Hydrogen Peroxide Mediates Brazilin-induced Glucose Transport in Adipocytes

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Abstract – Brazilin shows hypoglycemic effect in diabetic animals through enhancement of glucose metabolisms in insulin responsive tissues. One of the major mechanisms of brazilin to enhance glucose metabolism is stimulation of glucose transport in adipocytes. In this study, the essential molecular moiety of brazilin for the stimulation of glucose transport was investigated. We found that brazilin undergoes a structural change in physiological buffer and produces hydrogen peroxide. Methylation of hydroxyl group of brazilin or addition of catalase along with brazilin resulted in the complete inhibition of brazilin-induced glucose transport in adipocytes. Because hydrogen peroxide increases glucose transport by inhibition of phosphatases, we examined the effect of brazilin on phosphatase activity. Brazilin inhibited phosphatases in a wide range of acidity, and protein phosphatase 1 and 2A were also inhibited. These results suggest that the production of hydrogen peroxide by oxidation of catechol hydroxyl group of brazilin mediates glucose transport through inhibition of phosphatases which otherwise decrease glucose transport in adipocytes.

Keywords □ brazilin, hydrogen peroxide, glucose transport, adipocyte, phosphatase

In type 2 diabetes, hyperglycemia results from metabolic abnormalities including insulin resistance, defects in glucose-stimulated insulin secretion, and excessive hepatic glucose output (DeFronzo et al., 1992). In most genetically predisposed individuals, there is a slow progression from a normal state to insulin resistance followed by hyperinsulinemia, glucose desensitization, defects in insulin secretion, impaired glucose tolerance, and then to hyperglycemia (Jun et al., 1999).

Impaired glucose transport is one of the major factors contributing to insulin resistance in type 2 diabetic patients (James and Piper, 1994). The ability of insulin to mediate tissue glucose uptake is a critical step in maintaining glucose homeostasis and in clearing the post-prandial glucose load (DeFronzo, 1985; Kruszynska and Olefsky, 1996).

Brazilin, a major active principle of Caesalpinia sappan (Hikino et al., 1977), lowered the nonfasting plasma glucose level in normo- and hyperglycemic KK-mice, and potent hypoglycemic effect was observed in spontaneous diabetic KK mice without any change in plasma insulin level (Moon et al., 1990; Moon et al., 1993). Studies on the hypoglycemic mechanisms of brazilin have revealed that brazilin increases the rate of glucose transport, glucose oxidation and lipogenesis possibly through the regulation of phosphatidylinositol 3-kinase (Khil et al., 1999), glucose 6-phosphate dehydrogenase and fatty acid synthetase (Moon et al., 1993), respectively. In addition to the hypoglycemic effects, brazilin also showed positive effects on diabetic complications (Moon et al., 1985; Yang et al., 2000). For these reasons, brazilin is considered as a possible anti-diabetic agent.

Despite of intensive studies on the physiological effects of brazilin, the essential moiety of brazilin for its physiological roles is not yet clearly known. Flavonoids can produce reactive oxygen species through autoxidation (Bracke et al., 1988). Hematoxylin, the structural analogue of brazilin, is also known to produce superoxide anion during its oxidation (Martin et al., 1987). The reactive oxygen can be converted to hydrogen peroxide in cells (Fidelus, 1988), which is known to increase glucose transport (Mahadev et al., 2001). These facts suggest that brazilin can produce reactive oxygen, when it is oxidized to brazillein.

In this study, we examined the production of hydrogen peroxide by brazilin and its role in glucose transport in isolated rat epididymal adipocytes, in order to find the essential moiety of brazilin for the stimulation of glucose transport.
MATERIALS AND METHODS

Experimental animals and materials

Sprague-Dawley rats were purchased from the Animal Breeding Center of Seoul National University, Korea. The animals were fed with autoclaved rodent laboratory chow and water ad libitum in an air-filtered room. The study has been carried out along the “Principles of laboratory animal care” (NIH Publication no. 85-23, revised 1985).

Brazili(n[(6αS-cis)-7,11b-dihydrobenz[b]indenol[1,2-d]pyran-3, 6a, 9, 10(6H)-tetro] was used as (+) brazilin monohydrate ((6α)[3]D +123° (C=1, CH₂OH)), the product of Aldrich Chemical, USA. [γ-,32P]-ATP and [3H]-2-deoxyglucose (2-DOG) were obtained from Amersham Pharmacia Biotech, Buckinghamshire, UK. Collagenase type II, catalase, peroxidase, okadaic acid, silicone oil, phloretin, dihydrodihydramine 123 (DHR 123) were purchased from Sigma, St. Louis, MO, USA. Protein phosphatase assay system was the product of Gibco BRL, Gaithersburg, MD, USA. Other reagents were highest grade available.

Isolation of epididymal adipocytes

Epididymal adipose tissue was removed from 5-week-old male SD rats and digested with collagenase type II (1 mg/ml) in 1 ml Krebs-Ringer HEPES (KRH) buffer (131 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.25 mM MgCl₂, 2.5 mM NaH₂PO₄, 10 mM HEPES, pH 7.4) supplemented with 1% bovine serum albumin (BSA) and 2 mM pyruvate for 1 h at 37°C (Olesfsky, 1975). After digestion, adipocytes were separated by gentle massage with a rubber policeman followed by sieving through a 200 μm polycarbonate mesh. Isolated adipocytes were washed with fresh KRH buffer without collagenase three times. Adipocytes were counted under the microscope (Green, 1986).

Assay of glucose transport

Adipocytes (5×10⁵ cells/ml) in KRH buffer were incubated at 37°C for 20 min in a shaking water bath. Adipocytes were then treated with brasilin (50 μM or as indicated) or tetramethylbrazili(n[5 μM, TMB]) in the presence or absence of catalase or okadaic acid for 4h at 37°C. After incubation, [3H]-2-DOG (0.5 μCi, 0.125 mM) was added and the cells were incubated for 3 min. Three hundred μl of cell suspension was transferred to microtubes containing 100 μl of silicone oil. Cells were centrifuged for 30 sec, the oil layer was removed (Khil et al., 1999), and the radioactivity in the cell layer was measured. Trapped [3H]-2-DOG on the outer surface of the adipocytes was measured in the presence of 1.2 mM phloretin and the radioactivity was subtracted from that of the sample (Green, 1986).

Assay of protein phosphatase activity

Adipocytes were treated with brasilin for 4 hr and homogenized with teflon pestle in homogenizing buffer at 4°C, then centrifuged at full speed in microcentrifuge for 1 min. Supernatant (30 μl) was removed and incubated with 10 μl 1M p-nitrophenyl phosphate in 160 μl reaction buffer with 0.5 mM MgCl₂ at 30°C for 15 min. Reaction buffers used are 200 mM acetate buffer for pH 5.5; 50 mM phosphate buffer for pH 6.5; 50 mM HEPES buffer for pH 7.4; 50 mM phosphate buffer for pH 8.0; and 10 mM diethanolamine buffer for pH 9.5. The reaction was stopped by adding 1 ml 0.2N NaOH and 0.1 M EDTA. Released p-nitrophenol was measured by UV spectrometer at 410 nm (Tonks et al., 1988).

Protein phosphatase 1/2A (PP1/2A) activity was measured with protein phosphatase assay system (Cohen et al., 1988). Adipocytes treated with brasilin or okadaic acid were solubilized in extraction buffer (50 mM Tris pH 7.0, 0.1% β-mercaptoethanol, 0.1 mM EDTA, 0.1 mM EGTA, 0.5% Triton X-100, 20 μg/ml leupeptin, 20 μg/ml pepstatin, 20 μg/ml aprotinin) and sonicated for 30 sec. Cell extract was diluted with reaction buffer (0.1 mM EDTA, 1 mg/ml BSA, 20 mM imidazole, 0.1% β-mercaptoethanol, pH 7.63) and 20 μl of extract was mixed with 20 μl reaction buffer and 20 μl substrate solution, then incubated at 30°C for 10 min. After incubation, 180 μl 20% trichloroacetic acid was added and the mixture was incubated in ice for 10 min. The mixture was then centrifuged at 12000 g for 3 min at 4°C and radioactivity in supernatant was measured. Substrate solution was prepared by incubating phosphorylase b and phosphorylase kinase with [32P]-ATP (0.5 μCi) for 1 hr (approximately 3 mg/ml 32P-labeled phosphorylase a).

Assay of hydrogen peroxide

The amount of hydrogen peroxide was measured with DHR123 (Masaki et al., 1995). Brazili(n[50 μM) was added to BSA-free KRH buffer for the indicated times and the reaction mixture was divided to two 100 μl aliquots. DHR 123 (6 μM) and peroxidase (6.4 unit/ml) were added to one aliquot while catalase (40 unit/ml) was added to the other aliquot along with DHR123 and peroxidase. Aliquots were incubated at 37°C for 30 min in a dark place. Fluorescence was measured after 30 min with excitation wavelength of 488nm and emission wavelength of 530 nm.
**UV absorption spectra of brazilein**

Time dependent oxidation of brazilein was examined by adding brazilein to KRH buffer and UV absorption spectra were obtained at 0, 1, 5, 10, 20, 30, 60, 120, 180 and 240 min. To compare the oxidation of brazilein with TMB, brazilein (50 μM) or TMB (1, 5, 10, 20, 50 μM) were added to KRH buffer and UV absorption spectra were obtained at 60 min.

**Statistical analysis**

The statistical significance of the differences between groups was analyzed by Student’s t test or ANOVA test (Duncans multiplicity test). A level of $P < 0.05$ was accepted as significant.

**RESULTS**

**Brazilein stimulates glucose transport in adipocytes**

The structure of brazilein is shown in Fig. 1A. To confirm the effect of brazilein on glucose transport, adipocytes were treated with 25 or 50 μM brazilein for 4 hr at 37°C and 2-DOG uptake was measured. As shown in Fig. 1B, brazilein increased glucose transport in a dose dependent manner. At 50 μM, brazilein increased glucose transport more than 5 fold compared to untreated control group.

**Autoxidation of brazilein**

Schematic presentation of oxidation of brazilein to brazilein is shown in Fig. 2A. Catechol hydroxyl group can be oxidized to ketoone in the presence of metal and light. As shown in Fig. 2B, in the KRH buffer, brazilein underwent a structural change which increases absorbance at 480 nm wavelength. Absorption peak at 220 nm was observed only at the beginning of brazilein addition, and the absorbance at 480 nm was greatly increased in a time-dependent manner.

**Effect of tetramethylbrazilein on glucose transport**

To examine the role of hydroxyl group in brazilein-induced glucose transport, adipocytes were treated with TMB for 4 hr and 2-DOG uptake was measured. As shown in Fig. 3A, TMB did not stimulate glucose transport in adipocytes. Furthermore, TMB did not undergo any structural changes in KRH buffer and did not produce absorption at 480 nm as brazilein did (Fig. 3B).

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**Fig. 1.** Brazilein stimulates glucose transport in adipocytes. (A) Structure of brazilein. (B) Adipocytes were incubated with indicated amount of brazilein for 4 hr and 2-DOG uptake was measured. *$P<0.05$, **$P<0.01$ compared with untreated group. Data are means ± SEM of three independent experiments carried out in triplicate.

**Fig. 2.** Autoxidation of brazilein. (A) Oxidation of brazilein to brazilein. (B) Brazilein was added to KRH buffer and UV absorption spectra were obtained at various times.
HYDROGEN peroxide in brazilin-induced glucose transport

To determine if reactive oxygen produced by autooxidation of brazilin can be converted to hydrogen peroxide, hydrogen peroxide production was measured. As shown in Fig. 4A, hydrogen peroxide was produced from 30 min in KRH buffer by the addition of brazilin. The amount of hydrogen peroxide produced remained unchanged after 60 min.

To examine if hydrogen peroxide produced by brazilin induces glucose transport, 2-DOG uptake was measured after brazilin treatment of adipocytes in the presence of various amount of catalase. As shown in Fig. 4B, catalase significantly inhibited brazilin-induced glucose transport in a dose-responsive manner.

Brazilin inhibits phosphatases in adipocytes

To investigate the selective effect of brazilin on phosphatase activity, adipocytes were incubated with brazilin for 4 hr and phosphatase activity in the cell extract was measured in the medium under various pH conditions. As shown in Fig. 5, brazilin inhibited the phosphatase activities in all ranges of pH tested. Inhibition of phosphatase activity by brazilin at each pH is as follows; pH 5.5: 29.8%; pH 6.5: 38.2%; pH 7.4: 40.0%; pH 8.0: 44.2%; pH 9.5: 47.7%. This result indicates that the inhibition of phosphatases by brazilin may not be selective.

To examine the effect of brazilin on PP1/2A activity, adipocytes were incubated with brazilin for 4 hr in the presence or absence of okadaic acid (35 nM) and 2-DOG uptake was mea-
Fig. 5. Brazilin inhibits phosphatases in adipocytes. Adipocytes were incubated with 50 μM brazilin for 4 hr. The phosphatase activity in the cell extract was measured in the medium with indicated pH. *P<0.05 compared with the group treated with brazilin without catalase. Data are means ± SEM of three independent experiments carried out in triplicate.

Fig. 6. Brazilin inhibits protein phosphatase 1 and 2A activity. (A) Adipocytes were incubated with 50 μM brazilin for 4 hr in the presence or absence of okadaic acid (3 5nM). Data are means ± SEM of three independent experiments carried out in triplicate. (B) Adipocytes were incubated with 50 μM brazilin for 4 hr in the presence or absence of okadaic acid (35 nM) and cell extracts were obtained. (C) Cell extract was obtained from adipocytes and incubated with 50 μM brazilin for 30 min in the presence or absence of okadaic acid (35 nM). PP1/2A activity was measured. Data are means ± SEM of three independent experiments carried out in triplicate. Each letter indicates homogenous subset by ANOVA test (P<0.05, Duncans multiplicity test).

**DISCUSSION**

Brazilin is a major active principle of *Caesalpinia sappan* (Hikino et al., 1977), and it is one of γ-pyran derivatives. Brazilin lowered the nonfasting plasma glucose level in normo- and hyperglycemic KK-mice. Potent hypoglycemic effect was observed in spontaneous diabetic KK mice without any changes in plasma insulin level (Moon et al., 1990; Moon et al., 1993). The hypoglycemic mechanisms of brazilin were intensively studied in various insulin responsive tissues such as liver, muscle and adipose tissue. The studies have revealed that brazilin decreases hepatic glucose output and increases glycogen synthesis. It also increased the rate of glucose transport, glucose oxidation and lipogenesis possibly through the regulation of phosphatidylinositol 3-kinase (Khil et al., 1999), glucose 6-phosphate dehydrogenase and fatty acid synthetase (Moon et al., 1993), respectively. In addition to the hypoglycemic effects, brazilin also showed positive effects on diabetic complications (Moon et al., 1985; Yang et al., 2000).

Catechin, one of the flavonoids, is oxidized to its quinone form, catechin acid or epicatechin, producing reactive oxygen species (Bracke et al., 1988). Hematoxylin, which is 4-hydroxy-brazilin, also produces its oxidized form hematein releasing superoxide anion (Martin et al., 1987). Superoxide
anion can be converted into hydrogen peroxide by dismutases (Halliwell and Gutteridge, 1988). In this study, we found that brazilin undergoes oxidation forming brazilein, the ketone form of brazilin. We also found that the oxidation of brazilin in physiological buffer produces hydrogen peroxide, and blocking of the oxidation by methylation of hydroxyl groups resulted in the complete inhibition of brazilin-induced glucose transport. The site of oxidation in the structure of brazilin is the catechol hydroxyl group. Considering these facts, it is suggested that the active moiety of brazilin to induce glucose transport is the catechol hydroxyl group at carbon 9 and 10. It is not clear how brazilin produces hydrogen peroxide. However, it is assumed that the oxidation of brazilin also produces superoxide anion like hematoxylin and superoxide anion is dismutated into hydrogen peroxide by cellular dismutases. The present study suggested that peroxidase in the reaction buffer might produce hydrogen peroxide from reactive oxygen. These results imply that the oxidation of brazilin is critical for its stimulating effect on glucose transport.

Hydrogen peroxide is produced when cells were treated with insulin and mediates insulin action (Mahadev et al., 2001). Hydrogen peroxide was also known to increase glucose transport by inactivation of protein-tyrosine phosphatases (Koshio et al., 1988). We found that catalase, which scavenges hydrogen peroxide produced by brazilin, inhibited brazilin-induced glucose transport. Furthermore, phosphatase activities which are active in a wide range of pH conditions were inhibited by brazilin treatment. These results indicate that the inhibition of phosphatase activity by brazilin may play an important role in the stimulation of glucose transport.

Hydrogen peroxide inhibits serine/threonine phosphatases such as PP1 and PP2A (Sommer et al., 2002), which dephosphorylate Akt/PKB which stimulates the translocation of glucose transport 4 and glucose transport (Ugi et al., 2004). Okadaic acid, which is a PP1/2A inhibitor, increases glucose transport (Bialojan and Takai, 1988; Tanti et al., 1997). Brazilin and okadaic acid showed additional effects on glucose transport in adipocytes. Okadaic acid inhibited PP1/2A activity in intact cells and in cell extract. Brazilin also inhibited PP1/2A activity in intact cells. However, brazilin did not inhibit PP1/2A activity in a cell free system. These results indicate that brazilin does not directly inhibit PP1/2A activity but it requires cellular machinery to produce hydrogen peroxide and thereby inhibits phosphatases.

Although brazilin produces hydrogen peroxide and inhibits phosphatase activity, it is not clear if hydrogen peroxide or brazilin itself inhibits phosphatase activity. This should be further investigated carefully.

From the results obtained, it is suggested that the hydrogen peroxide produced from brazilin mediates glucose transport possibly through inhibition of phosphatases which otherwise decrease glucose transport in adipocytes.

REFERENCES


