

## Tannic Acid Stimulates Glucose Transport and Inhibits Adipocyte Differentiation in 3T3-L1 Cells<sup>1</sup>

Xueqing Liu,<sup>\*2</sup> Jae-kyung Kim,<sup>\*2</sup> Yunsheng Li,<sup>†</sup> Jing Li,<sup>†</sup> Fang Liu,<sup>†</sup>  
and Xiaozhuo Chen<sup>\*†\*\*3</sup>

<sup>\*</sup>Department of Biochemistry, <sup>†</sup>Edison Biotechnology Institute, and <sup>\*\*</sup>Department of Biomedical Sciences, College of Osteopathic Medicine, Ohio University, Athens, OH 45701

**ABSTRACT** Obesity is a major risk factor for Syndrome X and type II diabetes (T2D). However, most antidiabetic drugs that are hypoglycemic also promote weight gain, thus alleviating one symptom of T2D while aggravating a major risk factor that leads to T2D. Adipogenesis, the differentiation and proliferation of adipocytes, is a major mechanism leading to weight gain and obesity. It is highly desirable to develop pharmaceuticals and treatments for T2D that reduce blood glucose levels without inducing adipogenesis in patients. Previously, we reported that an extract from *Lagerstroemia speciosa* L. (banaba) possessed activities that both stimulated glucose transport and inhibited adipocyte differentiation in 3T3-L1 cells. Using glucose uptake assays and Western/Northern blot analyses as major tools and 3T3-L1 cells as a model, we showed that the banaba extract (BE) with tannin removed was devoid of the 2 activities, and tannic acid (TA), a major component of tannins, had the same 2 activities as BE. Inhibitors known to abolish insulin-induced glucose transport also blocked TA-induced glucose transport. We further detected that TA induced phosphorylation of the insulin receptor (IR) and Akt, as well as translocation of glucose transporter 4 (GLUT 4), the protein factors involved in the signaling pathway of insulin-mediated glucose transport. We also demonstrated that TA inhibited the expression of key genes for adipogenesis. Differences between samples with or without TA in all of the quantitative assays were significant ( $P < 0.05$ ). These results suggest that TA may be useful for the prevention and treatment of T2D and its associated obesity. TA may have the potential to become the lead compound in the development of new types of antidiabetic pharmaceuticals that are able to reduce blood glucose levels without increasing adiposity. *J. Nutr.* 135: 165–171, 2005.

**KEY WORDS:** • *type II diabetes* • *obesity* • *tannic acid* • *glucose transport* • *adipocyte differentiation*

Type II diabetes (T2D)<sup>4</sup> and obesity have become health problems of epidemic proportion in this and other developed countries (1). Obesity is intimately associated with syndrome X, which is characterized by hyperinsulinemia (insulin resistance), hyperlipidemia, and hyperglycemia, and which ultimately leads to T2D (2). Obesity is one of the most important contributing risk factors to syndrome X and T2D. Weight gain, particularly that associated with T2D, is due in part to adipogenesis (3,4). Adipogenesis is a 2-step process within which clonal expansion of preadipocytes precedes adipocyte differentiation (5). It leads to an increase in both the number and size (volume) of adipocytes (6,7). Overweight and obesity are the

result of excessive adipogenesis (3,4,7). Therapeutically and historically, for biomedical, economical, and convenience reasons, antidiabetic drugs have focused on hyperglycemia, while largely ignoring the overweight or obesity problem. Even worse, most of the antidiabetic drugs promote weight gain, i.e., adipogenesis, while reducing blood glucose (8). The current strategy, as effective as it may be for the short term, is not desirable for the long-term treatment of the large majority of the T2D patients who are overweight or obese. In comparison, reducing hyperglycemia, hyperlipidemia, and hyperinsulinemia without increasing adiposity or with a reduction of body weight would constitute a much preferred treatment alternative.

In a previous study, we reported that banaba extract (BE) possessed activities that both stimulated glucose transport and inhibited adipocyte differentiation in 3T3-L1 cells (9). The 2 activities working together seem to be an ideal combination for the prevention and treatment of syndrome X and T2D. However, the identity of the component in BE responsible for these 2 activities was not known. More recently, in the process of isolating and identifying effective antidiabetic compounds, we serendipitously discovered that the 2 activities reside in the tannin fraction of BE. Tannins are polyphenolic compounds found in foods such as legumes, vegetables, fruits, and bever-

<sup>1</sup> Supported in part by MetaCor Pharmaceuticals, by Central Ohio Diabetic Association, and by the Ohio Department of Development Thomas Edison Program.

<sup>2</sup> These authors contributed equally to this study, and should be considered as first authors.

<sup>3</sup> To whom correspondence should be addressed. E-mail: chenx@ohiou.edu.

<sup>4</sup> Abbreviations used: BE, banaba extract; BSA, bovine serum albumin; CHO, Chinese hamster ovary; FBS, fetal bovine serum; GLUT 4, glucose transporter 4; HNMPA-(AM)<sub>3</sub>, hydroxy-2-naphthalenylmethylphosphonic acid-tris acetoxymethyl ester; HRP, horseradish peroxidase; IBMX, 3-isobutyl-1-methyl-xanthine; IR, insulin receptor; KRP, Krebs-Ringer phosphate; MDCK, Madin-Darby canine kidney; MDI, IBMX, dexamethasone, and insulin; PPAR, peroxisome proliferator-activated receptor; TA, tannic acid; T2D, Type II diabetes.

ages (10). Tannins were reported to possess multiple biological activities including anticancer (10), antioxidant (11), and antimicrobial activities (12). Based on their chemical structures, tannins are further divided into hydrolyzable and condensed tannins (10). The main components of hydrolyzable tannins are gallotannins, also referred to as tannic acid (TA), and ellagitannins (10). TA has not been studied systematically for its potential functions in the processes of glucose transport or adipogenesis. Because commercially available TA covers a relatively well-defined class of structure within tannins, we decided to investigate its bioactivities. In this study, we used 3T3-L1 adipocytes as cell models and glucose uptake assays as a way in which to measure activities that stimulated glucose transport and inhibited adipocyte differentiation in an attempt to characterize the properties of TA. In addition, the mechanisms by which TA mediates its activities, especially glucose transport activity, were investigated.

## MATERIALS AND METHODS

**Materials.** Banaba leaves were obtained as a gift from Huagen Pharmaceuticals. DMEM, Dulbecco's PBS, and bovine calf serum were obtained from Gibco Life Technologies. Fetal bovine serum (FBS) was purchased from Cellgro, bovine insulin, 3-isobutyl-1-methyl-xanthine (IBMX), dexamethasone, hydroxy-2-naphthalenyl-methylphosphonic acid-tris acetoxymethylester [HNMPA-(AM)<sub>3</sub>], wortmannin, and cytochalasin B were purchased from Sigma Chemical; 2-deoxy-D-[<sup>3</sup>H]-glucose (1 Ci/L) was purchased from Amersham. The polyclonal antibodies against phospho-Akt (Ser473), phospho-insulin receptor (IR), SDS sample buffer, biotinylated protein marker, primary antibodies, horseradish peroxidase (HRP)-conjugated secondary antibody, and HRP-conjugated antibiotin antibody were purchased from Cell Signaling Technology; nitrocellulose membrane was purchased from Schleicher & Schuell BioScience, and the protein assay kit was purchased from Bio-Rad.

**Cells.** 3T3-L1 preadipocytes, Madin-Darby canine kidney (MDCK), and Chinese hamster ovary (CHO) cells were purchased from American Type Culture Collection. CHO-IR cells (13) were a gift from Dr. Alan Saltiel's laboratory. The 3T3-L1 preadipocytes were cultured in DMEM containing 10% (v:v) calf serum with 50 kIU/L penicillin, and 0.05 g/L streptomycin at 37°C in a 10% CO<sub>2</sub> cell incubator. MDCK and mouse L cells were maintained in DMEM with 10% FBS, 50 kIU/L penicillin, and 0.05 g/L streptomycin at 37°C in a 5% CO<sub>2</sub> cell incubator. CHO-IR cells were maintained in MEM containing nucleotides, 10% FBS, 2 mmol/L L-glutamine, 50 kIU/L penicillin, and 0.05 g/L streptomycin at 37°C in a 5% CO<sub>2</sub> cell incubator.

**BE and tannic acid.** BE was prepared as previously described (9). Tannin in BE was removed with either a gelatin (14) or a bovine serum albumin (BSA) procedure (15) with minor modifications. In both procedures, 100 g/L was prepared in 2 mL of distilled water. To use gelatin to remove tannin, BE was centrifuged for 5 min at 9200 × g and filtered with a 0.2-μm filter; 1 mL of 40 g/L gelatin was added and mixed with the filtered BE, which was then centrifuged for 5 min at 9200 × g. The supernatant was transferred to a new tube and the pellet, which contained tannin and gelatin, was discarded. This step was repeated once. The supernatant was collected in a new tube, and mixed with 1 mL of 40 g/L gelatin, and centrifuged at 30,000 × g for 20 min to remove residual tannin. The resulting supernatant was transferred to a new tube and the pellet discarded. A 5-fold volume of ethanol was added to remove excess gelatin in the remaining BE; this solution was then centrifuged at 30,000 × g for 10 min, and the final tannin-free supernatant was transferred to a new tube and vacuum-dried. The dried supernatant sample, BE (-TA)<sub>gelatin</sub>, was subsequently tested for glucose uptake activity.

Similarly, tannin was also removed from BE with 20 g/L BSA using an established procedure (15). Briefly, 200 μL of 10% BE was mixed with 200 μL of 20 g/L BSA, centrifuged at 30,000 × g for 10 min. To remove the excess BSA, 200 μL of ethanol was added to BE and the mixture was centrifuged at 30,000 × g for 10 min. The

resulting BE solution, BE (-TA)<sub>BSA</sub>, was dried and tested for its biological activity.

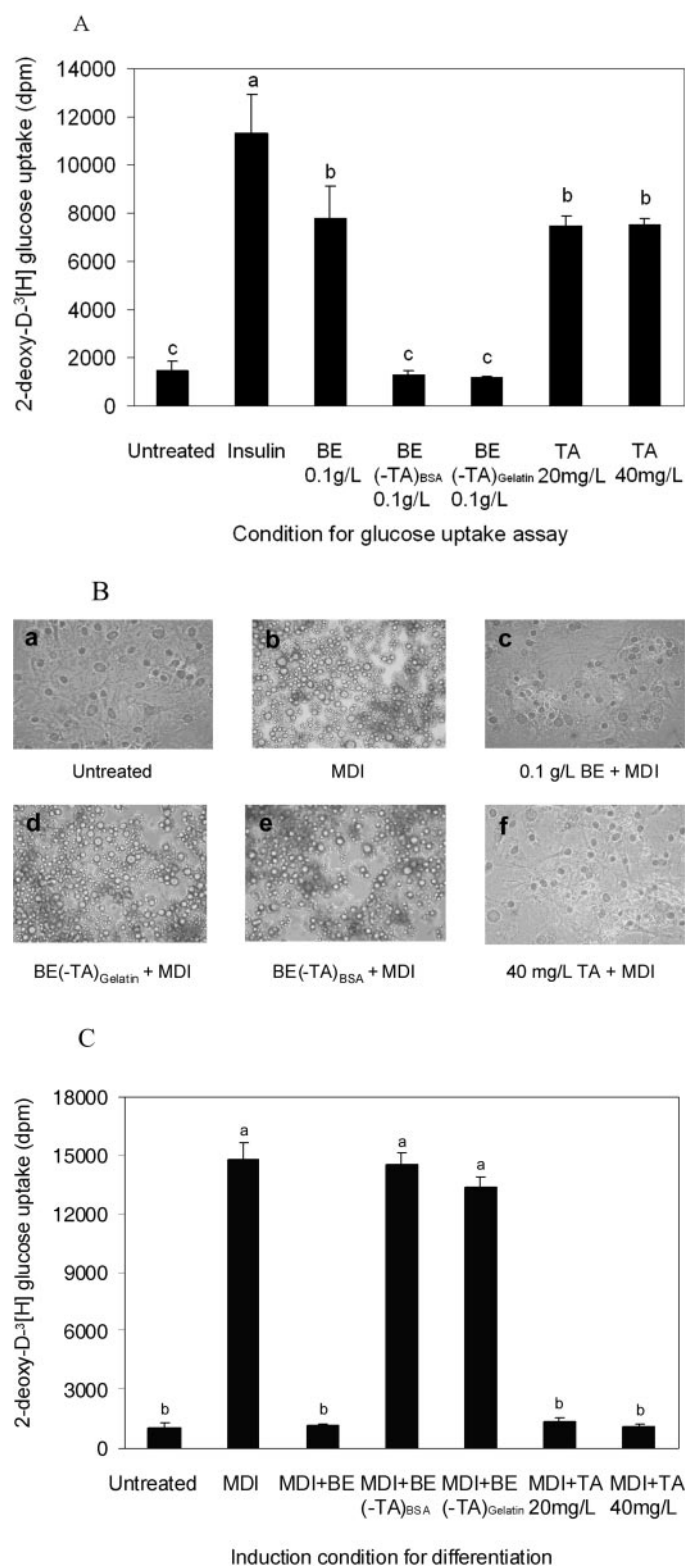
TA, a major constituent of tannins and a mixture of several structurally-related gallotannin compounds, was purchased from Sigma and dissolved in double distilled water as a 10 g/L stock solution.

**Glucose uptake assay.** The assay was performed essentially as previously described (9) with minor modifications. Briefly, 3T3-L1 preadipocytes grown in 24-well plates were induced by the combination of IBMX, dexamethasone, and insulin (MDI) to differentiate into adipocytes. Adipocytes were washed and incubated as described (9). Insulin, BE, BE (-TA), or TA were individually added to the cells at 1 μmol/L, 0.1 g/L, 0.1 g/L, and 20 mg/L, respectively, with each addition done in triplicate; then the adipocytes were incubated at 37°C for 15 min. Glucose uptake was initiated by the addition of 0.1 mL of Krebs-Ringer phosphate (KRP) buffer supplemented with 1 mCi/L [<sup>3</sup>H] 2-deoxy-D-glucose and 1 mmol/L cold regular glucose as the final concentration to the cells. After 10 min, the medium was aspirated and the plates were washed with ice-cold PBS to terminate the induced glucose uptake. The cells were lysed and the radioactivity taken up by the cells was determined using a scintillation counter (Beckman Instruments).

**Adipocyte differentiation assays.** 3T3-L1 preadipocytes were induced by MDI to differentiate into adipocytes as previous described (9). Two days after induction, the MDI-containing medium was replaced with 10% FBS-DMEM containing 1 mg/L insulin. The medium was replaced again with fresh culture medium every other day for 8 d. The degree of the differentiation of the cells was investigated by adding BE, BE (-TA), or TA for 15 min at 0.1 g/L, 0.1 g/L, and 20 mg/L, respectively, followed by a glucose uptake assay at the end of the induction period as described above. Alternatively, preadipocytes were induced with 0.1 g/L of BE, BE (-TA), or 20 and 40 mg/L of TA in the culture medium containing MDI-inducing cocktail for the first 2 d of adipogenesis. After the first 2 d of incubation, the medium was changed to fresh FBS-DMEM. The glucose uptake assay was performed to monitor the degree of differentiation 10 d after induction. The rationale behind this assay was that differentiated adipocytes are capable of insulin-mediated glucose transport, whereas undifferentiated preadipocytes are not (16; unpublished observation). Thus, glucose uptake can be used to measure indirectly the degree of adipocyte differentiation. To visualize directly the morphology of differentially treated cells, preadipocytes treated with MDI, BE + MDI, or TA + MDI were stained with Oil Red O (17).

**Mechanism study using inhibitors.** Fully differentiated adipocytes grown in 24-well plates were washed twice with serum-free medium, and placed in the same medium for 2 h in 10% CO<sub>2</sub> at 37°C before use. The cells were washed with 0.5 mL of KRP buffer 3 times and incubated for 30 min in 0.45 mL of KRP at 37°C in 10% CO<sub>2</sub>. The inhibitors, including HNMPA-(AM)<sub>3</sub> for IR tyrosine kinase activity (18), wortmannin for phosphatidylinositol-3-kinase (19), and cytochalasin for inhibiting glucose transporter 4 (GLUT 4) (20), were added individually to the cells at 200 μmol/L for HNMPA-(AM)<sub>3</sub>, 200 nmol/L for wortmannin, and 5 μmol/L for cytochalasin B. The cells were incubated at 37°C for 1 h in 10% CO<sub>2</sub>. After 1 h, the cells were induced with 1 μmol/L insulin or 40 mg/L of TA for 15 min in 10% CO<sub>2</sub> at 37°C. The glucose uptake assay was performed as described above.

**Mechanism study using protein phosphorylation assay.** Total protein (30–60 μg) isolated from BE- or TA-treated adipocytes, or the biotinylated protein marker were mixed with SDS sample buffer and heated at 95–100°C for 5 min. Each sample and a marker were subjected to 8% SDS-PAGE. After electrophoresis, the proteins on the gel were transferred to a nitrocellulose membrane, which was blocked and then incubated with the desired primary antibody overnight at 4°C with gentle agitation. After overnight incubation, the membrane was washed, and then incubated with the HRP-conjugated secondary antibody for 1 h at room temperature. After the membrane was washed again, the proteins on the membrane were detected by a Western blotting LumiGLO system (Cell Signaling Technology) and finally visualized by exposing the membrane to an X-ray film in a cassette for the proper time, usually from 1 to 10 min.



**FIGURE 1** TA stimulates glucose transport and inhibits adipocyte differentiation. Glucose transport-stimulatory and adipocyte differentiation-inhibitory activities of TA were induced under different conditions. (A) Glucose transport stimulatory activity of TA or BE after removal of tannins. Differentiated 3T3-L1 adipocytes were incubated individually with insulin, BE, TA, or BE (-TA) and the glucose taken up by the cells was measured. Data are means  $\pm$  SD,  $n = 8$ . Means with different letters differ,  $P < 0.05$ . (B) Oil Red O staining of 3T3-L1 cells treated under different conditions. Ten days after the induction of 3T3-L1 preadipocytes with different agents, the cells were stained with

**GLUT 4 translocation and confocal microscopy.** This assay was performed as described by Kupriyanova et al. (21) with minor modifications. 3T3-L1 preadipocytes grown in 2-well cell culture chambers were differentiated into adipocytes with the induction of MDI. Adipocytes in chambers were repeatedly washed and incubated as described (9,21). Insulin, BE, BE (-TA), or TA were added individually to the cells at the same concentrations as in the glucose uptake assay, and the cells were incubated at 37°C for 15 min for induction of GLUT 4 translocation. After incubation, the cells were washed with cold PBS, fixed with ice-cold 100% methanol, and incubated at -20°C for 20 min. The methanol was removed and the chambers were air-dried; 1 mL of 0.3% H<sub>2</sub>O<sub>2</sub> in methanol was added to each chamber to block endogenous peroxidase at room temperature. The cells were washed with PBS and incubated with 4% paraformaldehyde to fix cell surface antigens for 30 min at room temperature. The cells were washed and then blocked with 250  $\mu$ L of 5% normal serum (donkey serum) in PBS with 0.2% Triton X-100, 1% dimethyl sulfoxide each at room temperature for 50 min. The blocking solution was removed and the primary antibody against GLUT 4 was added to the cells, which were subsequently incubated overnight at 4°C. After overnight incubation, the cells were washed and then incubated with the secondary antibody [fluorescein isothiocyanate-conjugated affipure F(ad')<sub>2</sub> fragment Donkey anti-Mouse IgG] at 37°C for 30 min. The cells were finally washed, mounted, and photographed with a Zeiss LSM510 confocal microscope. The excitation wavelength was 488 nm, and detection occurred at 520 nm.

**Northern blot analyses.** Total RNA was isolated from 3T3-L1 cells induced with different reagents and conditions with a standard procedure. <sup>32</sup>P-labeled DNA fragments complementary to each mRNA were used individually as the probe for the detection of their respective mRNA; 10  $\mu$ g of total RNA was used per lane. The mRNA level of  $\beta$ -actin in the same samples was used as the sample loading reference.

**Statistical analysis.** All assay data were analyzed statistically using 1- or 2-way ANOVA. The experimental samples of the same treatment conditions were compared with negative control (untreated) samples, positive (insulin- or BE-treated) samples, or with experimental (TA-treated) samples under different conditions (different mixtures, concentrations, or cell types). In the figures, values are means and the standard deviations of samples. Samples were replicated either in duplicate or triplicate and each experiment was repeated at least 3 times.  $P < 0.05$  was set as the level of significant difference.

## RESULTS AND DISCUSSION

**BE without tannin does not have glucose transport-stimulatory or adipocyte differentiation-inhibitory activities.** To isolate and identify the effective antidiabetic compound(s), we started our study by removing tannin from BE because tannin constitutes up to 40% of all water-soluble materials in BE. Because tannin is not known to possess high biomedical activities or pharmaceutical potentials, we initially speculated that tannin was not responsible for the glucose transport or the differentiation inhibitory activities observed. Every time we removed the tannin fraction in BE by either the gelatin or BSA method (14,15), the remaining BE, either BE (-TA)<sub>gelatin</sub> or BE (-TA)<sub>BSA</sub>, completely lost the 2 activities in 3T3-L1 cells as revealed by the glucose uptake assay and the Oil Red O

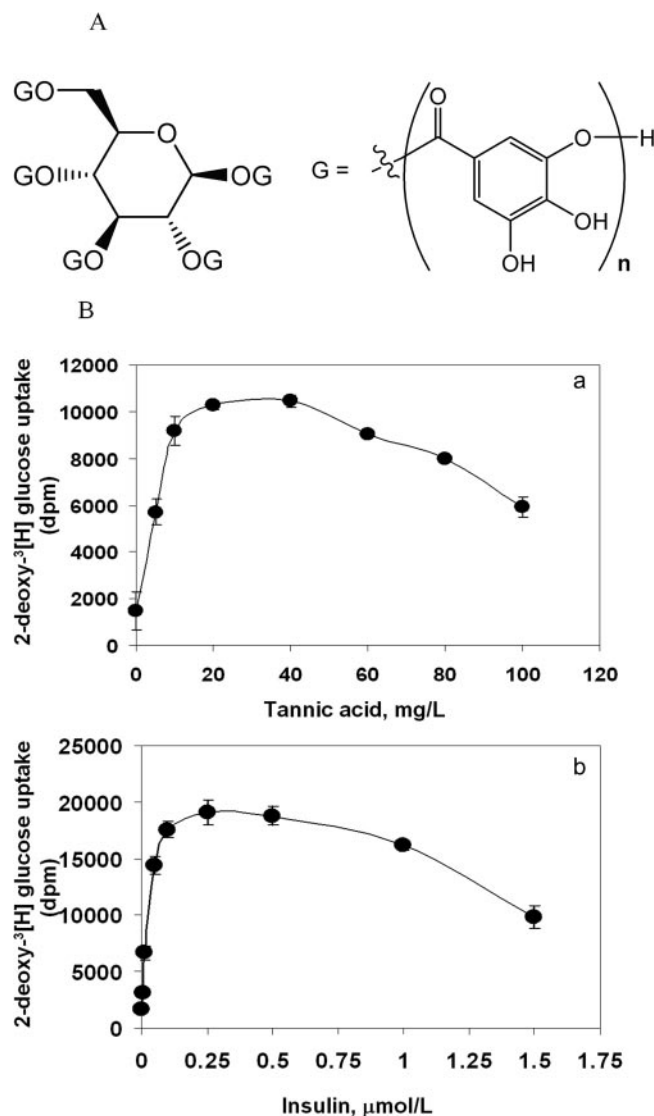
Oil Red O and photographed at magnification X200. Conditions for adipogenesis induction are as indicated under each photo. Differentiated adipocytes with accumulation of round-shaped fat vesicles can be distinguished from the undifferentiated cells by either morphology or staining. (C) TA inhibits adipocyte differentiation as revealed by indirect glucose uptake assays. Preadipocytes were separately induced by MDI or MDI plus other agents as indicated; 10 d after the induction, cells were assayed for their glucose transport activities as an indirect measurement for adipocyte differentiation.

staining of the treated cells, respectively [Fig. 1A and B (panels d and e)]. Furthermore, preadipocytes treated by MDI plus BE (-TA)<sub>gelatin</sub> or MDI plus BE (-TA)<sub>BSA</sub> differentiated into adipocytes as demonstrated by their adipocyte morphology [Fig. 1B (panels d and e)] and transported glucose after differentiation (Fig. 1C). This result indicates that the nontannin compounds, which were not removed by either gelatin or BSA and remained within the BE (-TA) samples, were not responsible for the activities we observed in 3T3-L1 adipocytes. Instead, it strongly suggests that components of the removed tannins induced the metabolic changes.

**TA possesses glucose transport-stimulatory and adipocyte differentiation-inhibitory activities.** Because tannin compounds removed by either gelatin or BSA were irreversibly precipitated and fully unrecoverable, we had to find and use indirect means to study the bioactivities of compounds in tannin. To confirm that tannin-related compounds indeed stimulate glucose transport and inhibit adipocyte differentiation, we used the glucose uptake assay to test commercially available TA. The assays showed that TA possesses the 2 activities, and that both are qualitatively and quantitatively similar to those found in BE (Fig. 1A-C). The results with commercial TA and the lack of activity for BE after removing tannin (Fig. 1A-C) suggest that tannin is responsible for both activities in BE. The components of TA, gallic acid and glucose, did not show any of the 2 activities when applied individually or in combination to 3T3-L1 cells (Xiao Chen, unpublished data). It appeared that only tannin molecules are required. However, it is presently unclear whether the 2 activities of BE are mediated only by TA or by other tannin molecules as well. Tannins are a very large group of plant-derived polyphenolic compounds (10). The exact composition of compounds is variable from plant to plant. Tannins can be further divided into 2 groups on the basis of their distinctive structures, i.e., condensed tannins and hydrolyzable tannins (10). Hydrolyzable tannins consist of 2 main groups, TA, also called gallotannins, Fig. 2A) and ellagitannins. TA comprise a relatively well-defined class of compounds that are commercially available. Because tannins constitute up to 40% of all water-soluble compounds in BE and tannins contain numerous structurally and functionally diversified polyphenolic compounds, it is possible that the 2 activities originate from >1 tannin compound. Our results do not exclude the possibility that other types of tannin, such as condensed tannin, may also possess the activities. Furthermore, Japanese researchers reported recently that some of the glucose transport-stimulatory compounds in BE were ellagitannins (22). This suggests that it is likely that multiple tannin compounds in BE are responsible for the 2 activities observed. Because of our successful preliminary experiments and the relatively well-defined chemical composition, commercial TA was used in all subsequent studies.

**TA stimulates glucose transport with a profile similar to that of insulin.** In a subsequent study, the determination of the concentration-activity relationship of TA-induced glucose transport revealed a dose-response curve similar in shape to that of insulin (Fig. 2B). This qualitative similarity between the 2 dose-response curves implies a potential similarity in the working mechanisms of the molecules. Similar dose-response profiles were also observed between BE and insulin (9). The demonstration of glucose transport activity of TA (Fig. 1A) and the comparison of the dose-response curves of TA and BE also suggest that BE's glucose transport activity originates from tannin-related compounds such as TA.

**3T3-L1 cells remain undifferentiated after treatment of TA.** Preadipocytes of 3T3-L1 cells differentiate into adipo-



**FIGURE 2** Changes in glucose transport in 3T3-L1 cells due to incubation with different concentrations of TA and insulin. (A) Chemical structure of tannic acid. TA consists of a glucose core, which covalently links to 3–5 gallic acid residues through ester bonds. In addition, each gallic acid residue can covalently link to other gallic acid molecules as shown. (B) Effect of TA and insulin on glucose transport activity. Adipocytes were incubated with different concentrations of TA, and then assayed for their respective intracellular 2-deoxy-D-[3H] glucose. Data are means  $\pm$  SD,  $n = 6$ .

cytes upon MDI induction. When TA was added to the preadipocytes in the presence of MDI, preadipocytes remained largely undifferentiated as shown by Oil Red O staining of the treated cells (Fig. 1B). The TA-treated cells remained in their undifferentiated cell morphology (Fig. 1B, panel f) and were incapable of insulin-mediated glucose transport (Fig. 1C). This result indicates that TA inhibits the differentiation induced by MDI. This observation is particularly puzzling in that TA has an insulin-like glucose transport stimulatory activity in adipocytes, whereas it exhibits “anti-insulin” activity in preadipocytes. At present it is unclear how TA mediates these 2 apparently contradictory activities. Some compounds were reported to exhibit similar apparently conflicting activities (23,24). It is possible that TA regulates these 2 activities in 2

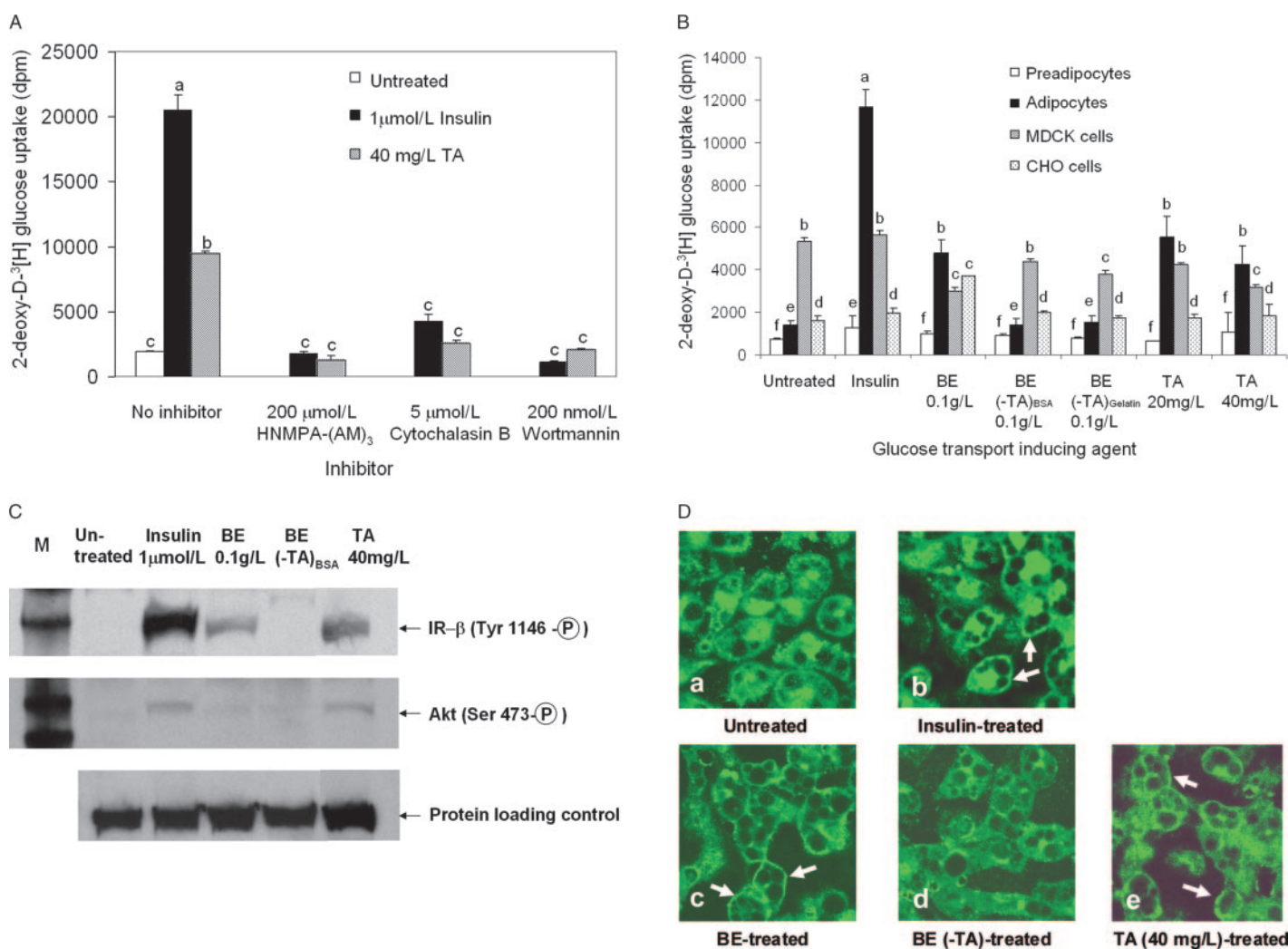
different cell types through 2 different targets and 2 different signaling pathways.

**Inhibitors of the insulin-mediated pathway also block TA-induced glucose transport.** To identify the signaling pathway used by TA to stimulate glucose transport, inhibitors of the insulin-mediated glucose transport cascade were selected and applied. The reason for the selection of certain inhibitors was based on the speculation that TA may use the same pathway as insulin for the induction of glucose transport. The study showed that the inhibitors abolished both the insulin- and TA-induced glucose transport to a similar degree (Fig. 3A). This result suggests that TA uses the glucose transport signaling pathway mediated by insulin for its glucose transport-stimulatory activity. More interestingly, HNMPA-(AM)<sub>3</sub>, an

inhibitor of the tyrosine kinase activity of the IR, also completely blocked the activity of TA (Fig. 3A). This result strongly suggests that TA stimulates glucose transport by activating the IR.

**TA does not induce glucose transport in IR-deficient cells.**

To further substantiate the pathway used by TA for its glucose transport-stimulatory activity, several cell types, which are either IR deficient (MDCK, 25) or expressing very low levels of IR (CHO, 26), were compared with 3T3-L1 cells. These cells were incapable of either insulin- or TA-induced glucose transport (Fig. 3B). This result indicates that TA did not induce any IR-independent glucose transport, strongly supporting the notion that TA uses the insulin-mediated pathway(s) exclusively for glucose transport.



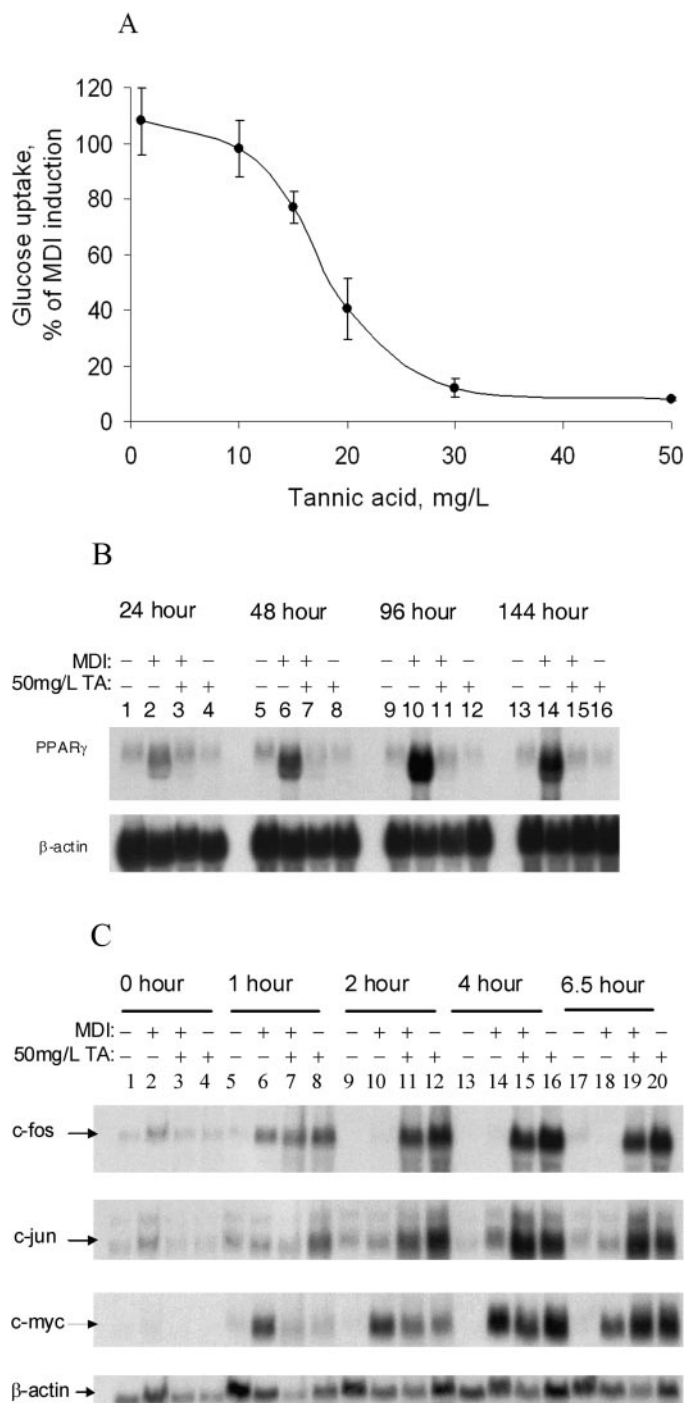
**FIGURE 3** Mechanisms underlying the effects of TA on glucose transport in 3T3-L1 cells. 3T3 adipocytes were treated under different conditions. (A) Inhibitors that suppress insulin's glucose transport signaling pathway also abolish TA's glucose transport activity. Insulin pathway inhibitors or TA were added individually to 3T3-L1 adipocytes at the concentrations indicated. Cells were then induced by insulin in the presence of 2-deoxy-D-<sup>3</sup>[H] glucose and assayed for the glucose taken up. Data are means  $\pm$  SD,  $n = 8$ . Means with different letters differ,  $P < 0.05$ . (B) TA and insulin induce glucose transport in 3T3-L1 adipocytes but not in IR-GLUT 4-deficient cells. IR-expressing 3T3-L1 adipocytes, and IR-deficient or low IR-expressing cells such as 3T3-L1 preadipocytes, CHO, and MDCK, were incubated with either insulin or TA in the presence of 2-deoxy-D-<sup>3</sup>[H] glucose. After the incubation, radioactive glucose taken up by the treated cells was measured and analyzed by 2-way ANOVA. Data are means  $\pm$  SD,  $n = 8$ . Means with different letters differ,  $P < 0.05$ . (C) TA induces phosphorylation of IR and Akt. CHO-IR cells or 3T3-L1 adipocytes were induced by insulin, BE, or TA for 15 min, and then lysed for total protein preparation. Protein samples were subjected to SDS-PAGE and then analyzed by Western blotting using antibodies specific against the phosphorylated form of the IR or Akt, respectively. M = protein markers. (D) TA induces GLUT 4 translocation. Differentiated adipocytes were induced with insulin, BE, BE (-TA), or TA. The treated cells were fixed and immunostained with an antibody specific against GLUT 4, and photographed with a confocal fluorescence microscope. Arrows indicate sites of GLUT 4 membrane translocation.

TA stimulates phosphorylation of protein factors in the insulin-mediated glucose transport pathway and induces GLUT 4 translocation. After demonstrating elimination of TA-induced glucose transport by inhibitors selective to the insulin-mediated pathway and lack of glucose transport activity in IR-deficient cells, we went further to determine the direct activation of the protein factors involved in the insulin cascade by TA. Western blots revealed that the IR and Akt, 2 important factors involved in the transmission of insulin signaling (27), were phosphorylated after cells were treated with TA (Fig. 3C). The phosphorylation of the IR further suggests that TA may induce glucose transport by directly or indirectly activating the IR. The effector of the insulin-induced glucose transport in adipocyte is GLUT 4 (27). Immunostaining with anti-GLUT 4 antibodies followed by confocal fluorescent microscopy revealed that TA induced translocation of GLUT 4 in a fashion similar to that of insulin (Fig. 3D). This result is consistent with the results of inhibitor and protein phosphorylation studies (Fig. 3A and C) that showed that TA uses the insulin-mediated signaling pathway for its glucose transport-stimulatory activity. It would be interesting to determine whether TA activates the insulin-mediated glucose transport pathway by binding directly to IR. Such a study has to wait until a single active compound in TA is identified and used in an IR binding assay. It is also important to investigate whether TA is capable of IR-dependent but GLUT 4-independent glucose transport such as GLUT 1-mediated glucose transport.

TA inhibits adipocyte differentiation and affects key genes involved in adipogenesis. Preadipocytes treated with MDI and TA as differentiation induction agents showed a decrease in glucose uptake with the increase of the TA concentration (Fig. 4A). This result, combined with the Oil Red O staining results of the cells treated with different concentrations of TA (data not shown), indicates that the more TA is added, the fewer preadipocytes differentiate into adipocytes, and the lower the glucose transport activity of the cells.

TA inhibited adipocyte differentiation induced by MDI (Figs. 1B and C), suggesting that TA must have altered the expression of genes involved in the adipogenesis program. Northern blot analysis using gene-specific probes revealed that, consistent with our speculations, the expression of peroxisome proliferator-activated receptor (PPAR)- $\gamma$  induced by MDI in preadipocytes was inhibited by TA (Fig. 4B). However, although the expression of the PPAR- $\gamma$  gene is absolutely required in adipogenesis, it is not a very early gene in the process (28). Additional Northern blot analyses showed that other earlier genes involved in cell proliferation of preadipocytes after induction of MDI (29), such as *c-fos*, *c-jun*, and *c-myc*, were all affected by the treatment of TA (Fig. 4C). These changes in patterns of gene expression clearly demonstrate the effect of TA on the adipogenesis induced by MDI, and suggest interesting but complicated signaling cascade(s) during the inhibition process. Further studies are required to identify the initial target(s) of TA and the signaling pathway(s) used by TA for the inhibition of adipocyte differentiation.

Tannins, as plant-derived compounds, are a part of our daily diet. TA was previously shown to be antilipogenic in an animal study (30) and antidiabetic in human T2D patients (31). However, the mechanism by which TA mediates these activities was not investigated in detail at molecular levels. This study is the first to demonstrate that TA induces glucose transport through activation of the insulin-mediated signaling pathway in adipocytes. In addition, TA inhibits adipocyte differentiation by inhibiting or altering the expression of key genes involved in the adipogenesis process. However, many



**FIGURE 4** TA inhibits adipocyte differentiation and alters the expression of genes involved in adipogenesis. (A) Dose dependence of TA's inhibitory effects on MDI-induced 3T3-L1 adipogenesis. TA was added to MDI-induced adipocytes at different concentrations; 10 d after the induction, the degree of 3T3-L1 adipogenesis was assessed indirectly by a glucose uptake assay and expressed as the percentage of the uptake in cells induced by MDI. Data are means  $\pm$  SD,  $n = 8$ . (B) TA inhibits the MDI-induced expression of PPAR $\gamma$  gene in 3T3-L1 cells. Gene expression of PPAR $\gamma$  in 3T3-L1 preadipocytes at different times after TA treatment was analyzed by Northern blots as shown by this representative gel. The mRNA of  $\beta$ -actin was used as the sample loading control. (C) TA alters the expression of genes involved in cell growth and differentiation in 3T3-L1 cells. Gene expression of *c-fos*, *c-jun*, *c-myc* in TA-treated cells was analyzed and shown by this representative Northern blot gel. Probes complementary to the *c-fos*, *c-jun*, *c-myc* genes were hybridized with the total RNA blotted on a nylon membrane. The mRNA of  $\beta$ -actin in the same samples was used as the loading reference.

questions remain to be answered. TA is still a mixture of hydrolyzable tannin compounds. What is the most effective compound in TA for the 2 activities? How does TA activate IR and mediate the inhibition of adipocyte differentiation? How is glucose metabolized in adipocytes after it is transported under the stimulation of TA? What are TA's effects on muscle and liver cells? Is TA bioavailable in vivo and can it be used to treat T2D? The answers to these questions will shed more light on how TA works as an antidiabetic and antiadipocyte differentiation compound. The combination of the 2 activities of TA makes it ideally suited as a prototypic compound for further structural and functional studies to develop novel pharmaceuticals that treat symptoms of syndrome X and T2D, such as hyperglycemia, hyperinsulinemia, and hypertriglyceremia, without concomitant weight gain or even with weight loss. A drug with such a combination of properties should be a much better option for up to 90% of T2D patients who have diabetes-associated weight problems.

### ACKNOWLEDGMENTS

We thank Klaus Himmeldirk and Yulin Ren for critical reading and valuable suggestions of the manuscript.

### LITERATURE CITED

1. Astrup, A. & Finer, N. (2000) Redefining type 2 diabetes: 'diabesity' or 'obesity dependent diabetes mellitus'? *Obes. Rev.* 2: 57-59.
2. Kahn, B. B. & Flier, J. S. (2000) Obesity and insulin resistance. *J. Clin. Invest.* 106: 473-481.
3. Bays, H., Mandarino, L. & DeFronzo, R. A. (2004) Role of the adipocyte, free fatty acids, and ectopic fat in pathogenesis of type 2 diabetes mellitus: peroxisomal proliferator-activated receptor agonists provide a rational therapeutic approach. *J. Clin. Endocrinol. Metab.* 89: 463-478.
4. Palou, A., Serra, F., Bonet, M. L. & Pico, C. (2000) Obesity: molecular bases of a multifactorial problem. *Eur. J. Nutr.* 39: 127-144.
5. Kopelman, P. G. (2000) Obesity as a medical problem. *Nature (Lond.)* 404: 635-643.
6. Hotamisligil, G. S. (2001) Molecular mechanisms of adipocyte differentiation. *Rev. Endocr. Metab. Disord.* 2: 349-355.
7. Camp, H. S., Ren, D. & Leff, T. (2002) Adipogenesis and fat-cell function in obesity and diabetes. *Trends Mol. Med.* 8: 442-447.
8. Moller, D. E. (2001) New drug targets for type 2 diabetes and the metabolic syndrome. *Nature (Lond.)* 414: 821-827.
9. Liu, F., Kim, J., Li, Y., Liu, X., Li, J. & Chen, X. (2001) An extract of *Lagerstroemia speciosa* L. has insulin-like glucose uptake-stimulatory and adipocyte differentiation-inhibitory activities in 3T3-L1 cells. *J. Nutr.* 131: 2242-2247.
10. Okuda, T., Yoshida, T. & Hatano, T. (1995) Hydrolyzable tannins and related polyphenols. *Fortschr. Chem. Org. Naturst.* 66: 1-117.
11. Hagerman, A. E., Riedl, K. M., Jones, J. A., Sovik, K. N., Ritchard, N. T., Hartzfeld, P. W. & Riechel, T. L. (1998) High molecular weight plant polyphenolics (tannins) as biological antioxidants. *J. Agric. Food Chem.* 46: 1887-1892.
12. Cowan, M. M. (1999) Plant products as antimicrobial agents. *Clin. Microbiol. Rev.* 12: 564-582.
13. Frattali, A. L., Treadway, J. L. & Pessin, J. E. (1991) Evidence supporting a passive role for the insulin receptor transmembrane domain in insulin-dependent signal transduction. *J. Biol. Chem.* 266: 9829-9834.
14. Riedl, K. M. & Hagerman, A. E. (2001) Tannin-protein complexes as radical scavengers and radical sinks. *J. Agric. Food Chem.* 49: 4917-4923.
15. Thoss, V., Baird, M. S., Lock, M. A. & Courty, P. V. (2002) Quantifying the phenolic content of freshwaters using simple assays with different underlying reaction mechanisms. *J. Environ. Monit.* 4: 270-275.
16. Lane, M. D., Tang, Q. Q. & Jiang, M. S. (1999) Role of the CCAAT enhancer binding proteins (C/EBPs) in adipocyte differentiation. *Biochem. Biophys. Res. Commun.* 266: 677-683.
17. Kasturi, R. & Joshi, V. V. (1982) Hormonal regulation of stearoyl coenzyme A desaturase activity and lipogenesis during adipose conversion of 3T3-L1 cells. *J. Biol. Chem.* 257: 12224-12230.
18. Saperstein, R., Vicario, P. P., Strout, H. V., Brady, E., Slater, E. E., Greenlee, W. J., Ondeyka, D. L., Patshett, A. A. & Hangauer, D. G. (1989) Design of a selective insulin receptor tyrosine kinase inhibitor and its effect on glucose uptake and metabolism in intact cells. *Biochemistry* 28: 5694-5701.
19. Tomiyama, K., Nakata, H., Sasa, H., Arimura, S., Nishio, E. & Watanabe, Y. (1995) Wortmannin, a specific phosphatidylinositol 3-kinase inhibitor, inhibits adipocytic differentiation of 3T3-L1 cells. *Biochem. Biophys. Res. Commun.* 212: 263-269.
20. Kletzien, R. F., Perdue, J. F. & Springer, A. (1972) Cytochalasin A and B. Inhibition of sugar uptake in cultured cells. *J. Biol. Chem.* 247: 2964-2966.
21. Kupriyanova, T. A. & Kandror, K. V. (2000) Cellugyrin is a marker for a distinct population of intracellular Glut4-containing vesicles. *J. Biol. Chem.* 275: 36263-36268.
22. Hayashi, T., Maruyama, H., Kasai, R., Hattori, K., Takasuga, S., Hazeki, O., Yamasaki, K. & Tanaka, T. (2002) Ellagitannins from *Lagerstroemia speciosa* as activators of glucose transport in fat cells. *Planta Med.* 68: 173-175.
23. Rocchi, S., Pichard, F., Vamecq, J., Gelman, L., Potier, N., Zeyer, D., Dubuquoy, L., Bac, P., Champy, M. F., Plunket, K. D., Leesnitzer, L. M., Blanchard, S. G., Desreumaux, P., Moras, D., Renaud, J. P. & Auwerx, J. (2001) A unique PPARgamma ligand with potent insulin-sensitizing yet weak adipogenic activity. *Mol. Cell* 8: 737-747.
24. Rieusset, J., Touri, F., Michalik, L., Escher, P., Desvergne, B., Niesor, E. & Wahli, W. (2002) A new selective peroxisome proliferator-activated receptor gamma antagonist with antiobesity and antidiabetic activity. *Mol. Endocrinol.* 16: 2618-2644.
25. Takeuchi, F., Seta, K. A., Omura, S. & Roth, R. A. (1997) Insulin degradation by Madin-Darby canine kidney cells expressing the insulin receptor. *Diabetes Res. Clin. Pract.* 37: 81-90.
26. Yamauchi, K. & Pessin, J. E. (1994) Enhancement or inhibition of insulin signaling by insulin receptor substrate 1 is cell context dependent. *Mol. Cell Biol.* 14: 4427-4434.
27. Sattiel, A. R. & Kahn, C. R. (2001) Insulin signalling and the regulation of glucose and lipid metabolism. *Nature (Lond.)* 414: 799-806.
28. Rosen, E. D., Walkey, C. J., Puigserver, P. & Spiegelman, B. M. (2000) Transcriptional regulation of adipogenesis. *Genes Dev.* 14: 1293-1307.
29. Ntambi, J. & Kim, Y.-C. (2000) Adipocyte differentiation and gene expression. *J. Nutr.* 130: 3122S-3126S.
30. Ong, C., Khoo, H. E. & Das, N. P. (1995) Tannic acid inhibits insulin-stimulated lipogenesis in rat adipose tissue and insulin receptor function in vitro. *Experientia* 51: 577-584.
31. Gin, H., Rigalleau, V., Caubet, O., Masquelier, J. & Aubertin, J. (1999) Effects of red wine, tannic acid, or ethanol on glucose tolerance in non-insulin-dependent diabetic patients and on starch digestibility in vitro. *Metabolism* 48: 1179-1183.