Original Article

Novel Mechanism for Plasma Glucose–Lowering Action of Metformin in Streptozotocin-Induced Diabetic Rats

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To better understand the insulin-independent plasma glucose–lowering action of metformin, we used streptozotocin (STZ)-induced diabetic rats to investigate the possible mechanisms. Oral intake of metformin decreased the plasma glucose of STZ-induced diabetic rats with a parallel increase of plasma β-endorphin–like immunoreactivity (BER). Mediation of opioid μ-receptors in the action of metformin was identified by the blockade of receptors with antagonist in STZ-induced diabetic rats and the failure of action in opioid μ-receptor knockout diabetic mice. Release of BER from adrenal glands by metformin was characterized, using bilateral adrenalectomy and the release of BER from isolated adrenal medulla of STZ-induced diabetic rats. Repeated treatment with metformin in STZ-induced diabetic rats increased the mRNA and protein levels of GLUT-4 in soleus muscle that was blocked by naloxonazine. Reduction of the mRNA or protein levels of hepatic PEPCK was also impeded in the same group of STZ-induced diabetic rats. In conclusion, our results provide novel mechanisms for the plasma glucose–lowering action of metformin, via an increase of β-endorphin secretion from adrenal glands to stimulate opioid μ-receptor linkage, leading to an increase of GLUT-4 gene expression and an attenuation of hepatic PEPCK gene expression in STZ-induced diabetic rats. *Diabetes* 55:819–825, 2006

Diabetes and its complications constitute a major health problem in modern societies. Both type 1 and type 2 diabetes comprise abnormalities of insulin action, including deficiency and insulin resistance (1).

Metformin is a widely prescribed antihyperglycemic agent for type 2 diabetes. Because it lowers blood glucose without increasing insulin secretion, metformin has been considered an insulin sensitizer (2). In fact, metformin showed beneficial effects in type 2 diabetes, including weight reduction, improved lipid profiles, and enhanced endothelial function (3). Thus, metformin is introduced for use in insulin-resistant states even before the development of hyperglycemia (4).

The mechanisms of metformin action have remained obscure, despite multiple pathways of action being proposed, including a decrease of hepatic glucose production, an increase of peripheral glucose disposal, and a reduction of intestinal glucose absorption (1). It has been documented that metformin activated 5′AMP-activated protein kinase (AMPK) in hepatocytes, thereby reducing activity of acetyl-CoA carboxylase and lowering expression of a lipogenic transcription factor as well as inhibiting hepatic gluconeogenesis (5,6). Thus, AMPK seems a major signal for the action of metformin to suppress lipogenesis and induce fatty acid oxidation (5,6). In fact, plasma glucose–lowering action is not entirely dependent on insulin (7). It has been mentioned that exercise causes an increase of glucose uptake in the skeletal muscle of diabetic and nondiabetic subjects through the translocation of GLUT-4 to cell membranes (8). This translocation of GLUT-4 is mediated through insulin-independent phosphorylation and activation of AMPK (9,10). However, direct evidence for the linkage of these two signals is lacking.

Additionally, it has been indicated that exogenous β-endorphin induces an increase of circulating insulin in humans with or without diabetes (11,12). The effect of opioids on glucose homeostasis may in fact be produced by other mechanisms in addition to insulin. Intravenous injection of synthetic β-endorphin lowered plasma glucose in streptozotocin (STZ)-induced diabetic rats, as observed in our previous study (13). Chemical agents such as loperamide or tramadol increased glucose utilization via activation of opioid μ-receptors to lower plasma glucose in STZ-induced diabetic rats (14,15). In obese Zucker rats, mediation of β-endorphin in exercise-induced improvement of insulin resistance has also been observed (16). These findings support a beneficial effect of opioid μ-receptor activation on plasma glucose regulation. Thus, the aim of this study is to clarify whether β-endorphin is involved in the plasma glucose–lowering action of metformin. The deficient functions of pancreas β-cells in STZ-induced diabetic rats has been documented (17), and rats with STZ-induced diabetes were thus used in the current study as an animal model of type 1–like diabetes.

RESEARCH DESIGN AND METHODS

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 the wild-type controls) and opioid μ-receptor knockout BDF1 mice (18), aged 8–10 weeks, all of which had been bred in the same animal center, were obtained from Dr. H.H. Loh (University of Minnesota Medical School, Minneapolis, MN). STZ-induced diabetic rats were prepared by intravenously injecting STZ (60 mg/kg) into the male Wistar rats at age 8–10 weeks. Mice

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Received for publication 22 July 2005 and accepted in revised form 28 November 2005.

AMPK, 5′AMP-activated protein kinase; BER, β-endorphin–like immunoreactivity; PKC, protein kinase C; STZ, streptozotocin.

with or without opioid μ-receptors also received an intraperitoneal injection of STZ at 50 mg/kg to induce diabetes (19). At 2 weeks after the injection of STZ, animals were considered to be diabetic if they had plasma glucose levels ≥20 mmol/l and other diabetic features, including polyuria, polydipsia, and hyperphagia. Also, plasma insulin in these STZ-induced diabetic rats was reduced to 1.23 ± 0.6 pmol/l (n = 8), a level markedly lower than that of normal rats (154.8 ± 5.2 pmol/l, n = 8). 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.

Effect of metformin on plasma glucose or β-endorphin level in STZ-induced diabetic rats. Metformin was dissolved in distilled water containing 0.9% (wt/vol) sodium chloride for oral administration into fasted rats at desired doses. It has been documented that rats that have received sodium pentobarbital show no changes of plasma glucose and glucagon (20). Thus, under anesthesia with sodium pentobarbital (30 mg/kg i.p.), blood samples (0.1 ml) were collected from the tail vein for the measurement of plasma glucose or β-endorphin–like immunoreactivity (BER). The time course of the effect of metformin on plasma glucose in STZ-induced diabetic rats was preliminarily determined; the plasma glucose–lowering effect of metformin at an oral dosage of 100 mg/kg reached a plateau within 60 min and was maintained for ≥120 min. Thus, the effects of metformin on plasma glucose and plasma BER were determined, using blood samples collected 60 min after oral administration. As controls, we used animals that had received a similar administration of saline used to dissolve metformin at the same volume. The opioid receptor antagonists known to antagonize μ-subtypes, including naloxonazine and naloxone, were injected into the tail vein of animals 30 min before oral treatment with metformin.

Effect of metformin on plasma glucose or BER level in opioid μ-receptor knockout diabetic mice. Fasting STZ-induced diabetic mice with or without opioid μ-receptors were given an oral administration of metformin at 100 mg/kg, the dose showing maximal effect in STZ-induced diabetic rats. After 60 min, blood samples (0.1 ml) were collected from the lower eyelid of mice under anesthesia with pentobarbital (30 mg/kg i.p.) for determination of plasma glucose and BER.

Isolation and incubation of adrenal medulla. Adrenal glands were removed from the killed STZ-induced diabetic rats, and medullas were immediately dissected after removal of the cortex as previously described (21). The tissues were cut into ~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 with metformin at indicated concentrations with continuous shaking at 40 cycles/min under 37°C for 30 min. Incubation was terminated by placing the tubes on ice. The medium from each incubated sample was collected and frozen at −70°C until the β-endorphin assay was performed.

Adrenalectomized rats. Bilateral adrenalectomy was performed in Wistar rats, using the dorsal approach as described previously (22). The adrenalectomized rats were also fed standard rat chow and 0.9% sodium chloride in their drinking water ad libitum. Sham-operated rats were fed standard rat chow and water ad libitum. Animals were allowed to recover for 2 weeks after the operation. The animals appeared alert and in good health. Then, diabetes was induced by an injection of STZ as described above.

Laboratory determinations. The concentration of plasma glucose was measured by the glucose oxidase method, using an analyzer (Quik-Lab; Ames, Miles, Elkhart, IN). An enzyme-linked immunosorbent assay for the determination of BER in plasma or medium incubating adrenal medulla was carried out, using a commercially available kit (Peninsula Laboratories, San Carlos, CA).

Determination of gene expression. STZ-induced diabetic rats were orally administered vehicle or metformin (100 mg/kg) every 8 h, three times daily. Naloxonazine was intravenously injected into another group of STZ-induced diabetic rats 30 min before receiving an oral administration of metformin in the same manner. In preliminary experiments, metformin was found to modify the mRNA and protein levels for GLUT-4 and hepatic PEPCK in STZ-induced diabetic rats after a 3-day treatment. Therefore, animals were killed after 3 days of treatment. Normal rats receiving a similar treatment of vehicle were used as controls. Liver and soleus muscle were immediately removed, frozen in liquid nitrogen, and stored at −70°C for the determination of gene transcripts. Changes in hepatic PEPCK mRNA were determined by RT-PCR. PEPCK-specific primers were 5′-AGTTGAATGTGTGGGTGATGACA-3′ and 5′-AAAACCGTTTTCTGGGTTGATG-3′ for forward and reverse primers, respectively (23). Prophobilinogene deaminase–specific primers were 5′-GGAGCCATGTCTGGTAACGGCA-3′ and 5′-GGTACCCACGCGAATCACTCT CA-3′ for forward and reverse primers, respectively (24). Quantification of the mRNA level was examined, using the ratio of PEPCK to prophobilinogene deaminase. The mRNA of GLUT-4 in soleus muscle was investigated by Northern blot analysis (15). The effects of testing agents on protein levels of PEPCK and GLUT-4 were evaluated by Western blot analysis (15). Anti–rat GLUT-4 antibody was purchased from Genzyme Diagnostics (Cambridge, MA). The antibody specific to PEPCK was a gift from Dr. D.K. Granner (Vanderbilt University School of Medicine, Nashville, TN). The blots were incubated with a goat polyclonal actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or a mouse monoclonal β-tubulin antibody (Zymed Laboratories, San Francisco, CA) as an internal control. Blood samples were also collected from the tail vein of these rats before death to assay the plasma levels of glucose and BER as described above. Also, the daily amount of food and water intake as well as changes of body weight in metformin-treated STZ-induced diabetic rats were measured to compare with vehicle-administered controls.

Statistical analysis. Plasma glucose–lowering activity was noted in the animals that had received metformin. Results of plasma glucose–lowering activity were calculated as the percentage reduction of the initial value according to the formula $(G_i - G_t)/G_i \times 100\%$, where G_i was the initial plasma glucose and G_t was the plasma glucose concentration after treatment with metformin.

Data are expressed as the means ± SE for the number (n) of animals in the group, as indicated in tables and figures. Repeated-measures ANOVA was used to analyze the changes in plasma glucose and other parameters. Dunnett range post hoc comparisons were used to determine the source of significant differences where appropriate. $P < 0.05$ was considered statistically significant.

RESULTS

Effects of metformin on plasma glucose and BER levels in STZ-induced diabetic rats. At 60 min after treatment, a dose-dependent decrease of plasma glucose was observed in STZ-induced diabetic rats receiving an oral administration of metformin (Fig. 1*A*). The plasma glucose–lowering activity produced by metformin at 100 mg/kg was 21.4 ± 1.8% in STZ-induced diabetic rats. Increasing the dosage of metformin to 115 mg/kg yielded a plasma glucose–lowering activity of 21.9 ± 2.3% that was not more effective. Plasma glucose decreased from 5.3 ± 0.9 mmol/l to 4.6 ± 0.8, 4.4 ± 0.5, and 3.9 ± 0.7 mmol/l at 60 min later in normal rats receiving oral administration of metformin at 50, 75, and 100 mg/kg, respectively. The plasma glucose–lowering activity of metformin in normal rats was as effective as that produced in STZ-induced diabetic rats. The effect of metformin at 100 mg/kg was investigated in subsequent experiments.

Plasma BER was raised ~30 min later in STZ-induced diabetic rats by oral administration of metformin. The action of metformin was maximal at 60 min, which was used as the optimal time in subsequent experiments. A dose-dependent elevation of plasma BER level was observed in the same group of STZ-induced diabetic rats receiving metformin (Fig. 1*B*).

Effect of bilateral adrenalectomy on the action of metformin in STZ-induced diabetic rats. The basal plasma glucose or BER in STZ-induced diabetic rats was not modified by adrenalectomy, compared with the sham-operated group (Table 1). However, both the decrease of plasma glucose and the increase of plasma BER by metformin (100 mg/kg) disappeared in STZ-induced diabetic rats with bilateral adrenalectomy, whereas these effects persisted in sham-operated STZ-induced diabetic rats receiving the same treatment (Table 1).

Effect of metformin on the secretion of BER from isolated adrenal medulla of STZ-induced diabetic rats. The amount of BER in the medium was increased by metformin in a concentration-dependent manner (Fig. 2). Metformin at 1 μmol/l increased BER in the medium and reached a plateau, and no further effect was produced by metformin at higher concentrations (Fig. 2).

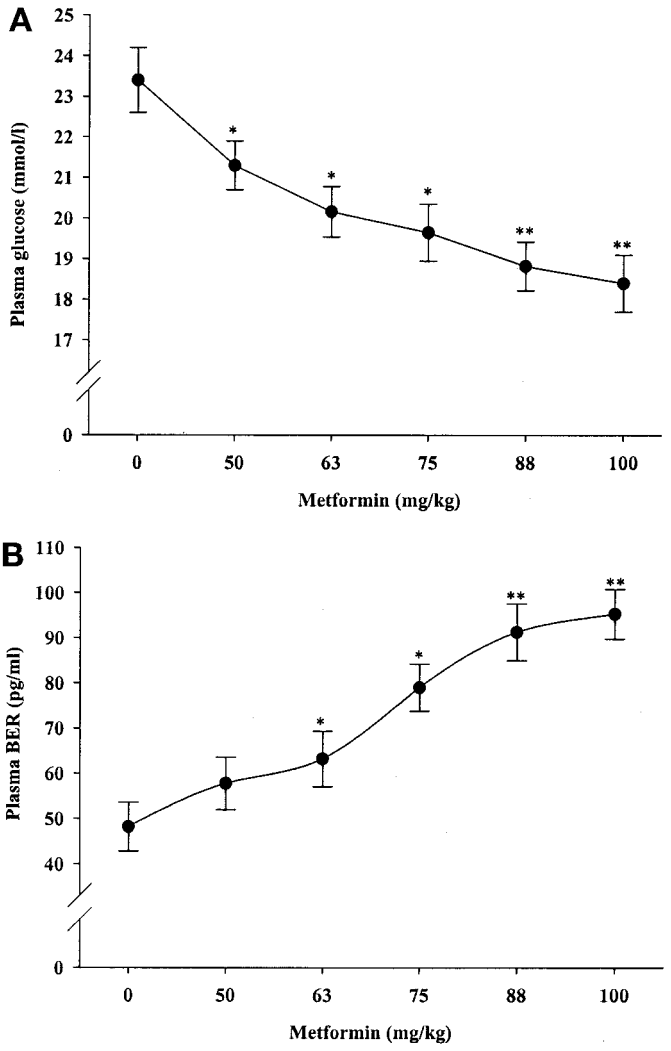

FIG. 1. *A*: The changes of plasma glucose in STZ-induced diabetic rats that received oral treatment of metformin. *B*: The change of plasma BER in the same group of animals. Values (means ± SE) were obtained from each group of eight animals. The vehicle (saline) used to dissolve metformin was given at the same volume. *$P < 0.05$ and **$P < 0.01$ vs. data from the vehicle-administered group (0 mg/kg metformin).

Effects of opioid μ-receptor antagonists on the action of metformin in STZ-induced diabetic rats. Table 2 shows the dose-dependent action of naloxone and naloxonazine to inhibit the plasma glucose–lowering activity of metformin in STZ-induced diabetic rats. In the presence of 1 mg/kg naloxone, the plasma glucose level in STZ-induced diabetic rats treated with 100 mg/kg metformin was not statistically different from the basal level. Also, naloxonazine (1 mg/kg) prevented the ability of metformin (100 mg/kg) to lower plasma glucose in STZ-induced diabetic rats. However, both naloxone and naloxonazine at the highest dose did not affect the basal plasma glucose level of STZ-induced diabetic rats (Table 2).

Change of metformin-induced plasma glucose–lowering activity in opioid μ-receptor knockout diabetic mice. Plasma glucose in opioid μ-receptor knockout diabetic mice was not modified by oral administration with metformin (100 mg/kg), whereas plasma BER was elevated (Fig. 3). Similar administration of metformin (100 mg/kg) in diabetic mice with opioid μ-receptors also increased plasma BER, but this action was in parallel with the plasma glucose–lowering action (Fig. 3). The plasma glucose-lowering activity of metformin in these wild-type diabetic mice was ~21.7 ± 2.1%, similar to the activity produced in STZ-induced diabetic rats.

TABLE 1
Effect of adrenalectomy on the metformin-induced changes of plasma glucose and BER in STZ-induced diabetic rats

	STZ-induced diabetic rats	
	Adrenalectomized	Sham-operated
n	8	8
Plasma glucose (mmol/l)		
Basal	22.9 ± 2.2	23.3 ± 1.8
Vehicle	22.7 ± 1.8	23.1 ± 1.7
Metformin (100 mg/kg orally)	22.4 ± 2.1	18.4 ± 1.9*
Plasma BER (pg/ml)		
Basal	50.1 ± 4.1	48.6 ± 4.8
Vehicle	50.6 ± 4.3	48.8 ± 4.2
Metformin (100 mg/kg orally)	52.1 ± 3.6	94.6 ± 3.7*

Data are means ± SE. The basal level shows the value from fasting animals without treatment. *$P < 0.01$ vs. the basal value in each group.

General characteristics of STZ-induced diabetic rats repeatedly receiving oral administration of metformin. Plasma glucose levels of STZ-induced diabetic rats were reduced after repeated oral administration of metformin (100 mg/kg) for 3 days (Table 3), showing a plasma glucose–lowering activity of 23.9 ± 2.3%. Elevation of plasma BER occurred in the same group of STZ-induced diabetic rats, and it was significantly ($P < 0.01$) higher than in the vehicle-administered group (Table 3). The 3-day treatment with metformin (100 mg/kg) did not influence the feeding behavior and/or body weight of STZ-induced diabetic rats (Table 3).

In the presence of naloxonazine (1 mg/kg), plasma BER levels in the STZ-induced diabetic rats treated with metformin (100 mg/kg) were still markedly raised (Table 3). However, the reduction of plasma glucose by metformin

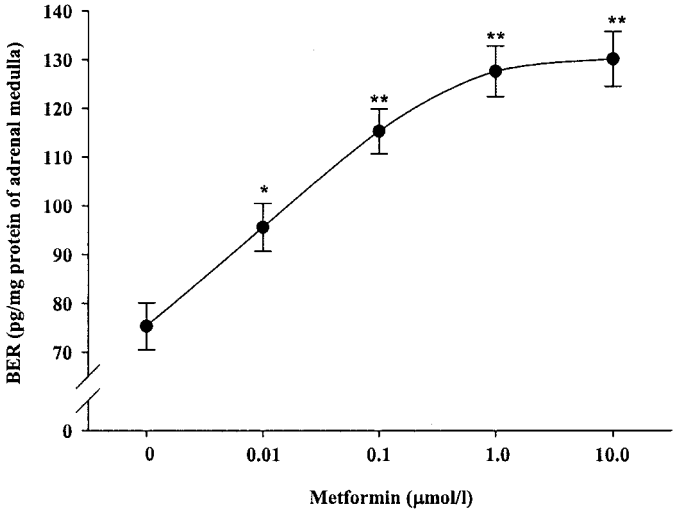

FIG. 2. Effect of metformin on BER secretion from the isolated adrenal medulla of STZ-induced diabetic rats. Results (pg/mg protein) are the means ± SE of seven determinations. *$P < 0.05$ and **$P < 0.01$ vs. data from samples treated with modified Krebs solution (0 μmol/l metformin).

TABLE 2
Effects of opioid μ-receptor antagonists on metformin-induced reduction of plasma glucose levels in STZ-induced diabetic rats

	Plasma glucose (mmol/l)
n	7
Basal	23.2 ± 1.8
Metformin (100 mg/kg orally)	
+ Vehicle	18.1 ± 1.9*
+ Naloxone (mg/kg i.v.)	
0.1	19.4 ± 1.7†
0.5	20.5 ± 2.1
1.0	22.8 ± 2.0
+ Naloxonazine (mg/kg i.v.)	
0.1	20.1 ± 2.1
0.5	22.6 ± 1.6
1.0	23.0 ± 2.2
Naloxone (1 mg/kg i.v.)	23.5 ± 2.3
Naloxonazine (1 mg/kg i.v.)	23.7 ± 2.6

Data are means ± SE. The basal level shows the value from fasting animals treated with vehicle. *$P < 0.01$ and †$P < 0.05$ compared with the basal value, respectively.

(100 mg/kg) in STZ-induced diabetic rats was abolished by naloxonazine (Table 3). The body weight of STZ-induced diabetic rats was not influenced by naloxonazine (Table 3).

Effect of metformin on the mRNA and protein levels of GLUT-4 in soleus muscle of STZ-induced diabetic rats. The mRNA level of GLUT-4 in soleus muscle isolated from vehicle-administered STZ-induced diabetic rats was ~47% of that from vehicle-administered normal rats (Fig. 4*A*). Repeated oral treatment of STZ-induced diabetic rats with metformin (100 mg/kg) for 3 days resulted in an elevation of GLUT-4 mRNA level in the soleus muscle to a level ~76% of that in vehicle-administered normal rats. Also, the effect of metformin (100 mg/kg) on GLUT-4 mRNA levels in STZ-induced diabetic rats was reversed by naloxonazine (1 mg/kg) to the same level as in the vehicle-administered STZ-induced diabetic rats (Fig. 4*A*).

The protein level of GLUT-4 in soleus muscle of vehicle-administered STZ-induced diabetic rats was significantly reduced to ~45% of that in the vehicle-administered normal rats (Fig. 4*B*). Repeated oral treatment with metformin (100 mg/kg) elevated the protein level of GLUT-4 in soleus muscle of STZ-induced diabetic rats to a level ~80% of that in vehicle-administered normal rats. Naloxonazine (1 mg/kg) reversed this action of metformin (Fig. 4*B*). However, naloxonazine alone did not affect basal GLUT-4 gene expression. Quantification of the mRNA and protein levels of GLUT-4 induced by these treatments is shown in Table 4.

Effect of metformin on the mRNA and protein levels of hepatic PEPCK in STZ-induced diabetic rats. Fig. 4*C* shows the level of mRNA encoding PEPCK that was elevated nearly 3.4-fold in untreated STZ-induced diabetic rats compared with normal rats. This increase of mRNA level in the liver of STZ-induced diabetic rats was reduced to ~33% of vehicle-administered STZ-induced diabetic rats by repeated oral treatment with metformin (100 mg/kg) for 3 days.

The protein levels of PEPCK in the liver of vehicle-administered STZ-induced diabetic rats were about threefold of the vehicle-administered normal rats. Repeated oral treatment of STZ-induced diabetic rats with metformin (100 mg/kg) for 3 days resulted in a marked reduction of the protein level of PEPCK to near the level in vehicle-administered normal rats; which was reversed on blockade of opioid μ-receptors by naloxonazine (1 mg/kg) (Fig. 4*D*). However, the basal levels of mRNA and protein in hepatic PEPCK were not influenced by naloxonazine alone. Quantification of all data from these treatments is also shown in Table 4.

DISCUSSION

Metformin has oral bioavailability, with peak plasma concentration reached after 2–3 h (25). We found that oral treatment with metformin for 1 h can dose-dependently lower plasma glucose in a manner parallel with an increase of plasma BER in STZ-induced diabetic rats. In fact, plasma insulin levels in STZ-induced diabetic rats was only ~1/120 of that in normal rats. Thus, mediation of endogenous insulin is negligible in this STZ-induced diabetic rat model. The effective dose of metformin in STZ-induced diabetic rats was similar to that used in type 2 diabetic rats (26). Also, the plasma glucose–lowering activity of metformin in STZ-induced diabetic rats was the same as that

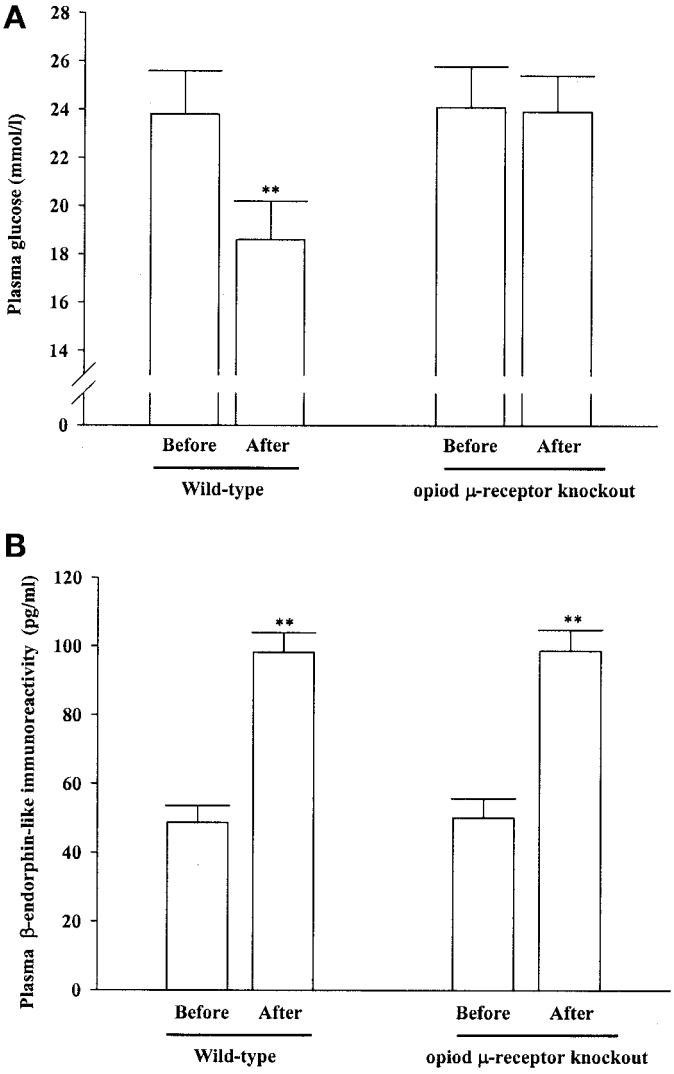

FIG. 3. *A*: The change of plasma glucose in opioid μ-receptor knockout diabetic mice and wild-type controls receiving an oral intake of metformin (100 mg/kg). *B*: The plasma BER in same group of mice. Values (means ± SE) were obtained from each group of seven animals. **$P < 0.01$ vs. data from animals before treatment.

TABLE 3
General characteristics of STZ-induced diabetic rats orally treated with metformin three times daily for 3 days

	Body weight (g per rat)	Food intake (g per day)	Plasma glucose (mmol/l)	Plasma BER (pg/ml)
Normal rats				
+ Vehicle	237.6 ± 12.4*	18.1 ± 4.5*	5.2 ± 1.8†	43.2 ± 5.2
STZ-induced diabetic rats				
+ Vehicle	179.3 ± 13.2	41.7 ± 6.2	23.4 ± 2.1	49.2 ± 4.8
+ Metformin (100 mg/kg orally)	175.3 ± 10.1	39.6 ± 7.1	17.2 ± 1.8*	96.4 ± 5.7*
+ Naloxonazine (1 mg/kg i.v.)	180.8 ± 11.4	40.1 ± 5.8	23.9 ± 2.4	93.8 ± 6.1*

Data are means ± SE from eight different animals in each group. *$P < 0.01$ and †$P < 0.001$ compared with the values from vehicle-treated STZ-induced diabetic rats.

in normal rats. These results indicated that metformin has an ability to lower plasma glucose without the help of endogenous insulin; elevation of β-endorphin seems related to this action of metformin.

Although β-endorphin is released with adrenocorticotrophic hormone from the pituitary gland (27), adrenal glands are also a source of β-endorphin (28,29). We have demonstrated that secretion of opioids from adrenal glands is associated with a decrease of plasma glucose in STZ-induced diabetic rats (19), which is consistent with the view that pituitary gland–independent release of endogenous opioids is operative in other organs (28,29). In an attempt to make certain that adrenal glands are the main source of metformin-induced release of β-endorphin, we used bilateral adrenalectomy. The plasma glucose–lowering action of metformin was eliminated by bilateral adrenalectomy in STZ-induced diabetic rats. Also, no increase of plasma β-endorphin was obtained in adrenalectomized diabetic rats receiving metformin at the effective doses. Thus, adrenal glands seem responsible for the secretion of β-endorphin by metformin. Moreover, metformin enhanced β-endorphin secretion from the isolated adrenal medullas of STZ-induced diabetic rats in a concentration-dependent manner. Taken together, one can conclude that a release of β-endorphin from adrenal glands by metformin is related to metformin's plasma glucose–lowering action in STZ-induced diabetic rats.

The actions of β-endorphins are mediated in part by opioid μ-receptors, which are believed to be expressed in specialized neurons for pain transmission at both spinal and supraspinal sites (30). Recently, we observed that β-endorphin enhanced the uptake of radioactive glucose into the isolated soleus muscle of STZ-induced diabetic rats and stimulated glycogen synthesis in the hepatocytes isolated from STZ-induced diabetic rats (13). Both actions of β-endorphin were naloxone and naloxonazine sensitive (13). Thus, opioid μ-receptors are also located in peripheral tissues that can be activated to lower plasma glucose by improving glucose utilization (13,15). The action of metformin in STZ-induced diabetic rats was inhibited by blockade of opioid μ-receptors, using naloxone or naloxonazine. In fact, two subtypes of the μ-receptors (μ_1 and μ_2) have been postulated, although the μ_1 subtype is naloxonazine sensitive (30). This suggests that the plasma glucose–lowering action of metformin in STZ-induced diabetic rats may be mediated by peripheral opioid μ_1-receptor activation, which is worthwhile to be investigated in advance. However, these antagonists may have nonspecific effects in addition to the blockade of opioid μ-receptors. Therefore, for further investigation, we used opioid μ-receptor knockout mice receiving STZ. The plasma glucose–lowering action of metformin was eliminated in opioid μ-receptor knockout diabetic mice, although the BER-elevating action of metformin was still observed. This result supports the essential role of opioid μ-receptors in plasma glucose–lowering action of metformin during the absence of insulin. The role of cerebral opioid receptors in the regulation of food intake has been well-established (31). Although the evidence indicating the cerebral action of metformin is not documented, chronic treatment with

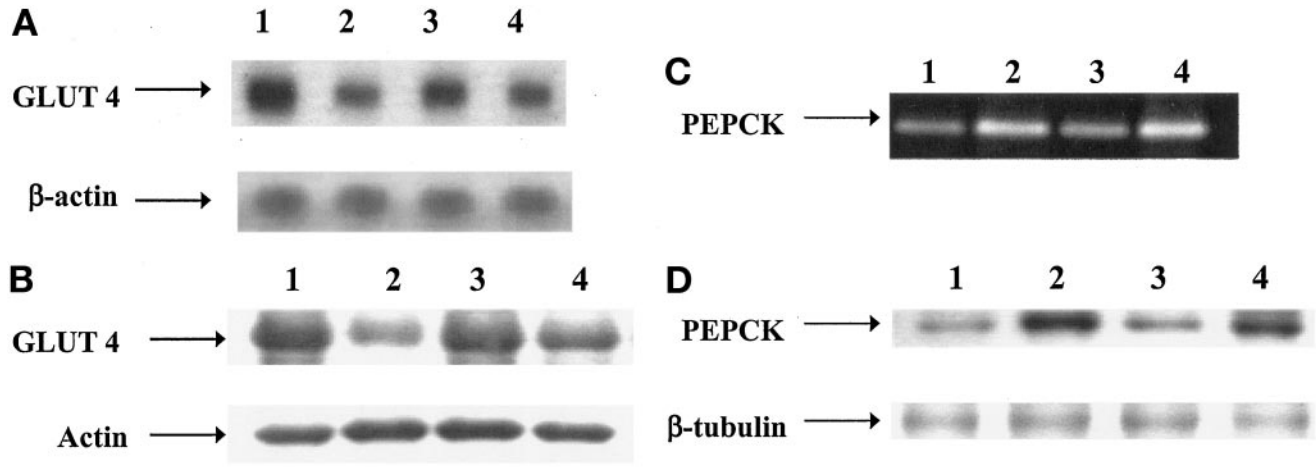

FIG. 4. Representative images indicating the mRNA level for GLUT-4 or β-actin in soleus muscle (*A*), the protein level for GLUT-4 or actin in soleus muscle (*B*), the gel electrophoresis of RT-PCR for PEPCK from the liver (*C*), and the protein level for PEPCK or β-tubulin in liver (*D*). *Lane 1*: Vehicle-administered normal rats. *Lane 2*: Vehicle-administered STZ-induced diabetic rats. *Lane 3*: Metformin-treated STZ-induced diabetic rats. *Lane 4*: Metformin plus naloxonazine-administered STZ-induced diabetic rats. Quantification of the data are shown in Table 4.

TABLE 4
Quantification of the mRNA and protein levels for GLUT-4 in soleus muscle or PEPCK in liver isolated from STZ-diabetic rats receiving repeated treatment with metformin alone or in combination of opioid μ-receptor antagonist for 3 days

	mRNA (arbitrary units)		Protein (arbitrary units)	
	GLUT-4/β-actin	PEPCK/PBGD	GLUT-4/actin	PEPCK/β-tubulin
Normal rats + vehicle	1.36 ± 0.05*	0.75 ± 0.08	0.65 ± 0.04*	0.25 ± 0.02
STZ-diabetic rats				
+ Vehicle	0.64 ± 0.04†‡	2.62 ± 0.23†‡	0.29 ± 0.02†‡	0.75 ± 0.02†‡
+ Metformin (100 mg/kg orally)	1.04 ± 0.04§	0.87 ± 0.09	0.52 ± 0.03§	0.29 ± 0.03
+ Naloxonazine (1 mg/kg i.v.)	0.75 ± 0.0*†	2.13 ± 0.18†‡	0.34 ± 0.04*†	0.61 ± 0.04†‡

Data are means ± SE and were obtained from each group of five samples. Vehicle was given at the same volume. *$P < 0.05$ compared with metformin-treated STZ-induced diabetic rats; †$P < 0.01$ compared with vehicle-treated normal rats; ‡$P < 0.01$ compared with metformin-treated STZ-induced diabetic rats; §$P < 0.05$ compared with vehicle-treated normal rats. PBGD, prophobilinogene deaminase.

metformin produced an anorectic effect to lower body weight in genetically obese Zucker rats (32). In fact, metformin did not influence the food intake of STZ-induced diabetic rats. This is probably because the β-endorphin release induced by metformin mainly exerts its effect on opioid μ-receptors located on peripheral tissues. However, this view shall be clarified in the future.

In diabetes, elevation of blood glucose is a consequence of increased hepatic glucose output in concert with reduced peripheral glucose utilization (33). PEPCK is one of the key enzymes in hepatic gluconeogenesis (33). Insulin deficiency is clearly associated with a change in hepatic metabolism (33). Moreover, a reduction in insulin-mediated glucose uptake caused by decreasing gene expression of GLUT-4 has been reported in skeletal muscle, a major site for glucose disposal, in diabetic rats (34). Recent studies strongly support the reduction in hepatic gluconeogenesis as the primary route through which metformin exerts its glucose-lowering action (5,6). In addition, metformin has been shown to facilitate the trafficking of GLUT-4 to membrane of skeletal muscle (8–10). Because long-term exposure is required for the activation of mRNA level, the gene expression associated with glucose regulation was examined in STZ-induced diabetic rats that had received a 3-day repeated treatment with metformin. Consistent with previous studies (5,6), the plasma glucose–lowering activity of metformin was associated with an attenuation of the raised hepatic PEPCK gene expression in STZ-induced diabetic rats. Meanwhile, an increase in the gene expression of GLUT-4 may contribute to plasma glucose regulation in metformin-treated STZ-induced diabetic rats. Both actions of metformin were reversed by blockade of opioid μ-receptors, using naloxonazine. This is consistent with the previous view that β-endorphin, via activation of opioid μ-receptors, is a positive regulator in glucose utilization and a negative modulator in hepatic gluconeogenesis in the insulin-deficient state (15). However, the effect of β-endorphin on the brain to affect these genes cannot be excluded, and this needs more studies in the future. Also, the effect of metformin on the regulation of hepatic PEPCK gene expression in STZ-induced diabetic rats seems more effective than that in the GLUT-4 gene expression. This finding suggests that metformin exerts its antihyperglycemic effect mainly through a reduction of hepatic gluconeogenesis; this view is consistent with previous reports (5,6).

It has been indicated that phospholipase C and protein kinase C (PKC) play a key role in opioid signals (13). Also, PKC is involved in a rate-limiting step in GLUT-4 mRNA expression (35). Therefore, the phospholipase C–PKC pathway is related to the signals of opioid μ-receptors in the regulation of GLUT-4 gene expression, although the detailed action mechanism needs further investigation. In fact, the suppression of PEPCK gene expression in STZ-induced diabetic rats by metformin was blocked by opioid μ-receptor antagonist, indicating the mediation of opioid μ-receptors. The gene expression of PEPCK in liver is regulated by a number of hormones (36), and signals for opioid μ-receptors to regulate the hepatic PEPCK gene expression need to be clarified in the near future. Nevertheless, we demonstrated that metformin possesses an ability to enhance the secretion of β-endorphin from adrenal glands of STZ-induced diabetic rats. Then, the released β-endorphin can activate peripheral opioid μ-receptors to modify gene expressions of GLUT-4 and PEPCK for lowering the plasma glucose level in the insulin-deficient state.

It has been documented that splanchnic nerve stimulation increases the release of opioids from adrenal glands (37,38). Activation of α_1-adrenoceptors on adrenal medulla in the regulation of β-endorphin secretion has also been mentioned (19). In fact, it has been proposed that metformin releases norepinephrine by an indirect sympathomimetic-like action (39). Also, metformin inducing the release of norepinephrine from postganglionic sympathetic nerve endings (40) has been demonstrated. The role of norepinephrine in the mechanism(s) for metformin to enhance β-endorphin secretion from adrenal grand needs further evidence.

In conclusion, our results suggest that metformin exerts its antihyperglycemic effect primarily through enhancement of β-endorphin secretion from adrenal glands to stimulate opioid μ-receptors located on peripheral tissue, thereby leading to the amelioration of GLUT-4 gene expression and an attenuation of raised hepatic PEPCK gene expression in rats with insulin-deficient diabetes. This finding provides a new insight on the mechanisms of metformin action.

ACKNOWLEDGMENTS

The current study was supported in part by a grant from the National Science Council (NSC93-2320-B006-010) of China.

We are grateful to Dr. H.H. Loh (Department of Pharmacology, University of Minnesota Medical School, Minneapolis, MN) for the kind supply of opioid μ-receptor knockout mice and the wild-type mice. We also thank Dr. C. Makepeace (Department of Cell Biology and Physiology, School of Medicine, Washington University, St. Louis,

MO) and Dr. S.S. Liu (Department of Microbiology and Immunology, National Cheng Kung University, Tainan City, Taiwan, China) for kindly providing us the plasmid containing cDNAs. Moreover, the kind donation of antibodies specific to PEPCK from Dr. D.K. Granner (Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, TN) is respectfully acknowledged.

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