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Full Paper

Activation of Insulin Receptors by Lagerstroemin

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Abstract. Lagerstroemin, an ellagitannin isolated from the leaves of *Lagerstroemia speciosa* (L.) Pers. (Lythraceae), was examined for its biological activities. In rat adipocytes, the compound increased the rate of glucose uptake and decreased the isoproterenol-induced glycerol release. In Chinese hamster ovary cells expressing human insulin receptors, it increased the Erk activity. These insulin-like actions were accompanied by the increased tyrosine-phosphorylation of the β-subunit of the insulin receptors. Tryptic digestion of the extracellular sites of the insulin receptors markedly increased the effective concentrations of insulin without changing those of lagerstroemin. Thus lagerstroemin was considered to cause its insulin-like actions by a mechanism different from that employed by insulin.

Keywords: insulin, lagerstroemin, lipolysis, glucose transport, mitogen-activated protein kinase

Introduction

The leaves of *Lagerstroemia speciosa* (L.) Pers. (Lythraceae), called "banaba" in the Philippines, are used as folk medicine for treatment of diabetes mellitus. Although critical evaluation for clinical use has not been reported, a recent study showed that the extract from the leaves decreases the blood glucose level of genetically diabetic (KK-AY) mice (1). As the basis of the hypoglycemic action, the banaba extract is shown to increase the glucose uptake of 3T3-L1 adipocytes (2). In efforts to identify the active ingredients, Murakami et al. isolated a triterpene, corosolic acid, from the methanol extract of the banaba leaves as an activator of glucose uptake of Ehrlich ascites tumor cells (3). The hypoglycemic effect of corosolic acid in experimental animals has also been reported (4). Another biologically active compound contained in the banaba leaves is lagerstroemin. The compound increases the glucose uptake of rat adipocytes, physiological target cells of insulin (5). However, it is not known whether the insulin-like action of lagerstroemin is elicited solely on the glucose transport system or broadly on the insulin-regulated cellular events. In the present study, we show that the compound possesses multiple insulin-like actions probably as a result of increased tyrosine-phosphorylation of the insulin receptors.

Materials and Methods

Materials

Lagerstroemin was isolated from the leaves of *Lagerstroemia speciosa* (Banaba) collected on a mountain in the Zambales, Philippines, as reported previously (5). Chinese hamster ovary cells (CHO cells) and the CHO cells overexpressing human insulin receptors (CHO-IR cells) were generously given to us by Dr. Yousuke Ebina (University of Tokushima). The monoclonal antibody against phosphotyrosine (PY99) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibody against the active form of Erk was from Cell Signaling Technology (Bevery, MA, USA). The ECL (enhanced chemiluminescence) detection system was purchased from Amersham Pharmacia Biotech Japan (Tokyo). Trypsin and HRP-conjugated goat anti-mouse IgG were from Wako Pure Chemical Industries (Osaka). Soybean trypsin inhibitor was purchased from Sigma-Aldrich Japan (Tokyo).

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Metabolic activities of rat adipocytes

The isolated adipocytes were prepared from the epididymal fat pads of male Wistar rats (100 – 150 g) by a collagenase digestion method. The uptake of [^{3}H]2-deoxy-D-glucose (2DG) and the release of glycerol were determined as reported previously (6, 7) as indices of the rate of glucose transport and the lipolytic activity of the cells, respectively.

Tyrosine phosphorylation

CHO cells and CHO-IR cells were maintained in Ham's F12 medium supplemented with 10% fetal calf serum. After being stimulated with insulin or lagerstroemin for 3 min, the cells were chilled by placing the culture dish on ice. The cells were washed twice with phosphate-buffered saline; incubated with 200 μl of an ice-cold solution consisting of 1% Nonidet P-40, 1 mM vanadate, and 50 mM Tris-HCl (pH 7.6); and then transferred into a centrifugation tube. After centrifugation of the tube at 13,000 rpm for 20 min, the supernatant was mixed with the SDS-PAGE sample buffer containing 50 mM dithiothreitol. The peptides were separated by SDS-PAGE (10.5% gel) and transferred to a PVDF membrane. After blocking in 3% fatty acid-free bovine serum albumin, the membrane was incubated with PY99, washed, and then incubated with HRP-conjugated goat anti-mouse IgG. The second antibody was located using the ECL detection system.

Results

In agreement with the result in a previous study (5), treatment of rat adipocytes with lagerstroemin increased the 2DG uptake of the cells in a dose-dependent manner (Fig. 1A). The uptake at 150 μM lagerstroemin was 68 ± 3.7% (n = 4) of that attained by 100 nM insulin. The increase was not observed in the presence of 20 μM cytochalasin B (data not shown), indicating that the uptake was via glucose transporter. The action of lagerstroemin was inhibited by wortmannin, a potent inhibitor of phosphoinositide (PI) 3-kinase, in a dose-dependent manner (Fig. 1B). Another inhibitor of PI 3-kinase, LY294002, also inhibited the action of lagerstroemin (data not shown). Treatment of the adipocytes with isoproterenol increased markedly the glycerol release of the cells (see Table 1). Insulin suppresses this increase in lipolysis by a PI 3-kinase-dependent mechanism (6). As shown in the Table 1, isoroterenol-

Table 1. The effect of lagerstroemin on the lipolytic activity of rat adipocytes

Treatment	Glycerol release (nmol for 30 min)
None	24.1 ± 2.1
Isoproterenol (Iso)	144.1 ± 5.6
Iso + Insulin	26.5 ± 1.9[a]
Iso + Insulin + Wortmannin	153.1 ± 8.1[b]
Iso + Lagerstroemin (Lag)	70.7 ± 4.0[a]
Iso + Lag + Wortmannin	156.3 ± 8.7[b]
Iso + Lag + LY294002	173.8 ± 18.5[b]

Rat adipocytes were incubated at 37°C for 10 min with or without 300 nM wortmannin or 300 μM LY294002. The cells were then incubated for 10 min with the addition of 150 μM lagerstroemin (Lag) or 100 nM insulin and then incubated for a further 30 min with 1 μM isoproterenol (Iso). The results are shown as the mean ± S.E.M. of triplicate determinations. [a] the effect of insulin or lagerstroemin is significant (P<0.01). [b] the effect of PI 3-kinase inhibitors is significant (P<0.01).

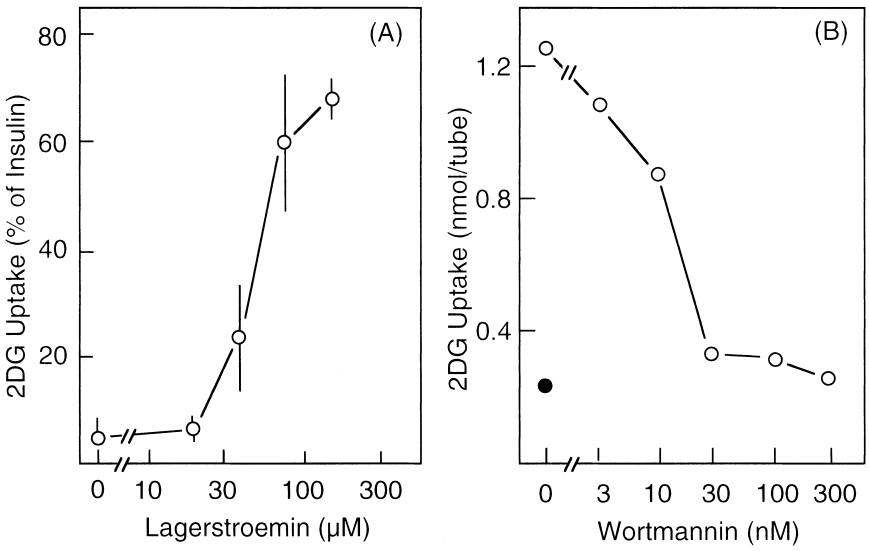


Fig. 1. The effect of lagerstroemin on the hexose uptake of rat adipocytes. A: Rat adipocytes were incubated at 37°C for 20 min with or without varied concentrations of lagerstroemin before the rate of [^{3}H]2DG uptake was determined. The results are shown as percent of the 2DG uptake in the cells treated without lagerstroemin but treated with 100 nM insulin. The vertical bars indicate S.E.M. from four separate experiments, each performed in duplicate. B: Rat adipocytes were treated with or without various concentrations of wortmannin for 10 min before stimulation with 150 μM lagerstroemin. The closed circle indicates the rate of 2DG uptake observed without lagerstroemin. Each point indicates the mean of duplicate determinations.

induced increase in glycerol release was suppressed by a prior treatment of the cells with lagerstroemin. The inhibitory action of lagerstroemin was not observed in the presence of wortmannin or LY294002. These results indicated that lagerstroemin produced the metabolic changes of rat adipocytes by a PI 3-kinase-dependent mechanism similar to that employed by insulin.

Another insulin-like action of lagerstroemin was observed in CHO-IR cells. Treatment of the cells with insulin or lagerstroemin increased the active form of cellular Erk as recognized by the specific antibody against the phosphorylated Erk (Fig. 2A). Stimulation of insulin receptors is known to cause phosphorylation of several proteins on tyrosine residues. Because this event is essential for insulin's actions on downstream signaling molecules (8), we next examined whether lagerstroemin induces tyrosine-phosphorylation of cellular proteins. Treatment of the CHO-IR cells with insulin caused tyrosine phosphorylation of several peptides including those of 160, 95, and 45 kDa (Fig. 2B). Lagerstroemin produced a similar set of tyrosine-phosphorylated proteins. In the parent CHO cells without overexpressing the insulin receptors, the phosphorylation of the 95-kDa peptide was hardly detectable, suggesting that the peptide is the β-subunits of the insulin receptors (IRβ) (Fig. 2C). Thus lagerstroemin was found to induce the tyrosine-phosphorylation of IRβ.

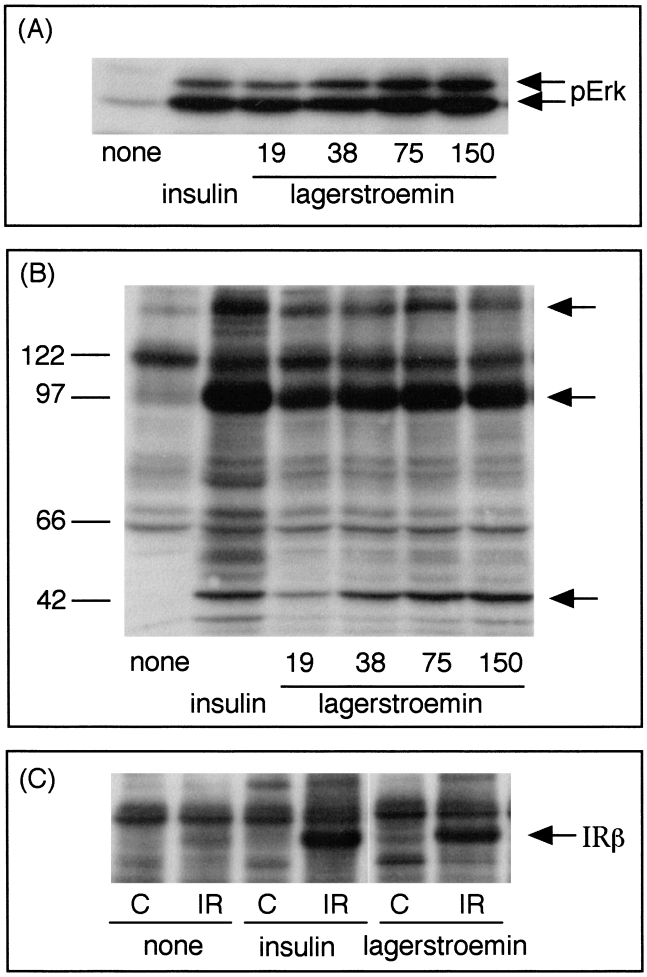


Fig. 2. Insulin-like actions of lagerstroemin in CHO-IR cells. A, B: CHO-IR cells were incubated with 100 nM insulin or the indicated concentrations of lagerstroemin. The lysate of the cells was analyzed by immunoblotting with the antibodies against the active Erk (A) and phosphotyrosine (B). C: CHO cells (C) or the CHO-IR cells (IR) were incubated with 100 nM insulin or 150 μM lagerstroemin. The lysate of the cells was analyzed by immunoblotting with the anti-phosphotyrosine.

Insulin causes its actions by binding to specific sites on the extracellular α-subunits of its receptors (IRα). The sites are sensitive to trypsin as shown by the fact that exposure of cells to the protease results in a dramatic decrease in the specific binding of insulin (9, 10). Because our findings suggested that lagerstroemin exerts its actions through activation of the insulin receptors, we next examined whether the trypsin treatment induces any change in the actions of lagerstroemin. In agreement with the previous reports (10, 11), trypsin increased the basal rate of glucose uptake but decreased markedly the insulin-stimulated glucose uptake (Fig. 3A). Trypsin did not affect the action of the higher concentrations of insulin, suggesting that the loss of insulin binding sites under the conditions was within the number of "spare receptors" (10). In contrast, vanadate-induced change in glucose uptake was unaffected by the trypsin treatment (Fig. 3B), indicating that trypsin did not injure the function of glucose transporters. Of interest, lagerstroemin-induced increase in glucose uptake was also unaffected by trypsin (Fig. 3C). Thus lagerstroemin was considered to cause its insulin-like actions by a mechanism different from that employed by insulin.

Discussion

We have reported that the plant product lagerstroemin is a potent activator of glucose uptake in rat adipocytes (5). In the present study, we observed that lagerstroemin possesses additional insulin-like actions, including effects on lipolysis and the Erk activity. The basis of these insulin-like actions is considered to be the activation of insulin receptor tyrosine kinase and/or the inhibition of tyrosine phosphatases, as discussed below.

Insulin causes its action through activation of the tyrosine kinase intrinsic to its receptors (8). The activation causes tyrosine-phosphorylation of several proteins, which in turn induces the functional change of proximate signaling molecules such as PI 3-kinase.

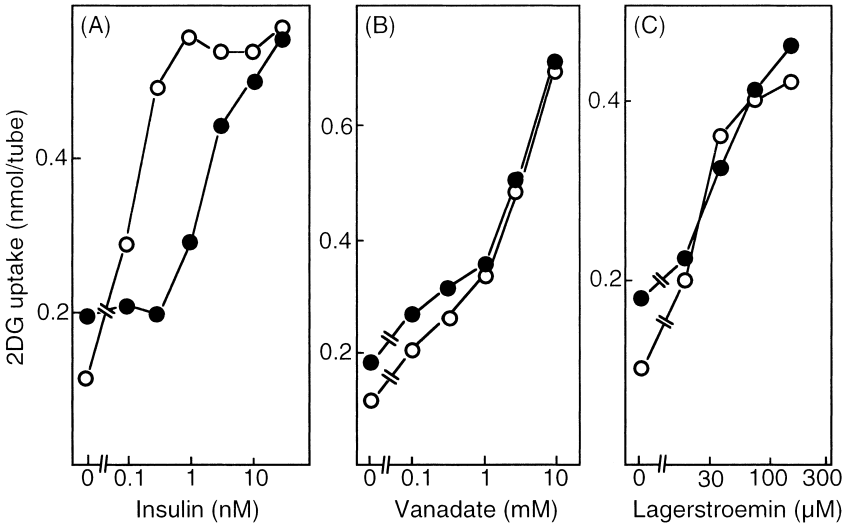


Fig. 3. The effect of trypsin-treatment on the action of lagerstroemin. Rat adipocytes were incubated with (closed circle) or without (open circle) 8.3 mg/ml of trypsin for 10 min. After addition of 0.1 mg/ml of soybean trypsin inhibitor, the cells were incubated with various concentrations of insulin (A), vanadate (B), or lagerstroemin (C) before measurement of their rate of 2DG uptake. Each point indicates the mean of duplicate determinations.

The inhibitors of the lipid kinase effectively prevent the insulin actions on glucose transport and lipolysis in rat adipocytes (6). In contrast, the effect of insulin on Erk activation in CHO-IR cells is unaffected or only partly inhibited by the inhibitors (12, 13). Because lagerstroemin shows insulin-like actions on the both types of cell response, the compound is considered to act at a point upstream of PI 3-kinase as a broad activator of the insulin signaling systems. Thus the action of lagerstroemin was expected to accompany the insulin-like effect on the tyrosine-phosphorylation of cell proteins. In fact, insulin and lagerstroemin produced the similar set of tyrosine-phosphorylated peptides (Fig. 2B).

It has been reported that vanadate increases glucose uptake of rat adipocytes by inhibiting the tyrosine phosphatase activities in the cells (11). The effect is accompanied with the tyrosine-phosphorylation of IRβ, is inhibited by wortmannin, and is unaffected by trypsin-treatment, as were the cases in the lagerstroemin action. Thus, one possible explanation for the lagerstroemin-induced tyrosine-phosphorylation is that the compound attenuates the tyrosine phosphatase activities in cells. In support of this hypothesis, we observed that lagerstroemin inhibits the ability of brain extract to hydrolyze *p*-nitrophenol phosphate with the half-maximal effect around 40 μM (K. Hattori et al., unpublished observation). However, it is not clear at present whether lagerstroemin, a polyphenol compound with molecular mass of 1,200, can accumulate in cells at such a higher concentration.

An alternative and a more probable explanation for the lagerstroemin actions is that the compound directly activates insulin-receptor tyrosine kinase. If lagerstroemin functions as an agonist of insulin receptors, it is not surprising that the compound possesses each of the insulin actions examined. In this case, however, the binding sites on the receptors must be different from those utilized by insulin, as indicated by the inability of trypsin to perturb the lagerstroemin actions (Fig. 3C). Lagerstroemin belongs to the ellagitannins, a class of plant polyphenols, which have been reported to bind several polypeptides such as the regulatory subunit of protein kinase A (14), although the precise nature of the interactions has not been understood. Thus, one possible explanation for the insulin-like actions of lagerstroemin is that the tannin binds the extracellular part of the insulin receptors in a manner causing the activation of IRβ. There must be further studies to explore this possibility.

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