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COMPARISON OF INSULIN AND INSULIN-LIKE GROWTH FACTOR I RECEPTORS FROM RAT SKELETAL MUSCLE AND L-6 MYOCYTES

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Insulin and IGF-I receptors were solubilized from fused L-6 myocytes, a rat skeletal muscle derived cell line, and compared to rat skeletal muscle receptors. In skeletal muscle, ^{125}I-insulin binding was competed by insulin > IGF-I > MSA, whereas in L-6 cells IGF-I > insulin > MSA. ^{125}I-IGF-I binding was competed by IGF-I > insulin = MSA in both tissues. On electrophoresis, differences in Mr were observed between skeletal muscle and L-6 derived receptors both in the α- and β-subunits. Six antibodies directed against the human insulin receptor β-subunit recognized the rat skeletal muscle insulin receptor, while only two reacted strongly with L-6 derived receptors. Skeletal muscle has receptors with relative specificity for insulin and IGF-I respectively; L-6 cells also have two classes of receptors, one is kinetically similar to the IGF-I receptor from skeletal muscle; the other, which binds insulin with relatively high affinity has even greater affinity for IGF-I. This unusual receptor may represent a developmental stage in muscle or the transformed nature of L-6 cells.

The development of cell culture models for skeletal muscle is an important advance in studying metabolic events in this tissue. L-6 myocytes, a transformed cell line derived from rat skeletal muscle, exhibit many characteristics of skeletal muscle upon differentiation from myoblast to myotube, including fusion to form multinucleated cells, expression of MB creatinine kinase activity and contractile activity in culture (1,2). In the myoblast stage there is a preponderance of IGF-I and IGF-II receptors

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Abbreviations used: IGF-I = insulin-like growth factor I; MSA = multiplication stimulating activity; PEG = polyethylene glycol; WGA = agarose bound wheat germ agglutinin; SDS-PAGE = sodium dodecyl sulphate polyacrylamide gel electrophoresis; TAME = N-tosyl-L-arginine methyl ester, BAEE = N-benzoyl-L-arginine ethyl ester; PMSF = phenylmethylsulfonyl fluoride; TIU = trypsin inhibitory units; HEPES = N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, TCA = trichloroacetic acid.

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(1,2). The number of IGF-I receptors decreases upon differentiation while the number of receptors with high affinity for insulin increases (2). In the myotube stage both insulin and IGF-I exert biological effects at physiological concentrations (2). In this report we compared the binding characteristics of insulin and IGF-I to receptors solubilized from L-6 myocytes and rat skeletal muscle. We also compared the structure of the subunits of these receptors and their ability to interact with various anti-receptor antibodies.

Methods: L-6 cells were obtained from The American Type Culture Collection and grown in Dulbecco's Modified Eagle's Medium with 10% fetal calf serum for 8-15 days; 5-10 days after reaching confluence, cells were harvested. In some experiments cells were treated with cytosine arabinoside on days 9 and 11 after plating and used on day 15 as described (1). In all studies at least 80% of cells were myotubes. Solubilization of receptors was achieved by scraping cells from four plates (30-40 mg protein) and gentle homogenization in 50 mM Hepes, pH 7.4, 1.0% Triton X-100 , 1 TIU/ml aprotinin, 1 mg/ml each of benzimidine, TAME, and BAEE, 2 mM PMSF and stirring at $4^{o}C$ for 30 min. Insoluble material was removed by centrifugation at 150,000 x g for 90 min and receptors further purified by adsorbing the supernatant to WGA and eluting with 50 mM Hepes, pH 7.4, 0.1% Triton X-100 (buffer A) supplemented with 0.3 M n-acetylglucosamine. Receptors from skeletal muscle were prepared as previously described (3).

The ability of antibodies directed to defined sequences of the human insulin receptor β-subunit (anti-P2, P4 and P5 (4), kindly provided by Dr. Ora Rosen, Cornell University) and monoclonal antibodies raised against human placental insulin receptors (1G2, 17A3 and 2G7, designated B_1, B_2 and B_3 respectively by Morgan and Roth (5), provided by Dr. Richard Roth, Stanford University) was assayed as described by Herrara and Rosen (6). Since some of the antibodies altered apparent receptor affinity, the ability of the antibodies to clear binding activity from the solution was used as an end point. Apparent affinity change was determined by incubation of WGA eluates with the indicated antibody in a standard binding assay, precipitation of receptors and bound ligand with PEG (3) and comparison to control sera, which in preliminary experiments were shown not to affect receptor binding. Non-specific binding was that occuring in the presence of 5 μg/ml unlabeled ligand and was subtracted from total binding. Insulin and IGF-I degradation in all experiments was less than 3% as judged by TCA precipitability.

Ligand binding assays, phosphorylation of receptors, crosslinkage of ^{125}I-ligands to their receptors and SDS-PAGE was performed as described previously (3,7). Human recombinant IGF-I was obtained from three different suppliers (Amgen, IMCELL and Lilly), yielding identical results. MSA was purchased from Collaborative Research Inc.

Results: Competition by unlabeled insulin for ^{125}I-insulin binding to receptors solubilized from rat skeletal muscle showed that 50% displacement occured at approximately 2 x 10^{-9}M unlabeled ligand. IGF-I was ~100 times less potent than insulin in competing for ^{125}I-insulin binding while MSA was ~1000x less potent (Figure 1A). ^{125}I-IGF-I binding in skeletal muscle extracts was displaced 100 x more readily by unlabled IGF-I than by insulin or MSA (Figure 1B).

In parallel studies using L6 derived receptors, performed at the same time and under identical conditions as those presented for skeletal muscle,

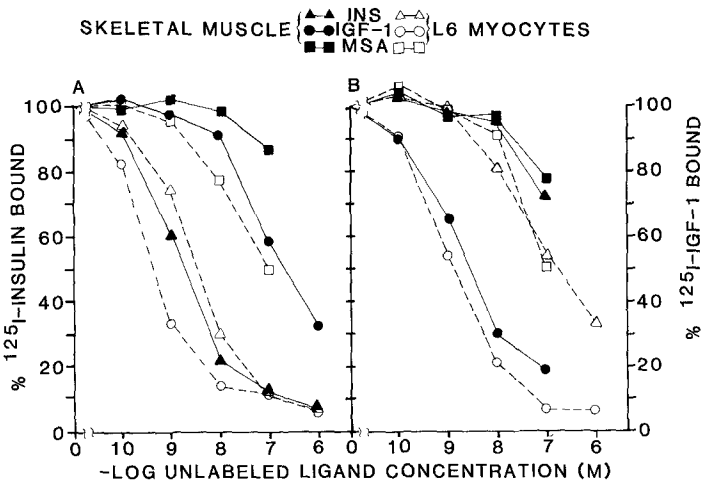

Figure 1. A) Competition of ^{125}I-insulin binding by insulin, IGF-I or MSA. WGA eluates from skeletal muscle (20 µl) and L6 myocytes (10 µl) were incubated with A14[^{125}I]-insulin (30,000 cpm, 1 x 10^{-11} M final conc.) and insulin, IGF-I or MSA at the indicated concentrations in 50 mM HEPES, pH 7.8, 0.1% Triton X-100, 100 mM NaCl and 1 mg/ml Bacitracin in a final volume of 65 µl for 16 hr at 4°C. The receptor and bound ligand were precipitated by adding bovine γ-globulin (0.1% final conc.) and PEG (12.5% final conc.), the precipitate was collected by centrifugation, washed once with 12.5% PEG and counted in a γ counter. Binding is expressed as % of that seen in the presence of trace ligand (100%). Skeletal muscle and L-6 cell preparations bound 17% and 11% of tracer ^{125}I-insulin, respectively.

B. Competition of ^{125}I-IGF-I binding by insulin, IGF-I or MSA. The experiment was performed in parallel and in an identical manner to that described in A except that 3[^{125}I]IGF-I (Amersham, 30,000 cpm, 1 x 10^{-11} M final conc.) was used as the tracer ligand. Skeletal muscle and L-6 preparations bound 9% and 61% of tracer ^{125}I-IGF-I respectively. The schematic designating the curves for the different ligands is presented at the top of the figure.

quite different results were obtained. ^{125}I-insulin binding was competed by unlabeled insulin with 50% displacement occuring at ~ 5 x 10^{-9} M, with MSA much less potent (ED_{50} ~1 x 10^{-7} M, Fig. 1A) in agreement with earlier studies of insulin binding to intact L6 myotubes (1). However, when ^{125}I-insulin was competed with unlabeled IGF-I, the latter was more efficient at displacing bound ^{125}I-insulin than was insulin itself, ~ 50% displacement occuring at ~5 x 10^{-10} M IGF-I (Fig. 1A). Insulin was 50-100x less effective in displacing ^{125}I-IGF-I bound to L6 myocyte receptors than unlabeled IGF-I which displaced 50% of ^{125}I-IGF-I at ~1 x 10^{-9} M, similar to the results observed in rat skeletal muscle extracts (Fig. 1B).

The results of the competition binding studies did not depend upon the time in culture after myotube formation, nor on treatment with cytosine arabinoside to decrease myoblast populations (1) nor upon a specific subclone of the L6 cell line used. Identical results were obtained 8-21 days after plating with or without treatment with cytosine arabinoside and

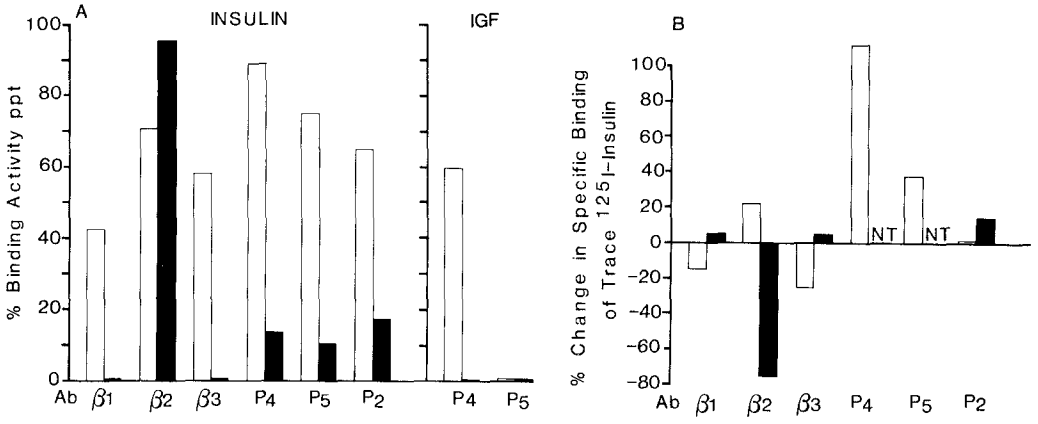

Figure 2. A) Immunoprecipitation of insulin and IGF-I binding activity. WGA eluates from skeletal muscle or L-6 myocytes (~3 fmol insulin binding activity) were incubated with 100,000 cpm ^{125}I-insulin or ^{125}I-IGF-I with or without 5 μg/ml unlabeled ligand (non-specific binding) in Buffer A supplemented with 0.05% BSA, 2 μg/ml each of leupeptin, soybean trypsin inhibitor, and bacitracin, 0.1 TIU/ml aprotinin, 1 mM PMSF, and designated antisera (b1,b2,b3=1:50; anti-P4, P5, P2=1:100) or appropriate dilutions of control sera in 35 μl at 4°C for 16 hr, then Pansorbin was added and incubated for an additional 2 hr. The immune complex was collected by centrifugation, washed twice and counted in a γcounter. The supernatant was made 0.1% in bovineγglobulin and receptors with bound ligand precipitated with an equal volume of 25% PEG, and processed as in Fig. 1. Results are presented as % removal of total ligand binding activity from the supernatant by the antibody as compared to non-immune sera.

B) Antibody induced changes in the apparent affinity of receptors towards insulin. WGA eluates were incubated as described in A and samples (insulin receptor with bound ligand and antibody) precipitated with PEG as in A. Results are presented as % change in the specific insulin binding activity precipitated as compared to non-immune sera. N.T.=not tested.

in two different subclones of the L-6 cell line (the second clone was kindly provided by Dr. Robert J. Smith, Joslin Res. Lab., Harvard Med. Sch., data not shown).

The interaction of receptors from rat skeletal muscle and L6 myocytes with a battery of anti-β-subunit specific antibodies was tested. Monoclonal antibodies B1 and B3 which were raised against the insulin receptor β-subunit from human placenta (5) precipitated most of the insulin binding activity from rat skeletal muscle extracts while they were ineffective in precipitating insulin binding activity from L-6 cells (Fig. 2A). Both antibodies decreased the apparent insulin binding affinity of the skeletal muscle insulin receptor by 14 and 29% respectively (Fig. 2B) but did not affect insulin binding by L-6 derived receptors. On the other hand, B2 precipitated insulin binding activity from L-6 cells more efficiently than from rat skeletal muscle, at the dilution used. This antibody markedly decreased the apparent insulin binding affinity in L-6 preparations while it increased the insulin binding affinity of receptors from rat skeletal muscle.

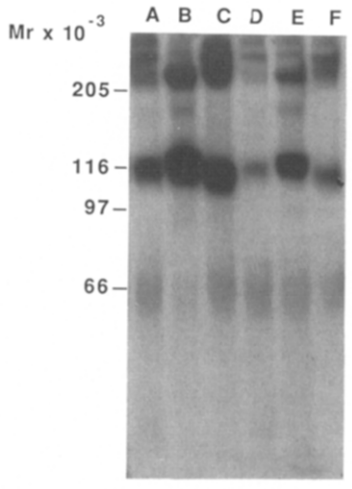

Figure 3. Affinity labeling with ^{125}I-insulin. WGA eluates (100 µl) from muscle and L-6 receptor preparations were incubated with 250,000 cpm of A14[^{125}]I-insulin without (Lanes A-C) or with 5 x 10^{-8} M unlabeled insulin (Lanes D-F) in 50 mM HEPES, pH 7.8, 0.05% Triton X-100, 0.025% BSA, 100 mM NaCl and 1 mg/ml Bacitracin for 16 hr at 4°C. Disuccinimidyl suberate was added (2mM final conc.) and incubated for an additional 15 min when electrophoresis buffer was added. The samples were boiled in the presence of 5 mM 2-mercaptoethanol and electrophoresed (SDS-PAGE, 6% polyacrylamide gel). An autoradiograph of a gel after drying is shown. Lanes A and D = Muscle extract; Lanes C and F = L6 extract. For comparison purposes rat liver derived insulin receptors are presented in lanes B and E.

As described for human placenta derived receptors (6), anti-P4 antibodies precipitated both insulin and IGF-I binding activity from rat skeletal muscle extracts while anti-P5 recognized only the insulin binding activity (Fig. 2A). Both anti-P4 and P5 antibodies recognized the L-6 insulin receptor only to a very limited degree. Neither antibody recognized the L-6 IGF-I receptor. In contrast to human derived insulin receptors, (Ref. 4 and our data not shown), anti-P4 and P5 affected the apparent insulin binding affinity, increasing trace insulin binding by 100 and 35% respectively (Fig. 2B). L-6 receptors were not studied in these experiments. Anti-P2 antisera, thought to interact with the domain involved in the tyrosine kinase activity of the receptor (6), had little effect on insulin binding affinity in either receptor preparation (Figure 1B). In contrast to human derived receptors (4) this antibody precipitated rat skeletal muscle insulin receptors to a significant extent in the non-phosphorylated state (Fig 2A), whereas non-phosphorylated L-6 derived insulin binding activity was poorly precipitated. Autophosphorylated insulin receptors from both skeletal muscle and L-6 cells precipitated completely with anti-P2 antibody at the dilutions used in these experiments (not shown).

In the presence of trace ^{125}I-insulin a protein of MWapp 135kDa (the α-subunit of the insulin receptor), was labeled in skeletal muscle extracts after crosslinking with disuccinimidyl suberate (Fig. 3). It's counterpart

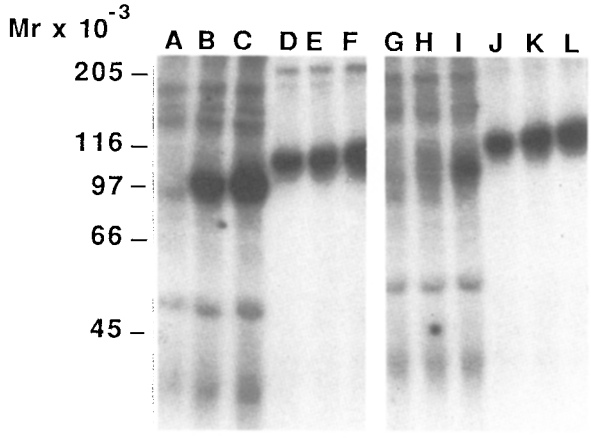

Figure 4. Autophosphorylation of insulin and IGF-I receptor β-subunits. WGA eluates from muscle (35 ul, Lanes A-C and G-I) and L6 myocytes (20 μl, Lanes D-F and J-L) were incubated without (Lanes A,D,G,J) or with 10^{-9}M (Lanes B,E,H,K) or 10^{-7}M (Lanes C,F,I,L) insulin (Lanes A-F) or IGF-I (Lanes (G-L)for 90 min in 50 mM HEPES, pH 7.4, 0.1% Triton X-100 and 0.25% BSA at 25°C and allowed to autophosphorylate in the presence of 5mM $MnCl_2$ and 10 μM γ[32P]ATP for an additional 10 min when the reaction was stopped with electrophoresis buffer. The samples were boiled in the presence of 5 mM 2-mercaptoethanol and electrophoresed. (SDS-PAGE, 7% polyacrylamide gel). An autoradiograph of a gel after drying is shown.

in L-6 myocte receptor preparations migrated at a MWapp of ~ 120kDa. The α-subunit of the rat liver derived insulin receptor is also shown. It migrates, as previously reported (7), at a slightly higher Mr than the muscle derived receptor. Crosslinking in the presence of 5×10^{-8} M unlabeled insulin resulted in decreased labeling of these bands.

Incubation of WGA eluate from skeletal muscle in the presence of 10^{-9} or 10^{-7} M insulin or IGF-I and γ[32P]ATP stimulated in a dose-dependent manner the phosphorylation of proteins of MWapp ~ 98kDa (presumably the β-subunits of the insulin and IGF-I receptors respectively, although at 10^{-7}M IGF-I may also bind to the skeletal muscle insulin receptor); stimulation by IGF-I was much less than that by insulin (Fig. 4). Under the same conditions in L-6 extracts both insulin and IGF-I stimulated the phosphorylation of protein(s) with a significantly higher Mr (~ 110kDa) than that seen in muscle derived receptor preparations. In the absence of ligand, basal autophosphorylation of this band was much greater in L6 extracts than in skeletal muscle (Fig. 4). These phosphoproteins were completely precipitable by antiphosphotyrosine antibodies (a generous gift of Dr. Morris White, Joslin Res. Lab., Harvard Med. Sch.,data not shown).

Discussion: Several reports characterized the receptors for insulin and insulin like growth factors on L6 myocytes (1,2,10,11). Ballard et al.

(11) demonstrated high affinity binding sites for both IGF-I and MSA (IGF-II) and suggested that the biological effects of these ligands are exerted primarily through binding to the IGF-I receptor. Recently, Beguinot et al (2) reported high affinity binding sites for insulin on L-6 myocytes, which increased in number after fusion, and had properties consistent with insulin receptors, e.g. high affinity for insulin with lower affinity for proinsulin and MSA, down regulation and competition for binding by sera from a patient with anti-insulin receptor antibodies (although at a relatively high concentration). To our knowledge, in no report was competition of IGF-I against labeled insulin examined. The observations presented here suggest that the receptor species responsible for binding insulin in L-6 cells may be a high affinity IGF-I receptor. It seems unlikely that the insulin binding observed reflects only the high abundance of a single class of IGF-I receptors in L-6 cells because insulin displaced ^{125}I-IGF-I ~ 75-100-fold less effectively than it displaced ^{125}I-insulin. This raises the possibility that two species of IGF-I receptors exist in L-6 cells, one with a high affinity for insulin (and by competition with IGF-I an even higher affinity for IGF-I) and the other with a relatively high affinity for IGF-I and a low affinity for insulin. The displacement curves suggest that the second form (which is similar to the IGF-I receptor of skeletal muscle) is more abundant. Morgan and Roth (8) identified two IGF-I receptor species in several transformed cell lines with monoclonal antibodies directed against the α-subunit, however their relative affinity for insulin and IGF-I was not indicated. Jonas and Harrison (9) reported that placenta contains two distinct receptor species for IGF-I, the species which was immunoprecipitable by B2 IgG had a higher affinity for insulin than the population which was not precipitable by this anti-insulin receptor antibody. In contrast to our data, the receptor species with a higher affinity for insulin had a lower affinity for IGF-I than the non-immunoprecipitable species.

The 'insulin' receptor of L-6 cells differs structurally and immunologically from the insulin receptor in skeletal muscle. Differences in protein structure and/or glycosylation that alter the migratory behavior of the presumed insulin and IGF-I receptor β-subunits on SDS-PAGE are likely responsible for their altered antigenicity towards defined antibodies to the human insulin recptor β-subunit. Antibodies directed towards domains away from the site believed responsible for the kinase activity of the receptor showed little or no reactivity with L-6 myocyte derived receptors whereas antibodies directed towards the tyrosine kinase domain were equally or more reactive towards the 'insulin receptor' of L-6 myocytes than toward rat skeletal muscle derived receptors. The conservation of this domain is consistent with its importance in transmembrane signalling.

Since the genes for the insulin and the IGF-I receptor are presumed to be separate entities, the receptor in L-6 cells which binds insulin and IGF-I with high affinity may represent a modification of either or both genes, their transcripts or translation products, or a separate gene. An intriguing question is whether this receptor species reflects the transformed nature of L-6 cells or their incomplete differentiation. Since IGF's may play an important role during fetal development (12), it remains to be established whether or not this type of receptor is expressed in normal muscle in the course of differentiation. The unusual characteristics of the 'insulin receptor' in L-6 cells should be considered when extrapolating data obtained in this cell line to fully differentiated skeletal muscle.

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