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IMPAIRED INSULIN RECEPTOR PHOSPHORYLATION IN SKELETAL MUSCLE MEMBRANES OF DB/DB MICE : THE USE OF A NOVEL SKELETAL MUSCLE PLASMA MEMBRANE PREPARATION TO COMPARE INSULIN BINDING AND STIMULATION OF RECEPTOR PHOSPHORYLATION

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SUMMARY: A method has been developed to isolate skeletal muscle plasma membranes from mice in good yield without harsh extraction procedures. The method involves perfusion of mouse hindquarters with a calcium-deficient buffer containing collagenase and hyaluronidase. This is followed by gentle disruption, filtration, and differential centrifugations. The entire procedure takes about six hours and the yield is approximately 4 mg. protein from 10 g. equivalent of hindquarter muscle. The preparation contained predominantly plasma membranes based on specific activities of marker enzymes, electron microscopic data, and specific binding sites for insulin and a -adrenergic ligand. Studies using such preparations from lean, 4-5 week old and 12-20 week old db/db mice showed marked reduction in the phosphorylation of the 95 kDa subunit of the insulin receptor of the obese mice with no change in insulin binding. In addition, there was a progressive reduction in insulin sensitivity in stimulating receptor phosphorylation in the db/db mice.

INTRODUCTION: Plasma membrane isolation from skeletal muscles for biochemical studies often involved lengthy extraction of tissue homogenates using solutions containing high concentrations of salt followed by lengthy sucrose gradient centrifugation. Various modifications of this procedure yielded plasma membranes of varying degrees of purity (1-6). These preparations have been used for characterization studies of plasma membrane enzymes such as (Na^+ ,K^+)ATPase (6,7), and adenylate cyclase (4,8), and membrane transport processes (5). Plasma membrane protein yields varied from 16 mg/ 100 g muscle (6) in enriched fractions, to 2.7 mg/ 50 g. muscle tissues of highly purified plasmalemmal vesicles (5).To study the role of the skeletal muscle in the etiology of insulin resistance in the obese mouse model, we needed to prepare skeletal muscle plasma membranes of good quality and in adequate yield to allow examination of plasma membrane-associated parameters such as insulin binding, insulin-receptor linked protein phosphorylation, and the activity of the glucose transporter. This

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communication describes (i) the method of isolation and characteristics of such a plasma membrane preparation, and (ii) the use of such preparations to compare insulin binding and receptor phosphorylation in the insulin resistant obese-diabetic mice.

MATERIALS AND METHODS

Isolation of muscle membrane vesicles. Procedures for the perfusion of mouse hindquarters were those described previously (9), except for the following modifications. The perfusion medium used was made up of Krebs Ringer bicarbonate buffer with $CaCl_2$ omitted, and the inclusion of 1 mM EDTA. Hindquarters were perfused at 1 ml/min for 15 min. without recirculation of perfusate. At this point collagenase and hyaluronidase were added to a final, respective concentration of 0.15%. Flowrate was then increased to 2 ml/min and perfusion was continued for 30 min with recirculation. The hindquarters at this point appeared grossly edemic. Skins were then removed and hindleg muscles were carefully excised and transferred to a petri dish containing a small volume of an ice-cold solution of 1 mM $NaHCO_3$, pH 7.4, 2% BSA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 10 ug/100 ml.each of aprotenin and leupeptin (Solution A). Bones and fat tissues were carefully removed and muscles were teased into fine pieces using surgical forceps. The volume of this muscle suspension was brought up to 25 ml. per hindquarter with cold solution A, and transferred to a Nalgene flask, which was then shaken at 120 strokes/min at 4° C for 15 min. The suspension was filtered through coarse nylon filter and centrifuged at 100 x g for 1 min. The resultant pellet containing predominantly broken myocyte fragments but devoid of 5'-nucleotidase and adenylate cyclase activities, was discarded. The supernatant was centrifuged at 5000 rpm in a Beckman B2-21 centrifuge for 10 min. The supernatant from this step was centrifuged at 105,000 x g for 30 min. The resultant pellet was washed 2 times with cold solution of 50 mM Tris buffer, pH 7.5 containing 1mM PMSF and 1mM dithiothreitol (DTT)(Solution B), rapidly frozen in liquid N_2 and stored at -70° C. Unless otherwise stated, all membrane preparations were suspended in a solution (Solution C) containing Hepes (50 mM), pH 7.6, and 0.5 mM PMSF, to protein concentrations of 1-2 mg/ml, for use in studies described below.

Determination of marker enzyme activities. Adenylate cyclase (E.C.4.6.1.1) activity assayed in the presence and absence of GTP and epinephrine, 5'-nucleotidase (E.C.3.1.3.5) , and N-acetylglucosamine galactosyl transferase (E.C.2.4.1.13) activities were determined as described previously (10). (Na^+ ,K^+)ATPase (E.C.3.6.1.3) activity was measured using the method of Reddy et al (4). K^+-stimulated p-nitrophenyl phosphatase (E.C.3.1.3.1) activity was assayed essentially as described by Kidwai et al (11).

Chromatographic purification of plasma membranes for insulin receptor studies. Muscle membranes from 16 mice were suspended in 2 ml of cold buffer containing 50 mM Hepes (pH 7.6), 1 mM PMSF, 1% Triton X-100 and 10 ug/ml leupeptin (Solution D) using a hand-driven Potter-Elvejhem homogenizer. Solubilization of the membrane suspension and the subsequent wheat germ lectin (WGA) purification was carried out by the method of Burant et al (12). Routinely, six-500 ul fractions from the WGA column were collected. Insulin binding activity was present mainly in fractions 2 through 5. These fractions were pooled and used for insulin binding and receptor phosphorylation experiments.

Binding of [^{125} I]-insulin, [^{125}I]-iodocyanopindolol, and [^{3} H]cytochalasin B. Binding of [^{125} I]-insulin was carried out according to the procedure described by Burant et al (12). Specific binding of the β-antagonist [^{125} I]-iodocyanopindolol was performed as described previously (13). Binding of [^{3}H]cytochalasin B was performed as described by Klip and Walker (14).

Plasma membrane phosphorylation, SDS-polyacrylamide gel electrophoresis and autoradiography. Phosphorylation of endogenous membrane proteins were carried out by incubating 50 ug membrane protein or WGA-purified preparations in a total volume of 65 ul of solution B containing magnesium acetate (5 mM), manganese acetate (2.5 mM), ATP (100 uM), and 3 uCi [γ-^{32} P]ATP, at 30° C for 10 min. The reaction was terminated by the addition of 65 ul of SDS-sample solution (15), and heated at 100° C for 5 min. SDS-polyacrylamide gel electrophoresis of these samples were performed according to the method of Laemmli (15), using 4% stacking gel and 7.5% resolving gel. Gels were then stained with Coomassie blue, destained and dried on a gel dryer (Bio-Rad). Phosphorylated proteins were detected by autoradiography at -70 °C with Kodak X-Omat AR film and Cronex lightning plus enhancing screen (Dupont).

RESULTS AND DISCUSSION: Plasma membranes isolated from various animal tissues have been extensively used to elucidate hormone and neurotransmitter actions which relate to interaction between these agents and their specific cell-surface receptors and, in some instances, subsequent modulation of plasma membrane associated biochemical events. Among the animal tissues frequently studied, the skeletal muscle has proved to be one of those less amenable to such an approach. The problem frequently encountered is in obtaining a plasma membrane fraction reasonably free of contaminations from structural components of myofibrillar origin, and membranes derived from cytoplasmic organelles. In addition, adequate disruption of the tissue by conventional methods was prevented by the extensive network of collagen encasing the muscle fibers. Over the last two decades, several laboratories, using a variety of approaches based on the theme of high salt extraction and lengthy sucrose gradient centrifugation, have succeeded in obtaining skeletal muscle plasma membrane preparations of varying degrees of purity (1-6). The yields in all instances were very low. Prompted by the good quality of plasma membranes prepared from intact hepatocytes (16) and adipocytes (17) isolated by collagenase digestion, we attempted to explore the possibility of preparing skeletal muscle membranes by perfusion of mouse hindquarters with collagenase and hyaluronidase. Our primary objectives were to prepare skeletal muscle plasma membranes in good yield and of acceptable quality to study both structural and functional aspects of the skeletal muscle insulin receptor in insulin resistant states. In this regard, this appraoch would allow studies focused on the cell surface-associated pool of the insulin receptor.

Activities of several plasma membrane marker enzymes, as well as that of 5'-nucleotidase, a less specific marker enzyme, are presented in Table 1. Specific activity of adenylate cyclase is comparable to that reported by Engel and co-workers (4,18), but lower than that found in highly purified membranes by Seiler and Fleischer (5). Conditions of assay in the latter report were not described however. Activity of this enzyme was increased three fold by GTP, and nearly ten fold by GTP plus epinephrine (Table 1, legend). Compared to those reported by Kidwai et al (2), and Schapira et al (3), our membrane preparation has higher 5'-nucleotidase activity while those of (Na^+, K^+)ATPase were comparable. The K^+-stimulated p-nitrophenyl phosphatase, which is a partial reaction of the (Na^+,K^+)ATPase, found in this preparation was predominantly ouabain sensitive. These comparisons suggest that our membrane preparation is rich with membranes of surface origin. Data in Table 1 also revealed minor contaminations of mitochondria, sarcoplasmic reticulum, and golgi membranes.

Table 1 : Activities of several membrane associated enzymes in mouse skeletal muscle membrane preparations

ENZYME	ACTIVITY
Adenylate cyclase [a]	23.2 ±0.11
5'Nucleotidase [b]	22.1 ±1.5
(Na^+ ,K^+)ATPase [c]	6.0 ±0.9
K^+-stimulated phosphatase [d]	500 ±25
Galactosyl transferase [e]	5.17 ±0.75
NADPH Cyt. c reductase [f]	0.025
NADH Cyt. c reductase [g] (rotenone insensitive)	3.02

a - pmoles cAMP formed/ min. mg. protein. Activities with GTP (1 mM) were 79.5 ±0.52, and with GTP + epinephrine (1 uM) were 237 ± 2.41.
b - umoles AMP converted/ hr. mg. protein.
c - umoles [γ^{32} P]ATP hydrolyzed/ hr. mg. protein.
d - nmoles p-nitrophenyl phosphate hydrolyzed/ hr. mg. protein.
e - UDP-galactose N-acetylglucosamine galactosyl transferase activity : pmoles substrate converted/ min. mg. protein.
f and g - Activities are expressed in Units, defined by Schapira et al. (3) as the amount of protein that caused a change of 1.0 O.D. per min. at 550 nm.

All data presented are averages of at least three preparations.

Morphological verification of this membrane preparation was carried out by electron microscopy (Fig. 1). The membrane preparation contains predominantly vesicles of heterogeneous sizes, ranging from roughly 0.1 to 1.0 u. This bears remarkable resemblance to the plasma membrane (F1) fraction isolated by Kidwai et al (2). Minor contamination with vesicles containing cristae-like internal structural elements (presumably mitochondria) can be visualized. The electrophoretic mobility profile of proteins separated by SDS-polyacrylamide gel electrophoresis (Fig. 2) is similar to that reported by Reddy et al (4) for their plasmalemmal preparation. Major protein bands of approximately 50-, 65-, and 100-kDa were also found in highly purified skeletal muscle plasma membrane preparations (5).

The conclusions drawn based on enzyme marker profile gains further support from surface membrane ligand binding studies. Binding sites for a β-adrenergic ligand were shown to reside predominantly in the plasmalemmal fraction (10). We found our membrane preparation to be rich in specific binding sites for the selective β-adrenergic antagonist [^{125}I]CYP, with a Bmax of 84 fmol/mg protein, a value comparable to that reported by Reddy and Engel (19) in their plasmalemmal fraction, and a K_d of 0.57 nM. These findings, in conjunction with data on adenylate cyclase (Table I), demonstrate the presence of functional β-adrenoreceptors coupled to their effector system, i.e., adenylate cyclase. D-glucose transporter was measured using

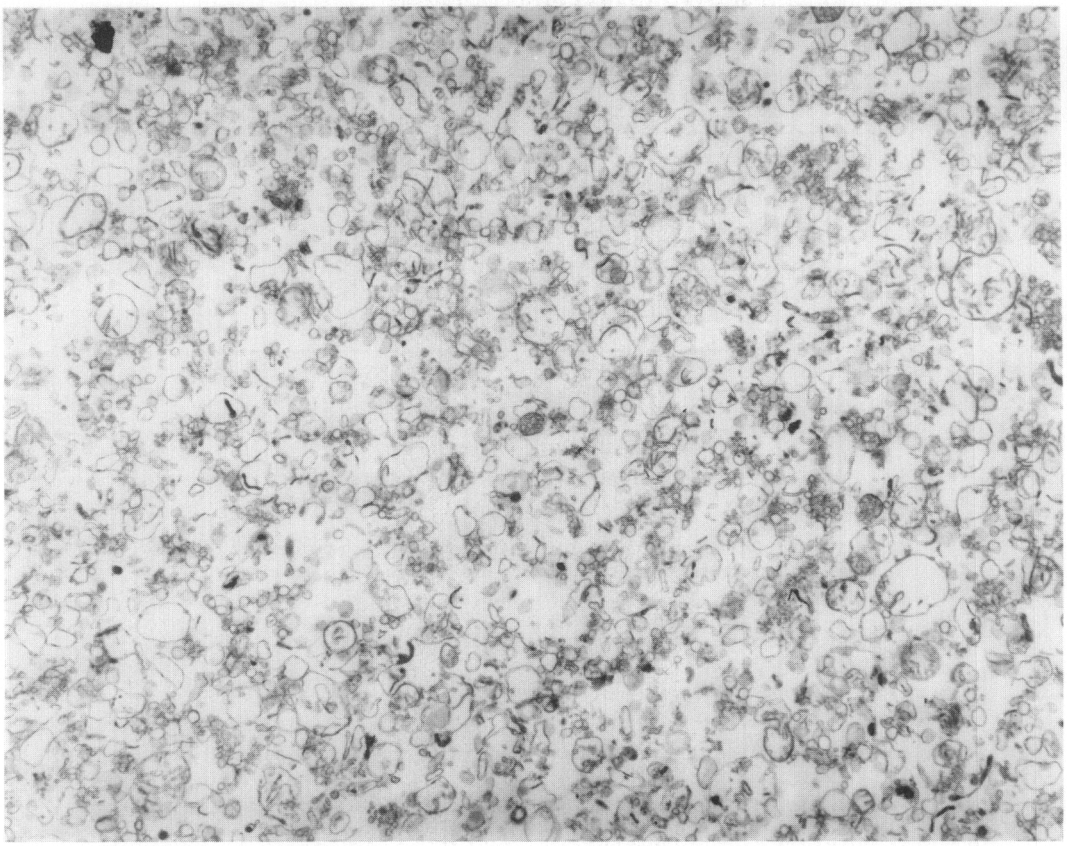

Figure 1 : Electron micrograph of mouse skeletal muscle membranes. Magnification shown is 13,500 X.

[^{3}H]cytochalasin B binding. The number of D-glucose-inhibitable [^{3}H]cytochalasin B binding sites in our preparation was 402 fmol/mg protein, which is in the range of those observed by Klip and Walker in their F1 fraction of the rat skeletal muscle homogenate (14), one containing plasma membranes and light membranes of T tubule origin.

The skeletal muscle is one of the key target tissues for insulin actions. Recent studies using plasma membranes from livers, adipose tissues and erythrocytes have provided significant insight relating to the molecular structure and functions of the cell surface insulin receptor. Similar information for the skeletal muscle is limited. Specific binding of ^{125}I-insulin in our plasma membrane preparation was 50% of total binding. Chromatographic purification using WGA-agarose resulted routinely in a 10-fold purification and a yield of 30% of specific insulin binding activity. A 10-fold purification of the human placental insulin receptor was reported by Fujita-Yamaguchi et al (20) using WGA-Sepharose. Scatchard analysis of binding data yields a

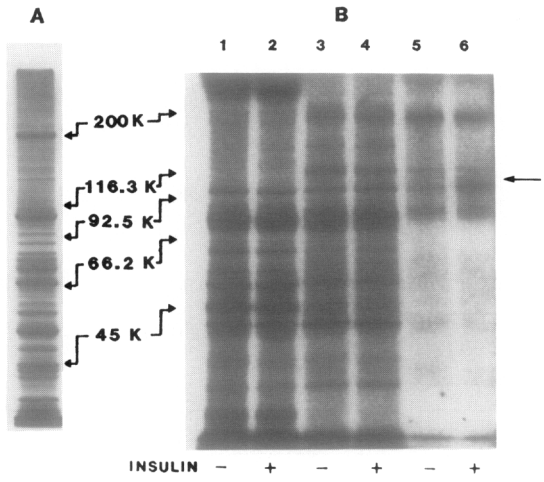

Figure 2 : Electrophoretic mobility profile of mouse skeletal muscle membrane proteins, and the effect of insulin on their phosphorylation by endogenous protein kinases. Lane A shows a typical profile of Coomassie Blue stained membrane proteins separated by SDS-gel electrophoresis. Lanes B1 through B6 are autoradiograms of phosphoproteins in crude membranes (lanes 1 and 2), Triton-solubilized membranes (lanes 3 and 4), and WGA-purified membranes (lanes 5 and 6). Each preparation was incubated with (+) or without (–) 10 mU/ ml insulin for 30 mins. at 24 C prior to incubation with [γ^{32}P]ATP as described in the methods. The arrow on the far right of the figure indicates a phosphoprotein band corresponding to apparent molecular weight of 95,000 daltons.

curvilinear plot with binding constants (K_d) of 2.58 x 10^{-9} M and 4.5 x 10^{-9} M and maximal number of binding sites of 1.43 and 12.1 pmol/ mg protein respectively. Along with the purification of insulin specific binding activity, insulin-stimulated protein phosphorylation can also be demonstrated in our preparation (Fig. 2). At least ten distinct phosphoprotein bands were observed in the crude membrane preparations, most of which were not affected by Triton solubilization. Protein phosphorylation in these preparations was nonetheless unaffected by insulin. In contrast, incubation of the WGA-purified preparation with insulin for only 30 min. resulted in significant increase in phosphorylation of a band of protein(s) with an apparent molecular weight of 95,000 dalton which corresponds to that of the β-subunit of the insulin receptor. Recent reports (21,22) strongly implicated this receptor subunit phosphorylation as an integral component of the biological function of the insulin receptor. Plasma membranes were thus prepared from lean, 4-5 week-old, and 12-20 week-old obese-diabetic (db/db) mice to correlate insulin receptor binding and phosphorylation with insulin biological actions which we previously described (9,23) in the development of skeletal muscle insulin resistance. Biochemical characteristics of all three preparations were similar except for adenylate cyclase activity which was 75% and 60% of the lean control in young and old db mice

Table 2 : Insulin binding and receptor phosphorylation activities in crude, and wheat germ -agglutinin (WGA) purified skeletal muscle membranes from lean, young and old db/db mice

	Lean	db/db (4-5 wks)	db/db (12-20 wks)
	Insulin binding (fmol/mg protein)		
Crude membrane	53 ± 5	61 ± 6	30 ± 4
WGA-purified	338 ± 50	274 ± 43	385 ± 48
	95 kDa Subunit phosphorylation (% of lean)*		
WGA-purified	100	50.4	39.5

Data presented are averages ±SEM of three experiments.
* Quantitation of phosphorylation of the 95 kDa subunit of the insulin receptor was carried out by densitometric scanning of the autoradiograms described in legend of Fig. 3.

respectively. Specific binding of insulin in membranes from the mildly hyperinsulinemic young db mice (9) was unaltered while that in membranes from the older, severely hyperinsulinemic db mice decreased to 62% of the lean control (Table 2). This is the first report of an unaltered insulin binding to a target tissue in the obese-diabetic mouse at this age. It is also important to point out that the status of insulin binding in the two age groups of db mice shown here reflect predominantly those of the plasma membrane origin. Insulin binding to WGA-purified receptors showed no significant difference among the three preparations (Table 2). Contrary to insulin binding, phosphorylation of the 95 kDa peptide in the absence of insulin was 50% and 39% of lean controls in the young and old db mouse preparations respectively. Furthermore, results in Figure 3 showed marked reductions in insulin sensitivity to stimulate receptor phosphorylation. Half-maximal effective concentration of insulin decreased from 3 nM in the lean to 8 nM and 10 nM in the young and old db mice respectively. This observed shift in insulin sensitivity in the obese mice was highly reproducible. The maximal stimulation was about 2-fold in the lean but only 70% in both the obese mouse preparations indicating a decrease in insulin responsiveness in this aspect of insulin action. These shifts in insulin sensitivity and responsiveness in receptor phosphorylation closely parallel those we previously described for insulin stimulation of glucose transport in the perfused hindlimb muscle of the two age groups of db mice (23). Our data also demonstrated a dissociation between insulin binding and stimulation of receptor phosphorylation as well as glucose transport in the young db mice. A similar defect in the insulin receptor kinase has been described for monocytes

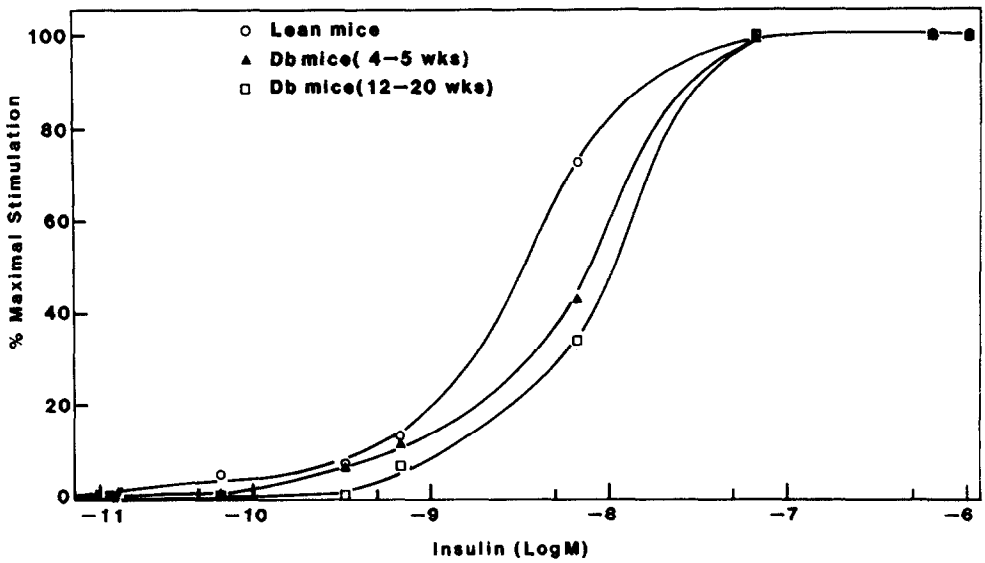

Figure 3 : Concentration-dependent stimulation of the phosphorylation of the 95 kDa subunit of the insulin receptor by insulin. WGA-purification, phosphorylation, electrophoresis, and autoradiography of all three preparations were performed simultaneously, with identical protein concentration. Autoradiography of radioactive gels from the three praparations were carried out for the exact same length of exposure. Data presented are based on densitometric determination of the phosphoprotein bands on the autoradiograms corresponding to a molecular weight of 95,000 Da. Results are from a typical experiment which has been repeated twice.

which had normal insulin binding activity from an insulin resistant patient (24). A recent study using gold thioglucose-induced obese mice (25), in contrast, showed a parallel decrease in both insulin binding and receptor kinase activity at a non-insulin resistant state, and a marked reduction in insulin responsiveness but not in sensitivity when the animal became insulin resistant. However, these investigators used whole homogenates of soleus muscle purified by WGA-lectin as a receptor source and a synthetic substrate was used to assay kinase activity (25). These and our studies suggest key differences between genetically transmitted and chemically induced insulin resistance in the relationship between insulin binding and post-receptor activity ,in particular receptor phosphorylation.

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