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Effects of Brazilin on Glucose Metabolism in Isolated Soleus Muscles from Streptozotocin Induced Diabetic Rats

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Abstract□The present study was performed to evaluate the hypoglycemic mechanism of brazilin. Brazilin significantly reduced plasma glucose level in streptozotocin induced diabetic rats and this effect seems to be mediated by extrapancreatic effects rather than by pacreatic effect because no significant changes were observed in plasma insulin levels. The rates of glycogen synthesis, glycolysis and glucose oxidation in soleus muscle were markedly increased following brazilin treatment to diabetic animals. Glucose transport seemed to be increased by the treatment of brazilin. Brazilin did not affect insulin binding to muscles from streptozotocin induced diabetic rats. These results suggest that potentiation of peripheral glucose utilization may be one of the major causes of hypoglycemic action of brazilin.

Keywords□Brazilin, hypoglycemic action, soleus muscle, glucose metabolism.

Diabetes mellitus is a representative metabolic disease, which is still remained as a controllable, but not a curable disease.

Since sulfonamide derivative, 2254RP, was found to have a hypoglycemic effect, a number of agents have been developed, that are able to modify metabolic processes so that the plasma glucose level is reduced[1]. However, many of them have serious toxicities and others are controversial as to whether they are sufficiently effective and safe enough to warrant their widespread usage[2,3]. From this reason, development of new antidiabetic agents, which have sufficient hypoglycemic effects as well as safety, is very important and desired.

As a consequence of our efforts to find out potential hypoglycemic agents from natural sources, brazilin, an active principle of *Caesalpinia sappan*, was proved to have a hypoglycemic activity. Based on this result, we have investigated the hypoglycemic mechanism of brazilin. In the present study, we examined the effects of brazilin on glucose metabolism in skeletal muscles isolated from streptozotocin induced diabetic rats, which play a major role *in vivo* in glucose utilization.

MATERIALS AND METHODS

Materials

Streptozotocin, crystalline porcine insulin, bovine serum and glycogen from oyster III were obtained from Sigma Chemical Co., U.S.A., D-[U-^{14}C]-glucose, 2-deoxy-[1,2-^{3}H]-D-glucose and D-[5-^{3}H]-glucose were purchased from New England Nuclear, U.S.A.; Brazilin monohydrate was from Aldrich Chemicals, U.S.A.; Dowex 1-X2 resin was from Bio-Rad Lab, U.S.A.. Other chemicals were of guaranteed grade.

Male Sprague-Dawley rats were supplied from the Experimental Animal Breeding Center of Seoul National University, and acclimated for 2 weeks.

Induction of diabetes mellitus

Male SD rats (160-180g) fasted overnight were injected with streptozotocin (40 mg/kg body weight) through a tail vein. Streptozotocin was dissolved in citrate buffer (pH 4.5), kept in ice bath, and administered within 10 min. After one week, blood samples were collected from retro-orbital plexus without fasting and blood glucose levels were determined by the glucose oxidase methods. Animals with blood glucose level of about 350 mg/dl were used as diabetic rats.

Animal grouping and treatment

Animals divided into normal control, normal brazilin treated, diabetic control and diabetic brazilin treated group. Animals were administered brazilin 10 and 100 mg/kg body weight intraperitoneally

and control animals were administered the same volume of saline.

Determination of plasma glucose and insulin

Blood samples were collected from the retro-orbital plexus in non-fasting state in the morning (a.m. 9:00-10:00). Plasma glucose levels were determined using glucose oxidase kit (Boehringer Manheim GmbH, Germany). Plasma insulin levels were determined by radioimmunoassay kit (Coat-A-count, Diagnostic Products Co., U.S.A.).

Isolation and preincubation of soleus muscle-strips

Rats were killed by cervical dislocation, and strips of soleus muscle weighing 25-35 mg were isolated and separated from the overall soleus muscle mass as described by Cretaz *et al.*[4]. Strips of soleus muscles were streched on a stainless steel holder as described by Cuendet *et al.*[5]. Muscles were preincubated for 30-60 minutes in Krebs-Ringer bicarbonate buffer (pH 7.4, supplemented with 1.0% defatted bovine serum albumin, 2 mM pyruvate) with or without insulin at maximally effective concentration (0.05 U/m*l*). The preincubation was carried out at 37°C in a shaking water bath and preceded by gassing with O_2:CO_2 (95:5 v/v) for 5 min. At the end of preincubation, media were replaced and the preparations were gassed as metioned above.

Determination of glucose metabolic rate and glucose uptake in/by soleus muscle

Following 2 hr incubation of soleus muscle with reaction mixture, glucose metabolic rates (glycogen synthesis, glycolysis and glucose oxidation) were measured as described by Cuendet *et al.*[5]. Glycogen synthesis was measured by ^{14}C incorporation from D-[U-^{14}C] glucose (5 mM, 20μCi/mmole) into glycogen. The rate of glycolysis was determined as the production of ^3H_2O from D-[5-^3H] glucose (5 mM, 20μCi/mmole) and glucose oxidation was measured by the quantitation of collected $^{14}CO_2$ produced from [U-^{14}C] glucose. Following 2 hr's incubation of muscle with reaction mixture, ^3H_2O in the medium ^{14}C-labeled glycogen and $^{14}CO_2$ were measured as described by Cuendet *et al.*[5]. The uptake of 2-deoxyglucose by muscles was used as an index of the rate of glucose transport and phosphorylation. Muscles were incubated for 30 min in the presence of 2-deoxy-D-[1,2-^3H] glucose (0.1 mM, 1 mCi/ mmole) and 2 mM pyruvate as energy source. The uptake of 2-deoxy-[^3H] glucose in muscle was measured as described by Cuendet *et al.*[5]. Results have been expressed per mg of muscle wet weight.

Statistical analysis

The data were subjected to analysis of variance followed by Duncan's Multiple Range Test to determine which means were significantly different from each other or controls. In all cases a p value of <0.05 was used to determine significance.

RESULTS AND DISCUSSION

The present study was undertaken to examine the effect of brazilin on glucose metabolism in soleus muscle of streptozotocin induced diabetic rats. Some characteristics of experimental animals used in this study are shown in Table I. In the preliminary experiments it was confirmed that plasma glucose levels were significantly decreased by the treatment of brazilin in streptotozocin induced diabetic rats but not in nor-

Table I. Characteristics of experimental animal-SD rats

	Blood sugar (mg%)		Body weight (g)		Plasma insulin (U/m*l*)	
	Initial	Final	Initial	Final	Initial	Final
Normal control (18)	111 ± 18	135 ± 23	208 ± 10	284 ± 12	31.7 ± 2.1	29.5 ± 3.4
Diabetic control (30)	375 ± 17	368 ± 28	205 ± 15	241 ± 12	21.0 ± 2.0	22.0 ± 3.0
Normal treat I (30)	114 ± 22	120 ± 13	200 ± 23	293 ± 21	27.4 ± 4.0	29.0 ± 3.0
II (30)	125 ± 21	105 ± 27	218 ± 15	282 ± 17	30.0 ± 7.0	30.0 ± 7.3
Diabetic treat I (28)	364 ± 22	282 ± 69	200 ± 11	269 ± 27	20.4 ± 4.0	21.3 ± 2.0
II (29)	368 ± 32	197 ± 68*	212 ± 10	257 ± 30	22.0 ± 2.0	23.3 ± 4.0

Brazilin was administered 10 mg/kg (I, III) or 100 mg/kg (II, IV) in physiological saline i.p. for 15 days. Control groups were administered the same volume of physiologic saline.
Each value represents mean ± SE. The number of experimental animals are given in parenthesis.
Initial and Final represent the values obtained before and after treatment, respectively.
*$p<0.05$ vs diabetic control

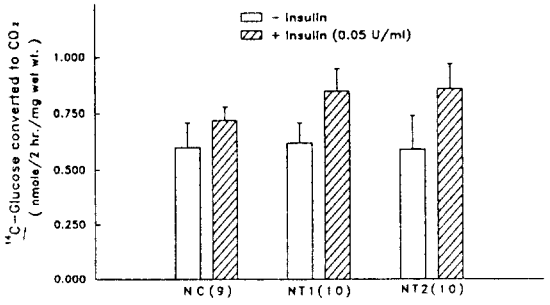

Fig. 1. ***In vivo*** **effect of brazilin on glucose oxidation in isolated soleus muscle from normal rats**
NC: Normal control group
NT 1: Normal treated group (10 mg/kg)
NT 2: Normal treated group (100 mg/kg)
Values are mean ± SE
The number of experimental animals is given in paranthesis. Samples were administered through i.p. for 15 days.

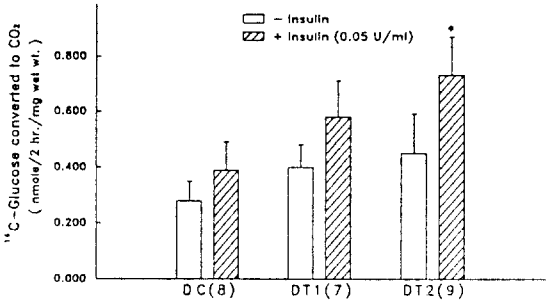

Fig. 2. ***In vivo*** **effect of brazilin on glucose oxidation in isolated soleus muscle from diabetic rats**
DC: Diabetic control group
DT 1: Diabetic treated group (10 mg/kg)
DT 2: Diabetic treated group (100 mg/kg)
Values are mean ± SE
The number of experimental animals is given in paranthesis. Samples were administered through i.p. for 15 days.

mal rats, in which any significant changes of plasma insulin levels were not detected. This fact suggests that hypoglycemic action of brazilin might be based on the increasing peripheral glucose utilization. In order to clarify this assumption, we examined the effects of brazilin on glucose metabolism in skeletal muscle. Different from adipose tissue, which plays only a minor role in the disposal of glucose load, skeletal muscle appears to be an important regulator of glucose homeostasis[6]. They are likely candidates to be involved in the overall insulin resistance in diabetic animals. As there is actually no preparation of isolated myocytes sufficiently sensitive to insulin *in vitro* to enable one to construct a dose response curve, soleus muscle is a typical skeletal muscle that has high and constant activity, and consists of homogenous fibers, and can be prepared intact[7]. However, there have been many reports that intact soleus muscle of adult rat presents critical problems of substrate diffusion[8]. Thus, we have used a strip of soleus muscle preparation developed more recently which overcomes this problem[4]. Indeed, it has been shown that glucose metabolism was inversely propotional to soleus muscle weight heavier than 25-35 mg[4]. Therefore, strips of soleus muscles weighing 25-35 mg were used in this study. At first, we examined the effects of brazilin on glucose utilization via glucose oxidation. As shown in Fig. 1 and 2, glucose oxidations were not influenced by brazilin but they were markedly increased in a dose dependent fashion in diabetic animals, although only high dose of insulin stimulated state was statistically different from paired control. Effects of brazilin on glycogen synthesis were shown in Fig. 3 and 4. Any significant effect of brazilin on glycogen synthesis were not observed in normal brazilin treated group. However, muscles from brazilin treated diabetic rats synthesized more glycogen than those from diabetic control rats in both basal and insulin stimulated state (116-132%, 134-156%, respectively). The magnitude of glycolysis in soleus muscle was determined by the measurement of ^3H_2O production from [5-^{3}H]-glucose. Fig. 5 and 6 show that brazilin did not affect glycolysis in normal rats but markedly increased in diabetic animals, especially in the insulin stimulated state. More effective results (147%) were obtained in high dose group (100 mg/kg brazilin). As shown in the obtained results, brazilin markedly increased overall glucose metabolic rates, which might be secondary to the effects on receptor binding or glucose transport and this fact might be important hypoglycemic mechanism of brazilin. Under this consideration we investigated the effects of brazilin on the insulin binding and the glucose transport. Although there were remarkable increases in insulin binding to muscles from normal rats treated with brazilin, no significant changes were found in muscles from diabetic treated animals (Table II and III). This result suggests that effects on insulin binding seemed to be minimally involved in enhancement of glucose metabolism in streptozotocin diabetic rats. It is well known that glucose transport is the rate limiting step for its utilization in soleus muscles[9]. Since glucose is rapidly phosphorylated and subsequently metabolized, 2-deoxyglucose, a non-metabolizable glucose analogue, was used for the determining rates of glucose transport[10]. 2-deoxyglucose is known to have

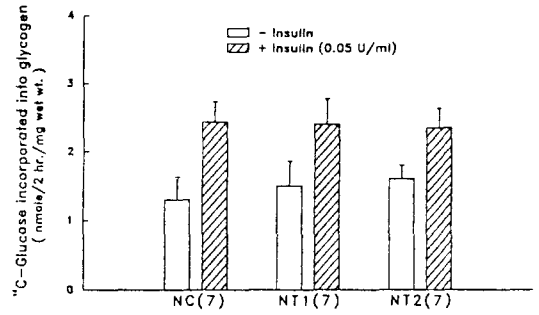

Fig. 3. *In vivo* effect of brazilin on glycogen synthesis in isolated soleus muscle from normal rats
NC: Normal control group
NT 1: Normal treated group (10 mg/kg)
NT 2: Normal treated group (100 mg/kg)
Values are mean ± SE
The number of experimental animals is given in paranthesis. Samples were administered through i.p. for 15 days.

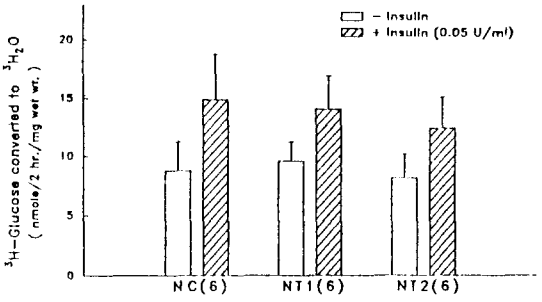

Fig. 5. *In vivo* effect of brazilin on glycolysis in isolated soleus muscle from normal rats
NC: Normal control group
NT 1: Normal treated group (10 mg/kg)
NT 2: Normal treated group (100 mg/kg)
Values are mean ± SE
The number of experimental animals is given in paranthesis. Samples were administered through i.p. for 15 days.

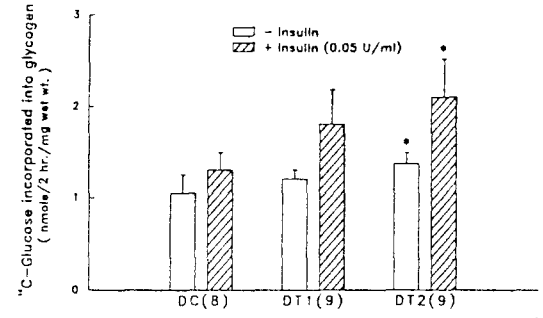

Fig. 4. *In vivo* effect of brazilin on glycogen synthesis in isolated soleus muscle from diabetic rats
DC: Diabetic control group
DT 1: Diabetic treated group (10 mg/kg)
DT 2: Diabetic treated group (100 mg/kg)
Values are mean ± SE
The number of experimental animals is given in paranthesis. Samples were administered through i.p. for 15 days.
*$p < 0.05$ *vs* DC

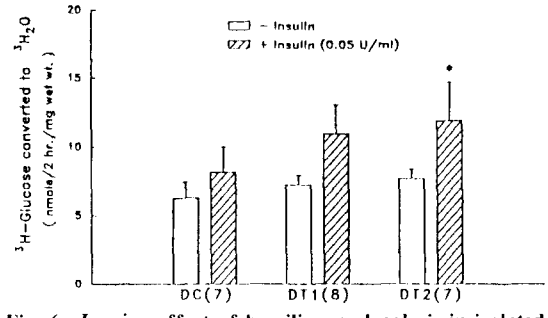

Fig. 6. *In vivo* effect of brazilin on glycolysis in isolated soleus muscle from diabetic rats
DC: Diabetic control group
DT 1: Diabetic treated group (10 mg/kg)
DT 2: Diabetic treated group (100 mg/kg)
Values are mean ± SE
The number of experimental animals is given in paranthesis. Samples were administered through i.p. for 15 days.
*$p < 0.05$ vs DC

affinities to the glucose carrier comparable to glucose. Therefore, the use of this analogue can ensure that the rate limiting step for glucose metabolism is not the intracellular metabolism subsequent to glucose transport but transport itself. Since it was reported that 2-deoxyglucose uptake of soleus muscles incubated with or without insulin was linear up to 60 min[11], 2-deoxyglucose uptake was measured for 30 min in this study. As shown in Fig. 7 and 8, both basal and insulin stimulated uptake seemed to be slightly increased by the treatment of brazilin, but statistical analysis revealed that there were no significant changes except basal uptake of muscles from animals treated with higher dose of brazilin (100 mg/kg). Considering the report that 2-deoxyglucose uptake in soleus muscle was dependent on its dose and thus, differences in uptake rates between normal and ob/ob mice increased in proportion to the dose of 2-deoxyglucose[5], more clear results might be obtained, if glucose transport assay was performed with more higher dose of 2-deoxyglucose.

Hypoglycemic effect of brazilin was more evidently

Table II. Affinity constant and binding sites in normal soleus muscle treated with brazilin
(per mg wet)

Group	K_1 ($\times 10^9$ M^{-1})	K_2 ($\times 10^8$ M^{-1})	R_1	R_2
Control	27.98	40.80	97.81	3,733
T-10	48.64	57.60	100.32	2,908
T-100	68.98	55.80	73.59	3,408

K_1: High affinity constant
K_2: Low affinity constant
R_1: High affinity binding sites
R_2: Low affinity binding sites
Brazilin was administered 10 mg/kg (T-10) or 100 mg/kg (T-100) for 15 days.

Table III. Affinity Constant and Binding Sites in Normal Soleus Muscle treated with Brazilin
(per mg wt)

Group	K_1 ($\times 10^9$ M^{-1})	K_2 ($\times 10^8$ M^{-1})	R_1	R_2
Control	46.89	50.70	95.48	5,638
DT-100	57.05	53.76	82.25	5,247

K_1: High affinity constant
K_2: Low affinity constant
R_1: High affinity binding sites
R_2: Low affinity binding sites
Brazilin was administered 100 mg/kg (DT-100) for 15 days.

observed in diabetic rats compared with normal rats. What caused this difference is not yet clear, but it is plausable that increased peripheral glucose utilization induced by brazilin was counterbalanced by the increased gluconeogenesis in the liver of normal subjects and therefore hypoglycemia was prevented. But in diabetic animals in contrast to normal animals, gluconeogenesis was already increased and thus, only little or no further increases in gluconeogenesis might be caused by brazilin administration, which resulted in the lowering of blood glucose in diabetic animals[12].

From the results obtained hitherto, we could find that enhancement of peripheral glucose utilization might be one of the major causes of hypoglycemic action of brazilin.

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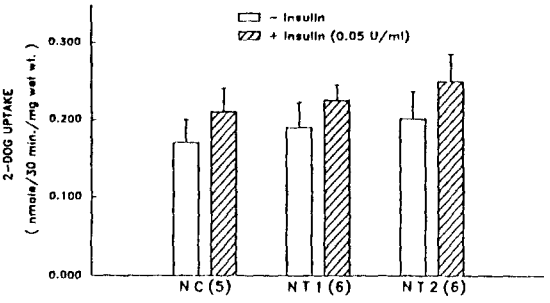

Fig. 7. *In vivo* effect of brazilin on 2-DOG uptake in isolated soleus muscle from normal rats
NC: Normal control group
NT 1: Normal treated group (10 mg/kg)
NT 2: Normal treated group (100 mg/kg)
Values are mean ± SE
The number of experimental animals is given in paranthesis. Samples were administered through i.p. for 15 days.

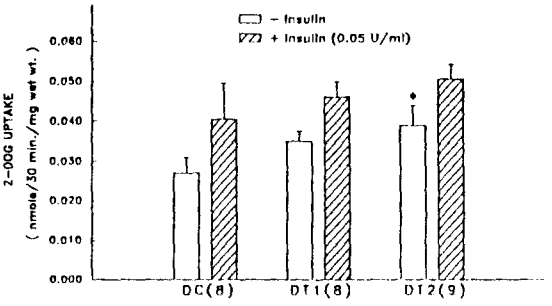

Fig. 8. *In vivo* effect of brazilin on 2-DOG uptake in isolated soleus muscle from diabetic rats
DC: Diabetic control group
DT 1: Diabetic treated group (10 mg/kg)
DT 2: Diabetic treated group (100 mg/kg)
Values are mean ± SE
The number of experimental animals is given in paranthesis. Samples were administered through i.p. for 15 days.

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