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Bitter melon fruit extract enhances intracellular ATP production and insulin secretion from rat pancreatic β-cells

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Abstract

Bitter melon (*Momordica charantia* L.) has been shown to have various health-promoting activities, including antidiabetic and hypoglycaemic effects. Improvement in insulin sensitivity and increase in glucose utilisation in peripheral tissues have been reported, but the effect on insulin secretion from pancreatic β-cells remains unclear. In this study, we investigated the effect of bitter melon fruit on insulin secretion from β-cells and the underlying mechanism. The green fruit of bitter melon was freeze-dried and extracted with methanol. The bitter melon fruit extract (BMFE) was fractionated using ethyl acetate (fraction A), *n*-butanol (fraction B) and water (fraction C). Insulin secretory capacity from INS-1 rat insulinoma cell line and rat pancreatic islets, as well as glucose tolerance in rats by oral glucose tolerance test (OGTT), was measured using BMFE and fractions. ATP production in β-cells was also examined. BMFE augmented insulin secretion from INS-1 cells in a dose-dependent manner. The significant augmentation of insulin secretion was independent of the glucose dose. Fraction A (i.e. hydrophobic fraction), but not fractions B and C, augmented insulin secretion significantly at the same level as that by BMFE. This finding was also observed in pancreatic islets. In OGTT, BMFE and fraction A decreased blood glucose levels and increased serum insulin levels after glucose loading. The decrease in blood glucose levels was also observed in streptozotocin-induced diabetic rats. In addition, BMFE and fraction A increased the ATP content in β-cells. We concluded that hydrophobic components of BMFE increase ATP production and augment insulin secretion from β-cells, consequently decreasing blood glucose levels.

Key words: **Insulin secretion: Bitter melon: Pancreatic β-cells: Hypoglycaemic effect**

Type 2 diabetes mellitus (T2DM) is characterised by impaired insulin secretion resulting from the dysfunction of pancreatic β-cells and/or reduction in β-cell mass, in addition to insulin resistance in peripheral tissues such as liver, muscle and adipose tissue[1–3]. Insulin secretagogues are widely used in the treatment of T2DM[4] and are beneficial to patients who retain sufficient β-cell mass. Insulin secretion from β-cells is regulated by intracellular glucose metabolism through metabolism-secretion coupling, in which glucose-induced ATP production in mitochondria plays an essential role[1,5]. Increased ATP concentration in the β-cell leads to closure of the ATP-sensitive K^+ channels, followed by membrane depolarisation and subsequent activation of voltage-dependent Ca^{2+} channels. Elevation of the intracellular Ca^{2+} concentration through the voltage-dependent Ca^{2+} channels triggers exocytosis of insulin granules[6]. Reduction in mitochondrial ATP production impairs glucose-induced insulin secretion[7], and the pathophysiological state is observed in humans and animals of T2DM[8,9]. Therefore, a novel insulinotropic agent that improves ATP production in β-cells might be helpful for future therapeutic strategies.

Various plants have been used as medicine worldwide since ancient times. Bitter melon (*Momordica charantia* L.), a member of the Cucurbitaceae family, is widely distributed in tropical and subtropical regions of Asia, Africa and South America[10,11]. The immature green fruit has been consumed as a vegetable and traditionally used in folk medicine in many developing countries. Bitter melon has been reported to have various health-promoting properties, such as antidiabetic, anti-cancer, antiviral, antioxidant and anti-inflammatory activities[10–12]. Regarding antidiabetic activity, the hypoglycaemic

Abbreviations: BMFE, bitter melon fruit extract; KRBH, Krebs–Ringer bicarbonate HEPES; STZ, Streptozotocin; T2DM, Type 2 diabetes mellitus.

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British Journal of Nutrition

British Journal of Nutrition

effect of bitter melon has been shown in studies using various diabetic animals[10–13]. The decrease in blood glucose levels, improvement in insulin sensitivity and increase in glucose utilisation in peripheral tissues by chronic administration of bitter melon juice or the extract have been reported[10–13]. However, its effect on insulin secretion from β-cells remains to be clarified.

Bitter melon contains numerous bioactive compounds, including triterpenoids, triterpene glycosides, phenolic compounds, alkaloids, flavonoids, polypeptides and polysaccharides[10–12]. However, the active components for each health-promoting property have not been fully identified. We recently reported that a bitter melon fruit extract (BMFE) by methanol suppresses induction of inducible nitric oxide synthase and production of the proinflammatory mediator nitric oxide in IL-1β-treated hepatocytes from rats and the bioactive components are included in the ethyl acetate-soluble fraction (i.e. hydrophobic fraction) of BMFE[14]. Furthermore, in our previous study, we found that cucurbitacin B, a cucurbitane-type triterpenoid, is present in the ethyl acetate-soluble fraction of BMFE and suppresses nitric oxide production in IL-1β-treated hepatocytes[15]. In addition, administration of the ethyl acetate-soluble fraction of BMFE to *ob/ob* mice for 7 d resulted in the reduction in hepatic lipid accumulation and improvement in hyperglycaemia[15]. The data indicated that hydrophobic substances included in BMFE, such as cucurbitacin B, have anti-inflammatory and antidiabetic effects.

In the present study, we investigated the effect of bitter melon fruit on insulin secretion from pancreatic β-cells and its underlying mechanism. We show here that the ethyl acetate-soluble fraction of BMFE augments insulin secretion from β-cells along with an enhancement of intracellular ATP production, consequently decreasing blood glucose levels.

Materials and methods

Extraction and fractionation of bitter melon fruit

The green fruit of bitter melon collected in Malang, East Java, Indonesia was freeze-dried and extracted by absolute methanol[14]. The methanol extract was filtered and evaporated *in vacuo*. The resultant extracts, which were dissolved in dimethyl sulfoxide, were used as BMFE. Extracts were resuspended in water and fractionated by hydrophobicity into an ethyl acetate-soluble fraction (A, hydrophobic), an *n*-butanol-soluble fraction (B, amphipathic) and a water-soluble fraction (C, hydrophilic) (Fig. 1), as previously described[16].

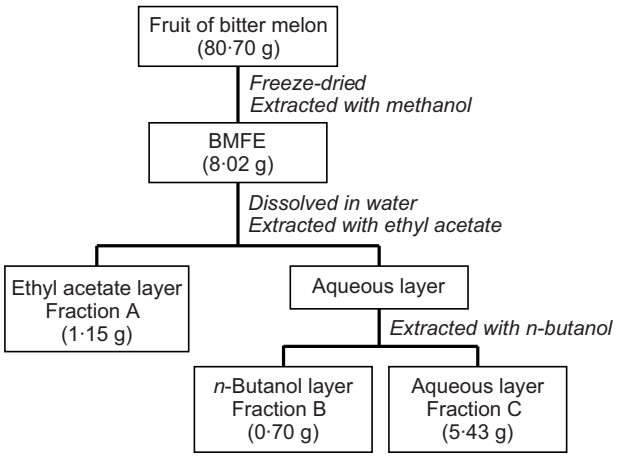

Fig. 1. A flow chart of extraction and fractionation of bitter melon fruit. The freeze-dried fruit of bitter melon was extracted by methanol. The extract (bitter melon fruit extract (BMFE)) was subsequently fractionated by ethyl acetate (fraction A), *n*-butanol (fraction B) and water (fraction C) by hydrophobicity.

Animals and in vivo experiments

Male Wistar ST rats (Japan SLC, Hamamatsu, Japan) were housed in a temperature-controlled environment under a 12 h light–12 h dark cycle with free access to water and standard laboratory chow (CRF-1; Charles River Laboratories Japan, Yokohama, Japan). The experiments were carried out with rats aged 6–7 weeks (180–230 g body weight). All animal care and experimental procedures were carried out in accordance with the guidelines and were approved by the Animal Care Committee of Ritsumeikan University, Biwako-Kusatsu Campus (BKC2017-020, BKC2019-034).

Streptozotocin (STZ)-induced diabetic model rats were made by intraperitoneal injection of STZ (40 mg/kg body weight) to rats. At 1 week after injection of STZ, the rats with high blood glucose levels (≥300 mg/dl) were used for experiments.

Oral glucose tolerance tests were performed after 16 h of fasting. After administration of glucose (1 or 2 g/kg body weight) at 09.00 hours, blood samples were collected from the tail vein at 0, 15, 30, 45, 60, 90 and 120 min. BMFE or its fraction was administered intraperitoneally 30 and 60 min before glucose administration. Blood glucose levels were measured using the glucose oxidase method (Glucocard GT-1820). The incremental AUC was calculated using the trapezoidal rule. Serum insulin levels were determined by ELISA (Morinaga Institute of Biological Science). Experiments using the same protocol were repeated at least three times to ascertain reproducibility.

Cell culture and islet isolation

Rat insulinoma cell line INS-1D cells were cultured as previously described[17].

Male Wistar ST rats were sedated by secobarbital sodium (100–150 mg/kg body weight, intraperitoneal) and euthanised by blood removal from carotid artery, and pancreatic islets were isolated from rats using the collagenase digestion technique[18]. Isolated islets were cultured overnight in the RPMI-1640 medium containing 5·5 mM glucose and 10 % fetal calf serum.

Insulin secretion

Insulin secretory capacity was measured by static incubation using INS-1D cells and isolated islets. INS-1D cells were cultured for 2 d on twenty-four-well plates coated with 0·001 % poly-L-ornithine. The cells were washed with Krebs–Ringer bicarbonate HEPES (KRBH) buffer (140 mM NaCl, 3·6 mM KCl, 0·5 mM $MgSO_4$, 0·5 mM NaH_2PO_4, 1·5 mM $CaCl_2$, 2 mM $NaHCO_3$, 0·1 % bovine serum albumin and 10 mM HEPES (pH 7·4)) containing 2 mM

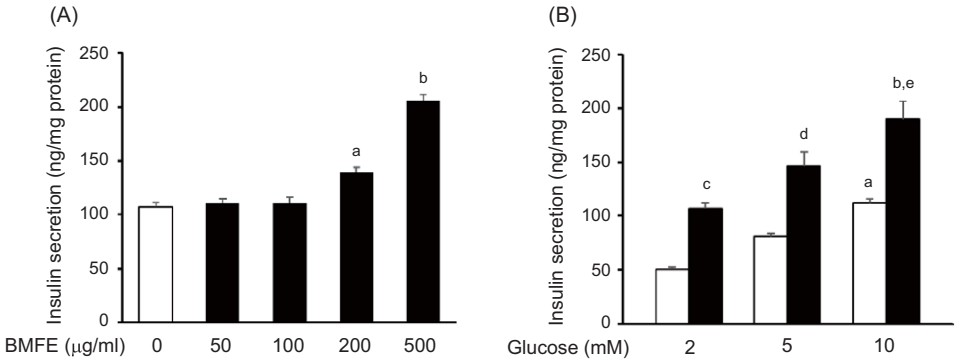

Fig. 2 Bitter melon fruit extract (BMFE) augments insulin secretion from INS-1D cells. (a) Dose-dependent effect of BMFE on insulin secretion at 5 mM glucose. Data were normalised by protein concentration (n 4). $^{a}P < 0{\cdot}05$ and $^{b}P < 0{\cdot}0001$ *v.* 0 μg/ml BMFE. (b) Effect of 500 μg/ml BMFE on insulin secretion at 2, 5 and 10 mM glucose. Data were normalised by protein concentration (n 4). $^{a}P < 0{\cdot}01$ and $^{b}P < 0{\cdot}001$ *v.* 2 mM glucose. $^{c}P < 0{\cdot}05$, $^{d}P < 0{\cdot}01$, and $^{e}P < 0{\cdot}001$ *v.* control. □, Control; ■, BMFE.

glucose, pre-incubated for 60 min at 37°C in KRBH buffer with 2 mM glucose and then incubated for 60 min at 37°C in KRBH buffer with BMFE or each fraction in the presence of various concentrations of glucose. Cultured islets were pre-incubated for 30 min at 37°C in KRBH buffer with 2·8 mM glucose and incubated for 30 min at 37°C in KRBH buffer with BMFE or its fraction in the presence of 5·5 mM glucose. Aliquots of supernatant from the incubation buffer were subjected to insulin ELISA (Morinaga Institute of Biological Science). After suction of the incubation buffer, the cells on the plate were treated with acid and frozen and cell lysates after neutralisation were subjected to measurement of protein concentration using a BCA method (FUJIFILM Wako Pure Chemical Corporation). Static experiments using the same protocol were repeated at least three times to ascertain reproducibility.

ATP production

ATP content was determined by the luminometric method. INS-1D cells were cultured for 2 d on forty-eight-well plates. The cells were washed, pre-incubated and incubated as described above. After suction of the incubation buffer, ATP content in the cells was measured using an Intracellular ATP measurement kit (Toyo B-Net). Protein content was measured as described above. Experiments using the same protocol were repeated at least three times to ascertain reproducibility.

Statistical analysis

Statistical analyses were conducted using Prism for windows (version 7). Data are expressed as mean values with their standard errors. Statistical significance of differences was evaluated by Student's t test or by ANOVA, followed by the Tukey's multiple comparison test. $P < 0{\cdot}05$ was considered as the level of significance.

Results

Preparation of extracts and their fractions of bitter melon fruit

To examine the effects of bitter melon fruit on the regulation of insulin secretion from β-cells, extraction of bitter melon fruit by methanol and subsequent fractionation based on hydrophobicity were performed. BMFE (8·02 g) was obtained from 80·70 g of the freeze-dried fruits. Although 0·74 g of insoluble matter within BMFE existed, 1·15 g of hydrophobic fraction A, 0·70 g of amphipathic fraction B and 5·43 g of hydrophilic fraction C were finally obtained and the yield was 15·80, 9·62 and 74·59 %, respectively (Fig. 1).

Augmentation of insulin secretion from β-cells by bitter melon fruit extract

We examined the effect of BMFE on insulin secretory capacity in pancreatic β-cells. In INS-1D cells, insulin secretion was stimulated by glucose in a dose-dependent manner between 2 and 10 mM (Fig. 2(b)). BMFE augmented insulin secretion dose-dependently in the presence of 5 mM glucose (Fig. 2(a)). Significant augmentation of insulin secretion by 500 μg/ml BMFE was also observed in the presence of 2 and 10 mM glucose (Fig. 2(b)). These results indicate that BMFE augments insulin secretion independent of the glucose dose.

Augmentation of insulin secretion from β-cells by fraction A

To investigate which components within BMFE augment insulin secretion, we next examined insulin secretory capacity using fractions A, B and C. Each concentration used was 80, 50 and 370 μg/ml in accordance with each yield from 500 μg/ml BMFE. Fraction A significantly augmented insulin secretion, and the degree of augmentation was similar to that of BMFE (Fig. 3(a)). In contrast, fractions B and C did not affect insulin secretion. Augmentation of insulin secretion by BMFE and fraction A, but not fraction B and C, was also observed in isolated islets (Fig. 3(b)). These results indicate that the active components of insulin secretion are hydrophobic materials included in fraction A.

Decrease in blood glucose levels by fraction A

To examine the effect of BMFE and fraction A on blood glucose levels *in vivo*, oral glucose tolerance tests were performed after they were administered. BMFE tended to decrease blood glucose levels at the peak, namely, at 30 and 45 min (Fig. 4(a)). The incremental AUC was slightly but not significantly decreased by BMFE (Fig. 4(b)). Fraction A significantly decreased blood glucose

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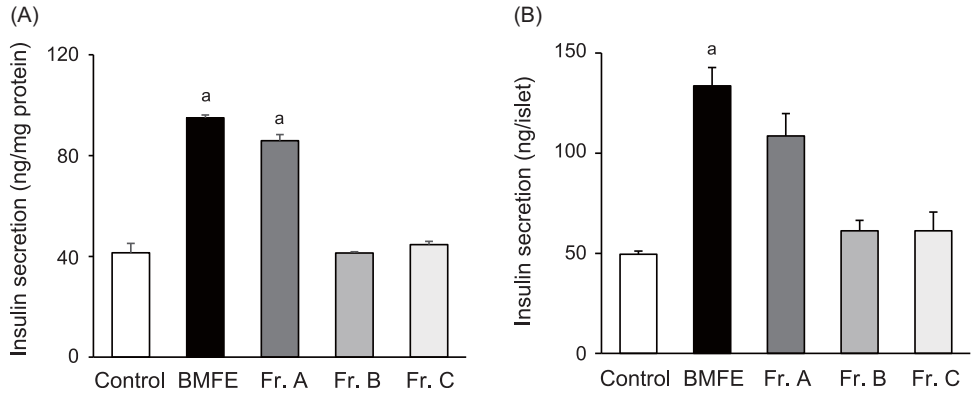

Fig. 3 Fraction A augments insulin secretion. (a) Effect of 500 μg/ml bitter melon fruit extract (BMFE), 80 μg/ml fraction A, 50 μg/ml fraction B and 370 μg/ml fraction C on insulin secretion at 5 mM glucose from INS-1D cells. Data were normalised by protein concentration (n 4). $^{a}P < 0{\cdot}0001$ *v.* control. (b) Effect of 500 μg/ml BMFE, 80 μg/ml fraction A, 50 μg/ml fraction B and 370 μg/ml fraction C on insulin secretion at 5·5 mM glucose from pancreatic islets (n 6). $^{a}P < 0{\cdot}05$ *v.* control.

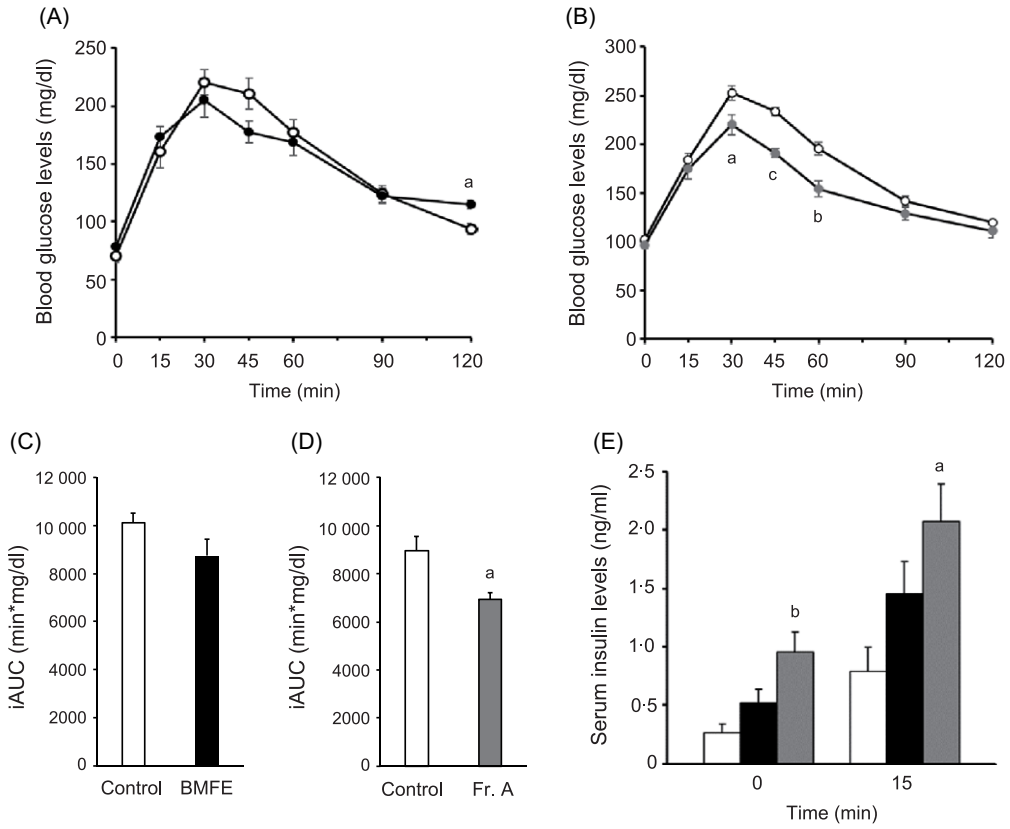

Fig. 4 Effects of bitter melon fruit extract (BMFE) and fraction A on glucose tolerance in normal rats *in vivo*. (a) Blood glucose levels during oral glucose tolerance test (OGTT) after twice intraperitoneal administration of BMFE (3·5 mg/kg body weight). (b) Incremental AUC (iAUC) of blood glucose levels in (a). Data were expressed as mean values with their standard errors (n 6). $^{a}P < 0{\cdot}01$ *v.* control. (c) Blood glucose levels during OGTT after twice intraperitoneal administration of fraction A (0·56 mg/kg body weight). (d) iAUC of blood glucose levels in (c). Data were expressed as mean values with their standard errors (n 6). $^{a}P < 0{\cdot}05$, $^{b}P < 0{\cdot}01$, and $^{c}P < 0{\cdot}0001$ *v.* control. (e) Serum insulin levels during OGTT after twice intraperitoneal administration of BMFE (3·5 mg/kg body weight) or fraction A (0·56 mg/kg body weight). Data were expressed as mean values with their standard errors (n 6). $^{a}P < 0{\cdot}05$ and $^{b}P < 0{\cdot}01$ *v.* control. —○—, Control; —●—, BMFE; —○—, Control; —●—, Fr. A; □, Control; ■, BMFE; ■, Fr. A.

levels at 30, 45 and 60 min (Fig. 4(c)), and there was a significant difference in incremental AUC between the control and fraction A (Fig. 4(d)). Serum insulin levels were slightly increased by BMFE and were significantly increased by fraction A (Fig. 4(e)). Increases were observed at both 0 and 15 min, which means that the effect is independent of glucose infusion. This finding is parallel with the effect on insulin secretion from β-cells. Thus, fraction A, which augments insulin secretion from β-cells, could greatly improve glucose tolerance *in vivo*.

Oral glucose tolerance test was also performed using STZ-induced diabetic non-obese rats. BMFE tended to decrease blood glucose levels at between 0 and 120 min (Fig. 5(a)). The incremental AUC was slightly but not significantly decreased by BMFE (Fig. 5(b)). These findings show that BMFE could also

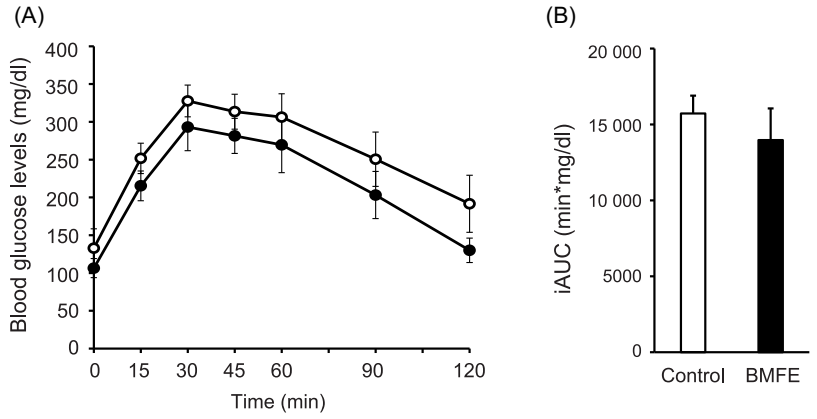

Fig. 5 Effects of bitter melon fruit extract (BMFE) on glucose tolerance in streptozotocin (STZ)-induced diabetic rats *in vivo*. (a) Blood glucose levels during oral glucose tolerance test (OGTT) after twice intraperitoneal administration of BMFE (3·5 mg/kg body weight). (b) Incremental AUC (iAUC) of blood glucose levels in (a). Data were expressed as mean values with their standard errors (*n* 6). —○—, Control; —●—, BMFE.

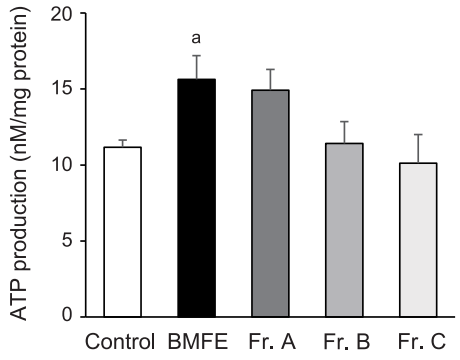

Fig. 6 Bitter melon fruit extract (BMFE) increases ATP content in INS-1D cells. The cells were incubated in the presence of 5 mM glucose with 500 μg/ml BMFE, 80 μg/ml fraction A, 50 μg/ml fraction B and 370 μg/ml fraction C. Data were normalised by protein concentration (*n* 5). $^{a}P < 0{\cdot}05$ *v.* control.

improve glucose tolerance in diabetic model which has a small mass of β-cells by STZ.

Increase in ATP content in β-cells by fraction A

To investigate the mechanism of augmentation of insulin secretion from β-cells by fraction A, ATP production, which plays an essential role in insulin secretory mechanism, was measured. BMFE significantly increased ATP content in β-cells. Fraction A, but not fraction B and C, increased ATP content at the same level as that by BMFE (Fig. 6). Consequently, some hydrophobic constituents of bitter melon fruit increase ATP production and augment insulin secretion from β-cells.

Discussion

In the present study, we demonstrated that the hydrophobic fraction of BMFE enhances insulin secretion from pancreatic β-cells and acutely lowers blood glucose levels in healthy animals. The lowering effect of BMFE on blood glucose levels was also observed in STZ-induced diabetic animals. Previous studies have shown multiple health-promoting effects, including the hypoglycaemic effect of bitter melon, in which the extraction methods of bitter melon fruit as well as the period (mostly long term) and dosage of administration of the extract to animals differ[10–13]. We have previously shown that the exposure of methanol extract of bitter melon fruit for 8 h suppresses inducible nitric oxide synthase induction and nitric oxide production in IL-1β-treated hepatocytes and that the hydrophobic fraction is responsible for the anti-inflammatory effect[14]. In addition, we found that 7 d of administration of the hydrophobic fraction to *ob/ob* mice reduces hepatic lipid accumulation and blood glucose levels[15]. Increases in insulin receptor expression in hepatocytes and serum insulin levels have also been observed[15]. These observations could be attributed to the anti-inflammatory effect of the hydrophobic fraction. The present study revealed that glucose-independent augmentation of insulin secretion from β-cells appears at 1 h, meaning that the effect also seems to be responsible for the hypoglycaemic effect in diabetic obese mice. Thus, multiple actions of the hydrophobic fraction are likely to have beneficial effects on diabetic conditions. In the present study, the greater effect of fraction A than that of BMFE was observed *in vivo*. The effect of the hydrophobic fraction might be weakened by the amphipathic or hydrophilic components included in BMFE *in vivo*.

In the present study, BMFE was shown to augment insulin secretion independently of the glucose dose. In addition, BMFE increased ATP production in β-cells, which seems to be a reasonable mechanism for the augmentation of insulin secretion. For the mechanism of an increase in ATP production, hydrophobic constituents of bitter melon fruit may activate glucose metabolism in β-cells, probably by up-regulation of enzymes involved in glucose metabolism and ATP production in mitochondria. Previous studies have shown that bitter melon extracts affect enzymes in glycolysis pathways[13]. Further examination to elucidate the detailed mechanism is required.

Bitter melon fruit contains various bioactive compounds with hydrophobicity[10–12]. Cucurbitane-type triterpenoids, cucurbitacins, which are a group of bitter-tasting substances, were originally isolated from Cucurbitaceae[19]. Cucurbitane-type triterpenoids are thought to be the main active compounds of bitter melon fruit and have some biological and

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pharmacological activities including antidiabetic activities[19,20]. We previously revealed that cucurbitacin B, which was identified from the ethyl acetate-soluble fraction of BMFE using HPLC, has an anti-inflammatory effect on hepatocytes[15]. Cucurbitacin B is found in many Cucurbitaceae species[19]; it has been isolated from *Ecballium elaterium* and exhibits anti-inflammatory and anti-hepatotoxic activity[21,22]. We then examined the effect of cucurbitacin B on insulin secretion from β-cells, but cucurbitacin B (about 1 μM) did not affect insulin secretion (data not shown). Over fifty cucurbitane-type triterpenoids have been isolated from bitter melon[20]. A previous study reported that new cucurbitane-type triterpenoids, 3β,25-dihydroxycucurbita-6,23(*E*)-diene and 3β,7β,25-trihydroxycucurbita-5,23(*E*)-dien-19-al, which were isolated from bitter melon extract, could lower blood glucose levels in diabetic mice[23]. Triterpenoids and triterpene glycosides including momordin and charantin have also been shown to have hypoglycaemic effect[13,20], and momordin also have anti-inflammatory and anti-cancer effects[24]. Thus, there are several active compounds on insulin secretion with low contents in the hydrophobic fraction, and they might synergistically induce hypoglycaemic activities.

Bitter melon has been used extensively in folk medicine for the treatment of T2DM[10–13]. It remains controversial whether bitter melon has beneficial properties for the treatment of T2DM, probably owing to the content of bioactive components included in the dosage materials; however, several studies on clinical efficacy and safety have been conducted in humans[12,25]. Although the hydrophilic fraction of BMFE did not have an effect on insulin secretion in the present study, other studies have shown that an aqueous extract of bitter melon fruit and hydrophilic compounds, such as polypeptide-p, has hypoglycaemic activity[12,26,27]. Future studies and clinical trials should focus on the identification of compounds which stimulate insulin secretion, as well as the extractives of bitter melon fruit, as it may be helpful in its application as a therapeutic agent.

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Author's responsibilities were as follows: T. S. and E. M. contributed to the conception and design of the research, data analysis, interpretation of data and writing of the manuscript. F. K., D. R. D., A. K. and T. N. contributed to data analysis and interpretation of data. T. O. and M. N. contributed to data analysis, interpretation of data and critical revisions of the manuscript for important intellectual content. All authors approved the final version of the manuscript.

The authors declare no conflicts of interest.

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British Journal of Nutrition

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