Deviated autophosphorylation of EGF receptor in insulin-deficient diabetic rats

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Okamoto, Motozumi, C. Ronald Kahn, Ruth Maron, and Morris F. White. Decreased autophosphorylation of EGF receptor in insulin-deficient diabetic rats. Am. J. Physiol. 254 (Endocrinol. Metab. 17): E429-E434, 1988.—We have previously reported that despite an increase in receptor concentration, there is a decrease in autophosphorylation and tyrosine kinase activity of the insulin receptor in insulin-deficient diabetic rats. To determine if other tyrosine kinases might be altered, we have studied the epidermal growth factor (EGF) receptor kinase in wheat germ agglutinin purified, Triton X-100-solubilized liver membranes from streptozotocin (STZ)-induced diabetic rats and the insulin-deficient BB rat. We find that autophosphorylation of EGF receptor is decreased in proportion to the severity of the diabetic state in STZ rats with a maximal decrease of 67% (P < 0.01). A similar decrease in autophosphorylation was observed in diabetic BB rats that was partially normalized by insulin treatment. Separation of tryptic phosphopeptides by reverse-phase high-performance liquid chromatography revealed a decrease in labeling at all sites of autophosphorylation. A parallel decrease in EGF receptor phosphorylation was also found by immunoblotting with an antiphosphotyrosine antibody. EGF receptor concentration, determined by Scatchard analysis of 125I-labeled EGF binding, was decreased by 39% in the STZ rat (P < 0.05) and 27% in the diabetic BB rat (not significant). Thus autophosphorylation of EGF receptor, like that of the insulin receptor, is decreased in insulin-deficient rat liver. In the case of EGF receptor, this is due in part to a decrease in receptor number and in part to a decrease in the specific activity of the kinase. Because tyrosine kinases are involved in the regulation of cellular growth and metabolism, alterations in the EGF and insulin receptor kinases may reflect a generalized change in this important class of proteins in diabetic animals.

Tyrosine protein kinase activity is relatively rare in cells and appears to be a property of proteins that are important in the regulation of cellular growth or metabolism (19, 28). Thus far the only known proteins with tyrosine kinase activity are receptors for insulin and other growth factors, including epidermal growth factor (EGF), platelet-derived growth factor (PDGF), insulin-like growth factor I (IGF I), colony stimulating factor I (CSF I), and the gene products of several of the transforming RNA viruses (for review, see Refs. 9, 28). This led us to further examine the characteristics of the 170-kDa protein, which is remarkably decreased in phosphorylation in STZ-induced and BB diabetic rats. The data of the present study indicate that this protein is the EGF receptor. Thus there are alterations in at least two different tyrosine kinases in insulin-deficient diabetic animals.

MATERIALS AND METHODS

Materials. 125I-labeled EGF was purchased from Collaborative Research (Lexington, MA), porcine insulin was from Elanco (Indianapolis, IN), EGF was from Bethesda Research Laboratories (Gaithersburg, MD), and other reagents for preparation of receptors and phosphorylation were as previously described (18). EGF receptor antibody was a generous gift of Dr. J. Schlessinger.

Animals. Mild and severe STZ induced diabetic rats were produced by injecting male Sprague-Dawley rats weighing ~200 g with 50 (mild) or 60 (severe) mg/kg of STZ (18). The rats were used for experiments 5 wk after injection. BB rats were kindly provided by Dr. Richard Jackson from a breeding colony at the Joslin Diabetes Center. Diabetes was diagnosed on the basis of glucosuria using TES-TAPE (Eli Lilly, Indianapolis, IN) and a plasma glucose in excess of 250 mg/dl. Diabetic BB rats were divided into two groups. One group was untreated and the other was insulin treated (3 wk, NPH insulin). All animals were fed ad libitum until the experiment.

The metabolic characteristics of each group have been previously summarized (18). The STZ rats showed a decrease in body weight gain and liver weight in proportion to the severity of the diabetic state. Blood glucose levels were markedly increased, whereas plasma insulin concentrations in both portal vein and inferior vena cava were decreased. Similar changes are observed in body and liver weight and blood glucose when the diabetic BB rat was compared with nondiabetic BB rats.
Phosphorylation with solubilized receptors. Solubilized insulin receptors and EGF receptors were prepared with 1% Triton X-100 as previously described and partially purified by chromatography on WGA agarose (18). Phosphorylation was studied with a modification of the method of Kasuga et al. (10). Aliquots from WGA eluate (3 μg protein) were added to a reaction mixture having a final concentration of 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (pH 7.4), 5 mM MnCl₂, and 0.1% Triton X-100 with or without insulin (1 μg/ml) or with or without EGF (2 μg/ml) in a total volume of 50 μl. These mixtures were incubated at 22°C for 30 min unless otherwise indicated. The phosphorylation reaction was initiated by adding 20 μCi [γ³²P]ATP and cold ATP to give a final concentration of 20 μM and terminated by adding fivefold concentrated Laemmli sample buffer. The mixture was heated immediately in boiling water for 3 min. The proteins were reduced with 100 mM dithiothreitol (DTT) and separated in 7.5% polyacrylamide gel by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (12). The gels were stained with Coomassie blue in 50% trichloroacetic acid, destained in 7% acetic acid, and dried, and the phosphoproteins were identified by autoradiography using Kodak X-Omat film. Molecular masses of proteins were calculated by using standard proteins (Bio-Rad, Cambridge, MA). The incorporation of ³²P into corresponding bands was quantitated by scanning densitometry of the film or by counting the corresponding bands of the gel in a scintillation counter.

Tryptic peptide mapping and phosphoamino acid analysis. Tryptic phosphopeptides were obtained from the corresponding bands in polyacrylamide gel fragments as previously described (10, 27, 29) and were separated with Waters high-performance liquid chromatography (HPLC) system equipped with a Bondapack C₁₈ reversed-phase column (Waters Chromatography, Milford, MA) (30). The phosphoamino acids were identified by a modification of the method of Hunter and Sefton, as described by Takayama et al. (24).

E₂₀⁻EGF binding. EGF binding to a microsomal fraction of rat liver was performed by the method of O'Keefe et al. (19) with minor modifications. Aliquots (150 μg protein in 200 μl) of the microsomal fraction were incubated with E₂₀⁻EGF (1.0 ng/ml; specific activity, 100 μCi/μg) and various concentrations of unlabeled EGF for 15°C for 4 h in a medium of 150 mM NaCl and 50 mM HEPES, pH 7.4, containing 0.1% bovine serum albumin. Bound and free E₂₀⁻EGF were then separated by centrifugation in a microfuge, and the pellet was counted.

RESULTS

Phosphorylation of EGF and insulin receptors. When the eluates from WGA affinity column from the livers of normal and diabetic rats were incubated with [γ³²P]ATP in the presence of 5 mM MnCl₂ but in the absence of hormones and analyzed by SDS-PAGE and autoradiography, three ³²P-labeled proteins were identified as 95-, 150-, and 170-kDa proteins (Fig. 1, lanes a, b). As previously reported (18), insulin stimulated ³²P incorporation into the 95-kDa protein that corresponds to the β-subunit of the insulin receptor but not into the 170-kDa protein. The 170-kDa protein has a mobility on SDS-PAGE similar to that previously described for the EGF receptor in liver (4, 6). Phosphorylation of the 170-kDa protein in these extracts was slightly stimulated with EGF (2 μg/ml) and DTT (1 mM), markedly stimulated by both combined, but not affected by insulin (Figs. 1 and 2), suggesting that the 170-kDa protein was the EGF receptor. As previously noted, ³²P incorporation into 170-kDa protein in STZ-induced diabetic rats was decreased ~60% (Fig. 1, cf. lanes c and d with a and b).

To confirm the nature of the 170-kDa protein, the WGA extract was phosphorylated with [γ³²P]ATP with
or without insulin (1 μg/ml), and the phosphoproteins were sequentially precipitated with anti-EGF receptor antibody (11) and anti-insulin receptor antibody (B-9) (10) and analyzed by SDS-PAGE. The 170-kDa protein was precipitated with anti-EGF receptor antibody confirming its identity as the EGF receptor (Fig. 3, lanes a, b). The 95-kDa protein, on the other hand, was stimulated by insulin and was precipitated with anti-insulin receptor antibody consistent with its identity as the β-subunit of the insulin receptor (Fig. 3, lanes c, d).

Effect of diabetes on autophosphorylation of EGF receptor. Phosphorylation of the EGF receptor was studied with WGA extracts from livers of control, mild STZ-diabetic (50 mg/kg), and severe STZ-diabetic (60 mg/kg) rats, as well as in diabetic and insulin-treated BB rats. Autoradiograms of the SDS gels were then subjected to densitometry scanning to quantitate the phosphorylation of the EGF receptor. In the STZ rat, there was a decrease in 32P incorporation into the 170-kDa protein in proportion to the severity of diabetes with a maximal decrease of 67% (P < 0.01) (Fig. 4, left; Table 1). A similar decrease in 32P incorporation into the EGF receptor was observed in untreated diabetic BB rats when compared with the control (P < 0.01) (Fig. 4, right; Table 1). A parallel decrease in EGF receptor phosphorylation was also found by immunoblotting, using an antiphosphotyrosine antibody (data not shown) (15). The decrease in EGF receptor phosphorylation in the BB rat was at least partially normalized by insulin treatment (Fig. 4).

Tryptic peptide mapping and phosphoamino acid analysis. To examine the mechanism underlying the decrease in autophosphorylation of the EGF receptor, tryptic peptide mapping and phosphoamino acid analysis of the 170-kDa protein were performed. Reverse-phase HPLC analysis of the tryptic peptides derived from the 170-kDa band revealed five major peaks, all of which contained exclusively phosphotyrosine. All phosphopeptides were decreased in the receptor from the STZ diabetic rats, suggesting that the decrease in phosphorylation of the 170-kDa protein in diabetic state affects all phosphorylation sites evenly (data not shown). Similar patterns were observed in control (Sprague-Dawley) rats and diabetic BB Wistar rats, suggesting that EGF-receptors of liver from Sprague-Dawley and BB Wistar rats are similar. There was no similarity in the pattern of tryptic peptide mapping and phosphoamino acid analysis between 170-kDa protein EGF receptor and 95-kDa protein β-subunit of the insulin receptor (18).

EGF binding. To determine if the decrease in autophosphorylation was due to a decrease in EGF receptor content, EGF binding was studied. The affinity of the EGF receptor in solubilized preparations of receptors is only ~10% of that in the membrane or microsomal fraction (4). This change in affinity does not significantly affect phosphorylation studies because they are performed with an excess of EGF, but the decrease in affinity will affect EGF tracer binding studies, making it difficult to do detailed competition curves. Therefore, EGF binding was assessed with microsomal fractions from the same liver used for the phosphorylation studies rather than the solubilized receptor.

In the STZ rat, specific EGF binding (150 μg protein of microsomal fraction at a tracer concentration of 1 μg/ml EGF was 5.5 ± 0.7% for control and 3.4 ± 0.8% for severe diabetic rats, respectively. Nondiabetic and diabetic BB rats revealed a similar difference (5.0 ± 0.6 vs. 3.6 ± 0.4%). Scatchard analysis (Fig. 5) revealed that the decrease in EGF binding to the microsomal fraction of the liver was mainly due to a decrease in receptor concentration. In the severe groups of STZ rats, a 39% decrease in EGF receptor concentration was found (P < 0.05), whereas in the diabetic BB rat there was only a 27% decrease in receptor concentration as compared with the control (not statistically significant) (Table 1).

A direct comparison of the decrease in receptor concentration and the decrease in autophosphorylation is presented in Fig. 6. In both the severe and mild STZ rats and the insulin-treated and untreated BB rats, the decrease in receptor concentration could account for only about one-quarter to one-half of the decrease observed in receptor autophosphorylation. Thus in all four types of insulin-deficient diabetes there was a modest decrease in EGF receptor concentration and a more marked decrease in EGF receptor autophosphorylation. This was confirmed by statistical analysis (Table 1).

DISCUSSION

EGF binds to specific receptors on a variety of cells and produces a number of different biological responses in intact animals, in organ cultures, and in cell culture systems (1–6, 16, 17, 19–22). The EGF receptor is a 170-kDa tyrosine-specific protein kinase with amino acid sequence homology to the catalytic domains of other protein kinases (8, 25). Most studies of the EGF receptor, however, have relied on A431 cells, a human epidermoid carcinoma in tissue culture, and thus few have defined
any disease state in which EGF receptor or kinase activity is increased or decreased (2).

In this study, we have demonstrated a decrease in autophosphorylation of EGF receptor on hepatocytes from insulin-deficient diabetic rats. The 170-kDa phosphorylated protein identified in this study was shown to be the EGF receptor based on molecular mass, stimulation of $^{32}$P incorporation by EGF and DTT (1), immu-

**TABLE 1. EGF receptor function in diabetic rats**

<table>
<thead>
<tr>
<th>Receptor Binding</th>
<th>Affinity, $\times 10^9$ M</th>
<th>Receptor concn, $\times 10^{-10}$ M</th>
<th>Relative receptor concn, % control</th>
<th>Autophosphorylation, % control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptozotocin diabetic rats</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.6±0.2</td>
<td>2.3±0.3</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Mild</td>
<td>2.6±0.2</td>
<td>1.7±0.2</td>
<td>73.9±8.7</td>
<td>44.8±6.4*</td>
</tr>
<tr>
<td>Severe</td>
<td>2.5±0.1</td>
<td>1.4±0.1†</td>
<td>61.0±3.3†</td>
<td>33.4±4.8*</td>
</tr>
<tr>
<td>BB diabetic rats (n = 3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.9±0.1</td>
<td>1.6±0.2</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Insulin-treated</td>
<td>2.9±0.1</td>
<td>1.3±0.2</td>
<td>88.9±11</td>
<td>58.4±10.4†</td>
</tr>
<tr>
<td>Untreated</td>
<td>2.9±0.1</td>
<td>1.3±0.2</td>
<td>72.9±9.4</td>
<td>36.8±6.2*</td>
</tr>
</tbody>
</table>

Values are means ± SE of 3-6 separate studies. Difference from control: * $P < 0.01$; † $P < 0.05$. All other differences are not statistically significant.
FIG. 6. Comparison of decreased EGF binding and decreased receptor autophosphorylation in diabetic rats. Data on binding and phosphorylation in Table 1 have been converted to percentage decrease from control and plotted. Diagonal line represents line of identity. Values below the line indicate a greater decrease in phosphorylation than in binding. • Severe STZ; ○, mild STZ; □, untreated BB rats; and △, treated BB rats. Bars represent ±SR.

noprecipitation with anti-EGF receptor antibody, and high content of phosphotyrosine (11).

Our data show that autophosphorylation of EGF receptor from rat liver is decreased in insulin-deficient diabetic states. The fact that the decrease is observed in both the STZ rat and the BB rat negates the possibility that these results are due to the toxic effect of STZ. The decrease is observed in proportion to the diabetic state, and the decrease is partially normalized by insulin treatment. The tryptic peptide map of the EGF receptor is similar in the Sprague-Dawley rat and BB rat (derived from Wistar rat), and all peaks in the peptide map are uniformly decreased in STZ rat.

In the microsomal fraction of the livers from four groups of insulin-deficient diabetic rats used to study phosphorylation, there was a decrease in EGF binding of up to 39%. In each case this was due to a decrease in EGF receptor concentration with no change in receptor affinity. A decrease in EGF receptor number has also been reported using placenta and lumps from fetuses of STZ-diabetic rats (23), suggesting that decreases in EGF receptor concentration may occur in multiple tissues in the insulin-deficient diabetic state. Autophosphorylation of the EGF receptor, however, was decreased almost 70% in the diabetic rats. These results suggest that the decrease in autophosphorylation of EGF receptor in insulin-deficient rat liver is due only partially to a decrease in receptor number and partially to a decrease in kinase activity. This point is made clear by the direct comparison of decreased binding and decreased phosphorylation (Fig. 6).

Although the role of the EGF receptor in hepatocytes is unknown, isolated hepatocytes have been shown to possess EGF receptors (14, 17), and EGF stimulates hepatic DNA synthesis both in vitro (14, 16, 20) and in vivo (3). In mouse liver, EGF also enhances guanylate cyclase activity (22), and cyclic GMP has been shown to stimulate DNA synthesis (26). Interestingly, KGF receptor number has been shown to decrease after partial hepatectomy (21). In primary cultures of hepatocytes, glucocorticoids increase EGF receptor binding, and this increase is inhibited by insulin (14). These data suggest that EGF plays a role in liver growth and suggest the possibility that the decrease in EGF receptor kinase activity in insulin-deficient diabetic states could lead to altered hepatic function.

Although further experiments are needed to understand the mechanisms of the decrease in binding and phosphorylation of EGF receptor, it is now clear that insulin-deficient states are associated with a decrease in two major tyrosine kinase activities, i.e., the insulin receptor kinase and EGF receptor kinase. In the former case this is associated with an increase in receptor concentration, whereas in the latter there is a decrease in the receptor concentration. Serine and threonine phosphorylation induced by phorbol esters results in a decrease in tyrosine phosphorylation of both the EGF (5) and insulin receptors (24), as well as a loss in EGF receptors (13), resembling some of the finding in diabetic animals. Whether protein kinase C could be activated in the diabetic animals and play a role in the alteration in the insulin and receptor, however, remains to be determined. Whatever the mechanism, these data suggest a possible generalized alteration in growth and metabolism in insulin-deficient states.

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REFERENCES

11. Kris, R. M., J. Lax, W. Gullick, M. D. Waterfield, A. Ullrich,


