Genistein Differentially Inhibits Postreceptor Effects of Insulin in Rat Adipocytes without Inhibiting the Insulin Receptor Kinase*

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Genistein, an isoflavone putative tyrosine kinase inhibitor, was used to investigate the coupling of insulin receptor tyrosine kinase activation to four metabolic effects of insulin: stimulation of glucose oxidation and substrate tyrosine phosphorylation in a concentration-dependent manner with an ID$_{50}$ of 25 μg/ml and complete inhibition at 100 μg/ml. Genistein also prevented insulin's (10$^{-6}$ M) stimulation of isoproterenol-stimulated lipolysis with an ID$_{50}$ of 15 μg/ml and a complete effect at 50 μg/ml. The effect of genistein (25 μg/ml) was not reversed by supraphysiological (10$^{-7}$ M) insulin levels. In contrast, genistein up to 100 μg/ml had no effect on insulin's (10$^{-6}$ M) stimulation of either pyruvate dehydrogenase or glycogen synthase activity. We determined whether genistein influenced insulin receptor β-subunit autophosphorylation or tyrosine kinase substrate phosphorylation either in vitro or in vivo by anti-phosphotyrosine immunoblotting. Genistein at 100 μg/ml did not inhibit insulin's (10$^{-7}$ M) stimulation of insulin receptor tyrosine autophosphorylation or tyrosine phosphorylation of the cellular substrates pp185 and pp60. Also, genistein did not prevent insulin-stimulated autophosphorylation of partially purified human insulin receptors from NIH 3T3/HIR 3.5 cells or the phosphorylation of histones by the activated receptor tyrosine kinase. In control experiments using either NIH 3T3 fibroblasts or partially purified membranes from these cells, genistein did not inhibit platelet-derived growth factor's stimulation of its receptor autophosphorylation. These findings indicate the following: (a) Genistein can inhibit certain responses to insulin without blocking insulin's stimulation of its receptor tyrosine autophosphorylation or the receptor kinase substrate tyrosine phosphorylation. (b) In adipocytes genistein must block the stimulation of glucose oxidation and the antilipolytic effects of insulin at site(s) downstream from the insulin receptor tyrosine kinase. (c) The inhibitory effects of genistein on hormonal signal transduction cannot necessarily be attributed to inhibition of tyrosine kinase activity, unless specifically demonstrated.

The predominant theory of insulin action proposes that the interaction of insulin with its receptor causes tyrosine autophosphorylation of the receptor β-subunit resulting in activation of the receptor tyrosine kinase (1–3). The tyrosine kinase then initiates the activation and inactivation of various kinases and phosphatases, leading to the pleiotropic effects of insulin. Evidence that the tyrosine kinase activity of the insulin receptor was required for insulin action came primarily from two types of studies (4–7). First, site-directed mutagenesis of the insulin receptor at either the ATP binding site (4, 5) or selected tyrosine residues (6) yielded mutant insulin receptors which failed to autophosphorylate and to mediate certain responses to insulin when expressed in Chinese hamster ovary cells or Rat1 fibroblasts. Second, insulin receptors with defective tyrosine kinase activity have been detected in insulin-resistant patients (7). Analysis of the biological function of such tyrosine kinase-defective receptors demonstrated that the insulin receptor tyrosine kinase domain must be intact for hormone-dependent signal transduction.

Although the data obtained from biochemical and mutational analysis of insulin receptors clearly indicate that mutations in the receptor kinase domain impair insulin receptor function, concluding that activation of the receptor tyrosine kinase activity alone triggers all responses to insulin may be an overinterpretation of the data. Such an interpretation does not consider the possibility that mutant receptors are nonfunctional because of conformational changes that prevent noncovalent coupling of the hormone receptor complex to effector proteins. That changes in receptor conformation may be important for receptor signal transduction is suggested by the observation that anti-insulin receptor antibodies can mimic insulin action without stimulating receptor autophosphorylation or tyrosine kinase activity (8–10) and that conformational changes of the insulin receptor β-subunit occur after insulin binding (11, 12). Additionally, two insulin receptors with defective tyrosine kinase activity have been isolated from insulin-resistant diabetics, but only one of these receptors fails to mediate certain insulin bioeffects when expressed in Chinese hamster ovary cells whereas the other kinase-defective receptor signals normally (13). Finally, Gottschalk (14) recently compared the effect of insulin on activation of pyruvate dehydrogenase in cell lines expressing normal human insulin receptors or those deficient in tyrosine kinase activity. He found that both normal and kinase-deficient receptors could mediate the activation of pyruvate dehydrogenase to similar levels. His findings show that the insulin signaling pathway that activates pyruvate dehydrogenase bypasses the insulin receptor tyrosine kinase.

Until recently, the role of insulin receptor tyrosine phosphorylation and substrate tyrosine phosphorylation in signal transduction was studied in cells transfected with cDNAs encoding wild-type or mutant receptors. By necessity, these studies utilized recipient cell lines that were insensitive to insulin and expressed low levels of endogenous receptors (1–

3946
3), two properties that distinguish these cell lines from bona-fide targets of insulin action such as muscle and adipose tissue. In addition, although the assumption was made that the recipient cell lines expressed the signal transduction machinery necessary to couple an occupied insulin receptor to a cellular response, it is now known that these cell lines do not express insulin-sensitive glucose transporter (GLUT4), which is a molecular marker of muscle and adipose tissue (15). Therefore, data obtained from the study of tyrosine kinase-deficient insulin receptors in transfected cells may not be directly applicable to physiological targets of insulin. The identification and synthesis of inhibitors of protein tyrosine kinases (16-18) allow a new biochemical approach to study the role of tyrosine phosphorylation in insulin action. This approach permits assessment of the potential anti-insulin effects of these inhibitors in physiologically meaningful target cells such as the rat adipocyte. We undertook such a study with the antibiotic genistein, which inhibits both EGF (18) and PDGF (19) receptor tyrosine kinase activity. We found that genistein inhibited insulin-stimulated glucose oxidation and blocked insulin’s inhibition of isoproterenol-stimulated lipolysis but had no effect on insulin’s stimulation of pyruvate dehydrogenase or glycogen synthase activity. However, genistein did not block insulin receptor or substrate tyrosine phosphorylation either in vivo or in vitro. These results indicate that beyond the insulin receptor there are multiple pathways for carrying out the pleiotropic effects of insulin and that in the adipocyte, genistein works downstream from the insulin receptor tyrosine kinase and not on the enzyme itself. These studies show that the inhibitory effects of genistein on hormonal signal transduction cannot necessarily be attributed to inhibition of tyrosine kinase activity, unless specifically demonstrated.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—Phenylmethanesulfonic acid, leupeptin, pepstatin, dithiothreitol, Triton X-100, Nonidet P-40, and prestained molecular weight standards were from Sigma. Collagenase was from Worthington. Bovine serum albumin was from U. S. Biochemical Corp. Porcine insulin was from Lilly. Genistein, I'-protein A, and Cysteine from ICN. 15-N labeled glucose and [**4C]pyruvate were from Amersham Corp. Male Sprague-Dawley rats were from ICN. Protein A beads (Trisacryl) were from Pierce Chemical Co. SDS and reagents for SDS-PAGE were from Bio-Rad. Nitrocellulose (BA85, 0.2 µm) was from Schleicher & Schuell. Anti-phosphotyrosine antibody, affinity purified from rabbit, was prepared as described previously by Rothenberg et al. (20). All other chemicals were of reagent grade or better and were purchased from standard vendors.

**Isolation of Adipocytes**—Male Sprague-Dawley rats (100-150 g) were fed ad libitum with Purina Laboratory Rodent Chow (5012). The epididymal fat pads were removed from rats killed by cervical dislocation and the cells isolated by the method of Rodbell (21) as was described in MezSO and refrigerated. All whole cell incubations to, the addition of other agents. Incubations were performed at 37 °C and the cells centrifuged for 1 h in a Beckman type 45 rotor (143,000 × g, 18 °C). The supernatant was acidified with 100% trichloroacetic acid to a final concentration of 10%. After 15 min on ice, the precipitate was recovered by centrifugation at 5,000 rpm in a Beckman JA-20 rotor for 5 min at 4 °C. Each pellet was washed three times with ethanol diethyl ether (1:1 v/v) at 4 °C. The precipitate was dissolved into a fine powder and used immediately for immunoprecipitation.

The method of Rothenberg et al. (20) was used for the immunoprecipitation of phosphotyrosyl proteins. Briefly, the dry protein precipitate was dissolved in 0.1 N NaOH, neutralized to pH 7.8 with 100 µM Tris-HCl, then made to 1 mm EDTA and 0.02% NaN3 and 1% MezSO and refrigerated. The cell suspension was added to solubilization buffer (2% SDS, 100 µM HEPES, pH 7.8, 32 °C, 100 mm NaCl, 10 mm EDTA, 50 mm dithiothreitol, and 2 mm Na3VO4 at 100 °C. The mixture was homogenized in a Brinkmann Polytron for 10 s at setting 10. The homogenate was heated at 100 °C for 3 min. It was cooled to 22 °C and then centrifuged for 1 h in a Beckman type 45 rotor (143,000 × g, 18 °C). The supernatant was acidified with 100% trichloroacetic acid to a final concentration of 10%. After 15 min on ice, the precipitate was recovered by centrifugation at 5,000 rpm in a Beckman JA-20 rotor for 5 min at 4 °C. Each pellet was washed three times with ethanol diethyl ether (1:1 v/v) at 4 °C. The precipitate was dissolved into a fine powder and used immediately for immunoprecipitation.

Soybean antigen- enriched fraction of insulin receptor was prepared by the method of Cuatrecasas et al. (21) as modified by Swol et al. (23) from murine 3T3-L1 adipocytes transformed with an expression vector containing human insulin receptor cDNA (NIH 3T3/HIR 3.5) (26). Receptor prepared from 3 X 106 cells was incubated for 10 min at 30 °C in 30 µl containing a final concentration of 33.3 mm HEPES, pH 7.4, 10 µM MgCl2, 3 mm MnCl2, 100 µM Na3VO4, 1% Me3SO, 50 µM N-acetyl-d-glucosamine, 25 mm NaCl, 0.017% Triton X-100, 0.017% BSA, and in the presence or absence of 25 µg of histone H2b, 10 µM insulin, in the presence or absence of 10-100 µg/ml genistein. At 22 °C, Na3ATP was added to a final concentration of 1 mM, and after 1 min the reaction was stopped by the addition of an equal volume of SDS-PAGE sample buffer (2 X) and heating to 100 °C for 2 min. SDS-PAGE and immunoblotting with anti-phosphotyrosine antibodies were carried out as described above.

**RESULTS**

**The Effect of Genistein on Insulin Action—Experiments** were designed to determine if genistein, a known tyrosine kinase inhibitor (18, 19), blocked insulin action in isolated adipocytes. Four acute responses to insulin were studied: stimulation of glucose oxidation, inhibition of isoproterenol-stimulated lipolysis, activation of pyruvate dehydrogenase, and activation of glycogen synthase.

The effect of genistein on the stimulation of glucose oxidation was the first response examined. As shown in Fig. 1, genistein caused a dose-dependent inhibition of insulin's abil-
production in isoproterenol-treated cells, and expressed as a percentage of glucose oxidation or inhibition of isoproterenol-stimulated lipolysis. The data were pooled from two experiments in the presence of genistein was determined at the end of a 30-min incubation.

The level that should saturate cell surface receptors did not overcome the inhibitory effect of genistein on insulin action. The data from two experiments that were performed in triplicate were pooled.

ability of adipocytes to respond to isoproterenol, which signals through a protein kinase A-dependent pathway.

We next tested the effect of genistein on the ability of insulin to stimulate the activity of pyruvate dehydrogenase and glycogen synthase. As shown in Fig. 4, the antibiotic at concentrations up to 100 µg/ml had no effect on insulin-dependent activation of pyruvate dehydrogenase. Concentrations of genistein as high as 250 µg/ml did not block the activation of pyruvate dehydrogenase by insulin in isolated adipocytes (data not shown). This result initially suggested that the activation of pyruvate dehydrogenase was independent of the insulin receptor tyrosine kinase activity. Since transfection studies had established that glycogen synthesis was dependent on insulin receptor tyrosine kinase activity (27), experiments were performed to determine if genistein would block the activation of glycogen synthase. Fig. 5 shows that genistein did not block insulin-dependent activation of glycogen synthase.

Although genistein did not inhibit insulin-dependent activation of either pyruvate dehydrogenase or glycogen synthase, incubation of cells with the drug did lower the basal levels of activity for each of these enzymes. The decreased basal activity had little effect on the magnitude of the insulin response for either enzyme. The observation that genistein did not inhibit either insulin-dependent activation of pyruvate dehydrogenase or glycogen synthase also indicated that genistein did not block insulin binding, eliminating this possible mech-
The presence and absence of 1.0 nM insulin. Genistein was added to the incubation, the cells were lysed, and the activity of glycogen synthase was determined as described under "Experimental Procedures." Total enzyme activity was determined in the presence of 10 mM glucose 6-phosphate. Active enzyme activity was determined in the presence of 0.1 mM glucose 6-phosphate. The effect of insulin was indicated by an increase in the ratio of active to total enzyme activity. The data from two experiments, which were performed in triplicate, were pooled. Each bar represents the mean ± S.D. of six samples.

The Effect of Genistein on Insulin Receptor Tyrosine Kinase—The experimental survey of genistein's effects on four metabolic responses to insulin revealed that genistein differentially inhibited two metabolic responses that are normally coordinately regulated in adipocytes. These results raised the possibility that the activation of pyruvate dehydrogenase and glycogen synthase was not mediated through the hormone-dependent tyrosine kinase activity of the receptor. Alternatively, these results might be explained if genistein acted downstream of the insulin receptor, along a pathway(s) necessary for activation of glucose oxidation and inhibition of isoproterenol-stimulated lipolysis but unnecessary for activation of pyruvate dehydrogenase and glycogen synthase. To resolve these two possibilities, experiments were performed to assess whether genistein inhibited the insulin receptor tyrosine protein kinase and to examine the effect of genistein on insulin-stimulated tyrosine phosphorylation of cellular proteins.

To investigate the effect of genistein on insulin-dependent protein tyrosine phosphorylation in isolated adipocytes, anti-phosphotyrosine antibody was used to immunoprecipitate the phosphotyrosyl proteins in adipocytes that had been incubated with or without insulin (10^{-7} M) in the presence or absence of 100 μg/ml genistein. Immunoprecipitated phosphotyrosyl proteins were separated by SDS-PAGE and detected by anti-phosphotyrosine immunoblotting. As shown in Fig. 6, insulin stimulated protein tyrosine phosphorylation in isolated adipocytes. Three distinct insulin-sensitive protein bands were detected, and these bands migrated with apparent molecular masses of 185, 95, and 60 kDa. The 95-kDa band is identified as the β-subunit of the insulin receptor (20). The 185- and 60-kDa proteins have been observed previously, and both are likely direct cellular substrates of the insulin receptor tyrosine kinase (20). The 120-kDa band constitutively contains phosphotyrosine and is unaffected by insulin in other tissues (20). The preincubation of adipocytes with genistein at concentrations that completely inhibited insulin-dependent activation of glucose oxidation and insulin-dependent inhibition of isoproterenol-stimulated lipolysis had no significant effect on insulin-stimulated receptor β-subunit (95-kDa) autophosphorylation. With both genistein and insulin present, the insulin receptor β-subunit band was 83.4% ± 9.2% (mean ± S.E., n = 8) as intense as this same band measured in the presence of insulin alone. Similarly, no consistent inhibitory effects of genistein were observed on the degree of tyrosine phosphorylation of pp185 or pp60. These results suggest that in the intact adipocyte, genistein has little, if any, effect on insulin activation of the insulin receptor tyrosine kinase or the tyrosine phosphorylation of the insulin receptor β-subunit and other endogenous substrates such as pp185 and pp60. As a positive control, parallel procedures were used to test the effect of genistein on PDGF receptor tyrosine kinase activity in NIH 3T3 mouse fibroblasts, a cell whose growth is regulated by PDGF (28). PDGF at 100 ng/ml markedly stimulated the tyrosine phosphorylation of the receptor. Genistein, in a dose-dependent manner, inhibited PDGF-stimulated tyrosine phosphorylation of the PDGF receptor with almost complete inhibition at 100 μg/ml genistein (data not shown). These findings are similar to those reported previously (19).

Genistein has been shown to inhibit the protein tyrosine kinase activity of the EGF receptor (18) and the PDGF receptor (19), both in vivo and in vitro. To determine whether genistein inhibited the hormone-dependent protein tyrosine kinase activity of the isolated insulin receptor, insulin receptors were extracted from NIH 3T3/HIR 3.5 mouse fibroblasts, a cell line stably transfected with a cDNA encoding the normal human insulin receptor. These partially purified receptors were used to study the effect of 100 μg/ml genistein on receptor protein tyrosine kinase activity in vitro. After a 1-min incubation with 1 mM ATP, the receptor preparation was separated by SDS-PAGE and immunoblotted with anti-phosphotyrosine antibody. As shown in Fig. 7, insulin (10^{-7} M) markedly stimulated tyrosine phosphorylation of the 95-kDa band, the β-subunit of the insulin receptor. Genistein had no effect on this tyrosine phosphorylation. In addition, insulin stimulated the tyrosine phosphorylation of added histones, but genistein at the same concentration had no effect on this exogenous substrate phosphorylation reaction (data not shown). As an additional positive control, a partially purified membrane preparation from NIH 3T3 cells was used to test the effect of genistein on PDGF receptor phosphorylation, using methods similar to those used for partially purified insulin receptors. In this in vitro system, PDGF markedly stimulated PDGF receptor phosphorylation, and this phos-
phosphorylation was partially inhibited by 10 μg/ml genistein and completely inhibited at 100 μg/ml (data not shown). These findings are similar to those reported previously (19).

**DISCUSSION**

The current hypothesis of insulin action proposes that interaction of insulin with its receptor leads to phosphorylation of the β-subunit of the receptor with subsequent activation of the receptor tyrosine kinase activity. Presumably, the receptor kinase then phosphorylates specific cellular substrates, leading to the regulation of various kinases and phosphatases that, in turn, mediate many of the pleiotropic effects of insulin (1–3). Most of the data supporting this hypothesis are derived from the study of mutated receptors expressed in cultured cell lines that are not normal targets for insulin action. As discussed in the Introduction, several lines of evidence raise questions as to whether all of the actions of insulin involve an active receptor tyrosine kinase. One recent study (14) clearly showed that in cells expressing kinase-deficient mutant human receptors, the insulin signaling pathway for activation of pyruvate dehydrogenase bypasses the insulin receptor tyrosine kinase. Another study (13) showed that a kinase-defective insulin receptor derived from an insulin-resistant diabetic patient responded normally to insulin and/or genistein at 30 °C. After 10 min Na3ATP was added to 1 mM. After 1 min the reaction was stopped by adding an equal volume of SDS-PAGE sample buffer (2 ×), boiled for 3 min, run on a 6% T gel, immunoblotted, and stained with anti-phosphotyrosine antibodies as described under “Experimental Procedures.”

The present study was designed to determine if in a physiological target cell, the rat adipocyte, all of the actions of insulin were dependent on an active insulin receptor tyrosine kinase. We approached this study using a putative tyrosine kinase inhibitor, genistein, and studied its effects on four well-represented insulin-stimulated processes in adipocytes: glucose oxidation and inhibition of isoproterenol-stimulated lipolysis. Recently, genistein has been shown to block biological effects of insulin receptor tyrosine kinase activity in vitro. The latter finding would suggest that genistein was working downstream from the receptor along a signal transduction pathway(s) that regulates glucose transport and antilipolyis but is not involved in insulin’s effects on pyruvate dehydrogenase and glycogen synthesis.

![Figure 7](https://example.com/figure7.png)

**FIG. 7.** The effect of genistein on insulin receptor (IR) autophosphorylation and substrate phosphorylation in vitro. Lanes are shown from an autoradiogram of a Western blot of samples immunostained with anti-phosphotyrosine antibody. C, no additions; G, 100 μg/ml genistein; G + I, 100 μg/ml genistein plus 10−4 M insulin; I, 10−4 M insulin. The receptor was incubated in a final concentration of 33.3 mM HEPES, pH 7.4, 10 mM MgCl2, 3 mM MnCl2, 100 μM Na3VO4, 1% Me2SO, 50 mM N-acetyl-D-glucosamine, 25 mM NaCl, 0.017% Triton X-100, and 0.017% BSA in the presence or absence of insulin and/or genistein at 30 °C. After 10 min Na3ATP was added to 1 mM. After 1 min the reaction was stopped by adding an equal volume of SDS-PAGE sample buffer (2 ×), boiled for 3 min, run on a 6% T gel, immunoblotted, and stained with anti-phosphotyrosine antibodies as described under “Experimental Procedures.”

Phosphorylation was partially inhibited by 10 μg/ml genistein and completely inhibited at 100 μg/ml (data not shown). These findings are similar to those reported previously (19).

Genistein most likely blocks insulin stimulation of glucose oxidation and inhibition of isoproterenol-stimulated lipolysis by inhibiting a process (or processes) downstream from the insulin receptor tyrosine kinase but required for both of these biological effects. Likely targets of genistein’s action are protein kinases, in which this antibiotic is known to compete with ATP binding at the catalytic site of these enzymes. Moreover, serine/threonine-specific protein kinases are strongly implicated in postreceptor insulin signaling mechanisms (3). Studies have shown recently that genistein can block S6 kinase activity (46) and topoisomerase I and II activity (47–49) by competing with ATP for binding. Thus, genistein as well as other putative tyrosine kinase inhibitors must be used carefully in trying to dissect the various kinases
or other pathways involved in the signal transduction pathway of insulin.

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