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Mediation of β-endorphin by myricetin to lower plasma glucose in streptozotocin-induced diabetic rats

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Abstract

Streptozotocin-induced diabetic (STZ-diabetic) rats were employed to investigate the mechanism(s) whereby myricetin, the active principle of *Abelmoschus moschatus* (Malvaceae), exerts its glucose-lowering effects. Myricetin was purified from the aerial portion of the plant and administered intravenously. A dose-dependent decrease in plasma glucose concentration was observed 30 min following injection, in parallel with increased plasma β-endorphin-like immunoreactivity (BER). Myricetin enhanced BER release similarly from isolated adrenal medulla. Plasma glucose-lowering and BER-elevating effects of myricetin were both eliminated after bilateral adrenalectomy. Myricetin failed to lower plasma glucose after treatment with opioid μ-receptor antagonists and in opioid μ-receptor knockout diabetic mice. Injection of myricetin three times daily for three consecutive days resulted in increased expression of the glucose transporter subtype 4 (GLUT 4) in soleus muscle and in reduced expression of phosphoenolpyruvate carboxykinase (PEPCK) in liver; these inductions were preventable by opioid μ-receptor blockade. Findings support the conclusion that the plasma glucose-lowering action of myricetin in insulin-deficient animals is mediated by activation of opioid μ-receptors of peripheral tissues in response to increased β-endorphin secretion. Opioid μ-receptor activation is held responsible for the enhancement of muscle GLUT 4 gene expression and the attenuation of hepatic PEPCK gene expression observed in these myricetin-treated diabetic animals.

Keywords: β-Endorphin; Myricetin; Opioid μ-receptors; STZ-diabetic rats

1. Introduction

Diabetes mellitus, which ranks among the top 10 causes of mortality world-wide, is a group of metabolic diseases characterized by abnormally high concentrations of glucose in blood and/or urine. In addition to neurological complications and premature death, consequences of these disorders include vascular complications such as coronary artery disease, cerebrovascular disorders, renal failure, blindness, and limb amputation (Jeffcoate, 2004). Dietary restrictions, exercise, and administration of oral glucose-lowering agents are applied widely to control blood glucose concentrations as tightly as possible (Lindstrom et al., 2003). Additionally, the use of herbal and other alternative medicines for treatment of diabetic disorders has recently increased.

Myricetin (3,5,7,3′,4′,5′-hexahydroxyflavone), a naturally occurring flavonol, is commonly found in tea, berries, fruits, and the herb named *Abelmoschus moschatus* Medic. (Malvaceae). This flavonol, which has been shown to possesses both antioxidative and cytoprotective properties (Mira et al., 2002; Dajas et al., 2003), has been applied successfully for the treatment of depression and anxiety in traditional Chinese medicine (Bown, 1995). Furthermore, myricetin has been reported to be of therapeutic benefit for cardiovascular diseases associated with diabetes mellitus (Ong and Khoo, 1997; Knekt et al., 2002). An insulinomimetic effect of myricetin on lipogenesis and glucose transport has been observed in adipocytes of rats with non-insulin-dependent diabetes mellitus (Ong and Khoo, 1996). In studies from this laboratory involving the streptozotocin-induced diabetic (STZ-diabetic) rat, a type-1 diabetes-like animal model, myricetin was found to lower plasma glucose through improved glucose utilization (Liu et

Abbreviations: BER, β-endorphin-like immunoreactivity; GLUT 4, glucose transporter subtype 4; i.v., intravenous; PEPCK, phosphoenolpyruvate carboxykinase; STZ-diabetic rats, streptozotocin-induced diabetic rats

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al., 2005). Myricetin was therefore proposed to be useful for treatment of certain diabetic disorders in humans. However, the mechanism(s) whereby myricetin exerts its effects on glucose homeostasis are not currently understood.

Exogenous β-endorphin is reported to promote an increase in circulating insulin in humans with or without diabetes mellitus (Curry and Li, 1987). However, the effects of β-endorphin or other opioid agonists on glucose homeostasis may not depend on insulin action. For example, β-endorphin was observed in this laboratory to increase glucose utilization via opioid μ-receptor activation and to lower plasma glucose in STZ-diabetic rats (Cheng et al., 2002). The present study was therefore undertaken to ascertain whether β-endorphin mediates the plasma glucose-lowering action of myricetin in diabetic animals lacking insulin.

2. Materials and methods

2.1. Animal models

Male Wistar rats weighing 200–250 g were obtained from the Animal Center of the National Cheng Kung University Medical College. Male BDF1 mice (as wild-type) and opioid μ-receptor knockout BDF1 mice (Loh et al., 1998) were obtained from Professor H.H. Loh. Both mouse strains were bred in the same animal center and were used at 8–10 weeks of age. Diabetes was induced in male rats by an intravenous (i.v.) injection of STZ (60.0 mg/kg; Sigma–Aldrich, Inc., St. Louis, MO, USA). Mice with or without opioid μ-receptors also received an intraperitoneal (i.p.) injection of STZ at 50.0 mg/kg to induce diabetes as described previously (Liu et al., 2001). Animals were considered to be diabetic if they had plasma glucose concentrations of 20 mmol/l or greater and exhibited polyuria in addition to other diabetic features. Plasma insulin concentrations of STZ-diabetic rats were reduced to 1.20 ± 0.7 pmol/l ($n = 8$) following STZ injection, a value markedly lower than that of vehicle-treated rats (158.7 ± 6.4 pmol/l; $n = 8$). These animals were considered to have type-1 diabetes mellitus. All studies were carried out 2 weeks after the injection of STZ. All animal procedures were performed according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, as well as the guidelines of the Animal Welfare Act.

2.2. Effect of myricetin on plasma concentrations of glucose and β-endorphin in STZ-diabetic rats

Abelmoschus moschatus purchased from the folk medicinal drug store in Ping-Tung, Taiwan in August of 2002 was identified by Dr. Hsien-Chang Chang (Division of Pharmacognosy, National Laboratories of Food and Drugs, Department of Health, Taiwan). A voucher specimen was deposited at the School of Pharmacy, Taipei Medical University (no. TMU 22082002). The powder of myricetin (purity = 98.6%) was extracted from the aerial portion of *Abelmoschus moschatu* as described previously (Liu et al., 2005). The powder was dissolved in 70% ethanol to serve as the stock solution. The vehicle (70% ethanol:saline, 1:19) used to prepare experimental myricetin solutions was found to have no effect on plasma glucose concentrations of STZ-diabetic rats (Liu et al., 2005). After an overnight fast, STZ-diabetic rats received an i.v. injection of myricetin at the desired doses. Animals were anesthetized with sodium pentobarbital (30.0 mg/kg, i.p.), and blood samples (0.1 ml) were collected from the tail vein for measurement of plasma glucose concentrations and plasma β-endorphin-like immunoreactivity (BER) as described below. In a previous study (Liu et al., 2005), a 1.0 mg/kg dose of myricetin was found to produce the maximal plasma glucose-lowering effect (reduction of $23.5 \pm 3.4\%$) in STZ-diabetic rats 30 min after a single i.v. injection. The effects of myricetin on plasma BER were therefore determined using blood samples collected at 30 min after a single i.v. injection. STZ-diabetic rats that received an i.v. injection of vehicle only were used as controls. Naloxone (Research Biochemicals, Inc., Natick, MA) or naloxonazine (Research Biochemicals, Inc.) was selected for experiments involving antagonism of opioid μ-receptor activation. These inhibitors were injected by the i.v. route into fasting rats 30 min prior to the i.v. injection of myricetin.

2.3. Effect of myricetin on plasma concentrations of glucose and β-endorphin in opioid μ-receptor knockout diabetic mice

Fasting STZ-diabetic mice with or without opioid μ-receptors were given an i.v. injection of myricetin at 1.0 mg/kg, the dose associated with maximal plasma glucose-lowering action in STZ-diabetic rats (Liu et al., 2005). After 30 min, blood samples (0.1 ml) were collected from the lower eye lids of mice under anesthesia with pentobarbital (30.0 mg/kg, i.p.) using a chilled syringe containing 10 IU heparin. Measurements of plasma glucose and BER concentrations were then performed as described below.

2.4. Isolation and incubation of adrenal medulla from STZ-diabetic rats

Adrenal glands were removed quickly from STZ-diabetic rats following sacrifice, and medullae were dissected immediately after removal of the cortex as previously described (Hwang et al., 2005). The tissues were cut into slices approximately 1 mm thick, and slices were transferred to glass tubes fitted at the bottom with nylon mesh to permit free interchange of components with the medium. The tissues were incubated for 15 min at 37 °C, pH 7.4, under aeration with 95% O_2/5% CO_2 and with continuous shaking in 2 ml of modified Krebs solution (MKS) ((mmol/l): NaCl, 118; KCl, 4.7; $MgCl_2$, 1.2; NaH_2PO_4, 1.0; $CaCl_2$, 2.5; EDTA-Na, 0.004; dextrose, 11.1; $NaHCO_3$, 25.0; and ascorbic acid, 0.11). The tissues were then incubated with myricetin at the concentrations indicated at 37 °C for 30 min with continuous shaking at 40 cycles/min. The incubation was terminated by placing the tubes on ice. The medium from each incubation sample was collected and stored at −70 °C until the β-endorphin assay was performed.

2.5. Adrenalectomized rats

Wistar rats to be adrenalectomized were fed standard rat chow and 0.9% sodium chloride in their drinking water ad libitum prior to surgery. Wistar rats to receive a sham operation (controls) were fed standard rat chow and water ad libitum prior to surgery. Bilateral adrenalectomy was performed using the dorsal approach under pentobarbital anesthesia (30.0 mg/kg, i.p.) as described previously (Hwang et al., 2005). Animals were allowed to recover for 2 weeks after the operations. The animals appeared alert and in good health. Following recovery, diabetes was induced by an injection of STZ as described above. To determine the effects of myricetin (1.0 mg/kg) on plasma glucose concentrations, blood samples were collected 30 min after a single i.v. injection of the preparation as described above.

2.6. Laboratory determinations

Plasma glucose concentration was determined according to the glucose oxidase method using an analyzer (Quik-Lab, Ames, Miles Inc., Elkhart, Indiana, USA). The enzyme-linked immunosorbent assay for the determination of BER present either in plasma or in the adrenal medulla incubation medium was conducted using a commercially available kit (Peninsula Laboratories, Inc., CA, USA).

2.7. Determinations of GLUT 4 and PEPCK gene expression

STZ-diabetic rats were injected i.v. every 8 h (three times daily) into the tail vein with vehicle, with myricetin (1.0 mg/kg), or with myricetin (1.0 mg/kg) plus naloxonazine (10.0 μg/kg). Naloxonazine was administered 30 min before the injection of myricetin. In preliminary experiments, myricetin was found to modify concentrations of the mRNA and protein for both the glucose transporter subtype 4 (GLUT 4) and hepatic phosphoenolpyruvate carboxykinase (PEPCK) in STZ-diabetic rats to significant degrees after 3 days of treatment. Animals were therefore sacrificed after 3 days of treatment. Rats receiving vehicle only served as controls. After the final treatment, no fasting period was imposed and animals were sacrificed immediately. Liver and soleus muscle were removed rapidly, frozen in liquid nitrogen, and stored at −70 °C prior to Northern and Western blot analyses. Blood samples were also collected from the tail vein prior to sacrifice for measurements of plasma glucose concentrations and BER as described above.

2.8. Northern blot analyses

Total RNA was extracted from soleus muscle or liver of experimental animals using the Ultraspec™-II RNA extraction system (Bioteck, Houston, TX, USA). The RNA (20 μg) was denatured in a solution containing 2.2 mmol/l formaldehyde and 50% formamide (v/v) at 55 °C for 15 min. Aliquots of total RNA were size-fractionated in a 1.2% agarose/formaldehyde gel. Gels were stained with ethidium bromide to identify the positions of the 18S and 28S rRNA subunits and to confirm that equivalent amounts of undegraded RNA had been loaded. The RNA then was transferred to a Hybond-N membrane (Amersham, Bucks, UK). GLUT 4 and PEPCK mRNA were detected using full-length cDNA probes radioactively labeled by the random primer method and were hybridized under stringent conditions. Intensity of the mRNA bands on the blot was quantified by scanning densitometry (Hoefer, San Francisco, CA, USA). β-Actin was used as an internal standard.

2.9. Western blot analysis

After homogenization with a glass/Teflon homogenizer, homogenates (50 μg) of muscle or liver were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Western blot analyses were performed using either anti-rat GLUT 4 antibody (1:1000; Genzyme Diagnostics, Cambridge, USA) for soleus muscle samples or anti-rat PEPCK antibody (1:1000) for liver samples. To ensure that equal amounts of protein were loaded into each lane of the gel, blots were also incubated with a goat polycolonal actin antibody (1:500; Santa Cruz Biotechnology, Inc., California, USA) or a mouse monoclonal β-tubulin antibody (1:500; Zymed Laboratories, Inc., San Francisco, California, USA). Blots were then treated with the appropriate peroxidase-conjugated secondary antibodies. Following removal of the secondary antibody, blots were washed and developed using the ECL-Western blotting system. Densities of the obtained immunoblots at 45 kDa for GLUT 4, 69.5 kDa for PEPCK, 43 kDa for actin and 50 kDa for β-tubulin were quantified using laser densitometry.

2.10. Statistical analyses

The plasma glucose-lowering activity of myricetin was calculated as the percentage decrease of the initial glucose value according to the formula: $(G_i - G_t)/G_i \times 100\%$, where G_i is the initial glucose concentration and G_t is the plasma glucose concentration after treatment of myricetin.

Data are expressed as the mean ± S.E.M. for the number (n) of animals in each treatment group as indicated in legends to tables and figures. Repeated measurement of analysis of variance (ANOVA) was used to analyze the changes in plasma glucose and other parameters. The Dunnett range post hoc comparisons were used to determine the source of significant differences where appropriate. A P-value <0.05 was considered statistically significant.

3. Results

3.1. Plasma glucose and β-endorphin-like immunoreactivity of myricetin-treated STZ-diabetic rats

The effects of administration of myricetin at varying doses on plasma BER and glucose concentrations of STZ-diabetic rats are shown in Fig. 1. A dose-dependent increase in BER was observed at 30 min following injection, in parallel with a dose-dependent decrease in plasma glucose concentration. When administered at 1.0 mg/kg, myricetin increased

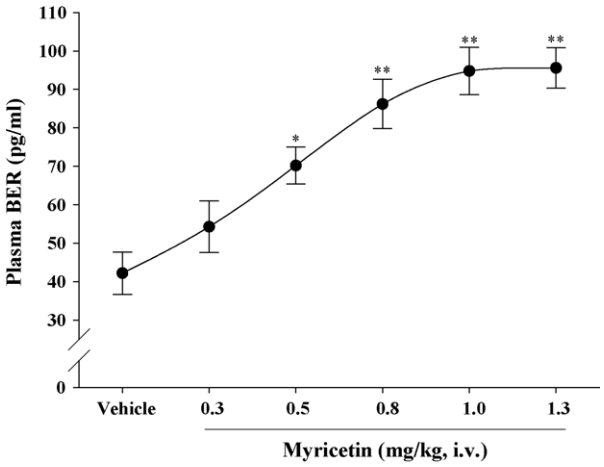

Fig. 1. Changes of plasma β-endorphin-like immunoreactivity (BER) in STZ-diabetic rats receiving an intravenous (i.v.) injection of myricetin. Values (mean ± S.E.M.) were obtained from each group of eight animals. The vehicle used to dissolve myricetin was given at the same volume. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. data from animals treated with vehicle.

the plasma BER value from 42.2 ± 5.5 to 94.8 ± 6.2 pg/ml and decreased the plasma glucose concentration from 24.2 ± 2.7 to 18.4 ± 3.1 mmol/l. Under these conditions, the plasma glucose-lowering activity of myricetin was found to be approximately 23.8 ± 3.4%.

3.2. Secretion of β-endorphin-like immunoreactivity from the adrenal medulla of myricetin-treated STZ-diabetic rats

The release of BER from adrenal medulla prepared from STZ-diabetic rats treated with vehicle or with myricetin at varying doses is illustrated in Fig. 2. Spontaneous secretion of BER was 86.4 ± 7.3 pg/mg protein, a value that was not changed by administration of vehicle. The amount of BER in the medium increased significantly in response to myricetin treatment, and this increase was concentration-dependent at doses between

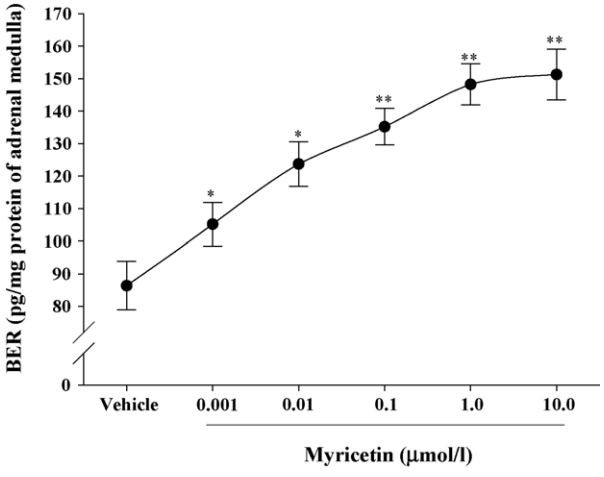

Fig. 2. Release of β-endorphin-like immunoreactivity (BER) from adrenal medulla isolated from myricetin-treated STZ-diabetic rats. Results expressed as pg/mg protein are the means ± S.E.M. of seven determinations. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. data from samples treated with vehicle, respectively.

Table 1
Effects of bilateral adrenalectomy on myricetin-induced changes in plasma glucose concentrations and β-endorphin-like immunoreactivity (BER) in STZ-diabetic rats

	STZ-diabetic rats	
	Adrenalectomized	Sham-operated
Plasma glucose (mmol/l)		
Basal	23.9 ± 1.8	24.2 ± 2.1
Vehicle	23.8 ± 2.0	24.1 ± 1.8
Myricetin (1.0 mg/kg, i.v.)	23.4 ± 2.1	18.4 ± 1.9**
Plasma BER (pg/ml)		
Basal	49.1 ± 4.1	44.3 ± 4.2
Vehicle	48.6 ± 4.5	44.8 ± 3.9
Myricetin (1.0 mg/kg, i.v.)	50.1 ± 4.4	93.2 ± 5.5**

Samples were taken 30 min following administration of myricetin or vehicle. Basal values were obtained from fasted animals receiving neither myricetin nor vehicle. Eight animals were used for each treatment condition, and values are expressed as the mean ± S.E.M.
** $P < 0.01$ compared with the respective basal value.

0.001 and 10.0 μmol/l myricetin. At 1.0 μmol/l, myricetin increased the BER of the medium to 148.2 ± 6.3 pg/mg protein with no further stimulatory effect observed at higher drug concentrations.

3.3. Effects of myricetin on adrenalectomized STZ-diabetic rats

The effects of bilateral adrenalectomy and sham operation on myricetin-dependent glucose lowering and BER release are shown in Table 1. Two weeks after performance of surgeries, basal plasma glucose concentrations and basal plasma BER did not differ significantly between the adrenalectomized and the sham-operated animals. Administration of vehicle to either group of animals had no effect on either of these parameters. By contrast, the decrease in plasma glucose concentrations and the increase in plasma BER in response to myricetin (1.0 mg/kg) in non-surgically treated animals were both abolished in the adrenalectomized rats but were retained in the sham-operated rats.

3.4. Effects of opioid μ-receptor antagonists on myricetin-induced plasma glucose lowering activity and BER in STZ-diabetic rats

The effects of pretreatment with an opioid μ-receptor antagonist on myricetin-induced plasma glucose-lowering activity and BER in STZ-diabetic rats are illustrated in Table 2. Following administration of 5 or 10.0 μg/kg of naloxone or naloxonazine, the plasma glucose-lowering action of myricetin (1.0 mg/kg) was almost fully eliminated. Lesser degrees of suppression of glucose-lowering activity were observed at 1.0 μg/kg of the antagonists. By contrast, myricetin-induced secretion of plasma BER was unaffected in animals pretreated with 1.0, 5.0 or 10.0 μg/kg of either antagonist. Neither naloxone nor naloxonazine at the highest doses tested had any significant effect on the basal plasma glucose concentrations of STZ-diabetic rats.

Table 2
Effects of opioid μ-receptor antagonists on myricetin-induced changes in plasma glucose concentrations and β-endorphin-like immunoreactivity (BER) in STZ-diabetic rats

	Plasma glucose (mmol/l)	Plasma BER (pg/ml)
Basal	24.1 ± 1.8	42.4 ± 6.5
Myricetin (1.0 mg/kg, i.v.)		
+Vehicle	18.3 ± 1.6**	94.2 ± 5.9**
+Naloxone (μg/kg, i.v.)		
1.0	19.8 ± 2.1*	93.8 ± 7.1**
5.0	21.5 ± 1.7	93.4 ± 6.2**
10.0	23.8 ± 2.0	92.6 ± 7.4**
+Naloxonazine (μg/kg, i.v.)		
1.0	20.1 ± 1.8*	94.0 ± 6.5**
5.0	22.8 ± 1.6	93.5 ± 7.2**
10.0	24.0 ± 2.1	92.8 ± 5.6**
Naloxone (10.0 μg/kg, i.v.)	24.3 ± 2.2	94.1 ± 6.0**
Naloxonazine (10.0 μg/kg, i.v.)	24.6 ± 2.3	93.9 ± 7.3**

Antagonist or vehicle was injected 30 min prior to administration of myricetin or vehicle. Basal values were obtained from fasted animals receiving vehicle only. Eight animals were used for each treatment condition, and values are expressed as the mean ± S.E.M.
* $P < 0.05$ compared with the respective basal value.
** $P < 0.01$ compared with the respective basal value.

3.5. Effects of myricetin on plasma glucose and β-endorphin-like immunoreactivity in opioid μ-receptor knockout diabetic mice

The plasma glucose concentrations and BER of wild-type diabetic and opioid μ-receptor knockout diabetic mice were examined before and after treatment with myricetin (1.0 mg/kg) or vehicle (Table 3). Resting plasma glucose and BER values for both strains of diabetic mice were comparable to values obtained in diabetic rats. Wild-type mice responded to myricetin, but not to vehicle, with a lowering of plasma glucose. The plasma glucose-lowering activity of myricetin was approximately 23.8 ± 2.9% in diabetic mice with opioid μ-receptors, an activity similar to that produced in diabetic rats. By contrast, the plasma glucose concentrations of opioid μ-receptor knockout diabetic mice were not altered by myricetin. Both strains of mice responded to myricetin with an enhancement of BER. For wild-type diabetic mice, this enhancement occurred concurrently with the decline in plasma glucose concentrations.

Table 3
Plasma glucose concentrations and β-endorphin-like immunoreactivity (BER) in opioid μ-receptor-knockout and wild-type diabetic mice following injection of myricetin

	Diabetic mice	
	Wild-type	Opioid μ-receptor-knockout
Plasma glucose (mmol/l)		
Basal	23.9 ± 2.4	24.8 ± 2.1
Vehicle	23.7 ± 2.1	24.9 ± 1.9
Myricetin (1.0 mg/kg, i.v.)	18.2 ± 1.7**	24.2 ± 2.3
Plasma BER (pg/ml)		
Basal	41.6 ± 4.2	47.6 ± 3.8
Vehicle	43.2 ± 3.8	48.2 ± 5.2
Myricetin (1.0 mg/kg, i.v.)	94.2 ± 5.2**	95.4 ± 4.7**

Samples were taken 30 min following administration of myricetin or vehicle. Basal values were obtained for fasted animals receiving vehicle. Eight animals were used for each treatment condition, and values are expressed as the mean ± S.E.M.
** $P < 0.01$ compared with the respective basal value.

3.6. Plasma glucose concentrations and β-endorphin-like immunoreactivity in response to repeated injections of myricetin

Plasma glucose concentrations and BER of STZ-diabetic rats were also measured following repeated treatments with myricetin (1.0 mg/kg). Following injections of drug three times daily for three consecutive days, plasma glucose concentrations decreased to 17.6 ± 1.6 mmol/l. This value was significantly lower as compared with that for vehicle-treated STZ-diabetic rats (23.6 ± 1.8 mmol/l; $P < 0.01$) and represented a plasma glucose-lowering activity of 25.5 ± 2.2%. A concurrent increase in plasma BER to 96.8 ± 5.6 pg/ml was observed; this value was significantly higher than that observed with the vehicle-treated group (48.4 ± 5.2 pg/ml; $P < 0.01$). Neither feeding behavior nor body weight was affected by the repeated myricetin treatments. Naloxonazine (10.0 μg/kg) antagonized the effects of repeated myricetin treatments on BER such that values (51.2 ± 5.8 pg/ml) approached those due to treatment with vehicle (48.6 ± 6.1 pg/ml). The opioid μ-receptor antagonist also abolished the effects of repeated myricetin treatment on plasma glucose concentrations in these diabetic rats.

3.7. Effects of myricetin on expression of hepatic PEPCK in STZ-diabetic rats

Relative changes in expression of hepatic PEPCK mRNA and protein as a consequence of diabetes induction and myricetin treatment are illustrated in Fig. 3. PEPCK mRNA and protein values were increased approximately three-fold in livers of STZ-diabetic as compared with non-diabetic animals (Fig. 3A). After treatment of STZ-diabetic rats repeatedly with myricetin (1.0 mg/kg, three times daily for 3 days), hepatic PEPCK mRNA was reduced to approximately 38% of that seen in these rats in response to vehicle (Fig. 3A). These effects of myricetin were abolished in the presence of naloxonazine (10.0 μg/kg). Repeated myricetin treatments reduced hepatic PEPCK protein values in STZ-diabetic rats in parallel with PEPCK mRNA such that near-basal values were obtained (Fig. 3B). These effects of repeated myricetin treatment were also eliminated in the presence of naloxonazine. Quantification of PEPCK mRNA and protein values before and after treatments is presented in Table 4.

3.8. Effects of myricetin on expression of GLUT 4 in soleus muscle of STZ-diabetic rats

Relative changes in expression of soleus muscle GLUT 4 mRNA and protein as a consequence of diabetes induction and myricetin treatment are illustrated in Fig. 4. The value for

Table 4
Quantification of hepatic PEPCK mRNA, hepatic PEPCK protein, soleus muscle GLUT 4 mRNA, and soleus muscle GLUT 4 protein in non-diabetic and STZ-diabetic rats receiving vehicle and in STZ-diabetic rats receiving repeated treatment with myricetin alone or combined with naloxonazine

Groups	PEPCK (arbitrary units)		GLUT 4 (arbitrary units)	
	mRNA/β-actin	Protein/β-tubulin	mRNA/β-actin	Protein/actin
Non-diabetic rats				
+Vehicle	0.39 ± 0.04	0.53 ± 0.07	1.88 ± 0.06	1.28 ± 0.05
STZ-diabetic rats				
+Vehicle	1.17 ± 0.07**	1.51 ± 0.05**	0.70 ± 0.05**	0.38 ± 0.04**
+Myricetin	0.44 ± 0.06	0.47 ± 0.06	1.82 ± 0.07	1.33 ± 0.06
+Naloxonazine	1.21 ± 0.07**	1.38 ± 0.09**	0.84 ± 0.08**	0.56 ± 0.07**

Five animals were used for each treatment condition, and values are expressed as the mean ± S.E.M. Vehicle and myricetin were administered at equivalent volumes. STZ-diabetic animals received myricetin (1.0 mg/kg, i.v.) three times daily for 3 days. Naloxonazine was administered i.v. at 10.0 μg/kg, 30 min prior to myricetin.
** $P < 0.01$ compared to vehicle-treated non-diabetic rats.

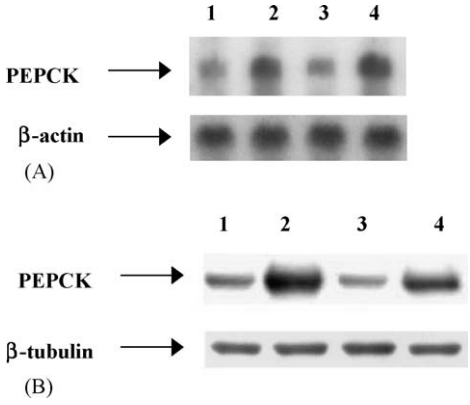

Fig. 3. Relative changes in expression of hepatic PEPCK mRNA and protein as a consequence of diabetes induction and myricetin treatment. (A) Northern blot analysis of the mRNAs for PEPCK and β-actin in livers isolated from non-diabetic and STZ-diabetic rats after receiving treatment with either myricetin alone or myricetin with naloxonazine, three times daily for 3 days. (B) Western blot analysis of PEPCK and β-tubulin in livers isolated from same groups of rats. Naloxonazine was injected into the veins of animals 30 min before injection of myricetin. The vehicle used to dissolve the testing drugs was given at equivalent volumes. Lane 1: vehicle-treated non-diabetic rats; lane 2: vehicle-treated STZ-diabetic rats; lane 3: myricetin (1.0 mg/kg)-treated STZ-diabetic rats; lane 4: myricetin (1.0 mg/kg) plus naloxonazine (10.0 μg/kg)-treated STZ-diabetic rats. Findings have been reproduced on four separate occasions. Quantification of the data is presented in Table 4.

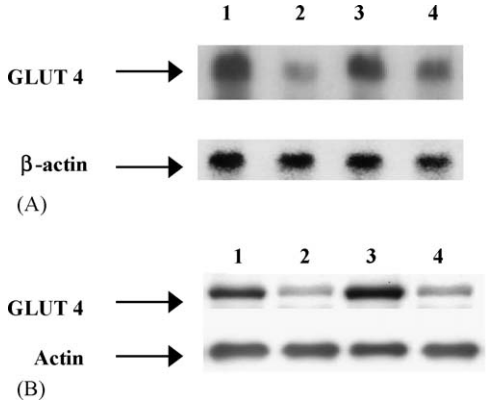

Fig. 4. Relative changes in expression of soleus muscle GLUT 4 mRNA and protein as a consequence of diabetes induction and myricetin treatment. (A) Northern blot analysis of the mRNAs for GLUT 4 and β-actin in soleus muscle isolated from non-diabetic and STZ-diabetic rats after receiving treatment with either myricetin alone or myricetin with naloxonazine, three times daily for 3 days. (B) Western blot analysis of GLUT 4 and actin in soleus muscle isolated from same groups of rats. Naloxonazine was injected into the veins of animals 30 min before an injection of myricetin. The vehicle used to dissolve the testing drugs was given at equivalent volumes. Lane 1: vehicle-treated non-diabetic rats; lane 2: vehicle-treated STZ-diabetic rats; lane 3: myricetin (1.0 mg/kg)-treated STZ-diabetic rats; lane 4: myricetin (1.0 mg/kg) plus naloxonazine (10.0 μg/kg)-treated STZ-diabetic rats. Findings have been reproduced on four separate occasions. Quantification of the data is presented in Table 4.

GLUT 4 mRNA in soleus muscle isolated from STZ-diabetic rats was approximately 35% of that for non-diabetic rats (Fig. 4A). Similarly, GLUT 4 protein in soleus muscle of STZ-diabetic rats was reduced to approximately 30% of that of non-diabetic rats (Fig. 4B). Repeated treatment of STZ-diabetic rats with myricetin (1.0 mg/kg; three times daily for 3 days) resulted in an elevation of GLUT 4 gene expression in soleus muscle to values approaching those of non-diabetic rats (Fig. 4A and B). The effects of myricetin on GLUT 4 mRNA and protein expression were blocked by naloxonazine (10.0 μg/kg; Fig. 4A and B). Quantification of GLUT 4 mRNA and protein values before and after treatments is presented in Table 4.

4. Discussion

The present study reveals that myricetin promotes an increase in plasma BER concurrently with a lowering of plasma glucose concentrations when administered to STZ-diabetic animals. Both effects of myricetin are dose-dependent and are observed over comparable dose ranges. Pancreatic β-cells of STZ-diabetic rats are established to be insulin-deficient (Wohaieb and Godin, 1987). The plasma glucose-lowering and BER-elevating actions of myricetin, therefore, are concluded to occur independently of insulin. As illustrated by the findings of the present study, STZ-diabetic rats proved a suitable model to explore the possibility that increased BER and the reduction of plasma glucose concentrations are related phenomena in diabetic subjects with treated with myricetin.

Splanchnic nerve stimulation is known to promote the release of opioids from the adrenal gland into the adrenal vein (Hanbauer et al., 1982). In previous studies from this laboratory (Hwang et al., 2005), secretion of opioids from the adrenal gland was observed to foster a decrease in plasma glucose in STZ-diabetic

rats. This observation is consistent with the view that endogenous opioids can be released into the blood stream from glands other than the pituitary (Vargo et al., 1977). Bilateral adrenalectomy was therefore performed in the present study to verify that the source of the increased plasma BER observed in STZ-diabetic rats in response to myricetin was the adrenal gland. Accordingly, the plasma glucose-lowering activity of myricetin and the increased BER observed in response to the drug in non-adrenalectomized animals were both abolished by adrenalectomy. These observations are consistent with the proposal that secretion of endogenous β-endorphin from the adrenal gland is involved in the plasma glucose-lowering action of myricetin. To test this hypothesis, the effects of myricetin on the secretion of β-endorphin were investigated in adrenal medulla isolated from STZ-diabetic rats. Myricetin enhanced β-endorphin secretion from the isolated adrenal medulla in a concentration-dependent manner. Secretion of endogenous β-endorphin from the adrenal gland is therefore concluded to be essential to the plasma glucose-lowering action of myricetin in STZ-diabetic rats.

Many of the physiologic actions of endogenous β-endorphin are recognized to be mediated by opioid μ-receptors (Pasternak, 1993). Opioid μ-receptor blockade was therefore employed to evaluate the involvement of these receptors in the actions of myricetin in STZ-diabetic rats. The ability of myricetin to lower plasma glucose was suppressed by blockade of opioid μ-receptors with naloxone or naloxonazine. By contrast, neither antagonist was found to modify myricetin-induced secretion of plasma BER. These findings strongly implicate the activation of opioid μ-receptors through increased circulating β-endorphin concentrations in the plasma glucose-lowering action of myricetin in diabetic rats. The possibility existed, however, that the observed effects of naloxone and naloxonazine were exerted at sites other than the opioid μ-receptor. Opioid μ-receptor knockout mice were therefore utilized to confirm the involvement of opioid μ-receptor activation in the action of myricetin. In contrast to wild-type diabetic mice possessing opioid μ-receptors, the plasma glucose-lowering action of myricetin in opioid μ-receptor knockout diabetic mice was abolished. The BER-elevating effects of myricetin were nonetheless retained in the knockout mice. These findings support the essential role of opioid μ-receptors in the plasma glucose-lowering action of myricetin in the insulin-deficient state. The elevation of BER in response to myricetin is concluded to promote opioid μ-receptor activation which, in turn, mediates the plasma glucose-lowering action of the drug. These actions are accomplished without the involvement of insulin.

In classical forms of diabetes, elevation of blood glucose is held to be the consequence of increased hepatic glucose output in concert with reduced peripheral glucose utilization (Consoli et al., 1989). PEPCK, which catalyzes a regulatory step in gluconeogenesis, is a key enzyme of hepatic carbohydrate metabolism (Consoli et al., 1989). Insulin deficiency is clearly associated with changes in hepatic metabolism including increased expression of PEPCK (Consoli et al., 1989). Additionally, decreased expression of skeletal muscle GLUT 4 was proposed previously (Berger et al., 1989) to mediate the reduction of insulin-mediated glucose uptake into skeletal muscle in diabetes. It was of interest, therefore, to ascertain whether myricetin exerted its glucose-lowering action in diabetic rats by overturning the diabetes-dependent reduction of GLUT 4 gene expression and/or increase in PEPCK expression. To provide ample time for alterations in gene expression to be exerted, STZ-diabetic rats received repeated myricetin treatments over 3 days. Under these conditions, the increase in hepatic PEPCK gene expression due to induction of diabetes was found to be attenuated by myricetin. The decrease in GLUT 4 expression due to induction of diabetes, similarly, was overturned by repeated myricetin treatments. These findings reveal that myricetin retains its glucose-lowering actions over extended time periods and that this drug alters the expression of hepatic PEPCK and muscle GLUT 4 in an insulin-independent manner.

In previous studies from this laboratory (Cheng et al., 2002) endogenous β-endorphin, via activation of opioid μ-receptors located on peripheral tissues, was found to serve as a positive regulator of glucose utilization and a negative modulator of hepatic gluconeogenesis in the insulin-deficient state. Opioid μ-receptor antagonists were therefore used to test the involvement of opioid μ-receptors in effects of myricetin on specific gene expression in diabetic rats. Suppression of PEPCK gene expression in STZ-diabetic rats by myricetin was blocked by naloxonazine. Furthermore, myricetin failed to elevate GLUT 4 mRNA and protein concentrations in the soleus muscle of STZ-diabetic rats in the presence of opioid μ-receptor antagonists. It is likely, therefore, that the normalizations of hepatic PEPCK and muscle GLUT 4 concentrations in STZ-diabetic rats following myricetin treatments are both mediated through an activation of opioid μ-receptors. These opioid μ-receptor-dependent actions of myricetin are anticipated to improve glucose utilization by peripheral tissues and to decrease hepatic gluconeogenesis, such that glucose concentrations are maintained at more desirable values in diabetic subjects. Myricetin clearly possesses the potential to serve as a valuable therapeutic intervention or as an attractive adjuvant for treatment of diabetic disorders. Further investigations with this interesting agent are therefore warranted.

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