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Differential Modification of Activities of the High-Affinity and Low-Affinity Insulin Receptors of 3T3-L1 Fibroblasts by Phosphonolipids *in Vivo*[1]

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The low-affinity and high-affinity forms of the insulin receptor respond differently to modifications of cellular phospholipid content in mouse 3T3-L1 fibroblasts *in vivo.* When cells are cultured with 2-aminoethylphosphonate the resulting phosphonolipid, which has previously been demonstrated to prevent the insulin-induced differentiation of the fibroblasts into adipocytes [J. D. Smith *et al., Biochem. Arch.* **8,** 339–344 (1992)] results in alterations in both the affinity for insulin and receptor number of the low-affinity receptor while leaving the high-affinity receptor unaffected. That this phospholipid modification induces a specific change in the cellular insulin effect is demonstrated by the lack of alteration in the mobilization of GLUT-4 and glucose transport in the lipid modified cells. The results suggest that this specific cellular phospholipid modification will be useful in dissecting the specific functions of the two forms of the mammalian insulin receptor.

The insulin receptor is one of a family of membrane-associated hormone receptors whose mode of action involves an autophosphorylation event upon hormone binding, followed by phosphorylation of secondary substrates and internalization of the hormone-receptor complex (1, 2). In many animals the primary effects of insulin are: to increase the uptake of glucose, to increase the rate of glycogen synthesis and fatty acid synthesis, and to inhibit gluconeogenic pathways in target tissues (3, 4). In addition, insulin is known to induce differentiation in embryonic tissues (5, 6). Among the most studied of such systems are the mouse fibroblast 3T3 cell lines, most of which, when exposed to insulin, differentiate into adipocytes (7).

The activity of the insulin receptor has been shown to be affected by its lipid environment (8-10). Both insulin binding and insulin receptor signalling have been found to be sensitive to the fatty acid composition of the membrane phospholipids, to the head-group composition of the phospholipids themselves and to the physical environment of the membrane itself (8-13).

We have previously used the protozoan *Tetrahymena thermophila* for studies on the roles of individual phospholipids in cell structure and function by culturing the organism with different aminoalcohols and aminophosphonic acids to observe changes in the phospholipid composition and cell physiology (14-19). In expanding these studies into animal cell systems, we were able to demonstrate that when 3T3-L1 fibroblasts were cultured in the presence of the naturally-occurring 2-aminoethylphosphonate, the presence of the phosphonolipids prevented the insulin-induced differentiation of the fibroblasts into adipocytes (20). The present

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study explores the effect of this phospholipid modification on insulin binding and action in order to characterize the nature of the phosphonolipid effect on insulin action.

MATERIALS AND METHODS

3T3-L1 fibroblasts, received from Dr. Howard Green, Harvard University, were cultured in Dulbecco's-modified Eagle's medium (DMEM) supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), nyastatin (200 units/ml) and either 10% heat-inactivated calf serum for the cell growth phase or 10% heat-inactivated fetal bovine serum for cellular differentiation (21, 22). When AEP was present, sufficient 0.25 M AEP filter-sterilized in phosphate-buffered saline (PBS) pH 7.4 was added to growth medium to give a final concentration of 10 mM AEP. AEP was maintained in the medium throughout the differentiation phase as well during as the growth phase of the experiment (20).

The DMEM and AEP were purchased from Sigma Chemical Co. (St. Louis, MO). All the supplements and PBS were purchased from Life Technologies/Gibco-BRL (Grand Island, NY) or Sigma.

Trypsinized 3T3-L1 fibroblasts ($0.5\text{-}1 \times 10^6$ cells) from stock cultures were inoculated into 25 ml of medium in 150 cm^2 flasks and grown for 6-7 days to confluence at 37°C under a 5%/95% CO_2/air atmosphere. The medium was changed every 48 hours with the cells rinsed with PBS before fresh medium was added to each of the flasks. For experiments studying the down regulation of the insulin receptor, confluent cells were cultured in differentiation media for an additional 24 hours with 1.73 μM insulin before harvesting. For harvesting, cells were rinsed with PBS and scraped in cold, fresh PBS and frozen at -20°C until used. For binding studies frozen material was pooled and resuspended with a glass homogenizer to give a uniform suspension. Glucose transport studies were carried out using freshly-harvested cells.

Insulin binding was measured by incubation of 250 μg of the resuspended cellular membrane with insulin concentrations varying from 62.5 pM to 2.0 nM; a 6.7 μM insulin concentration was used to measure non-specific background binding. The experiments were performed in a 96 well, polystyrene, microtiter plate (Corning Glass Works, Corning, NY). Each well also contained 0.125 μCi of [^{125}I]insulin (NEX-196, DuPont-New England Nuclear, Boston, MA). Incubations were performed at room temperature in a buffer of 50mM Tris-HCl, pH 7.8, 50 mM NaCl, 5mM KCl, 10 mM $CaCl_2$, 50 mM $MgCl_2$, 2 mM EDTA, 10 mM D-glucose, and 10 mg/ml BSA for one hour on a rocking platform. RIA grade BSA was purchased from Sigma. Triplicate determinations were performed for the control and for the AEP-grown membrane fractions with each concentration of insulin.

After one hour the cellular material was filtered on a Skatron filtration unit through glass-fiber filter paper. Each sample was washed with 4 ml of cold (4°C) wash buffer (pH 7.8, 50 mM Tris-HCl, 50 mM NaCl, 5mM KCl, 10 mM $CaCl_2$, 50 mM $MgCl_2$, 2 mM EDTA, and 10 mM D-glucose), and dry aspirated for 10 seconds between each wash. This process was repeated twice for a total of three washes of four milliliters and three 10-second dry aspirations. The filtered cellular material and filter paper were air dried and radioactivity determined with a Beckman Scintillation Counter (LS-5801). Specific insulin binding was calculated for each of the samples by subtracting the counts obtained from the background well from each of the experimental wells. Scatchard plots were created for both the control and AEP-containing membranes. For the down-regulation studies, the same procedure was performed for cellular material that had been exposed to insulin for 24 hours.

For glucose transport studies, a freshly-harvested cell suspension, was incubated at room temperature in buffer consisting of cell suspension buffer plus 10 mg/ml of RIA grade BSA in the microtiter plate for 1 hour. Each well had a different insulin concentration ranging from 2.0 nM to 31.2 pM along with one well without insulin serving as the measure of non-induced transport. After a one hour incubation with insulin, [5,6 ^{3}H] D-glucose (American Radiolabeled Chemicals Inc., St. Louis, MO) was added with cold D-glucose at a final concentration of 0.125 mM D-glucose with a specific activity of 1.6 μCi/mmole D-glucose. Cold glucose was added to the well without insulin to create a final glucose concentration of 0.183 M D-glucose to measure non-induced transport.

Cells were incubated with the glucose for 5 min and harvested using a Skatron Filtration apparatus, with a cold wash buffer at 4°C. Four milliliters of wash buffer were used to rinse the cells followed by a 10 second dry aspiration. The wash and aspiration were repeated for a total of two washes and two dry aspirations. Bound radioactivity on the dried filter disks was determined as before. Glucose uptake in control or AEP-containing cells from each insulin concentration were assayed in triplicate. Protein concentrations were determined using the Lowry procedure. Cellular integrity for the glucose transport studies was determined with the Trypan Blue exclusion procedure (23).

RESULTS

Insulin binding to plasma membranes prepared from control and AEP-grown 3T3-L1 cells was measured by binding of radiolabeled insulin. The Scatchard plot (Fig. 1) shows the biphasic curve typical of tissues that express both the high-affinity and low-affinity isoforms of the insulin receptor (24). Linear regression analysis was performed on the curves of this biphasic

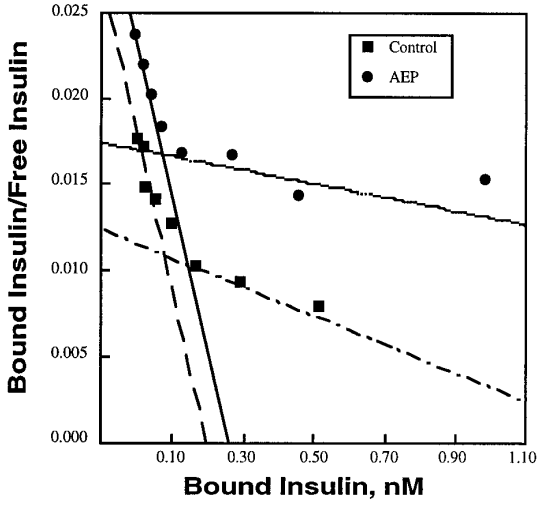

FIG. 1. Scatchard plot of insulin binding to membranes of control and AEP-containing 3T3-L1 fibroblasts with linear regression analysis. High-affinity form from control cells, (■); low-affinity form from control cells (■); high-affinity form from AEP-grown cells (●); and low-affinity form from AEP-grown cells (●).

plot as described by Winzor and Sawyer (25) and the asymptotes drawn through the terminal portions of each curve.

The insulin affinities (Fig. 2) and receptor numbers (Fig. 3) for each type of receptor were derived from the binding curves (Fig. 1) . There is a decreased affinity of the low-affinity receptor for insulin from 8.2×10^6 M^{-1} to 1.6×10^6 M^{-1} while the binding affinity of the high-affinity receptor is unaffected at 1.8×10^8 M^{-1}. There is a four-fold increase from 6.0 to 26 nmol/mgprotein in the number of low-affinity receptors while there is a slight, but not statistically-significant increase from 0.78 to 0.95 nmol/mg protein in the number of high-affinity receptors.

When confluent cells were cultured in the presence of insulin for an additional 24 hours to determine down-regulation of the insulin receptor levels, the receptor levels in control

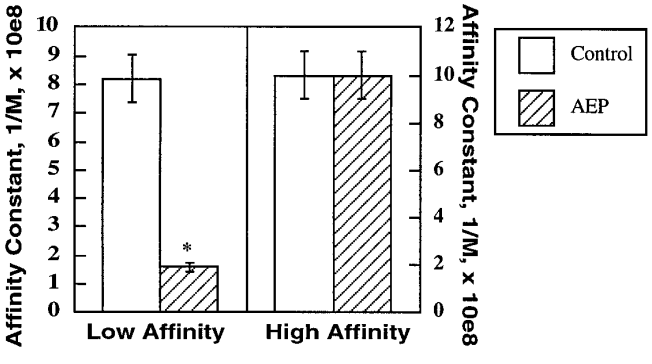

FIG. 2. Affinity constants for the A isoform and the B isoform of the insulin receptor in control and AEP-containing membranes of 3T3-L1 fibroblasts. The figure shows the differences in the affinity constants for the A isoform receptors and the B isoform receptors as well as the differences in binding constants for the control and AEP-containing membranes. *Difference from control based on Student's *t*-test ($p < 0.001$); means ± S.E. for triplicate determinations from 12 independent experiments.

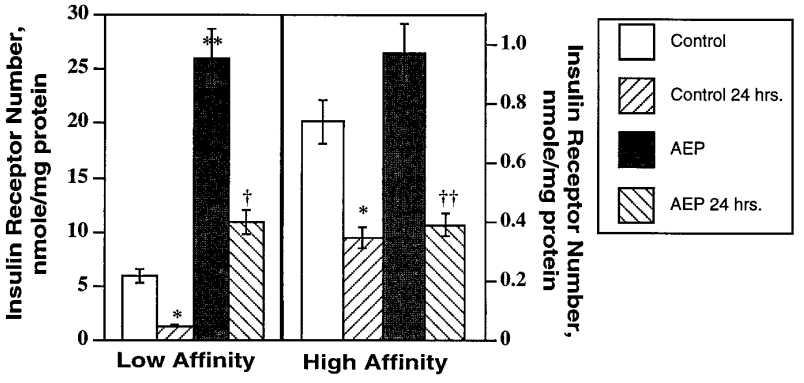

FIG. 3. Comparison of insulin receptor numbers in control and AEP-containing membranes. The graph shows the number of insulin receptors for both the high-affinity and low-affinity forms on insulin binding to control and AEP-grown membranes and after 24 hour exposure to insulin to determine down-regulation. *Significance compared to control ($p < 0.01$), **compared to control ($p < 0.001$), †compared to AEP and to control 24 hours ($p < 0.01$), ††compared to AEP ($p < 0.01$) based on Student's *t*-test; means ± S.E. for triplicate determinations from 12 independent experiments. The difference between the AEP-grown and control values for the high-affinity receptor is not statistically significant.

cells decreased by 70% and 50% (Fig. 3) for the low-affinity and high-affinity receptors, respectively, in agreement with literature values (26). The levels of the high-affinity receptor in the AEP-grown cells decrease to control levels when the cells are exposed to insulin. In contrast, while the levels of the low-affinity receptor in the AEP-grown cells decrease about 60% after down-regulation, the receptor levels are still six-fold higher than those of the control cells which have been down-regulated and remain twice the level of the low-affinity receptor in the control cells.

The glucose uptake studies indicate that there is no apparent change in the rate of activation of the GLUT-4 transporter (Fig. 4). In addition, the glucose uptake induced with increasing concentrations of insulin shows the dose-dependent increase in glucose uptake with increasing insulin concentration which reaches the maximum rate of about 25.0 pmole glucose per milli-

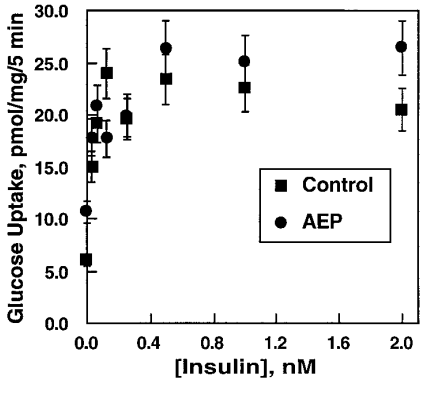

FIG. 4. Glucose uptake in control and AEP-grown cells. Confluent 3T3-L1 fibroblast cultures were incubated with increasing concentrations of insulin for one hour, the cells harvested and glucose uptake determined over a five-minute period using [^{3}H]glucose. Control cells (■); AEP-grown cells (●). Values are means ± s.d. for three determinations. Differences are not statistically significant by Student's *t*-test.

gram of protein per five minutes at an insulin concentration of about 0.5 nM. There is no apparent difference in the glucose transport between AEP-containing cells and control cells.

DISCUSSION

Most reports on the Insulin Receptor do not distinguish between its two forms: the high-affinity and low-affinity forms. Beside the reports on the relative affinities of the different insulin receptor forms and several observations on the relative tissue distributions of the two forms (27), there have been no reports on clear functional differences between them.

The results presented here clearly indicate that the high-affinity and low-affinity forms of the insulin receptor are affected differently in vivo by their phospholipid environments. The low-affinity receptor shows both a four-fold decrease in affinity for insulin (Fig. 2) as well as a four-fold increase in number (Fig. 3) in response to the presence of phosphonolipids in 3T3-L1 fibroblasts. In contrast, the high-affinity receptor is unaffected. Both receptor forms down-regulate in response to prolonged exposure to insulin (Fig. 3). However even after down-regulation, the levels of the low-affinity receptor are still considerably higher than they are in the unexposed cells.

This phospholipid modification has previously been demonstrated to prevent the insulin-induced differentiation of 3T3-L1 fibroblasts into adipocytes (20). That this lipid modification does not interfere with all insulin effects is demonstrated by the observation that GLUT-4 mobilization in response to insulin and subsequent glucose transport are unaffected (Fig. 4). It is probably premature to speculate that the high-affinity insulin receptor is involved in the activation of glucose transport while the low-affinity receptor in involved in potentiating the mitogenic affects of insulin, but the results reported here provide the basis for further experimentation in differentiating physiological roles for the insulin receptor.

ACKNOWLEDGMENTS

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