An Extract of *Lagerstroemia speciosa* L. Has Insulin-Like Glucose Uptake–Stimulatory and Adipocyte Differentiation–Inhibitory Activities in 3T3-L1 Cells[1]

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ABSTRACT The effects of extracts isolated from *Lagerstroemia speciosa* L. (banaba) on glucose transport and adipocyte differentiation in 3T3-L1 cells were studied. Glucose uptake–inducing activity of banaba extract (BE) was investigated in differentiated adipocytes using a radioactive assay, and the ability of BE to induce differentiation in preadipocytes was examined by Northern and Western blot analyses. The hot water BE and the banaba methanol eluent (BME) stimulated glucose uptake in 3T3-L1 adipocytes with an induction time and a dose-dependent response similar to those of insulin. Furthermore, there were no additive or synergistic effects found between BE and insulin on glucose uptake, and the glucose uptake activity of insulin could be reduced to basal levels by adding increasing amounts of BE. Unlike insulin, BE did not induce adipocyte differentiation in the presence of 3-isobutyl-1-methylxanthine (IBMX) and dexamethasone (DEX). BE inhibited the adipocyte differentiation induced by insulin plus IBMX and DEX (IS-IBMX-DEX) of 3T3-L1 preadipocytes in a dose-dependent manner. The differences in the glucose uptake and differentiation inhibitory activities between untreated cells and those treated with BE were significant ($P < 0.01$). The inhibitory activity was further demonstrated by drastic reductions of peroxisome proliferator-activated receptor γ2 (PPARγ2) mRNA and glucose transporter-4 (GLUT4) protein in cells induced from preadipocytes with IS-IBMX-DEX in the presence of BE. The unique combination of a glucose uptake stimulatory activity, the absence of adipocyte differentiation activity and effective inhibition of adipocyte differentiation induced by IS-IBMX-DEX in 3T3-L1 cells suggest that BE may be useful for prevention and treatment of hyperglycemia and obesity in type II diabetics. J. Nutr. 131: 2242–2247, 2001.

KEY WORDS: • *type II diabetes* • *banaba extract* • *insulin* • *adipocyte differentiation* • *glucose uptake*

Obesity is considered to be the most important risk factor for noninsulin-dependent (type II) diabetes mellitus (NIDDM).[3] NIDDM has been increasing at an alarming rate (projecting to increase from 135 million in 1995 to 300 million worldwide in 2025), and has become a serious public health problem, particularly in developed countries (1–5). NIDDM is a complicated disease, involving both genetic and nongenetic factors (5–7). Although the causes of NIDDM are not completely known, obesity, hyperinsulinemia, hyperglycemia and insulin resistance are closely associated with NIDDM (8–11). Insulin plays multiple physiologic roles in the human body, including the reduction of blood glucose levels and the promotion of lipid biosynthesis in adipocytes (12–14). It is highly desirable to find antidiabetic agents that induce glucose uptake in cells, but, unlike insulin, do not simultaneously up-regulate lipogenesis.

Leaves of the tropical plant *Lagerstroemia speciosa L.* (banaba in the Tagalog dialect in the Philippines) have been used as a folk medicine for treatment of diabetes and kidney diseases. The extract from banaba significantly reduced blood glucose and insulin levels in type II KK-Ay diabetic mice (15). It was reported recently that the extract exhibited an anti-adipogenic activity by effectively reducing weight gain and parametrial adipose tissue in female diabetic mice (16). However, no study has reported the mechanisms of action by the extract at the cellular and molecular levels, and it is not known how the extract exerts these effects.

In this study, the ability of the banaba extract (BE) to stimulate glucose uptake in 3T3-L1 adipocytes was examined and compared with that of insulin. In addition, the effects of BE on differentiation of preadipocytes into adipocytes, a process induced by an insulin/3-isobutyl-1-methylxanthine/dexamethasone (IS-IBMX-DEX) cocktail were also investigated. The expression of peroxisome proliferator-activated receptor γ2 (PPARγ2), a nuclear protein that turns genes on and off upon binding to molecules that belong to a group of compounds called peroxisome proliferators and is essential for adipocyte differentiation (17,18), and of glucose transporter-4

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[3] Abbreviations used: BE, banaba hot water extract; BME, banaba HP-20 methanol eluent; BWE, banaba HP-20 water eluent; DEX, dexamethasone; DMEM, Dulbecco's modified Eagle's medium; DPBS, Dulbecco's PBS; FBS, fetal bovine serum; GLUT4, glucose transporter-4; IBMX, 3-isobutyl-1-methylxanthine; IS, insulin; KRP, Krebs-Ringer-Hepes; NIDDM, noninsulin-dependent diabetes mellitus; PPARγ2, peroxisome proliferator-activated receptor γ2; TZD, thiazolidinedione.

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(GLUT4), a hallmark of adipocyte differentiation and the molecule that mediates insulin-stimulated glucose transport (12,13), were studied in 3T3-L1 preadipocytes treated with BE in the presence or absence of insulin, IBMX or DEX. These studies were designed to characterize the effects of BE in 3T3-L1 cells at cellular and molecular levels, and to identify an extract that may be used for prevention and treatment of obesity and NIDDM without the undesirable side effects of insulin therapy (19).

MATERIALS AND METHODS

Materials. 3T3-L1 fibroblasts were purchased from American Type Culture Collection (ATCC, Rockville, MD). Banaba leaves were obtained as a gift from Huagen Pharmaceuticals (Hong Kong). Dulbecco's modified Eagle's medium (DMEM) and Dulbecco's PBS (DPBS) were from Gibco Life Technologies (Grand Island, NY). Fetal bovine serum (FBS) was from Atlanta Biologicals (Norcross, GA). Bovine IS, IBMX and DEX were from Sigma Chemical (St. Louis, MO). 2-Deoxy-D-[^{3}H] glucose and XK 50 column were from Amersham Pharmacia Biotech (Piscataway, NJ). Dianion HP-20 resin was from Mitsubishi Chemical (Tokyo, Japan). Anti-mouse GLUT4 monoclonal antibody was from Biogenesis (Brentwood, NH).

Banaba extract preparation. Banaba extract was prepared by the method described previously (15) with modifications. Banaba hot water extract (BE) was isolated by boiling 50 g of banaba tea in 1 L distilled water for 30 min, followed by ultracentrifugation at 30,000 $\times$ *g* for 30 min, filtration with a 0.4-μm filter, concentration by heat evaporation and freeze-drying. The BE was further separated by passage through a Dianion HP-20 resin column. The BE was loaded on the column packed with Dianion HP-20 resin, then washed with distilled water (BWE), and the absorbed fraction was eluted with methanol (BME). These two eluted fractions from the column were individually concentrated and freeze-dried. The powder of the banaba extracts (BE and BME) was dissolved in sterile distilled H_2O, and then further sterilized with 0.2-μm filters for the adipocyte differentiation study. Unless otherwise stated, BE was used in the study.

Cell culture and adipocyte differentiation. 3T3-L1 cells were maintained in DMEM and supplemented with 10% FBS at 37°C in a 10% CO_2 cell incubator. Preadipocyte 3T3-L1 cells were grown in 12-well plates until 2 d postconfluence. The differentiation was induced as previously described (20) by addition of 1 mg/L IS, 0.5 mmol/L IBMX and 0.25 μmol/L DEX (IS-IBMX-DEX). Two days after induction, the IS-IBMX-DEX–containing medium was replaced with medium containing 1 mg/L IS alone. The medium was subsequently replaced again with fresh culture medium (DMEM supplemented with 10% FBS) after 2 d and then every other day thereafter. To determine the roles of banaba extract in adipocyte differentiation, BE was added to the medium either to substitute for insulin (BE-IBMX-DEX) or to supplement IS-IBMX-DEX (BE-IS-IBMX-DEX). The differently induced cells were assayed for glucose uptake activity 9–12 d after the initiation of induction.

Glucose uptake activity assay. Glucose uptake activity was analyzed by measuring the uptake of 2-deoxy-D-[^{3}H] glucose as described previously (21,22). Briefly, confluent 3T3-L1 adipocytes grown in 12-well plates were washed twice with serum-free DMEM and incubated with 1 mL of the same medium at 37°C for 2 h. The cells were washed 3 times with Krebs-Ringer-Hepes (KRP) buffer and incubated with 0.9 mL KRP buffer at 37°C for 30 min. Insulin and/or BE were then added and adipocytes were incubated at 37°C for 15 min. Glucose uptake was initiated by the addition of 0.1 mL KRP buffer and 37 MBq/L 2-deoxy-D-[^{3}H] glucose and 1 mmol/L glucose as final concentrations. After 10 min, glucose uptake was terminated by washing the cells 3 times with cold PBS. The cells were lysed with 0.7 mL of 1% Triton X-100 at 37°C for 20 min. The radioactivity retained by the cell lysates was determined by a scintillation counter. Nonspecific glucose uptake was measured at a glucose concentration of 100 mmol/L. Assay data were analyzed statistically using Student's *t* test by comparison of experimental samples of the same treatment conditions as a group with negative control (untreated) samples, or positive (insulin-treated) samples, or with experimental samples with different treatment conditions. To compensate for multiple *t* tests, $P < 0.01$ was set as the level of significant difference.

Adipocyte differentiation assay. Undifferentiated 3T3-L1 preadipocytes were induced to differentiate into adipocytes as described above. The degree of the differentiation of the cells induced by different agents was evaluated by microscopic observation of lipid accumulation, as well as by the glucose uptake activities they exhibited at the end of the induction as described above. The glucose uptake assay was chosen and performed here for determination of the degree of adipocyte differentiation on the basis of the observation that differentiated adipocytes can be induced by insulin to take up glucose, whereas preadipocytes cannot (23,24).

Northern blot and Western blot analyses. Total RNA or total protein was isolated with standard procedures from 3T3-L1 cells induced by different combinations of insulin, IBMX and DEX, and BE. For detection of PPARγ2 mRNA expression, a ^{32}P-labeled fragment of 308 bp, corresponding to the nucleotides from + 29 to + 336 of the coding region of the PPARγ2 cDNA, was used as a probe and 10 μg of total RNA (isolated 144 h postinduction) was used per sample. For Western blot analysis, an anti-mouse GLUT4 monoclonal antibody was used and 100 μg of total protein (isolated 10 d post induction) was loaded per lane.

RESULTS AND DISCUSSION

Although to a lesser extent than insulin, both banaba extracts (BE and BME) stimulated glucose uptake in 3T3-L1 adipocytes, whereas BWE did not ($P < 0.01$; **Fig. 1**), suggesting that the effective component(s) with glucose transport-inducing activity in the banaba extract are water-soluble but relatively nonpolar. Also, the maintenance of the activity through boiling and heat evaporation during extract preparation indicated that the effective component(s) is heat stable and is unlikely to be a protein(s).

The effect of the concentration of BE on glucose uptake was compared with that of insulin. The concentration-dependent curve of glucose uptake activity of BE (**Fig. 2*A***) is very similar to that of insulin (Fig. 2*B*). The concentration range of BE that stimulated the greatest glucose uptake was ~0.1–0.25 g/L (Fig. 2*A*). The similarity between the two dose-response curves and the observation that the induction time required by BE for stimulating glucose uptake activity was similar to that of insulin, i.e., no >15 min (refer to the glucose uptake activity assay in Materials and Methods), suggest that BE may

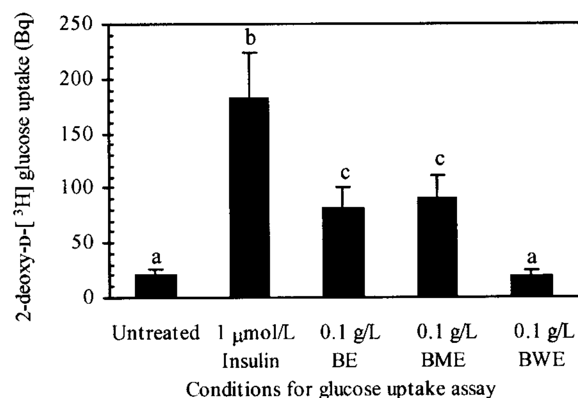


FIGURE 1 The effect of banaba extract (BE) on glucose uptake in 3T3-L1 adipocytes. Adipocytes in 12-well plates were incubated for 15 min with different BE [hot water extract (BE), HP-20 methanol eluent (BME) and HP-20 column water eluent (BME)], or with insulin as a positive control, or without treatment as a negative control, then assayed for 2-deoxy-D-[^{3}H]glucose uptake. Data are means $\pm$ SD, $n = 8$. Means with different letters differ, $P < 0.01$.

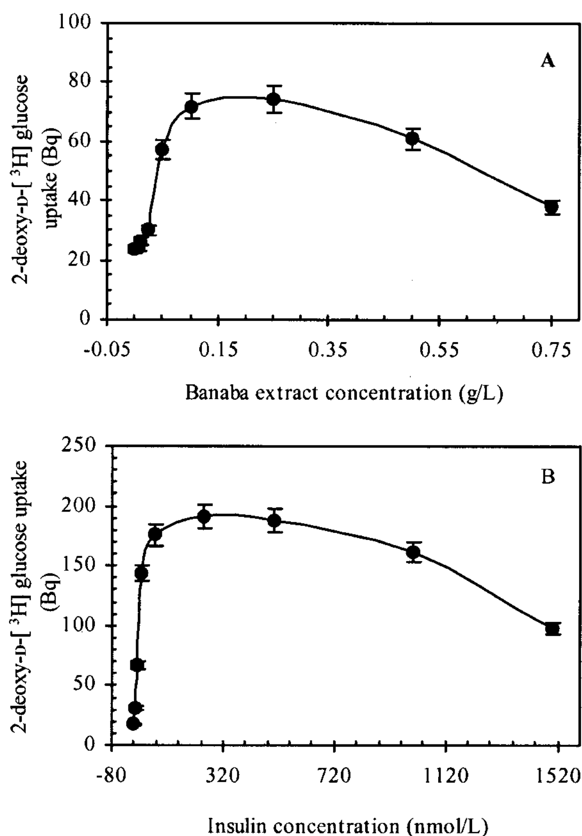


FIGURE 2 The effect of the concentration of banaba extract (BE, *panel A*) or insulin (*panel B*) on glucose uptake in 3T3-L1 adipocytes. Differentiated 3T3-L1 cells in 12-well plates were incubated with different concentrations of BE or insulin for 15 min, and then assayed for 2-deoxy-D-[^{3}H]glucose uptake. Data are means ± SEM, $n = 6$.

stimulate glucose uptake by a mechanism that is similar to that of insulin (23–25) and different from those utilized by other chemicals (24,26–28).

To test whether BE could further enhance insulin's glucose uptake activity, 0.1 g/L BE was added to insulin at various concentrations (0–1000 nmol/L). Glucose uptake was not different from that of insulin alone (**Fig. 3***A*), indicating that no additive or synergistic effects exist between BE and insulin. To the contrary, in comparison to insulin alone (the leftmost data point on the insulin + BE curve of Fig. 3*B*), reduction of the glucose uptake activity by addition of BE to insulin was observed (Fig. 3*B*). On the basis of the observations of glucose uptake–inducing activity of BE and inhibitory activity of BE on insulin-induced glucose uptake, a mechanism of action of BE may be hypothesized. BE may interact with a protein factor that is directly involved in the insulin-mediated glucose transport signaling pathway that starts with the insulin receptor and terminates with GLUT4, consequently activating glucose uptake. On the other hand, the interaction between BE and the protein factor may structurally alter the conformation of the factor, preventing it from properly receiving the glucose uptake signal initiated from insulin-insulin receptor binding. Further studies of insulin receptor binding and receptor and intracellular protein phosphorylation are needed for the final elucidation of the site of action and the glucose uptake induction mechanism mediated by BE. BE apparently stimulates glucose uptake through a mechanism that is very different from that used by other common antidiabetic drugs such as the thiazolidinediones (TZD) (29,30). TZD stimulate glucose uptake using a slow and indirect mechanism by activating PPARγ, which in turn up-regulates GLUT4 gene expression (29–31). In contrast, the glucose uptake–stimulatory activity of BE seems to be much faster and more direct. The effective compound(s) in BE may represent a new group of chemicals that could be used as alternatives to TZD to induce glucose uptake in both cells and animals.

Undifferentiated 3T3-L1 preadipocytes can be converted to adipocytes by addition of a cocktail containing insulin, IBMX and DEX (20). However, when 1–100 mg/L BE was substituted for insulin and added to preadipocytes in the presence of IBMX and DEX, no adipocyte differentiation of 3T3-L1 cells was observed as revealed by glucose uptake assays (**Fig. 4**). This result indicates that BE does not induce adipocyte differentiation in 3T3-L1 cells. Interestingly, both BE and BME, when co-incubated with IS-IBMX-DEX, inhibited adipogenesis, whereas BWE did not (**Fig. 5**). This indicates that both the adipocyte differentiation inhibition activity and the glucose uptake activity were due to BME (Fig. 1). The inhibition of adipocyte differentiation by BE was time (**Fig. 6***A*) and concentration dependent (Fig. 6*B*). These results are consistent with the microscopic observations of fat accumulation (Fig. 5). The 3T3-L1 preadipocytes, whose differentiation was blocked by co-incubation of BE and IS-IBMX-DEX, retained the capacity to reenter the differentiation process when the IS-IBMX -DEX induction cocktail was reintroduced into the cells (data not shown). It is interesting to note that BE showed an

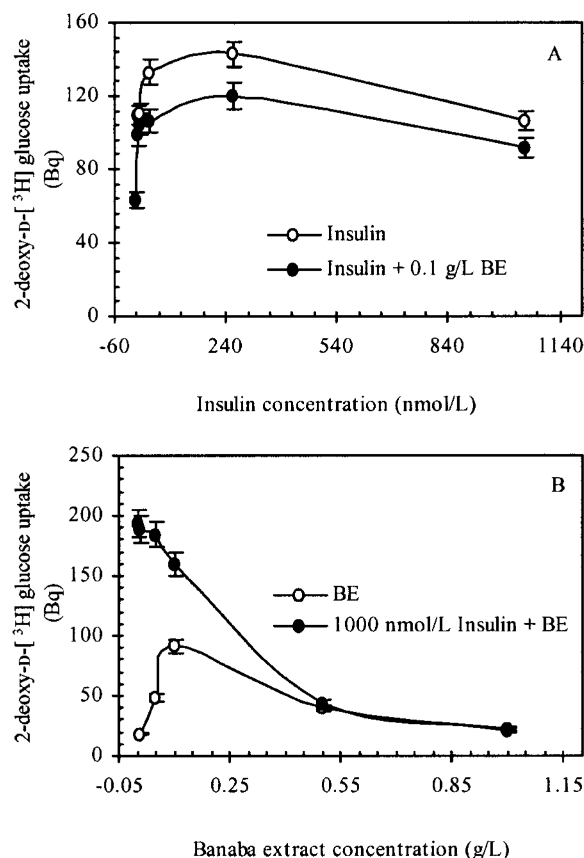


FIGURE 3 Combined effects of insulin and banaba extract (BE) on glucose uptake in 3T3-L1 adipocytes. Differentiated 3T3-L1 cells were incubated with insulin in the presence or absence of 0.1 g/L BE (*A*), or incubated with BE in the presence or absence of 1 μmol/L insulin (*B*) for 15 min, and then assayed for the glucose uptake activities. Data are means ± SEM, $n = 6$.

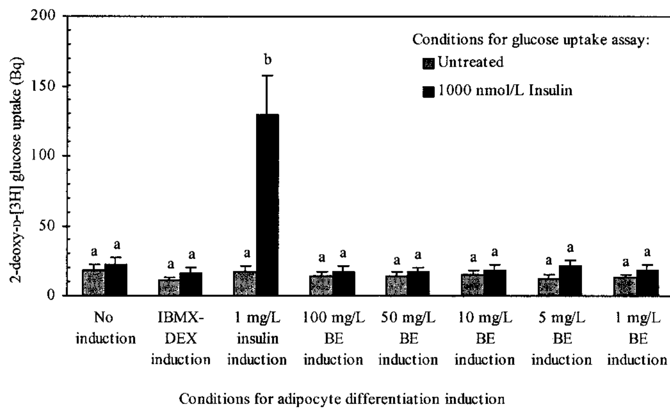


FIGURE 4 The effect of banaba extract (BE) on adipocyte differentiation in the absence of insulin in 3T3-L1 cells. Undifferentiated 3T3-L1 preadipocytes were induced by either insulin or BE in the presence of dexamethasone (DEX) and 3-isobutyl-1-methylxanthine (IBMX). Ten days after induction, the degree of adipocyte differentiation was assayed by the glucose uptake activities of the cells. Data are means ± SD, $n = 6$. Means with different letters differ, $P < 0.01$.

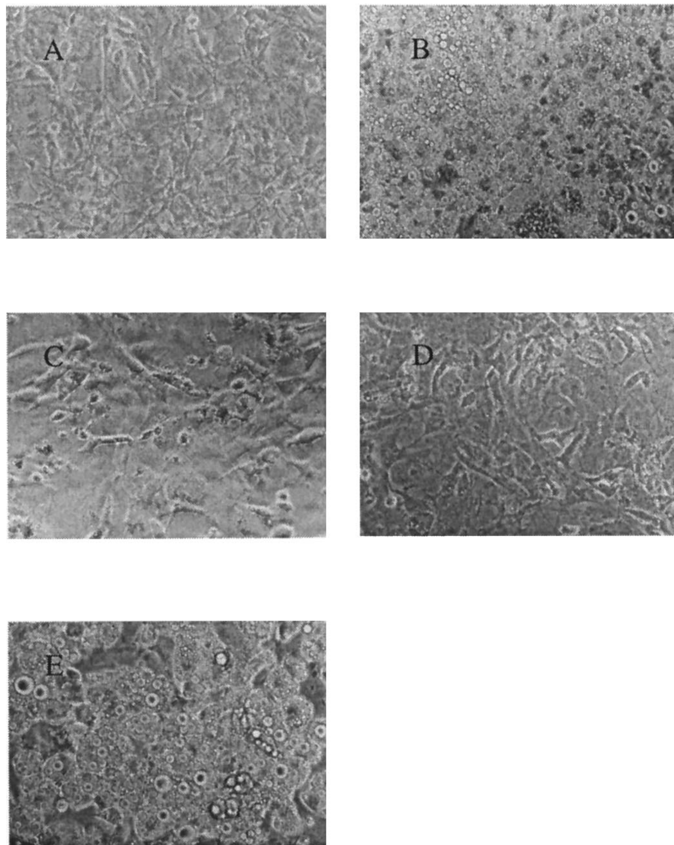
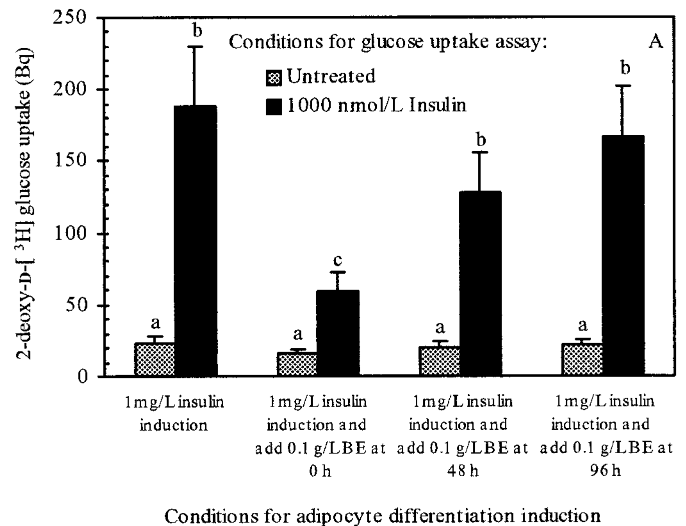


FIGURE 5 The effect of different banaba extract (BE) on adipocyte differentiation in the presence of insulin (IS) in 3T3-L1 cells. In the presence of dexamethasone (DEX) and 3-isobutyl-1-methylxanthine (IBMX), undifferentiated 3T3-L1 preadipocytes were induced by either insulin, (BE + IS), (BME + IS) or (BWE + IS). Ten days after the induction, the cells were photographed at magnification X200. (*A*) No induction; (*B*) IS; (*C*) BE (0.5 g/L) and IS; (*D*) BME (0.5 g/L) and IS; (*E*) BWE (0.5 g/L) and IS. Figure shown represents one of four independent experiments. All four experiments showed similar results. Abbreviations: BME, banaba HP-20 methanol eluent; BWE, banaba HP-20 water eluent.



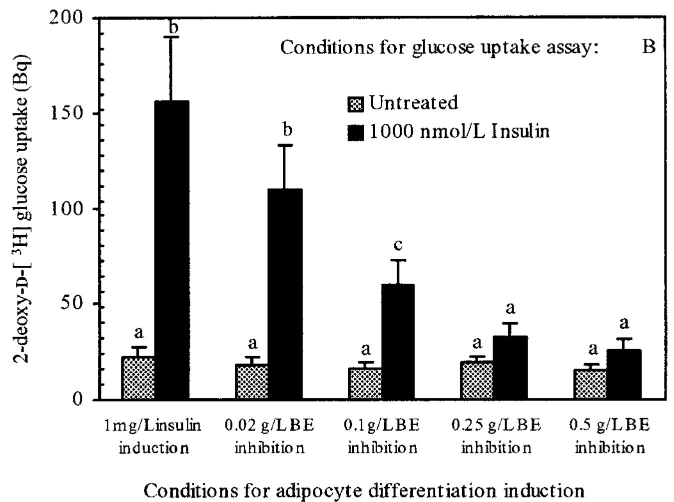


FIGURE 6 The effects of introduction time and concentration of banaba extract (BE) on adipocyte differentiation in the presence of insulin (IS) in 3T3-L1 cells. Preadipocyte 3T3-L1 cells were induced with IS in the presence of dexamethasone (DEX) and 3-isobutyl-1-methylxanthine (IBMX) at d 0. BE was added to the media either at various times after the initial induction (*A*) or at various concentrations at the time of the initial induction (*B*). The degree of differentiation of differently treated 3T3-L1 cells was assayed by the glucose uptake activities of the cells. Data are means ± SD, $n = 6$. Means with different letters differ, $P < 0.01$.

insulin-like glucose uptake–inducing activity in adipocytes but did not show an insulin-like differentiation-inducing activity in preadipocytes. This difference may be explained by the facts that these two activities involve two distinct signaling pathways and that the insulin receptor is involved in glucose transport in adipocytes, whereas the insulin-like growth factor 1 receptor, which is homologous to the insulin receptor, is used by insulin in preadipocytes for induction of differentiation (18,32,33).

To investigate how BE inhibits adipocyte differentiation induced by IN-IBMX-DEX, two important differentiation markers, PPARγ2 and GLUT4, were used to monitor the progress of differentiation in the preadipocytes that were induced by either IBMX-DEX or IS-IBMX-DEX in the presence or absence of BE. Northern blot analysis revealed that BE greatly inhibited the mRNA expression of PPARγ2 induced

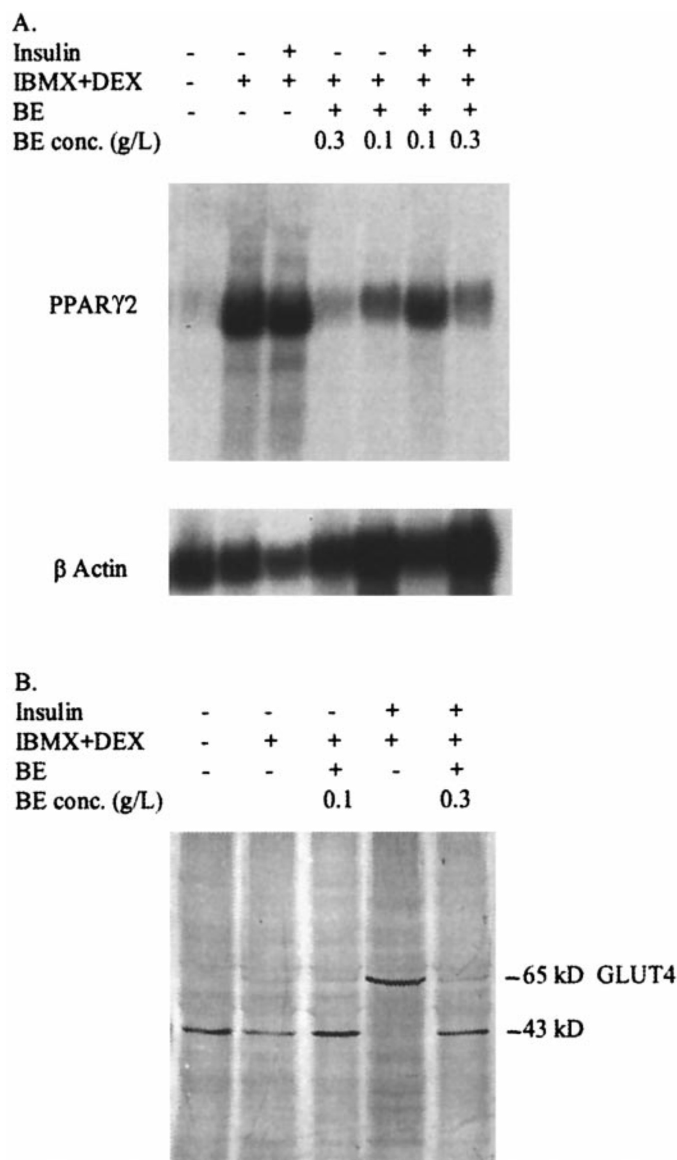


FIGURE 7 Expression of peroxisome proliferator-activated receptor γ2 (PPARγ2) or glucose transporter 4 (GLUT4) in 3T3-L1 cells induced under different conditions. Preadipocytes were induced with or without insulin in the presence or absence of banaba extract (BE). Total RNA or protein was isolated from the differently treated cells and analyzed for the expression of PPARγ2 mRNA and GLUT4 protein. (*A*) Northern blot analysis of PPARγ2 expression in 3T3-L1 cells 144 h postinduction; 10 μg of total RNA was used per lane. (*B*) Western blot analysis of GLUT4 expression in 3T3-L1 cells 10 d post induction; 100 μg of total protein was used per lane. GLUT4 has a molecular mass of ~65 kDa. Figure shown here represents one of three independent experiments. All three experiments showed similar results.

by either IBMX-DEX or IS-IBMX-DEX in a dose-dependent manner (**Fig. 7***A*). Furthermore, as shown by a Western blot analysis, the protein production of GLUT4 (~65 kDa) was inhibited more in the cells induced by IS-IBMX-DEX in the presence of BE than in the cells induced in the absence of BE (Fig. 7*B*). These results are consistent with our other differentiation inhibition results studied with glucose uptake assays (Figs. 4 and 6), and are not inconsistent with other glucose uptake assays (Figs. 1–3) because the glucose uptake–inducing activity of BE was examined in fully differentiated adipocytes (Figs. 1–3), whereas the GLUT4 inhibitory effect of BE was observed in undifferentiated cells (Fig. 7*B*). GLUT4 expression was used as an indicator of differentiation in this study to monitor the ability of BE to induce differentiation in preadipocytes, not glucose transport in adipocytes. All of these results suggest that the specific target of the differentiation inhibition exerted by BE is PPARγ2 or factor(s) that directly or indirectly regulate the expression of PPARγ2. Further experiments are necessary to determine the site of the inhibition. The identity and function of the 43-kDa protein (Fig. 7*B*) are not known at this time. The presence of such a protein band in the Western blot was likely due to the specific monoclonal antibody, and possibly the cells used in this study.

It is also interesting and important to determine whether the glucose uptake and adipocyte differentiation activities are mediated by the same or different compounds in BE. We used HPLC to analyze and isolate the effective compound(s) from BE. Up to now, the differentiation-inhibitory activity was always associated with the glucose uptake–inducing activity in a single fraction or peak isolated by HPLC (data not shown). These observations strongly suggest that the two activities come from the same compound in BE. The final isolation and characterization of the effective compound(s) are in progress.

We have demonstrated that BE stimulates glucose uptake activity and inhibits the adipocyte differentiation activity of IS-IBMX-DEX in 3T3-L1 cells. These new findings are consistent with the previous observations that BE lowered blood glucose levels in diabetic mice (15) and reduced weight gain and adipose tissue mass in female diabetic mice (16).

Antidiabetic drugs such as insulin or TZD up-regulate both glucose transport and lipid biosynthesis in adipocytes (29,30). Weight gain is a frequent side effect of insulin therapy in type II diabetic patients (19). Therefore, drugs with glucose-lowering activity, but lacking adipogenic activity are highly desirable. The effective component(s) of BE seem to have such an advantageous combination. A new polypeptide hormone, resistin, has recently been found in adipocytes to be one of the potential links between obesity and type II diabetes (34,35). Resistin may be responsible for insulin resistance, and its gene expression profile appears to be very similar to that of PPARγ (34), a gene that BE down-regulates (Fig. 7*A*). Thus, an understanding of the mechanism of BE action will be valuable for the study, prevention, and treatment of obesity, insulin resistance and type II diabetes.

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