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Effects of Brazilin on Glucose Metabolism in Primary Cultured Rat Hepatocytes

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Abstract□In order to investigate the cellular mechanisms of hypoglycemic action of brazilin, hepatocyte monolayer culture was introduced and, glycogen synthesis rate and insulin binding were measured as parameters. Glycogen synthesis and insulin sensitivity were remarkably augmented by the treatment of brazilin. Brazilin slightly increased insulin binding. Scatchard analysis revealed that this increase in insulin binding was not due to increase in the binding capacity but in binding affinity. These results suggest that the augmentation of hepatic glycogenesis and insulin sensitivity by brazilin may play an important role in the improvement of hyperglycemia.

Keywords□Brazilin, hepatocyte, monolayer culture, glycogenesis, insulin binding.

In spite of extensive searches for hypoglycemic compounds, only two classes of compounds have been successfully developed and used clinically, sulfonylureas and biguanides. But the exact mechanisms of hypoglycemic action of these drugs are still unclear and their side effects are too serious for either of these agents to be a choice of drug[1-3]. From this reason, exploration of new hypoglycemic agents, of which effects are definite and safe enough, is still required. In the search of new hypoglycemic agents, brazilin, an active principle of *Caesalpinia sappan,* was proved to have hypoglycemic effect in our laboratory. The heart wood of *C. sappan* has long been used as emmenagogue, analgesic, astringent, antidiarrhetic and microcirculatory stimulating agnet[4]. In order to elucidate the hypoglycemic mechanism of brazilin, we began to investigate in this study the effects on glucose metabolism in primary cultured rat hepatocytes.

MATERIALS AND METHODS

Materials

Porcine monocomponent insulin, oyster glycogen, Hank's balanced salt solution, collagenase and Waymouth's medium were purchased from Sigma Chemical Co., U.S.A. Dulbecco's MEM was obtained from Gibco, U.S.A; [1-^{14}C]-glucose from ICN radioisotope Co., U.S.A; receptor grade ^{125}I-insulin from New England Nuclear, U.S.A and brazillin monohydrate was from Aldrich Chemicals, U.S.A. Other chemicals were guaranteed grade. Male Sprague-Dawley rats were supplied from the Experimental Animal Breeding Center of Seoul National University.

Isolation of hepatocytes

Rat hepatocytes were isolated using a modified collagenase perfusion technique as described by Dickins and Peterson[5]. Isolated cell suspension was diluted to 1.0×10^6 cell/m*l* in the WO/BA-M2 medium containing 10% FBS and 3 m*l* was pipetted into 60×15 mm plastic Petri dishes precoated with rat tail collagen. The viability of isolated cell suspension was routinely above 80%. After hepatocytes were inoculated, they were incubated at 37°C in a humidified 5% CO_2/95% air incubator. The medium was changed 4 hr's after initial plating with serum free Dulbecco's MEM containing 11 mM glucose in order to remove unattached cells and cellular debris. After 24 hr's cultivation, the medium was aspirated off and the plate was replenished with fresh medium (serum free Dulbecco's MEM supplemeted with 11 mM glucose) with or without brazilin (10^{-4} M ~ 10^{-6} M) or metformin hydrochloride (10μg/m*l*) and incubated for 22 hours.

Determination of D-[U-^{14}C]-glucose incorporation into glycogen

At the end of incubation medium was aspirated off, and plates were incubated for 3 hr in fresh medium supplemented with labelled glucose (final concentration 11 mM, total activity 1.2 mCi/*l*) and insulin (0-100 mU/m*l*). Incubation was stopped by three washes with ice cold phosphate buffered saline containing 20 mM cold glucose. The hepatocyte monolayers were scraped off and cell pellets were obtained by rapid centrifugation (12,000g, 1 min). Each cell pellet was suspended in 1.5 m*l* of 30% KOH solution, and 5 mg of carrier glycogen was added to cell suspension, and then, glycogen was precipitated by treatment. Glycogen precipitate was finally resuspended and radioactivity was measured.

Insulin binding assay

At the end of 22 hr's treatment, fresh culture medi um was added to the monolayer before the binding experiments. The temperature was lowered to 20°C and after 1 hr, the medium was exchanged against an identical culture medium (2 m*l*) which contained in addition labelled insulin. In order to prevent degradation of insulin, bacitracin was added at the concention of 1.5 mg/m*l*. Equilibrium binding was achieved after 2 hr's incubation at 20°C[6]. The suspernatant was then aspirated to remove unbound insulin and the monolayer was washed five times with chilled phosphate buffered saline (pH 7.4). The cells were taken off in 1 m*l* of 1% Sodium dodesyl sulfate solution and counted radioactivity. The binding data were corrected for protein content, insulin degradation and non specific binding, *i.e.*, the binding of labelled insulin in the presence of insulin 50 μg/m*l*. ^{125}I-labelled insulin degradation was determined by trichloroacetic acid precipitation[7].

Statistical analysis

The data were subjected to analysis 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

Brazilin, an active principle of *Caesalpinia sappan*, has been reported to have several biological activities including antidiabetic action[8-14]. For the investigation of cellular mechanisms of hypoglycemic action of brazilin, hepatocyte monolayer culture was introduced and glycogen synthesis rate was measured as a parameter. In order to obtain optimal experimental condition, glucose concentration and incubation time were determined in preliminary study. In this study, Dulbecco's MEM supplemented with 11 mM glucose was chosen for the experiment because this particular concentration was known to balance positive effect of glucose itself on glycogen synthesis against the ongoing glycogenolysis[15,16], and increased sufficiently the stimulation of glycogen synthesis by insulin[17]. The rate of glycogen synthesis was measured for 3 hr, because under this condition, insulin stimulated the glycogen synthesis sufficiently (223 ± 9% stimulation vs. basal). In contrast, with 1.5 hr's incubation, insulin did not significantly enhance glycogen synthesis (124 ± 24% stimulation vs. basal). As shown in Fig. 1, brazilin significantly increased the glycogen synthesis in primary cultured rat hepatocytes. Metformin hydrochloride, a most widely used biguanide, also increased the glycogen synthesis, but compared to brazilin (10^{-5} M), the effect was somewhat lower in this experimental condition. High dose of brazilin (10^{-4} M) strikingly inhibited glycogen synthesis. This effect seems to be due to unusuall high concentration of brazilin compared to physiological state. But the correct reason why this effect was evoked should be further investigated. Effects on glycogen synthesis in the presence of various concentrations of insulin are also shown in Fig. 2. Consistent with the previous findings, brazilin enhanced glycogen synthesis at all insulin concentrations used in this experiment. To analyze the effect of brazilin on the insulin sensitivity, these data are replotted as the percent of maximal insulin stimulation. Fig. 3 shows that insulin sensitivities were augmented by the treat-

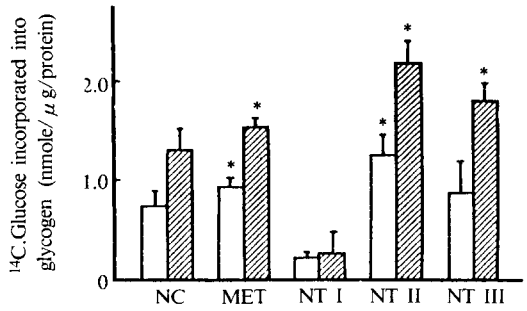

Fig. 1. Effect of brazilin on glycogenesis in primary cultured rat hepatocytes.

NC: Normal control group
Met: Metformin treated group (10^{-5} M)
NT I: Brazilin treated group (10^{-4} M)
NT II: Brazilin treated group (10^{-5} M)
NT III: Brazilin treated group (10^{-6} M)
Values are mean ± SE of three separate experiments.
□ – Insulin ▨ + Insulin (100 mU/m*l*)
*$p<0.05$ vs NC

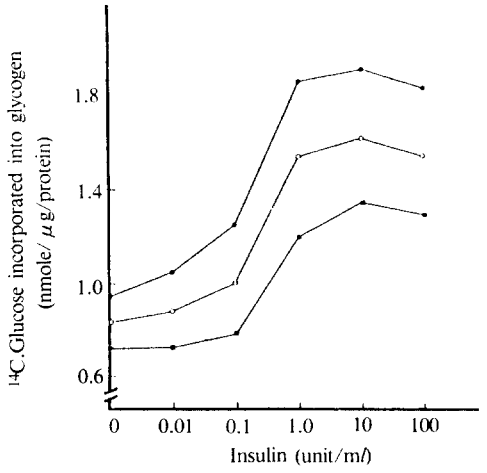

Fig. 2. Effect of brazilin on glycogenesis in primary cultured rat hepatocytes at various concentrations of insulin.
(■) Control group
(○) Metformin treated group (10^{-5} M)
(●) Brazilin treated group (10^{-5} M)

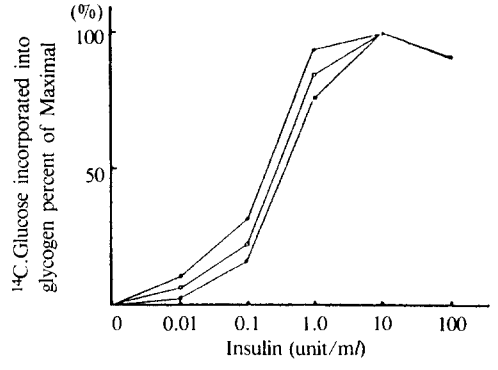

Fig. 3. Effect of brazilin on insulin sensitivity in primary cultured rat hepatocytes.
(■) Control group
(○) Metformin treated group (10^{-5} M)
(●) Brazilin treated group (10^{-5})

ment of brazilin and metformin. From the foregoing findings, increase in the insulin binding could be predicted. In order to assess the effects of brazilin on insulin binding to insulin target tissues, first of all, incubation time for binding equilibrium should be determined. In the case of isolated adipocytes, binding equilibrium was achieved within 2 hr under this experimental condition. By the *in vitro* treatment of brazilin, slight increase in insulin binding (114%) were observed, but statistic analysis revealed that there were no significant changes (data are not shown). In order to characterize the insulin binding, Scatchard analysis was performed. Since insulin receptor shows negative cooperativity, curvilinear plots are usually obtained. Although it is difficult to calculate the exact binding affinity from curvilinear Scachard plots[18], for convenience' sake, apparent affinity constants were calculated by fitting the data to two linear components over the same range of insulin concentrations[19]. The intercept on the abscissa of the Scatchard plot approximates the two kinds of receptor site concentration. But it must be born in mind that this curve fitting method gives quite large variations[19]. Scachard analysis of insulin binding data were summarised in Table I. While there were no significant alterations in the number of receptor sites, affinity constants, especially high affinity constants were markedly increased. Metformin also altered the affinity constant but not the number of receptor sites. These results well coincide with other published studies[20]. To ensure the optimal conditions for binding studies degradation of insulin was measured. Under the condition used in this study, only 2-5% of total ^{125}I-insulin was degraded during the experiment (data are not shown). Therefore, degradation of insulin was thought to give minimal effect on insulin binding. Diabetes is not a single diease, but rather represents a heterogenous constellation of disease syndroms, all leading to a final common pathway, *i.e.*, hyperglycemia[21]. Three major metabolic abnormalities co-exist in dibetes mellitus, each contributing to the hyperglycemic state. These include impared insulin secretion, peripheral insulin resistance, and increased basal hepatic glucose production[22,23]. It is well known that target tissue defects in insulin action is the major cause of insulin resistance. Defects in the binding of insulin to receptor as well as in any effector system distal to receptor binding can lead to impared insulin action and resistance. In this study, we found that brazilin increased glycogen synthesis and insulin sensitivity in primary cultured rat hepatocytes. These results imply some special senses, because insulin resistance, a prominent feature of diabetes, is considerably related to hepatic function[21,24]. In dia-

Table I. Affinity constant and binding sites in primary cultured rat hepatocytes

(per mg protein)

Group	K_1($\times 10^9$ M^{-1})	K_2($\times 10^8$ M^{-1})	R_1	R_2
Control	59.69	10.74	272.72	4,596
Brazilin	71.88	10.80	264.44	4,533
Metformin	68.76	13.68	261.43	4,941

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

betes, hepatic glycogensis is seriously inhibited and thus, considering these facts, the augmentation of hepatic glycogenesis by brazilin may play an important role in the improvement of hyperglycemia. Generally, the regulation of hepatic glucose output is regarded as the most important factor in maintaining euglycemia. In fact, hepatic glucose output is markedly increased in diabetes[24]. For further elucidation of hypoglycemic action mechanism of brazilin, effects of brazilin on hepatic glucose output are being now undertaken in our laboratory.

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