Glucose Regulation of Insulin Receptor Affinity in Primary Cultured Adipocytes*

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The mechanism by which insulin regulates cell surface insulin receptors in primary cultured adipocytes is unique from that in most other cell types. For example, in an earlier study (1) we found that a lag time of 4-6 h precedes insulin-induced loss of cell surface receptors and that insulin triggers receptor down-regulation such that receptor loss continues after removal of insulin. Thus, when cells were exposed to insulin for 12 h, washed, and then resuspended in insulin-free medium, a progressive loss of surface receptors was observed for up to 36 h. Basically, we interpreted both the triggering effect of insulin and the lag time preceding insulin-induced receptor loss as indicative that a signal is generated by the initial binding event that, in turn, mediates insulin receptor down-regulation such that receptor loss continues following the effect of insulin and the lag time preceding insulin-induced receptor down-regulation.

In more recent studies, we found that insulin-induced desensitization of the glucose transport system requires three components: glucose, insulin to facilitate glucose uptake in cells, and selective amino acids (2). In the absence of any one component, little or no desensitization was observed. Since all three of these components were present in the medium when we conducted our earlier studies on receptor down-regulation (1), we felt it necessary to examine whether glucose or amino acids are required for the expression of insulin-induced receptor down-regulation and whether either of these two components could influence 125I-insulin binding into isolated adipocytes.

**EXPERIMENTAL PROCEDURES**

Materials—Sources of materials were as follows. Porcine monocomponent insulin was from Dr. R. Chance, Lilly, Indianapolis, Indiana; bovine serum albumin (CRG-7), from Armour; silicone oil, from A. H. Thomas Co.; Dulbecco’s modified Eagle’s medium (DMEM) and DMEM specially formulated without insulin, from Gibco; 2-[1,2-3H]-deoxy-o-glucose, from Du Pont-New England Nuclear; 5-[[3H]lodoisyl]A14-insulin, from Amersham Corp.; and Hepes, phloretin, and all other chemicals, from Sigma.

Preparation of Sterile Isolated Adipocytes—Isolated adipocytes were obtained from the epididymal fat pads of male Sprague-Dawley rats (160-225 g) by collagenase digestion (4) as previously described (5, 6). After digestion, cells were washed 3 times in a sterile Hepes-buffered balanced salt solution (pH 7.4) containing 20 mM Hepes, 120 mM NaCl, 1.2 mM MgSO4, 2 mM CaCl2, 2.5 mM KCl, 1.0 mM NaH2PO4, 1% bovine serum albumin, and 1 mM sodium pyruvate (HBSS) plus 20 units/ml penicillin and 20 mg/ml streptomycin. Cells were then diluted to a final concentration of ~1 x 10^6 cells/ml (4 ml of HBSS/1 g of initial fat weight).

Primary Culture of Adipocytes and Washing Procedures—Freshly prepared adipocytes were added to HBSS (200 μl of cells to 2 ml of HBSS, final concentration of ~1 x 10^6 cells/ml in either 17 x 100-mm sterile polystyrene tubes (to assess insulin binding) or 50-mm tubes (to assess insulin binding affinity). Cells were incubated at 37°C for various times from 2 to 36 h in the absence (control) or presence of 20 mM glucose and 25 ng/ml insulin, alone or in combination. After culture, control and treated cells were washed 3 times at 37°C with glucose-free and insulin-free HBSS containing 0.4% bovine serum albumin (v/v). The cells were then incubated for 30 min at 37°C to allow bound insulin to dissociate. During the final wash, cells were concentrated to about ~1 x 10^6 cells/ml (4 ml of HBSS/1 g of initial fat weight).

1 The abbreviations used are: DMEM, Dulbecco’s modified Eagle’s medium containing 20 mM glucose, 25 mM Hepes, and 1% bovine serum albumin (pH 7.4); Hepes, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; HBSS, Hepes-buffered salt solution containing 25 mM Hepes and 1% bovine serum albumin (pH 7.4).

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0.5 × 10^6 cells/ml. This procedure, as previously shown (7), effectively removes all extracellular and surface-bound insulin. All experiments were derived from a common pool of freshly isolated cells, were subjected to an equal number of washes, and were concentrated to the same final volume with HBSS. Thus, any loss of cells due to washing was identical among control and treated cells.

Measurement of 125I-Insulin Binding to Intact Cells—Control and treated cells were cultured as described above, washed 4 times, and then incubated in a total volume of 200 μl of DMEM buffer (pH 7.8) with 0.2 ng/ml 125I-insulin in the absence or presence of excess unlabeled insulin (nonspecific binding tubes). Incubations were performed in 12 × 75-mm polystyrene tubes for 3 h at 16 °C in triplicate, unless otherwise noted. Specific 125I-insulin binding to intact adipocytes was performed at 16 °C because insulin internalization is negligible at this low temperature. Thus, cell-associated 125I-insulin reflects binding to cell surface receptors (8). The binding assay was terminated, and free 125I-insulin was separated from cell-bound radioactivity by removing 180-μl aliquots from the cell suspension and rapidly centrifuging the cells in plastic microcentrifuge tubes to which 60 μl of silicone oil had been added. The cell pellets were then removed, and the amount of cell-associated radioactivity was measured in a y counter. Specific 125I-insulin binding was determined by subtracting the counts in the nonspecific binding tubes from the amount of radioactivity in the total binding tubes.

An estimate of insulin receptor affinity was obtained by coincubating cells with 0.2 ng/ml 125I-insulin and a full range of unlabeled insulin concentrations (0-500 ng/ml). Complete insulin binding competition curves were constructed using a third-degree polynomial fit. Since high concentrations of unlabeled insulin reduced specific 125I-insulin binding to 0, the insulin concentration that resulted in a 50% reduction in specific binding was used as a measure of receptor affinity (insulin ED50).

Insulin Dissociation Studies—Cells were incubated in HBSS and incubated with 0.2 ng/ml 125I-insulin at 16 °C in a total volume of 3 ml at 2 °C. Specific 125I-insulin binding was determined by removing the amount of cell-associated radioactivity that was measured at the start of the dissociation experiment was negligible (∼4% of the total cell-associated radioactivity). The medium was then incubated at 37 °C to initiate dissociation, and aliquots of 180 μl were removed at various times. The amount of 125I-insulin nonspecifically associated with cells at the start of the dissociation experiment was negligible (∼4% of the total cell-associated radioactivity). Thus, when 125I-insulin was prebound to cells at 16 °C, more than 96% of the cell-associated counts could be displaced by an excess of unlabeled insulin.

Glucose Transport Assay—Basal and maximally insulin-stimulated rates of glucose transport were determined by preincubating adipocytes (0.2 ml in 12 × 75-mm polystyrene tubes) in the absence (basal uptake) or presence of 25 ng/ml insulin for 30 min at 37 °C (maximal insulin responsiveness). Initial rates of glucose uptake were measured by adding 20 μl of HBSS containing 0.12 μCi of 2-deoxy-D-[3H]glucose and 2-deoxyglucose (final substrate concentration, 0.1 mM). At the end of 3 min, the reaction was terminated by separating cells (180-μl aliquots) from buffer by centrifugation through silicone oil as previously described (9). To correct the 2-deoxyglucose uptake values for uptake of hexose by simple diffusion and for nonspecific trapping of radioactivity in the cell pellet, we assessed glucose uptake in the presence of 0.3 mM phloretin (10). Insulin sensitivity (ED50) of the glucose transport system was determined by preincubating adipocytes (200 μl) at 37 °C for 30 min with a full range of insulin concentration from 0 to 25 ng/ml. In each experiment, glucose uptake was derived from the mean of triplicate determinations.

RESULTS

To determine the individual effects of insulin and glucose on insulin binding capacity, we incubated adipocytes for various times from 2 to 30 h in the absence (HBSS) or presence of 20 mM glucose, 50 ng/ml insulin, or glucose plus insulin (Fig. 1). Results from these time course studies revealed that specific 125I-insulin binding continually increases over a 36-h period in glucose-treated cells, with little or no discernible lag period at early times (Fig. 1, inset). In contrast, a 0-h lag period preceded insulin-induced down-regulation of cell surface receptors, a finding in agreement with our earlier report (1). On the basis of these temporal differences, we conclude that the glucose-mediated increase in insulin binding occurs by a mechanism distinct from that mediating insulin-induced receptor down-regulation. We noted that, in cells cultured in the presence of maximally effective concentrations of both glucose and insulin, little change in binding capacity was observed. This finding is consistent with the idea that the extent of insulin binding may represent a balance between the actions of glucose and insulin.

The ability of other hexoses to enhance insulin binding was examined by treating cells with 20 mM of either L-glucose, D-3-O-methylglucose, D-mannose, D-fructose, D-galactose, or D-glucose. All groups were then washed after a 24-h incubation, and specific 125I-insulin binding was measured (data not shown). Of these sugars, only D-mannose and D-glucose were capable of inducing an increase in specific insulin binding, which is interesting since these are the primary sugars known to induce desensitization of the glucose transport system in conjunction with insulin and amino acids (11). The remaining hexoses were ineffective in enhancing insulin binding, indicating that osmolarity was not a causative factor in altering binding.

The extent to which 125I-insulin specifically binds to intact adipocytes is determined by both the number of cell surface receptors and their affinity for insulin. To determine which of these two factors was affected by glucose and insulin treatment, we analyzed insulin binding competition curves using cells incubated for 24 h under the conditions depicted in Fig. 2. Although a full range of unlabeled insulin concentrations (0-500 ng/ml) was used, only the 0-10 ng/ml region is plotted, since the ED50 values were in the low ng/ml range. Using the concentration of unlabeled insulin that decreases insulin binding by 50% (ED50) as a relative estimate of receptor affinity, we found that exposure to 20 mM glucose for 24 h increased receptor affinity by 63%, a finding which agrees with the observed increase in specific 125I-insulin binding capacity (average of 65%). Thus, the increased insulin binding capacity of cells incubated with glucose appears to be accounted for entirely by a corresponding increase in receptor affinity. Similar conclusions were drawn when 125I-insulin binding was measured at 37 °C, indicating that the glucose-induced increase in binding affinity is not an artifact of measuring insulin binding at low temperatures (16 °C).

In contrast to the glucose-induced increase in receptor affinity, insulin treatment of adipocytes down-regulated the
The half-maximally effective dose of glucose was 4.6 mg/ml, and insulin (25 ng/ml), or a combination of glucose and insulin (Ins). After 24 h, cells were washed and then incubated with 125I-insulin and various concentrations of unlabeled insulin (from 0 to 500 ng/ml) for either 3 h at 16 °C or 15 min at 37 °C (inset). Following incubation, cells were separated from buffer by centrifugation through silicone oil, and the specific 125I-insulin binding was determined. From each curve, the concentration of insulin that inhibited specific binding by 50% was calculated (insulin ED_{50}), and this value was used to estimate receptor affinity. Data points and ED_{50} values represent the mean ± S.E. of three experiments (two experiments at 37 °C (inset). The receptor affinity (ED_{50}) of treated groups was statistically compared with that of control cells (HBSS), using Student's t test. **, p < 0.01.

Glucose Regulation of Insulin Receptor Affinity

In an effort to confirm the idea that glucose enhances receptor affinity, we assessed the rate at which prebound 125I-insulin dissociates from cell surface insulin receptors. These results (data not shown) revealed that 125I-insulin dissociated from 24-h glucose-treated adipocytes in a significantly slower rate (p < 0.05) than from control cells incubated for 24 h under glucose-free conditions (t_{1/2} of 3.6 ± 0.24 min, n = 3, versus 5.2 ± 0.12 min, n = 3, respectively). Thus, it appears that the predominant effect of prolonged glucose treatment is to slow the rate of insulin dissociation, thereby increasing receptor affinity.

Fig. 3 depicts a dose-response study in which specific 125I-insulin binding was measured after adipocytes were incubated for 24 h in the presence of various concentrations of glucose or insulin. As can be seen in Fig. 3A, 20 mM glucose was maximally effective in enhancing insulin binding capacity and the half-maximally effective dose of glucose was 4.6 mM, a value well within the physiological range of glucose concentrations found in the circulation. On the other hand, insulin treatment (Fig. 3B) led to a substantial loss of binding capacity with an ED_{50} of 7.4 ng/ml. These findings suggest that the glucose-mediated increase in insulin binding is related to glucose metabolism, whereas down-regulation is associated with receptor occupancy.

To explore the idea that glucose induces the biosynthesis of a regulatory protein that modulates insulin receptor affinity, we blocked de novo protein synthesis with cycloheximide, incubated cells for 24 h with 20 mM glucose, and then measured specific 125I-insulin binding (Fig. 4). Cycloheximide completely prevented the glucose-mediated enhancement of insulin binding (ED_{50} = 560 ng/ml), and this effect was specific in that cycloheximide failed to alter insulin binding in either control (HBSS) or insulin-treated cells. Since inhibition of protein synthesis by cycloheximide failed to block insulin-induced receptor down-regulation but did effectively prevent the glucose-mediated increase, it appears that different mechanisms are operative.

On the basis of the data in Fig. 4, we postulated that glucose rapidly induces the synthesis of a protein(s) that enhances the affinity of cell surface insulin receptors. To obtain additional evidence to support this idea, we assessed whether cycloheximide could restore insulin receptor affinity to control values in glucose-treated cells. In control cells, cycloheximide had a minimal effect on receptor affinity (Fig. 5A), whereas, in glucose-treated cells, cycloheximide lowered receptor affinity to control values (Fig. 5B). Thus, we conclude that de novo protein synthesis is involved in the glucose-mediated enhancement of receptor affinity.

The predicted functional consequence of increased receptor affinity is a leftward shift in the insulin dose-response curve toward greater insulin sensitivity. Therefore, to determine whether the increase in 125I-insulin binding seen in glucose-treated cells (24 h) is accompanied by enhanced insulin sensitivity of the glucose transport system, we obtained complete insulin dose-response curves under the conditions depicted in Fig. 6. Clearly, glucose does indeed increase insulin sensitivity of the glucose transport system (by 63%), from an insulin ED_{50}
of 0.22 ng/ml in 24 h control cells to 0.14 ng/ml in cells treated with glucose for 24 h), and the enhanced sensitivity of the glucose transport system is proportional to the observed increase in insulin binding capacity (average of 65%). Thus, it appears that the glucose-mediated enhancement of insulin binding is functionally coupled to insulin action. Insulin treatment, either alone or in combination with glucose, also affected insulin sensitivity of the glucose transport system but in the opposite direction (a 75% reduction in insulin sensitivity).

**DISCUSSION**

The individual roles of insulin and glucose in regulating insulin binding capacity were assessed using primary cultured adipocytes. When cells were exposed to 20 mM glucose (in the absence of either insulin or amino acids), a progressive increase in specific $^{125}$I-insulin binding was observed that began almost immediately (no lag period) and culminated in a 60% increase by 24 h. This effect was dose-dependent (glucose ED$_{50}$ of 4.6 mM) and mediated by both D-glucose and D-mannose. Since other hexoses, such as L-glucose, 3-O-methyl-d-glucose, D-fructose, and D-galactose, were unable to alter binding capacity, it appears that osmolarity was not a causative factor and that glucose metabolism is required to elicit an increase in insulin binding.

To determine whether glucose enhances insulin binding capacity by altering the number of cell surface receptors or by increasing receptor affinity, complete insulin binding competition curves were constructed. The results clearly revealed that glucose increases receptor affinity to the same extent that it enhances specific $^{125}$I-insulin binding, a finding consistent with the idea that enhanced binding capacity is mediated predominantly by increased receptor affinity. Moreover, it appears that glucose enhances receptor affinity by slowing the rate of insulin dissociation, based on the finding that $^{125}$I-insulin dissociated from cell surface receptors with a $t_{1/2}$ of 3.6 ± 0.24 min in control cells versus a $t_{1/2}$ of 5.2 ± 0.12 min in glucose-treated cells. The glucose-induced increase in insulin receptor affinity was mediated by glucose alone. This in marked contrast to our recent studies on glucose-induced desensitization of the glucose transport system (3) in which we found that induction of insulin resistance requires three components: glucose, insulin to facilitate glucose uptake, and amino acids (particularly glutamine). Thus, it appears that glucose can influence the insulin action pathway through multiple effects at diverse cellular sites by acting either individually (to enhance receptor affinity) or in concert with other factors such as insulin and amino acids (to desensitize the insulin-responsive glucose transport system).

When the effects of insulin were examined, we found that insulin altered insulin binding capacity but in a manner that was temporally, directionally, and mechanistically distinct from glucose-mediated changes in insulin binding. Specifically, insulin treatment of cells for 24 h profoundly down-regulated the number of cell surface insulin receptors (>60% loss of binding capacity without change in insulin receptor affinity) in a dose-dependent manner (insulin ED$_{50}$ of 7.4 ng/ml). In general, these findings are consistent with those of previous studies demonstrating insulin-induced receptor down-regulation in a number of cell types (10, 12–22). Although the individual actions of glucose and insulin on specific insulin binding are relatively straightforward in the current study, complex interactions do occur when the two are present together. Insulin was found to completely block the glucose-induced increase in receptor affinity, while glucose prevented the insulin-induced down-regulation of cell surface receptors (Fig. 2). Thus, no net changes in binding capacity or receptor affinity were seen in cells cotreated with maximally effective concentrations of both glucose and insulin. The cellular mechanisms underlying these antagonistic effects remain to be elucidated.

A prominent finding from the current study was that cycloheximide selectively inhibited the glucose-induced increase in insulin binding capacity in a dose-dependent manner (ED$_{50}$ of 360 ng/ml) and restored receptor affinity to control values (Figs. 4 and 5). On the basis of these results, we hypothesize that glucose modulates the affinity of cell surface insulin receptors by inducing the biosynthesis of an affinity regulatory protein. Such a regulatory role for glucose is not unheard of. Glucose is known to exert both direct and indirect control over several biological processes. For example, in pancreatic tissue, glucose exerts both translational and transcriptional control over the biosynthesis of insulin (22–24), and glucose has been shown to accelerate the conversion of proinsulin to insulin by inducing a 2-fold increase in the synthesis of the converting enzyme, proconvertin B (25). Moreover, glucose has been implicated in the regulation of gene expression...
leading to the synthesis of a unique set of proteins collectively known as the glucose-regulated proteins (20, 27).

The concept of a protein that regulates insulin receptor affinity is not unique, and evidence for such a regulator has been obtained by others (28-31). For example, Harmon et al. (28-30), using liver membranes and the technique of radiation inactivation, have shown that the insulin receptor behaves as if it is composed of at least two functional components, a binding component (M, 100,000) and an affinity regulatory component (M, 300,000). The interaction between the affinity regulator and binding component results in a decrease in receptor affinity. Maturo and Hollenberg (31) have also demonstrated the existence of an affinity regulator in liver cell membranes, although their regulator appears to differ from that of Harmon et al. in that it functions to elevate binding affinity. More recently, Crettaz et al. (32) proposed that insulin induces the loss of different oligomeric forms of the insulin receptor, resulting in enhanced binding affinity. However, such a mechanism is not applicable to our adipocyte studies since the glucose-induced increase in binding affinity is not accompanied by receptor loss.

In addition to enhancing insulin binding, glucose treatment of adipocytes also increased the sensitivity of the insulin-responsive glucose transport system by 63%, a value proportional to the observed increase in insulin binding capacity (average of 65%). Thus, it appears that the action of glucose on insulin binding is functionally coupled to insulin action. These in vitro adipocyte results agree with the results of a number of in vivo studies. For example, in humans, glucose ingestion transiently increases circulating levels of glucose and insulin and is associated with a rise in insulin receptor affinity after 3–5 h, as well as a concomitant increase in insulin sensitivity of lipolysis (33). A similar enhancement of insulin receptor affinity has been observed in adipocytes obtained from rats after oral glucose administration (34).

A number of studies employing monocytes have reported similar findings (35–37), adding credence to the idea that glucose is involved in regulating insulin action by altering insulin receptor affinity. The one exception to this general finding was a study in which glucose administered intravenously to rats did not appear to enhance insulin receptor affinity in adipocytes, although a rapid increase in the insulin sensitivity of the glucose transport system was observed (38).

It is becoming increasingly evident that metabolic substrates such as glucose and amino acids play a key role in regulating the cellular actions of insulin. In a recent study, we reported that amino acids acutely modulate the ability of insulin to enhance protein synthesis in isolated adipocytes by acting at a site within the insulin action-protein synthesis cascade, and this effect was manifested by a 3-fold increase in insulin sensitivity and a marked enhancement of maximal insulin responsiveness (39). More recently, we found that amino acids are directly involved in modulating glucose-induced desensitization of the glucose transport system (3). Thus, when adipocytes were treated for 6 h in a defined buffer containing 25 mg/ml insulin and 2 mM glucose plus the 15 amino acids found in DME, we observed marked desensitization of the glucose transport system, manifested by a 60% decrease in maximal insulin responsiveness and a 2.5-fold reduction in insulin sensitivity. In contrast, little or no desensitization was seen under similar conditions in the absence of amino acids. These studies led us to conclude that desensitization of the glucose transport system occurs at a postreceptor site and requires three components, glucose, insulin, and selective amino acids. The current finding that glucose can selectively enhance insulin receptor affinity independent of amino acids extends to the receptor level the concept of substrate regulation of insulin action and adds another level of complexity to the carefully orchestrated sequence of events that modulate the pleiotropic actions of insulin.

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