished data). Therefore, intestinal transit might be enhanced by the high concentration of glucose itself in the suspension, which could conceal a GIP-evoked inhibitory effect on intestinal transit. However, limitations of this study must be considered. While GIP was found to inhibit intestinal transit under the conditions of this study, the effect of GIP on intestinal transit may differ among the constituents of the food or nutrient. Further investigations are required.

Regarding the GIP dosage applied in the *in vivo* experiments, low GIP dosage has been used when applied by the route of continuous intravenous administration; GIP (0.25 nmol/kg body weight) was reported to stimulate insulin secretion by intravenous administration in rat [17] and (GIP 4 pmol/kg body weight/min) in human [18]. However, high GIP dosage has been used when applied by the other routes of administration than intravenous administration. Indeed, one group has reported that subcutaneous pre-administration of 100 μg GIP (approximately 800 nmol/kg body weight) lowered glucose excursion in oral glucose tolerance test in mice [15] and another group has reported that intraperitoneal administration of [D-Ala^2]GIP (48 nmol/kg body weight/day), a DPP4-resistant analogue, lowered glucose excursion in intraperitoneal glucose tolerance test in mice [19]. In this study, we applied GIP intraperitoneally at a dosage of 50 nmol/kg body weight to demonstrate the pharmacological effects of GIP on intestinal transit and glucose absorption, which dosage is comparable to those used in the latter reports.

Regarding the mechanism of inhibition of intestinal transit by GIP, SST secretion has been reported to be stimulated by GIP [20–22] and to prolong intestinal transit [23,24]. The SST receptor has five isoforms (sst1-5) and all five receptors have been shown to be expressed in gastrointestinal tract, with high levels of sst2 receptor in intestine [25]. The sst2 receptors in intestine have been shown not to be expressed on enterocytes or muscle cells, but on myenteric and submucosal plexuses and on neuroendocrine cells in epithelium [26] and also on interstitial cells of Cajal in deep muscular plexus [27]. Thus, the mechanisms by which exogenous GIP inhibits intestinal motility through two SST-mediated pathways may be as follows. In the first, exogenous GIP binds to the GIP receptors on the cell surface membrane in SST-containing enteric neurons and/or in mucosal endocrine cells of D cells in gastrointestinal tract and/or in pancreatic islets, resulting in the release of SST. Subsequently, the released SST acts as a neurotransmitter and binds to sst2 receptors expressed on other neurons in myenteric plexus, parts of which nerve fibers are distributed to muscular cells, permitting inhibition of intestinal motility. In this pathway, the local SST concentration in interneural synaptic space may be increased prominently. In an alternate pathway, SST secreted from D cells flows into systemic circulation through submucosal vessels to reach the neurons in myenteric plexus. Indeed, in this study, intraperitoneally-administered GIP induced a small but significant increase in plasma SST levels, suggesting involvement of the latter pathway.

In our experiment of intestinal perfusion, GIP was found to inhibit intestinal glucose absorption primarily by reducing intestinal motility. On the other hand, the tissue of everted intestinal ring is set inside-out and distended far from the physiological condition, and thus incapable of reflecting general intestinal motility. Thus, the lack of GIP action on glucose uptake in the tissues of everted intestinal ring in this study may be expected.

Several studies have found that the inhibitory effect of SST on intestinal glucose absorption may be attributable to either the effect of SST on the splanchnic hemodynamics [28] or a direct effect of SST on enterocytes [29]. However, consistent with this study, another study has found that SST delays intestinal glucose absorption by its inhibitory effect on intestinal motility [24]. SST exerts its inhibitory effect on intestinal glucose absorption by several mechanisms; our results indicate that the inhibitory effect of SST is mediated, at least in part, by alteration of intestinal motility.

In this study, the somatostatin receptor antagonist CSS was found to reduce the inhibitory effect of GIP on intestinal transit, suggesting that GIP stimulates SST release. In addition, we show that SST itself inhibits intestinal transit and glucose absorption in perfused intestine. Consistently, a recent study has reported that SST inhibits intestinal glucose absorption [29]. Considered together with previous reports, we conclude that exogenous GIP inhibits intestinal transit and glucose absorption indirectly through a somatostatin-mediated pathway.

One of the physiological roles of GIP is known to be facilitation of nutrient uptake into adipose tissue and bone. In this study, exogenous GIP was found to inhibit intestinal glucose absorption by reducing intestinal motility. Since this observation was obtained by the action of a supraphysiological level of plasma GIP, it is unclear whether or not the action is associated with already known physiological actions of GIP. In the point of delay of intestinal carbohydrate absorption, however, the biological action of GIP found in this study appears to be similar to that of medical medicine α-glucosidase inhibitor, which does not influence the regulation of energy accumulation in adipose tissue or bone.

Acknowledgments

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Exendin-4 Protects Pancreatic Beta Cells from the Cytotoxic Effect of Rapamycin by Inhibiting JNK and p38 Phosphorylation

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Abstract
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It has been reported that the immunosuppressant rapamycin decreases the viability of pancreatic beta cells. In contrast, exendin-4, an analogue of glucagon-like peptide-1, has been found to inhibit beta cell death and to increase beta cell mass. We investigated the effects of exendin-4 on the cytotoxic effect of rapamycin in beta cells. Incubation with 10 nM rapamycin induced cell death in 12 h in murine beta cell line MIN6 cells and Wistar rat islets, but not when coincubated with 10 nM exendin-4. Rapamycin was found to increase phosphorylation of c-Jun amino-terminal kinase (JNK) and p38 in 30 minutes in MIN6 cells and Wistar rat islets while exendin-4 decreased their phosphorylation. Akt and extracellular signal-regulated kinase (ERK) were not involved in the cytoprotective effect of exendin-4. These results indicate that exendin-4 may exert its protective effect against rapamycin-induced cell death in pancreatic beta cells by inhibiting JNK and p38 signaling.

Introduction
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Exendin-4 is presently being used in patients with type 2 diabetes [1]. The glucagon-like peptide-1 (GLP-1) analogue improves blood glucose levels by increasing insulin secretion. Exendin-4 is also suggested to promote beta cell proliferation and neogenesis and to inhibit beta cell apoptosis, thereby increasing beta cell mass, at least in rodents [2]. Apoptosis induced by inflammatory cytokines [3] or endoplasmic reticulum (ER) stress [4] was shown to be prevented by exendin-4 in primary rat beta cells and INS-1, a murine beta cell line. Treatment with exendin-4 markedly attenuates beta cell apoptosis in db/db mice [5] and in male C57BL/6 mice exposed to streptozotocin [6]. The molecular mechanism of the cytoprotective effect of exendin-4 is mediated by increased levels of cyclic AMP (cAMP) that lead to activation of protein kinase A (PKA), enhanced insulin receptor substrate-2 (IRS-2) activity, and activation of Akt and extracellular signal-regulated kinase (ERK) [7]. Recently it was reported that exendin-4 inhibits cytokine-induced apoptosis, which involves electron transport chain proteins of mitochondria with a reduction of oxidative stress in INS-1 cells [8].

Rapamycin, an immunosuppressant used to prevent rejection in organ transplantation, is reported to impair glucose-stimulated insulin secretion in rat islets and to decrease viability of rat and human islets [9]. A recent study suggested that rapamycin reduces beta cell mass by 50% under diabetic conditions in *Psammomys obesus*, an animal model of nutrition-dependent type 2 diabetes, by increasing c-Jun amino-terminal kinase (JNK) phosphorylation [10].
We investigated whether exendin-4 might ameliorate the cytotoxic effects of rapamycin. In the present study, we found that rapamycin induces cell death in beta cells through an increase in phosphorylation of JNK and p38, and that exendin-4 prevents such rapamycin-induced cell death by inhibiting these molecules.

Materials and Methods
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Materials
Tissue culture media, DMEM and RPMI1640, rapamycin, exendin-4, exendin (9–39) (exendin-9), H89, LY294002, PD98059, SP600125, SB203580, RNaseA, propidium iodide (PI), and anti-α-tubulin antibody (Ab) were obtained from Sigma Aldrich (St Louis, MO, USA). Fetal bovine

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serum (FBS) was from Invitrogen (Carlsbad, CA, USA). Anti-phospho-Akt (Ser473) Ab, anti-Akt Ab, anti-phospho-JNK (Thr183/Tyr185) Ab, anti-JNK Ab, anti-phospho-p38 (Thr180/Tyr182) Ab, anti-p38 Ab, anti-caspase-3 Ab, and anti-cleaved caspase-3 Ab were obtained from Cell Signaling (Danvers, MA, USA). Anti-phospho-ERK Ab and anti-ERK Ab were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Methods

Cell culture and stimulation of MIN6 cells

MIN6 cells were maintained in 25 mM glucose DMEM media supplemented with 13% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 5 µl β-mercaptoethanol at 37 °C in humidified air containing 5% CO_2. For the experiment, the cells (1.2×10^6) were plated into 35 mm dishes, incubated in DMEM media with or without protein kinase inhibitors for 30 min, and rapamycin and/or exendin-4, and/or forskolin, and/or exendin-9 were then added into the media. After the indicated time periods, the cells were collected and analyzed for cell death and protein phosphorylation.

Islet isolation and stimulation

Pancreatic islets were isolated from Wistar rats by collagenase digestion as described previously [11], and preincubated in RPMI 1640 medium containing 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 5.5 mM glucose at 37 °C in humidified air containing 5% CO_2 for 60 min. After preincubation, the islets were cultured in the media with 10 nM rapamycin and/or 10 nM exendin-4 for the indicated time periods and then analyzed for cell death, protein phosphorylation, and insulin secretion and content.

Insulin secretion and content

Insulin secretion from islets was monitored using batch incubation as previously described [11]. After islets were cultured with 10 nM rapamycin and/or 10 nM exendin-4 for 12 h, the islets were collected, washed, and preincubated at 37 °C for 30 min in Krebs-Ringer bicarbonate buffer (KRBB) medium supplemented with 2.8 mM glucose, and groups of 5 islets were then batch-incubated for 30 min in 0.7 ml KRBB medium containing 2.8 mM and 16.7 mM glucose. At the end of the incubation period, the islets were pelleted by centrifugation, and aliquots of the buffer were sampled to determine the amount of immunoreactive

Fig. 1 Exendin-4 inhibits rapamycin-induced cell death in MIN6 cells. **a**: Dose-dependency and **b**: time-dependency of rapamycin-induced cell death in MIN6 cells. MIN6 cells were treated with 0.1, 1, 10, or 100 nM rapamycin for 12 h (**a**) or with 10 nM rapamycin for 6, 12, 24, or 48 h (**b**), and dead cells with sub-G1 DNA content were counted by flowcytometer. Data are means ± SE of five independent experiments. ** p<0.01. **c**: Quantification of dead cells 12 h after treatment with DMSO (control), 10 nM rapamycin, 10 nM rapamycin and 1, 10, or 100 nM exendin-4, 10 nM rapamycin and 5 µM forskolin, 100 nM exendin-9, and 100 nM exendin-9 and 10 nM rapamycin with or without 10 nM exendin-4. Data are means ± SE of four independent experiments. ** p<0.01. **d**: The percentage of annexin-V-FITC positive cells. MIN6 cells were cultured with 10 nM rapamycin and/or 10 nM exendin-4, and annexin-V-FITC positive cells were counted by flowcytometer. Data are means ± SE of three independent experiments. ** p<0.01. **e**: Western blot analysis of caspase-3 and cleaved caspase-3 in MIN6 cells treated with 10 nM rapamycin and/or 10 nM exendin-4 for 12 h. Images are representative of three independent experiments. Graphs show relative ratio of cleaved caspase-3 versus caspase-3 or α-tubulin, respectively. * p<0.05. C: control (DMSO); R: rapamycin; Ex4: exendin-4; F: forskolin; Ex9: exendin-9.

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insulin by RIA. After an aliquot of incubation medium for insulin release assay in 2.8 mM glucose was taken, the remaining islets were lysed and insulin contents were determined.

Quantification of cell death

MIN6 cells incubated under the conditions indicated were collected from both attached and floating cell populations and fixed with 70% ethanol for 4h. The cells were then washed with PBS, incubated in phosphate-citrate buffer for 30 min, resuspended in 10 µg/ml RNaseA containing PBS, and stained with 10 µg/ml PI. Dead cells containing sub-G1 DNA content were identified and analyzed by a FACS Calibur instrument (BD Biosciences, San Jose, CA, USA). Annexin-V positive cells were analyzed by FACS Calibur using annexin-V-FITC Apoptosis Detection Kit (BD Pharmingen, San Diego, CA, USA). Primary islets were cultured in the indicated conditions for 12 h. DNA fragmentation was measured by quantification of cytosolic oligonucleosome-bound DNA using Cell Death Detection ELISA (Roche, Mannheim, Germany) as previously described [12]. To detect caspase-3 activity, MIN6 cells and primary islets were cultured with 10 nM rapamycin and/or 10 nM exendin-4 for 12 h, and 50 µg of the proteins were subjected to western blot analysis.

Western blot analysis

Cells were collected and washed twice with PBS, and then sonicated in lysis buffer. Equivalent amounts of protein were resolved by SDS/PAGE on 4–12% acrylamide gels (Invitrogen) and transferred to PVDF membranes (Invitrogen), followed by immunoblotting with antibodies to detect respective proteins.

Fluorescence labeling with Newport Green

Islet beta cells from Wistar rat were cultured with 10 nM rapamycin and/or 10 nM exendin-4 for 12 h and stained with Newport Green (Invitrogen) for 30 min. The labeled beta cells were visualized by fluorescence microscopy (Keyence, New Jersey, USA).

Statistics

Data are expressed as mean±SE. Statistical comparisons were made between groups using one-way analysis of variance. Significant differences were evaluated using Tukey-Kramer post hoc analysis.
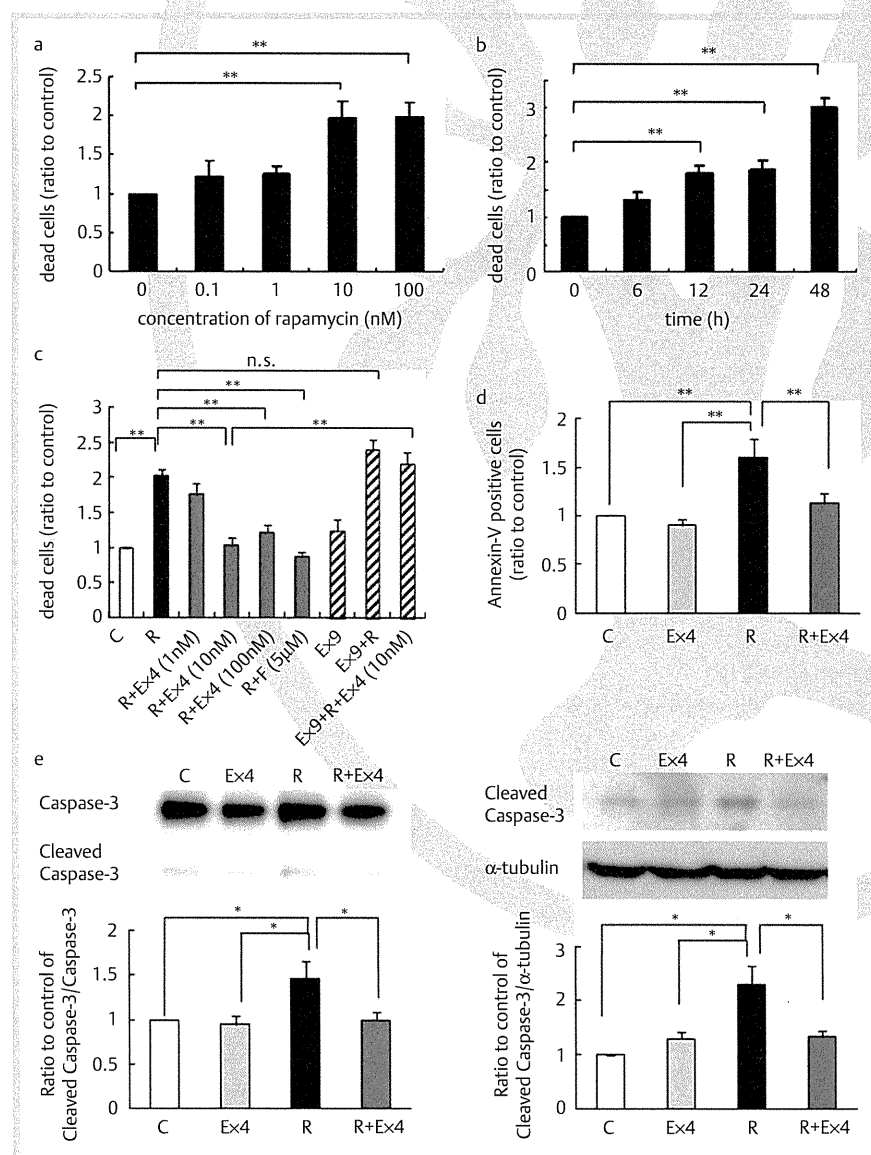
Results

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Exendin-4 inhibits rapamycin-induced cell death in MIN6 cells

We first examined the dose-dependency and time-dependency of rapamycin-induced cell death in MIN6 cells. The number of dead cells was significantly increased in 10 and 100 nM rapamycin-treated MIN6 cells compared to that in DMSO-treated cells (control) in 12 h (○ **Fig. 1a**). The number of dead cells induced by 10 nM rapamycin, a therapeutic concentration in blood [13], was significantly increased from 12 h and maximized at 48 h (○ **Fig. 1b**), indicating cytotoxicity in MIN6 cells in 12 h.

MIN6 cells were then treated with 1, 10, or 100 nM exendin-4 in the presence of 10 nM rapamycin for 12 h. The number of rapamycin-induced dead cells was significantly reduced by 97.0±14.9% and 78.1±14.5% by coincubation with 10 and 100 nM exendin-4, respectively (○ **Fig. 1c**). In addition, 5 µM forskolin, an adenylyl cyclase activator, completely blocked rapamycin-induced cell death (○ **Fig. 1c**). In addition, exendin-9, an
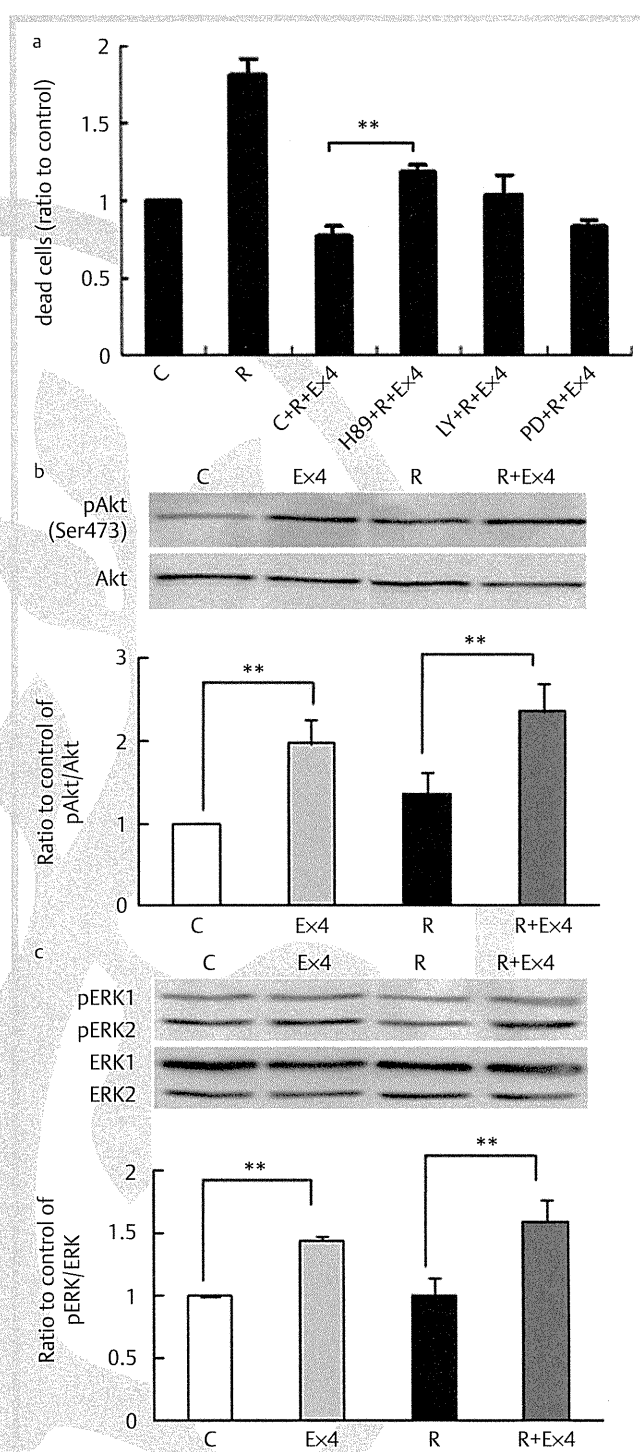
Fig. 2 Cytoprotective effect of exendin-4 is partially mediated by PKA, but not by PI3K/Akt and ERK. **a:** The percentage of dead cells with sub-G1 DNA content. DMSO (control), 15 µM H89, 10 µM LY294002, or 50 µM PD98059 was added into the media 30 min before treatment with 10 nM rapamycin and 10 nM exendin-4 for 12 h in MIN6 cells, and dead cells were counted by flowcytometer. Data are means±SE of four independent experiments. **p<0.01. **b, c:** Western blot analysis of Akt and ERK in MIN6 cells treated with 10 nM rapamycin and/or 10 nM exendin-4 for 30 min. Images are representative of three independent experiments. Graphs show relative ratio of phosphorylated Akt or ERK vs. total Akt or ERK, respectively, by treatment with rapamycin and/or exendin-4 compared to control (DMSO). Data are means±SE of three independent experiments. **p<0.01. C: control (DMSO); R: rapamycin; Ex4: exendin-4; LY: LY294002; PD: PD98059.

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Fig. 3 Exendin-4 inhibits rapamycin-induced cell death by decreasing JNK and p38 phosphorylation in MIN6 cells. **a**: The percentage of dead cells with sub-G1 DNA content. DMSO (control), 10 µM SP600125 or 10 µM SB203580 was added into MIN6 cells 30 min before treatment with 10 nM rapamycin for 12 h, and dead cells were counted by flowcytometer. Data are means ± SE of four independent experiments. ** p < 0.01.
b, c: Western blot analysis of JNK and p38 in MIN6 cells treated with 10 nM rapamycin and/or 10 nM exendin-4 for 30 min. **d, e**: Western blot analysis of JNK and p38 in MIN6 cells to which 15 µM H89 was added 30 min before incubation with 10 nM rapamycin and/or 10 nM exendin-4 for 30 min. Images are representative of three independent experiments. Graphs show relative ratio of phosphorylated JNK or p38 v.s. total JNK or p38, respectively, by treatment with rapamycin and/or exendin-4 compared to control (DMSO). Data are means ± SE of three independent experiments. * p < 0.05 ** p < 0.01. C: control (DMSO); R: rapamycin; Ex4: exendin-4; SP: SP600125; SB: SB203580.

antagonist of the GLP-1 receptor, inhibited the cytoprotective effect of exendin-4 on rapamycin-induced cell death (○ **Fig. 1c**). Annexin-V-positive apoptotic cells were also significantly increased in rapamycin-treated MIN6 cells compared to those in control cells, which increase was prevented by treatment with exendin-4 (○ **Fig. 1d**). Furthermore, rapamycin increased caspase-3 activity, while exendin-4 decreased it in rapamycin-treated MIN6 cells (○ **Fig. 1e**). These results indicate that exendin-4 inhibits rapamycin-induced cell death through the GLP-1 receptor by an increase in the intracellular cAMP concentration in MIN6 cells.
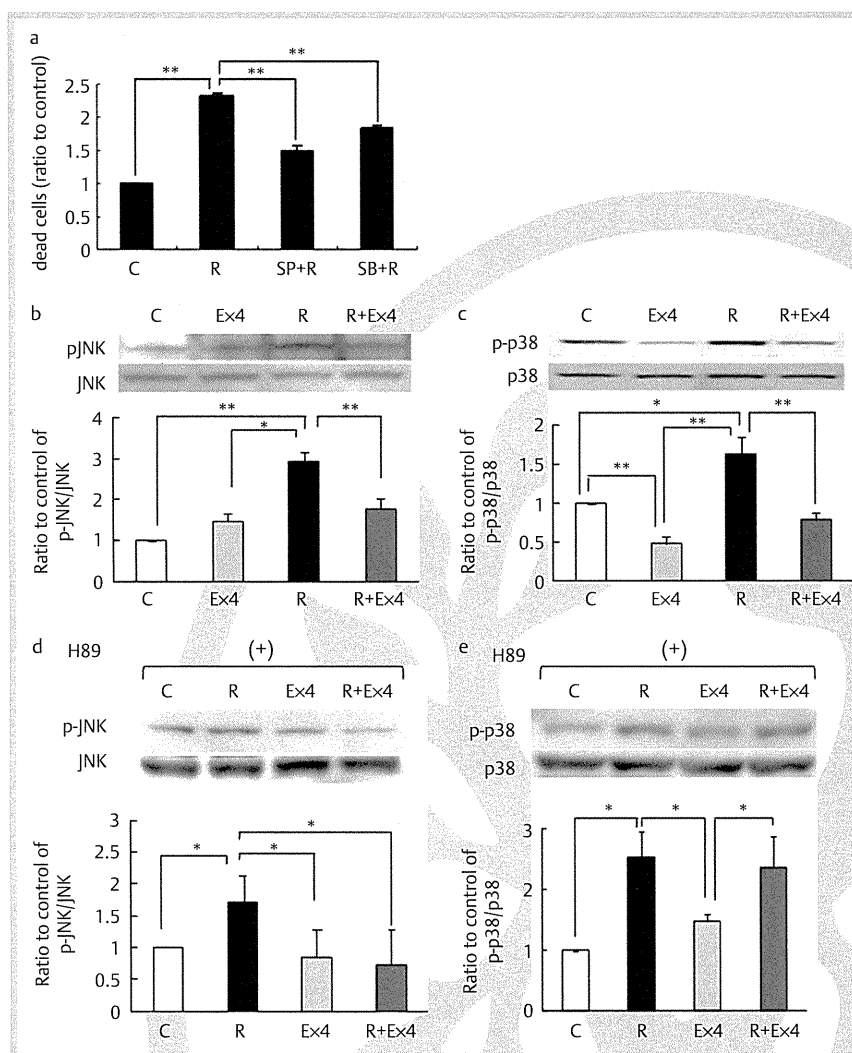
Cytoprotective effect of exendin-4 is mediated in part by PKA, but not by PI3K and ERK

We then examined the involvement of PKA, phosphatidylinositol 3-kinase (PI3K)/Akt, and ERK, downstream molecules of the GLP-1 receptor signaling pathways. DMSO (control), 15 µM H89, a PKA inhibitor, 10 µM LY294002, a PI3K inhibitor, or 50 µM PD98059, an ERK inhibitor, was added into the media 30 min before treatment with 10 nM exendin-4 and 10 nM rapamycin for 12 h in MIN6 cells, and dead cells were counted by flowcytometer. H89, a PKA inhibitor, partially but significantly blocked the cytoprotective effect of exendin-4 (○ **Fig. 2a**). On the other hand, neither LY294002 nor PD98059 altered the cytoprotective effect of exendin-4 against rapamycin-induced cell death (○ **Fig. 2a**), although phosphorylation of Akt and ERK was sig-

nificantly increased by treatment with exendin-4 (○ **Fig. 2b** and **c**). These results indicate that PKA is involved, at least in part, in the cytoprotective effect of exendin-4 against rapamycin-induced cell death.

JNK and p38 are involved in rapamycin-induced cell death in MIN6 cells

Previous studies found that rapamycin induces apoptosis in rh30, a human rhabdomyosarcoma cell line [14], and in islets isolated from P. Obesus [10], by activating JNK. In contrast, rapamycin inhibits palmitate-induced ER stress and apoptosis in INS-1E cells [15]. To clarify the involvement of JNK in rapamycin-induced cytotoxicity in MIN6 cells, 10 µM SP600125, a JNK inhibitor, was added into the media 30 min before treatment with 10 nM rapamycin for 12 h, when dead cells were counted by flowcytometer. SP600125 significantly inhibited rapamycin-induced cell death by 62.6 ± 9.1 % (○ **Fig. 3a**). We also examined the involvement of p38, the other stress-activated protein kinase [16]. Ten µM SB203580, a p38 inhibitor, added to MIN6 cells 30 min before treatment with 10 nM rapamycin for 12 h resulted in significant inhibition of rapamycin-induced cell death by 36.8 ± 6.4 % (○ **Fig. 3a**). These results indicate that rapamycin-induced cell death is mediated by JNK and p38 in MIN6 cells.
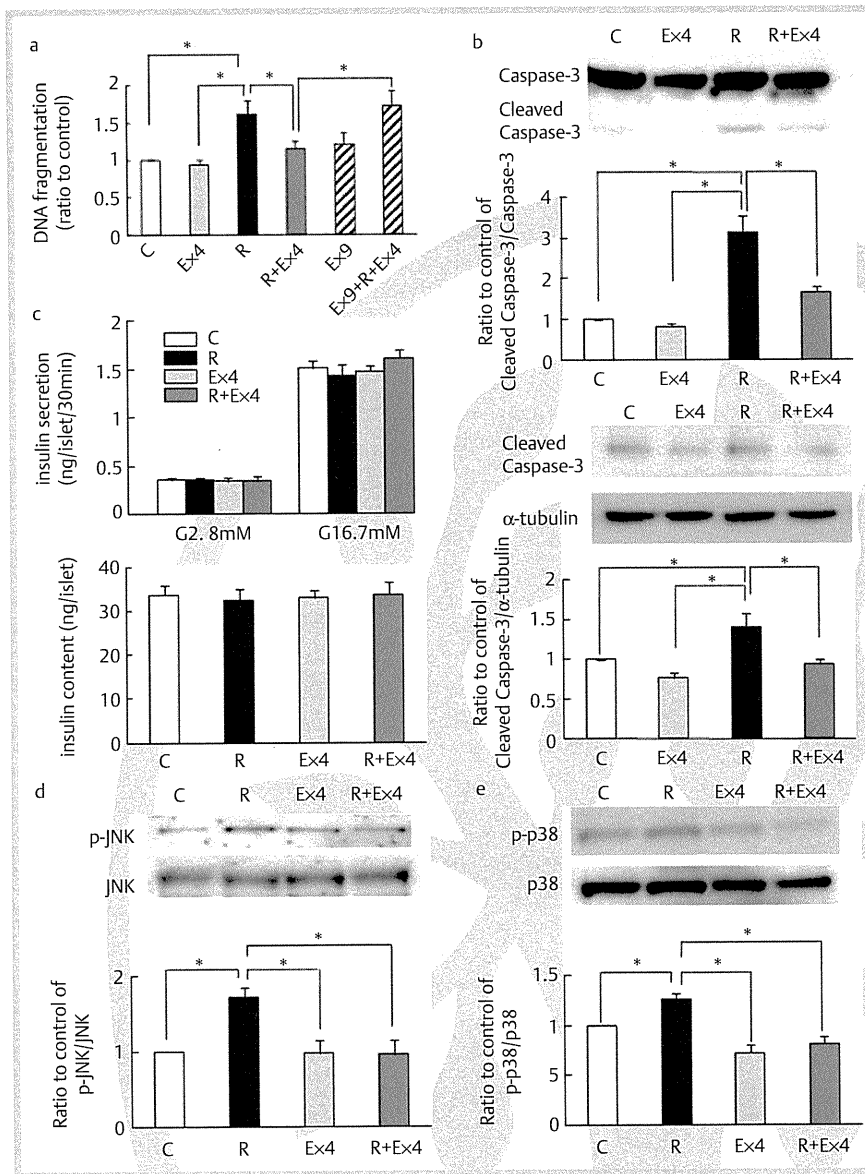
Fig. 4 Exendin-4 inhibits cell death and phosphorylation of JNK and p38 induced by rapamycin in Wistar rat primary islets. **a**: Primary islets were treated with 10 nM rapamycin and/or 10 nM exendin-4 and/or 100 nM exendin-9 for 12 h, and dead cells were detected by DNA fragmentation. Data are means ± SE of three independent experiments. * p < 0.05 **b**: Western blot analysis of caspase-3 and cleaved caspase-3 in primary islets treated with 10 nM rapamycin and/or 10 nM exendin-4 for 12 h. Images are representative of three independent experiments. Graphs show relative ratio of cleaved caspase-3 v.s. caspase-3 or α-tubulin, respectively. * p < 0.05. **c**: Glucose-stimulated insulin secretion and insulin content in Wistar rat islets treated with 10 nM rapamycin and/or 10 nM exendin-4 for 12 h. Data are means ± SE of three independent experiments. **d, e**: Primary islets were treated with 10 nM rapamycin and/or 10 nM exendin-4 for 30 min, and phosphorylation of JNK and p38 was detected by western blot. Images are representative of three independent experiments. Graphs show relative ratio of phosphorylated JNK or p38 vs. total JNK or p38, respectively, by treatment with rapamycin and/or exendin-4 compared to control (DMSO). Data are means ± SE of four independent experiments. * p < 0.05. C: control (DMSO); R: rapamycin; Ex4: exendin-4; Ex9: exendin-9; G: glucose.

Exendin-4 inhibits phosphorylation of JNK and p38 induced by rapamycin

To confirm the involvement of JNK and p38 in rapamycin-induced cell death, MIN6 cells were incubated with 10 nM rapamycin for 30 min, and phosphorylation of JNK and p38 was detected by western blot analysis. Phosphorylation of JNK and p38 was significantly increased 2.9 ± 0.2-fold and 1.6 ± 0.3-fold, respectively, compared to that in nontreated cells (○ **Fig. 3b** and **c**). In contrast, 10 nM exendin-4 decreased phosphorylation of both JNK and p38 in rapamycin-treated MIN6 cells (○ **Fig. 3b** and **c**). However, rapamycin and exendin-4 were found not to affect phosphorylation of mitogen-activated protein kinase kinase (MKK) 3/6, a kinase of p38, or MKK4, a kinase of JNK (data not shown), indicating that dephosphorylation of both JNK and p38 by exendin-4 is not mediated by these upstream kinases.

PKA is involved in dephosphorylation of p38 by exendin-4

Because PKA partially inhibited rapamycin-induced cell death, we examined the effect of H89 on JNK and p38 phosphorylation. Fifteen μM H89 was added into the media of MIN6 cells 30 min before treatment with 10 nM rapamycin and/or 10 nM exendin-

4 for 30 min, when phosphorylation of JNK and p38 was detected by western blot analysis. H89 did not affect decreased phosphorylation of JNK by exendin-4 (○ **Fig. 3d**). By contrast, H89 significantly inhibited dephosphorylation of p38 by exendin-4 (○ **Fig. 3e**), indicating that PKA regulates p38 phosphorylation.

Exendin-4 inhibits rapamycin-induced cell death and phosphorylation of JNK and p38 in Wistar rat islets

We lastly investigated whether exendin-4 inhibits cell death induced by rapamycin in primary islets by affecting phosphorylation of JNK or p38. Wistar rat islets were treated with 10 nM rapamycin and/or 10 nM exendin-4 for 12 h, and the number of dead cells was determined by DNA fragmentation. Rapamycin significantly increased DNA fragmentation by 1.6 ± 0.2-fold compared to that of control (DMSO) (○ **Fig. 4a**), while exendin-4 significantly inhibited rapamycin-induced DNA fragmentation (○ **Fig. 4a**). Exendin-9 again completely blocked the cytoprotective effect of exendin-4 (○ **Fig. 4a**), indicating that such cytoprotective effect of exendin-4 is mediated by the GLP-1 receptor in primary islets. Caspase-3 activity also was significantly increased in rapamycin-treated islets, which was prevented by treatment with exendin-4 (○ **Fig. 4b**). To determine whether the surviving

cells treated with exendin-4 were actually beta cells, islet beta cells were stained with Newport Green, a fluorescent indicator of zinc that allowed visualization of islet beta cells [17] after treatment with 10 nM rapamycin and/or 10 nM exendin-4 for 12 h. Fluorescence microscopy revealed Newport Green positive cells treated with rapamycin and exendin-4 that are almost the same as those treated with DMSO (control) or exendin-4 alone (data not shown). In addition, insulin content and glucose-stimulated insulin secretion (GSIS) were compared among rapamycin- and/or exendin-4-treated Wistar rat islets to determine whether or not these islets are still functional. After islets were incubated with 10 nM rapamycin and/or 10 nM exendin-4 for 12 h, the rapamycin and exendin-4 were washed out and the islets were then stimulated with 2.8 mM or 16.7 mM glucose for 30 min. The amounts of GSIS and insulin content were almost the same in rapamycin- and exendin-4-treated islets, indicating that islets treated with rapamycin and exendin-4 for 12 h are still functional (○ **Fig. 4c**).

Finally, regulation of JNK and p38 by rapamycin or exendin-4 in primary islets was examined. Incubation with 10 nM rapamycin for 30 min significantly increased phosphorylation of JNK and p38 by 1.7±0.2-fold and 1.3±0.1-fold, respectively, which was reversed to control level by coincubation with exendin-4 (○ **Fig. 4d** and **e**). These results indicate that exendin-4 protects primary islets from rapamycin-induced cytotoxicity by decreasing phosphorylation of JNK and p38.

Discussion

▽

In the present study, we have demonstrated that rapamycin induces cytotoxicity in MIN6 cells and Wistar rat islets by phosphorylating JNK and p38, and that exendin-4 inhibits this effect by inhibiting phosphorylation of JNK and p38. This is the first report indicating that rapamycin and exendin-4 regulate phosphorylation not only of JNK but also of p38 in primary islets.

The molecular mechanism of the cytoprotective effect of exendin-4 is mediated by increased levels of cAMP that lead to activation of PKA, enhanced IRS-2 activity, and activation of Akt and ERK [7]. Our data also reveal that an increase in the cAMP concentration completely blocked rapamycin-induced cell death. However, LY294002 and PD98059 did not affect rapamycin-induced beta cell death, even though exendin-4 increased Akt and ERK phosphorylation. Phosphorylation of Akt and ERK also were not affected by treatment with rapamycin, suggesting that these kinases are not directly involved in the cytoprotective effect of exendin-4 on rapamycin-induced beta cell death.

On the other hand, exendin-4 and rapamycin both regulate phosphorylation of JNK and p38 in islet beta cells. Rapamycin increases JNK and p38 phosphorylation; exendin-4 decreases their phosphorylation. JNK and p38 are activated by cellular stress, and lead the cells to apoptosis [18]. In islet beta cells, phosphorylation of JNK and p38 are involved in cell death induced by IL-1β [19,20] and islet isolation [21,22]. Conversely, inhibition of JNK or p38 with specific inhibitors prevents cell death induced by islet isolation [22,23], indicating that JNK and p38 are significant molecules in pancreatic beta cell death. In addition, when Wistar rat islets were preincubated for 24 h, rapamycin decreased GSIS, most likely due to reduction in mitochondrial ATP production in the islets of these rats [24]. JNK and p38 are involved in beta-cell function, their activation also affecting insulin biosynthesis. JNK activates c-Jun, a downstream

protein, and inhibits insulin gene transcription [25]. Furthermore, the p38 molecule is reported to repress rat insulin gene 1 promoter activated by GLP-1 [26]. Although insulin secretion and insulin content were not affected by pretreatment with 10 nM rapamycin and/or 10 nM exendin-4 for 12 h, inhibition of JNK and p38 activity by treatment with exendin-4 for a longer time may not only inhibit beta cell apoptosis but also increase insulin biosynthesis.

In summary, exendin-4 can inhibit rapamycin-induced beta cell death via a decrease in phosphorylation of both JNK and p38, and dephosphorylation of p38 by exendin-4 is accomplished at least partially through PKA signaling pathways. These results demonstrate that regulation of both JNK and p38 is important in the cytoprotective action of exendin-4 against rapamycin in pancreatic beta cells.

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Coffee, green tea, black tea and oolong tea consumption and risk of mortality from cardiovascular disease in Japanese men and women

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ABSTRACT

Background The effects of coffee and green, black and oolong teas and caffeine intake on cardiovascular disease (CVD) mortality have not been well defined in Asian countries.

Methods To examine the relationship between the consumption of these beverages and risk of mortality from CVD, 76 979 individuals aged 40–79 years free of stroke, coronary heart disease (CHD) and cancer at entry were prospectively followed. The daily consumption of beverages was assessed by questionnaires.

Results 1362 deaths were documented from strokes and 650 deaths from CHD after 1 010 787 person-years of follow-up. Compared with non-drinkers of coffee, the multivariable HR and 95% CI for those drinking 1–6 cups/week, 1–2 cups/day and ≥3 cups/day were 0.78 (0.50 to 1.20), 0.67 (0.47 to 0.96) and 0.45 (0.17 to 0.87) for strokes among men (p=0.009 for trend). Compared with non-drinkers of green tea, the multivariable HR for those drinking 1–6 cups/week, 1–2 cups/day, 3–5 cups/day and ≥6 cups/day were 0.34 (0.06–1.75), 0.28 (0.07–1.11), 0.39 (0.18–0.85) and 0.42 (0.17–0.88) for CHD among women (p=0.038 for trend). As for oolong tea, the multivariable HR of those drinking 1–6 cups/week and ≥1 cups/day were 1.00 (0.65–1.55) and 0.39 (0.17–0.88) for total CVD among men (p=0.049 for trend). Risk reduction for total CVD across categories of caffeine intake was most prominently observed in the second highest quintile, with a 38% lower risk among men and 22% among women.

Conclusions Consumption of coffee, green tea and oolong tea and total caffeine intake was associated with a reduced risk of mortality from CVD.

Both coffee and tea are one of the most frequently consumed beverages worldwide. They are rich in caffeine and polyphenols, which have potent antioxidant properties and have been observed to play a protective role against cardiovascular disease (CVD).[1][2] Although the relationship between coffee consumption and CVD has been extensively studied,[3–7] few studies have examined the relationship between tea consumption and CVD.[8–11] Instead, most studies have examined the association between coffee consumption and CVD and have focused on coronary heart disease (CHD) in which the association of coffee consumption with stroke remains unclear. In addition, most studies on

the link between coffee consumption and CVD have been conducted in western countries, and only a few studies have been undertaken in Asian countries. The purpose of the present study was thus to examine comprehensively the relationship of the consumption of coffee, green, black and oolong tea with mortality from CVD among Japanese men and women in a large prospective cohort study with 1 010 787 person-years of follow-up. We also examined whether caffeine can be used to explain the effects of these beverages.

METHODS

Study population

The Japan Collaborative Cohort Study for Evaluation of Cancer Risk (JACC Study) started between 1988 and 1990. Details of our research methods have been published elsewhere.[12] The study consisted of 110 792 individuals (46 465 men and 64 327 women) who were 40–79 years of age and living in 45 communities across Japan. Among them, data on the consumption of all beverages (coffee, green tea, black tea and oolong tea) were available for 36 332 men and 50 925 women. A total of 1977 men and 2615 women was then excluded from the study because of a history of stroke, CHD, or cancer at baseline. Therefore, 34 345 men and 48 310 women were involved in the present study. The ethical committees at Nagoya University and the University of Tsukuba approved the study.

Assessment of cardiovascular disease

For mortality surveillance in each community, investigators conducted a systematic review for death certificates, all of which were forwarded to the public health centre in the area of residency.[13] Mortality data were sent centrally to the Ministry of Health and Welfare, and the underlying causes of death were coded according to the International Classification of Diseases, 9th revision, from 1988 to 1994 and the 10th revision from 1995 to 2003 for the National Vital Statistics. In Japan, registration of death is required by the Family Registration Law and is believed to be followed across Japan. Therefore, all deaths that occurred in the cohort were ascertained by death certificates from a public health centre, except for subjects who died after they had moved from their original community, in which case subjects were treated as censored cases.

The follow-up was conducted until the end of 2003, and the average follow-up for the participants was 13.1 years. Cause-specific mortality was determined by total CVD (International Classification of Disease, 9th revision codes 390—459, 10th revision codes I01—I99), total CHD (codes 410—414 and I20—I25) and total stroke (430—438 and I60—I69), separately.

Assessment of consumption of coffee and caffeine intake

At baseline, consumption of coffee and teas was assessed using a self-administered dietary questionnaire as described previously.[14] Briefly, participants were asked to state their average consumption of coffee, green, black and oolong teas during the previous year. They could select any of four frequency responses: 'less than once a week', 'about one to two times a week', 'about three to four times a week' and 'almost every day'. Participants who selected the response of 'almost every day' were also asked to state their average consumption of these beverages in numbers of cups per day. The consumption of decaffeinated coffee or tea was not recorded because these products were not commercially available in Japan in the early 1990s. The estimated caffeine content was 153 mg per cup (170 ml) of coffee, 30 mg per cup (200 ml) of green tea, 51 mg per cup (170 ml) of black tea and 38 mg per cup (190 ml) of oolong tea. The mean caffeine intake was 287 mg/day for men and 254 mg/day for women. Relative proportions of caffeine intake by beverage were 45—49% from coffee, 47—48% from green tea, 1—2% from black tea and 3—6% from oolong tea.

The reproducibility and validity of this dietary questionnaire was reported previously.[15] Briefly, the Spearman correlation coefficients between the two questionnaires, administered 1 year apart for 85 participants (eight men and 77 women), were 0.87 for coffee, 0.79 for green tea, 0.77 for black tea and 0.56 for oolong tea.[15] The coefficients between the average of two questionnaires and four 3-day dietary records and four 1-week dietary records were 0.79 (8.0 cups and 7.1 cups per week) for coffee, 0.47 (25.4 cups and 30.1 cups per week) for green tea, 0.70 (1.4 cups and 1.6 cups per week) for black tea and 0.55 (1.8 cups and 1.2 cups per week) for oolong tea. When we restricted the data to the 77 women, the results were essentially the same.

Statistical analysis

We presented baseline characteristics according to the frequency of consumption of each beverage. Tests for trends were conducted using the median values of confounding variables in each category of beverage. The linear regression model was used for continuous variables and the logistic regression model was used for categorical variables.

The HR and 95% CI for CVD were calculated in each category of beverage and in each quartile of caffeine intake; less than one cup per week or the lowest quartile was used as the reference category. We estimated age and body mass index (BMI)-adjusted HR and multivariable HR using the Cox proportional hazards model, adjusting for age (in years), sex-specific quintiles of BMI (weight in kilograms divided by the square of height in meters), smoking status (never, former, or current (one to 19, 20—29, or ≥30 cigarettes/day)), alcohol intake (never, former, or current (one to 22, 23—45, 46—68, or ≥69 g/day)), hours of walking (<0.5, 0.5, 0.6—0.9 and ≥1.0 h/day), hours of participation in sports (<1, 1—2, 3—4 and ≥5 h/week), use of hormone therapy for women, history of hypertension (yes or no), history of diabetes mellitus (yes or no), perceived mental stress (low, medium and high), and educational level (primary school, junior high school, high school and college or higher). Furthermore, we adjusted for the consumption of other beverages, multivitamin

use, vitamin E supplement use, consumption of total fruits, total vegetables, total bean products, total meats (continuous variable, servings/week for each food) and daily total energy intake (continuous variable, kcal/day). Sex-specific quintiles of BMI were used to account for different distributions between the sexes. We conducted a test for trend by treating median values of each category of beverage or caffeine intake as continuous variables. We also examined a possible non-linear relationship between mortality from total CVD and caffeine intake, coffee consumption or green tea consumption with cubic spline analysis using SAS macro.[16 17] Four knots were set at quintiles 0.05, 0.35, 0.65 and 0.95.

We examined the association of each beverage consumption and total caffeine intake with the risk for CVD stratified by age group (40—59 years and 60—80 years), smoking status (non or ex-smokers and current smokers), alcohol intake (non or ex-drinkers and current drinkers), history of hypertension (yes and no), history of diabetes (yes and no), BMI (<25.1 kg/m² and ≥25.1 kg/m²), educational level (before college and college or higher), total fruit intake (<3 servings/week and ≥3 servings/week), total vegetable intake (<3 servings/week and ≥3 servings/week) and total bean intake (<3 servings/week and ≥3 servings/week). The interactions with these stratified variables were tested by using cross-product terms of caffeine intake and the stratified variables. All analyses were conducted using the SAS statistical package, version 8.2. p Values for statistical tests were two-tailed, and 95% CI were estimated.

The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

RESULTS

The baseline characteristics of the study cohort according to beverage consumptions are given in table 1 and table 2. During the 13.1-year follow-up of 34 345 men and 48 310 women aged 40—79 years (mean age of 57.1 years), we documented 1807 deaths from total CVD (404 total CHD and 782 total strokes) among men and 1557 (292 total CHD and 704 total strokes) among women. Age was associated with higher consumption of green tea and black tea and lower consumption of coffee and oolong tea for both men and women. BMI was positively associated with the consumption of oolong tea for men and women but were inversely associated with the consumption of coffee for men and women, green tea for men and black tea for women. A history of hypertension was inversely associated with the consumption of coffee and black tea, but it was not associated with the consumption of green tea and oolong tea. A history of diabetes was inversely associated with the consumption of coffee, green tea and black tea, but it was inversely associated with oolong tea consumption. Higher stress, higher vitamin E intake, higher multivitamin intake and less walking time were associated with a higher consumption of coffee, black tea and oolong tea and lower consumption of green tea. Achieving a higher educational level was associated with a higher consumption of coffee, black tea and oolong tea, but it was not associated with green tea consumption. In general, the consumption of vegetables, fruits and beans were positively associated with the consumption of teas but were inversely associated with coffee in both men and women. The association of current smoking and alcohol drinking with the consumption of the beverages varies among each beverage and among men and women.

A U-shaped association was found between coffee consumption and mortality from total CVD (table 3). In men, compared

Table 1 Baseline characteristics of men according to the consumption of coffee, green tea, black tea, and oolong tea: Japan Collaborative Cohort (JACC) Study, Japan, 1988

Variable	Men Participants, n	Age, years	BMI, kg/m²	History of HT, %	History of DM, %	Current smoker, %	Current drinker, %	Vitamin E use, %	Multivitamin use, %	Hormone use, %	High mental stress, %	College or higher education, %	Walking >0.5 h/day, %	Sports participation >3 h/week, %
Coffee														
<1 cup/week	11 680	59.5	22.6	20.6	8.9	46.7	75.3	2.7	12.5	−	17.4	13.4	71.8	15.1
1−6 cups/week	8837	57.4	22.8	21.3	7.0	51.3	78.7	3.3	16.1	−	19.0	15.6	70.4	14.7
1−2 cups/day	8012	55.5	22.7	16.6	6.0	58.9	76.1	3.6	16.4	−	27.1	20.5	68.2	14.5
≥3 cups/day	3061	51.7	22.6	10.7	5.5	76.8	67.4	3.9	16.7	−	34.2	22.4	66.2	13.2
p for trend		<0.001	0.026	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	−	<0.001	<0.001	<0.001	0.014
Green tea														
<1 cup/week	3076	56.7	22.6	21.4	8.4	51.2	71.7	3.5	12.9	−	25.2	14.7	68.1	12.9
1−6 cups/week	3415	55.3	22.9	18.8	7.3	52.8	79.4	3.6	15.2	−	24.3	17.7	67.3	13.0
1−2 cups/day	4510	55.4	22.6	18.8	6.3	55.6	77.4	3.4	15.6	−	27.1	20.5	67.9	14.7
3−5 cups/day	12 151	57.0	22.6	19.5	6.9	54.0	76.8	3.4	15.1	−	23.9	19.1	68.4	14.9
≥6 cups/day	8115	58.7	22.5	20.2	6.1	55.2	73.3	2.7	13.3	−	19.1	17.1	73.7	15.1
p for trend		<0.001	<0.001	0.936	<0.001	<0.001	0.115	0.009	0.431	−	<0.001	0.234	<0.001	<0.001
Black tea														
<1 cup/week	30 459	56.9	22.6	20.2	6.6	54.5	75.8	3.0	14.1	−	22.7	17.0	69.2	13.8
1−6 cups/week	3363	56.3	27.1	17.9	6.0	49.9	74.3	5.2	18.5	−	28.0	28.0	67.4	17.0
≥1 cups/day	523	57.1	22.4	17.1	6.3	45.2	72.0	5.5	17.3	−	29.6	31.7	65.2	16.2
p for trend		<0.001	0.098	0.001	0.266	<0.001	0.007	<0.001	<0.001	−	<0.001	<0.001	0.007	<0.001
Oolong tea														
<1 cup/week	26 973	57.1	22.5	20.0	6.2	53.9	75.4	2.8	13.7	−	22.4	17.5	69.4	13.9
1−6 cups/week	3273	54.4	23.3	18.3	6.9	55.1	77.2	4.9	19.6	−	28.7	22.9	66.8	16.1
≥1 cups/day	1611	54.9	23.4	19.8	10.8	51.1	74.0	6.9	17.2	−	30.0	22.3	65.9	16.3
p for trend		<0.001	<0.001	0.170	<0.001	0.294	0.944	<0.001	<0.001	−	<0.001	<0.001	<0.001	<0.001

Variable	Men Total vegetables, servings/week	Total fruits, servings/week	Total beans, servings/week	Total fish, servings/week	Total meat, servings/week	Total energy intake, kcal/day
Coffee						
<1 cup/week	2.83	2.42	2.37	2.49	1.03	1773
1−6 cups/week	2.7	2.4	2.36	2.47	1.12	1785
1−2 cups/day	2.61	2.91	2.21	2.31	1.11	1697
≥3 cups/day	2.44	2.77	2.01	2.15	1.13	1691
p for trend	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Continued

Table 1 Continued

Variable	Men Total vegetables, servings/week	Total fruits, servings/week	Total beans, servings/week	Total fish, servings/week	Total meat, servings/week	Total energy intake, kcal/day
Green tea						
<1 cup/week	2.49	2.40	2.22	2.23	0.98	1653
1–6 cups/week	2.52	2.39	2.18	2.27	1.02	1712
1–2 cups/day	2.66	2.78	2.22	2.32	1.11	1680
3–5 cups/day	2.70	2.87	2.30	2.38	1.10	1721
≥6 cups/day	2.95	3.02	2.47	2.60	1.14	1842
p for trend	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Black tea						
<1 cup/week	2.71	2.71	2.31	2.40	1.07	1743
1–6 cups/week	2.95	3.35	2.51	2.49	1.27	1750
≥1 cups/day	2.89	3.33	2.40	2.37	1.19	1627
p for trend	<0.001	<0.001	<0.001	0.024	<0.001	0.079
Oolong tea						
<1 cup/week	2.70	2.71	2.29	2.39	1.07	1746
1–6 cups/week	2.84	3.12	2.39	2.40	1.17	1716
≥1 cups/day	2.91	3.21	2.53	2.49	1.16	1649
p for trend	<0.001	<0.001	<0.001	0.012	<0.001	<0.001

with those who drank less than one cup per week, the multi-variable HR for total CVD among those drinking one to six cups a week, one to two cups a day and three or more cups a day were 0.71 (95% CI 0.53 to 0.96), 0.84 (95% CI 0.64 to 0.99) and 1.17 (95% CI 0.77 to 1.76). The corresponding data for women were 0.87 (95% CI 0.62 to 1.23), 0.77 (95% CI 0.55 to 0.99) and 2.30 (95% CI 1.31 to 4.02). The non-linear relationship was confirmed by cubic spline analysis (figure 1; non-linear p<0.001 for both men and women). On the other hand, coffee consumption was associated with linear risk reduction for mortality from stroke in men: the multivariable HR across categories of coffee consumption were 1.0, 0.78 (95% CI 0.50 to 1.20), 0.67 (95% CI 0.47 to 0.96), 0.45 (95% CI 0.17 to 0.87, p=0.009 for trend). The inverse association was more prominently observed among men, although interaction with sex was not statistically significant (p=0.204). We found no significant association of coffee consumption with CHD for either men or women.

We found that green tea consumption was associated with a lower risk of mortality from total CVD (table 4). In women, the multivariable HR of mortality due to total CVD based on green tea consumption compared with non-drinkers were 1.13 (95% CI 0.66 to 1.93) for one to six cups a week, 0.77 (95% CI 0.48 to 1.26) for one to two cups a day, 0.81 (95% CI 0.56 to 1.18) for three to five cups a day and 0.62 (95% CI 0.40 to 0.98) for six or more cups a day (p=0.031 for trend). The inverse association was not observed in men (p=0.047 for interaction). The corresponding data for CHD among women were 0.34 (95% CI 0.06 to 1.75), 0.33 (95% CI 0.07 to 1.11), 0.39 (95% CI 0.18 to 0.85) and 0.42 (95% CI 0.15 to 0.92, p=0.038 for trend).

Black tea consumption showed no association with CHD, stroke or total CVD either among men and women (see supplementary table 1 (available online only), p=0.467 for interaction with sex). Oolong tea consumption of one or more cups per day was associated with a reduced risk of mortality from total CVD. Compared with those who drank less than one cup a week, the multivariable HR for total CVD among those drinking one to six cups a week and one or more cups per day were 1.00 (95% CI 0.65 to 1.55) and 0.39 (95% CI 0.17 to 0.88) in men (see supplementary table 2 (available online only), p=0.049 for trend). The inverse association was more strongly observed in men, although the interaction was not significant (p=0.261).

The relationship between caffeine intake and the risk of mortality from total CVD and stroke was U-shaped, in which the highest risk reduction was observed among persons with the second highest quintile (table 5). In men, the HR for mortality from total CVD across categories of caffeine intake were 1.0, 0.83 (95% CI 0.61 to 1.13), 0.70 (95% CI 0.50 to 0.98), 0.62 (95% CI 0.43 to 0.92) and 0.95 (95% CI 0.86 to 1.05; p=0.083 for trend). The inverse association was more pronounced in men than women (p=0.040 for interaction). A U-shaped association was also observed between caffeine intake and stroke. The multivariable HR for those with the second highest quintile for caffeine intake compared with those with the lowest quintile was 0.65 (95% CI 0.37 to 1.13) for men and 0.50 (95% CI 0.26 to 0.94) for women. The non-linear relationship was confirmed by cubic spline analyses (figure 1; non-linear p<0.001 for both men and women). No significant association was observed between caffeine intake and CHD.

We conducted stratified analyses to evaluate whether the association between CVD and caffeine intake or the consumption of coffee or teas varied according to age, smoking status, alcohol intake, history of hypertension, history of diabetes, BMI, educational level, total fruit intake, total vegetable intake and

Table 2 Baseline characteristics of women according to the consumption of coffee, green tea, black tea, and oolong tea: Japan Collaborative Cohort (JACC) Study, Japan, 1988

Variable	Women Participants, n	Age, years	BMI, kg/m²	History of HT, %	History of DM, %	Current smoker, %	Current drinker, %	Vitamin E use, %	Multivitamin use, %	Hormone use, %	High mental stress, %	College or higher education, %	Walking >0.5 h/day, %	Sports participation >3 h/week, %
Coffee														
<1 cup/week	18 214	60.2	23	28.1	5.5	3.5	17.7	7.2	11.5	4.1	17.8	7.6	73.6	11.0
1–6 cups/week	11 966	56.9	23.1	22.0	3.2	3.8	26.9	9.1	13.5	4.5	18.5	9.9	74.8	11.2
1–2 cups/day	12 477	55.3	22.8	18.3	3.2	6.5	30.5	9.3	14.0	5.1	22.3	11.4	72.1	9.8
≥3 cups/day	2732	50.9	22.7	12.1	2.5	18.3	34.3	9.1	13.2	5.8	28.3	13.8	71.0	8.2
p for trend		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Green tea														
<1 cup/week	5118	56.9	22.9	23.4	5.4	6.5	22.6	8.0	11.8	5.3	21.6	8.6	69.7	8.7
1–6 cups/week	5017	55.6	23.1	20.9	4.0	5.7	28.2	10.1	12.8	5.0	21.8	9.2	71.0	9.4
1–2 cups/day	5424	55.9	22.8	19.5	3.8	6.3	26.5	8.6	13.2	5.0	22.3	11.4	71.0	9.7
3–5 cups/day	17 665	57.6	22.8	21.9	3.8	4.7	23.3	7.8	12.7	4.7	19.9	10.9	72.5	10.6
≥6 cups/day	10 288	58.5	23.0	21.7	3.7	5.3	22.1	7.3	12.1	5.0	17.9	10.1	75.1	10.1
p for trend		<0.001	0.605	0.592	<0.001	<0.001	<0.001	<0.001	0.918	0.307	<0.001	0.004	<0.001	0.001
Black tea														
<1 cup/week	41 605	57.3	22.9	22.6	4.1	5.1	22.5	7.5	11.8	4.5	20.0	9.2	72.0	9.6
1–6 cups/week	5761	56.1	22.7	19.4	2.8	4.0	27.3	11.3	16.4	7.0	21.7	17.0	71.8	12.3
≥1 cups/day	944	57.4	22.2	21.5	4.0	7.0	30.6	11.9	18.7	8.3	25.3	23.0	69.3	10.2
p for trend		<0.001	<0.001	<0.001	0.002	0.298	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.165	<0.001
Oolong tea														
<1 cup/week	35 575	57.6	22.8	22.1	3.7	4.7	21.8	6.7	11.8	4.4	19.7	9.6	72.4	9.7
1–6 cups/week	4760	55.0	23.5	21.7	4.5	6.2	29.4	11.6	15.9	6.2	23.8	14.0	70.6	11.3
≥1 cups/day	3694	55.5	23.6	22.8	4.9	8.1	29.8	14.4	15.8	7.4	23.3	14.5	70.3	10.2
p for trend		<0.001	<0.001	0.581	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.0

Variable	Women Total vegetables, servings/week	Total fruits, servings/week	Total beans, servings/week	Total fish, servings/week	Total meat, servings/week	Total energy intake, kcal/day
Coffee						
<1 cup/week	3.29	3.11	2.68	2.65	1.04	1636
1–6 cups/week	3.21	3.35	2.59	2.68	1.14	1674
1–2 cups/day	3.06	3.73	2.5	2.52	1.17	1621
≥3 cups/day	2.95	3.56	2.27	2.41	1.23	1665
p for trend	<0.001	<0.001	<0.001	<0.001	<0.001	0.325

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