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Insulinomimetic Effects of Myricetin on Lipogenesis and Glucose Transport in Rat Adipocytes but not Glucose Transporter Translocation

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ABSTRACT. Myricetin is a naturally occurring flavonol that is commonly found in tea, berries, fruits, and medicinal plants. It mimics insulin in stimulating lipogenesis and glucose transport in rat adipocytes *in vitro*. It was found to stimulate lipogenesis in rat adipocytes and enhance the stimulatory effect of insulin. The EC_{50} was estimated to be about 65 μM. Myricetin did not have any effect on insulin receptor autophosphorylation nor on the tyrosine kinase activity of the receptor. However, myricetin stimulated both D-glucose and D-3-O-methyl-glucose uptake in rat adipocytes. The V_{max} of glucose transport was increased, but the K_m did not change significantly. Immunoblot analysis of Glut4 in rat adipocyte plasma membrane showed that the stimulation of glucose transport was not a consequence of glucose transporter translocation. Instead, the stimulation in glucose uptake probably was due to a change in the intrinsic activity of the glucose transporter possibly caused by alterations in membrane fluidity or transporter–lipid interactions as a result of the insertion of myricetin into the membrane bilayer. Thus, myricetin may have therapeutic potential in the management of non-insulin-dependent diabetes mellitus by stimulating glucose uptake without the presence of fully functional insulin receptors. BIOCHEM PHARMACOL 51;4:423–429, 1996.

KEY WORDS. myricetin; lipogenesis; glucose transport; bioflavonoid; rat adipocyte

Current management of NIDDM† involves the combination of a dietary plan, an exercise program, and the use of drugs such as sulfonylureas and biguanide. Sulfonylureas stimulate the release of insulin from pancreatic β-cells [1], while biguanides, such as metformin, reduce hepatic glucose production [2]. Acarbose, an α-glucosidase inhibitor, has been reported to be of therapeutic potential in the management of NIDDM by reducing the rate of carbohydrate absorption in the gastrointestinal tract [3] and is now undergoing clinical trials.

There is growing interest in the therapeutic applications of bioflavonoids and other naturally occurring polyphenols for the treatment and prevention of diseases in humans. Among the best-documented medical applications of flavonoids are their use in the treatment and prevention of allergy, asthma, and inflammation [4]. Their beneficial effects have also been implicated in a number of diseases including cancer [5, 6], cardiovascular diseases [7, 8], diabetic cataracts [9], and cirrhosis [10].

Several naturally occurring polyphenols have been shown to exert an effect on glucose transport and on insulin–receptor function, both of which play essential roles in diabetes. Phloretin and genistein inhibit glucose transport in rat adipocytes [11, 12], while phloridzin inhibits glucose transport in vesicles derived from the plasma membrane of rat adipocytes [13]. Quercetin inhibits IR tyrosine kinase without having any inhibitory effect on IR autophosphorylation [14]. Tannic acid, on the other hand, inhibits IR autophosphorylation but only partially inhibits the tyrosine kinase activity of the receptor [15].

Myricetin (Fig. 1) is a naturally occurring flavonol commonly found in tea, berries, fruits, and medicinal plants. Earlier studies showed that it could potentially be an anticarcinogenic agent [16] as well as an antiviral and antimicrobial agent [17, 18]. It was also shown to prevent the aggregation of platelets [19]. In our study of over 30 bioflavonoids, myricetin was the only compound that was found to stimulate lipogenesis and enhance insulin-stimulated lipogenesis. Thus, it was studied in more detail.

MATERIALS AND METHODS

Materials

Fed male Wistar rats (180–220 g) were obtained from the Animal Lab. Centre, National University of Singapore. D-[U-^{14}C]Glucose (300 mCi/mmol), D-3-O-[*methyl*-^{14}C]glucose (200 mCi/mmol), [γ-^{32}P]ATP (>5000 Ci/mmol), monoclonal anti-phosphotyrosine antibody, the ECL Western Blotting

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† *Abbreviations:* NIDDM, non-insulin-dependent diabetes mellitus; IR, insulin receptor; PMSF, phenylmethylsulfonyl fluoride; WGA-agarose wheat germ agglutinin-agarose; and KHB, Krebs–Henseleit buffer.

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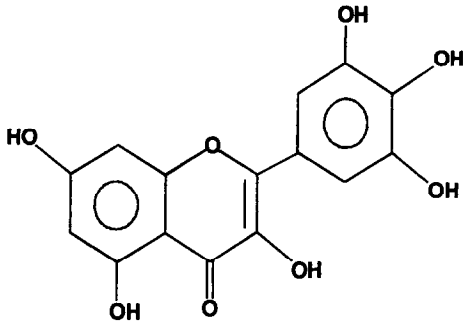

FIG. 1. Structure of myricetin.

Analysis System, and biodegradable liquid scintillant were purchased from Amersham (Amersham, U.K.). Recombinant human insulin was obtained from Lilly France SA (Fegersheim, France). The Protein Tyrosine Kinase Assay System was purchased from GIBCO BRL (Gaithersburg, MD, U.S.A.). Myricetin was obtained from Extrasynthase (Ganey, France). BSA fraction V, collagenase type II (290 U/mg), aprotinin, *N*-acetyl-D-glucosamine, PMSF, ATP, SDS, Tris–HCl, EDTA, HEPES, WGA-agarose, D-3-O-methylglucose, anti-mouse IgG and a 5′-Nucleotidase Assay Kit were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Monoclonal mouse anti-rat Glut4 antibody was purchased from the Genzyme Corp. (Cambridge, MA, U.S.A.). Silicone oil was from General Scientific (Singapore). KHB, pH 7.4, consisted of 120 mM NaCl, 1.2 mM KH_2PO_4, 4.7 mM KCl, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$ and 2.5 mM $CaCl_2$. All other reagents and chemicals used in this study were of analytical grade.

Preparation of Isolated Adipocytes

Isolated rat adipocytes were prepared from epididymal fat pads of male Wistar rats by collagenase digestion (2 mg/mL) in KHB supplemented with 3% BSA and 0.51 mM glucose [20]. Packed cell volume of the concentrated adipocyte stock was estimated by pipetting 100-μL aliquots into microfuge tubes, layering with 50 μL silicon oil, and centrifuging at 8500 *g* for 2 min [21]. The volume of the aqueous phase was measured and the packed cell volume inferred. Cell viability was determined by measuring the incorporation of [U-^{14}C]glucose into lipids as described by Moody *et al.* [22]. The incorporation was linear for at least 2 hr after collagenase digestion.

Lipogenesis Assay

Lipogenesis in isolated rat adipocytes was measured by the incorporation of [U-^{14}C]glucose into lipids [22]. Briefly, rat adipocytes were diluted to a final packed cell volume of 2–3% with KHB containing 3% BSA and 0.51 mM glucose. The adipocytes were preincubated in various concentrations of myricetin for 15 min at 37°. The assay was initiated by the addition of 0.51 mM [U-^{14}C]glucose (0.2 μCi), and lipogenesis was allowed to proceed for 1 hr. Control samples were incubated in the presence or absence of insulin (0.1 U/mL) but in the absence of myricetin. The assay was terminated by the addition of toluene-based scintillation liquid. The radioactivity in the lipids that were extracted into the toluene phase was determined by liquid scintillation counting.

Purification of Insulin Receptors

WGA-purified insulin receptors were prepared from rat liver membrane as described by Meyerovitch *et al.* [23] and Yarden and Schlessinger [24] with modifications. Briefly, rat liver was homogenized and solubilized in 50 mM HEPES (pH 7.4), 1% Triton X-100 supplemented with 2 mM PMSF and aprotinin (10 μg/mL). The suspension was centrifuged at 100,000 g for 60 min, and the supernatant was applied to WGA-agarose beads. The WGA-agarose beads were pelleted and washed extensively with 50 mM HEPES, 0.1% Triton X-100. The partially purified receptors were eluted by resuspending the beads in 2 vol. of 50 mM HEPES (pH 7.4), 0.1% Triton X-100 supplemented with 0.3 M *N*-acetyl-D-glucosamine. The receptors obtained after centrifugation were stored at −80°.

In Vitro *Autophosphorylation of Insulin Receptors*

Autophosphorylation and immunoprecipitation of WGA-purified insulin receptors were carried out by modifying the procedures described by Shisheva and Shechter [14] and Carrascosa *et al.* [25]. Insulin receptors (~50 μg total protein) were preincubated in 50 mM HEPES (pH 7.4), 0.1% Triton X-100, 2 mM $MnCl_2$, 10 mM $MgCl_2$ and various concentrations of myricetin at 25° for 50 min. Control samples were preincubated in the presence or absence of insulin (5 mU/mL) but in the absence of myricetin. Autophosphorylation was initiated by the addition of 25 μM [γ-^{32}P]ATP (5 μCi) and allowed to proceed for 10 min. The reaction was terminated by adding 3 vol. of 50 mM HEPES (pH 7.4), 0.1% Triton X-100, 100 mM NaF, 10 mM $Na_4P_2O_7$, 5 mM EDTA, and 2 mM $NaVO_3$. Phosphorylated insulin receptors were incubated overnight with anti-phosphotyrosine antibody (5 μg) at 4°. Receptor–antibody complexes were immunoprecipitated with secondary antibody for 2 hr at 4°. The pellet obtained after centrifugation was resolubilized in electrophoresis treatment buffer, resolved by 7.5% SDS–PAGE [26], and identified by autoradiography.

Phosphorylation of RR-SRC Substrate

WGA-purified receptors were preincubated in 50 mM HEPES (pH 7.4), 0.1% Triton X-100, 2 mM $MnCl_2$, 10 mM $MgCl_2$, 100 μM ATP, various concentrations of myricetin and in the presence or absence of insulin (10 mU/mL) for 30 min at 22°. Phosphorylation of RR-SRC peptide was assayed using the Protein Tyrosine Kinase Assay System and [γ-^{32}P]ATP (1 μCi) as previously described [15].

D-*Glucose Transport*

D-Glucose transport was measured under extremely low glucose concentrations as described by Kashiwagi *et al.* [27] and

Murer *et al.* [28]. Rat adipocytes were diluted in KHB containing 3% BSA to a final packed cell volume of 5%. The adipocytes were preincubated in the presence or absence of 200 μM myricetin at 37° for 20 min. D-Glucose uptake was initiated by adding 0.3 μM [U-^{14}C]glucose (0.1 μCi/mL). The assay was stopped by centrifugation through a layer of silicon oil. The radioactivity in the adipocyte islets above the silicon oil layer was measured by liquid scintillation counting.

Preparation of Plasma Membrane Vesicles

Rat adipocyte plasma membrane was prepared as described by Cushman and Wardzala [29], and vesicles were prepared from the plasma membrane fraction as described by Carter *et al.* [30] with modifications. Adipocytes were incubated in the presence or absence of myricetin (250 μM) and insulin (0.1 U/mL) for 30 min at 37°. The cells were then washed extensively and homogenized in a buffer containing 20 mM Tris–HCl (pH 7.4), 1 mM EDTA, 255 mM sucrose and 1 mM PMSF. Subsequent preparative steps were carried out at 4°. The homogenate was centrifuged at 16,000 *g* for 15 min. The fat cake was discarded, while the supernatant was aspirated and saved for preparation of the microsomal membrane fraction. The pellet was washed several times and resuspended in buffer. The suspension was applied to a discontinuous 1.12 M sucrose cushion and centrifuged at 23,000 *g* for 60 min. The plasma membrane, which appeared as a white fluff at the interface, was washed and resuspended in buffer. An aliquot was removed and stored at −80° for marker enzyme assays. The remainder was subjected to brief sonication in an ice-water bath and pelleted by centrifugation at 100,000 *g* for 30 min. The plasma membrane vesicles were stored at −80°. The microsomal membrane fraction was prepared from the initial supernatant by centrifugation at 160,000 *g* for 70 min. The pellet was washed several times, resuspended, and stored at −80°. 5′-nucleotidase and NADH–cytochrome *c* reductase activities in the plasma membrane and microsomal membrane fractions were measured using a 5′-Nucleotidase Assay Kit and the method described by Williams and Kamin [31]. The 5′-nucleotidase activity in the plasma membrane fraction was at least 7 times higher than that in the microsomal membrane fraction. The NADH–cytochrome *c* reductase activity in the microsomal membrane fraction was about 3.4 times that in the plasma membrane fraction.

Translocation of Glut4 Glucose Transporters

Plasma membrane fractions (100 μg total protein) prepared from rat adipocytes incubated in the presence or absence of myricetin (250 μM) and insulin (0.1 U/mL) were separated by 10% SDS–PAGE [26] and transferred to nitrocellulose membrane [32]. The membrane was blocked with Tris-buffered saline containing 5% BSA (w/v) and 0.5% Tween-20 (v/v), incubated with anti-Glut4 antibody and detected using an ECL Western Blotting Analysis System.

3-O-Methylglucose Transport

3-O-Methylglucose transport in rat adipocytes was measured under equilibrium conditions as previously described by Whitesell and Gliemann [12]. Rat adipocytes were preincubated in KHB containing 3% BSA, various concentrations of methylglucose and with or without 250 μM myricetin for 20 min at 25°. The assay was initiated by the addition of 3-O-[*methyl*-^{14}C]glucose (0.2 μCi) and terminated after 10 sec by the addition of KHB containing 0.5 mM phloretin followed by centrifugation through silicon oil. Zero time was determined by the addition of stop buffer to the cells before the isotope was added followed by the addition of the remaining stop buffer. Preliminary studies showed that methylglucose uptake was linear for the time interval used.

Protein concentrations were estimated using the method of Bradford [33]. All assays were performed either in duplicate or triplicate, and all data are presented as means ± SEM ($N \geq 3$). Data were analyzed using Student's *t*-test for significance.

RESULTS

The effect of myricetin on lipogenesis in rat adipocytes was studied in both the presence and the absence of insulin (Fig. 2). Myricetin (250 μM) stimulated the incorporation of [U-^{14}C]glucose into lipids in rat adipocytes. The stimulation of lipogenesis by myricetin was approximately 86 ± 3% of the maximal stimulation by insulin (0.1 U/mL). Myricetin further enhanced the stimulatory effects of insulin, increasing the stimulation from 100 ± 9 to 157 ± 13%. The stimulatory effect of myricetin on lipogenesis in rat adipocytes was concentration dependent (Fig. 3). Half-maximal stimulation by myricetin in the absence of insulin was obtained at approximately 65 μM, and maximal stimulation by myricetin was achieved at concentrations above 125 μM.

Myricetin did not stimulate insulin receptor autophosphorylation for the range of concentrations studied (Fig. 4). The 95 kDa band was observed in the lane containing the control sample that was incubated in the presence of insulin. The effect of myricetin on insulin receptor kinase activity was studied using the RR-SRC peptide as substrate. This synthetic substrate is specific for tyrosine kinases and has been used for measuring insulin receptor tyrosine kinase activity [15, 34]. Myricetin did not exhibit any inhibitory or stimulatory effects on insulin receptor tyrosine kinase activity in either the presence or the absence of insulin for the various concentrations of myricetin studied (Fig. 5).

D-Glucose transport in rat adipocytes was determined under very low glucose conditions. This was based on the premise that at extremely low glucose concentrations, the rate of glucose uptake gave a measure of glucose transport. At such low glucose concentrations, the rate of glucose transport was limiting compared with the rate of glucose utilization [27, 28, 35]. Rat adipocytes incubated in the presence of myricetin showed an increased rate of glucose uptake as compared with those incubated in the absence of myricetin (Fig. 6).

Plasma membrane fractions were prepared from rat adipocytes that were incubated in the presence or absence of myricetin and insulin. Western blot analysis of the plasma membrane fractions showed no apparent increase in Glut4 concentration upon treatment with myricetin in either the

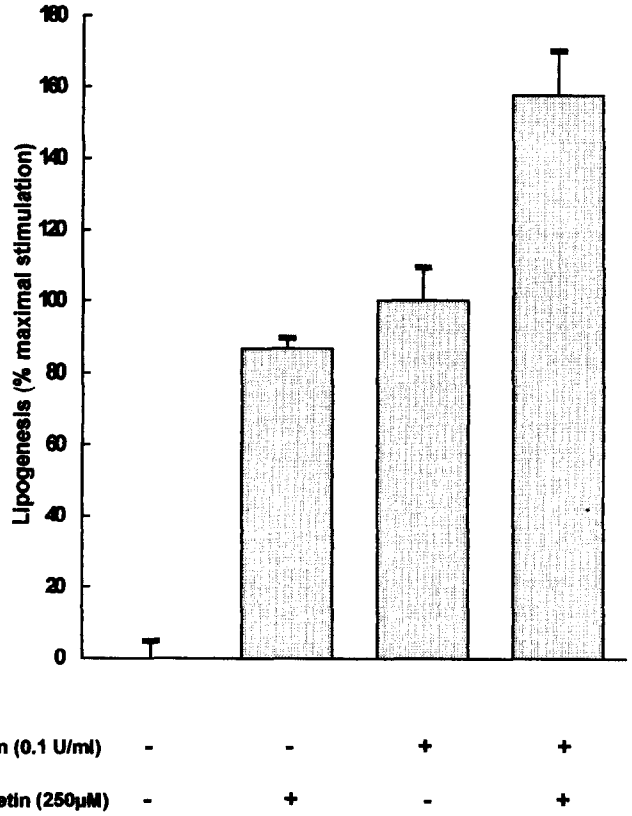

FIG. 2. Effect of myricetin on basal and insulin-stimulated lipogenesis in isolated rat adipocytes. Adipocytes were preincubated with or without myricetin (250 μM) for 15 min at 37°. Lipogenesis was assayed as described in Materials and Methods and measured with respect to the maximal stimulation by insulin (0.1 U/mL). Values for basal and insulin-stimulated incorporation of glucose into triglycerides were 85.9 ± 6.3 and 209.1 ± 18.3 nmol/100 μL packed cells, respectively. Values are means ± SEM of 3 experiments, each performed in triplicate.

presence or the absence of insulin as compared with their respective controls (Fig. 7).

The uptake of 3-O-methylglucose into rat adipocytes under equilibrium conditions was higher in adipocytes incubated in the presence of myricetin than in adipocytes that were incubated in the absence of the bioflavonoid (data not shown). The uptake of 3-O-methylglucose into rat adipocytes was also measured at several substrate concentrations in the presence or absence of myricetin. A preliminary study was done to ensure that the uptake was linear for the time interval used to measure initial velocity. The data were linearized and plotted according to the Hane's form of the flux equation: $S/v = K_m/V_{max} + S/V_{max}$ (Fig. 8). The K_m determined in five experiments was 3.1 ± 0.7 mM in the absence of myricetin and 3.8 ± 0.9 mM in the presence of myricetin. The V_{max} was found to be 45.9 ± 3.8 nmol/sec/50 μL packed cells in the absence of myricetin and increased to 61.4 ± 5.5 nmol/sec/50 μL packed cells in the presence of myricetin.

DISCUSSION

In our preliminary study of over 30 bioflavonoids, we found that most of these compounds inhibited either basal or insulin-

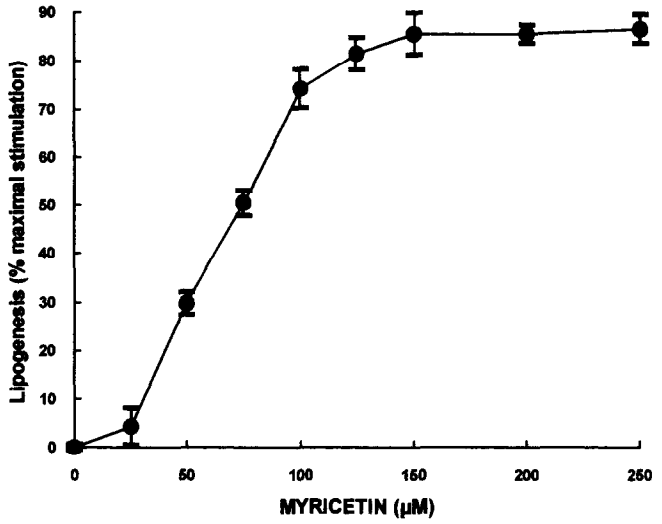

FIG. 3. Effect of myricetin concentration on basal lipogenesis in rat adipocytes. Adipocytes were preincubated with the indicated concentrations of myricetin for 15 min at 37°. Lipogenesis was assayed as described in Materials and Methods and measured with respect to the maximal stimulation by insulin (0.1 U/mL). Values for basal and insulin-stimulated incorporation of glucose into triglycerides were 66.3 ± 6.6 and 180.8 ± 11.5 nmol/100 μL packed cells, respectively. Values are means ± SEM of 3 experiments, each performed in triplicate.

stimulated lipogenesis or both (data not shown). An exception, however, was myricetin. Myricetin was found to mimic the action of insulin in stimulating lipogenesis in rat adipocytes (Fig. 2). It also enhanced the stimulation of lipogenesis by insulin substantially. The stimulation of lipogenesis was concentration dependent, increasing with the concentration of myricetin. The EC_{50} was estimated to be about 65 μM, while maximum stimulation was achieved at concentrations above 125 μM (Fig. 3).

The binding of insulin to its receptor stimulates the auto-

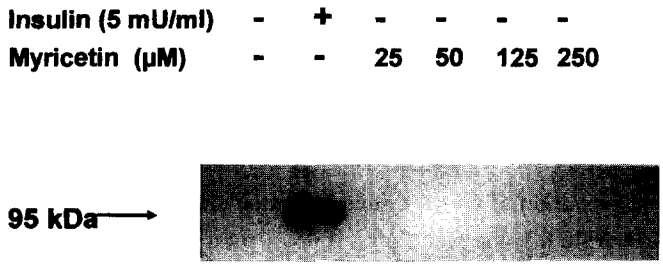

FIG. 4. Effect of myricetin on the autophosphorylation of the insulin receptor. WGA-purified insulin receptors (~50 μg total protein) were preincubated with various concentrations of myricetin at 25° for 50 min. Control samples were preincubated in the presence or absence of insulin (5 mU/mL) but in the absence of myricetin. Autophosphorylation was initiated by the addition of 25 μM [γ-^{32}P]ATP (5 μCi) and allowed to proceed for 10 min. The reaction was terminated by the addition of stop buffer containing protease, kinase, and phosphatase inhibitors. Phosphorylated insulin receptors were identified by autoradiography as described in Materials and Methods.

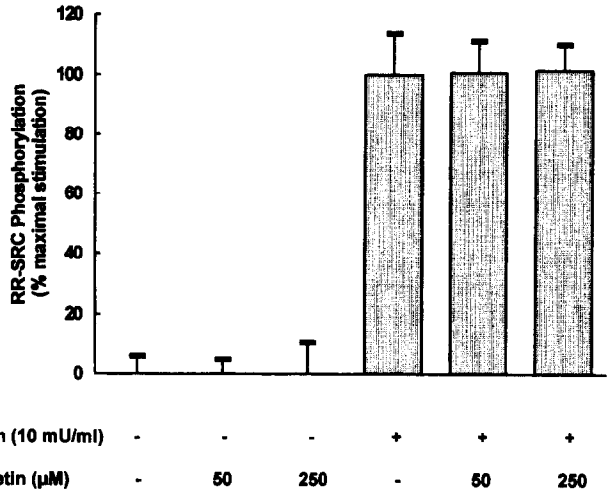

FIG. 5. Effect of myricetin on the phosphorylation of RR-SRC substrate by insulin receptor kinase. WGA-purified receptors (~1 μg total protein) were preincubated in 50 mM HEPES (pH 7.4), 0.1% Triton X-100, 2 mM $MnCl_2$, 10 mM $MgCl_2$, 100 μM ATP, various concentrations of myricetin, and in the presence or absence of insulin (10 mU/mL) for 30 min at 22°. Phosphorylation of RR-SRC peptide was assayed using the Protein Tyrosine Kinase Assay System and [γ-^{32}P]ATP (1 μCi) and expressed as percent maximal stimulation by insulin. Values for basal and insulin-stimulated phosphorylation were 3300 ± 360 and 9200 ± 820 cpm/μg protein, respectively. Values are means ± SEM of 3 experiments, each performed in duplicate.

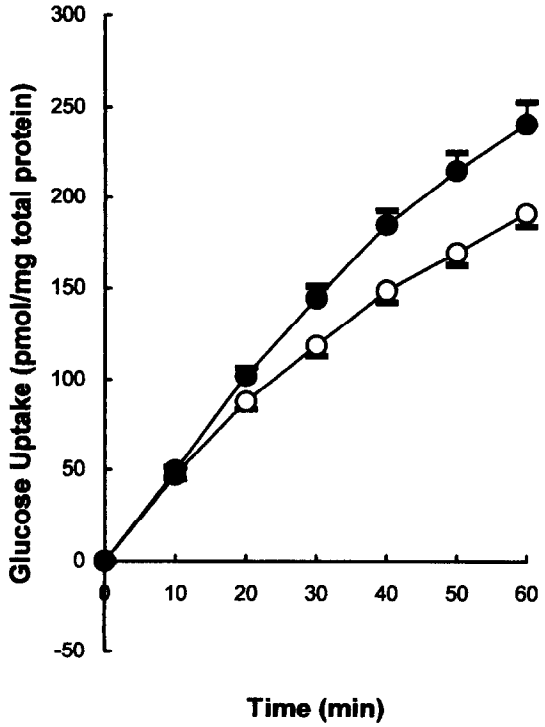

FIG. 6. Effect of myricetin on D-glucose transport in rat adipocytes. Rat adipocytes were preincubated in the presence (●) or absence (○) of 200 μM myricetin at 37° for 20 min. D-Glucose uptake was measured as described in Materials and Methods. Values are means ± SEM of 4 experiments, each performed in duplicate.

phosphorylation of the receptor at specific residues in its β-subunit [36, 37]. This, in turn, enhances the tyrosine kinase activity of the receptor [38–40], the consequence of which is the propagation of biological responses. The blocking of this signal pathway with inhibitors such as quercetin results in the inhibition of insulin-stimulated lipogenesis [14]. The possibility that the stimulatory effect of myricetin on lipogenesis could have been a consequence of its effect on insulin receptor function was investigated in this study. Incubation of partially purified insulin receptor with myricetin did not result in the autophosphorylation of the receptor for the range of concentrations studied (Fig. 4). Myricetin was also found not to affect the tyrosine kinase activity of the receptor in either the presence or the absence of insulin (Fig. 5). The action of myricetin was thus not via the activation or enhancement of insulin receptor functions.

The rate of glucose transport affects the rate of lipogenesis. Myricetin was found to stimulate both D-glucose (Fig. 6) and 3-O-methylglucose (data not shown) uptake. The kinetic parameters of glucose uptake were determined by measuring the rate of uptake at various concentrations of 3-O-methylglucose (Fig. 8). The V_{max} of the transport was found to have increased in the presence of myricetin, whereas the K_m did not differ significantly. Myricetin could have achieved this effect by stimulating the translocation of transporters from microsomal membranes to the plasma membrane or by changing the intrinsic property of the transporters or both.

Insulin enhances glucose uptake in rat adipocytes by stimulating the translocation of glucose transporters to the plasma membrane [41]. The V_{max} for glucose transport in the presence of insulin is increased greatly without any significant changes in the K_m [12], similar to the changes in kinetic parameters observed when rat adipocytes were incubated with myricetin. However, immunoblot analysis of Glut4 distribution in plasma membrane fractions did not show any increase in Glut4 population upon treatment with myricetin (Fig. 7). Thus, myricetin did not effectively stimulate the translocation of glucose transporters to the plasma membrane.

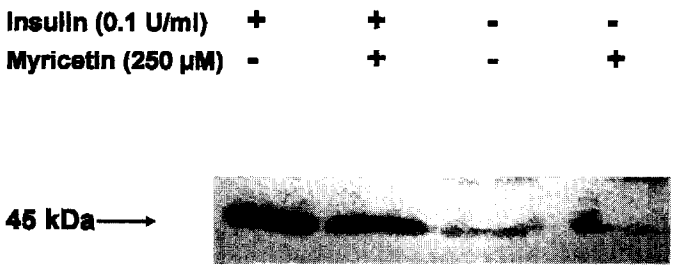

FIG. 7. Effect of myricetin on Glut4 translocation to the plasma membrane in rat adipocytes. Isolated rat adipocytes were incubated in the presence or absence of myricetin (250 μM) and in the presence or absence of insulin (0.1 U/mL). Plasma membrane fractions were then prepared from the adipocytes as described in Materials and Methods. The plasma membrane fractions (100 μg protein) were separated by 10% SDS–PAGE, transferred onto nitrocellulose membrane, and immunodetected using mouse anti-rat Glut4 antibody and the ECL Western Blotting Analysis System.

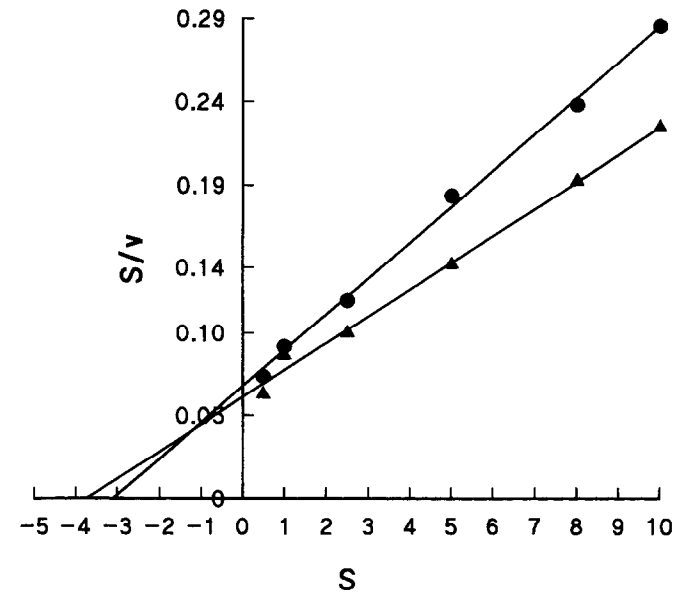

FIG. 8. Effect of myricetin on the kinetic parameters of 3-O-methylglucose transport in rat adipocytes. Rat adipocytes were preincubated with the indicated concentrations of methylglucose in the presence (▲) or absence (●) of myricetin (250 μM) for 20 min at 25°. 3-O-methylglucose transport was measured as described in Materials and Methods. The data were linearized and plotted according to the Hane's form of the flux equation: $S/v = K_mN_{max} + S/V_{max}$. Values are means of 5 experiments, each performed in triplicate.

Glucose transport is sensitive to the environment provided by the surrounding lipid bilayer. The lipid composition strongly governs the activity of glucose transporters [42] reflecting the importance of protein-lipid interaction in glucose transport. A fluid bilayer is also required for optimal activity [43]. The change in V_{max} was found to be the predominant influence in hexose transfer when there is a change in either the lipid composition of the bilayer or the fluid state of the membrane [42, 44]. In a recent study employing electron spin resonance techniques by Das and Morel,* myricetin was found to insert into the membrane. The insertion of myricetin into the membrane could affect transporter–lipid interactions or the fluidity of the bilayer or both, which, in turn, could affect glucose transport.

Our study indicates that myricetin mimics insulin in its ability to stimulate lipogenesis and glucose transport in rat adipocytes. However, it does not affect insulin receptor functions nor does it stimulate the translocation of glucose transporters to the plasma membrane. Instead, it exerts its effects by altering the intrinsic property of the glucose transporter, possibly by affecting the interaction between glucose transporters and the surrounding lipid bilayer or by changing the physical state of the surrounding lipid bilayer. Further work, however, has to be done to investigate the effect of myricetin on protein–lipid interactions and the fluidity of the membrane bilayer. Further studies are also necessary to investigate the *in vivo* effects of myricetin on the modulation of glucose levels in serum. As can be seen, myricetin may be of therapeutic potential particularly in the treatment of non-insulin-dependent diabetes mellitus since it does not require a functional insulin receptor to exert its effect, does not mediate its effects by the translocation of glucose transporters, and is able to enhance the stimulatory effects of insulin on glucose uptake.

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