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Effects of Brazilin on Glucose Metabolism in Epididymal Adipocytes from Streptozotocin induced Diabetic Rats

Soo Hwan LEE, Hyeon Soon WON, Lee Yong KHIL, Chang-Hyun MOON[1] Jin Ho CHUNG and Chang-Kiu MOON*

College of Pharmacy, Seoul National University, Seoul 151-742, Korea
[1]College of Medicine, Ajou University, Suwon 442-749, Korea

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Abstract—Hypoglycemic mechanism of brazilin was investigated in the streptozotocin induced diabetic rats. Plasma glucose levels of diabetic rats were significantly ameliorated by the treatment of brazilin, but there were no changes in plasma insulin levels. Brazilin increased insulin binding capacity to epididymal adipocytes, and Scatchard analysis revealed that this increase in insulin binding was not due to the increase of insulin binding sites but that of binding affinity. 2-Deoxyglucose uptake of epididymal adipocytes was significantly augmented by the intraperitoneal administration of brazilin and the same result was obtained in *in vitro* study. Glucose oxidation and lipogenesis in epididymal adipocytes were significantly enhanced by the treatment of brazilin.

Keywords □ brazilin, insulin binding, glucose transport, glucose oxidation.

Since sulfonylamide 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. However, many of them have serious toxicities and others are controversial as to whether they are sufficiently effective and safe enough to warrant their wide spread usage (Krall, 1986; Lebovitz, 1990). For this reason, development of new antidiabetic agents, which have sufficient hypoglycemic effects as well as safety, is very important and desired in the management of diabetes mellitus. Based on this point of view, we started to search for potential hypoglycemic agents from natural sources and found that brazilin, a natural dye, has a potent hypoglycemic effect (Moon *et al.*, 1993) and could improve several diabetic complications (Moon *et al.*, 1985, 1989). In this study, we tried to elucidate the hypoglycemic action mechanism of brazilin in streptozotocin induced diabetic rats and found that it increased the glucose metabolism in peripheral tissues.

Materials and Methods

Materials

Streptozotocin, crystalline porcine insulin, collagenase (type II) and Parker's medium 199 were obtained from Sigma Chemical (St. Louis, Missouri, USA). 2-Deoxy-[1,2-^{3}H]-D-glucose (specific activity 40 Ci/mmol), [1-^{14}C]-D-glucose (specific activity 40 Ci/mmol) and ^{125}I-insulin (specific activity<2,200 Ci/mmol, receptor grade) were obtained from New England Nuclear (Boston, Massachusettes, USA). Brazilin monohydrate was purchased from Aldrich Chemical Co. (Milwakee, Wisconsin, USA). All other reagents were of analytical grade.

Animals

Male Sprague-Dawley rats (160~180 g) fed *ad libitum* were used in all studies. Nonketotic diabetes was induced by tail vein injection of streptozotocin (40 mg/kg) dissolved in citrate buffer (pH 4.5). Animals with plasma glucose level of about 350 mg/d*l* were used as diabetic rats.

Isolation of Epididymal Adipocytes

Rats were sacrificed by cervical dislocation and epididymal fat pads were removed. Isolated fat cells were prepared by shaking at 37℃ for 60 min in Krebs-Ringer Hepes buffer (pH 7.4) containing collagenase (1 mg/m*l*), glucose (2 mM) and bovine serum albumin (10 mg/m*l*) according to the method of Rodbell (1964). Adipocyte counts were performed using a microscope according to the method of Gliemann *et al.* (1967).

* To whom correspondence should be addressed.

Primary Culture of Epididymal Adipose Tissue

Epididymal fat pads were removed under sterile conditions and placed in Parker's medium 199 containing 1.0 mM HEPES and 10% fetal bovine serum, pH 7.4 (1971). Approximately 20 pieces, each weighing 5~10 mg, were placed in a culture dish and incubated at 37℃ for 20 hours in a humidified 5% CO_2/95% air incubator. During this period, the fat tissue was incubated in the absence or presence of 10^{-5} M or 10^{-6} M of brazilin. Metformin hydrochloride (10^{-5} M) was used as positive control.

Insulin Binding Assay

Isolated fat cells were suspended in Krebs-Ringer Hepes buffer, pH 7.0 containing 1.0 mM sodium pyruvate and 1% bovine serum albumin. The cells were then incubated in this buffer for 30 minutes at 37℃ in order to dissociate any remaining receptor bound insulin (Garvey W. T. *et al.*, 1987). Cells were resuspended in Krebs-Ringer Hepes buffer, pH 7.6, supplemented with 5 mM glucose and 0.5 mg/m*l* bacitracin. One m*l* aliquots of adipocytes were incubated in a shaking water bath at 16℃ with 0.2 ng of ^{125}I-insulin (sp. act. 2,200 Ci/mmol, receptor grade) and unlabeled insulin at indicating concentration in plastic test tubes. Optimal steady state for binding conditions was achieved after 120 minutes of incubation. The incubations were terminated by removing 300 μl aliquots from the cell suspension and rapidly centrifuging the cells in plastic microtubes to which 100 μl of dinonyl phthalate oil had been added as described by Gammeltoft and Gliemann (1973). All data were corrected for nonspecific binding, which means the amount of radioactivity remaining bound in the presence of excess (50 μg/m*l*) of native hormone. Degradation of ^{125}I-insulin was determined by measuring its precipitability in 0.75 M trichloroacetic acid (Garvey W. T. *et al.*, 1987).

Glucose Transport Assay

Glucose transport studies were performed using oil floatation technique described for the binding studies (Gammeltoft and Gliemann, 1973). Isolated adipocytes were incubated with 2-deoxy-[1,2-^{3}H]-D-glucose (sp. act. 2 mCi/mmol) at a concentration of 0.125 mM in Krebs-Ringer Hepes buffer, pH 7.4 containing 2 mM pyruvate following a half hour preincubation in the absence or the presence of insulin (25 ng/m*l*). Exactly 3 minutes after, 200 μl aliquots from the assay mixture were transferred to plastic microtubes containing 100 μl of dinonyl phthalate oil. Medium and cells were separated by centrifugation for 30 seconds through oil. The incubation was terminated when centrifugation began. Additional incubations which had contained phloretin were carried out to correct for sugar trapped but not transported by the cell pellets.

Glucose Oxidation and Lipogenesis

The ability of adipocytes to metabolize glucose was determined by measuring incorporation of [1-^{14}C]-D-glucose into $^{14}CO_2$ and [^{14}C]-triglycerides (Rodbell, 1964). Adipocytes were incubated at 37℃ for 1 hour with [1-^{14}C]-D-glucose at a total glucose concentration of 5 mM in Krebs-Ringer Hepes buffer, pH 7.4, containing bovine serum albumin (10 mg/m*l*). The conversion of glucose into its metabolites was terminated by the addition of 6N H_2SO_4 and the generated $^{14}CO_2$ was collected. The remaining medium was extracted by the Dole's procedure to determine total lipids (Dole and Meinertz, 1960).

Statistical Analysis

All data were analyzed by Duncan's multiple range test.

Results and Discussion

The pathogenesis of diabetes mellitus remains unclear, despite an enormous literature developed over many years. One important feature of this disease obscuring the etiologic picture is that it is not a single disease process, but rather, represents a heterogenous constellation of disease syndromes all leading to a final common pathway, *i.e.* hyperglycemia (Olefsky *et al.*, 1985). Three major metabolic abnormalities co-exist in diabetes mellitus, each contributing to the hyperglycemic state (Ward *et al.*, 1984; Olefsky *et al.*, 1982). These include impaired insulin secretion, peripheral insulin resistance, and increased basal hepatic glucose production. Abnormal beta cell secretion is apparently one cause of insulin resistance, but available evidence points to a target tissue defect in insulin action represents a multistep sequence; the binding of insulin to receptor is only the initial event. A defect in any effector systems distal to receptor binding can also lead to impaired insulin action and resistance. These defects can involve abnormal coupling between insulin receptor complexes and the glucose transport system *per se*, or a variety of intracellular enzymatic defects located in various pathways of glucose metabolism.

Based on these points of views, we tried to elucidate hypoglycemic mechanism of a natural dye, brazilin, which has various positive biological activities, especially in the hyperglycemia associated state. Firstly, we tried to elucidate whether the action site of brazilin is pancreas or extrapancreatic tissues. As shown in Table I, brazilin treatment did not elicit insulin release

Table I. Characteristics of Experimental Animals

	Blood Sugar (mg %)		Body Weight (g)		Plasma Insulin (μU/ml)	
	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 Treated I (30)	114± 22	120± 13	200± 23	293± 21	27.4± 4.0	29.0± 3.0
Normal Treated II (30)	125± 21	105± 27	218± 15	282± 17	30.0± 7.0	30.0± 7.3
Diabetic Treated I (28)	364± 22	282± 69	200± 11	269± 27	20.4± 4.0	21.3± 2.0
Diabetic Treated II (29)	368± 32	197± 68*	212± 10	257± 30	22.0± 2.0	23.3± 4.0

Brazilin was intraperitoneally administered 10 mg/kg (I) or 100 mg/kg (II) in physiologic saline for 15 days. Control groups were administered the same volume of physiologic saline. Each value represents mean± S.D. 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.

Table II. Effects of brazilin and metformin on the number and the affininty constant of insulin receptor of primary cultured epididymal adipocytes (per cell)

Group	K_1 ($\times 10^9 M^{-1}$)	K_2 ($\times 10^8 M^{-1}$)	R_1	R_2
Control	4.21	5.00	1,780	8,390
Brazilin	6.20	4.46	1,500	7,830
Metformin	5.50	6.31	1,910	8,130

K_1: High affinity constant K_2: Low affinity constant
R_1: High affinity binding sites R_2: Low affinity binding sites
Cells were treated with brazilin (10^{-5} M) or metformin (10^{-5} M) during 22 hrs cultivation.

from pancreas in spite of the reduction of blood glucose levels. From these results, we deduced that hypoglycemic effect of brazilin was not caused by direct action to pancreas thus peripheral tissues might be regarded as action sites. Thus, in order to get informations on the hypoglycemic action of brazilin, effects on receptor- and postreceptor-events in epididymal adipocytes were accessed.

First of all, in order to ensure the optimal conditions for binding studies, degradation of insulin was measured. Under our experimental conditions for binding studies, only 2%~5% of ^{125}I-insulin was degraded during experiment. Thus, degradation of insulin was thought to give minimal effect on insulin binding study. *In vivo* treatment of brazilin slightly increased insulin binding in diabetic animals. The increment in total insulin binding ranged from 110%~120%, depending on the dose of brazilin (data are not shown). But the effects might be somewhat underestimated due to the insulin deficiency in streptozotocin induced diabetic rats. It is well known that insulin binding is significantly

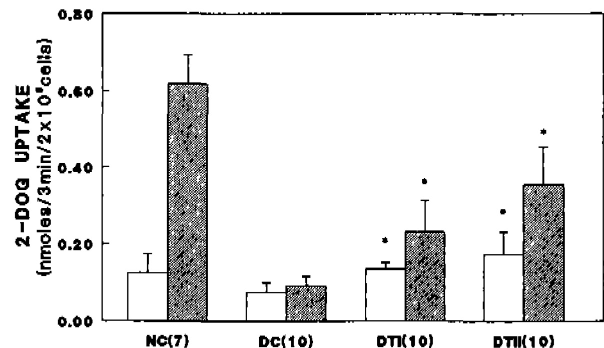

Fig. 1. Effects of brazilin on the glucose uptake in epididymal adipocytes. Brazilin was intraperitoneally administered for 15 days. Control groups were administered the same volume of physiologic saline. The number of experimental animals are given in parenthesis. Adipocytes were incubated for 1 hr with [1-^{14}C]-D-glucose in the presence (hatched bar) or absence (open bar) of maximally effective concentration of insulin (25 ng/ml). NC: normal control DC: diabetic control DTI: diabetic brazilin treated (10 mg/kg) DTII: diabetic brazilin treated (100 mg/kg). Values are means± S.E. *; $p<0.05$ vs DC.

increased in insulin deficient state (Kasuga *et al.*, 1978). Scatchard analysis revealed that increase in insulin binding was mainly due to increase in binding affinity rather than the number of binding sites (Table II). In this sense, brazilin shared common feature with metformin which is clinically used as hypoglycemic agent.

In order to evaluate the effects of brazilin on postreceptor events, glucose transport activity was primarily investigated. As shown in Fig. 1, the treatment of brazilin in diabetic animals resulted in the marked impro-

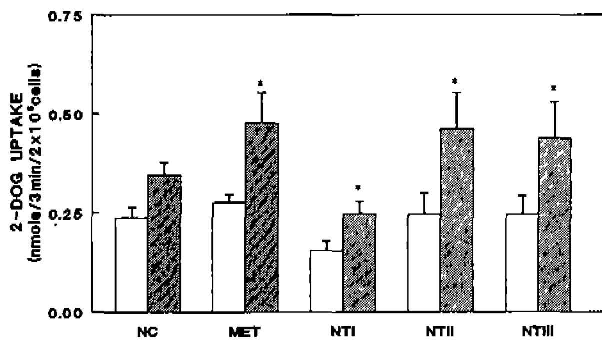

Fig. 2 Effects of brazilin on the glucose uptake in primary cultured epididymal adipocytes. Adipocytes isolated from noraml SD rats were incubated with samples for 22 hrs. The maximally effective concentration of insulin (25 ng/ml), hatched bar) or saline (open bar) was added 30 min before assay. NC: normal control MET: metformin treated (10^{-5} M) NTI: brazilin treated (10^{-4} M) NTII: brazilin treated (10^{-5} M) NTIII: brazilin treated (10^{-6} M). Values are means± S.E. for four separated experiments. *; $p<0.05$ vs NC.

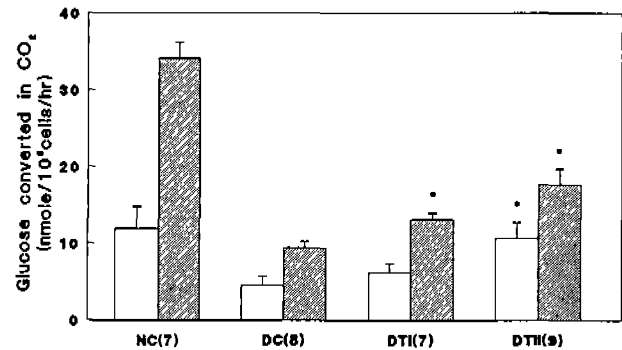

Fig. 3. Effects of brazilin on the glucose oxidation in epididymal adipocytes. Brazilin was intraperitoneally administered for 15 days. Control groups were administered the same volume of physiologic saline. The number of experimental animals are given in parenthesis. Adipocytes were incubated for 1 hr with [1-^{14}C]-D-glucose in the presence (hatched bar) or absence (open bar) of maximally effective concentration of insulin (25 ng/ml). NC: normal control DC: diabetic control DTI: diabetic brazilin treated (10 mg/kg) DTII: diabetic brazilin treated (100 mg/kg). Values are means± S.E. *; $p<0.05$ vs DC.

vement of 2-deoxyglucose transport activity of epididymal adipocytes. It is well known that glucose *per se* (*i.e.* hyperglycemia) might be a cellular toxin (Unger and Grundy, 1985) and chronic hyperglycemia could lead to the development of insulin resistance both *in vivo* and *in vitro* through down regulation of the glucose transport system (Rossetti *et al.*, 1987; Sasson and Cerasi, 1986; Van Putten and Krans, 1985). Thus, it could not be excluded that above result might be simply due to secondary effect of amelioration of hyperglycemia. To clarify this possibility, adipose tissue culture was adopted, and during the cultivation, adipose tissue was exposed to brazilin or metformin. After 20 hour's incubation, 2-deoxyglucose uptake was measured and the results are shown in Fig. 2. Insulin stimulated 2-deoxyglucose uptake was significantly increased by the treatment of brazilin (10^{-5} M and 10^{-6} M) and 10^{-5} M of metformin (135%, 126% and 139%, respectively), but the basal uptake was not significantly altered in any group. These results suggest that restoration of glucose transport in diabetic treated animals is mediated by not only improvement of hyperglycemia but also direct effects on glucose transport *per se* or related factors. Insulin is known to have three separate effects on glucose transport process; (a) direct activation of glucose transporters within the plasma membrane (an acute effect); (b) recruitment of glucose transporters from the cytoplasm to the plasma membrane (an acute effect); (c) stimulation of synthesis of glucose transport units (a chronic effect) (Pilch, 1991). According to Lockwood *et al.* (1983), sulfonylurea potentiated insulin stimulated hexose transport by its *in vitro* treatment and Jacobs *et al.* (1985) concluded that this effect was mainly due to a potentiation of the insulin induced recruitment of carriers. Likewise, *in vitro* effect of brazilin might be explained by one of the above three possibilities. Another study suggests that one of the cell membrane component, cis-unsaturated fatty acid (*e.g.* oleic acid) might participate in the fusion of glucose transporter and cell membrane so that finally lead to increased glucose entry and metabolism (Shechter and Henis, 1984). Previous study performed in this laboratory demonstrated that the content of oleic acid in cell membrane of diabetic mice was markedly increased following treatment of brazilin (Ha, 1985). Thus, it is quite possible that the hypoglycemic action of brazilin could be partially explained by the enhancement of the recruitment of glucose transport system.

The effect of brazilin on glucose oxidation and lipogenesis were presented in Fig. 3 and Fig. 4. Brazilin significantly improved the glucose oxidation and lipogenesis in diabetic adipocytes. These results could be in part, explained by the improvement of glucose transport activity but our previous study revealed that activities of several lipogenic enzymes in diabetic animals were increased by the treatment of brazilin (Moon *et al*, 1993). Thus, increases in glucose metabolism, we guess, might be not only due to increased glucose uptake but also improvements of activities of glucose metabolic enzymes.

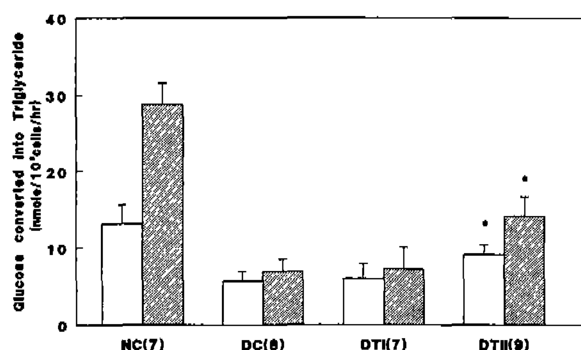

Fig. 4. Effects of brazilin on the lipogenesis in epididymal adipocytes. Brazilin was intraperitoneally administered for 15 days. Control groups were administered the same volume of physiologic saline. The number of experimental animals are given in parenthesis. Adipocytes were incubated for 1 hr with [1-^{14}C]-D-glucose in the presence (hatched bar) or absence (open bar) of maximally effective concentration of insulin (25 ng/m*l*). NC: normal control DC: diabetic control DTI: diabetic brazilin treated (10 mg/kg) DTII: diabetic brazilin treated (100 mg/kg). Values are means± S.E. *; $p<0.05$ vs DC.

There have been many reports on the multiplicity of insulin action and many researchers tried to suggest evidences explaining this multiplicity. In fact, the effect of insulin in increasing hexose transport were reported to be dissociated from the effects in inhibiting lipolysis (Shechter, 1986). And also, many investigators have reported several putative mediators of insulin action. Bernier *et al.* (1987) reported the low molecular weight polypeptide was phosphorylated by insulin tyrosine kinase and suggested that phosphorylation of this peptide on tyrosine may control glucose transport. Malchoff *et al.* (1987) isolated two putative mediators—one stimulates pyruvate dehydrogenase and the other inhibits cAMP dependent protein kinase and adenyl cyclase. These mediators were identified as separate molecules in spite of similar composition. More recently, on the basis of above results, Larner (1988) and Saltiel (1989) tried to correlate insulin receptor kinase activity with mediator formation and through this speculation, multiplicity of insulin action was tried to be explained.

Considering insulin signaling mechanisms, it may be possible to get insights into the hypoglycemic action mechanism of brazilin. In fact, it is well known that flavonoids have diverse biological activities including hypoglycemic activity and many of them are thought to be due to the generation of H_2O_2 (Farkas *et al.*, 1985). It was reported that hydrogen peroxide increased insulin receptor associated tyrosine kinase activity (Hayes, 1987). Thus, although many questions remain to be elucidated, it is possible that brazilin, a flavonoid analogue, might share some of mechanisms of insulin action or affect signal transduction processes. Of course, it could not be excluded that, at least in some aspects, brazilin act via other process than that of insulin. In order to understand the action mechanism of brazilin more precisely, effects on several biochemical processes including signal transduction are under investigation.

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