Muscle Cell Differentiation Is Associated with Increased Insulin Receptor Biosynthesis and Messenger RNA Levels

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Abstract

Muscle is a major tissue for insulin action. To study the effect of muscle differentiation on insulin receptors, we employed cultured mouse muscle BC3H-1 and C2 cells. In both cell lines differentiation from myoblasts to myocytes was associated with a 5–10-fold increase in specific ^{125}I-insulin binding to intact cells. When ^{125}I-insulin binding was carried out on solubilized myocytes and myoblasts, ^{125}I-insulin binding to myoblasts was low. After differentiation the number of insulin receptors increased 5–10-fold. In contrast to insulin binding, insulin growth factor I receptor binding was elevated in myoblasts and was decreased by 50% in myocytes. Specific radioimmunoassay of the insulin receptor indicated that the increase in insulin binding to myocytes was due to an increase in insulin receptor content. Studies employing [^{35}S]methionine indicated that this increase in insulin-binding sites reflected an increase in insulin receptor biosynthesis. To study insulin receptor gene expression, myoblast and myocyte mRNA was isolated and analyzed on Northern and slot blots. Differentiation from myoblasts to myocytes was accompanied by a 5–10-fold increase in insulin receptor mRNA. These studies demonstrate, therefore that differentiation in muscle cells is accompanied by increased insulin receptor biosynthesis and gene expression.

Introduction

Muscle is a major target tissue for insulin action. In muscle, insulin regulates the transport of glucose which leads to major changes in blood glucose concentrations (1). In addition, in muscle, insulin regulates a variety of other functions including transport of amino acids, ions, and nucleotides; electrical potential; protein synthesis and degradation; and RNA metabolism (2).

The initial interaction of insulin with muscle and other target cells is via a receptor located in the plasma membrane (3–6). This receptor serves a role both in directing insulin to specific target tissues and in initiating the response of tissues to the hormone. The insulin receptor is a tetrameric glycoprotein that is composed of two extracellular α subunits (130 kD) that bind the hormone and two transmembrane β subunits (95 kD) that contain tyrosine kinase activity in their intracellular domains (3–6). One α and one β subunit are derived from a common precursor protein that has a minimum molecular mass of 153 kD as derived from cDNA analysis (7, 8).

In a variety of tissues the insulin receptor has been demonstrated to be under the regulation of hormones, metabolites, and differentiation (3–6, 9). Studies in adult muscle, however, have been limited by two technical difficulties. First, insulin receptor protein and mRNA levels in muscle are relatively low compared to other major insulin responsive tissues such as liver (10). Secondly, it is relatively difficult to subfractionate muscle in order to obtain receptor-rich plasma membrane fractions (11).

Cultured mouse and rat cell lines have been employed in order to study the in vitro regulation of muscle insulin receptors (12, 13). These cells grow as undifferentiated myoblasts when kept at low densities in media with high serum concentrations (14–16). However, when confluent cells are grown in media with low serum concentrations, the cells morphologically differentiate into myocytes (14–17). In addition to morphologic changes, there are changes in the content of muscle specific proteins (18, 19). Concomitant with differentiation into myocytes, there is also an increase in insulin binding to its receptor (12, 13).

BC3H-1 cultured mouse muscle cells have been studied extensively and show a 5–10-fold increase in insulin receptors when myoblasts differentiate into nonfusing myocytes (12). In myocytes there are enhanced effects of insulin on the regulation of several biological functions including glucose and amino acid transport, protein kinase activity, and phospholipid turnover (12, 20, 21). The mechanism that accounts for the increased binding of insulin to differentiated BC3H-1 cells has not previously been elucidated.

In the present study we measured insulin receptor content by ligand binding and radioimmunoassay studies. Moreover, we measured insulin receptor biosynthesis and insulin receptor mRNA content. The data indicate that differentiation in BC3H-1 cells is accompanied by increased insulin receptor mRNA levels and increased receptor biosynthesis. Similar data were also obtained in C2 cultured muscle cells that differentiate into fusing myocytes (19).

Methods

Chemicals

The following chemicals were purchased: bacitracin, Hepes, and phenylmethylsulfonyl fluoride from Sigma Chemical Co., St. Louis, MO; bovine plasma albumin (fraction V) was from Reheis, Chicago, IL; ^{125}I-labeled insulin and insulin growth factor I (IGF-I),[1] and [^{35}S]-methionine from New England Nuclear, Boston, MA; porcine insulin from Elanco Products, Indianapolis, IN; Bio-Rad protein assay reagent, dithiothreitol, and molecular weight markers from Bio-Rad Laboratories, Richmond, CA; IgG Sorb (protein A) from Enzyme Center Inc., Boston, MA; Dulbecco's modified Eagle's medium (DME H-21) with minimal Eagle's medium amino acid supplement, with and

1. *Abbreviations used in this paper:* IGF-I, insulin growth factor I.

without methionine, penicillin, streptomycin, amphotericin B, fetal bovine serum (FBS), and trypsin from the Cell Culture Facility, University of California, San Francisco, CA; tissue culture flasks and dishes from both Falcon Labware, Los Angeles, CA and Lux tissue culture dishes from Miles Laboratories, Inc., Naperville, IL; and IGF-I from Amgen, Thousand Oaks, CA.

Insulin receptor cDNA probes were a gift from Dr. G. I. Bell (University of Chicago). Ribosomal RNA gene pDF15 was a gift from Dr. A. P. Bollon (Westley Institute of Molecular Medicine). Antireceptor antiserum B-8 was a gift of Dr. S. I. Taylor (National Institutes of Health), and antireceptor antiserum Bd was a gift of Dr. G. Boden (Temple University).

Cell culture

The BC3H-1 and C2 mouse muscle cells were routinely grown in DME H-21 with 20% FBS. For experiments, cells were plated at an initial density of 6×10^3 cells/cm^2 in either 75-cm^2 tissue culture flasks or 35-mm tissue culture dishes. To initiate differentiation, the media containing 20% FBS was removed on day 3 and replaced with media containing 1% FBS. The cells were then cultured for up to 16 d and the media changed every 3 d. For experiments with undifferentiated BC3H-1 and C2 myoblasts, cells were plated in a similar manner and used only after 3 d in culture with 20% FBS.

Cell number was determined after cell dissociation with 0.25% trypsin and 0.5% EDTA at 37°C.

Hormone binding assays

^{125}I-insulin and ^{125}I-IGF-I binding to intact cells. At the indicated times, cells attached to 35-mm tissue dishes were rinsed with 3 ml of binding buffer containing 120 mM NaCl, 1.2 mM MgSO$_4$, 15 mM Na acetate, 2.5 mM KCl, 10 mM glucose, 1 mM EDTA, 1% bovine serum albumin, and 100 mM Hepes, pH 7.6, and incubated in 2 ml of the same buffer containing 40 pM ^{125}I-insulin for 20 h at 4°C (12). The incubation medium was then removed, the monolayers were washed twice, the cells were lysed with 0.1 M NaOH, and the radioactivity associated with the cell lysate was measured in a gamma-scintillation counter (Beckman Instruments, Inc., Palo Alto, CA). Binding was corrected for nonspecific ^{125}I-insulin binding (< 5% of total) as determined in the presence of an excess (1 μM) of unlabeled insulin. Specific ^{125}I-IGF-I binding was carried out in the same manner with 10 pM labeled hormone and 100 μM unlabeled hormone.

^{125}I-insulin and ^{125}I-IGF-I binding to solubilized myoblasts and myocytes. Cells were gently scraped from the surface of the flasks and solubilized with 0.4% Triton X-100 for 30 min at 4°C (22). Solubilized insulin and IGF-I receptors from BC3H-1 cells were precipitated and concentrated by the addition of 30% polyethylene glycol at 4°C. The pellet was reconstituted in binding buffer with 0.02% Triton X-100 at a concentration of 560 μg cell protein/ml. Solubilized receptor binding from myoblasts and myocytes was assayed in triplicate by adding either 40 pM of either ^{125}I-insulin or 10 pM ^{125}I-IGF-I to solubilized receptors in binding buffer with 0.02% Triton X-100 in the absence or presence of unlabeled hormones. The binding reaction was terminated after 20 h of incubation at 4°C by adding 150 μl of 30% polyethylene glycol and centrifuging the mixture at 11,000 g for 3 min. In this manner the free ^{125}I-ligands were separated from receptor-bound ligands present in the pellet. In all assays nonspecific binding was < 2% of total radioactivity.

Insulin receptor radioimmunoassay

This assay (23) employs purified human insulin receptor and high-affinity rabbit polyclonal antibody to the human insulin receptor (24). Highly purified human placental insulin receptor was obtained by sequential affinity chromatography with insulin receptor monoclonal antibody-agarose and wheat germ agglutinin-agarose (24). Purified human placental insulin receptor was labeled with ^{125}I-Bolton Hunter reagent. The specific activity of the labeled insulin receptor preparations ranged from 2,000 to 3,000 Ci/mmol. Unlabeled receptor (0–20 ng) or unknown sample and anti-insulin receptor antiserum were

incubated for 16 h at 4°C. ^{125}I-labeled insulin receptor (0.5 ng) was added for 24 h and then goat-rabbit γ-globulin serum was added. After 18 h at 4°C, the tubes were centrifuged, the supernatants aspirated, and the radioactivity in the tubes was counted. To measure insulin receptor content in BC3H-1 myoblasts and myocytes, cells were solubilized in 50 mM Hepes, 0.4% Triton X-100, 0.2 mM phenylmethysulfonyl-fluoride, and 0.5 mg/ml bacitracin for 30 min at 4°C. The lysate was then centrifuged for 3 min at 11,000 g and the supernatant was used for assay without further purification.

Insulin receptor biosynthesis

BC3H-1 and C2 cells were plated in 75-cm^2 flasks (Falcon Labware) at 6×10^3 cells/cm^2 in DME H-21 supplemented with 20% fetal bovine serum and differentiation induced as described above. To study insulin receptor biosynthesis, the flasks were washed twice in phosphate-buffered saline (PBS) and 5 ml of methionine-free medium was added. The cells were labeled by the addition of 1 mCi [^{35}S]methionine. After 4 h at 37°C, the monolayers were washed twice in PBS, and the cells were scraped off and solubilized for 60 min with Triton X-100 plus protease inhibitors (phenylmethylsulfonyl fluoride and bacitracin) (24). After solubilization the suspensions were centrifuged at 100,000 g for 60 min to remove insoluble components and the solubilized insulin receptors were partially purified by wheat-germ agglutinin chromatography. Labeled receptors were then immunoprecipitated using polyclonal antibodies, denatured under reducing conditions, and subjected to polyacrylamide gel electrophoresis (PAGE) and autoradiography (24).

Preparation of RNA

Total cellular RNA was extracted from both undifferentiated and differentiated BC3H-1 and C2 cells according to the guanidinium/thiocyanate/cesium chloride gradient method (25). Culture media was aspirated and the cells were removed by scraping into 10 ml of PBS at 4°C. Cell suspensions were centrifuged at 1,000 rpm and cell pellets were resuspended in buffer containing 4.5 M guanidinium thiocyanate, 50 mM EDTA (pH 7.8), 25 mM sodium citrate, pH 7.0, 0.1 mM 2-mercaptoethanol, and 2% lauryl sarcosine. The lysate was homogenized in a glass homogenizer for 10 strokes and cesium chloride was added at a concentration of 0.2 g/ml. 9 ml of this solution was layered over 4.5 ml of 5.7 M CsCl in a 13.5-ml polyallomer Quick-Seal tube (Beckman Instruments, Inc.). Sedimentation was performed in a type 70.1 Ti rotor (Beckman Instruments, Inc.) at 70,000 rpm for 4.5 h at 22°C. After centrifugation, the RNA pellet was removed and dissolved in 10 mM Tris-HCl, pH 7.6, 2 mM EDTA, 0.5% SDS. Further purification was performed by extracting the sample with phenol and chloroform and then the RNA was precipitated with ethanol. Poly(A)$^+$ RNA was isolated by oligo(dT)-cellulose chromatography according to the procedure described by Maniatis et al. (26).

Slot blots and Northern transfers

For Northern transfers poly(A)$^+$ RNA was denatured in formaldehyde, subjected to electrophoresis in 1% agarose and transferred to nitrocellulose paper (27). For "slot blots" total RNA was denatured as described by Thomas (28) and various dilutions of RNA were immobilized to nitrocellulose using a Schleicher & Schuell slot blot apparatus (Schleicher & Shuell, Inc., Keene, NH). Yeast tRNA was added as a carrier to samples when the final RNA concentration was below 1 μg/ml.

DNA labeling and RNA-DNA hybridization

Two human insulin receptor cDNA probes (a gift from Dr. G. I. Bell, University of Chicago), spanning the entire open reading frame of the receptor and extending into the 3'-untranslated region (29), were labeled by nick translation (27) to a specific activity of 10^9 cpm/μg. The nitrocellulose filters were prehybridized and hybridized and washed as previously described (27). Probes for the 5' portion of the rat 18S ribosomal RNA gene (30) and for β-actin (31) were nick translated and also hybridized as described for the insulin receptor.

Results

Studies with BC3H-1 cells

Increased insulin binding during BC3H-1 differentiation.
When cells were kept at subconfluent densities and grown in
20% FBS, they maintained a typical myoblast appearance with
a flat morphology and numerous small processes (18) (Fig. 1
A). When the cells were grown in 1% FBS and became con-
fluent, they differentiated into myocytes with a typical spindly
appearance (18) (Fig. 1 *B*). Concomitant with this differentia-
tion into myocytes there was an increase in ^{125}I-insulin binding
which was maximal at 14–16 d (Fig. 2 *A*). When cells were
placed into medium with 1% FBS, cell growth ceased and
receptor binding increased. Myoblasts bound 0.25% of total
radioactivity per 10^6 cells; in contrast, myocytes bound
3.65±0.2% per 10^6 cells ($n = 3$, mean±SEM).

*Comparison of ^{125}I-insulin and ^{125}I-IGF-I binding in myo-
cytes.* The insulin receptor has structural similarity to the
IGF-I receptor (32), and ^{125}I-insulin can weakly bind to the
IGF-I receptor. To determine whether the observed increase in
^{125}I-insulin binding to myocytes was due to an increase in
insulin receptors, insulin and IGF-I receptor binding were di-
rectly compared (Fig. 3 *A*). In myoblasts, specific ^{125}I-insulin
binding was relatively low whereas specific ^{125}I-IGF-I binding
was elevated. When myoblasts differentiated into myocytes,
^{125}I-IGF-I binding decreased whereas ^{125}I-insulin binding in-
creased.

To investigate the receptor specificity of ^{125}I-insulin and
^{125}I-IGF-I binding to BC3H-1 cells, competition-inhibition
studies were carried out (Fig. 4). Specific ^{125}I-insulin binding to
mature myocytes was inhibited by unlabeled insulin one-half-
maximally at ~ 1 nM; IGF-I only weakly interacted with the
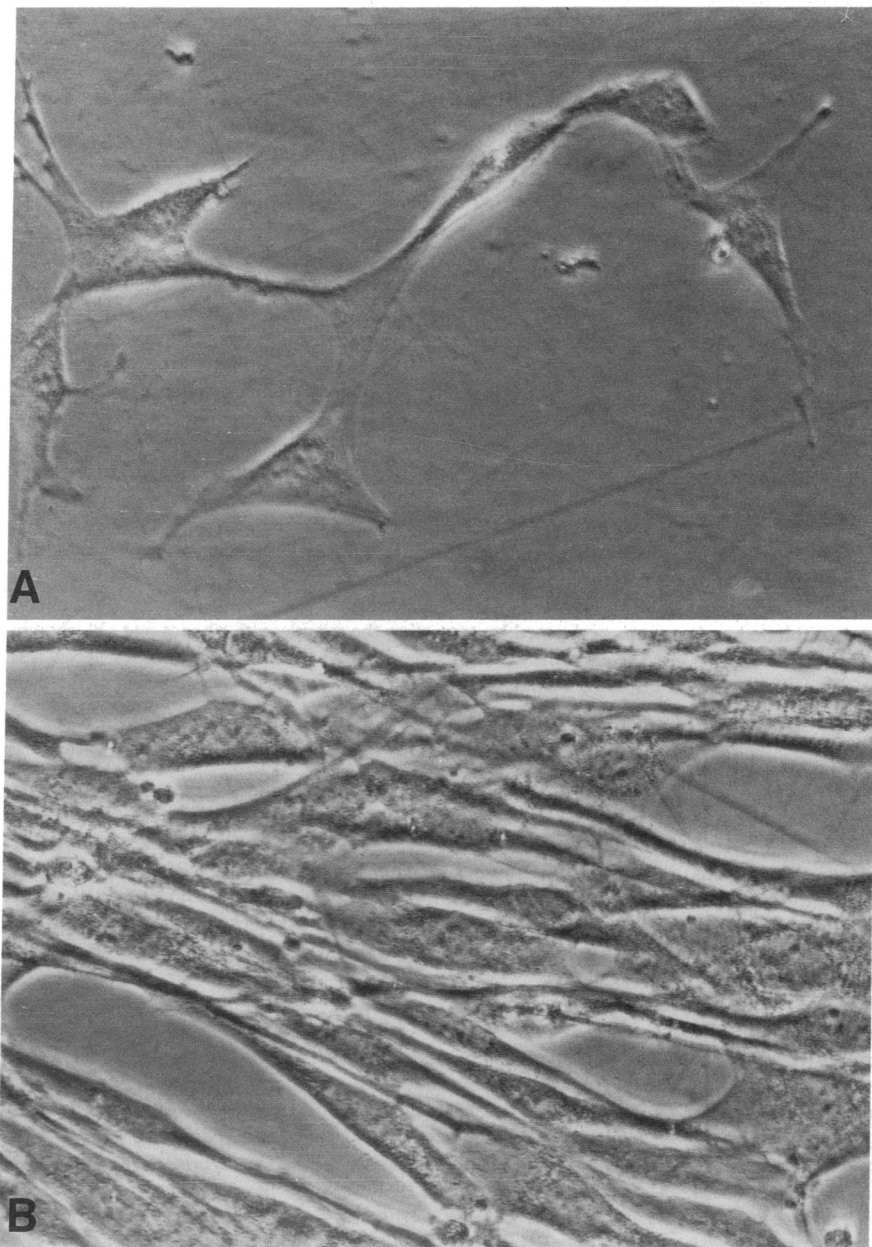insulin receptor. One-half-maximal inhibition of insulin bind-

Figure 1. Morphological characteristics of
BC3H-1 myoblasts and myocytes. Cells were
placed in 35-mm tissue culture dishes at 6
$\times 10^3$ cells/cm^2 and cultured either in DME
H-21 containing 20% FBS (to produce myo-
blasts) or DME H-21 containing 1% FBS for
16 d (to produce myocytes). Photographs
show the morphology of (*A*) myoblasts and
(*B*) myocytes. In *A*, the cells are separated
and have a flat morphology with numerous
small processes extending from the cell sur-
face, whereas in *B*, the cells are at con-
fluency and have a spindly morphology typi-
cal of differentiated myocytes. Cultures were
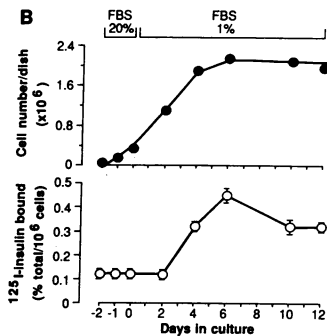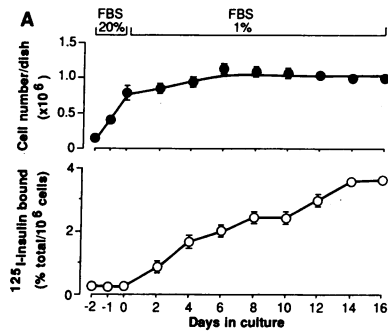photographed with an inverted Nikon phase
microscope. $\times 40$.

Figure 2. Effect of differentiation on insulin binding to receptors in (A) BC3H-1 and (B) C2 muscle cells. Cells were placed on 35-mm tissue culture dishes at 6×10^3 cells/cm^2 in DME H-21 plus 20% FBS. On day 0, cells were incubated with medium that contained 1% serum and cultured for up to 12–16 d. Specific ^{125}I-insulin binding was expressed per 10^6 cells. The cell number per dish as a function of time was determined by cell counting after dissociation with 0.25% trypsin and 0.5% EDTA at 37°C. Values are the mean±SEM for three separate experiments.

Figure 4. Specificity of insulin and IGF-I binding to their receptors in BC3H-1 myocytes. Cells were plated in 30-mm tissue culture dishes and specific ^{125}I-insulin and ^{125}I-IGF-I binding was then measured. Binding is expressed as a percentage of either maximal specific ^{125}I-insulin or ^{125}I-IGF-I binding. Each point is the mean of two separate experiments performed in triplicate.

ing occurred at > 100 nM IGF-I. IGF-I inhibited the binding of labeled IGF-I to its receptor with a one-half-maximal effect occurring at ~ 10 nM. Insulin was significantly less potent at inhibiting IGF-I binding.

Effect of differentiation on insulin receptor content of myocytes. The possibility was considered that ^{125}I-insulin binding to receptors was increased in mature myocytes because differentiation either changed the affinity of the insulin receptor, or altered insulin receptor compartmentalization such that receptors that were previously unavailable for hormone binding in myoblasts became available for binding in myocytes. To study the effect of BC3H-1 differentiation on insulin receptor affinity, Scatchard analyses were performed (Fig. 5). Binding
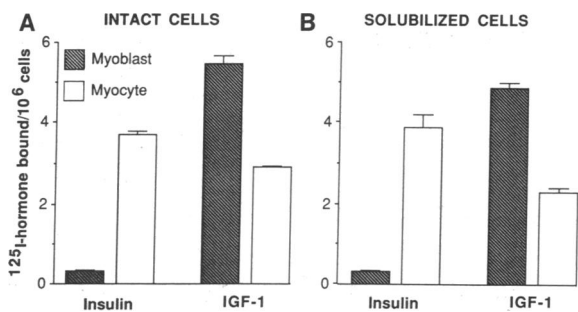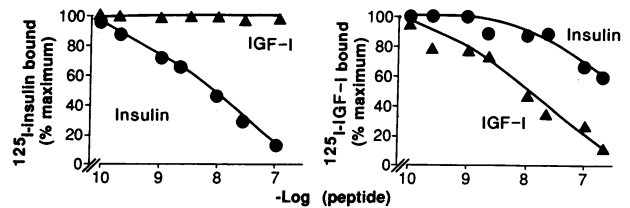
of insulin to both myocytes and myoblasts was best fit by a two-site model. Differentiation increased the number of insulin receptors in both classes of binding sites (high affinity, 26,000±3,000 binding sites per cell in myocytes, 1,000±120 binding sites per cell in myoblasts; low affinity, 300,000±20,000 binding sites per cell in myocytes, 20,000±2,000 binding sites per cell in myoblasts). In contrast, affinity was not markedly altered (high affinity 0.28±0.06 nM in myocytes, 0.25±0.09 nM in myoblasts; low affinity 56.6±15 nM in myocytes, 18.8±4.72 nM in myoblasts).

To measure total cellular insulin binding sites, myocytes and myoblasts were solubilized and ^{125}I-insulin binding was carried out on solubilized fractions (Fig. 3 B). Insulin binding to solubilized myoblasts was low and very similar to that of intact myoblasts. After differentiation, there was a 5–10-fold increase in the binding of ^{125}I-insulin to solubilized myocytes. For comparison ^{125}I-IGF-I binding was studied in solubilized cells. IGF-I binding was higher in myoblasts than in myocytes.

An alternative approach to measuring insulin receptor content was carried out by using a recently described radioimmunoassay of the insulin receptor (23). Solubilized extracts of myocytes were sevenfold more potent in displacing ^{125}I-insulin receptor binding from the antibody than were solubilized extracts of myoblasts (Fig. 6). By radioimmunoassay it was estimated that myoblasts had ~ 1 ng of receptors per 10^6 cells whereas mature myocytes had 7 ng of receptors per 10^6 cells.
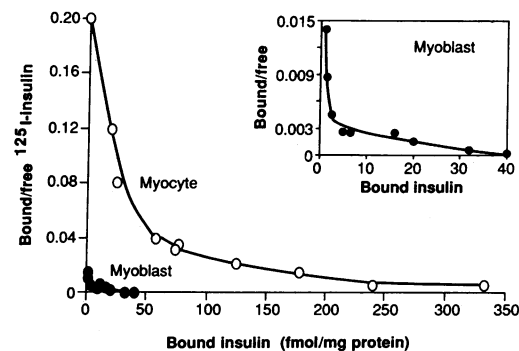
Figure 3. Comparison of insulin and IGF-I receptors in BC3H-1 myoblasts and myocytes. Cells were placed on 60-mm tissue culture dishes and cultured for 3 or 16 d in DME H-21 supplemented with either 20% or 1% serum, respectively. Specific ^{125}I-insulin and ^{125}I-IGF-I binding expressed per 10^6 cells was determined in myoblasts and myocytes both (A) in intact cells and (B) with soluble receptors. Each bar is the mean±SEM of triplicate dishes of three separate experiments.

Figure 5. Scatchard plot of insulin binding to BC3H-1 myoblasts and myocytes. Specific ^{125}I-insulin binding was measured, in the presence of increasing concentrations of insulin (to 1 μM). Each point is the mean±SEM of three separate experiments. Inset shows the binding data for myoblasts with an expanded scale.
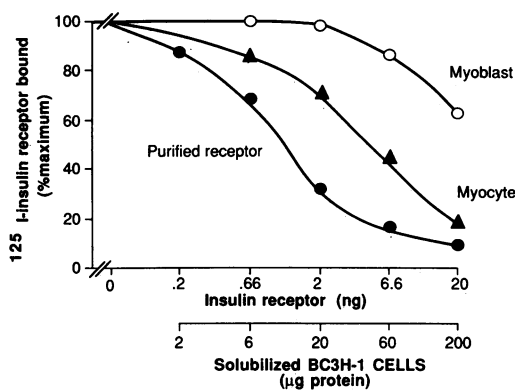
Figure 6. Insulin receptor radioimmunoassay in myoblasts and BC3H-1 myocytes. Ability of increasing concentrations of solubilized mouse muscle insulin receptors to compete with labeled insulin receptor for antireceptor antibody. (●) Insulin receptor standard, (○) myoblasts, (▲) myocytes. A representative of three experiments is shown.

Insulin receptor biosynthesis. To study whether the increase in insulin receptor content was due to a change in receptor biosynthesis, labeling studies were carried out with [^{35}S]methionine. In these studies myoblasts and myocytes were labeled for 4 h with [^{35}S]methionine, the cells were solubilized, and the insulin receptor was immunoprecipitated with two potent polyclonal antireceptor antisera. Studies with both antisera indicated that differentiation was associated with a marked increase in the biosynthesis of the receptor α and β subunits (135 and 95 kD, respectively) (Fig. 7 *A*).

Insulin receptor mRNA levels. To determine whether the increase in insulin receptor biosynthesis was associated with an increase in insulin receptor mRNA levels, slot blot and Northern blot analyses were carried out. Total RNA was extracted from myoblasts and myocytes and slot blotted, and the filters probed with labeled insulin receptor cDNA (Fig. 8 *A*). Differentiation was associated with an approximately sixfold increase in insulin receptor mRNA content. Other filters were also probed with a DNA probe for the 18S ribosomal RNA. As previously reported, differentiation was associated with a decrease in ribosomal RNA content (Fig. 8 *B*) (14, 17).
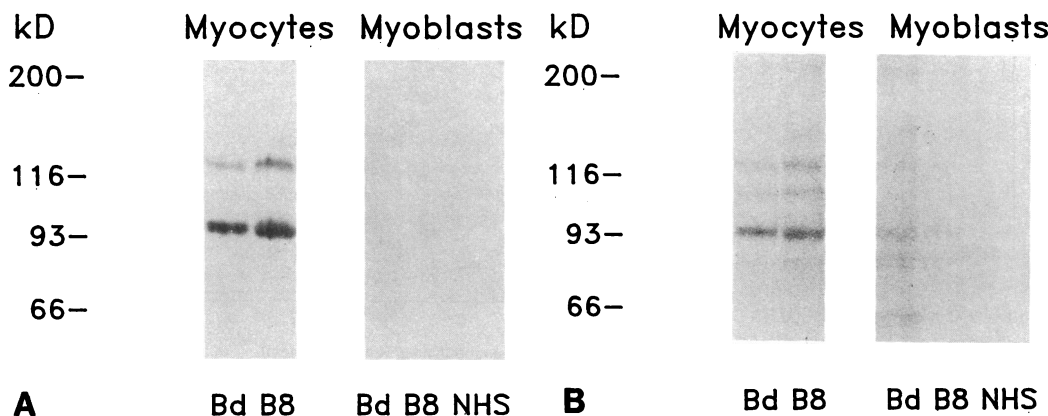
Insulin receptor mRNA consists of several species of different molecular sizes (7, 8, 10, 27). To determine whether differentiation was associated with qualitative difference in insulin receptor mRNA, poly(A)$^+$ RNA was prepared from myoblasts and myocytes. The poly(A)$^+$ RNA was then subjected to agarose gel electrophoresis followed by transfer to nitrocellular filters. The filters were then probed with labeled insulin receptor cDNA. In the BC3H-1 cells, two major bands were seen on autoradiography representing the two major species of insulin receptor mRNA at 7.0 and 9.5 kilobases (Fig. 9). Differentiation into myocytes was associated with a significant increase in both species. In contrast, a probe for β actin, a protein known to decrease with muscle differentiation (33), showed a decrease in mRNA levels (data not shown).

Studies with C2 cells

C2 myoblasts, unlike BC3H-1 myoblasts, fuse when they differentiate (19). In these cells, when the media was changed from 20% FBS to 1% FBS the cells differentiated. With differentiation insulin binding increased from ~ 0.12±0.01% of total radioactivity per 10^6 cells ($n = 3$, mean±SEM) to approximately 0.45±0.03% (Fig. 2 *B*). In contrast to BC3H-1 cells, however, C2 cells continued to grow for several days in 1% FBS. Specific ^{125}I-insulin binding for C2 cells was typically 10–15% that of BC3H-1 cells.

In the C2 cells, as in BC3H-1 cells, there was an increase in insulin receptor biosynthesis as determined by [^{35}S]-methionine labeling studies (Fig. 7 *B*). Furthermore, C2 insulin receptor mRNA levels, as determined by slot blot analysis, increased after differentiation (Fig. 8 *B*).

Discussion

In the present study we have employed two lines of cultured muscle cells, BC3H-1 and C2, to study the effect of muscle differentiation on insulin receptor expression. In both cell lines, myoblasts differentiated into myocytes upon reaching confluence and having the serum concentration reduced from 20% to 1%. BC3H-1 cells are derived from a nitrosoethylurea-induced brain neoplasm (18) and the exact cell of origin is unknown. These cells have the major morphological properties of smooth muscle cells but also have some properties of

Figure 7. Biosynthesis of insulin receptors in myoblasts and myocytes in both (*A*) BC3H-1 and (*B*) C2 cells. Flasks (75 cm^2) of cells were washed twice in PBS. 5 ml of methionine-free medium was added and the pulse commenced by the addition of 1 mCi [^{35}S]methionine. After 4 h of incubation at 37°C, the cells were then washed with PBS, collected by scraping, and solubilized in 1% Triton X-100. The insulin receptor was partially purified over a wheat-germ agglutinin column and immunoprecipitated with either two different polyclonal antireceptor antisera (*Bd* and *B8*) or normal human serum (*NHS*). The immunoprecipitate was then reduced with dithiothreitol, denatured with SDS and subjected to electrophoresis on a denaturating PAGE and autoradiography (24).

A   Insulin Receptor mRNA   Ribosomal RNA   **Kb**

1.25   2.5 μg   2.5   5.0 ng

Myoblast

Myocyte

9.5—
7.5—

B   Insulin Receptor mRNA   Ribosomal RNA

1.25   2.5 μg   2.5   5.0 ng
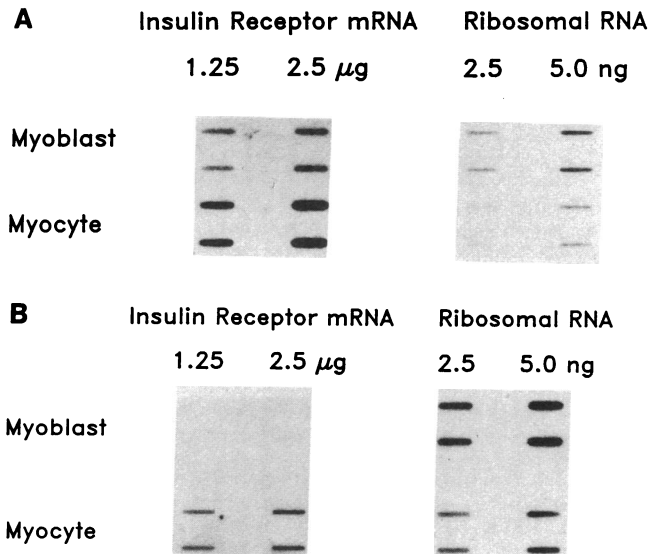
Myoblast

Myocyte

Figure 8. Slot blot analysis of insulin receptor mRNA and ribosomal RNA. Total RNA was isolated from either (*A*) BC3H-1 or (*B*) C2 cells and analyzed in duplicate.
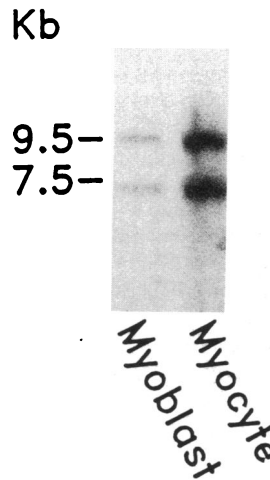
Figure 9. Northern blot analysis of insulin receptor mRNA. Poly(A)$^+$ RNA was purified from myoblasts and BC3H-1 myocytes and analyzed. An RNA ladder was used for molecular weight standards.

skeletal muscle such as the opposition of the sarcoplasmic reticulum with invaginations of the cell membrane (18). In these cells α actin is of the smooth muscle type. BC3H-1 cells do not fuse after differentiation.

C2 cells were derived from muscle cells of a normal mouse and have the characteristics of skeletal muscle (19). In contrast to BC3H-1 cells, these cells irreversibly fuse after differentiation. In both BC3H-1 and C2 cells differentiation is accompanied by morphological and biochemical changes (18, 19). Typical muscle enzymes and proteins present in normal muscle, such as creatine phosphokinase, myosin and α actin (14, 33, 34), increase during differentiation. Cell surface receptors, such as the nicotinic acetylcholine receptor (17, 35), also increase.

In both cell lines, we found that differentiation was associated with an increase in insulin receptor binding. Because BC3H-1 cells had more insulin receptors in the differentiated state than C2 cells, they were studied more extensively. Several lines of evidence indicated that the increased insulin binding seen after differentiation reflected an increase in receptor biosynthesis.[2] First, Scatchard analysis and radioimmunoassay indicated an increased receptor content. Secondly, pulse labeling of cellular proteins with [35S]methionine followed by immunoprecipitation with antireceptor antisera indicated that receptor biosynthesis was markedly enhanced. Thirdly, insulin receptor mRNA levels were increased.

The insulin receptor has structural similarity to the IGF-I receptor (32). Since IGF-I receptors were known to be present in BC3H-1 cells, we also examined IGF-I binding in myocytes

and myoblasts both in intact cells and in solubilized receptors. In contrast to insulin receptors (which increased during differentiation), IGF-I receptors were expressed at their highest level in myoblasts and decreased by 50% after differentiation. Similar changes in insulin and IGF-I receptors were also seen in rat L-6 muscle cells (36). The precise nature of this decrease remains to be established.

To study insulin receptor gene expression, myoblast and myocyte mRNA were isolated and analyzed by both Northern and slot blot analysis. During the terminal differentiation of BC3H-1 and C2 myoblasts, there was an increase in the levels of insulin receptor mRNA. The molecular mechanism involved in the increase in insulin receptor mRNA accumulation during myogenic differentiation is unknown. Control of insulin receptor mRNA in BC3H-1 and C2 cells most likely occurs at the level of mRNA transcription, however posttranscriptional processes such as selective mRNA transport from the nucleus or differential mRNA stability (and/or turnover) may also occur. Regulation of muscle gene expression has recently been studied (35, 37). With the use of purified cDNA probes, it has been shown that the accumulation of mRNAs for the thick and thin filament proteins of the contractile apparatus is coordinated with myoblast fusion. Studies of gene transcription have suggested that this mRNA accumulation was induced by new mRNA synthesis. Furthermore, studies of acetylcholine receptor expression in C2 myocytes have demonstrated an increase in gene transcription (35).

Muscle tissue is a predominant peripheral site of insulin action and insulin resistance in muscle is a feature of several pathological states including non–insulin-dependent (type II) diabetes mellitus and obesity (38, 39). However the in vitro regulation of insulin sensitivity in muscle remains to be studied. Thus, studies of insulin receptor expression during muscle cell differentiation in cell types such as those used herein may be useful in providing insights into the development of insulin resistance in these and other pathological states.

Acknowledgments

2. In BC3H-1 cells it has been reported that after placing differentiated myocytes into media containing high serum concentrations, the cells rapidly dedifferentiate into myoblasts (15). In preliminary studies, we find that 24 h after placing myocytes into culture medium with 20% FBS, insulin receptor biosynthesis decreases by ~ 50% with a concomitant decrease in ^{125}I-insulin binding.

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