Communication

Structural Differences between Liver- and Muscle-derived Insulin Receptors in Rats*

(Received for publication, July 7, 1986)
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The structure of insulin receptors, solubilized from rat skeletal muscle and liver, was studied. The α subunit was identified by specific cross-linking to A14 125I-insulin with disuccinimidyl suberate. Muscle- and liver-derived α subunits migrated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a M\(_r\) of 131,000 and 135,000, respectively. There was no significant difference in insulin binding affinity. Treatment of cross-linked, immunoprecipitated receptors with either neuraminidase or endoglycosidase H decreased the M\(_r\) of muscle- and liver-derived α subunits but did not affect the difference in M\(_r\). Autophosphorylated β subunits migrated with a M\(_r\) of 98,000 for muscle and 101,000 for liver. After partial V8 digestion of autophosphorylated, immunoprecipitated receptors the major phosphopeptide fragment migrated on SDS-PAGE at M\(_r\) 57,000 from muscle and 60,000 from liver. Glycosidase digestion of autophosphorylated receptors suggested that M\(_r\) heterogeneity was due in part to differences in the sialic acid content of β subunits. Muscle and liver are the major target organs of insulin; the apparent heterogeneity of insulin receptor structure may be relevant to tissue-specific differences in insulin action.

The insulin receptor is a heterotetramer glycopeptide composed of two α and two β subunits which are linked by disulfide bonds (for review see Refs. 1–3). Pulse-chase studies (4, 5) and recent analysis of cDNA clones (2, 6) suggest that the insulin receptor is synthesized as a M\(_r\) ~ 180,000 precursor which is subsequently glycosylated and cleaved to yield subunits of ~135,000 (α) and ~95,000 (β). Heterogeneity in the structure of the insulin receptor has been reported. In brain both the α (7) and β (8) subunits may have smaller M\(_r\) than those observed in adipocyte and liver, respectively; the difference in the α subunit is apparent due to a decrease in sialic acid content (7). Gordon’s group (9) reported that both the α and β subunits of insulin receptors isolated from a human monocyte cell line have larger M\(_r\) than the corresponding subunits from IM-9 lymphocytes. The β subunit of endothelial cells had a larger M\(_r\) than that derived from a hepatoma cell line (10). Only in the first instance (7) was a molecular basis for the heterogeneity suggested. In this report we compared in rats the structure of the insulin receptor derived from the two main in vivo target organs of insulin, skeletal muscle, and liver.

EXPERIMENTAL PROCEDURES

Materials—Polyclonal anti-insulin receptor antibody (B9) was a gift from Dr. C. Ronald Kahn (Joslin Research Laboratories, Boston, MA). Monocomponent, crystalline pork insulin and moniodinated A14 35S-insulin were kindly provided by Dr. Ronald Chance and Dr. B. H. Frank, respectively. Lilly Research Laboratories, Indianapolis, IN. Insulin-like growth factor-1 was purchased from AMGen Biologicals, Thousand Oaks, CA. All other reagents and chemicals were obtained as previously reported (11). Animals—Male Wistar rats weighing 80–150 g were used; food was withdrawn 24 h before decapitation by guillotine.

Receptor Purification—The liver and hindlimb skeletal muscles were rapidly removed, immediately frozen in liquid nitrogen, and processed as described previously (12). In some experiments, additional protease inhibitors (1 mM N-ethylmaleimide, 1 mg/ml bacitracin, 20 μg/ml leupeptin, 10 μg/ml papainin, 1 mg/ml benzimidrone, and 1 mg/ml benzoyl arginine ethyl ester) were added to the homogenization buffer. After adsorption to wheat germ agglutinin (WGA) and washing, receptors were eluted from the WGA column with buffer containing 50 mM HEPES, pH 7.4, 0.1% Triton X-100, and 0.3 M N-acetylgalactosamine (approximately 1 ml/g original tissue weight for muscle and liver, respectively) and used directly or further purified by immunoprecipitation. In separate experiments previously prepared WGA eluate from liver was dialyzed against 50 mM HEPES, pH 7.4, 0.1% Triton X-100, to remove N-acetylgalactosamine; Triton X-100 was added to the retentate (final concentration 1%) and the receptors were added to frozen powdered muscle for resolubilization.

Insulin Binding—Insulin binding was performed on the WGA eluate as previously described (12) using A14 125I-insulin as the tracer (specific activity 200–300 μCi/μg, 50,000 cpm/200 μl final volume). Nonspecific binding (in the presence of 10 μg/ml unlabeled insulin) was 4–6% of total binding in muscle and 0.5–2% in liver-derived receptor preparations.

Cross-linking—A14 35S-insulin was cross-linked to solubilized insulin receptors with disuccinimidyl suberate as described (11). Insulin binding was measured prior to cross-linking, and equal insulin binding activities (11) from muscle- and liver-derived WGA eluates were used. In some studies the cross-linked insulin receptor was immunoprecipitated and digested with glycosidases as described below.

Autophosphorylation—Autophosphorylation was carried out as described (12) using approximately equal insulin binding activities (11). In experiments utilizing immunoprecipitated insulin receptors, the autophosphorylation reaction was terminated by the addition of either 4 × Laemmli’s sample buffer or a “Stop solution” (11) with anti-receptor antibodies. In some experiments the insulin receptors were adsorbed to insulin-agarose beads prior to autophosphorylation. WGA eluates from muscle and liver were concentrated by incubating with 200 μl of insulin-agarose (13) in 50 mM HEPES, 0.1% Triton X-100, 150 mM NaCl, and 1.5 mg/ml bacitracin overnight at 4 °C. The resin was washed and resuspended in 150 μl of 50 mM HEPES, pH 7.4, 5 mM MnCl₂, and 0.025% bovine serum albumin and the autophosphorylation was initiated with 20 μl of [γ-32P]ATP (100 μCi, 50 μM final concentration). After 5 min the resin was washed,
Laemmli's sample buffer was added, and the samples were submitted to electrophoresis.

Immuno precipitation and V8 Protease and Glycosidase Digestion—Cross-linked or phosphorylated insulin receptors were immunoprecipitated as described (12), selected samples were subjected to V8 protease and/or glycosidase digestion as described (4, 11).

Electrophoresis—Under reducing conditions (5% 2-mercaptoethanol), electrophoresis was performed using the discontinuous system of Laemmli (14) with a 6.5, 7, or 8% resolving gel for analysis of α subunits, intact β subunits, and V8 protease-treated β subunits, respectively. A 4.5% stacking gel was used in all cases. Autoradiography was performed as described (11). The M₀ of labeled bands was assigned by regression analysis of log molecular weight versus relative mobility of standard proteins supplied by Sigma, e.g., myosin, 205,000; β-galactosidase, 116,000; phosphorylase b, 97,400; bovine albumin, 66,000; egg albumin, 45,000; and pepsin, 35,000. The assignments are approximate since glycoproteins do not migrate according to their true M₀ on SDS-PAGE (15).

RESULTS AND DISCUSSION

As previously reported, (12) insulin receptors can be extracted directly by homogenizing frozen, powdered tissues in the presence of buffered 1% Triton X-100 and protease inhibitors. The receptors, thus isolated, retain insulin binding activity and, after further purification on WGA, insulin stimulatable tyrosyl kinase activity (12) (see below). Recovery of insulin binding activity from the 150,000 × g supernatant in the WGA eluate was 65–85%.

The α subunit of the insulin receptor solubilized from muscle and liver was identified by specific covalent coupling to A14 [¹²⁵I]-insulin using disuccinimidyl suberate and examination of the reaction product by SDS-PAGE and autoradiography. When examined under reducing conditions, a protein of M₀, 131,000 from WGA eluate of muscle homogenates was labeled (Fig. 1, lane G) while a protein of M₀, 135,000 was labeled in WGA eluates from liver (Fig. 1, lane E). These differences were observed without exception in seven separate receptor preparations (Table I). The labeling of these two proteins was prevented by adding excess unlabeled insulin to the radioligand prior to cross-linking (Fig. 1, lanes A and B).

The A14 [¹²⁵I]-insulin cross-linked protein was identified as the insulin receptor α subunit (as opposed to the structurally similar IGF-I receptor α subunit (16)) by incubating the WGA eluates with A14 [¹²⁵I]-insulin and increasing concentrations of unlabeled insulin or IGF-1 prior to cross-linking. IGF-1 was approximately 100 times less potent at inhibiting A14 [¹²⁵I]-insulin cross-linking than native insulin (Fig. 2A). Since the smaller M₀ of the muscle-derived insulin receptor α subunit could be obscured by protease degradation during isolation, insulin receptors from muscle and liver were isolated in the presence of additional protease inhibitors as described under "Experimental Procedures," but the difference between the α subunits of the muscle- and liver-derived insulin receptors persisted (Fig. 1, lanes C and D). Furthermore, insulin receptors isolated from rat liver were dialyzed to remove N-acetylglucosamine and added to powdered frozen muscle prior to homogenization. The mixture was purified by the standard procedure and the WGA eluate was cross-linked with radiolabeled insulin; SDS-PAGE under reducing conditions followed by autoradiography revealed an approximately 2-fold widened band, the top of which comigrated with the liver-derived, and the bottom with the muscle derived, α subunit (Fig. 1, lanes F and F').

Equilibrium binding of A14 [¹²⁵I]-insulin to receptors in the WGA eluate was performed to determine whether the difference in the M₀ of the α subunits resulted in functional alterations. The yield of insulin receptors per gram of original tissue weight (pmol of insulin specifically bound/g of tissue) was 7–10 times greater from liver than from skeletal muscle. Binding data were plotted in the form (B/F)/(B₀/F₀) versus insulin concentration to compare the relative affinities of the receptors for insulin directly (Fig. 2B). The affinity for insulin of receptors from liver and muscle was not significantly different when data from seven separate sets of receptor preparations were compared, although the apparent affinity of

**Table I.**

<table>
<thead>
<tr>
<th>Enzyme (n)</th>
<th>Muscle</th>
<th>Liver</th>
<th>Δm post-enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>α subunit</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- (7)</td>
<td>131.0</td>
<td>135.0</td>
<td></td>
</tr>
<tr>
<td>Neuraminidase (3)</td>
<td>125.5</td>
<td>129.0</td>
<td>5.5</td>
</tr>
<tr>
<td>Endo H (2)</td>
<td>124.5</td>
<td>128.0</td>
<td>6.5</td>
</tr>
<tr>
<td>β subunit</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- (5)</td>
<td>98.0</td>
<td>101.0</td>
<td></td>
</tr>
<tr>
<td>Neuraminidase (5)</td>
<td>95.5</td>
<td>96.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Endo H (4)</td>
<td>92.0</td>
<td>95.5</td>
<td>6.0</td>
</tr>
<tr>
<td>Neuraminidase +</td>
<td>90.5</td>
<td>92.5</td>
<td>7.5</td>
</tr>
<tr>
<td>Endo H (3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V8 protease-treated β subunit (major phosphopeptide)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- (4)</td>
<td>57.0</td>
<td>60.0*</td>
<td></td>
</tr>
<tr>
<td>Neuraminidase (4)</td>
<td>57.0</td>
<td>57.0</td>
<td>0</td>
</tr>
<tr>
<td>Endo H (4)</td>
<td>52.5</td>
<td>55.5*</td>
<td>4.5</td>
</tr>
<tr>
<td>Neuraminidase +</td>
<td>52.5</td>
<td>54.0</td>
<td>4.5</td>
</tr>
<tr>
<td>Endo H (3)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Liver M₀, significantly different from muscle M₀; (p < 0.05).  
| Change in liver M₀, significantly greater than the change in muscle M₀; (p < 0.05) by Student's t-test.  

**Fig. 1.** Autoradiogram of muscle- and liver-derived insulin receptor α and β subunits. WGA eluates were prepared by the standard procedure (lanes E, F, F', G, H, and J) or in the presence of additional protease inhibitors (lanes A–D, I, and K) or from muscle homogenates to which previously purified liver-derived insulin receptors were added (lanes F and F', see "Experimental Procedures"). In experiments shown in lanes A–G, WGA-purified receptors were incubated with A14 [¹²⁵I]-insulin without or with (lanes A and B) 50 ng/ml unlabeled insulin at 4°C for 16 h. Disuccinimidyl suberate (2 mM) was added for an additional 15 min when 4 × concentrated Laemmli's sample buffer was added. Lanes C and G illustrate cross-linked α subunits derived from muscle, and lanes D and E from liver; lanes F and F' illustrate the mixed population from muscle and liver. Electrophoresis was performed under reducing conditions on a 6.5% resolving gel. Lanes A–D and E–G are derived from separate experiments; the two autoradiograms were aligned according to the molecular weight markers. In experiments shown in lanes H–K, WGA eluates from muscle (lanes H and J) or liver (lanes D and K) were incubated with insulin-agarose (13) for 16 h at 4°C, phosphorylated, and electrophoresed as described under "Experimental Procedures."
Insulin Receptor Structural Heterogeneity: Liver Versus Muscle

To investigate whether the carbohydrate side chains were responsible for the differences in $M_r$, receptors were immunoprecipitated and treated with either neuraminidase or endoglycosidase H. Treatment of $^{125}$I-insulin cross-linked receptors with either enzyme increased the electrophoretic mobility of the $\alpha$ subunits from both tissues similarly (Table I) but failed to eliminate the difference between their $M_r$. Since neuraminidase treatment did not affect the difference in $M_r$ between muscle- and liver-derived $\alpha$ subunits, it is probably not caused by differences in sialic acid content. Neither could the difference be attributed to altered processing of high mannose to low mannose oligosaccharides since the $M_r$ of muscle- and liver-derived $\alpha$ subunits decreased similarly after endo-$\beta$-N-acetylglucosaminidase H treatment.

The difference in $M_r$ between insulin receptor $\beta$ subunits could not be completely eliminated by treating autophosphorylated, immunoprecipitated insulin receptors with neuraminidase, endoglycosidase H, or a combination of both (Fig. 3A). However, after neuraminidase treatment, the decrease in $M_r$ of the liver-derived $\beta$ subunit was greater than that observed in muscle (Table I). The decrease in $M_r$ of $\beta$ subunits after treatment with endoglycosidase H was similar in both tissues.

Brief digestion of immunoprecipitated insulin receptors with V8 protease reproducibly generated 3–4 phosphopeptides. The major phosphopeptide from muscle-derived insulin receptors had a $M_r$ of 57,000 while from liver it migrated at a $M_r$ of 60,000 (Fig. 3B). Treatment of the V8 protease-generated fragments with endoglycosidase H increased the mobility of the major phosphopeptide from muscle- and liver-derived receptors to the same extent (Fig. 3B, lanes E and F, and Table I). Thus a majority of the autophosphorylation sites as well as some of the glycosylation sites on the $\beta$ subunit can be localized to a domain with $\sim60\%$ of the $M_r$ of the intact $\beta$ subunit.

Treatment of the insulin receptor fragments with neuraminidase did not affect the migration of the major phosphopeptide generated from muscle-derived receptors (Fig. 3B, lanes A and C, and Ref. 11) but decreased its $M_r$ in liver-derived receptors which resulted in its apparent comigration with the same peptide generated from muscle (Fig. 3B, lanes B and D and Table I). This observation is consistent with a difference in sialic acid content between the major phosphopeptides generated by V8 digestion from muscle and liver. However, after sequential digestion with neuraminidase and endo-$\beta$-N-acetylglucosaminidase H of the V8 protease-treated insulin receptors, a difference in the $M_r$ of the major fragment from muscle and liver was still discernable but appeared reduced when compared to the difference between the non-glycosidase digested phosphopeptides (Fig. 3B, lanes G and H, and Table I). The reason for the inability to completely eliminate the differences in $M_r$ between insulin receptor $\beta$ subunits by sequential digestion with neuraminidase and endo-$\beta$-N-acetylglucosaminidase H is not clear. For example, it may be due to differences in the oligosaccharides that remain after removal of the sialic acids, which may affect the electrophoretic mobility of the phosphopeptides after the low mannose oligosaccharide sidechains are removed. Studies of IM-9 cell-derived insulin receptors suggest the presence of $O$-linked oligosaccharides on the $\beta$ subunit in addition to the predominant $N$-linked side chains (18). Our attempts at complete removal of the oligosaccharides from the $\beta$ subunit by either enzymatic or chemical means was unsuccessful using the autophosphorylated subunit (not shown).

The $\beta$ subunit of the insulin receptor from muscle and liver was identified by autophosphorylation in the presence of $\left[\gamma^32P\right]$ATP with subsequent SDS-PAGE and autoradiography. When the muscle-derived insulin receptor was allowed to autophosphorylate, a protein of $M_r \sim 98,000$ was labeled. This protein increased its labeling in the presence of insulin in a dose-dependent manner (12), was specifically immunoprecipitable with anti-insulin receptor antibodies, and was thus identified as the $\beta$ subunit of the insulin receptor. In parallel experiments, insulin stimulatable labeling of a protein of $M_r \sim 100,000$ was observed in WGA eluates prepared from liver, which was also immunoprecipitable with anti-insulin receptor antibodies (not shown). To further purify the insulin receptor prior to autophosphorylation, WGA eluates were incubated with insulin-agarose, and after subsequent washing the insulin-agarose bound receptors were allowed to autophosphorylate in the presence of $50\mu M\left[\gamma^32P\right]$ATP. The same $M_r$ differences between muscle- and liver-derived receptor $\beta$ subunits were observed (Fig. 1, lanes H and J). When the insulin receptors were isolated in the presence of excess protease inhibitors, as described above for the $\alpha$ subunit, the $\beta$ subunit of the insulin receptor isolated from muscle still had a smaller $M_r$ than that derived from liver (Fig. 1, lanes I and K, and Table I). In the liver-derived WGA eluate, phosphorylation of higher $M_r$ bands was also observed but these bands were not consistently immunoprecipitable (not shown) and did not become labeled in insulin cross-linking experiments (Fig. 1, lanes D and E). Thus, it is unlikely that they represent different forms of the liver-derived insulin receptor, but rather associated proteins which were co-purified.

It is difficult to compare the $M_r$ presented here to those reported in the literature for insulin receptors isolated from various tissues, due to minor differences in SDS-PAGE technique between laboratories and the variability in the $M_r$ assigned to phosphorylase b, the commonly used standard which migrated near the $\beta$ subunit. In the present study it was assigned $97,400$ (17), but this value varies between 92,000 and 97,500 in various papers characterizing insulin receptor subunits. It was not until the muscle- and liver-derived subunits were electrophoresed next to each other that the differences in electrophoretic mobility became discernible.

![Graph](image-url)
activity/insulin binding site of the liver-derived insulin receptor may be less than that derived from muscle; these observations are currently under investigation.

Two-dimensional separation of phosphopeptides generated by trypsin digestion of autophosphorylated insulin receptor $\beta$ subunits revealed no major discernible differences between the phosphopeptide maps derived from muscle and liver (not shown). It also should be noted that the hind limb muscles used consist of varying proportions of fast-twitch glycolytic and slow-twitch oxidative fibers (19); insulin receptor heterogeneity between muscles enriched in certain fiber types, while possible, was not investigated.

Recent studies of insulin receptor gene structure suggest that while there appears to be only one copy of the insulin receptor gene, there is mRNA heterogeneity; in man, certain mRNA species predominate in liver versus other tissues (e.g. placenta and IM-9 lymphocytes). A salient difference between insulin’s action in liver and a number of other tissues is a lack of insulin-stimulated glucose transport in the former. Morphological studies (20) highlight differences in the distribution of insulin receptors on the cell membrane in various tissues (e.g. liver versus adipocytes) which may reflect in part structural heterogeneity of the receptors. Whether or not the structural differences described here are reflected in differences in transmembrane signalling and/or have functional implications remains to be determined.

Acknowledgments—We thank Dr. B. H. Frank and Dr. R. Chance for A125I-insulin and crystalline porcine insulin, respectively. Dr. Ronald Kahn for the gift of anti-receptor antibody, Margaret Sowell for technical advice, and Barbara Whillock for help in preparing the manuscript.

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