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Plasma Glucose Lowering Mechanisms of Catalpol, an Active Principle from Roots of *Rehmannia glutinosa*, in Streptozotocin-Induced Diabetic Rats

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ABSTRACT: Catalpol is one of the active principles from roots of *Rehmannia glutinosa* Steud (Scrophulariaceae) that is widely used to treat diabetic disorders in Chinese traditional medicine using the name of Di-Huang, which is used to investigate the mechanisms for lowering of plasma glucose in streptozotocin-induced diabetic rats (STZ-diabetic rats). Catalpol decreased plasma glucose in a dose-related manner, and this action was reduced by pretreatment with naloxone or naloxonazine. An increase of plasma β-endorphin by catalpol was also observed in parallel. The plasma glucose lowering action of catalpol was deleted in bilateral adrenalectomized rats. Moreover, catalpol enhanced β-endorphin release from the isolated adrenal medulla of STZ-diabetic rats. Otherwise, plasma glucose lowering action of catalpol failed to produce in opioid μ-receptor knockout mice. Also, repeated administration of catalpol for 3 days in STZ-diabetic rats resulted in a marked reduction of phosphoenolpyruvate carboxykinase (PEPCK) expression in liver and an increased expression of glucose transporter subtype 4 (GLUT 4) in skeletal muscle. These effects were also reversed by blockade of opioid μ-receptors. Our results suggested that catalpol increased glucose utilization through increase of β-endorphin secretion from adrenal gland in STZ-diabetic rats.

KEYWORDS: β-Endorphin, blood glucose, catalpol, diabetes, opioid μ-receptor

INTRODUCTION

Diabetic disorders often lead to disability through the vascular complications of coronary artery disease and cerebrovascular disease, renal failure, blindness, and limb amputation in addition to neurological complications and premature death.[1,2] Recently, the management of diabetic disorders and its complications became an important health subject in clinics.[2] Dietary restrictions, exercise, and administration of oral glucose-lowering agents are applied widely to control blood glucose concentrations as tightly as possible.[3] Moreover, herbal supplements and other alternative medicines for treatment of diabetic disorders have gradually increased.

In Chinese traditional medicine, *Rehmannia glutinosa* named as Di-Huang was widely applied for handling of diabetic disorders [4,5] Catalpol is introduced as an active ingredient in the roots of *Rehmannia glutinosa*.[6] In recent years, catalpol showed a plasma glucose-lowering action in streptozotocin-induced diabetic rats (STZ-diabetic rats), the animal model used as type-1 like diabetes.[4] Thus, catalpol seems effective for handling of diabetic disorders. However, mechanism(s) for the effect of catalpol on glucose homeostasis remained obscure. Otherwise, mediation of β-endorphin or endogenous opioids in glucose lowering action of natural products has been mentioned.[7]

Actually, an activation of opioid μ-receptors by either exogenous β-endorphin or chemical agents, such as loperamide and tramadol,[8,9] might improve glucose homeostasis in diabetic rats lacking insulin. Thus, the present study is designed to clarify the role of β-endorphin in plasma glucose lowering action of catalpol in STZ-diabetic rats.

MATERIALS AND METHODS

Plant Materials. Catalpol (purity >98.6%) extracted from the roots of *Rehmannia glutinosa* Steud (Scrophulariaceae) as described previously[4] was obtained from Professor Hsu F.L., and voucher specimen (TMU27308) was deposited in the herbarium of the College of Pharmacy, Taipei Medical University, Taiwan.

Animal Models. Male Wistar rats weighing 250−300 g were obtained from the Animal Center of National Cheng Kung University Medical College. Male wild-type (BDF1 mice) and opioid μ-receptor knockout mice, 8−10 weeks of age, were obtained from Professor H. H.

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Loh (Department of Pharmacology, University of Minnesota Medical School, Minneapolis, MN).[10] The diet for animals in this study was the standard chow. The number of animals in each group was eight rats. STZ-diabetic rats were induced by intravenous injection (i.v.) of STZ (65 mg/kg) into Wistar rats. Mice with or without opioid μ-receptors received an intraperitoneal injection (i.p.) of STZ at 50 mg/kg to induce diabetes according to the previous method.[11] Animals were considered to be diabetic if they had plasma glucose concentrations of 20 mM or greater in addition to polyuria and other diabetic features. 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.

Laboratory Determinations. The determination of plasma glucose was carried out according to a previous study.[12] The concentration of plasma glucose was measured by the glucose oxidase method using an analyzer (Quik-Lab, Ames; Miles Inc., Elkhart, IN). The determination of BER in samples was carried out using a commercially available enzyme-linked immunosorbent assay (Peninsula Laboratories, Belmont, CA).

Effect on Plasma BER Level in STZ-Diabetic Rats. After fasting overnight, STZ-diabetic rats received an oral treatment of catalpol at the desired doses. It has been documented that rats receiving sodium pentobarbital showed no significant change in the parameters measured in plasma.[13] Thus, animals were anesthetized with sodium pentobarbital (35 mg/kg i.p.), and blood samples (0.1 mL) were collected from the femoral vein for measurement of plasma glucose concentrations and BER. In our previous study, catalpol at 0.1 mg/kg was found to produce the maximal plasma glucose-lowering effect in STZ-diabetic rats 30 min after intravenous injection. Thus, the effect of catalpol on plasma BER was determined using blood samples collected at 30 min after the treatment. STZ-diabetic rats that received an intravenous treatment of vehicle only (0.9% saline) were used as controls. Further experiments were performed with the pretreatment of inhibitors, the antagonists of opioid μ-receptors including naloxone (1 mg/kg) or naloxonazine (1.5 mg/kg) purchased from Tocris Cookson, Bristol, UK. These inhibitors were injected intravenously into rats 30 min before the treatment of catalpol.

Effect on Plasma Glucose Concentrations in Opioid μ-Receptor Knockout Diabetic Mice. Fasting STZ-diabetic opioid μ-receptor knockout mice and STZ-diabetic wild-type mice were employed to receive an intravenous treatment of 0.1 mg/kg catalpol as mentioned above. After 30 min, blood samples (0.1 mL) were collected from the lower eye lid of mice under anesthesia with pentobarbital (30.0 mg/kg, i.p.) using a chilled syringe containing 10 IU of heparin. Next, blood samples were used to estimate the concentration of plasma glucose as described above.

Isolation and Incubation of Adrenal Medulla from STZ-Diabetic Rats. Adrenal glands were quickly removed from sacrificed STZ-diabetic rats, and medullae were immediately dissected after removal of cortex as described previously.[14] The tissues were cut into approximately 1 mm thick slices and transferred to a glass tube fitted with a mesh of nylon at the bottom to permit free interchange 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 (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. Next, the tissue was incubated with catalpol at the indicated concentrations at 37 °C for 30 min with continuous shaking at 40 cycles/min. Incubation was terminated by placing the tubes on ice. The medium from each incubation sample was collected and frozen at −80 °C until the β-endorphin assay was performed.

Adrenalectomy of STZ-Diabetic Rats. Bilateral adrenalectomy was performed using the dorsal approach under pentobarbital anesthesia (30 mg/kg, i.p.) as described previously.[14] 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. 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. The effect of catalpol at 0.1 mg/kg was determined using blood samples collected at 30 min after the treatment.

Determination of mRNA Level by Northern Blot Analysis. Northern blotting analysis was performed as described previously,[14] and the quantification was obtained from various individual experiments indicated in figure legends. Total RNA was extracted from soleus muscle or liver of experimental animals using the Ultraspec-II RNA extraction system. The concentration of RNA was measured using the absorbance at 260 nm. STZ-diabetic rats and normal rats received the oral treatment of catalpol at an effective dose (0.1 mg/kg), or with pharmacological inhibitors, such as opioid μ-receptor blocker (naloxonazine), three times daily. The inhibitor was injected intravenously into STZ-diabetic rats 30 min before the treatment of catalpol.

For Northern blot analysis, total RNA (40 μg) was denatured in a solution containing 2.2 mmol/L formaldehyde and 50% formamide (v/v) by heating at 55 °C for 15 min. Aliquots of total RNA were then size-fractionated in a 1.2% agarose/formaldehyde gel. Ethidium bromide staining was used to identify the position of the 18S and 28S rRNA subunits and to confirm that equivalent amounts of undegraded RNA had been loaded. The fractionated RNA was transferred to a Hybond-N membrane (Amersham Corp., Bucks, UK) and cross-linked by UV irradiation (Stratagene, CA). Probes were labeled with [γ-^{32}P]dCTP (New England Nuclear, Boston, MA) using the Medaprime labeling system kit (Amersham Corp., Bucks, UK). Hybridizations were carried out in medium containing denatured salmon sperm DNA (100 μg/mL) at 65 °C for 2 h. The membrane was washed twice for 20 min in 2 × sodium saline citrate (SSC)/0.1% SDS at room temperature and once for 20 min in 0.1 × SSC/0.1% SDS at 40 °C. Autoradiograms were prepared on Kodak X-ray (Rochester, NY, USA) film using a single enhancing screen at −80 °C. Intensities of the mRNA bands on the blot were quantified by scanning densitometry (Hoefer, San Francisco, CA,). The response of β-actin was used as an internal standard.

Western Blot Analysis. Western blotting analysis was also carried out as by our previous method,[14] and the quantification was obtained in the same manner. After homogenization of liver and soleus muscle using a glass/Teflon homogenizer, the homogenates (50 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and Western blot analysis was performed using either an antirat GLUT 4 antibody (1:1000) purchased from R&D Systems Inc. (Minneapolis, MN; Cat. #BAM1262) in soleus muscle or an antirat PEPCK antibody (1:1000) from Professor D. K. Granner (Vanderbilt University, Nashville, TN) in liver. The blots were probed with a goat polyclonal actin antibody (1:500) or a mouse monoclonal β-tubulin antibody (1:500) from Zymed Laboratories (South San Francisco, CA; Cat. # 32-2600) to ensure that the amount of protein loaded into each lane of the gel was constant. Blots were incubated with the appropriate peroxidase-conjugated secondary antibodies. After removal of the secondary antibodies, the 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 a laser densitometer.

Statistical Analysis. The plasma glucose-lowering activity of catalpol 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 catalpol. Data are expressed as the mean ± SEM for the number (n) of animals in the group as indicated in tables and figures. Differences among groups were analyzed by one-way ANOVA. The Dunnett range post hoc comparisons were used to determine the source of significant differences where appropriate. A p-value of 0.05 or less was considered statistically significant.

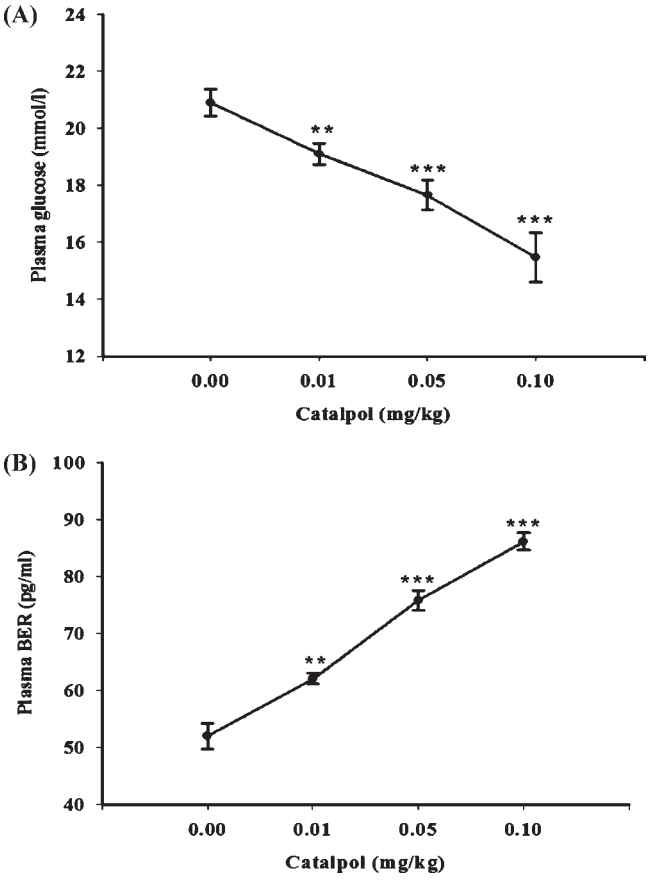

Figure 1. (A) The plasma glucose lowering activity produced by an intravenous (i.v.) injection of catalpol into STZ-diabetic rats. (B) Plasma β-endorphin-like immunoreactivity (BER) level in STZ-diabetic rats receiving i.v. injection of catalpol. Values of mean and bar of SEM were obtained from each group of eight rats. Vehicle only (0.9% NaCl in distilled water) was given at the same volume. **, $p < 0.01$ and ***, $p < 0.001$ versus data from animals treated with vehicle (0).

RESULTS

Effects of Catalpol on Plasma Glucose Concentration and Plasma β-Endorphin-like Immunoreactivity (BER) Level in STZ-Diabetic Rats. Thirty minutes after treatment, the plasma glucose-lowering activities were 8.5 ± 1.1%, 13.7 ± 1.6%, and 23.4 ± 2.3% in STZ-diabetic rats receiving i.v. injections of catalpol at 0.01, 0.05, and 0.1 mg/kg, respectively ($n = 8$). Catalpol at 0.1 mg/kg significantly decreased the plasma glucose concentration to 15.5 ± 0.9 mmol/L ($p < 0.001$; $n = 8$) (Figure 1A). Similar to our previous report,[4] the maximal plasma glucose lowering activity in STZ-diabetic rats was achieved using catalpol at 0.1 mg/kg, and increasing the dose to 0.15 mg/kg had no further effect. As positive control agent, metformin at an oral dose of 100 mg/kg decreased the plasma glucose and produced a lowering activity of 32.4 ± 8.8% ($n = 8$) in STZ-diabetic rats. Otherwise, a dose-dependent elevation of the plasma BER level was observed in the same group of STZ-diabetic rats after single i.v. injection of catalpol at doses ranging from 0.01 to 0.1 mg/kg (Figure 1B). Catalpol at 0.1 mg/kg increased the plasma BER level from basal level of 52.0 ± 2.2 to 86.1 ± 1.5 pg/mL in STZ-diabetic rats; no additional increase of plasma BER was observed using higher concentrations of catalpol. Also, metformin increased plasma BER from 49.6 ± 3.1 to 87.4 ± 2.7 pg/mL at an oral dose of 100 mg/kg in a way similar to a previous report.[15]

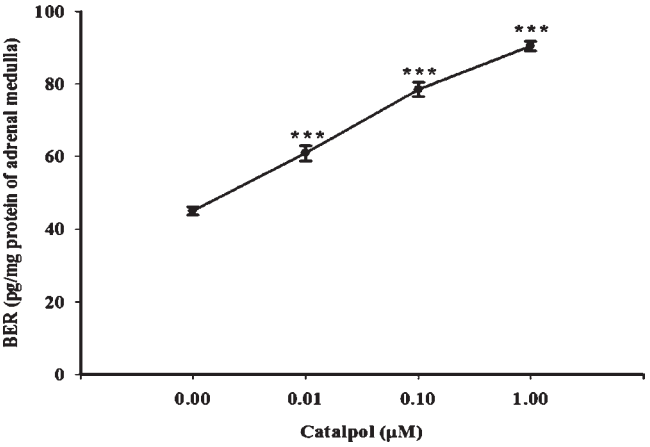

Figure 2. Effect of catalpol on β-endorphin-like immunoreactivity (BER) secretion from isolated adrenal medulla from STZ-diabetic rats. Results are expressed as pg/mg protein of adrenal medulla and represent the mean ± SEM of eight independent experiments. ***, $p < 0.001$ versus data from samples treated with vehicle (0).

Table 1. Effect of Adrenalectomy on the Catalpol-Induced Changes of β-Endorphin-like Immunoreactivity (BER) and Plasma Glucose Concentration in STZ-Diabetic Rats[a]

	adrenalectomized group	sham-operated group
plasma glucose (mmol/L)		
basal	18.7 ± 1.3	22.3 ± 0.9
vehicle	18.0 ± 2.0	21.8 ± 0.3
catalpol (0.1 mg/kg)	18.6 ± 2.3	16.3 ± 0.5**
plasma BER (pg/mL)		
basal	53.5 ± 1.7	55.1 ± 3.4
vehicle	52.2 ± 1.5	56.0 ± 2.2
catalpol (0.1 mg/kg)	52.6 ± 2.4	89.7 ± 2.9**

[a] Blood samples from STZ-diabetic rats receiving an intravenous injection of catalpol or a vehicle-treated control were used for determination of β-endorphin-like immunoreactivity (BER) and the plasma glucose concentration. Vehicle (0.9% NaCl in distilled water) was given at the same volume. Values (mean ± SEM) were obtained from each group of eight rats. Basal level shows the value from fasted animals without treatment. **$p < 0.01$ versus basal value in each group.

Effect of Catalpol on the Secretion of β-Endorphin-like Immunoreactivity (BER) from Adrenal Medulla Isolated from STZ-Diabetic Rats. Figure 2 shows the effect of catalpol on the secretion of BER from adrenal medulla isolated from STZ-diabetic rats. Catalpol increased the levels of BER in the culture medium in a concentration-dependent manner from 0.01 to 1.0 μmol/L.

Bilateral Adrenalectomy in STZ-Diabetic Rats Abolishes the Effect of Catalpol on Plasma Glucose and BER Levels. Bilateral adrenalectomy was performed in STZ-diabetic rats. Two weeks after adrenalectomy, there were no significant differences in the basal plasma levels of glucose and BER between adrenalectomized STZ-diabetic rats and sham-operated control (Table 1). However, the ability of catalpol to lower plasma glucose levels and to elevate plasma BER levels was abolished in STZ-diabetic rats with bilateral adrenalectomy but was unaltered in the sham-operated STZ-diabetic rats (Table 1).

Effects of Opioid μ-Receptor Antagonists on Catalpol-Induced Plasma Glucose Lowering Activity in STZ-Diabetic Rats. Table 2 shows the inhibitory effect of naloxone and naloxonazine on

Table 2. Effects of Opioid μ-Receptors Antagonists on the Catalpol-Induced Lowering of Plasma Glucose in STZ-Diabetic Rats[a]

	plasma glucose (mmol/L)
basal	20.1 ± 0.7
catalpol (0.1 mg/kg)	
+vehicle	15.5 ± 0.9**
+naloxone (1 mg/kg)	18.5 ± 0.6
+naloxonazine (1.5 mg/kg)	20.6 ± 0.6
naloxone (1 mg/kg)	19.0 ± 0.6
naloxonazine (1.5 mg/kg)	20.4 ± 0.8

[a] The antagonists were given by intravenous (i.v.) injection 30 min before the injection of catalpol. Vehicle (0.9% NaCl in distilled water) was given at the same volume. Values (mean ± SEM) were obtained from each group of eight rats. Basal level shows the value from fasted animals treated with vehicle. **$p < 0.01$ versus basal value, respectively.

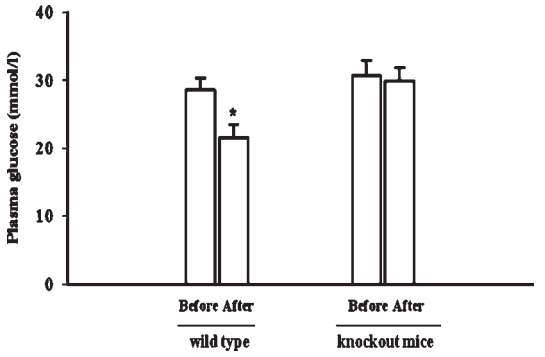

Figure 3. Effect of catalpol on plasma glucose in diabetic mice with (wild-type) or without (knockout) opioid μ-receptors. Values of mean and standard error (SE) bar were obtained for each group of eight mice. *, $p < 0.05$ as compared to data obtained from animals before treatment in each group.

the plasma glucose-lowering activity of catalpol in STZ-diabetic rats. In the presence of 1.0 mg/kg naloxone, the plasma glucose concentration when treated with 0.1 mg/kg catalpol was 18.5 ± 0.6 mmol/L, which was not statistically different from the basal level (20.1 ± 0.7 mmol/L). The effects of naloxonazine (1.5 mg/kg) were similar to those of naloxone; the plasma glucose level in naloxonazine (1.5 mg/kg)-pretreated STZ-diabetic rats before treatment with 0.1 mg/kg catalpol was 20.6 ± 0.6 mmol/L, a level near the basal level. Neither naloxone nor naloxonazine alone had any effect on basal plasma glucose levels in STZ-diabetic rats.

Effect of Catalpol on Plasma Glucose Levels in Opioid μ-Receptor Knockout Diabetic Mice. The plasma glucose level in opioid μ-receptor knockout diabetic mice was not significantly influenced by catalpol (from 30.7 ± 2.1 to 29.9 ± 1.9 mmol/L, $n = 8$, $p > 0.05$) (Figure 3). However, in the presence of opioid μ-receptors in diabetic mice, catalpol (0.1 mg/kg) decreased the plasma glucose from 28.6 ± 1.7 to 21.6 ± 1.9 mmol/L. The plasma glucose-lowering activity of catalpol in these wild-type diabetic mice was approximately 25.0 ± 2.5%, similar to the action of catalpol in STZ-diabetic rats.

Effects of Opioid μ-Receptor Antagonist on Catalpol-Induced Changes of mRNA and Protein Levels of GLUT 4 in Skeletal Muscle of STZ-Diabetic Rats. Treatment of STZ-diabetic rats with catalpol (0.1 mg/kg) three times daily for 3 days resulted in

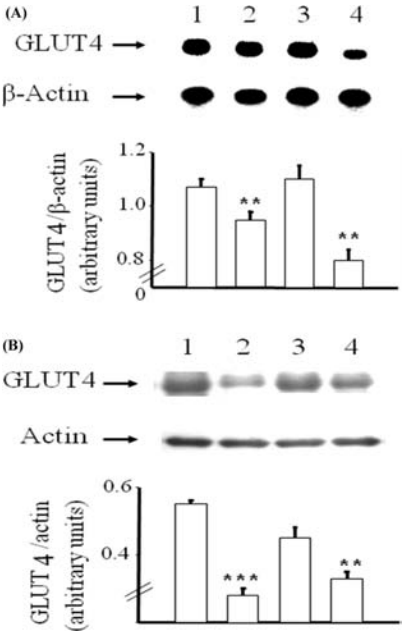

Figure 4. (A) Upper panel shows the representative response of mRNA level for GLUT 4 or β-actin in skeletal muscle isolated from STZ-diabetic rats receiving treatment with catalpol or catalpol and naloxonazine in combination, three times daily for 3 days. Lane 1, vehicle-treated Wistar rats; lane 2, vehicle-treated STZ-diabetic rats; lane 3, catalpol (0.1 mg/kg)-treated STZ-diabetic rats; lane 4, catalpol (0.1 mg/kg) plus naloxonazine (1.5 mg/kg)-treated STZ-diabetic rats. Quantification of mRNA level using GLUT 4/β-actin expressed as mean with standard error (SE) ($n = 4$ per group) in each column is indicated in the lower panel. **, $p < 0.01$ as compared to data obtained from lane 1. (B) Upper panel shows the representative response of protein level for GLUT 4 or actin in skeletal muscle isolated from STZ-diabetic rats receiving treatment with catalpol or catalpol and naloxonazine in combination, three times daily for 3 days. Lane 1, vehicle-treated Wistar rats; lane 2, vehicle-treated STZ-diabetic rats; lane 3, catalpol (0.1 mg/kg)-treated STZ-diabetic rats; lane 4, catalpol (0.1 mg/kg) plus naloxonazine (1.5 mg/kg)-treated STZ-diabetic rats. Quantification of protein level using GLUT 4/actin expressed as mean with standard error (SE) ($n = 4$ per group) in each column is indicated in the lower panel. **, $p < 0.01$ and ***, $p < 0.001$ as compared to data obtained from lane 1.

an elevation of GLUT 4 mRNA level in skeletal muscle. Western blot analysis showed a similar effect of catalpol (0.1 mg/kg) on the GLUT 4 protein level in skeletal muscle. Pretreatment of diabetic rats with naloxonazine (1.5 mg/kg) abolished the effect of catalpol on GLUT 4 mRNA (Figure 4A). Similarly, elevation of GLUT 4 protein level produced by catalpol was markedly reduced by the same treatment of naloxonazine in diabetic rats (Figure 4B). The data for quantification of GLUT4 mRNA and protein values were presented in Figure 4.

Effects of Opioid μ-Receptor Antagonist on Catalpol-Induced Changes of the mRNA and Protein Levels of Hepatic PEPCK in STZ-Diabetic Rats. In the present study, the mRNA level of PEPCK in the liver of STZ-diabetic rats was increased to about 6.5 fold of that in nondiabetic rats. The reduction of PEPCK mRNA by catalpol in diabetic rats was observed, and this action was also attenuated by naloxonazine (1.5 mg/kg) (Figure 5A). Similarly, the protein level of PEPCK in the liver of STZ-diabetic rats was raised to approximately 1.9 fold of that in nondiabetic rats. The protein level of PEPCK in diabetic rats was reversed by catalpol to the normal level, and the action could be abolished by naloxonazine (Figure 5B). The data for quantification of PEPCK mRNA and protein values were indicated in Figure 5.

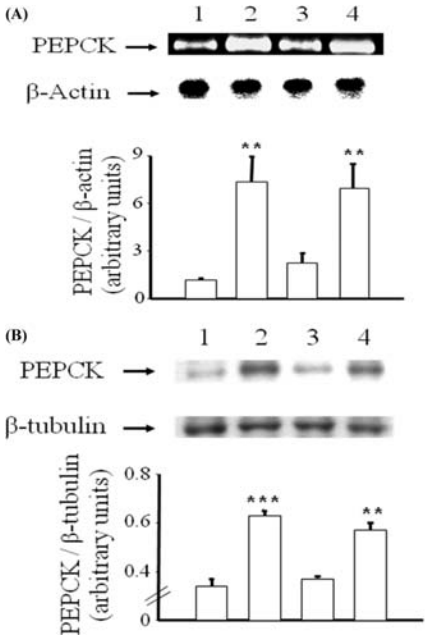

Figure 5. (A) Upper panel shows the representative response of mRNA level for PEPCK or β-actin in liver isolated from STZ-diabetic rats receiving treatment with catalpol or catalpol and naloxonazine in combination, three times daily for 3 days. Lane 1, vehicle-treated Wistar rats; lane 2, vehicle-treated STZ-diabetic rats; lane 3, catalpol (0.1 mg/kg)-treated STZ-diabetic rats; lane 4, catalpol (0.1 mg/kg) plus naloxonazine (1.5 mg/kg)-treated STZ-diabetic rats. Quantification of mRNA level using PEPCK/β-actin expressed as mean with standard error (SE) (n = 4 per group) in each column is indicated in the lower panel. **, $p < 0.01$ as compared to data obtained from lane 1. (B) Upper panel shows the representative response of protein level for PEPCK or β-tubulin in liver isolated from STZ-diabetic rats receiving treatment with catalpol or catalpol and naloxonazine in combination, three times daily for 3 days. Lane 1, vehicle-treated Wistar rats; lane 2, vehicle-treated STZ-diabetic rats; lane 3, catalpol (0.1 mg/kg)-treated STZ-diabetic rats; lane 4, catalpol (0.1 mg/kg) plus naloxonazine (1.5 mg/kg)-treated STZ-diabetic rats. Quantification of protein level using PEPCK/β-tubulin expressed as mean with standard error (SE) (n = 4 per group) in each column is indicated in the lower panel. **, $p < 0.01$ and ***, $p < 0.001$ as compared to data obtained from lane 1.

DISCUSSION

The present study found that catalpol promotes an increase of plasma BER concurrently with a reduction of plasma glucose in STZ-diabetic rats. Both effects of catalpol are dose-dependent and are observed over comparable dose ranges. STZ-diabetic rats are widely used as type-1 like diabetic animal model.[16] Therefore, the plasma glucose-lowering and BER-elevating actions of catalpol 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 when treated with catalpol.

Previous studies indicated that the secretion of opioids from the adrenal gland was observed to foster a decrease in plasma glucose in STZ-diabetic rats.[14] This observation is consistent with the view that endogenous opioids can be released into the bloodstream from glands other than the pituitary gland.[17] Therefore, bilateral adrenalectomy was performed in the present study to verify that the source of the increased plasma BER observed in STZ-diabetic rats in response to catalpol was the adrenal gland. As shown in Table 1, the plasma glucose-lowering activity of catalpol and the increased BER observed in response to catalpol in STZ-diabetic rats 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 catalpol. To confirm this hypothesis, the effects of catalpol on the secretion of β-endorphin were investigated by the adrenal medulla isolated from STZ-diabetic rats. Catalpol enhanced β-endorphin secretion from the isolated adrenal medulla in a concentration-dependent manner (Figure 2). Therefore, the action of catalpol on the adrenal gland is thought to be essential for the glucose-lowering effect in STZ-diabetic rats. Similar to our results, another hypoglycemic principle from same herb named as *Rehmannia glutinosa* oligosaccharide was lost of effectiveness in adrenalcetomized animals.[18]

It has been indicated that the expression of β-endorphin sensitive receptor on skeletal muscle is vastly increased in type 1 and type 2 diabetic animals.[19,20] Actually, many actions of endogenous β-endorphin are recognized to be mediated by the opioid μ-receptors including the regulation of plasma glucose,[21,22] but the mediation of opioid delta-receptor located in skeletal muscle regarding to the hypoglycemic effect of BER has also been demonstrated.[23,24] Opioid μ-receptor blockade was therefore employed to evaluate the involvement of this receptor in the actions of catalpol in STZ-diabetic rats. The ability of catalpol to lower plasma glucose was suppressed by blockade of opioid μ-receptors with naloxone or naloxonazine. These findings strongly implicate the activation of opioid μ-receptors through increased circulating β-endorphin concentrations in the glucose-lowering action of catalpol in diabetic rats. In addition, the opioid μ-receptor knockout mice were employed to confirm the involvement of opioid μ-receptor activation in the action of catalpol. In contrast to wild-type diabetic mice, the plasma glucose-lowering action of catalpol in opioid μ-receptor knockout diabetic mice was abolished (Figure 3). These findings support the essential role of opioid μ-receptors in the plasma glucose-lowering action of catalpol in the insulin deficient state. The elevation of BER in response to catalpol 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.[25] Insulin deficiency is clearly associated with changes in hepatic metabolism including increased expression of PEPCK, which is a key enzyme of hepatic carbohydrate metabolism.[25] Additionally, decreased expression of skeletal muscle GLUT 4 was proposed previously to mediate the reduction of insulin-mediated glucose uptake into skeletal muscle in diabetes.[26] It was interesting to ascertain whether catalpol exerted its glucose-lowering action in diabetic rats by overturning the diabetes-dependent reduction of GLUT 4 expression and/or increase in PEPCK expression. To provide ample time for alterations in gene expression, STZ-diabetic rats receive repeated catalpol treatments for 3 days. Under these conditions, the increase in hepatic PEPCK gene expression due to the induction of diabetes was found to be attenuated by catalpol. The decrease in GLUT4 expression due to induction of diabetes, similarly, was reversed by repeated catalpol treatments. These findings indicated that catalpol retains its glucose-lowering actions over extended time periods and that this agent alters the expression of hepatic PEPCK and muscle GLUT 4 in an insulin-independent manner.

Previous studies indicated that endogenous β-endorphin via activation of opioid μ-receptors located in 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.[27] Opioid μ-receptors antagonists were therefore used to test the involvement of opioid μ-receptors in effects of catalpol on specific gene expression in diabetic rats. Catalpol failed to elevate GLUT 4 mRNA, and protein concentrations in skeletal muscle of STZ-diabetic rats received the pretreatment of opioid μ-receptor antagonist (Figure 4). Furthermore, suppression of PEPCK gene expression in STZ-diabetic rats by catalpol was blocked by naloxonazine (Figure 5). Thus, the normalizations of hepatic PEPCK and muscle GLUT 4 expressions in STZ-diabetic rats by catalpol are both mediated through an activation of opioid μ-receptors. In the present study, the action of catalpol was investigated by intravenous administration, and the pharmacokinetic factors can be ruled out. Actually, it has been indicated that oral administration of catalpol could effectively ameliorate diabetic encephalopathy by attenuating oxidative stress.[28] In addition, some bioactive iridoid glucosides including verproside, isovanilloylcatalpol, catalposide, and 6-*O*-veratroyl catalpol have been documented as metabolites of catalpol.[29] The bioavailability of catalpol-like compound (verproside) has been mentioned,[30] but the transferable in humans is still not indicated. Moreover, there is no report showing the obvious side effects of catalpol. However, it shall be investigated in the future.

In conclusion, our results suggest that catalpol may enhance the secretion of endogenous β-endorphin from adrenal gland of STZ-diabetic rats. The plasma glucose-lowering action of catalpol was mainly obtained by β-endorphin release via an activation of opioid μ-receptors to achieve the enhancement of GLUT 4 gene expression and/or the amelioration of the elevated hepatic PEPCK gene expression. Therefore, catalpol can be used as the adjuvant for improvement of hyperglycemia in diabetic disorders.

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