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The Role of the Insulin Receptor in Skeletal Muscle Insulin Resistance: Evaluation of Two Animal Models

Charles F. Burant
Medical University of South Carolina

THE ROLE OF THE INSULIN RECEPTOR IN SKELETAL MUSCLE INSULIN RESISTANCE: EVALUATION OF TWO ANIMAL MODELS

by

Charles F. Burant

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TABLE 2. Taken from Reference 1.

Table 1. Stable changes in enzyme and carrier activity following short term exposure of tissues to insulin

Enzyme or carrier	Tissue	Change in activity	Possible mechanism	References
Glucose uptake				
Glucose transport	W, (SM, H)	increase (V*)	? translocation	26–30
Cyclic AMP metabolism				
Phosphodiesterase (low K_m)	W, L	increase (V*)	? (W); phosphorylation (L)	34–44
Protein kinase (Cyclic AMP dep.)	W, SM	decrease (V^+ in W; Ka* in SM)	association of R and C subunits	45–48
Glycogen metabolism				
Glycogen synthase	H, SM, W, L	increase (K_a*)	dephosphorylation	49–54, 86
Phosphorylase kinase	L	decrease (V^+)	dephosphorylation	51, 55
Phosphorylase	W, L	decrease ($K_a{}^+$)	dephosphorylation	52, 53, 55–57, 86
Glycolysis and pyruvate oxidation				
Pyruvate kinase	L, W	increase ($K_s{}^+$ in L; K_s* in W)	dephosphorylation (L); ? (W)	58–62
Pyruvate dehydrogenase	W, B, L (MG)	increase (V*)	dephosphorylation	63–71
Lipid metabolism				
Acetyl CoA carboxylase	W, B, L	increase (V*)	polymerisation and phosphorylation	71–82
Hydroxymethyl glutaryl CoA reductase	L	increase (V*)	dephosphorylation	83, 84
Triacylglycerol lipase	W	decrease (V^+)	dephosphorylation	85–86

Column 2 and elsewhere: *W*, white fat; *B*, brown fat; *H*, heart muscle; *SM*, skeletal muscle; *L*, liver; *MG*, mammary gland. Column 3: *V*, change in V_{max}; K_s, change in K_m for substrate; K_a change in sensitivity to appropriate activator; * change in activity most marked in absence of other hormones; $^+$, change in activity most marked under conditions of increased cell cyclic AMP concentrations

CHARLES F. BURANT. The Role of the Insulin Receptor in Skeletal Muscle Insulin Resistance: Evaluation of Two Animal Models. (Under the direction of MARIA G. BUSE.)

The role of the insulin receptor in muscle insulin resistance was studied. Denervation of skeletal muscle resulted in the rapid appearance of a decrease in basal and insulin stimulated glucose transport, glycogen synthesis and a defect in the activation of glycogen synthase which was independent of levels of glycogen in the muscle. The effect was seen in both predominantly red and predominantly white muscle. There was no change in the denervated muscles' sensitivity to epinephrine-mediated decrease in glycogen synthesis or glycogen content, nor was there a major change in the insulin binding capacity of denervated muscles.

A technique was developed to investigate the properties of the insulin receptor solubilized from skeletal muscle. Freezing hindlimb muscles with subsequent powdering and homogenization with buffered Triton X-100 produced a fraction with specific insulin binding. Further purification on agarose-bound wheat germ agglutinin yielded insulin receptors with intact insulin binding, as well as insulin stimulatable autophosphorylation and exogenous substrate kinase activity. The autophosphorylation of the insulin receptor β-subunit in intact tissues was also demonstrated.

The binding and kinase domains of insulin receptors solubilized from denervated skeletal muscle were identical to that from contralateral controls. No significant changes were seen in the autophosphorylation of the β-subunit in intact, denervated solei. Injection of

insulin in the rat and subsequent partial purification of insulin receptors in the presence of phosphatase inhibitors allowed the demonstration of an increased kinase activity towards histone H_2b as compared to saline treated rats. The increase was due to an increase in tyrosine phosphorylation attributed to the activation of the insulin receptor kinase. Again, no change in the insulin receptors from denervated muscles was observed.

Streptozotocin diabetes resulted in an increase in specific insulin binding but a decrease in insulin-stimulatable autophosphorylation and tyrosine kinase activity. Structural analysis showed no change in the α-subunit but the β-subunit attained a component with a slower migration on electrophoresis. The β-subunit change was shown to be, in part, due to increased sialic acid content of the receptor subunit. The kinetic and structural changes were reversible with insulin treatment of the rat.

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Robert Lefkowitz has identified the attributes of the ideal scientific mentor. They include the ability to respond to new findings, to be creative and at times going outside the accepted scientific paradigms for explanations of data. The ideal mentor should be a strict taskmaster, blessed with an off-beat sense of humor, but at the same time very serious about research. The mentor should guide the student, but allow latitude for individual goals. He left out two important points. The mentor should be Hungarian and female. One of the better things that has happened to me is my "adoption" by Dr. Maria Buse. It will be my priviledge for the rest of my life to declare that I am an S.O.B. I hope our friendship and collaboration will continue for a long time to come. Thanks, Maria, for all the encouragement, advice and prodding you have given me.

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minimum of complaints, shared my happiness and frustrations and gave constant encouragement to be the best I could be. She has helped and supported me above and beyond what she probably should have. This dissertation is as much hers as it is mine, and it is to her I would like to dedicate this work.

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LIST OF ABBREVIATIONS

AII: Angiotensin II

DMSO: dimethyl sulfoxide

EDL: extensor digitorum longus

EDTA: ethyleledіamine tetraacetic acid disodium salt

Hepes: N-2-hydroxyethylpiperazine-n'-2-ethanesulfonic acid

KD: kilodalton

MW: molecular weight

PIC: protease inhibitor cocktail

PMSF: phenylmethylsulfonylfluoride

SDS-PAGE: sodium dodecylsulfate polyacrylamide gel electrophoresis

TCA: trichloroacetic acid

TIU: Trypsin Inhibitory Units

TLC: thin layer chromatography

TPCK: L-1-Tosylamide-2-phenylethyl chloromethyl ketone

WGA: agarose-bound wheat germ agglutinin

CHAPTER I: INTRODUCTION & LITERATURE REVIEW

Insulin has varied and profound effects upon its target tissue. Even with the immense amount of effort to elucidate the mode of action of insulin, the exact pathway leading from insulin binding to the changes in intracellular metabolism remain a mystery. Many theories on the mechanism by which insulin acts upon the cell, including searches for 'second messengers' analogous to cAMP have been proposed (Table 1). Most, however, have not withstood the test of time (1).

INSULIN MEDIATORS: Two areas of investigation into the mode of action of insulin have recently received much attention: the generation of small peptide mediators and the recently discovered kinase activity of the insulin receptor. This latter aspect is discussed in detail below. By taking extracts of cells treated with insulin, several investigators have been able to demonstrate insulin-like effects on certain enzyme systems in broken cell preparations (2,3,4,5). Two commonly used end point assays are the activation of pyruvate dehydrogenase and the inhibition of cAMP dependent protein kinase (6). Since the insulin-like activity of the substance is eliminated by protease treatment, the mediator is thought to be a protein. Gel filtration has shown that the molecular weight of the substance to be small - ~ 1000-2000 daltons (5). Further data revealed that some of the 'mediator' effects could be abolished by treatment with glycosidases (6), suggesting a glycoprotein nature of the substance.

Because cells treated with low concentrations of trypsin show insulin-like responses, and this same treatment can reproduce the generation of the small peptide 'mediators', Larner has proposed that insulin acts to produce these mediators by the initiation of a proteolytic event (6). However, due to complications such as multiple peaks of activity when the mediator extract is separated by chromatography, the nonspecificity of the end point assays and the fact that the exact chemical nature of the peptide has not been determined, it is difficult to assess at this time the merits of the mediator theory in insulin action.

GLUCOSE TRANSPORT: Upon exposure of a cell to insulin, one witnesses an increase in the influx of glucose. Glucose enters the cell by facilitated diffusion and the effect of insulin is to increase the Vmax of the transport reaction. In independent observations Suzuki and Kono (7) and Cushman and Wardzala (8) have provided a mechanism for the increase in glucose transport. By isolating plasma membranes from insulin treated cells they showed there is an increase in the ability of the membranes to transport glucose and bind [^{3}H]cytochalsain B, which tightly binds hexose transporters. In parallel to the observed increase in glucose transporters on the surface membranes there was a concommittant decrease in glucose transporters in the low density microsome fraction of these cells. These results suggested that insulin causes the translocation of the glucose transporters from the interior of the cell to the cell surface.

This redistribution theory has been elegantly confirmed by Oka and Czech (9). Cytochalasin B binds to the "inside" hexose binding site of the glucose transporter while the non-transportable glucose analogue ethylidene glucose binds to the "outside" hexose binding site and

changes the transporters conformation such that the "inside" site now loses its ability to bind the cytochalasin. Incubation of control cells with ethylidene glucose along with Cytochalasin B (which can be photo-crosslinked to the glucose transporter) allowed the demonstration of labeling of only the low density microsomal fraction of the glucose transporters. Furthermore, upon stimulation of the cells with insulin there was an increase in the number of photo-crosslinked transporters isolated from the plasma membrane.

In addition to increasing glucose transport insulin also causes the stimulation of intracellular glucose metabolism such as increases in glucose oxidation and glycogen synthesis as well as an increase in pentose shunt activity. The immediate effects of insulin on the intracellular metabolism of glucose occur primarily through the increase in activity of certain sets of enzymes and inhibition of others. For example, the increase in glycogen accumulation seen in cells after insulin treatment is due to the activation of the enzyme glycogen synthase which incorporates glucose into glycogen. Dephosphorylation of certain sites on the enzyme increases its affinity for glucose-6-phosphate which is an allosteric activator of the enzyme (10). The concentration of glucose-6-phosphate also increases in the cell as a result of the insulin stimulation of glucose transport. The enzyme phosphorylase, which breaks down glycogen into glucose monomers is inactivated in cells treated with insulin also by the dephosphorylation of sites on the enzyme (11). This cycle of phosphorylationdephosphorylation is repeated on numerous other metabolic pathways that are modulated by insulin (Table 2).

In addition to the effects on glucose metabolism, insulin also has effects on transport of amino acids, ion fluxes across cell membranes, the synthesis and degradation of protein and triglycerides and influences RNA synthesis (1). These effects of insulin are also seen relatively rapidly after insulin exposure and occurs at relatively low concentrations of insulin. This is in contrast to the growth promoting effects of insulin which require higher concentrations of insulin and occurs in a time frame of hours to days. This growth promoting effect of insulin may be mediated through a pathway distinct from that associated with the well-characterized changes in intermediary metabolism (see below).

INSULIN BINDING: Although the results of insulin exposure to cells are well-characterized, the mechanism by which insulin causes changes in the metabolic state of the target tissues are unknown. The initial event in insulin action is binding to specific cell surface receptors. The binding of insulin to this receptor is specific and saturable with a dissociation constant of approximately 10^{-12}M. The number of these high affinity receptors is low on most cells and in the most abundant case represents only 0.01% of the protein in plasma membrane preparations (12). Even with this small number of receptors it has been observed in a number of cell types that the maximal biological response can be elicited when only a fraction of the high affinity receptors are occupied (13). Binding isotherms of insulin to receptors on intact cells, on membranes and to receptors solubilized by the use of non-ionic detergents are non-linear when plotted by the method of Scatchard. Debate exists for the reason behind this curvilinearity, which may be due on one hand to the existence of two different classes of receptor molecules

that differ in their affinity for insulin or to a negative cooperativity in insulin binding where the bivalent receptor, after binding one insulin molecule, has a reduced affinity for the second. The negative cooperativity model was given strong experimental support by the experiments of DeMeyts (14) who showed that the dissociation of previously bound [^{125}I]insulin was increased in the presence of unlabeled insulin. Evidence also exists for the first model however. Pollet et al. (15) have demonstrated that the increase in the dissociation of labeled insulin from its receptor induced by added insulin to the media occurs at the same rate regardless of the degree of receptor occupancy. He also points out that univalent receptors such as the prolactin receptor also show curvilinear Scatchard binding profiles.

BINDING INDUCED CONFORMATIONAL CHANGES: The binding of insulin to its receptor appears to result in change in the conformation of the receptor. By the use of radiation inactivation it was shown that the occupied receptor was altered in rat liver membranes (16). The occupied receptor is more susceptible to the digestive actions of trypsin (17) suggesting a conformational change upon insulin binding that exposes trypsin sensitive sites. In a series of reports, Donner and co-workers have demonstrated a time and temperature dependent increase in insulin receptor affinity. They suggest that the receptor exists in two affinity states and that binding of insulin shifts the equilibrium towards the higher affinity conformation (18,19,20,21). In a recent report, this group used affinity cross-linking techniques to show that a biphasic pattern of trypsin sensitivity of the receptor occurred upon insulin binding. After an initial increase in trypsin sensitivity, components of the receptor became less trypsin labile. This shift in

protease sensitivity was correlated to an increase in the amount of insulin receptor in the higher affinity state (22).

LIGAND INDUCED AGGREGATION AND INTERNALIZATION: In an analogous fashion to that seen with surface immunoglobulin molecules which are exposed to antigen, the insulin receptor, upon binding insulin, aggregates to form patches and caps on the cell surface. This has been directly observed using fluorescent microscope techniques (23). The importance of microaggregation in the signalling of the receptor was demonstrated by the use of fragments of antibodies directed towards the insulin receptor. It was shown that bivalent Fab fragments of these antibodies were insulinomimetic in that they could stimulate the uptake of glucose into the target cells (in this case lymphocytes) but the monovalent Fab did not change glucose flux. The addition of bivalent antibodies towards the monovalent antibodies resulted in aggregation and an increase in glucose transport. Bivalent antibodies directed towards insulin were synergistic with insulin in eliciting a metabolic response (23,25).

Several morphological and biochemical studies have shown that the aggregated insulin receptor and associated insulin are internalized and the insulin subsequently degraded in a time and temperature dependent fashion. This is known as receptor mediated endocytosis and is common to many cell surface receptors. For low density lipoproteins, transferrin and certain toxins (i.e. tetanus toxin) this endocytotic event is required for these substances to alter cellular metabolism (26). The receptors for these substances are seemingly the shuttle for transfer of the ligands to the interior of the cell. In most cells the endosomes fuse with lysosome like vesicles (receptosomes) and the bound insulin is

rapidly degraded. The unoccupied receptor is then either degraded or recycled back to the surface of the cell (27,28). The exact role of the endocytotic event in peptide hormone action remains unclear. It has been suggested that some of insulin's effects may be mediated through binding of intracellular insulin receptors which reside on various organelles (29). This is unlikely in that substances which interfere with the internalization of insulin have little effect on insulin's biological effects (30). Furthermore, microinjected insulin had no insulin-like effects on the cells treated.

STRUCTURE OF THE INSULIN RECEPTOR: Various methodologies that have evolved over the last 15 years have allowed the determination of the structure of the insulin receptor. By using affinity chromatography after solubilizing membrane components in non-ionic detergents, Jacobs and Cuatrecasas were able to isolate receptor-rich fractions that were analyzed by gel electrophoresis (31). They identified a component of 135,000 daltons, but the affinity of the fractions isolated had a much lower affinity for insulin than the crude receptor preparation making interpretation of the data difficult. A major step in elucidating the structure of the insulin receptor was the development of ways to label the insulin receptor with [^{125}I]insulin either by the use of photo-activable insulin analogues (32) or by cross-linkage with chemical reagents such as disuccinimidyl suberate as used by Pilch and Czech (33). Combining these techniques with affinity chromatography allowed the identification of the molecular weight and subunit composition of the insulin receptor (33,34,35). As shown in Figure 1 the insulin receptor is considered to be a heterotetramer composed of 2 α and 2 β subunits of molecular weight of ~ 125,000 daltons and ~ 95,000 daltons

respectively. The subunits are held together by disulfide bonds. Using various concentration of reducing agents Massague and Czech (36) have shown that the disulfide bonds that link the β-subunits to the α-subunits are much stronger than the α-S-S-α bond. This proposed structure of the insulin receptor was confirmed in a report by Fujita-Yamaguchi (37) who used affinity chromatography to obtain an apparently homogeneously pure preparation of the insulin receptor. She identified subunits of 135,000 and 95,000 daltons and determined by peptide mapping that an often identified subunit of approximately 50,000 daltons was a proteolytic degradation product of the β-subunit.

On the basis of their relative labeling after cross-linkage of insulin, it has been suggested that the α-subunit of the insulin receptor is the primary binding subunit with perhaps only a small domain of the β-subunit involved in insulin binding (38). This is consistent with the orientation of the subunits in the membrane of intact cells. The α-subunit is sensitive to digestion when cells are treated with trypsin added to the media while the β-subunit remains intact (38). Likewise, the α-subunit is preferentially labeled when membrane proteins are surface iodinated with much weaker labeling of the β-subunit (39,40), indicating that the β-subunit has less of its protein sequence on the outer face of the cell. It has been shown that the β-subunit of the insulin receptor is very susceptible to proteolytic cleavage by elastase type enzymes (41). Using this property, Roth et al. (42) demonstrated that the β-subunit could be preferentially degraded while leaving the α-subunit intact. The α-subunit in these preparations could bind insulin nearly as well as the insulin receptor before degradation.

Since most, if not all surface proteins and receptors are glycoproteins(43), it is not surprising that the insulin receptor too is a glycoprotein. This was first assumed based on studies that showed that insulin binding to isolated adipocytes was severely impaired after treatment with neuraminidase and other glycosidases (44). Subsequently it was shown that concanavalin A and wheat germ agglutinin could modulate insulin binding (45) as well as specifically adsorb insulin binding activity when the lectins were immobilized on an insoluble matrix (46). Direct demonstration of the glycoprotein nature of the insulin receptor was carried out by Hedo et al. (39) by metabolically labeling IM9-lymphocytes with [^{3}H]monosaccharides and specifically immunoprecipitating the insulin receptor with anti-insulin receptor antibodies. The results presented suggest that both the α- and β-subunits contain complex carbohydrate side chains since all the major [^{3}H]monosaccharides known in complex carbohydrate sugar side chains were incorporated into both subunits. It appears that the α-subunit has a relatively greater abundance of galactose and N-acetylglucosamine residues whereas the β-subunit has a higher sialic acid content.

Tunicamycin, a potent inhibitor of core oligosaccharide addition to asparagyl residues (n-linked core glycosylation) effectively reduces cell surface insulin binding capacity (47) but has no effect on recycling of previously synthesized receptors (48). Furthermore, tunicamycin causes the accumulation of intracellular aglycoreceptor precursors which will appear on the cell surface after removal of tunicamycin from the media (49). Using pulse chase labeling techniques in 3T3-L1 adipocytes Ronnett et al. (50) have proposed a model for the synthesis and post-translational processing of the insulin receptor. They proposed

that in these cells both the α- and β-subunits are synthesized as a 180-190kDa pro-receptor peptide that is rapidly converted to a 210kDa core glycosylated peptide. Specific proteolytic cleavage forms an immature α(125kDa) and β(83kDa) subunit which through further side chain modifications (conversion from a 'high mannose' to a 'low mannose' state and subsequent addition of monosaccharides) are converted to the mature 135kDa α and 95kDa β subunits. This latter glycosylation event appears to occur immediately prior to the mature receptors' appearance on the cell surface. Some refinements of the results presented in this paper were provided in a report by Salzman et al. (51). Using different concentrations of the ionophore monensin, the investigators were able to block processing of the core glycosylated pro-receptor (210 kDa above) or with a lower concentration block the maturation of the immature forms of the insulin receptor subunits. They were able to show that the uncleaved, pro-receptor and the immature subunits could accumulate on the cell surface, suggesting that the signal for transit to the cell surface resides in the core peptide or the core glycosylated peptide. The 210 kDa peptide and the immature α-subunit were able to bind insulin and the former peptide and the immature β-subunit were able to autophosphorylate. The invesgigators in this report were also able to show that the mature α- and β-subunits were sensitive to the enzyme endoglycosidase H, suggesting that not all the carbohydrate side chains on the receptor subunits are processed to a 'low mannose' state.

Indirect evidence for the existence of possible O-link carbohydrate side chains on the insulin receptor β-subunit was provided by Herzberg et al. (52). Using both enzymatic and chemical deglycosylation methods, they were able to show that both the α- and β-subunits of the insulin

receptor were sensitive to digestion with endoglycosidase H and endoglycosidase F. The former endoglycosidase removes 'high mannose' carbohydrate side chains at the chitobiose core (the cleavage takes place between the two N-acetylglucosamine residues that are attached to the asparigyl group in N-linked glycoproteins) while endoglycosidase F removes both high and low mannose sugar side chains of most N-linked glycoproteins, also at the chitobiose core. The α-subunit was apparently completely deglycosylated by treatment with endoglycosidase F as judged by its mobility shift when compared to insulin receptors deglycosylated by chemical means (treatment in this case was performed with trifluoromethanesulfonic acid, TFMS) and resolved at a molecular weight of ~ 98 kDa. The β-subunit on the other hand did not achieve the same decrease in molecular weight after digestion with Endoglycosidase F as was observed after treatment with TFMS. This suggested to the authors that there may be O-linked oligosaccharide side chains on the β-subunit of the insulin receptor. Data relevant to this point are presented in Chapter 5 of this dissertation.

The role of the carbohydrate moiety of the insulin receptor in insulin binding and signal transmission has been studied by several groups (53,54,55). In general it was shown that removal of sialic acid residues with neuraminidase did not affect insulin binding. Cuatrecasas and Illiano (56) showed however that the treatment affected the ability of insulin to promote glucose transport and inhibit lypolysis. A recent report by Fujita-Yamaguchi (57) using highly purified insulin receptor preparations showed that the removal of the sialic acids from the receptor resulted in an increase in the binding activity and the tyrosine kinase activity of the insulin receptor (the tyrosine kinase activity of

the insulin receptor will be discussed in detail below).

Two groups have recently produced a cDNA clone of the insulin receptor which allowed the peptide sequence of the receptor to be deduced (58,59). The data suggest that the insulin receptor is synthesized as a single polypeptide and is post-translationally cleaved to form the α and β-subunits. This is exactly the results that are obtained in the pulse-chase studies of Ronnett et al. (50) and Salzman et al. (51). The predicted molecular weight of the unglycosylated α-and β-subunits are ~84,000 and ~70,000 kDa respectively. The α-subunit occupies the N-terminus of the precursor and is rich in cysteine residues that may compose a cross-linking domain on the external face of the membrane. This region is probably distinct from the insulin binding domain that also is predicted to lie in the C-terminus region of the α-subunit. At the end of the α-subunit coding sequences a run of basic amino acids are predicted which may code for the cleavage site of the polypeptide precursor which would then form α- and β-subunits. The β-subunit coding region predicts a transmembrane region and a concensus sequence for an ATP binding site and an area that may code for phosphotyrosyl transferase activity. It is of considerable interest that even though the two reported cloned insulin receptor cDNAs came from placental mRNA, the sequence that were obtained were different in 22 nucleotide residues and a block of additional nucleotides that were found in the Ebina paper that was not found in the Ulrich paper. The basis for these differences are not apparent. It is also of interest to note that while the Ulrich et al. found 15 possible glycosylation sites on the α-subunit, Ebina et al. found only 13, while on the βsubunit 7 or 4 poten-

tial glycosylation sites were quoted by the groups respectively. Part of these differences are due to differences in the predicted amino acid sequence and to the assessment of the groups of asparagine residues that are potentially glycosylated. Because of the absence of a hydrophobic domain in the α-subunit that is long enough to span the membrane, Ulrich et al. (58) have suggested that the α-subunit is entirely extracellular and anchored to the cell via disulfide linkages with the β-subunit. The schematic representations of the insulin receptor as derived from sequence data is presented in Figure 2.

By comparing predicted sequences, it was noted that there is a great deal of homology between the insulin receptor and the EGF receptor and, in the region that codes for the phosphotransferase activity, there is a great deal of homology with the src family of oncogene products. The consequences of these homologies will be discussed below. Jacobs et al. have recently published data on the structure and synthesis of the IGF I receptor (60). It shows that there is remarkable structural similarities between the receptors for IGF I and insulin. The hormones themselves are also homologous (61) and they can cross react, with lower affinity, with each others' receptors. These observations have lead to the suggestion that the growth promoting effects of insulin may be mediated through the IGF I receptor.

INSULIN RECEPTOR KINASE ACTIVITY: Perhaps the most important recent discovery in the field of insulin research is the identification of the insulin stimulated protein kinase activity that appears to be an intrinsic part of the insulin receptor. The distinguishing feature of the insulin receptor associated protein kinase is that the phosphoryl-transferase reaction uses tyrosine as the acceptor amino acid (62,63).

This tyrosine kinase activity was first recognized as a unique feature of the transforming gene product of the Rous sarcoma virus (64,65) and has been identified as a feature of many of the transforming peptides associated with retroviruses (64). In most cases the protein carrying out the phosphotransferase activity are themselves substrates for their own kinase activity (i.e. undergo autophosphorylation). Evidence suggests that this tyrosine kinase activity is related to the transformation of cells infected with these viruses. The tyrosine content of cells that are transformed with the retroviruses increases substantially (65). Rous sarcoma virus that is temperature sensitive for the induction of the transformed phenotype also show a temperature lability of the tyrosine kinase activity associated with the transformation associated peptide, pp60src (66). Mutation of the gene that codes for the tyrosine kinase containing peptide such that the tyrosine kinase activity is abolished (as assessed by in vivo tyrosine content of the cells thus infected and by in vitro assays) show a marked decrease or an elimination of the transforming activities of the altered viruses (65,66,67). A recent report (68) demonstrated that the addition of vanadate ion, a potent inhibitor of phosphotyrosyl phosphatases, to the culture medium of several different cell types resulted in the morphological transformation of these cells. The transformation was dose-dependent and correlated with an increase in the phosphotyrosine content of the cells. Thus, it seems that there ia evidence that the phosphorylation of tyrosine is correlated with cell multiplication and growth.

The fact that the transforming proteins of the retroviruses originated in the genome of eukaryotic cells strongly suggests that the tyrosine kinase activity is important for normal cellular growth and

differentiation. It is then perhaps not surprising in retrospect that many of the surface receptors for polypeptide hormones contain tyrosine kinase activity. Tyrosine kinase activity as an integral aspect of a normal cellular component was first identified in the epidermal growth factor (EGF) receptor (69) and has since been identified in the receptors for the platelet derived growth factor receptor (70), the insulin like growth factor I receptor (71) and as mentioned above, the insulin receptor.

The tyrosine kinase activity of the insulin receptor has been well-characterized and has been identified in numerous tissues (Table 3). The initial characterization of the skeletal muscle insulin receptor was carried out as part of this dissertation (Ref. 72) and the data is presented in Chapter 3. The autophosphorylation event is seen in both the intact tissues as well as in the detergent solubilized system (62,63) and occurs on the β-subunit which also contains the ATP binding site of the receptor (80,81). Evidence that the β-subunit is the actual moiety that carries out the phosphotransferase reaction comes from the experiments of Roth et al. (42) who preferentially digested the β-subunit of the insulin receptor and showed while binding remained intact the ability of the solubilized receptor to act as a kinase was eliminated. In the solubilized receptor the autophosphorylation is stimulated by insulin binding, is ATP dependent, requires Mn++ of Co++ cations and occurs almost exclusively on tyrosine residues (76). Avruch et al. (76) have shown that the autophosphorylation event parallels insulin receptor occupancy and is saturated when insulin binding is saturated. In an elegant series of experiments White et al. (82) showed that insulin's effect on the reaction is to increase the Vmax while the

required cation reduces the Km. Using High Performance Liquid Chromatography (HPLC) to separate peptides generated by trypsin digestion of the autophosphorylated receptor, this group showed that the solubilized receptor contained approximately 5 sites that were phosphorylated in response to insulin. Using a similar system Yu and Czech (78) identified 3 sites on the β-subunit that was labeled by $\gamma[^{32}P]$ATP in response to insulin.

In addition to its ability to autophosphorylate, the insulin receptor, in the solubilized system, has an insulin stimulatable exogenous substrate kinase activity. First identified by Rosen et al. (83) a wide variety of substrates have been identified for the tyrosine kinase of the solubilized insulin receptor system (Table 4). While many of these substrates are obviously not possible intracellular targets of the insulin receptor, they serve as useful tools in examining the exogenous substrate kinase activity of the insulin receptor. The identification of physiologically relevant intracellular targets for the insulin receptor has been elusive. The activation of the exogenous substrate kinase activity of the insulin receptor is dependent on the autophosphorylation of the insulin receptor β-subunit (78,84) and is thought to be due to the phosphorylation of a specific tyrosine residue on the insulin receptor β-subunit (78). The cation requirement of the exogenous substrate kinase activity is complex and seemingly depends upon the substrate, the phosphoacceptor, as well as on the concentration of ATP used in the kinase reactions (90,91). As expected from their similarity in structure and function, the properties of the EGF receptor and the IGF I receptor associated kinases are very similar to those of the insulin receptor (Table 5).

In the intact cell, the observed insulin stimulated autophosphorylation of the insulin receptor β-subunit is different than that seen in the solubilized system. The formation of phospho-tyrosine, serine and threonine is observed in the immunoprecipitated insulin receptor β-subunit after insulin stimulation. Recently it has been shown that the labeling of the serine and threonine residues are likely due to other kinases, distinct from the insulin receptor, and the initial autophosphorylation events occur almost exclusively on tyrosine residues (92). In this same report it was demonstrated that insulin receptors that were phosphorylated on serine residues prior to insulin binding were poorly labeled on tyrosine subsequent to insulin binding. This was done by first precipitating the solubilized proteins from the labeled cells with anti-phosphotyrosine antibodies and then precipitating with anti-insulin receptor antibodies. Within seconds after the exposure of the cells to insulin, the proteins that were precipitated with the anti-phosphotyrosine sera were almost exclusively labeled on tyrosine with the content of phosphoserine and phoshhothreonine increasing with longer incubations. On the other hand the receptors precipitated with anti-insulin receptor serum after the first immunoprecipitation were labeled almost exclusively on serine residues.

The modulation of insulin receptors by serine phosphorylation was directly demonstrated by White et al. (93) wherein treatment of hepatoma cells with the phorbolester 12-0-tetradeconoyl phorbol acetate (TPA) resulted in decreased phosphorylation of the insulin receptor on tyrosine residues upon exposure to insulin. It had previously been shown (94) that the treatment of lymphocytes with TPA resulted in a decrease in insulin receptor affinity. It is interesting to note that Pessin and

Czech (95) have recently reported that the treatment of fat cells with isoproterenol (a β-adrenergic agonist) greatly reduces the affinity of insulin receptors for insulin and in a preliminary report Czech has stated that this treatment decreases the ability of the insulin receptor to autophosphorylate (62) possibly due to the cAMP mediated phosphorylation of the insulin receptor. It has been previously demonstrated that cAMP mediated hormones have the ability to phosphorylate the EGF receptor (96).

White et al. (97) compared the sites of autophosphorylation on the insulin receptor β-subunit in the intact cell as compared to the solubilized receptor. In the hepatoma cell line employed insulin was able to induce the autophosphorylation 3-4 fold. The increase in labeling occurred on all the phospho-accepting amino acids. Time course analysis showed that the stimulation of tyrosine phosphorylation was at steady state after 20 sec and that no change in the amount of phosphoserine or phosphothreonine occurred during this initial stage of autophosphorylation. The phosphotyrosine content of the receptor remained constant for one hour. Two in vivo sites of tyrosine phosphorylation on the β-subunit were autophosphorylated, one minor site corresponded to the major site seen in vivo, while a major site seen in the in vitro system was a minor site observed in vivo. One of the sites autophosphorylated in the solubilized system did not occur in the whole cell labeling experiments. This report points out that while there are similarities in the tyrosine autophosphorylation event in the intact cell and in the solubilized system, there are also differences that could make interpretation of experiments performed in vitro difficult.

INSULIN RESISTANCE: This dissertation is concerned with the role of the insulin receptor in insulin resistance. As seen in the above discussion, the insulin receptor is a complex molecule that has two domains, a binding domain which presumably lies in the α-subunit, and a kinase domain which lies in the β-subunit. It is not then surprising that pathologies exist that show alterations in one or another of these functional domains of the insulin receptor.

Flier (98) has defined insulin resistance as "a state in which a given concentration of insulin produces less than the expected response". Two experimentally defineable endpoints of insulin action can be used to quantitate insulin resistance, (a) the dose of insulin that produces half-maximal response and (b) the maximal effect achievable with the hormone. Kahn (99) has proposed that an alteration in the dose to give half maximal effect is defined as a change in sensitivity whereas a change in maximal achievable effect is a change in responsiveness.

A wide variety of physiological and pathological states exist which manifest themselves, at least in part, with alterations in the insulin receptor (Table 6). The most common alteration of the insulin receptor is down-regulation that occurs in the hyperinsulinemic state. With chronic exposure to insulin in the plasma, the number of insulin receptors decreases on the cell surface without an apparent change in the affinity of the insulin receptors which remain. Using lymphocytes this decrease in insulin receptor number was shown to be due to an increase in the rate of degradation with no alteration in the rate of synthesis (100). This is a common occurence and is a major part of the insulin resistance which is seen in obesity.

Experimental studies with obese animals have shown that all tissues respond to hyperinsulinemia with a decrease in receptor numbers and that the decrease is proportional to the degree of hyperinsulinemia (101). This decrease in insulin receptors, by mass action should only result in a shift in the dose response curve i.e. a decrease in sensitivity. However, in adipocytes there also develops a decrease in responsiveness. The nature of this defect is unclear but experiments directed towards understanding this phenomenon have recently been carried out by Garvey et al. (102). They showed that concommittant with a decrease in surface insulin receptors in adipocytes chronically exposed to insulin there occurred a decrease in the number of surface glucose transporters. These "co-down-regulated" transporters could not be recruited to the cell surface upon further stimulation of the adipocytes with insulin. This suggests that in the case of glucose transport the mechanism of down-regulation of the receptors is related to the insulin resistance seen in these cells. The relationship of down-regulation and defects in intracellular glucose metabolism is still to be determined.

In many patients obesity is associated with the development of non-insulin dependent (type II) diabetes. These patients have fasting hyperglycemia and normal or high fasting insulin levels that are sub-normal after the administration of an oral glucose load (103). These subjects have approximately the same degree of impairment of insulin binding as compared to obese individuals but a greater defect in insulin action thus the appearance of overt diabetes (104). That fibroblasts from obese type II diabetics bind and respond to insulin normally after culturing (105) suggests that the defect is due to an alteration in the metabolic milieu of the subject rather than a genetically defined defect

expressed constitutively in all cells.

A rare subset of patients with severe insulin resistance have a defect in the action of insulin at the cellular level. Characterized as a syndrome with virilization, polycystic ovaries and acanthosis nigricans, most of these patients have a genetic defect in the structure of the insulin receptor which results in defective insulin binding (106) or decreased or eliminated insulin receptor kinase activity (73,107). This has been designated the Type A syndrome of insulin resistance. Patients with Type B syndrome exhibit the acanthosis nigricans and rheumatic type symptoms but not the virilization of the Type A patients and are characterized by a high titer of anti-insulin receptor antibodies (106).

There has also been described a defect in the action of insulin in insulinopenic diabetes mellitus (Type I diabetes). A discussion of this problem and our experiments in trying to understand this is contained in Chapter 5 of this dissertation.

DENERVATION INDUCED INSULIN RESISTANCE: Patients with peripheral neuropathies or paralysis often have glucose intolerance (108,109). In early work from this laboratory, it was shown that the glucose intolerance could come from an alteration in the ability of skeletal muscle to respond to insulin. Buse and Buse (110) showed that in the absence of insulin there was no change in xylose uptake of denervated diaphragms but the response of the denervated diaphragms to insulin was significantly less than controls. This effect was seen within twelve hours after denervation. Using relatively unrefined techniques there was no demonstrable change in insulin binding to the denervated muscle. In a later report it was shown that in addition to decreased hexose transport, the denervated diaphragm was also resistant to insulin stimulated

accumulation of glycogen (111). Recently, numerous reports have appeared in the literature investigating the effects of denervation on glucose and protein metabolism in the denervated skeletal muscle (112-118). Our own efforts in this regard have been published (118) and are contained in Chapter 2 of this dissertation.

Our observations and those of others on the insulin resistance of skeletal muscle following denervation can be summarized as follows: 1) The ability of skeletal muscle to transport glucose, both basal and insulin stimulated, is decreased and a change in both sensitivity and responsiveness is observed. 2) There is a decrease in basal and insulin stimulated glycogen synthesis. 3) As expected, due to decreased glucose transport, there is a decrease in glucose-6-phosphate levels. 4) There is a decrease in the active form of glycogen synthase and a decrease in the activation by insulin which is independent of the decrease in glucose-6-phosphate concentration. 5) There is an impairment of pyruvate dehydrogenase activity. 7) Both fast twitch, white and slow twitch, red skeletal muscle are affected. The effect of denervation has been seen in the soleus, diaphragm, extensor digitorum longus, the levator ani, and the epitrochlearis muscles. 8) Even with the severe insulin resistance there is no apparent effect of denervation on the muscles' ability to respond to epinephrine as assessed by a decrease in glycogen synthesis and increase in glycogenolysis in response to epinephrine.

Identical results have been observed in the immobilized muscle (119,120). In this latter case it should be noted that the electromyogenic activity of immobilized muscles drops to 15-20% of control levels and thus, is very similar to the denervated state. Using the immobilized muscle system, Butler and Booth (121) saw a decrease in the

basal rate of protein synthesis in the extensor digitorum longus and the soleus muscle after 24 hrs but did not observe any impairment of insulin stimulated increase in the rate of synthesis, although in absolute terms the immobilized leg still synthesized significantly less protein than the contralateral control muscle. This raises the interesting possibility that the pathway leading to an alteration in glucose transport and intracellular metabolism, thus the differences in the two system's response to denervation.

In contradistinction to the decrease in action of insulin on denervated skeletal muscle the exact opposite is true of exercised muscle. There is an apparent insulin-like effect of exercise on glucose transport (122). In addition to increasing the basal state of these activities (which apparently can occur in the absence of insulin (125), exercise also increases the sensitivity of muscle to insulin (122-124).

Changes which occur in the membrane of denervated skeletal muscles may have some bearing on the insulin resistance. There is a rapid appearance of extra-junctional acetylcholine receptors (126,127) which is due to an increase in the transcription of a subclass of these receptors (128). There is an increase in phospholipid turnover but no change in the total content of phospholipids (129). The membrane content of glycoproteins are increased as assessed by concanavalin A, ricin, and wheat germ agglutinin binding (130). The activities of sialyl-galactosyl- and N-acetylglucosaminyltransferases in the sarcolemmal fraction was increased while there was a decrease in fucosyl-glucoprotein transferase. The changes were observed to be due to an increase in the Vmax of the enzyme with no change in affinities. There is no change in the membrane associated Na/K ATP ase activity seen after

denervation (130), however, a decrease in the resting membrane potential of denervated muscles has been described (131).

Even with these alterations in the membrane of skeletal muscle after denervation, there has been no observable change in insulin binding to its receptor after denervation that could account for the severe insulin resistance (114,118,132). Thus, two groups have suggested that the insulin resistance be classified as a 'post receptor' defect (114,132). This may have been premature considering the above mentioned cases of normal insulin binding associated with severe insulin resistance and defects in the insulin receptor kinase activity. Studies investigating the possible alteration in activation of the insulin receptor kinase in denervated skeletal muscle are contained in Chapter 4 of this dissertation.

In summary, the mode of insulin action is unknown but the effects of insulin on many systems affected by insulin treatment are beginning to be well characterized. These include the glucose transport system, the various phosphorylation-dephosphorylation cascades and the insulin receptor associated tyrosine kinase activity. Inroads are also being made in elucidating the mechanism of various insulin resistant states (i.e. obesity, Type A and Type B syndromes of insulin resistance) and into the structural characteristics of the glycoprotein insulin receptor. In this dissertation I present data on the role of the insulin receptor and associated kinase activity in two models of insulin resistance. In Chapter 2 I present the characterization of the insulin resistance seen shortly after denervation. In Chapter 3 I present a method that was developed for the purification of functionally active insulin receptors from skeletal muscle. In Chapters 4 and 5 this method

was used to investigate what, if any, role the insulin receptor plays in the insulin resistance seen in denervated skeletal muscle and in diabetic skeletal muscle from rats with insulinopenic diabetes.

TABLE 1. Taken from Reference 63.

Proposed second messengers for insulin action
Cyclic nucleotides (cAMP, cGMP)
Monovalent cations; altered membrane potential
Calcium^{2+}
H_2O_2
Membrane phospholipids
Degradation fragments of insulin
Peptides of membrane origin that modify protein kinases and alter phosphorylation

TABLE 3

Tissues and Cells where Insulin Receptor and Associated Kinase Have Been Identified

Tissue or Cell	Species	Reference
Fibroblasts	Human	73
Erythrocytes	Human	73
Lymphocytes	Human	74
Monocytes	Human	75
Placenta	Human	76
Skeletal Muscle	Rat	73
Liver	Rat	77
Adipocyte	Rat	78
Brain	Rat	79

TABLE 4

Identified Substrates for the Insulin Receptor Kinase

Substrate	Reference
Angiotensin II	84
Casein	85
Histone H^2b	86
Src related peptide	86
Tyrosine containing synthetic peptides	86
Fodrin	87
Tubulin	88
Microtubule-associate proteins	88
Actin	89

TABLE 6. Taken from Reference 63.

Alterations in insulin receptors in disease states

	Receptor number	Receptor affinity
Type II diabetes mellitus	↓	—
Obesity	↓	—
Uremia	↓	—
Insulin resistance and acanthosis nigricans	↓	—
Acidosis	—	↓
Glucocorticoid excess	—	↓
Antireceptor antibody	—	↓
Lipoatrophic diabetes (some)	—	↓
Leprechaunism (some)	—	↓
Growth hormone excess	↓	↑
Insulinoma	↓	↑
Obese–72 hr fast	↓	↑
Lipoatrophic diabetes (some)	—	—
Leprechaunism (some)	—	—
Variants, Type A syndrome	—	—

FIGURE 1. Taken from Reference 62.

Proposed structural features of the heterotetrameric insulin receptor complex. See text for detailed description of supporting data.

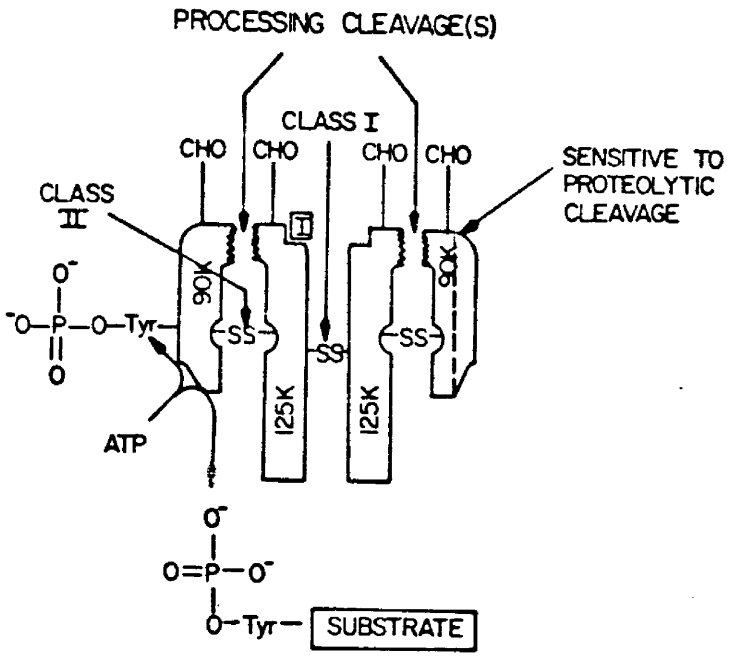
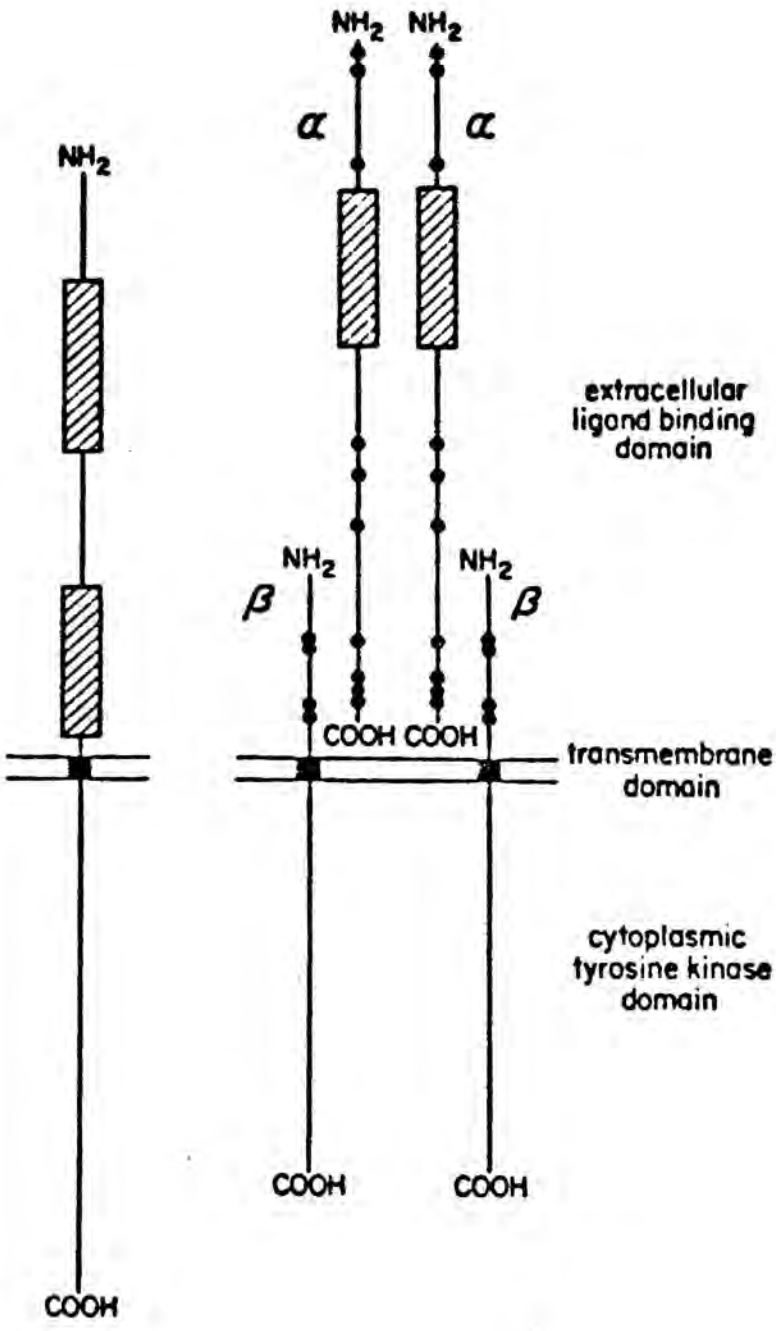

PROCESSING CLEAVAGE(S)
CLASS I
CHO
CHO
CHO
CHO
SENSITIVE TO PROTEOLYTIC CLEAVAGE
CLASS II
90K
90K
125K
125K
SS
SS
SS
ATP
O⁻
O=P—O⁻
O—Tyr—
SUBSTRATE

FIGURE 2. TAKEN from Reference 58.

Schematic comparison of insulin and EGF receptors. Regions of high Cys-residue concentration are shown as hatched boxes, transmembrane domains as black boxes and single cysteine residues, possibly involved in formation of the α_2-β_2 insulin receptor complex, as black circles.


NH2
NH2 NH2
α α
NH2 NH2
β β
COOH COOH
COOH
COOH
COOH
extracellular ligand binding domain
transmembrane domain
cytoplasmic tyrosine kinase domain

REFERENCES:

1. Denton, R.M., R.W. Brownsey and G.J. Belsham. "A Partial View of Insulin Action." Diabetologia 21; 347-362, 1981.

2. Larner, J., G. Galaski, K. Cheung, A.A. DePaoli-Roach, and A.A. Huang. "Generation by Insulin of a Chemical Mediator that Controls Protein Phosphorylation and Dephosphorylation." Science 206; 1408-1410, 1979.

3. Seals, J.R. and L. Jarett. "Activation of Pyruvate Dehydrogenase by Direct Administration of Insulin to an Isolated Plasma Membrane/ Mitochondrial Mixture: Guidance for Generation of Insulin's Second Messenger in a Subcellular System." Proc. Natl. Acad. Sci. (USA) 77; 77-81, 1980.

4. Cheng, K., G. Galaski, L. Huang, J. Kellog and J. Larner. "Studies on the Insulin Mediator: II. Separation of Two Antagonistic Biologically Active Materials from Fraction II." Diabetes 29; 659-664, 1980.

5. Seals, J.R. and M.P. Czech. "Characterization of a Pyruvate Dehydrogenase Activator Released by Adipocyte Plasma Membranes in Response to Insulin." J. Biol. Chem 256; 6529-6535, 1981.

6. Larner, J., K. Cheng, C. Schwartz, K. Kikuchi, S. Tamura, S. Creacy, R. Dubler, G. Galasko, C. Pullin and M. Katz. "Insulin Mediators and their Control of Metabolism through Protein Phosphorylation." Res. Prog. Hormone Res. 38; 511-556, 1982.

7. Suzuki, K. and T. Kono. "Evidence that Insulin Causes Translocation of Glucose Transport Activity to the Plasma Membrane from an Intracellular Site." Proc. Natl. Acad. Sci. (USA) 77; 2542-2545, 1980.

8. Cushman, S.W. and L.J. Wardzala. "Potential Mechanism of Insulin Action of Glucose Transport in the Isolated Rat Adipocyte: Apparent Translocation of Intracellular Glucose Transporters to the Plasma Membrane." J. Biol. Chem. 255; 4758-4762, 1980.

9. Oka, Y. and M.P. Czech. In Methods in Diabetes Research, Vol. I, edited by S.L. Pohl and J. Larner. New York: Wiley, 1984.

10. Lawrence, J.C., J.F. Hiken, A.A. DePaoli-Roach, and P.J. Roach. "Hormonal Control of Glycogen Synthase in Rat Hemidiaphragms." J. Biol. Chem. 258; 10710-10719, 1983.

11. Steinberg, D., S.E. Mayer, J.C. Khoo, E.A. Miller, R.E. Miller, B. Fredholm, and R. Eichner. "Hormonal Control of Lipase, Phosphorylase and Glycogen Synthase in Adipose Tissue." Adv. Cyclic Nucleotide Res. 5; 549-568, 1975.

12. Cuatrecases, P. "Affinity Chromatography and Purification of the Insulin Receptor of Liver Cell Membranes." Proc. Natl. Acad. Sci. USA 69; 1277-1281, 1972.

13. Kono, T. and F.W. Barham. "The Relationship between the Insulin-binding Capacity of Fat Cells and the Cellular Response to Insulin." J. Biol. Chem. 246; 6210-6216, 1971.

14. DeMeyts, P., J. Roth, D.M. Neville, J.R. Gavin and M. Losniak. "Insulin Interaction with its Receptors: Experimental Evidence for Negative Cooperativity." Biochem. Biophys. Res. Comm. 55; 154-160, 1973.

15. Pollet, R.J., M.L. Standaert and B.A. Hasse. "Insulin Binding to the Human Lymphocyte Receptor. Evaluation of the Negative Cooperativity Model." J. Biol. Chem. 252;5828-5834.

16. Harmon, J.T., E.J. Kempner, and C.R. Kahn. "Demonstration by Radiation Inactivation that Insulin Alters the Structure of the Insulin Receptor in Rat Liver Membranes." J. Biol. Chem. 256; 7719-7722, 1981.

17. Pilch, P.F. and Czech, M.P. "Hormone Binding Alters the Conformation of the Insulin Receptor." Science 120; 1152-1153, 1980.

18. Donner, D.B. and R.E. Covin. "Formation of a Receptor State from which Insulin Dissociates Slowly in Hepatic Cells and Plasma Membranes." J. Biol. Chem. 255; 9005-9008, 1980.

19. Covin, R.E. and D.B. Donner. "Insulin Receptors Convert to a Higher Affinity State Subsequent to Hormone Binding." J. Biol. Chem. 257; 104-110, 1982.

20. Covin, R.E. and D.B. Donner. "Insulin Binding to Liver Plasma Membranes from Rats Rendered Diabetic by Alloxan." Biochem. J. 202; 259-262, 1982.

21. McCaleb, M.L. and D.B. Donner. "Affinity of the Hepatic Insulin Receptors Is Influenced by Membrane Phospholipids." J. Biol. Chem. 256; 11051-11057, 1981.

22. Donner, D.B. and K. Yonkers. "Hormone Induced Conformational Changes in the Hepatic Insulin Receptor." J. Biol. Chem. 258; 9413-9418, 1983.

23. Schlessinger, J., E. Van Obberghen and C.R. Kahn. "Insulin and Antibodies against the Insulin Receptor Cap on the Membrane of Cultured Lymphocytes." Nature 286; 729-731, 1980.

24. Kahn, C.R., K.L. Baird, D.B. Jarrett and J.S. Flier. "Direct Demonstration that Receptor Crosslinking or Internalization is Important in Insulin Action." Proc. Natl. Acad. Sci. 75; 4209-4213, 1978.

25. Schechter, Y., K. Chang, S. Jacobs and P. Cuatrecasas. "Modulation of Binding and Bioactivity of Insulin by Anti-insulin Antibody: Relation to Possible Role of Receptor Self Aggregation in Hormone Action." Proc. Natl. Acad. Sci. 76; 2720-2724, 1979.

26. Brown, M.S., R.G.W. Anderson and J.L. Goldstein. "Recycling Receptors: The Round-Trip Itinerary of Migrant Membrane Proteins." Cell 32; 663-667, 1983.

27. Fehlmann, M., J.L. Carpentier, E. Van Obberghen, P. Freychet, P. Thamn, D. Saunders, D. Brandenberg and L. Orci. "Internalized Insulin Receptors are Recycled to the Cell Surface in Rat Hepatocytes." Proc. Natl. Acad. Sci. 79; 5921-5925, 1982.

28. Marshall, S. and J.M. Olefsky. "Tris (hydroxymethyl) aminomethane Permits the Expression of Insulin-induced Receptor Loss in Isolated Rat Adipocytes." Biochem. Biophys. Res. Comm. 102; 646-653, 1981.

29. Goldfine, I.D., R. Vigneri, D. Cohen, N.B. Pilam and C.R. Kahn. "Intracellular Binding Sites for Insulin are Immunologically Distinct from those on the Plasma Membrane." Nature 269; 698-700, 1977.

30. King, G.C. and C.R. Kahn. The Growth Promoting Effects of Insulin in Growth and Growth Maturation Factors. G.Guroff. New York: Wiley, 1984, pp 224-265.

31. Jacobs, S., Y. Shechter, K. Bissel, and P. Cuatrecasas. "Purification and Properties of Insulin Receptors from Rat Liver Membranes." Biochem. Biophys. Res. Comm. 77; 981-986, 1977.

32. Yip, C.C., M.L. Moule, and C.W.J. Young. "Characterization of Insulin Receptor Subunits in Brain and Other Tissues by Photoaffinity Labeling." Biochem. Biophys. Res. Comm. 96; 1671-1676, 1980.

33. Pilch, P.F. and M.P. Czech. "Interaction of Cross-linking Agents with the Insulin Effector System of Isolated Fat Cells." J. Biol. Chem. 254; 3375-3380, 1979.

34. Massague, J., P.F. Pilch, and M.P. Czech. "Electrophoretic Resolution of Three Major Insulin Receptor Structures with Unique Subunit Stoichometries." Proc. Natl. Acad. Sci. (USA) 77; 7137-7140, 1980.

35. Yip, C.C., C.W.T. Yeung and M.L. Moule. "Photoaffinity Labeling of Insulin Receptor Proteins of Liver Plasma Membrane Proteins." Biochemistry 19; 70-76, 1980.

36. Massague, J. and M.P. Czech. "Multiple Redox Forms of the Insulin Receptor in Native Liver Membranes." Diabetes 29; 945-947, 1980.

37. Fujita-Yamaguchi, Y. "Characterization of Purified Insulin Receptor Subunits." J. Biol. Chem. 259; 1206-1211, 1984.

38. Kasuga, M., J.A. Hedo, K.M. Yamada and C.R. Kahn. "The Structure of Insulin Receptor and Its Subunits." J. Biol. Chem. 257; 10392-10399, 1982.

39. Hedo, J.A., M. Kasuga, E. Van Obberghen, J. Roth and C.R. Kahn. "Direct Demonstration of Glycosylation of Insulin Receptor Subunits by Biosynthetic and External Labeling. Evidence for Heterogeneity." Proc. Natl. Acad. Sci. 78; 4791-4795, 1981.

40. Kasuga, M., C.R. Kahn, J.A. Hedo, E. Van Obberghen, and K.M. Yamada. "Insulin-induced Receptor Loss in Cultured Lymphocytes is Due to Accelerated Receptor Degradation." Proc. Natl. Acad. Sci (USA) 78; 6917-6921, 1981.

41. Massague, J., P.F. Pilchand and M.P. Czech. "A Unique Proteolytic Cleavage Site on the β-Subunit of the Insulin Receptor." J. Biol. Chem. 256; 3182-3190, 1981.

42. Roth, R.A., M.L. Mesivow and D.J. Cassell. "Preferential Degradation of the β-Subunit of Purified Insulin Receptor." J. Biol. Chem. 258; 14456-14460, 1983.

43. Hubbard, S.C. and R.J. Ivatt. "Synthesis and Processing of Asparagine-Linked Oligosaccharides." Ann. Rev. Biochem. 50; 555-583, 1981.

44. Cuatrecasas, P. ??? In: Insulin Action, edited by I.B. Fritz. New York: Academic Press, 1981, pp 137-163.

45. Cuatrecasas, P. and G.P.E. Tell. "Insulin-like Activity of Concanavalin A and Wheat Germ Agglutinin: Direct Interaction with the Insulin Receptors." Proc. Natl. Acad. Sci. USA 70; 485-489, 1973.

46. Hedo, J., L.C. Harrison and J. Roth. "Binding of Insulin Receptors to Lectins. Evidence for Common Carbohydrate Determinants on Several Membrane Receptors." Biochemistry 20; 3385-3393, 1981.

47. Ronnett, G.V. and M.D. Lane. "Post-translational Glycosylation Induced-Activation of a Glycoinsulin Receptor Accumulated during Tunicamycin Treatment." J. Biol. Chem. 256; 4704-4707, 1981.

48. Rosen, O.M., G.H. Chia, C. Fung. and C.S. Rubin. "Tunicamycin-mediated Depletion of Insulin Receptors in 3T3-L1 Adipocytes." J. Cell. Physiol. 99;37-42, 1979.

49. Reed, B.C., G.V. Ronnett and M.D. Lane. "Role of Glycosylation and Protein Synthesis in Insulin Receptor Metabolism by 3T3-L1 Mouse Adipocytes." Proc. Natl. Acad. Sci. 78; 2908-2912, 1981.

50. Ronnett, V., P. Knutsin, R.A. Kohanski, T.L. Simpson and M.D. Lane. "Role of Glycosylation in the Processing of Newly Translated Insulin Proreceptors in 3T3-L1 Adipocytes." J. Biol. Chem. 259; 4566-4575, 1984.

51. Salzman, A., C.F. Wan and C.S. Rubin. "Biogenesis, Transit and Functional Properties of the Insulin Proreceptor and Modified Insulin Receptors in 3T3-L1 Adipocytes. Use of Monensin to Probe Proreceptor Cleavage and Generate Altered Receptor Subunits." Biochemistry 23; 6555-6565, 1984.

52. Herzberg, V.L., F. Grigorescu, A.S.B. Edge, R.G. Spiro and C.R. Kahn. "Characterization of Insulin Receptor Carbohydrate by Chemical and Enzymatic Deglycosylation." Biochem. Biophys. Res. Comm. 129; 789-796, 1985.

53. Kasuga, M., O. Ezaki, Y. Okanuma, and K. Kosaka. "Effects of Neuraminidase Treatment on Solubilized Insulin Receptor." Horm. Met. Res. 12; 494-496, 1980.

54. Clark, S., M. Deluise, R.G. Larkins, R.A. Melick and L.C. Harrison. "The Effects of Digestive Enzymes on Characteristics of Placental Insulin Receptor." Biochem. J. 174; 37-43, 1978.

55. Caron, M., J. Picard and P. Kern. "Involvement of Glycoconjugates in Insulin-Receptor Interactions." Biochim. Biophys. Acta 572; 29-40, 1978.

56. Cuatrecasas, P. and G. Illiano. "Membrane Sialic Acid and the Mechanism of Insulin Action in Adipose Tissue Cells." J. Biol. Chem. 246; 4938-4946, 1971.

57. Fujita-Yamaguchi, Y., Y. Sato and S. Kathuria. "Removal of Sialic Acids from the Purified Insulin Receptor Results in Enhanced Insulin-binding and Kinase Activities." Biochem. Biophys. Res. Comm. 129; 739-745, 1985.

58. Ullrich, A., J.R. Bell, E.Y. Chen, R. Herrera, L.M. Petruzzeli, T.J. Dull, A. Gray, L. Coussens, Y-C Liao, M. Tsubokawa, A. Mason,

P.H. Seeburg, C. Grunfeld, O.M. Rosen and J. Ramachandran. "Human Insulin Receptor and its Relationship to the Tyrosine Kinase Family of Oncogenes." Nature 313; 756-761, 1985.

59. Ebina, Y., L. Ellis, K. Jarnagin, M. Edery, L. Graf, E. Clauser, J. Ov, F. Masiarz, Y.W. Kan, I.D. Goldfine, R. Roth and W.J. Rutler. "The Human Insulin Receptor. DNA. The Structural Basis for Hormone-activated Transmembrane Signalling." Cell 40; 747-758, 1985.

60. Jacobs, S., F.C. Kull and P. Cuatrecasas. "Monensin Blocks the Maturation of Receptors for Insulin and Somatomedin C: Identification of Receptor Precursors." Proc. Natl. Acad. Sci. USA 80; 1228-1231, 1983.

61. Rinderknecht, E., and R.E. Humbel. "The Amino Acid Sequence of Human Insulin-like Growth Factor I and Its Structural Homology with Proinsulin." J. Biol. Chem. 253; 2769-2776, 1978.

62. Czech, M.P. "New Perspectives on the Mechanism of Insulin Action." Rec. Prog. Hormone. Res. 40; 347-377, 1984.

63. Kahn, C.R. "The Molecular Mechanism of Insulin Action." Ann. Rev. Med. 36; 429-451, 1985.

64. Bishop, J.M. "Cellular Oncogenes and Retroviruses." Ann. Rev. Biochem. 52; 301-354, 1983.

65. Sefton, B.M., T. Hunter, K. Beeman and W. Eckhart. "Evidence that Phosphorylation of Tyrosine is Essential for Cellular Transformation by Rous Sarcoma Virus." Cell 20; 807-816, 1980.

66. Levinson, A., O. Hoppermann, H.-E. Varmus and J.M. Bishop. "The Purified Product of the Transforming Gene Activity of Avian Sarcoma Virus Phosphorylates Tyrosine." J. Biol. Chem. 255; 11973-11980, 1980.

67. Pawson, T., J. Guyden, T.-H. Kung, K. Radke, T. Gilmore, and G.S. Martin. "A Strain of Fujinamin Sarcoma Virus which is Temperature-sensitive in Protein Phosphorylation and Cellular Transformation." Cell 22; 767-775, 1980.

68. Klarlund, J.K. "Transformation of Cells by an Inhibitor of Phosphatases Acting on Phosphotyrosine in Proteins." Cell 41; 707-717, 1985.

69. Ushiro, H. and S. Cohen. "Identification of phosphotyrosine as a Product of Epidermal Growth Factor-activated Protein Kinase in A-431 Cell Membranes." J. Biol. Chem. 255; 8363-8365, 1985.

70. EK, B., B. Westermark, A. Wastesom and C.H. Heldin. "Stimulation of Tyrosine-specific Phosphorylation by Platelet-derived Growth Factor." Nature 295; 419-420, 1982.

71. Rubin, J.B., M.A. Shia, and P.F. Pilch. "Stimulation of Tyrosine-specific Phosphorylation in vitro by Insulin-like Growth Factor I." Nature 305; 438-440, 1983.

72. Burant, C.F., M.K. Treutelaar, G.E. Landreth and M.G. Buse. "Phosphorylation of Insulin Receptors Solubilized from Rat Skeletal Muscle." Diabetes 33; 704-708, 1984.

73. Grigorescu, F., J.S. Flier and C.R. Kahn. "Defect in Receptor Phosphorylation in Erythrocytes and Fibroblasts Associated with Severe Insulin Resistance." J. Biol. Chem. 259; 15003-15006, 1984.

74. Kasuga, M., F.A. Karlsson and C.R. Kahn. "Insulin Stimulates the Phosphorylation of its Own 95,000 Dalton Subunit." Science 215; 185-186, 1982.

75. Grunberger, G., Y. Zick, J. Roth and P. Gordon. "Protein Kinase Activity of the Insulin Receptor in Human Circulating and Cultured

Mononuclear Cells." Biochem. Biophys. Res. Comm. 115; 560-566, 1983.

76. Auruch, J. and R.A. Nemenoff. "Insulinstimulated Tyrosine Phosphorylation of the Insulin Receptor in Detergent Extracts of Human Placental Membranes." J. Biol. Chem. 257; 1516215166, 1982.

77. Van Obberghen, E. and A. Kowalski. "Phosphorylation of the Hepatic Insulin Receptor." FEBS Lett. 143; 179-182, 1982.

78. Hu, K.-T. and Czech, M.P. "Tyrosine Phosphorylation of the Insulin Receptor β-subunit Activates the Receptor Associated Tyrosine Kinase Activity." J. Biol. Chem. 259; 5277-5286, 1984.

79. Heidenreich, K.A., N.R. Zahniser, P. Berhanv, D. Brandenberg and J.M. Olefsky. "Structural Differences between Insulin Receptors in the Brain and Peripheral Target Tissues." J. Biol. Chem. 258; 8527-8530, 1983.

80. Roth, R. and D.J. Cassell. "Insulin Receptors: Evidence That it is a Protein Kinase." Science 219; 299-301, 1983.

81. Shia, M.A. and P.F. Pilch. "The β-subunit of the Insulin Receptor is an Insulin-activated Protein Kinase." Biochem. 22; 717-721, 1983.

82. White, M., H.V. Haring, M. Kasuga and C.R. Kahn. "Kinetic Properties of the Partially Purified Insulin Receptor from Hepatoma Cells." J. Biol. Chem. 259; 255-264, 1984.

83. Petruzzelli, S. Ganguly, C.J. Smith, M.H. Cobb, C.S. Rubin and O.M. Rosen. "Insulin Activates a Tyrosine-specific Protein Kinase in Extracts of 3T3-L1 Adipocytes and Human Placenta." Proc. Natl. Acad. Sci. USA 79; 6792-6796, 1982.

84. Rosen, O.M., R. Herrera, Y. Olowe, L.M. Petruzzelli and M.H. Cobb. "Phosphorylation Activates the Insulin Receptor Tyrosine Protein Kinase." Proc. Natl. Acad. Sci. USA 80; 3237-3240, 1983.

85. Zick, Y., R.W. Rees-Jones, G. Grunberger, S.I. Taylor, V. Moncada, P. Gordon, and J. Roth. "The Insulin-stimulated Receptor Kinase is a Tyrosine-specific Casein Kinase." Eur. J. Biochem. 137; 631-637, 1983.

86. Braun, S., W.E. Raymond and E. Racker. "Synthetic Tyrosine Polymers as Substrates and Inhibitors of Tyrosine-specific Protein Kinases." J. Biol. Chem. 259; 2051-2054, 1984.

87. Kadowaki, T., E. Nishida, M. Kasuga, T. Akiyama, F. Takaku, M. Ishikawa, H. Sakai, S. Kathuria and Y. Fujita-Yamaguchi. "Phosphorylation of Fodrin by the Purified Insulin Receptor Kinase." Biochem. Biophys. Res. Comm. 127; 493-509, 1985.

88. Kadowaki, T., Y. Fujita-Yamaguchi, E. Nishida, F. Takaku, T. Akiyama, S. Kathuria, Y. Akanuma and M. Kasuga. "Phosphorylation of Tubulin and Microtubule-associated Proteins by the Purified Insulin Receptor Kinase." J. Biol. Chem. 260; 4016-4020, 1985.

89. Machicao, F., T. Urumov and O.H. Weiland. "Evidence for Phosphorylation of Actin by the Insulin Receptor-associated Protein Kinase from Human Placenta." FEBS Lett. 163; 76-80, .

90. Nemenoff, R.A., Y.C. Kwok, G.F. Shulman, P.J. Blackshear, R. Osathanondh, and J. Auruch. "Insulin-stimulated Tyrosine Protein Kinase." J. Biol. Chem. 259; 5058-5065, 1984.

91. Sasaki, N., R.W. Rees-Jones, Y. Zick, S.P. Nissley and M.M. Rechler. "Characterization of Insulin-like Growth Factor I-stimulated Tyrosine Kinase Activity Associated with the β-subunit

of Type I Insulin-like Growth Factor Receptors of Rat Liver Cells." J. Biol. Chem. 260; 9793-9804, 1985.

92. Pang, D.T., B.R. Sharma, J.A. Shafer, M.F. White and C.R. Kahn. "Predominance of Tyrosine Phosphorylation of Insulin Receptors during the Initial Response of Intact Cells to Insulin." J. Biol. Chem. 260; 7131-7136, 1985.

93. Takayama, S., M.F. White, V. Louris and C.R. Kahn. "Phorbol esters Modulate Insulin Receptor Phosphorylation and Insulin Action in Cultured Hepatoma Cells." Proc. Natl. Acad. Sci. USA 81; 7797-7801, 1984.

94. Grunberger, G. and P. Gordon. "Affinity Alteration of Insulin Receptor Induced by a Phorbol Ester." Am. J. Physiol. 243; E319-E324, 1982.

95. Pessin, J.E., W. Gitomer, Y. Oka, C.L. Oppenheimer and M.P. Czech. "β-Adrenergic Regulation of Insulin and Epidermal Growth Factor Receptors in Rat Adipocytes." J. Biol. Chem. 258; 7386-7394, 1983.

96. Ghosh-Dastidar, P. and C.F. Fox. "cAMP-dependent Protein Kinase Stimulates Epidermal Growth Factor-dependent Receptors. J. Biol. Chem. 259; 3864-3869, 1984.

97. White, M.F., S. Takayama and C.R. Kahn. "Differences in the Sites of Phosphorylation of the Insulin Receptor in vivo and in vitro." J. Biol. Chem. 260; 9470-9478, 1985.

98. Flier, J.F. "Insulin Receptors and Insulin Resistance." Ann. Rev. Med. 34; 145-160, 1983.

99. Kahn, C.R. "Insulin Resistance, Insulin Insensitivity and Insulin Unresponsiveness: A Necessary Distinction." Metabolism 27; 1893-1902, 1978.

100. Kasuga, M., C.C. Kahn, J.A. Hedo, E. Van Obberghen, and K.M. Yamada. "Insulin Induced Receptor Loss in Cultured Lymphocytes is Due to Accelerated Receptor Degradation." Proc. Natl. Acad. Sci. USA 78; 6917-6913, 1981.

101. Kahn, C.R. "Role of Insulin Receptors in Insulin-resistant States." Metabolism 29; 455-466, 1980.

102. Garvey, T.W., J.M Olefsky, and S. Marshall. "Insulin Receptor Down-regulation is Linked to an Insulin-induced Postreceptor Defect in Glucose Transport System in Rat Adipocytes." J. Clin. Invest. 76; 22-30, 1985.

103. Reaven, G.M., R. Bernstein, B. Davis and Jim Olefsky. "Nonketotic Diabetes Mellitus: Insulin Deficiency or Insulin Resistance?" Am. J. Med. 60; 80-88, 1976.

104. Kelterman, O.G., R.S. Gray, P. Griffin, J. Burstein, J.A. Insel, J.A. Scarlett, and J.M. Olefsky. "Receptor and Postreceptor Defects Contribute to the Insulin Resistance in Noninsulin-dependent Diabetes Mellitus." J. Clin. Invest. 68; 957-969, 1981.

105. Prince, M.J., P. Tai and J.M. Olefsky. "Insulin Binding, Internalization, and Insulin Receptor Regulation in Fibroblasts from Type II, Non-insulindependent Diabetic Subjects." Diabetes 596-600, 1981.

106. Kahn, C.R., J.S. Flier, R.S. Bar, J.A. Archer and P. Gorden. "The Syndromes of Insulin Resistance and Acanthosis Nigricans: Insulin Receptor Disorders in Man." N. Engl. J. Med. 294; 739-745, 1976.

107. Grunberger, G., Y. Zick, and P. Gorden. "Defect in Phosphorylation of Insulin Receptors on Cells from an Insulinresistant Patient with Normal Insulin Binding." Science 223; 932-934, 1984.

108. Barbosa, J., F.Q. Nuttal, and W. Kenedy. "Plasma Insulin in Patients with Myotonic Dystrophy and their Relatives." Medicine 53; 307-323, 1974.

109. Collins, W.J. and W.K. Engel. "Glucose Metabolism in Five Neuromuscular Disorders." Neurology 18; 915-925, 1968.

110. Buse, M.G. and J. Buse. "Glucose Uptake and Response to Insulin of the Isolated Rat Diaphragm. The Effect of Denervation." Diabetes 8; 718-225, 1959.

111. Buse, M.G. and J. Buse. "Effect of Denervation and Insulin on Penetration of D-xylose into Rat Hemidiaphragms." Diabetes 10; 134-141, 1961.

112. Bergamini, E., R. Pagni and C. Pellegrino. "Sugar uptake in Acutely Denervated Levator Ani Muscle of Rat." Biochim. Biophys. Acta 203; 344-347, 1970.

113. Turner, L.V. and K.L. Manchester. "Effects of Denervation on the Glycogen Content and on the Activities of Enzymes of Glucose and Glycogen Metabolism in Rat Diaphragm Muscle." Biochem. J. 128; 789-801, 1972.

114. Forsayeth, J.R. and M.K. Gould. "Inhibition of Insulinstimulated Xylose Uptake in Denervated Rat Soleus Muscle: A Post-receptor Effect." Diabetologia 23; 511-516, 1982.

115. Moruzzi, E.V. and E. Bergamini. "Effect of Denervation on Glycogen Metabolism in Fast and Slow Muscle of Rat." Muscle and Nerve 6; 356-366, 1983.

116. Evans, O.B. "Muscle Pyruvate Oxidation Following Denervation and Reinnervation." Muscle and Nerve 6; 557-560, 1983.

117. Smith, R.L. and J.C. Lawrence. "Insulin Action in Denervated Rat Hemidiaphragms." J. Biol. Chem. 259; 2201-2207, 1984.

118. Burant, C.F., S.K. Lemmon, M.K. Treutelaar and M.G. Buse. "Insulin Resistance of Denervated Rat Muscle: A Model for Impaired Receptor-function Coupling." Am. J. Physiol. 247; E657-E666, 1984.

119. Seider, M.J., W.F. Nicholson and F.W. Booth. "Insulin Resistance for Glucose Metabolism in Disused Soleus Muscle of Mice." Am. J. Physiol. 242; E12-E18, 1982.

120. Nicholson, W.F., P.A. Watson and F.W. Booth. "Glucose Uptake and Glycogen Synthesis in Muscles from Immobilized Limbs." J. Appl. Physiol.: Respirat. Environ. Exercise Physiol 56; 431-435, 1984.

121. Butler, D.T. and F.W. Booth. "Muscle Atrophy by Limb Immobilization is Not Caused by Insulin Resistance." Horm. Metab. Res. 16; 172-174, 1984.

122. Richter, E.A., L.P. Garretto, M.N. Goodman and N.B. Ruderman. "Muscle Glucose Metabolism Following Exercise in the Rat: Increased Sensitivity to Insulin." J. Clin. Invest. 69; 785-793, 1982.

123. Kochan, R.G., D.R. Lamb, E.M. Reimann and K.K. Schlender. "Modified Assay to Detect Activation of Glycogen Synthase Following Exercise." Am. J. Physiol. 240; E197-E202, 1981.

124. Zorzano, A., T.W. Balon, L.P. Garetto, M.N. Goodman and N.B. Ruderman. "Muscle α-aminoisobutyric Acid Transport after Exercise: Enhanced Stimulation by Insulin." Am. J. Physiol. 248; E546-E552, 1985.

125. Plough, T., H. Galbo and E.A. Richter. "Increased Muscle Glucose Uptake during Contractions: No Need for Insulin." Am. J. Physiol. 247; E726-E731, 1984.

126. Hartzel, H.C. and P.M. Fambrough. "Acetylcholine Receptors. Distribution and Extrajunctional Density in Rat Diaphragm after Denervation Correlated with Acetylcholine Sensitivity." J. Gen. Physiol. 60; 248-262, 1972.

127. Almon, R.R. and S.H. Appel. "Cholinergic Sites in Skeletal Muscle. I. Denervation Effects." Biochemistry 15; 3662-3667, 1976.

128. Merlie, J.P., K.E. Isenberg, S.D. Russell and J.R. Janes. "Denervation Supersensitivity in Skeletal Muscle: Analysis with a Cloned cDNA Probe." J. Cell. Biol. 99; 332-335, 1984.

129. Appel, S.H., C.G. Andrew and R.R. Almon. "Phosphatidyl Inositol Turnover of Membranes Following Denervation." J. Neurochem. 33; 1077-1080, 1974.

130. Leung, W.N., P.L. Jeffery and J.A.P. Rostas. "The Effect of Denervation on Mammalian Sarcolemmal Proteins and Glycoproteins." Muscle and Nerve 7; 35-49, 1984.

131. Axelsson, J. and S.J. Thesleff. "A Study of Supersensitivity in Denervated Mammalian Skeletal Muscle." J. Physiol. (Lond) 147; 178-195, 1959.

132. Smith, R.L. and J.C. Lawrence. "Insulin Action in Denervated Skeletal Muscle. Evidence that the Reduced Stimulation of Glycogen Synthesis Does Not Involve Decreased Insulin Binding." J. Biol. Chem. 260; 273-278, 1985.

CHAPTER II: INSULIN RESISTANCE OF DENERVATED RAT MUSCLE: A MODEL FOR IMPAIRED RECEPTOR-FUNCTION COUPLING

Glucose metabolism and insulin response of skeletal muscle is profoundly affected by antecedent activity. In animals and humans, depleted glycogen stores are rapidly replenished after exercise to levels that exceed resting values. This phenomenon, termed supercompensation, involves activation of glycogen synthase (1). Richter et al. (2) observed that prior exercise lowered the insulin-dose required for half-maximal stimulation of glucose transport and glucose utilization by skeletal muscle, suggesting that at least one of the mechanisms is enhanced insulin sensitivity. On the other hand, when muscles were denervated (3,48) or immobilized (5) for 24 hrs before experiments, their subsequent response to insulin was markedly impaired. Others reported that the impaired insulin response required 3 days of denervation (6). Early studies from this laboratory (3,4) using hemidiaphragms 1-7 days after cutting the phrenic nerve suggested that denervation impaired the response to insulin without affecting insulin binding. However, at that time methods for assessing insulin-receptor interactions were relatively unsophisticated. Since short-term denervation and immobilization affected the insulin responsiveness of muscles similarly (3-5), we felt that muscles studied early after denervation may serve as useful models to elucidate how work and/or nervous stimuli modify the

insulin receptor or its coupling to the glucose transport and glycogen synthase system. We addressed the following questions: a) Does short-term denervation affect primarily insulin binding or coupling of the receptor to intracellular events; b) is there decreased insulin sensitivity and/or impaired maximal response, c) are glucose transport and glycogen synthesis affected in parallel, d) are red and white muscles similarly affected by denervation? Our results indicate that 24 hours after denervation, specific insulin binding to muscles is minimally decreased, while insulin sensitivity and response of glucose transport and glycogen synthesis are markedly impaired, suggesting that in red and white muscles, contractile activity and/or nervous stimuli are needed for efficient coupling of the insulin receptor to intracellular responses.

MATERIALS AND METHODS

Denervation and muscle preparation. Male Wistar rats (50-100 g) were purchased from Charles River Breeding Laboratories (Wilmington, Mass.). One hind limb was denervated under methoxyflurane (Metofane, Pitman-Moore, Inc., Washington Crossing, N.J.) anesthesia via a small incision in the posterior aspect of the upper thigh 6 or 24 hrs before experiments. A small section (approx. 3mm) of the sciatic nerve was removed and the incision closed with a stainless steel clip. Rats were either allowed food and water ad libitum or food was withheld for 24 hrs before experiments.

Rats were killed by decapitation and the soleus and/or extensor digitorum longus (EDL)[1] were dissected free, split in half longitudinally, rapidly weighed and placed either directly into 30% KOH for measurement of tissue glycogen or attached to stainless steel clips,

care being taken to attach muscles at in vivo resting length. Muscles were removed within 2 min. after decapitation and the time required between killing the animal and start of incubation was 4-5 min. Following decapitation, spontaneous muscle contractions were observed in non-denervated legs; the rapid removal of the muscles was aimed at minimizing their metabolic effects.

Muscle incubation. Gey and Gey's balanced salt solution (7) (bicarbonate buffer), pH 7.4 containing 5 mM glucose or 2 mM pyruvate as indicated, gas phase 95% O_2 + 5% CO_2 was used in all experiments. Bovine serum albumin, 1 mg/ml, was included in all media except where indicated in Methods. In insulin binding studies higher concentrations of albumin (10 mg/ml) were used. Muscles were incubated with shaking (80 cycles/min.) at 37°C, except for insulin binding studies which were carried out at 25°C to minimize insulin degradation (8).

Glycogen synthesis. Muscles were pre-incubated for 30 min. in 2 ml medium containing 5 mM glucose, with or without insulin at the doses indicated, then transferred to identical media supplemented with [$U^{14}C$]glucose (5mM, 0.2μCi/ml) and incubated for 1 hr. In experiments testing epinephrine responsiveness all media contained 5 mM glucose and 1 mg/ml ascorbic acid; after 30 min. preincubation in this medium the muscles were transferred to media supplemented with [$U^{14}C$]glucose with or without epinephrine (10^{-9} - 10^{-6}M) and incubated for 60 min.

[^{14}C]glucose incorporation into glycogen was assessed by two methods which yielded identical results. The first method was used in initial experiments, the second was adopted to allow the simultaneous measurement of tissue glycogen. In the first method (2) muscles were digested in 0.5ml 1 N NaOH containing 20 mg/ml oyster glycogen to which

1 ml absolute ethanol was added and glycogen was allowed to precipitate at -80°C overnight. The precipitate was centrifuged and washed twice with 1 ml 66% ethanol, dissolved in 0.5 ml H_2O and an aliquot counted in Scintiverse scintillation cocktail (Fisher Scientific) in a Beckman LS-7000 β-scintillation counter. In the second method (10), muscles were digested in 0.5 ml 30% KOH and glycogen precipitated by the sequential addition of 50 µl 10% Na_2SO_4 and 1 ml absolute ethanol and cooled at -20°C for 1 hr. The precipitate was washed twice by sequential addition of 0.5ml 30% KOH and 1 ml absolute ethanol, dissolved in 0.75 ml 4 N H_2SO_4 and an aliquot counted in Scintiverse. Dpm in muscle glycogen was related to the specific activity of the medium, and expressed as nmol of glucose incorporated/mg tissue. No change in muscle weight was observed within 24 hrs after denervation, in either fed or fasted animals.

Glycogen content. Glycogen extracted by the second method (37) was hydrolysed in 4 N H_2SO_4 at 100°C for 2 hrs. The solution was neutralized with 4N NaOH and glucose assayed by the glucose oxidase method. Glycogen recovery was greater than 95%. Values were expressed as glucose equivalents, nmol/mg wet weight.

2-deoxyglucose uptake. Muscles were preincubated for 30 min. in buffer containing 2 mM pyruvate with or without insulin at varying concentrations, then transferred to identical media supplemented with 0.1 or 5 mM [^{3}H]2-deoxyglucose (0.5 µCi/ml) and 0.1 mM [^{14}C]sucrose (0.2 µCi/ml). After 15 or 30 min. incubations (with 5 and 0.1 mM 2-deoxyglucose respectively) the muscles were extracted for 10 min. in 1 ml of boiling water (11). Aliquots of the incubation media and of the tissue extracts were counted as described above. In preliminary

Glycogen synthase assay. Half-solei or EDL were preincubated for 15 min. in buffer containing 2 mM pyruvate without albumin then transferred to identical media, except for the presence of 10 mU/ml insulin in alternate vials. After 30 min. incubation the individual muscles were frozen in liquid nitrogen, placed in a precooled Potter-Elvejhem homogenizer and crushed in 1.2 ml of Tricene buffer (25 mM) containing 50 mM KF; 20 mM EDTA; 1 mg/ml glycogen and 10% w/v glycerol pH 7.1 at 4°C. The frozen slurry was homogenized as it thawed and the mixture placed in 1.5 ml microfuge vials and centrifuged at 15,000Xg for 5 minutes in a Fisher Model 235 microcentrifuge at 4°C. The supernate (30 µl) was assayed for glycogen synthase activity by the method of Thomas et al. (13) as modified by Kochan et al. (14) using 0.075 mM [^{3}H]UDP-glucose (6.75 Ci/mole) as the final substrate concentration. The reaction was run for 10 min at 30°C in 90 µl final volume in the absence or presence of 100 or 1000 µM G-6-P; the latter concentration of G-6-P resulted in complete activation of the enzyme. Results are expressed as activity ratio (-G-6-P/+1000 µM G-6-P) and as fractional velocity (+100 µM G-6-P/1000 µM G-6-P) (14). In some experiments the assay was also performed using 4.4 mM UDP-glucose at pH 7.8, without or with increasing concentrations of G-6-P up to 10 mM (13).

The low (0.075 mM) ^{3}[H]UDP-glucose concentration in the assay was selected based on considerations discussed (1,15), indicating that the assay becomes more sensitive to changes in the phosphorylation state of the enzyme when subsaturating concentrations of UDP-glucose are used. With respect to muscle pretreatments (e.g. denervation and insulin) we obtained qualitatively identical results in solei with low or high substrate assays although baseline activity ratios were higher in the

latter system. Studies in EDL were done only with the low UDP-glucose concentration. The data presented are those obtained with 0.075 mM UDP-glucose, except for assays of total enzyme activity, where both measurements are shown; the total activity measured with 4.4 mM UDP-glucose represents Vmax (15).

Total enzyme activity was measured as UDP-glucose incorporation into glycogen during incubation with 1 or 10 mM G-6-P and related to the protein concentration. Maximal enzymatic activity was observed with 1 mM G-6-P in the low substrate assay and with 10 mM in the high substrate assay. The assay was linear with time for 15 min, UDP-glucose was depleted less than 10% during either assay. Protein was measured by the method of Lowry et al. (16) against bovine serum albumin standards.

Insulin Binding. Monocomponent porcine insulin was labeled with ^{125}I by the lactoperoxidase method to a specific activity of 100-200mCi/mg and was purified before each experiment as previously described (17) by adsorption to and elution from a Whatman CF11 cellulose column. Before use ^{125}I-insulin was 99% precipitable with trichloroacetic acid (TCA). ^{125}I-insulin binding was measured by the method of LeMarchand-Brustel et al. (23). Briefly, muscles were preincubated for 30 min. at 25°C in 3 ml buffer containing 2 mM pyruvate and 10 mg/ml bovine serum albumin; the muscles were then transferred to vials containing the same buffer supplemented with 0.135 μCi/ml ^{125}I-insulin and increasing concentrations of non-labeled insulin and incubated for an additional 2 hrs. The muscles were then washed with 5 ml 0.9% NaCl containing 5 mg/ml albumin at 4°C for 15 min three times, digested in 1 N KOH and counted in a Beckman Gamma 4000 scintillation counter. Non-specific binding was estimated in the presence of 50 μg/ml

unlabelled insulin and was subtracted from all measurements to calculate specific binding. Non-specific binding was typically 30-40% of total binding in the presence of tracer only. The degradation of ^{125}I-insulin in the medium during 2 hrs. incubation was 5-7% as assessed by TCA precipitation.

Although this insulin binding assay was originally developed for mouse soleus muscles (8) it has been adapted with various modifications (6,18) to rat solei. We found the hemisected soleus of young rats (muscle weight 17.2 ± 0.5 mg, n = 128) to be suitable for the assay. Experiments varying the time of incubation with ^{125}I-insulin between 1 and 3 hrs at 25°C indicated that most of the specific insulin binding occurred during the first hour of incubation; with gradual smaller increases thereafter. After 2 hrs incubation, specific binding was 80% of that observed at 3 hrs., although the difference between binding at 2 and 3 hrs was not statistically significant. Concommitantly the % of degraded insulin in the medium increased significantly ($p < 0.001$), reaching 15% of total in some experiments. The 2 hrs. incubation time was selected as a compromise between the achievement of binding equilibrium and minimizing insulin degradation. When muscles were homogenized in 6% TCA after incubation with ^{125}I-insulin (1 ng or 50 μg/ml) for 1, 2 or 3 hrs. followed by 45 min. washing at 4°C as described above, it was found that 85 ± 1% (n = 12) of the muscle associated radioactivity was TCA precipitable at 2 hrs. The respective values after 1 and 3 hrs. incubation were 86 ± 1 and 83 ± 1.5. Because the fraction of TCA soluble radioactivity associated with the muscles appeared constant and relatively small and the additional manipulations involved in homogenizing, precipitating and washing with TCA may introduce errors of similar

magnitude in the measurements the TCA precipitation step was not routinely applied.

Time course experiments as described above were run in parallel using control and denervated solei of rats 24 hrs. after unilateral denervation. There was no significant difference in the time course of specific or non-specific insulin binding or in the rate of insulin degradation (assessed by muscle associated TCA precipitable radioactivity) between control and denervated solei.

Materials. Monocomponent porcine insulin was a gift from Dr. Ronald Chance, Eli Lilly Co. (Indianapolis, Ind.) $Na^{125}I$ (carrier free), [1-^{3}H]UDP-glucose, D-[U-^{14}C]glucose, [1,2-^{3}H]2-deoxy-D-glucose, and [U-^{14}C]sucrose were purchased from New England Nuclear (Boston, MA). Oyster glycogen, bovine serum albumin (Fraction V), and l(-) epinephrine(+)bitartrate were from Sigma (St. Louis, MO). Lactoperoxidase beads were from Bio-Rad Laboratories (Richmond, CA) and glucose-6-phosphate dehydrogenase (Type XII) from Boehringer-Mannheim (Indianapolis, IN). Stainless steel clips (Autoclips) and Scintiverse were purchased from Fisher Scientific Co. (Pittsburgh, PA).

Statistical Analysis. Data are presented as Mean ± SE. Statistical analysis was performed by paired or unpaired Student's t test where appropriate.

RESULTS

Glycogen Synthesis. Fig. 1A illustrates the insulin dose response curves of intact and denervated solei, isolated following a 24 hr fast and 24 hrs after cutting one sciatic nerve. Control muscles showed a three-fold stimulation of [^{14}C]glucose incorporation into glycogen in response to physiological concentrations of insulin (50 μU/ml).

Denervated solei synthesized less glycogen than controls in the absence of insulin ($p < 0.01$). While insulin at concentrations of 250 μU/ml or greater caused a significant stimulation of glycogen synthesis in denervated solei, both the relative stimulation (% of baseline) and the absolute increment over baseline (Δ insulin) were significantly smaller in denervated than control solei, e.g. maximal rates of glycogen synthesis in the presence of 500 μU/ml insulin were five-fold greater in control than denervated muscles. The insulin dose required for ½ maximal stimulation was ~ 30 μU/ml in control muscles and ~ 100 μU/ml in their denervated pairs. Increasing the insulin concentration to 2.5 and 5 mU/ml failed to cause further stimulation in intact or denervated muscles (data not shown).

Similar results were obtained in rats fed ad libitum (Fig. 1B). Although basal rates of glycogen synthesis were lower in 24 hr denervated solei, they were not significantly different from controls. Insulin (500 μU/ml) increased glycogen synthesis four-fold in control solei, while no significant stimulation was observed in their denervated pairs up to 1 mU/ml, the highest dose tested.

The impaired insulin response was already detectable 6 hrs after denervation using rats fed ad lib (Fig. 1C). Stimulation of glycogen synthesis by insulin in control solei was less than that observed in the preceding studies (Fig. 1A and 1B), which may be attributed to the relatively recent stress of anesthesia and surgery. Basal rates of glycogen synthesis were identical in denervated and control solei, while stimulation by insulin was greater ($p < 0.05$-0.01) in controls than in denervated muscles at all insulin concentrations tested. The lowest dose of insulin tested (100 μU/ml) was ineffective in denervated

muscles, while it caused 70% of apparent maximal stimulation in controls.

We used the non-denervated contralateral leg as control, to minimize rat to rat variation, and expose the denervated and control limbs to the same nutritional and hormonal milieu. In preliminary experiments we compared glucose incorporation into glycogen by non-denervated solei of unilaterally denervated rats 24 hrs. after operation to those of sham operated controls and found no difference in either basal or insulin stimulated glycogen synthesis (data not shown).

Soleus muscle contains predominantly slow-twitch red fibers (19). To determine whether or not insulin resistance after denervation occurred in a muscle with predominantly fast-twitch white fibers (19), EDL muscles were studied 24 hrs after unilateral denervation (Fig. 2). Control EDL appeared to be less responsive to insulin than solei; and this was particularly apparent in muscles obtained from fed rats. Such differences in insulin responsiveness between red and white muscles have been reported (20). Basal rates of glycogen synthesis were lower in denervated EDL than in their control pairs ($p < 0.01$) in fed and in fasted rats. The most significant finding was that while insulin stimulated glucose incorporation into glycogen ~2 fold in control EDL, insulin was completely ineffective in their denervated pairs, in both fed and fasted rats.

Effect of denervation on the uptake of 2-deoxyglucose. Resistance of glycogen synthesis to stimulation by insulin could represent the inability of insulin to stimulate the transport of glucose and/or a block in a subsequent reaction e.g. phosphorylation of glucose or activation of glycogen synthase. 2-deoxyglucose uptake was measured to

assess the response to insulin of the glucose transport system, since this analogue enters muscle on the same transporter as d-glucose, and upon entry is phosphorylated to 2-deoxyglucose-6-PO_4 but not further metabolized. The analogue was tested at 0.1 and at 5 mM concentrations in the medium. Pyruvate was substituted for glucose as an energy source, to avoid competition of glucose with the labelled analogue for the transport system.

The highest dose of insulin tested (1 mU/ml) increased 2-deoxyglucose uptake ~ 2-fold in control solei of fasted rats during incubations with 0.1 mM or with 5 mM 2-deoxyglucose (Fig. 3A and 3B). Their denervated pairs showed decreased uptake in the absence of insulin, as well as decreased insulin sensitivity ($p < 0.01$) when incubated in the presence of either 0.1 mM or 5 mM 2-deoxyglucose. While a complete dose-response curve was not obtained decreased insulin sensitivity in denervated solei is suggested by the fact that ~ 40 μU/ml insulin was required for ½ maximal stimulation of 2-deoxyglucose uptake in control muscles, while only the highest dose tested (1000 μU/ml) caused a significant stimulation in denervated solei. With 0.1 mM 2-deoxyglucose the maximal insulin response over baseline (Δ insulin) was not significantly different between control (83 ± 15nmol/g/30 min) and denervated muscles, (60 ± 16nmol/g/30 min). After 15 min. incubation in 5 mM 2-deoxyglucose the increment in 2-deoxyglucose uptake induced by 1 mU/ml insulin was significantly greater in controls than in denervated solei, (1150 ±224 vs. 310 ±186nmol/g/15 min., $p < 0.01$).

Hexokinase activity did not appear to be limiting 2-deoxyglucose uptake by denervated solei, since the ratio of 2-deoxyglucose-6-PO_4/ [free + phosphorylated 2-deoxyglucose] in the intracellular compartment

was essentially identical in intact and denervated muscles. In all experiments more than 80% of the intracellular analogue was in the phosphorylated form (Fig. 3C and 3D).

Denervation for 24 hrs affected 2-deoxyglucose uptake in EDL muscles similarly to solei (Table 1). Using 5 mM 2-deoxyglucose insulin sensitivity and insulin response were significantly decreased. In control muscles 50 μU of insulin caused near maximal stimulation, while this dose was ineffective in their denervated pairs. 1000 μU stimulated 2-deoxyglucose uptake by denervated and control EDL but the response of denervated muscles was smaller than that of the controls ($p < 0.01$). Basal uptake was lower than that of controls, although the difference was not significant.

Glucose-6-phosphate content was measured in control and 24 hr denervated solei of fasted rats following incubations in 5 mM glucose identical to those used in studies of glycogen synthesis. Since phosphorylation at C-6 is the first and obligatory reaction for glucose metabolism after entry into the cell, impaired glucose transport may result in decreased intracellular G-6-P. Following incubation without insulin, G-6-P in denervated muscles was 45% lower than in controls ($p < 0.05$). Following incubation with 1 mU/ml insulin, G-6-P increased in control muscles more than two-fold ($p < 0.01$) while there was no significant change in their 24 hr. denervated pairs (Table 2).

Activation of glycogen synthase by insulin. (Table 3) While impaired stimulation of glucose transport by insulin could account for the observed insulin resistance of glycogen synthesis in denervated muscles, the magnitude of the defect suggested that a second step may also be involved such as the activation of glycogen synthase by insulin.

Total glycogen synthase activity was greater in soleus than in EDL and was not affected by 24 hrs denervation in either muscle. The fractional velocity of the enzyme was lower in denervated than in control solei after incubation without insulin ($p < 0.05$); while the trend was similar in EDL muscles, it was not significant. When solei and EDL were incubated with 10 mU/ml insulin both the activity ratio and the fractional velocity of glycogen synthase increased slightly (20-30%) but significantly ($p < 0.05$) in control muscles whereas changes were not significant in their denervated pairs.

Glycogen content. The glycogen content of muscles may control the rate of glucose transport and glycogen synthesis, possibly through a feedback mechanism (2,21). Since work induced glycogenolysis is abolished in denervated muscles, they may contain more glycogen than controls before incubation. Table 4 shows that this was the case in denervated solei, obtained from fed or 24 hr fasted rats. However, in EDL the glycogen content of control and 24 hr denervated muscles was not significantly different either in the fed state or after a 24 hr fast. Since glycogen synthesis and glucose transport in denervated EDL and solei was equally resistant to insulin, increased initial glycogen content does not appear to be the cause of the insulin resistance.

Effect of denervation on the binding of insulin (Fig. 4). Specific insulin binding by intact and 24 hrs denervated solei of rats fasted overnight was studied. In initial experiments half-solei of the intact and the denervated leg of 6-9 rats per experiment were randomly distributed between media containing increasing concentrations of insulin, 3 control and 3 denervated muscles were used in each experiment for determination of non-specific binding. Each dose was tested in at

least 6 separate muscles. While denervated muscles tended to bind slightly less insulin than controls at all concentrations up to 50 ng/ml (1.3 mU/ml) using this experimental design differences between control and denervated muscles were not significant. In one series of experiments (Insert, Fig. 4) a paired experimental design was used; only one dose of insulin was tested (1 ng/ml) and one hemisoleus of each control and each denervated leg was used for measurement of non-specific binding. The means of 13 paired measurements yielded a significant difference ($p < 0.05$), demonstrating that in the presence of a physiological insulin concentration (26 μU/ml), denervated muscles bound 30% less insulin than the controls. Scatchard analysis of the data (not shown) yielded the classical curvilinear plot reported for insulin binding in a number of tissues. The differences in binding between control and denervated muscles are not large enough to allow meaningful kinetic analyses; the data would be compatible with a small decrease in binding affinity or decreased number of high affinity binding sites in denervated muscles.

Effect of epinephrine in denervated muscles. To assess whether or not the insulin resistance of denervated muscles extends to other hormones we examined the response of control and denervated solei to epinephrine in vitro. Two well-characterized effects of the hormone served as end points: the ability to accelerate glycogen degradation in vitro and to inhibit the incorporation of [^{14}C]glucose into muscle glycogen (22).

Intact and 24 hrs denervated solei were removed from ad lib fed rats and half-solei incubated with or without increasing concentrations of epinephrine (10^{-9} - 10^{-6}M) in 5 mM [U-^{14}C]glucose (Fig. 5). The

glycogen content of intact and denervated solei was similar following incubation without epinephrine. Increasing concentrations of epinephrine resulted in progressive depletion of tissue glycogen. The sensitivity and the response of glycogenolysis to epinephrine appeared identical in control and denervated muscles (Fig. 5A).

As shown previously (Fig. 1), denervated solei incorporated less glucose into glycogen during incubation than controls. However, significant inhibition by epinephrine was observed with identical doses (10^{-7}M). The highest dose of epinephrine used (10^{-6}M) decreased glucose incorporation into glycogen to approximately 50% of that observed in the absence of epinephrine in both intact and in denervated muscles respectively (Fig. 5B).

DISCUSSION

Short-term denervation affects multiple parameters of glucose metabolism in skeletal muscle. We observed diminished incorporation of glucose into glycogen, decreased transport of 2-deoxyglucose reduced glucose-6-PO_4 content and decreased activation of glycogen synthase. While in many instances these effects were demonstrable without insulin, insulin resistance was the most salient characteristic of denervated muscles.

Isolated solei and EDL seemed to be appropriate models for studies of insulin responsiveness, since control muscles responded to physiological doses of insulin, and the sensitivity and magnitude of the responses were similar to those reported for perfused hindlimbs (2). Yet, after 24 hr. denervation, when assessed by glucose incorporation into glycogen, solei of fed rats, and EDL of fed and fasted rats showed no response to even pharmacological doses of insulin and the response of

solei from fasted rats was greatly diminished. Thus, insulin resistance after denervation is a feature of both red and white muscles. Whether or not the decreased baseline observed in a number of experiments in denervated muscles represented an impaired response to endogenous insulin which persisted during incubation or an insulin-independent deceleration of glucose transport following denervation, needs further investigation. Vigorous exercise has been reported to stimulate glucose transport in skeletal muscle by an insulin-independent mechanism and the effect persists for many hours after the cessation of work (21). However, insulin may play a permissive role, since no stimulation of glucose uptake is observed when hindlimbs of diabetic rats are exercised without insulin in the perfusion medium (23).

The insulin resistance of denervated muscles seemed to entail both decreased sensitivity and impaired maximal response to the hormone. This seemed evident in those studies of glucose incorporation into glycogen, where a significant response to supraphysiological doses of insulin was obtainable in denervated muscles (Fig. 1A and 1C). In studies of glucose transport, the decreased sensitivity to insulin of denervated solei was evident using 0.1 or 5 mM 2-deoxyglucose. (Figs. 3A, 3B, and Table 1). Using 5 mM 2-deoxyglucose a significant diminution of the maximal insulin response was also demonstrated in denervated muscles incubated with 1 mU/ml insulin (Fig. 3B and Table 1). Since this dose exceeds the in vivo physiological range ~5-fold, and since further increments in insulin concentration were ineffective in our in vitro studies of glycogen synthesis, higher doses were not tested.

In contrast to the markedly impaired insulin response, we observed a small decrease in insulin binding which was demonstrable only in paired experiments. Since denervated muscles were resistant to 20-50 fold higher doses of insulin than those which elicited maximal response in control muscle, it seems likely that the major defect caused by denervation is not decreased insulin binding, but a defect in signal transmittal from the receptor to intracellular events. Note however that because of technical considerations, insulin binding was measured after longer incubations at a lower temperature (25°C vs 37°C) than was used to test metabolic responses; therefore the above conclusions need further validation. Forsayeth and Gould (6) observed no significant change in insulin binding by solei after 7 days denervation, although binding by denervated muscles was slightly lower. The values of fmoles insulin bound per mg muscle in their study and ours are in remarkable agreement.

The results presented here are in essential agreement with our earlier studies in denervated diaphragms (3,4) and with a recent study of Forsayeth and Gould (19) who reported that in solei, 3-7 days after denervation insulin-stimulated sugar transport was inhibited at some early post-receptor step. In contrast to the latter study (6), we found that insulin resistance develops very early after denervation in muscles, is detectable after 6 hrs., and profound at 24 hrs.

Our results also indicate that 24 hrs after denervation total glycogen synthase activity in red and white muscles is unchanged, but there is a significant decrease in the enzyme's basal activation state in the soleus as well as impaired activation in response to insulin in soleus and EDL. These observations differ from those in Ref. 19 but are congruent with a preliminary

report of Smith and Lawrence who studied glycogen synthase activity of rat diaphragms 24 hrs after denervation (24), and with the reported increase in glycogen synthase kinase activity in denervated muscles (25). The total glycogen synthase activity of incubated solei measured here at saturating substrate concentrations is similar to that reported for red vastus lateralis muscles of rats in vivo (14).

The measured changes in the activity ratio of glycogen synthase following incubation of control muscles with insulin were relatively small, in view of the marked stimulation of [^{14}C]glucose incorporation into glycogen by insulin. Similar discrepancies have been noted using the perfused rat hindlimb (26) as well as a number of isolated tissues (for additional references and theoretical considerations see 14, 15, 26). Relatively small changes in the activity ratio reflect larger changes in the phosphorylation state of the enzyme, the effects of which may be magnified under the conditions prevailing in the intact tissue. Further optimization of incubation or assay conditions may have yielded larger responses. However, since all measurements in denervated and control muscles were carried out in parallel, we believe the data demonstrate the denervation induced relative insensitivity of glycogen synthase to activation by insulin.

Seider et al. (5) reported severely impaired sensitivity and response to insulin in disused mouse solei as assessed by 2-deoxyglucose uptake and glucose incorporation into glycogen 24 hrs. after hindlimbs were immobilized in a plaster cast. The striking similarity between our results indicate that the insulin resistance which develops rapidly after denervation is not due to the lack of a "neurotrophic factor" but to decreased electromyogenic and/or contractile activity. Note that

when solei are immobilized at resting or shorter than resting length, their electromyogenic activity decreases to 10-15% of normal resting activity (27).

Other changes that occur rapidly in immobilized or denervated muscles include an increase in the number of cytosolic cortisol receptors (28,29) as well as an increase in the number of extrajunctional acetylcholine receptors (30). We postulated that a denervation induced change in membrane fluidity and/or permeability may cause generalized non-responsiveness of membrane associated hormone receptors. This appears not to be the case since epinephrine mediated glycogenolysis in isolated solei was not affected by 24 hours denervation.

Impaired glucose tolerance with peripheral insulin resistance has been demonstrated in healthy humans after one week bed rest (reviewed in 31) as well as in patients with spinal cord injuries (32) and with a number of musculoskeletal disorders (33). It is tempting to speculate that the early morning insulin resistance observed in insulin dependent diabetic patients (34) may be related in part to decreased muscular tone and activity during sleep. On the other hand, physical activity decreases the insulin requirements of diabetic patients (for review see 35). Antecedent work enhances glucose transport in muscles (21) increases muscle sensitivity to insulin (21,26,35) without affecting insulin binding to muscle (36,37) and induces activation of glycogen synthase (1). All of these findings taken together suggest that nervous stimuli and/or contractile activity regulate the insulin response of skeletal muscle, resulting in a continuum of graded responses, which include at the two extremes the insulin resistance observed shortly after denervation or immobilization, and enhanced sensitivity after

work. Such a mechanism would assure the rapid replenishment of glycogen stores after exhaustive work, and spare glucose, by limiting its supply to non-working muscles; it appears to operate primarily at the level of receptor-function coupling (36,37). The observed inverse relationship between insulin response and glycogen levels in control and denervated solei would support the proposed negative feedback of muscle glycogen content on the insulin response (2,21); however, since severe insulin resistance was observed in EDL muscles 24 hrs after denervation without a detectable change in glycogen content, it appears that, at least in the EDL, glycogen content is not the major regulator of the insulin response.

The mechanism by which nervous stimuli and/or contractile activity modulate the response to insulin in muscle is conjectural. Denervation may inhibit the generation or the propagation of a signal(s) from the occupied receptor or, alternatively, changes in the intracellular milieu may impair the response. Since at least three separate insulin regulated systems appear affected (glucose transport, glycogen synthase activation and in Ref.19 amino acid transport), impaired signal-transmission seems to be the more attractive hypothesis.

TABLE 1

Uptake of [^{3}H]-2-deoxyglucose (5mM) by control and denervated EDL.

	2-deoxyglucose uptake (nmoles/mg wet weight/15 min)	
Insulin (μU/ml)	Control	Denervated
0	1.42 ± 0.12	1.22 ± 0.08
50	2.37 ± 0.22‡	1.36 ± 0.13*
1000	2.56 ± 0.21‡	1.81 ± 0.09*‡

Half EDL from fasted rats, unilaterally denervated for 24 hrs, were preincubated, incubated and processed as described in Fig. 4 for solei. *$p < 0.01$ vs. nondenervated pairs. ‡ $p < 0.01$ vs. muscles incubated without insulin.

TABLE 2

Effect of insulin on glucose-6-phosphate content of control and denervated solei.

Insulin	Glucose-6-phosphate	
uU/ml	nmol/g tissue	
	Denervated	Control
0	87 ± 19 (9)	159 ± 25 (9)*
50	94 ± 40 (4)	207 ± 24 (5)*
1000	98 ± 33 (4)	351 ± 52 (5)*†

Control and 24 hr denervated solei from 24 hr fasted rats were preincubated for 30 min in 5 mM glucose with or without insulin and for an additional 60 min in identical medium. Means ± SE are shown. The number of observations is in parentheses.

* $p < 0.05$ vs. non-denervated pairs

† $p < 0.01$ vs. control without insulin

TABLE 3

Effect of denervation and insulin on glycogen synthase activity.

Muscle	Total Activity (pkat/mg protein)	Activity Ratio	Fractional Velocity
Solei			
Control, -I	297 ± 18 (656 ± 71)	0.14 ± 0.02	0.50 ± 0.03
Control, +I	297 ± 25 (668 ± 110)	0.18 ± 0.02‡	0.61 ± 0.04‡
Denervated, -I	285 ± 18 (733 ± 93)	0.11 ± 0.01	0.43 ± 0.03*
Denervated +I,	290 ± 25 (688 ± 87)	0.12 ± 0.02*	0.48 ± 0.04*
EDL			
Control,-I	155 ± 25	0.16 ± 0.01	0.57 ± 0.03
Control, +I	121 ± 18	0.19 ± 0.01‡	0.66 ± 0.03‡
Denervated, -I	129 ± 15	0.12 ± 0.02	0.52 ± 0.03
Denervated, +I	144 ± 22	0.14 ± 0.02*	0.58 ± 0.04

Muscles were obtained from fasted rats 24 hrs after unilateral denervation. They were preincubated for 15 min in buffer containing pyruvate without insulin or albumin and incubated for 30 min ± 10 mU/ml insulin. Glycogen synthase activity of the 15,000xg supernate of muscle homogenates was measured, and activity ratios (-G-6-P/1mM G-6-P) and fractional velocities (0.1 mM G-6-P/1mM G-6-P) calculated as described in Methods. pkat = pkatals, amount of enzyme activity converting 1 pmol of glucose from UDP glucose into glycogen per second under standard assay conditions. All data shown are measurements made using subsaturating concentrations of UDP glucose (0.075 mM) except for data in p arentheses where UDP-glucose was 4.4 mM. Total activity was measured as described in Methods. Results are expressed as mean ± S.E. of 6-7 observations. *$p < 0.05$ vs. non-denervated pairs. ‡ $p < 0.05$ vs. incubation without insulin. I = insulin, 10 mU/ml.

TABLE 4

Initial glycogen content of muscles

	Glycogen (glucose equivalents) nmol/mg wet weight
Soleus	
24 hr. Fasted Rats	
Control	17.5 ± 1.38
Denervated	34.8 ± 1.38*
Ad Lib. Fed Rats	
Control	22.3 ± 1.42
Denervated	27.5 ± 1.60*
EDL	
24 hr. Fasted Rats	
Control	19.1 ± 1.46
Denervated	17.7 ± 1.21
Ad Lib. Fed Rats	
Control	24.0 ± 2.00
Denervated	23.3 ± 1.82

Control or 24 hr. denervated soleus and extensor digitorum longus muscles from fasted or fed rats were dissected and glycogen extracted immediately as described in Methods. Values are Means ± SE of 8-24 observations.

* $p < 0.05$ vs. paired control. Fed and fasted animals were studied in separate experiments.

FIGURE LEGENDS

Fig. 1. Glucose incorporation into glycogen by solei. Half solei were preincubated for 30 min in 5 mM glucose with or without increasing concentrations of insulin and incubated for 1 hr in identical media supplemented with [U-^{14}C]glucose (0.2 μCi/ml). Each point represents the mean ± SE of 6-8 observations. Controls , Denervated Panel A: Rats fasted and one sciatic nerve severed 24 hrs before experiments. B: Rats fed ad lib.; one sciatic nerve severed for 24 hrs. C: Fed rats, unilateral denervation 6 hrs. before experiment.

* control >denervated, $p < 0.01$. Insulin effects were significant at all doses tested in control muscles in each panel ($p < 0.01$); in the denervated, insulin effects were not significant in panel B, but were significant at 250 μU/ml or higher doses in panels A and C ($p < 0.01$).

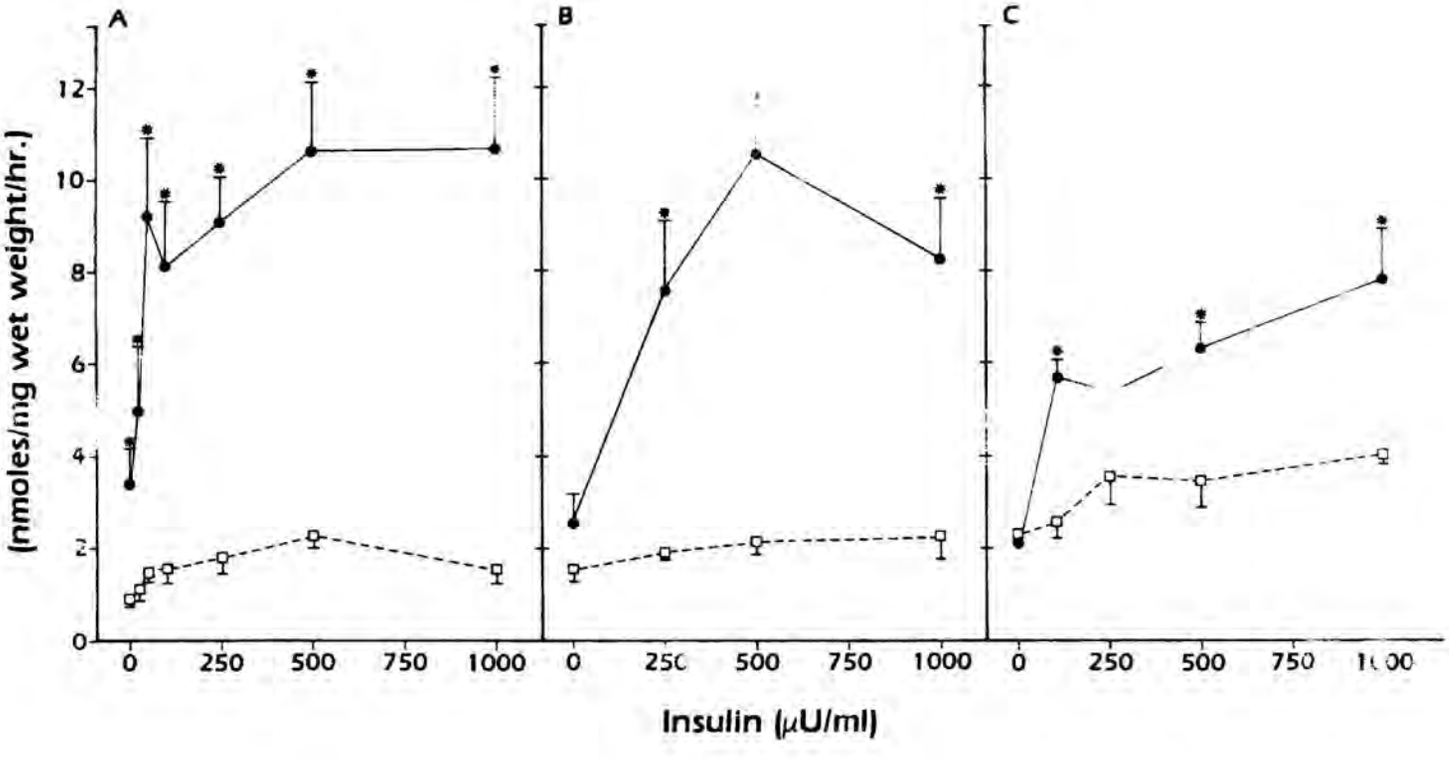

A
B
C
(nmoles/mg wet weight/hr.)
0
2
4
6
8
10
12
0
250
500
750
1000
Insulin (μU/ml)

Fig. 2. Glucose incorporation into glycogen by extensor digitorum longus (EDL)muscle. Half EDL were incubated as described in Fig. 1. Bars are Means ± SE of 6 observations. Panel A: Rats fasted and one sciatic nerve severed 24 hrs. before experiment. B: Fed rats, unilateral denervation 24 hrs. Black bars indicate denervation, clear bars controls.

* Control > denervated, $p < 0.01$. Insulin effects were significant at both doses in control muscles in A ($p < 0.01$) and at 500 μU/ml in B ($p < 0.05$); no insulin effect in denervated muscles.

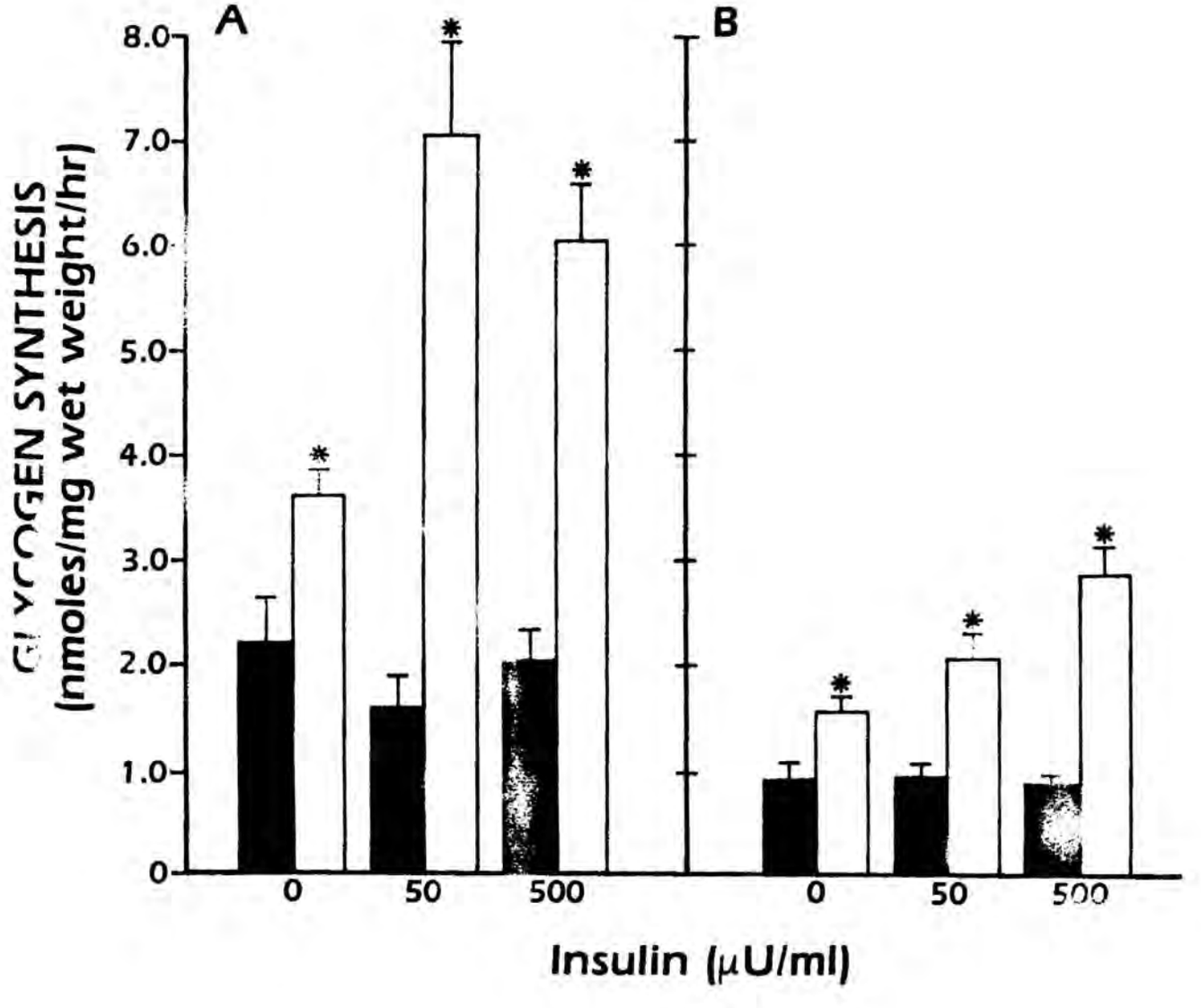

A
B
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
0
50
500
0
50
500
Insulin (μU/ml)

Fig. 3. Uptake and phosphorylation of 2-deoxyglucose. Half solei from rats, fasted and unilaterally denervated for 24 hrs, were preincubated and incubated as described in Fig. 3 but [^{14}C]sucrose served as an extracellular marker and [^{3}H]2-deoxyglucose as the transported sugar. Panel A: intracellular 2-deoxyglucose after 30 min. incubation in 0.1 mM 2-deoxyglucose; B: 15 min incubation in 5 mM 2-deoxyglucose. Control ; denervated . The % of intracellular 2-deoxyglucose as 2-deoxyglucose-6-phosphate after incubation is shown in Panel C and D for experiments A and B respectively. Black bars represent denervated, clear bars control muscles. Means ± SE of 6-8 observations are shown in A and B, 8-10 observations in C and D.

* Control > denervated, $p < 0.05$. In A and B insulin effects in control muscles were significant at each dose ($p < 0.05$ - $p < 0.001$); in denervated muscles the only significant insulin effect is in Panel A at 1000 μU/ml, $p < 0.05$.

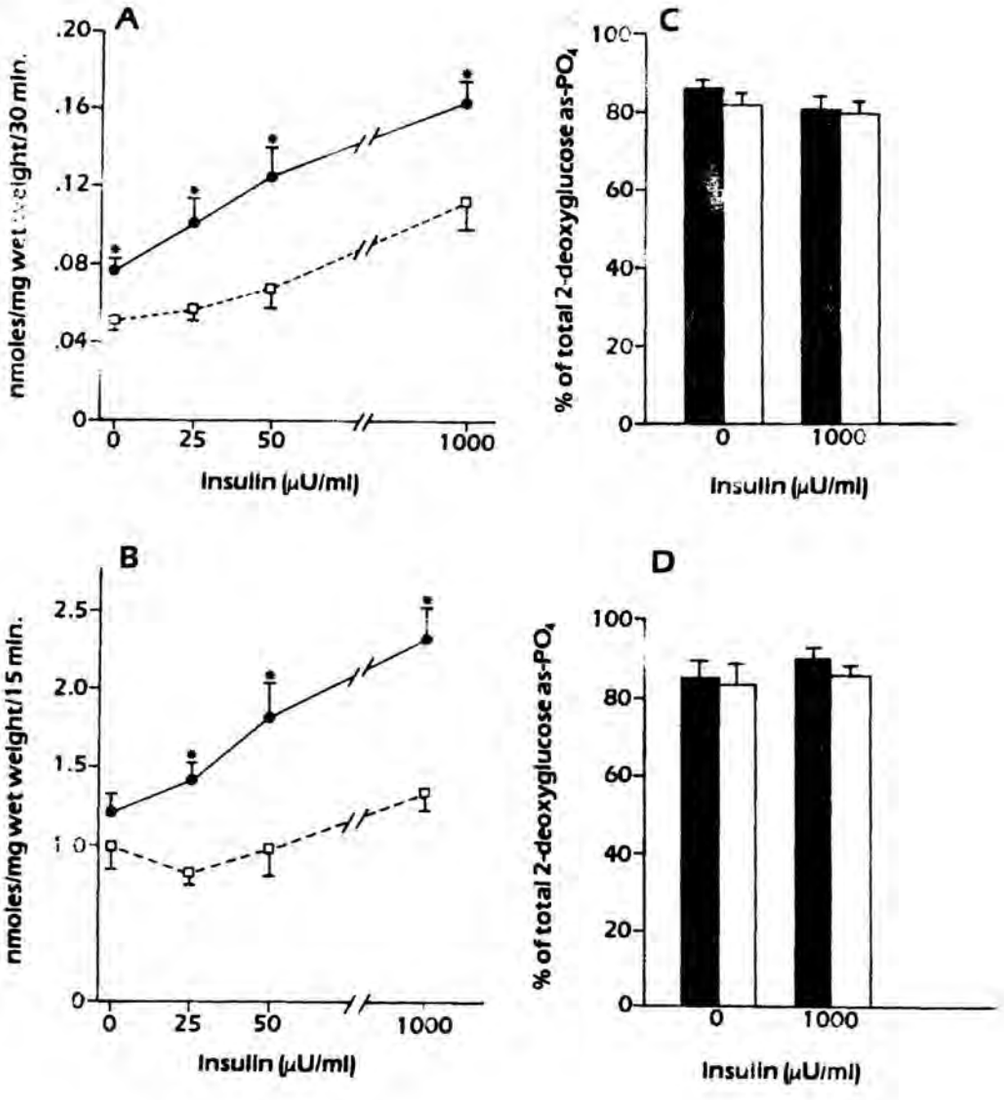

A
.20
.16
.12
.08
.04
0
nmoles/mg wet weight/30 min.
0
25
50
1000
Insulin (μU/ml)
C
100
80
60
40
20
0
% of total 2-deoxyglucose as-PO4
0
1000
Insulin (μU/ml)
B
2.5
2.0
1.5
1.0
0
nmoles/mg wet weight/15 min.
0
25
50
1000
Insulin (μU/ml)
D
100
80
60
40
20
0
% of total 2-deoxyglucose as-PO4
0
1000
Insulin (μU/ml)

Fig. 4. Insulin binding by solei. Rats were fasted and one sciatic nerve severed 24 hrs before experiment. Half solei were preincubated for 30 min in 2 mM pyruvate, then incubated for 2 hrs in identical media supplemented with 100,000 dpm ^{125}I-insulin and increasing concentrations of unlabeled insulin. Specific insulin binding was measured as described in Methods, each point is the Mean ± SE of 6-14 observations in control and in denervated muscles.

Inset: One half-soleus of each side was incubated with 1 ng/ml insulin, the other with 50 μg/ml for non-specific binding. Each bar represents the mean specific binding ± SE of 13 control (clear bars) and 13 denervated solei (black bars). *$p < 0.05$ vs. control.

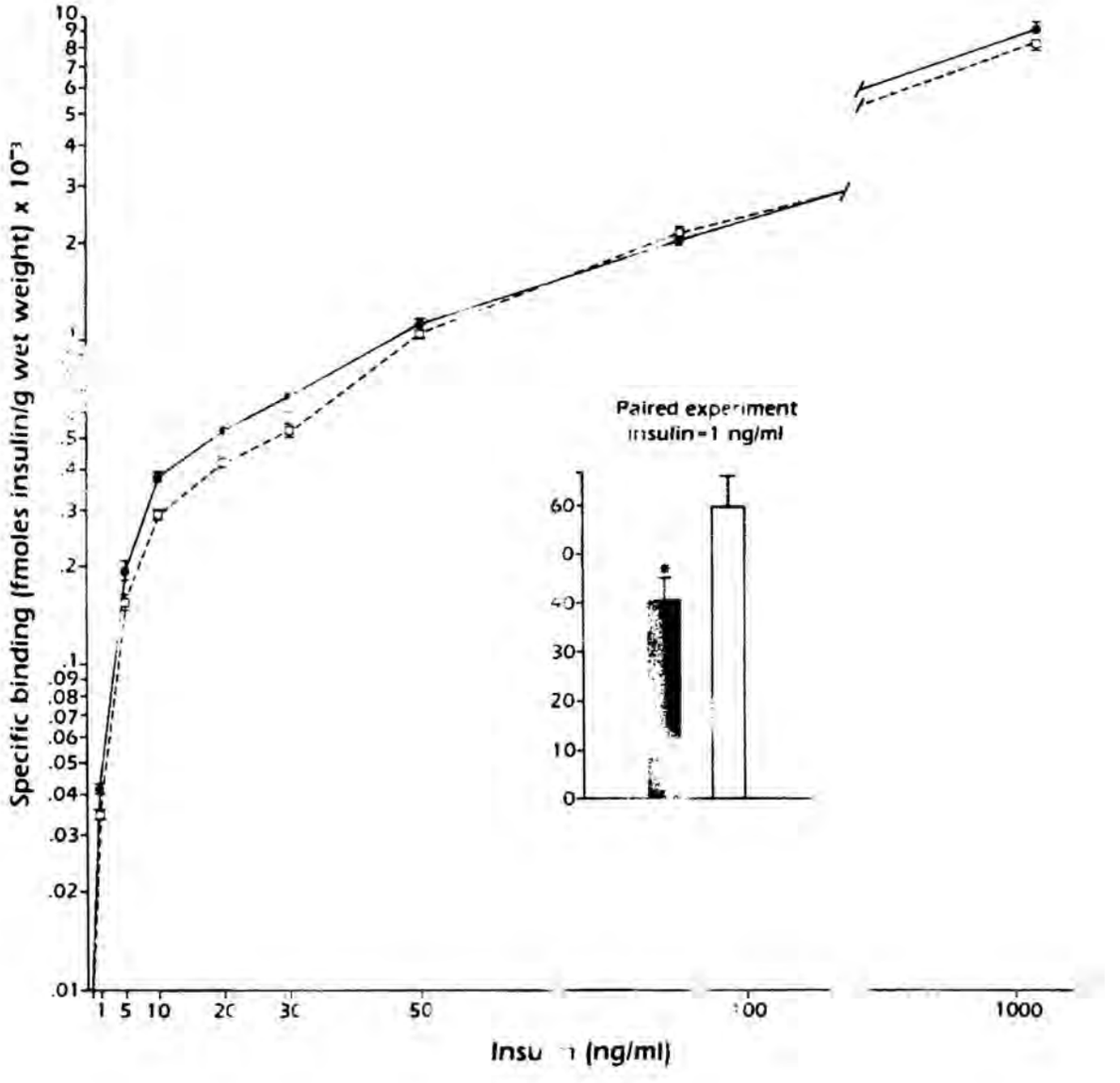
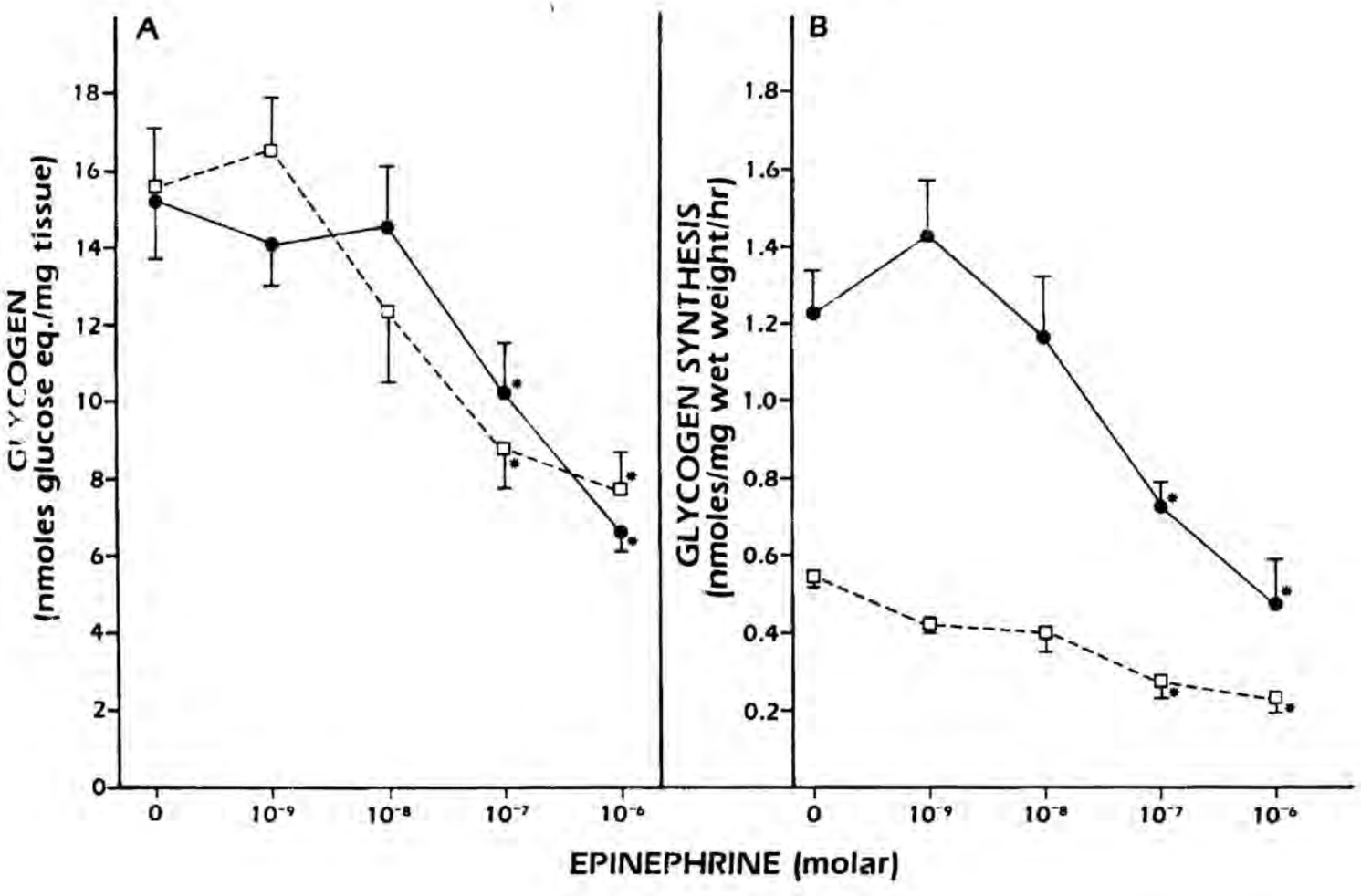

Specific binding (fmoles insulin/g wet weight) x 10⁻³
10
9
8
7
6
5
4
3
2
1
.5
.4
.3
.2
.1
.09
.08
.07
.06
.05
.04
.03
.02
.01
1 5 10 20 30 50 100 1000
Insulin (ng/ml)
Paired experiment
Insulin=1 ng/ml
60
0
40
30
20
10
0

Fig. 5. Effect of epinephrine on glycogen content and glucose incorporation into glycogen. Solei were obtained from ad lib. fed rats 24 hrs. after unilateral denervation. Half-solei were preincubated for 30 min in 5 mM glucose, then incubated for 60 min in identical media supplemented with [U-^{14}C]glucose (0.2 μCi/ml), with and without increasing concentrations of epinephrine. At the end of incubation muscles were processed for measurement of glycogen content (Panel A) and [^{14}C]glucose incorporation into glycogen (Panel B). Means ± SE of 6-10 observations are shown. * $p < 0.05$ vs. muscles incubated without epinephrine. Control ; denervated .


A
B
GLYCOGEN
(nmoles glucose eq./mg tissue)
GLYCOGEN SYNTHESIS
(nmoles/mg wet weight/hr)
EPINEPHRINE (molar)
0
10⁻⁹
10⁻⁸
10⁻⁷
10⁻⁶

REFERENCES

1. Kochan, R.G., D.R. Lamb, S.A. Lutz, C.V. Perrill, G.M. Reimann and K.K. Schlender. "Glycogen Synthase Activation in Human Skeletal Muscle: Effects of Diet and Exercise. Am. J. Physiol. 236;E660-E666, 1979.

2. Richter, E.A., L.P. Garretto, M.N. Goodman, and N.B. Ruderman. "Muscle Glucose Metabolism following Exercise in the Rat: Increased Sensitivity to Insulin. J. Clin. Invest. 69;785-793, 1982.

3. Buse, M.G. and J. Buse. :Glucose Uptake and Response to Insulin of the Isolated Rat Diaphragm. The Effect of Denervation. Diabetes 8;218-225, 1959.

4. Buse, M.G. and J. Buse. "Effect of Denervation and Insulin on the Penetration of D-xylose into Rat Hemidiaphragms. Diabetes 10;134-141, 1961.

5. Seider, M.J., W.F. Nicholson and F.W. Booth. "Insulin Resistance for Glucose Metabolism in Disused Soleus Muscle of Mice." Am. J. Physiol. 242;E12-E18, 1982.

6. Forsayeth, J.R. and M.K. Gould. "Inhibition of Insulin-stimulated Zylose Uptake in Denervated Rat Soleus Muscle: A Post-receptor Effect." Diabetologia 23; 511-516, 1982.

7. Gey, G.O. and M.K. Gey. The Maintenance of Human Normal and Tumor Cells in Continuous Culture. Am. J. Cancer 27;45-76, 1936.

8. LeMarchand-Brustel, Y., B. Jeanrenaud, P. Freychet. "Insulin Binding and Effects in Isolated Soleus Muscles of Lean and Obese Mice." Am. J. Physiol. 234;E348-E358, 1978.

9. Cuendet, G.S., E.G. Loten, B. Jeanrenaud, and A.E. Renold. "Decreased Basal, Non-insulin Stimulated Glucose Uptake and

Metabolism by Skeletal Soleus Muscle Isolated from Obese-Hyperglycemic (ob/ob) Mice. J. Clin. Invest. 58;1078-1088, 1976.

10. Van Handel, E. 1965. "Estimation of Glycogen in Small Amounts of Tissue. Anal. Biochem. 11;256-265, 1965.

11. Olefsky, J.M. "Mechanisms of the Ability of Insulin to Activate the Glucose-transport System in Rat Adipocytes. Biochem. J. 172; 137-145, 1978.

12. Lowry, O.H., and J.V. Passoneau. "ATP, Phosphocreatine and Glucose-6-phosphate Assay, Method B." In: A Flexible System of Enzymatic Analysis. New York: Academic, 1972, p. 151-153.

13. Thomas, J.A., K.K. Schlender, and J. Larner. "A Rapid Filter Paper Assay for UDP-glucose-glycogen Glucosyltransferase, Including an Improved Synthesis of UDP-^{14}C Glucose. Anal. Biochem. 25;486-499, 1968.

14. Kochan, R.G., D.R. Lamb, E.M. Reimann, and K.K. Schlendler. "Modified Assays to Detect Activation of Glycogen Synthase following Exercise." Am. J. Physiol. 240 (Endocrinol. Metab. 3);E197-E202, 1981.

15. Guinovart, J.J., A. Salavert, J. Massague, C.J. Cuidad, E. Salsas, and E. Itarte. "Glycogen Synthase: A New Activity Ratio Assay Expressing a High Sensitivity to the Phosphorylation State." FEBS Lett. 106;284-288, 1979.

16. Lowry, O.H., N.Y. Rosebrough, A.L. Farr, and R.J. Randall. "Protein Measurement with the Folin Phenol Reagent." J. Biol. Chem. 193;265-275, 1951.

17. Galbraith, R.A., C. Wise, and M.G. Buse. "Insulin Binding and Response in Erythroblastic Leukemic Cells." Diabetes 29;571-578, 1980.

18. Crettaz, M., M. Prentki, D. Zaninetti, and B. Jeanrenaud. "Insulin Resistance of Soleus Muscle from Obese Zucker Rats." Biochem. J. 186;525-534, 1980.

19. Ariano, M.A., R.B. Armstrong, and V.R. Edgerton. "Hindlimb Muscle Fiber Population in Five Mammals. J. Histochem. Cytochem. 21;51-55, 1973.

20. Bonen, A., M.H. Jan, W.M. Watson-Wright. "Insulin Binding and Glucose Uptake Differences in Rodent Skeletal Muscles." Diabetes 30;702-704, 1981.

21. Ivy, J.L. and J.O. Holloszy. "Persistent Increase in Glucose Uptake by Rat Skeletal Muscle following Exercise. Am. J. Physiol. 241;C200-C203, 1981.

22. Davidheiser, S., E.F. Haugaard and N. Haugaard. "Effects of Epinephrine and the Cyclic AMP Phosphodiesterase Inhibitor SQ 2009 on Glucose and Glycogen Metabolism in kSeletal Muscle." Biochem. Pharmacol. 28;807-813, 1979.

23. Berger, M., S.A. Hagg and N.B. Ruderman. "Glucose Metabolism in Perfused Skeletal Muscle. Interaction of Insulin and Exercise on Glucose Uptake." Biochem. J. 146;231-238, 1975.

24. Smith, R.L. and J.C. Lawrence. "Mechanism of Denervation-induced Insulin Resistance in Skeletal Muscle. Diabetes 32 (Suppl. 1), 128A (Abstract), 1983.

25. DePaoli Roach, A.A., C. Villar-Palasi and E. Bergamini. "Effect of Denervation on Glycogen Synthase Kinase Activity." Exp. Neurol. 62;494-496, 1978.

26. Chiasson, J.-L., M.R. Dietz, H. Shikama, M. Wooten and J.H. Exton. "Insulin Regulation of Skeletal Muscle Glycogen Metabolism." Am. J. Physiol. 239; E69-E74, 1980.

27. Fischbach, G.D. and N. Robbins. "Effect of Chronic Disuse of Rat Soleus Neuromuscular Junctions on Post-synaptic Membrane." J. Neurophysiol. 34;562-569, 1971.

28. DuBois, D.C. and R.R. Almon. "Disuse Atrophy of Skeletal Muscle is Associated with an Increase in Number of Glucocorticoid Receptors." Endocrinology 107;1649-1651, 1980.

29. DuBois, D.C. and R.R. Almon. "A Possible Role for Glucocorticoids in Denervation Atrophy." Muscle and Nerve 4;370-373, 1981.

30. Booth, F.W. "Effect of Limb Immobilization on Skeletal Muscle." J. Appl. Physiol: Respirat. Environ. Exercise Physiol. 52;1113-1118, 1982.

31. Dolkas, C.B. and J.E. Greenleaf. "Insulin and Glucose Responses during Bed Rest with Isotonic and Isometric Exercise." J. Appl. Physiol.: Respirat. Environ. Exercise Physiol. 43;1033-1038, 1977.

32. Duckworth, W.C., S.S. Solomon, P. Jallepalli, C. Heckemeyer, J. Finnern and A. Powers. "Glucose Intolerance Due to Insulin Resistance in Patients with Spinal Cord Injuries." Diabetes 29;906-910, 1980.

33. National Diabetes Data Group. "Classification and Diagnosis of Diabetes Mellitus and Other Categories of Glucose Intolerance." Diabetes 28;1039-1057, 1979.

34. Bright, J.M., T.W. Melton, A.D. Rogol and W.L. Clarke. "Failure of Cortisol Blockade to Inhibit Early Morning Increases in Basal Insulin Requirements in Fasting, Insulin-dependent Diabetics." Diabetes 29;662-664, 1980.

35. Berger, M., P. Berchtold and F.W. Kemmer. "Metabolic and Hormonal Effects of Exercise in Diabetic Patients." In: Diabetes Mellitus, Vol. III, Intermediary Metabolism and Its Regulation, edited by M. Brownlee. New York: Garland STPM Press, 1981, pp 273-305.

36. Garetto, L.P., E.A. Richter, M.N. Goodman, and N.B. Ruderman. "Enhanced Insulin Sensitivity of Skeletal Muscle following Exercise." In: Biochemistry of Exercise, edited by J. Poortmans. Champaign, Ill.: Human Kinetics Publishers, 1983, 681-687.

37. Horton, E.S., E.D. Horton, M. Hirshman, E. Ravussin. "Increased Insulin Sensitivity without Altered Insulin Binding in Rat Soleus Muscle following Exercise." Abstract, Program of XIth International Diabetes Federation Congress, 1982.

CHAPTER 3: PHOSPHORYLATION OF INSULIN RECEPTORS SOLUBILIZED FROM RAT SKELETAL MUSCLE

Introduction: Elucidation of the mechanism of insulin action has advanced significantly with the recent characterization of the structure of the insulin receptor, and the discovery of its autophosphorylation and associated protein kinase activity upon binding of insulin. These studies have been performed in adipocytes (1), hepatocytes (2), erythrocytes (3), monocytes (4), brain (5), placenta (6), and various cells in culture (7); however, direct study of the insulin receptor in skeletal muscle has been hampered by technical difficulties.

In view of its mass, skeletal muscle is the most important target tissue of insulin action. Regulation of the insulin receptor in muscle is of particular interest, since contractile activity modulates its sensitivity and response to insulin, apparently at an early step after insulin binding (8). Furthermore, insulin resistance of skeletal muscle, a salient feature of several pathological conditions e.g. type II diabetes, obesity and uremia, appears to involve primarily defective coupling between insulin binding and response (for review see 9). The methods reported here should provide a system for probing the actions of insulin and the effect of modulators of insulin action in skeletal muscle.

Materials and Methods

Receptor Purification. Male Wistar rats (Charles Rivers Breeding Laboratories, Wilmington, Mass.), weighing 60-100 g, were decapitated after an overnight fast. Hind limb muscles were trimmed of fat and quickly frozen in liquid nitrogen. The frozen muscle was finely ground in a mortar precooled to -80°C and to it was added ice-cold 3.5 v/w of a buffer consisting of 25 mM Hepes*, 1% Triton-X 100, 4 mM EDTA, 1 TIU/ml aprotinin and 2 mM PMSF, pH 7.4. The frozen slurry was homogenized as it thawed in a Potter-Elvejhem homogenizer then centrifuged at 10,000 x g for 10 min at 4°C. The resulting supernate was slowly stirred at 21°C for 60 min. then centrifuged at 4°C (150,000xg for 90 min.) and the supernate (150K supernatant) applied to an agarose-bound wheat germ agglutinin column (WGA, approx. 3 g original muscle weight/ml resin). The column was washed with 25 mM Hepes, 0.1% Triton X-100 pH 7.4 (Buffer A, 75 ml/ml settled gel) and receptors eluted with this buffer supplemented with 0.3 M N-acetylglucosamine (WGA eluate). Typically 1 ml of WGA eluate/g original muscle weight was collected.

Insulin binding to solubilized receptors was carried out according to Taylor et al. (10). 150 K supernatant (20 µl) or WGA eluate (100 µl) were incubated with either 10,000 or 100,000 cpm of ^{125}I-insulin (11) (spec. act. 125 µCi/µg) in 50 mM Hepes (pH 7.8), 0.1% Triton X-100, 150 mM NaCl, 0.1% bovine serum albumin (fatty acid free, Sigma), 100 U/ml bacitracin and varying concentrations of unlabeled insulin ($3x10^{-11}$ - $8x10^{-6}$M) for 16 hrs at 4°C in a final volume of 0.2 ml. The receptor was precipitated by sequential additions of 0.1 ml bovine γ-globulin (3 mg/ml) and 0.3 ml polyethylene glycol (25% wt/vol.). Each point was assayed in triplicate, non-specific binding.

In vitro phosphorylation of purified insulin receptors. WGA eluate (50 µl, approx. 0.03 mg protein) was preincubated in 25 mM Hepes (pH 7.4), 0.1% Triton X-100, 5 mM $MnCl_2$ and 0.1% bovine serum albumin (buffer B) with or without varying concentrations of insulin for 40 min. at 21°C in 75 µl final vol. [γ-^{32}P]ATP was prepared using [^{32}P]H_3PO_4 (ICN) and Gammaprep vials, (Promegabiotec, Madison, WI.). [γ-^{32}P]ATP (5 µl, final conc. 5 µM, 5-15 mCi/µmole) was added to each sample and incubation continued for 20 min. The reaction was stopped with 20 µl 25 mM Hepes buffer pH 7.4 containing 0.1% Triton X-100, 10 mM Na pyrophosphate, 20 mM ATP and 2 mM PMSF at 4°C. Polyclonal anti-rat insulin receptor antibody diluted 1:500 (kindly provided by Dr. Steve Jacobs, Burroughs-Wellcome) or control rabbit serum was added and the mixture incubated overnight at 4°C. The immune complex was precipitated with Protein A (Pansorbin, Calbiochem) (6),washed 3 times in buffer A and boiled for 5 min. in Laemmli's sample buffer (13) with or without 5 mM 2-mercaptoethanol. In studies of the time-course of autophosphorylation and of histone phosphorylation, this protocol was modified, as described in the Legends of Fig. 3 and Fig. 4, respectively.

In situ phosphorylation of insulin receptors from solei. Solei from 18 hr fasted (50-70 g) rats were removed, divided longitudinally and mounted on stainless steel frames at resting length. Individual half-solei were incubated for 1 hr at 25°C in 3 ml Earle's Minimal Essential Medium (MEM) modified to contain 0.1 mM Na_2HPO_4. Groups of 6 muscles were then transferred to flasks containing 9 ml of phosphate free MEM supplemented with 0.25-0.4 mCi/ml of 0.1 mM Na_2[^{32}P]PO_4 and incubated for 3 hrs at 37°C. After labelling endogenous ATP pools the muscle groups were incubated for 30 min at 37°C in 9 ml Gey and Gey's

balanced salt solution (bicarbonate buffer,15) containing 0.1 mM $Na_2H[^{32}P]PO_4$ of identical spec. act., with or without 10 mU/ml insulin. All incubations were under 95% O_2 + 5% CO_2 atmosphere. The muscles were quickly removed from the supporting frames, immediately frozen in liquid nitrogen, and each group of 6 muscles crushed in a glass Potter-Eljevhem homogenizer precooled in liquid nitrogen. Buffer (2 ml) containing 50 mM Hepes, 1.5% Triton X-100, 10 mM NaF, 0.2 mM Na_3VO_4, 10 mM sodium pyrophosphate, 40 mM ATP, 1 TIU/ml aprotinin, 100 mU/ml Bacitracin and 2 mM PMSF, pH 7.5 at 4°C was added and the frozen slurry homogenized as it thawed. The homogenate was centrifuged at 200,000 x g for 60 min at 4°C and the supernate applied to 0.75 ml WGA. The column was washed with 75 ml Buffer A containing 5 mM EDTA, 10 mM sodium pyrophosphate, 1 TIU/ml Aprotinin and 2 mM PMSF and eluted with 1.8 ml of this buffer supplemented with 0.3 M N-acetylglucosamine. The insulin receptor was then immunoprecipitated and washed as described above.

Electrophoresis and Autoradiography. Reaction samples or immunoprecipitates were applied to either a 5% (for non-reduced receptors), or 7.5% (for reduced receptor samples) or a 5-25% gradient polyacrylamide gel (for histone samples) with molecular weight standards (Sigma) according to Laemmli (12). Following electrophoresis gels were fixed, stained with Coumassie blue, destained and dried under vacuum. Autoradiography was performed with Kodak AR Film with or without a DuPont Lightening Plus intensifying screen. For quantitation of radioactivity gel slices were digested overnight at 55°C in 1 ml 30% H_2O_2 and then counted in Scintiverse (Fisher).

RESULTS AND DISCUSSION.

Insulin binding by receptors solubilized from hind limb skeletal muscle display classic curvilinear Scatchard plots (Fig. 1a). The 150,000 x g supernate reproducibly yielded approximately 0.4 pmoles of insulin binding activity/mg protein; assuming that each insulin receptor bound two insulin molecules (17), the calculated yield was approximately 0.5 pmol solubilized receptor/g wet weight of original muscle mass. Subsequent WGA chromatography resulted in approximately 8-10 fold purification (per mg protein) and 75-80% recovery of binding activity (Fig. 1b).

Incubation of WGA purified receptors with increasing concentrations of insulin and [γ-^{32}P]ATP followed by immunoprecipitation with polyclonal anti-insulin receptor serum revealed that insulin stimulates the incorporation of ^{32}P into a 95KD protein when the immunoprecipitate is electrophoresed under reducing conditions (Fig. 2). As reported, for other tissues, this corresponds to the β-subunit of the insulin receptor (16). Autophosphorylation of the 95KD subunit was stimulated half-maximally at approximately 5×10^{-9}M insulin with a four-fold increase at 10^{-7}M insulin. Insulin stimulated phosphorylation of the 95KD subunit showed an absolute requirement for Mn^{++} (data not shown). A labelled protein with apparent MW of 130KD (α-subunit) was also observed in the immunoprecipitate but its phorphorylation was not stimulated by insulin. Under nonreducing conditions, receptors incubated with 10^{-6}M insulin showed ^{32}P incorporation into bands with apparent MW of 360, 290, 95KD; probably corresponding to $\alpha_2\beta_2$, $\alpha_2\beta\beta_1$ and β subunit stoichometries (17) (Fig. 2, Lanes I and J).

Determination of the time required for autophorphorylation of the

insulin receptor β-subunit (Figure 3) necessitated rapid quenching of the reaction, the concommitant denaturation of the receptor precluded immunoprecipitation. Since on autoradiograms of reduced gels only the 95KD protein showed increased labelling after addition of insulin, we were confident that this was the β-subunit of the insulin receptor (Fig. 2, lanes G and H). The $t_{\frac{1}{2}}$ for autophorphorylation in the presence of 5 mM $MnCl_2$ and 5 μM ATP was approximately 100 sec; maximal phosphorylation remained relatively constant between 4 and 10 min. and gradually decreased thereafter.

Insulin induced autophosphorylation of the receptor, probably through an intramolecular event (18), stimulates its activity as a kinase towards exogenous substrates (14,19). This property was also observed with insulin receptors purified from skeletal muscle, using histone H2B as the exogenous substrate, (Fig. 4). The incorporation was linear for 5 min but decreased by 15 min suggesting the presence of phosphoprotein phosphatase activity. The phosphorylation events probably occurred on tyrosine residues since incubation of gels in 0.5 N NaOH did not diminish the ^{32}P content of the 95KD protein nor that of histones (data not shown). Mild base treatment has been reported to selectively hydrolyse phosphoserine and phosphothreonine residues leaving most phosphotyrosine residues intact (20).

Long term incubation of soleus muscle in phosphate poor minimal essential medium supplemented with $[^{32}P]PO_4$ was required to equilibrate endogenous ATP pools with ^{32}P. Preliminary experiments showed that even after 4 hrs in low phosphate medium, solei were still responsive to 10 mU/ml insulin as shown by a 4-fold increase in $[^{14}C]$glucose incorporation into glycogen (data not shown). By quickly freezing,

grinding and homogenizing muscles which were incubated for 3.5 hours with $[^{32}P]PO_4$, the last half hour with or without insulin, we were able to observe an approximately 2-fold increase in ^{32}P labelling of the insulin receptor β-subunit of muscles stimulated with insulin (Fig. 5).

As alluded to above, the most common conditions associated with insulin resistance of skeletal muscle, have been classified as "post-receptor" events, since decreased insulin binding could not account for the observed decrease in insulin sensitivity and/or response. However, intact insulin binding (primarily a function of the α-subunit) does not preclude the possibility of receptor abnormalities (of structure or function due to an altered microenvironment) which may result in impaired propagation of signals. Indeed, in a subgroup of patients with type A insulin resistance, insulin binding to monocytes was normal, but insulin mediated receptor autophosphorylation was impaired (21). The demonstration of insulin stimulated in situ phosphorylation of the β-subunit of the insulin receptor in intact muscles and the easy availability of solubilized receptors from this tissue should further the investigation of factors which modulate receptor-function coupling in muscle, such as disuse and exercise.

Fig. 1. Scatchard plot of insulin binding by solubilized receptors. Receptors were solubilized as described in Methods; 20 µl of 150,000xg supernatant (A) or 100 µl of WGA eluate (B) were incubated with 0.4-4ng ^{125}I-insulin and increasing concentrations of non-labelled insulin. Non-specific binding (20-25%) was subtracted from each point. Data represent the mean of 3-7 determinations from 3 separate experiments.

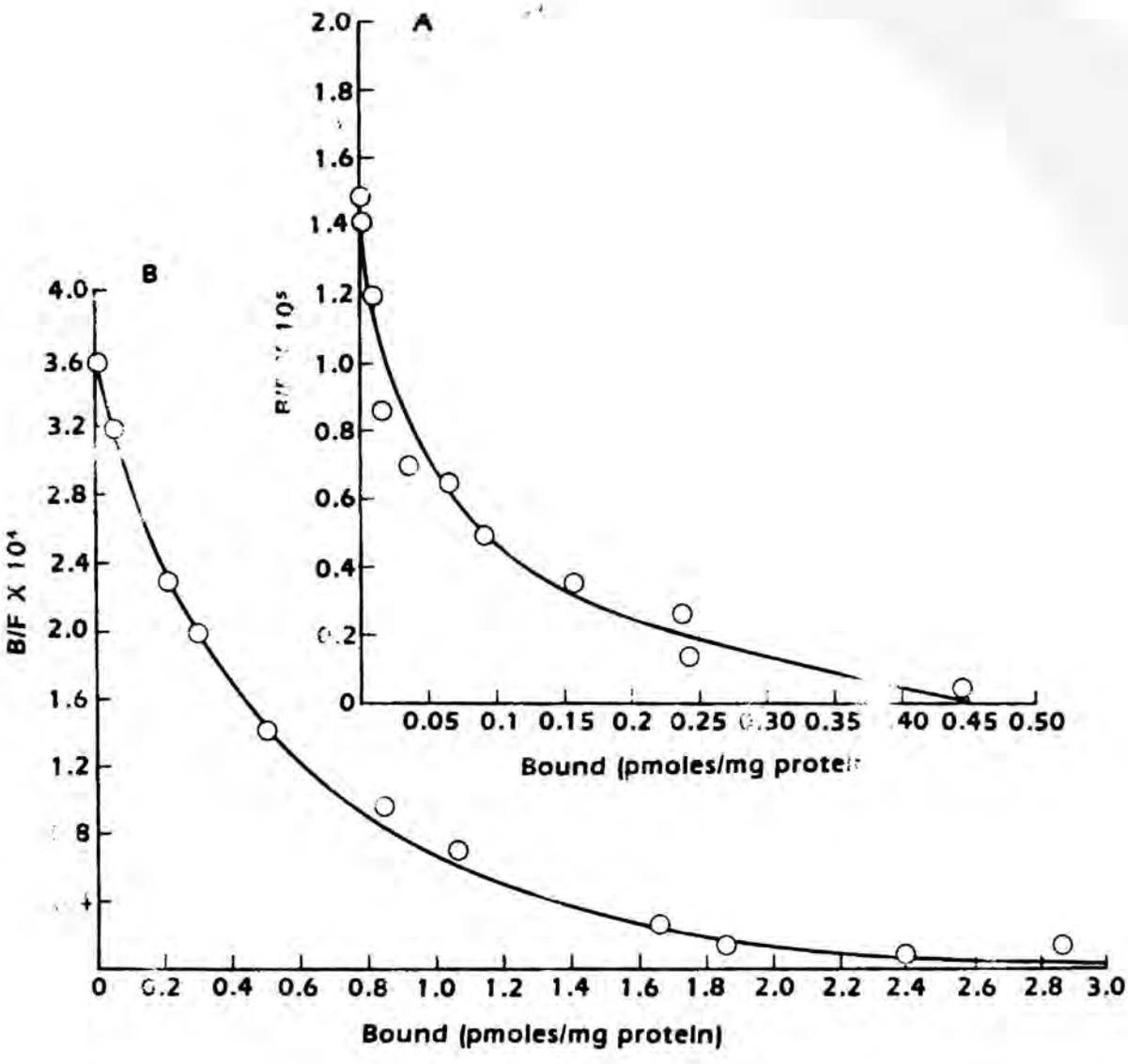

A
B
B/F X 10⁵
B/F X 10⁴
Bound (pmoles/mg protein)
Bound (pmoles/mg protein)

Fig. 2. Identification of muscle insulin receptor. Wheat germ eluate (50µl) was incubated with or without insulin for 40 min at 21°C after which [γ^{32}P]ATP was added for an additional 20 min. Proteins were separated by SDS-PAGE (7.5%). Autoradiographs of ^{32}P-labeled proteins are shown. Lanes A-E:SDS-PAGE under reducing conditions of insulin receptors immunoprecipitated by polyclonal anti-insulin receptor antibody or non-immune rabbit serum (Lane F) after incubation with 0(A), 10^{-9}(B), 10^{-8}(C), 10^{-7}(D), 10^{-6}(E&F) M insulin. Lanes G and H:SDS-PAGE under reducing conditions of the complete reaction mixture (without immunoprecipitation) after incubation without (G) or with (H) 10^{-6}M insulin. Lane I: SDS-PAGE under non-reducing conditions of WGA after incubation with 10^{-6} M insulin followed by immunoprecipitation. Lane J: Same as I but using non-immune rabbit serum. Graph represents relative ^{32}P incorporation into the β-subunit of the insulin receptor incubated as described for Lanes A-E and immunoprecipitated by anti-receptor antibody. Points are means ± SE of 3 observations. Arrow indicates the β-subunit of the insulin receptor.

Fig 3. Time course of ^{32}P incorporation into the β-subunit of the insulin receptor. Wheat germ eluate (300 µl) was preincubated with $2x10^{-7}$ M insulin for 40 min at 21°C in a final volume of 360 µl followed by the addition of 10 µl [γ-^{32}P]ATP (final conc. 5 µM, 5-15 mCi/µmole). At the times indicated 35 µl aliquots were transferred into 35 µl twice concentrated Laemmli's sample buffer and boiled for 5 min. After SDS-PAGE under reducing conditions the 95 KD bands were identified by autoradiography, excised and counted for radioactivity. Bars represent means ± SE for 3 experiments. Inset: Representative autoradiogram of first 9 time points plotted on graph. Arrow indicates β-subunit of the insulin receptor.

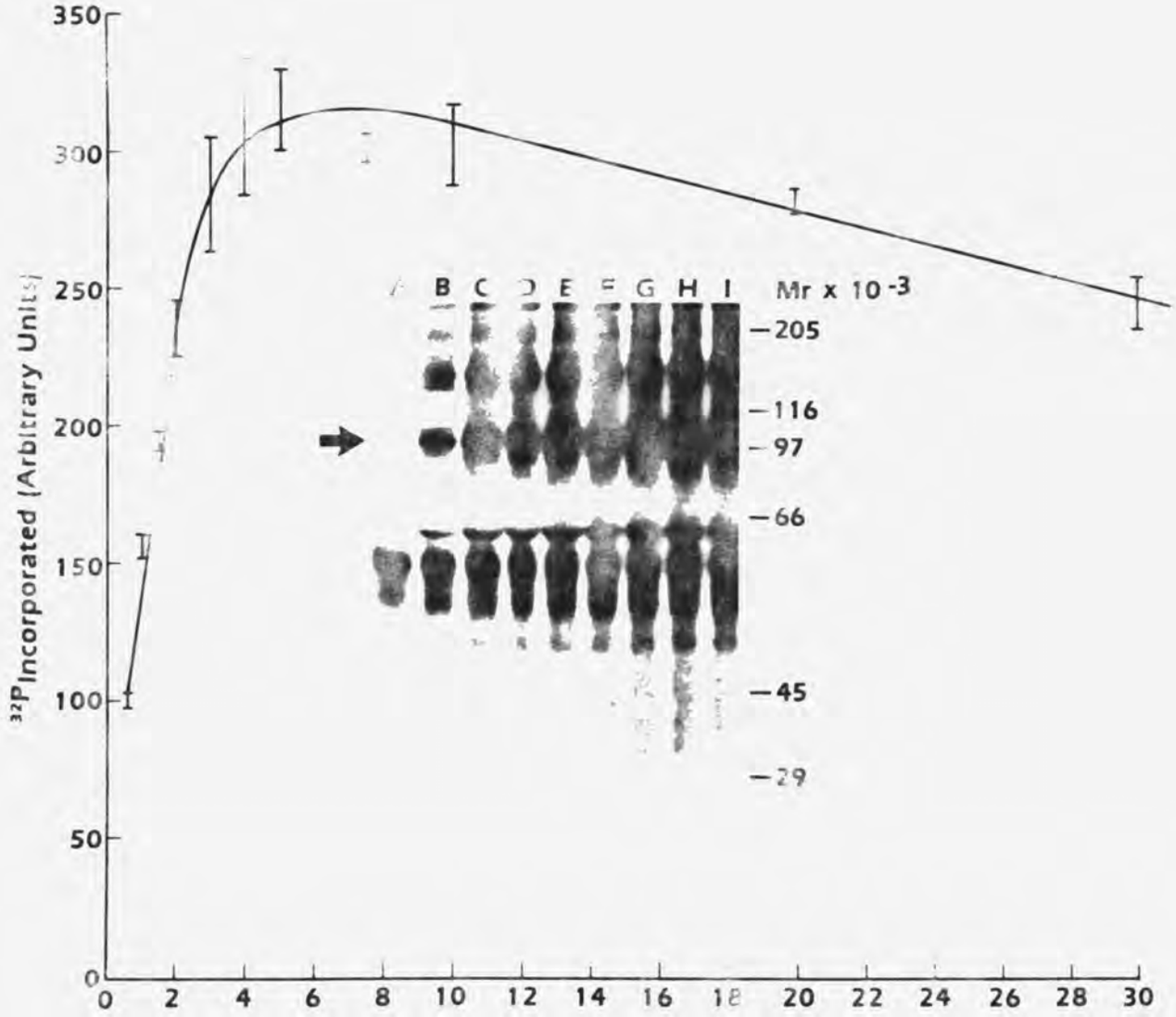

350
300
250
200
150
100
50
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
32P Incorporated (Arbitrary Units)
B C D E F G H I
Mr x 10-3
205
116
97
66
45
29

Fig. 4. Insulin stimulated phosphorylation of Histone H2B. WGA eluate (150 µl) was preincubated in Buffer B (final volume 700 µl) with or without $2x10^{-7}$ M insulin for 46 min at 21°C; $\gamma[^{32}P]$ATP (5µM, 5-15mCi/µmole) was included for the last 6 min. Aliquots (35 µl) were then added to 5µl histone H2B (Worthington Biochemical,final concentration 0.4 mg/ml) and further incubated for the times indicated. The samples were then either boiled in Laemmli's solution (for subsequent gel electrophoresis) or precipitated with 40 µl of 25% trichloroacetic acid. The precipitates were hydrolysed at 55°C in 0.5 M NaOH for 1 hr (14), reprecipitated, collected on glass filter papers and counted for radioactivity. Points are means of 2 experiments. Inset: Autoradiogram of SDS-PAGE (reducing conditions) of incubation mixture after 15 min in the absence (Lane A) or the presence of insulin (Lane B). Arrow indicates β-subunit of the insulin receptor. Histones are between MW markers of 14 and 20 KD.

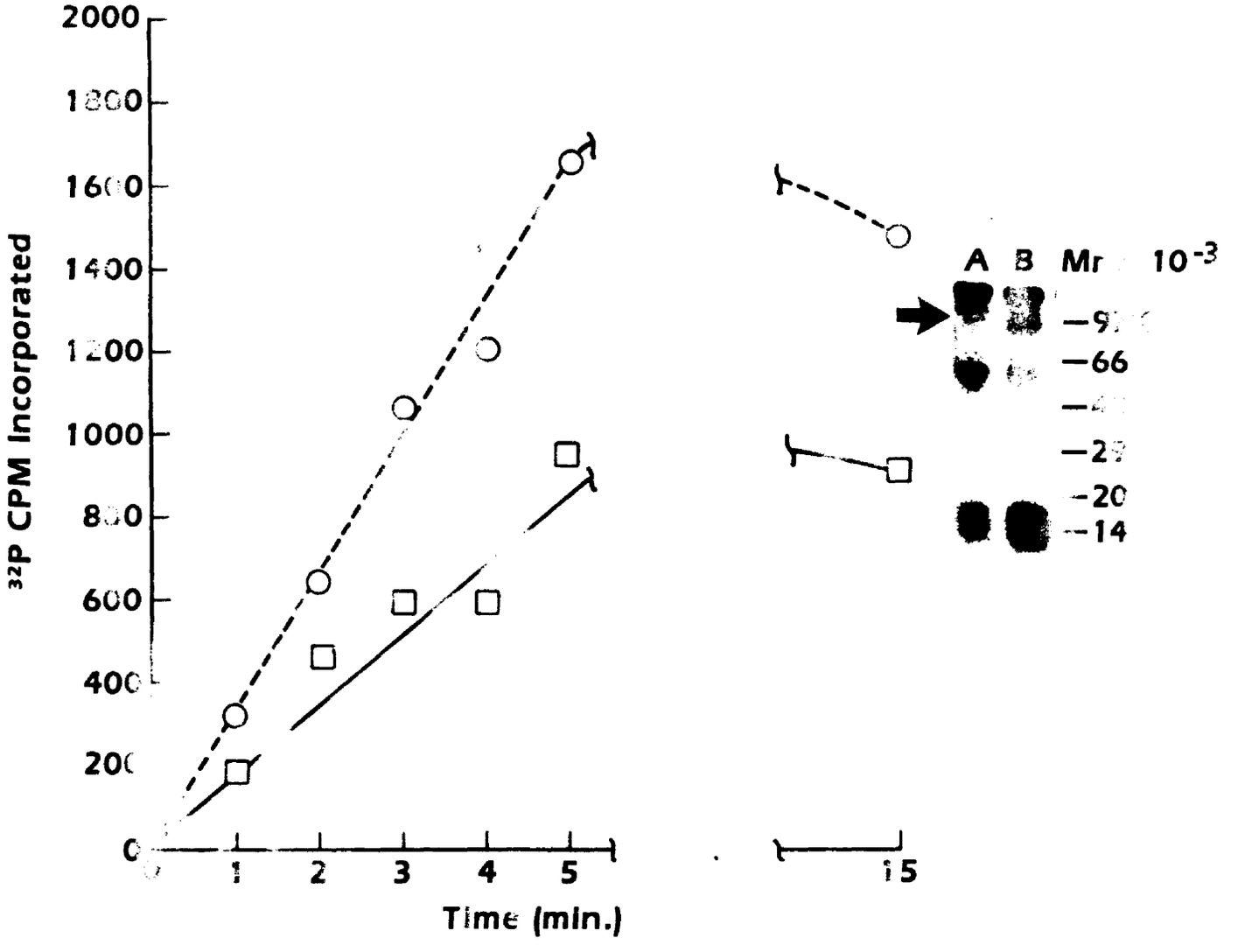
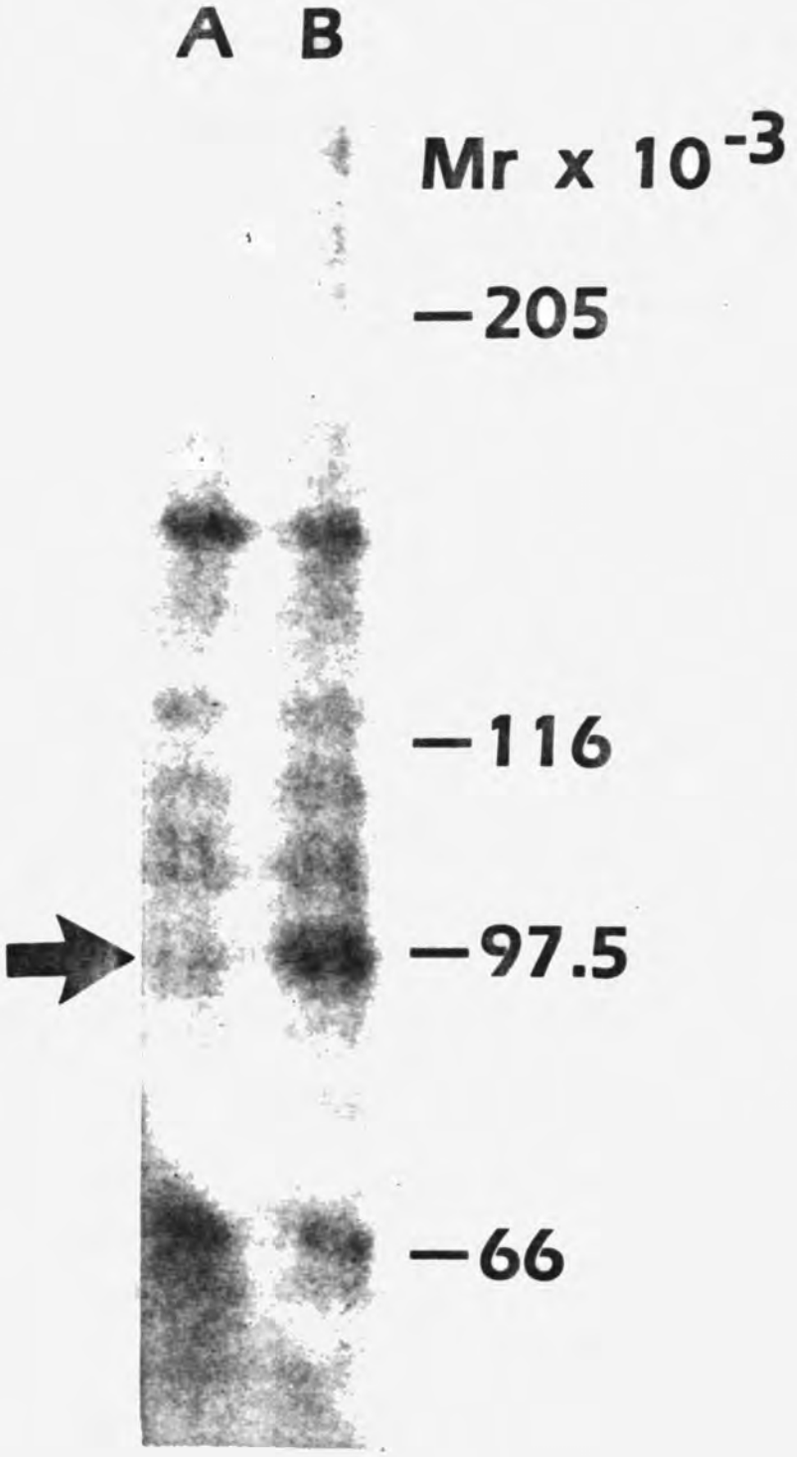

2000
1800
1600
1400
1200
1000
800
600
400
200
0
1
2
3
4
5
15
Time (min.)
32P CPM Incorporated
A
B
Mr
10-3
-66
-20
-14

Fig. 5. Autoradiogram of insulin receptors phosphorylated in situ. Half solei were incubated for 3 hours in MEM with $Na_2H[^{32}P]PO_4$ and then an additional ½ hour in bicarbonate medium supplemented with $Na_2H[^{32}P]$ without (Lane A) or with (Lane B) 10 mU/ml insulin. Receptors were then solubilized, partially purified by WGA chromatography and immunoprecipitated as described. Arrow indicates β-subunit of insulin receptor.


A B
Mr x 10-3
—205
—116
—97.5
—66

1. Tamura, S., Brown, T.A., Dubber, R.E., and Larner, J. "Insulin-like Effect of Vanadate on Adipocyte Glycogen Synthase and on Phosphorylation of 95,000 Dalton Subunit of Insulin Receptor." Biochem. Biophys. Res. Comm. 113;80-86, 1983.

2. Van Obberghen, E. and Kowalski, A. "Phosphorylation of the Hepatic Insulin Receptor." FEBS Lett. 143;179-182, 1982.

3. Grigoresev, F., White, M.F., and Kahn, C.R. "Insulin Binding and Insulin-dependent Phosphorylation of the Insulin Receptor Solubilized from Human Erythrocytes." J. Biol. Chem. 258;13708-13716, 1983.

4. Grunberger, G., Zick, Y., Roth, J., and Gorden, P. "Protein Kinase Activity of Insulin Receptors in Human Circulating and Cultured Mononuclear Cells." Biochem. Biophys. Res. Commun. 115;560-566, 1983.

5. Heindenrich, K.A., Zahnisev, N.R., Berhanu, P., Brandenberg, D. and Olefsky, J. "Structured Differences between nIsulin Receptors in the Brain and Peripheral Target Tissues." J. Biol. Chem. 258;8527-8550, 1982.

6. Kasuga, M., Fujita-Yamaguchi, Y., Blithe, D.L., White, M.F., and Kahn, C.R. "Characterization of the Insulin Receptor Purified from Human Placental Membranes." J. Biol. Chem. 258;10973-10980, 1983.

7. Kasuga, M., Karlseson, F.A., and Kahn, C.R. "Insulin Stimulates the Phosphorylation of the 95,000-Dalton Subunit of its Own Receptor." Science 215; 185-187, 1982.

8. Richter, E.A., Garrett, L.P., Goodman, M.N., and Ruderman, N.B. "Muscle Glucose Metabolism following Exercise in the Rat: Increased Sensitivity to Insulin." J. Clin. Invest. 69;785-793.

9. Olefsky, J.M. and Ciaraldi, T.P. In: Diabetes mellitus, edited by M. Brownlee. New York: Garland STPM Press, 1981, pp 73-116.

10. Taylor, S.I., Hedo, J.A., Underhill, L.H., Kasuga, M., Elders, M.J. and Rath, J. "Extreme Insulin Resistance in Association with Abnormally High Binding Affinity of Insulin Receptors from a Patient with Leprechaunism: Evidence for a Defect Intrinsic to the Receptor." J. Clin. Endocrin. and Metab. 55;1108-1113, 1982.

11. Galbraith, R.A., Wise, C., Buse, M.G. "Insulin Binding and Response in Erythroblastic Leukemic Cells." Diabetes 29;571-578, 1980.

12. Bradford, M.M. "A Rapid and Sensitive Method for Quantitation of Microgram Quantities of Protein Utilizing the Principal of Protein-binding." Anal. Biochem. 976; 72:248-254, 1976.

13. Laemmli, V.K. "Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4." Nature (Lond.) 227;680-685, 1970.

14. Rosen, O.M., Herrera, R., Olowe, Y., Petruzzeli, L.M., Cobb, M.H. "Phosphorylation Activates the Insulin Receptor Tyrosine Protein Kinase." Proc. Natl. Acad. Sci. USA 80;3237-3240, 1983.

15. Gey, G.O. and Gey, M.K. "The Maintenance of Human Normal and Tumor Cells in Continuous Culture." Am. J. Cancer 27;45-76, 1936.

16. Jacobs, S. and Cuatrecasas, P. "Insulin Receptor: Structure and Function." Endocrin. Reviews 251-263, 1981.

17. Massague, J., Pilch, P.F. and Czech, M.P. "Electrophoretic Resolution of Three Major Insulin Receptor Structures with Unique Subunit Stoichiometries." Proc. Natl. Acad. Sci. U.S.A. 77;7137-7141, 1980.

18. White, M.F., Haring, H.U., Kasuga, M. and Kahn, C.R. "Kinetic Properties and Sites of Autophosphorylation of the Partially Purified Insulin Receptor from Hepatoma Cells." J. Biol. Chem. 259; 255-264, 1984.

19. Stadtmauer, L.A. and Rosen, O.M. "Phosphorylation of Exogenous Substrates by the Insulin Receptor-associated Protein Kinase." J. Biol. Chem. 258;6682-6685, 1983.

20. Cooper, J.A. and Hunter, J. "Changes in Protein Phosphorylation in Rous Sarcoma Virus." Mol. Cell. Biol. 1;165-178, 1981.

21. Grunberger, G., Zick, Y. and Gorden, P. "Defect in Phosphorylation of Insulin Receptors in Cells from an Insulin-resistant Patient with Normal Insulin Binding." Science 932-934, 1984.

CHAPTER 4: IN VITRO AND IN VIVO ACTIVATION OF THE INSULIN RECEPTOR KINASE IN CONTROL AND DENERVATED SKELETAL MUSCLE

Introduction:

Antecedent activity modulates the insulin response of skeletal muscle. Following exercise the muscle's sensitivity to insulin is increased as assessed by insulin-stimulated glucose transport (1) glycogen synthase activation (2) and amino acid transport (3). Basal glucose transport into muscle cells is also stimulated following vigorous exercise (4). The converse is also true, e.g. muscles which have been denervated (5-9) or immobilized (10) develop within 6-24 hrs decreased sensitivity and response to insulin. The insulin resistance encompasses multiple parameters of insulin action e.g. glucose transport (5-9), glycogen synthesis (7-9), glycogen synthase activation (7-9) and amino acid transport (7). Baseline glucose transport and glucose incorporation into glycogen are also decreased in denervated muscles. Several changes occur in the membrane of skeletal muscle after denervation. Extra junctional acetylcholine receptors appear (11) as well as sodium channels that are insensitive to tetrodotoxin (12). The resting membrane potential is decreased (13) and phospholipid turnover accelerates (14). However, no significant change in the binding of insulin to its receptor has been observed shortly after denervation, to account for the severe insulin resistance (7,9,15) and thus the defect in denervated skeletal muscle may be classified as a post-binding

defect. It has recently become evident that in some insulin resistant states there is little or no change in insulin binding yet there is impaired insulin-stimulated autophosphorylation of the insulin receptor β-subunit (16-18). In the intact cell and in the solubilized insulin receptor system the initial, insulin stimulated autophosphorylation occurs on tyrosine residues of the β-subunit and this intramolecular event activates an intrinsic, tyrosyl phospho-transferase to exogenous substrates. The latter may initiate the cascade of phosphorylation-dephosphorylation which ensues upon insulin binding to the cell surface (for review see 19, 20).

Since the insulin resistance in denervated muscles encompasses multiple parameters, we postulated that the defect involves an early step in insulin's action (9). The autophosphorylation of the β-subunit is the earliest recognized event after insulin binds to its receptor. We therefore addressed the following questions: a) Is the insulin stimulated receptor autophosphorylation and/or insulin-activation of the receptor tyrosyl kinase (using exogenous substrate) impaired after denervation in receptors solubilized from skeletal muscle? b) Is the insulin-stimulated receptor autophosphorylation affected in situ, in incubated muscles, after denervation? c) In the intact animal, is the activation of muscle insulin receptor exogenous substrate tyrosyl kinase impaired after denervation? The latter question led to the development of methods to assess the in vivo activation of the skeletal muscle insulin receptor kinase activity by the hormone. Our data indicate that insulin-mediated receptor-kinase activation is unimpaired in muscle 24 hrs after denervation, suggesting that in this model impaired receptor-function coupling is a true post-receptor defect.

MATERIALS

Monoiodinated A[^{14}I]insulin was kindly provided by Dr. B.H. Frank (Lilly Research Laboratories, Indianapolis, IN). Monocomponent, crystalline pork insulin was a gift of Dr. Ronald Chance (Lilly Research Laboratories). Polyclonal anti-insulin receptor antibody was a gift of Dr. Steve Jacobs (Wellcome Research Laboratories). Wheat germ agglutinin was from Vector, Aprotinin was from FBA Pharmaceuticals, Bacitracin from Pfizer. Electrophoresis reagents were from Serva and apparatus from LKB. Protein A bearing S. aureus was from Calbiochem. CnBr-activated Sepharose 4B was from Pharmacia. Precoated cellulose TLC plates were from Brinkmann Instruments. Autoradiography Film, X-omat AR, was from Kodak and Lightening + intensifying screens from duPont. γ[^{32}P]ATP was prepared from ^{32}P-orthophosphate (ICN) in Gammaprep vials (Promegabiotec) as described by the manufacturer. [^{14}C]glucose was from New England Nuclear. All other reagents and chemicals were from Sigma unless otherwise specified.

METHODS[1]

Animals. Male rats, of the Wistar strain, weighing 60-110g were used in all experiments. They were allowed access to food and water ad libitum until 24 hr before experimentation when food was removed. In all experiments rats were unilaterally denervated 22-26 hr before experiments, by cutting the sciatic nerve through a small incision in the posterior aspect of the thigh as previously described (9).

Insulin receptor solubilization. Insulin receptors were solubilized from the muscle groups innervated by the sciatic nerve (lower leg muscles). The muscles were rapidly removed and quickly frozen in liquid nitrogen, powdered and the insulin receptors solubilized by

homogenization in 25 mM Hepes, pH 7.4, 1% Triton X-100, 1 TIU/ml aprotinin, 5 mM EDTA, and 2 mM Phenyl methyl sulfonyl fluoride (PMSF). After 10 min. centrifugation at 10,000 x g the supernatant was stirred for 30 min at 22°C to complete solubilization and then centrifuged at 150,000 x g for 90 min. (Method 1). The supernatant was passed three times over an agarose bound wheat germ agglutinin (WGA) column and the resin washed with 25 bed volumes of 25 mM Hepes, pH 7.4, 0.1% Triton X-100 (buffer A) and the partially purified glycoproteins eluted with buffer A supplemented with 0.3M N-acetylglucosamine. The WGA eluate was aliquoted and stored at -80°C without loss of binding or kinase activity. In some experiments the insulin receptors were isolated in the phosphorylated state by homogenization in at least 10 volumes of a buffer containing 50 mM Hepes, pH 7.4, 1.5% Triton X-100, 1 TIU/ml aprotinin, 5 mM EDTA, 10 mM NaF, 10 mM Na pyrophosphate, 1.5 mg/ml bacitracin, 100 μM Na_3VO_4, and 2 mM PMSF. After centrifugation at 10,000 x g for 10 min the supernatant was immediately centrifuged at 150,000 x g for 1 hr (Method 2). The resulting supernatant was applied to a WGA column pre-equilibrated with buffer A supplemented with 10 mM Na pyrophosphate, 5 mM EDTA, 1 TIU/ml aprotinin and 2 mM PMSF (buffer B) and recycled three times. The column was then washed with 25 bed volumes of Buffer B and the insulin receptors eluted with buffer B supplemented with 0.3 M N-acetylglucosamine.

Insulin binding to the solubilized insulin receptors was according to Taylor et al. (21) using A14[^{125}I]insulin. The binding data were normalized to the protein content of the wheat germ eluate as determined by the Bradford reaction using bovine γ-globulin standards (22). All studies of receptor-kinase activities were carried out using equal

insulin binding activities from WGA eluates prepared from control and denervated muscles. The latter were always prepared and assayed in parallel with the contralateral control muscle insulin receptors.

Insulin induced autophosphorylation of the solubilized insulin receptor was performed as previously described (23). Briefly, WGA eluate from control and denervated skeletal muscles were incubated without or with 10^{-9} or 10^{-7} insulin in 65 µl Buffer A supplemented with 5 mM $MnCl_2$ and 0.05% BSA (phosphorylation buffer), for 1 hr at 25°C. The autophosphorylation was initiated by the addition of 10 µl $\gamma[^{32}P]ATP$ (50 µM final concentration, 7.5 µCi/vial) and the reaction continued for 4 min. when 4X concentrated Laemmli's sample buffer (24) (supplemented in all experiments with 5 mM 2-mercaptoethanol) was added and the samples placed in boiling water for 3 min. The proteins were separated by $NaDodSO_4$ polyacrylamide gel electrophoresis (SDS-PAGE), the β-subunit was localized by autoradiography, excised and digested, and the radioactivity determined, as described in "Electrophoresis".

Estimation of exogenous substrate kinase activity of the insulin receptors purified by Method 1 above was performed with histone H_2b as the phosphoacceptor after the receptors were bound to insulin-agarose using the protocol of Yu and Czech (25). Wheat germ eluates from control and denervated skeletal muscles were incubated first with 50 µl dithiothreitol-heat inactivated insulin-agarose (150 µl wheat germ eluate in a final volume of 500 µl) at 25°C for 1 hr and the supernatant then incubated with active insulin-agarose for an additional 1 hr. After washing twice with 1 ml Buffer A supplemented with 0.5 M NaCl and once with 1 ml Buffer A alone the exogenous substrate kinase activity of the insulin-agarose bound receptor was activated by incubating it in

buffer A supplemented with 5 mM $MnCl_2$ and 750 μM unlabeled ATP at 25°C for 20 min. After washing twice in 1 ml Buffer A, the resin was resuspended in 65 μl Buffer A supplemented with 5 mM $MnCl_2$, 0.05% BSA and 1 mg/ml Histone H_2b. The kinase reaction was initiated by adding 10 μl γ[^{32}P]ATP (5 μM final concentration, 5 μCi/vial) and continued for 4 min, when 4X concentrated Laemmli's sample buffer was added and the solution placed in boiling water for 3 min. After separation of the proteins on SDS-PAGE the histones were localized by autoradiography and the radioactivity incorporated determined as described under "Electrophoresis".

Autophosphorylation of the insulin receptor in the intact soleus was carried out as previously described (23). Briefly, hemisolei (groups of 5-6 muscles) were incubated in Dulbecco's Modified Eagles Medium (DMEM) containing 0.1 mM Na phosphate for 1 hr at 25°C to lower endogenous phosphate pools. The muscles were then transferred to DMEM with 0.2 mCi/ml (0.1 mM) Na [^{32}P]phosphate and incubated at 37°C for an additional 3 hrs. (preliminary experiments showed this was sufficient time to reach steady state labeling of nucleotide pools, as determined below). The muscles were then transferred to Gey and Gey's balanced salt solution (26) which was modified to yield a concentration and specific activity of phosphate identical to that of the modified DMEM medium described above, with or without 10 mU/ml of insulin and the incubation continued for ½ hour. The individual hemi-solei were then frozen with metal tongs precooled in liquid nitrogen and the insulin receptors solubilized and partially purified by Method 2 described above except that 40 mM unlabeled ATP was added to the homogenization buffer. Immunoprecipitation of the phosphorylated insulin receptor was achieved

by incubating the WGA eluate overnight at 4°C with polyclonal anti-insulin receptor antibody (1:250 dilution), followed by protein A bearing, formalin fixed Staphylococcus for an additional 2 hrs. After centrifugation, the precipitated complex was washed 3X in Buffer A, and the pellet boiled in Laemmli's sample buffer. Electrophoresis and quantitation of ^{32}P incorporated into the β-subunit was as described above.

A parallel set of control and denervated hemi-solei were incubated in an identical fashion in the modified Dulbecco's MEM with 1/100 the specific activity of ^{32}P, to estimate the concentration and specific activities of the nucleotide pools prior to the transfer to the insulin containing medium. After freezing, the individual muscles were powdered with a glass rod in a microfuge vial and the nucleotides extracted by the addition of 60 µl of 3M perchloric acid and incubated for 20-30 min at -10°C (27). Cold distilled water (200 µl) was added and incubation continued for an additional 10 min at 4°C. The protein precipitate was removed by centrifugation for 15 min in a microfuge at 4°C and the supernate neutralized with 65 µl 2N $KHCO_3$, and clarified by centrifugation. The supernatant was filtered and 20 µl injected onto a 5 cm C_{18} reverse phase column on a Perkin-Elmer Series 4 high pressure liquid chromatograph. The nucleotides (ATP, ADP, and GTP) were eluted isocratically using a buffer composed of 350 mM phosphoric acid brought to pH 6.5 with triethylamine (98%) and methanol (2%) (28). The location of the eluted nucleotides was determined by the injection of appropriate standards monitored by absorbance at 259 nm. Each peak was collected in duplicate determinations and counted in a liquid scintillation counter. The concentration of nucleotides was estimated by the peak area as

determined by a Perkin-Elmer LCI-100 integrator. The specific activity was calculated as cpm/nmole nucleotide, nucleotide content of the tissue was normalized to the protein content, measured by the Lowry reaction using bovine serum albumin standards (29).

In vivo activation of the exogenous kinase activity of the insulin receptor was studied as follows. Unilaterally denervated rats were anesthetized with metophane and divided into two groups. One group received an intravenous injection of 5U regular insulin (Humulin, recombinant DNA origin, Eli Lilly Co.) via the femoral vein, the other received an equal volume of saline. Five or 30 min later the hindleg muscles were exposed, the soleus muscles rapidly removed, mounted on wire frames and incubated for ½ hour in [^{14}C]glucose containing buffer to estimate glycogen synthesis. The remaining leg muscles (primarily gastrocnemius and anterior compartment muscles) were removed, frozen in liquid nitrogen, homogenized and the insulin receptors solubilized by Method 2 above. After elution from the WGA column, the exogenous kinase activity of the receptors was estimated by incubation in media containing 50 mM $MgSO_4$ (which was added first to precipitate inorganic phosphate and EDTA), 5 mM $MnCl_2$, 0.05% BSA, 1mg/ml histone H_2b, with or without 10^{-7}M insulin for 10 min at 25°C. γ[^{32}P]ATP was then added (5 or 50 μM final concentration, 7.5-10 μCi/vial), in a final reaction volume of 75 μl. After 4 min. the reaction was stopped by the addition of 4X concentrated Laemmli's sample buffer and the solution placed in boiling water for 3 min. The proteins were separated by electrophoresis, autoradiographed and ^{32}P incorporated into histones determined as described under "Electrophoresis".

In a second assay WGA eluted receptors were incubated with insulin-agarose (50 µl/150 µl WGA eluate, the latter diluted with 2 vol buffer A) for 15 min at 22°C after which the resin was washed twice with 50 mM Hepes, pH 7.4, 0.1% Triton X-100, 0.5 M NaCl and once with the same buffer without NaCl, according to Yu and Czech (25). The insulin-agarose bound receptors were then resuspended in 75 µl Buffer A, supplemented with 5 mM $MnCl_2$, 0.05% BSA and 1 mg/ml histone H_2b. The kinase reaction was initiated by adding 10 µl $\gamma[^{32}P]$ATP (5 µM final concentration, 7.5 µCi/vial). In a parallel reaction the receptor kinase was maximally activated by first incubating the washed insulin-agarose bound receptors in the above phosphorylation buffer (without histone) with 750 µM unlabeled ATP for 20 min at 25°C. After washing twice in 1 ml Buffer A the kinase activity against histone H_2b was measured as described above.

Plasma glucose determination. Blood obtained via cardiac puncture was used for plasma glucose determinations with a Beckman Glucose Analyzer 2. Only those animals whose plasma glucose fell below 65 mg% were considered "treated" in the in vivo activation experiments. The mean plasma glucose of insulin treated rats was 39 ± 4 mg/dl at 5 min and 33 ± 11 mg/dl at 30 min after insulin injection (n = 4 and 10 respectively), while the mean plasma glucose of saline treated controls was 85 ± 6 mg/dl.

Glycogen synthesis by control and denervated solei. Solei were divided lengthwise and mounted on wire frames as described previously (9) and incubated in 2 ml Gey and Gey's balanced salt solution (26) supplemented with 0.1% BSA and 5 mM $[^{14}C]$glucose (0.3 µCi/ml) under 95% O_2/5% CO_2 atmosphere at 37°C for 30 min. The muscles were then digested

in 0.5 ml 30% KOH for 20 min at 100°C and the glycogen precipitated by the sequential addition of 50 µl 10% Na_2SO_4 and 1 ml 100% ethanol. The precipitate was collected by centrifugation, washed twice with 66% ethanol, dissolved in 1 ml 1N HCl and the radioactivity determined by scintillation counting.

Phosphoaminoacid analysis. The position of the histone samples for phosphoamino acid analysis was determined by autoradiography of an unfixed, undried gel and aligned with fluorescent dye marks (Ultemit, New England Nuclear). The desired portion of the gel was excised and placed in a dialysis bag with 2 ml electrophoresis buffer. The bag was closed with dialysis clips and the gel slice trapped between the plates of the vertical electrophoresis unit, one end plugged with acrylamide. A voltage of 300 V was applied for 40 min and the eluted protein precipitated with 20% trichloroacetic acid using 100 µg bovine gamma globulin as a carrier. The precipitate was hydrolysed in 6 N HCl, lyophilized, absorbed to 500 µl Dowex 1-X8 and eluted with 0.25 M HCl (30). After lyophilization the samples were resuspended in a small volume of mixed phosphoamino acid standards (0.33 mg/ml each of phosphotyrosine, phosphoserine and phosphothreonine), spotted on 0.1 mm cellulose coated plates and electrophoresed at pH 3.5 (pyridine/acetic acid/water, 1:10:189) at 1000 V for 40 min (30). The positions of the phosphoaminoacids were located by ninhydrin staining and the radioactivity detected by autoradiography.

Electrophoresis was performed using the discontinuous system of Laemmli (24) under reducing conditions. For samples identifying only the β-subunit of the insulin receptor a 7.5% resolving gel was employed. For samples identifying both the β-subunit of the insulin receptor and

histone H_2b a linear 6-20% polyacrylamide gel system was used. In all cases a 4.5% stacking gel was used. After electrophoresis, the gels were stained in 50% methanol, 10% acetic acid with 0.125% Coomassie brilliant blue R, destained in 30% methanol, 10% acetic acid, and dried in vacuo with heat. Phosphorylated peptides were localized by autoradiography using Kodak AR-5 film, in some cases in the presence of a duPont lightening + intensifying screen. Phosphorylated bands of interest were excised and the radioactivity estimated by digestion of the gel slice for 10 hrs in 1 ml 30% H_2O_2 and subsequent counting in a Beckman LS 7000 β-counter after dissolving the sample in Scintiverse E (Fisher).

RESULTS

Equilibrium binding of ^{125}I-insulin to insulin receptors in the WGA eluate solubilized from control and denervated hind limb muscles yielded typical curvilinear binding kinetics (Fig. 1). When expressed per mg of protein in the WGA eluate, there was no significant difference in the number of insulin receptors isolated from control and denervated muscles (although the latter was slightly lower), nor was there a difference in the apparent insulin binding affinity or in the amount of glycoprotein eluted from the WGA column/tissue weight.

Solubilized insulin receptor kinase activities. Upon binding insulin, the receptor, both in the intact cell and in the solubilized system, undergoes rapid autophosphorylation (for review see 19,20). This property of the solubilized receptor was tested, by incubating WGA eluates, prepared from control and denervated muscles, with increasing concentrations of insulin, using equal insulin binding activities from each preparation. Insulin induced a dose-dependent increase in ^{32}P incorporation into the β-subunit of the receptor (Fig. 2a). The latter

was identified as the β-subunit by its apparent molecular weight (~97,000), its insulin dependent labeling with ^{32}P and its precipitability with specific antibodies (23 and Figure 2c). There was no apparent difference in autophosphorylation between receptors derived from control and denervated muscles at all insulin doses tested (Fig. 2a). The metal ion dependence of the autophosphorylation reaction (Mn++>Mg++>>Ca++) was also identical in the two groups, as was the time course of the autophosphorylation (data not shown). Similarly, the exogenous substrate kinase activity of the fully activated insulin receptor was not altered by denervation using histone H_2b as the phosphoacceptor (Figure 2b).

Phosphorylation in the intact soleus. When insulin receptors were isolated by Method 1, no attempt was made to prevent dephosphorylation of the receptor. In vivo, the basal state of phosphorylation of the insulin receptor β-subunit, especially phosphorylation on serine residues, is thought to modulate the autophosphorylation on tyrosine residues upon binding of insulin (31,32). To investigate whether or not denervation affects the insulin stimulated autophosphorylation of the receptor in the intact cell, soleus muscles from control and denervated hind limbs were incubated in $[^{32}P]PO_4$ containing media for 3 hrs to label intracellular nucleotide pools and then placed into media with or without insulin for ½ hr to stimulate receptor autophosphorylation. The muscles were quickly frozen, and the insulin receptors solubilized and partially purified in a buffer which, as determined in preliminary experiments, minimized dephosphorylation. After immunoprecipitation and electrophoresis under reducing conditions the β-subunit was localized by autoradiography, excised, and radioactivity determined. Table 1

summarizes the results of five such experiments. While in each case denervated and control muscles responded to insulin with increased labeling of the insulin receptor β-subunit, ^{32}P incorporation in the presence of insulin was 19% less in the denervated group. When Δ insulin, e.g. ^{32}P-incorporation above baseline was calculated, the magnitude of the insulin effect was decreased in denervated muscles, but the decrease was not significant by paired analysis. The concentration and specific activities of ATP, ADP, and GTP were determined immediately before insulin stimulation. The concentrations of the three nucleotides were identical in denervated and control solei after three hours of incubation (Table 1) but in both muscle groups the ATP concentration decreased 20% from pre-incubation levels (data not shown). The specific activity of the ATP pool on the other hand was 11% lower in the denervated muscle groups. Thus, the slight decrease in insulin stimulated labeling of the β-subunit from denervated muscles may reflect the decrease in the specific activity of intracellular ATP; this would suggest, however, that there may be a slight increase in the basal phosphorylation of the β-subunit from denervated muscles. Unfortunately, the low receptor associated radioactivity precluded the analysis of the labelled phosphoaminoacids before and after in situ insulin stimulation.

Kinase activity of in vivo activated receptors. A method was developed to solubilize muscle insulin receptors in the presence of phosphoprotein phosphatase and kinase inhibitors and determine their in vivo activation state by assessing their ability to phosphorylate exogenous substrates, in vitro. Since the exogenous substrate kinase activity of the receptor is dependent on its phosphorylation state (25),

if the sites on the insulin receptor which were phosphorylated in response to in vivo insulin remained phosphorylated during receptor isolation, the exogenous kinase activity should reflect the degree of in vivo activation. Rats were anesthetized and one group was given an i.v. bolus of insulin via the femoral vein. After 5 or 30 min, the hind limb muscles innervated by the sciatic nerve were removed and quickly frozen in liquid nitrogen. The procedure used to solubilize and partially purify insulin receptors from hind limb muscles was similar to that used previously in isolating the autophosphorylated insulin receptors from incubated soleus muscle (Method 2). In an initial attempt to quantitate the exogenous substrate kinase activity $MgSO_4$ was added to the insulin receptor containing WGA eluate to precipitate inorganic phosphate and EDTA. The receptor kinase activity was then assessed with 5 μM $\gamma[^{32}P]$ATP and 1 mg/ml histone H_2b as the substrate. Using equal insulin binding activities the histone kinase activity of muscle insulin receptors from rats which had been treated in vivo with insulin was ~ 2-fold greater than that of untreated controls (Fig. 3) with no significant difference between receptors isolated 5 or 30 minutes after the administration of insulin (30 min point not shown). The insulin receptor kinase from denervated skeletal muscles activated to the same level as that derived from the innervated pairs after i.v. insulin (Fig. 3). When insulin ($2x10^{-7}$ M) was added to the incubation mixture for 10 min before initiating the kinase reaction, stimulation of histone kinase activity was not observed in any group of receptors. When the same series of experiments were performed with 50 μM ATP, the receptor associated histone kinase activity prepared from rats which had received insulin was still greater than that of receptors prepared from saline

treated controls. However, when WGA eluates were preincubated with insulin before the addition of 50 μM ATP, histone phosphorylation by receptors derived from saline treated controls increased two-fold and a smaller but significant increase was observed using the WGA eluate derived from insulin treated animals. Again no difference was observed between receptors prepared from denervated and innervated muscles. These results demonstrate that 5 μM ATP is too low to allow the in vitro activation of the exogenous substrate kinase by insulin, at least in the time frame investigated. Increasing the concentration of ATP ten-fold allowed the in vitro activation of histone kinase by insulin in both the control and denervated derived WGA eluates, but this increase in kinase activity was not as great as that seen in the in vivo activated receptors. Phosphoamino acid analysis of histones showed that the increase in labeling after in vivo activation of the insulin receptor occurred exclusively on tyrosine residues (not shown).

The differences in kinase activity between insulin receptors derived from insulin and saline treated rats may have been due to insulin carried through the receptor purification procedure or to a co-purified histone kinase activity that was independent of the insulin receptor. To test this, a second assay was performed. The WGA purified receptors were incubated with insulin-bound agarose beads and after centrifugation and washing the kinase activity towards histone H_2b was assessed in a reaction using 5 μM $\gamma[^{32}P]$ATP, a concentration which, as shown above, does not allow the further activation of the insulin receptor kinase. As in the previous assay, receptors isolated from insulin treated rats showed increased histone kinase activity, and in vivo activation by insulin was not affected by denervation (Fig. 4).

When these insulin-agarose bound receptors were first allowed to autophosphorylate with 750 μM unlabeled ATP, the histone kinase activity increased to identical levels in all groups (Fig. 4). Histone phosphorylation after activation with 750 μM ATP was slightly lower than that observed after in vivo activation with insulin, this difference, although not significant, likely represents small losses of receptors during washing and/or slight dilution of the labeled ATP, resulting in decreased specific activity. When WGA eluates were incubated with dithiothreitol-heat inactivated insulin-agarose beads the histone kinase activity observed was less than 10% of that seen in any of the experiments using fully activated beads suggesting that the bulk of the kinase activity pertained to insulin receptors bound to the insulin agarose. Phosphoamino acid analysis of the histones phosphorylated in the presence of insulin-agarose bound receptors revealed considerable ^{32}P incorporation into serine residues, which however was not affected by pretreating the rats with insulin in vivo, or by preincubating receptors from saline treated controls with 750 μM ATP, while both maneuvers approximately doubled ^{32}P incorporation into tyrosine residues (Fig. 5). Identical increments were observed using receptors prepared from denervated muscles (not shown).

The intravenous insulin bolus stimulated the metabolism of innervated muscles, as assessed by the in vitro incorporation of [^{14}C]glucose into glycogen. Denervated muscles were resistant to intravenously administered insulin in the same way as they are resistant to insulin in vitro (7-9). Innervated solei from non-treated animals had a low basal rate of glycogen synthesis (Fig. 6) which was stimulated by adding insulin in vitro. The 24 hr denervated solei from saline

treated controls demonstrated a markedly reduced basal rate of glycogen synthesis and this did not increase significantly upon addition of insulin to the incubation medium. On the other hand, solei from the innervated legs of insulin treated animals synthesized twice as much glycogen than those from saline treated controls when incubated without insulin and this rate was not increased further by adding insulin in vitro. Stimulation of glycogen synthesis in non-denervated solei was apparently complete within 5 min. after the i.v. bolus of insulin (Fig. 6) which correlates with the increase in the exogenous kinase activity of the receptors isolated from the ipsilateral muscle groups at this time. Attempts at investigating the activation after shorter time periods proved difficult in that at least 1 min. was required to remove both solei and excise and freeze the remaining muscles. We could demonstrate nearly complete activation of both glucose incorporation into glycogen and exogenous kinase activity within 30-45 sec after the i.v. injection of insulin, this result was variable, however (data not shown).

Discussion. Insulin resistance of peripheral tissues, including muscle, is associated with a number of physiological and pathological conditions which include hormonal imbalance (e.g. excess glucocorticoids, catecholamines or growth hormone), obesity, type II diabetes, uncontrolled insulinopenic (type I) diabetes, uremia, the catabolic state which accompanies severe trauma, burns and sepsis, some genetic abnormalities and in skeletal muscle, denervation and immobilization. Conditions of insulin resistance associated with increased circulating insulin levels are usually accompanied by a decrease in the number of insulin receptors on the cell-surface, a phenomenon termed "down-

regulation". However, in most conditions, the ensuing decrease in insulin binding does not account for the magnitude of the insulin resistance, indicating that post-binding defects play an important role (for review, see 33). Recent findings suggest that in some conditions e.g. a genetic abnormality of the insulin receptor resulting in severe insulin resistance in man (16,17) as well as in uncontrolled insulin deficient diabetes in the streptozotocin treated rat (18) and in the goldthioglucose treated obese, diabetic rat (34) insulin binding (which involves primarily the α-subunit of the receptor) is unimpaired, but the insulin activated autophosphorylation and tyrosine kinase activity of the β-subunit are diminished upon insulin binding in vitro. Thus, post-binding insulin resistance may include receptor abnormalities resulting in abnormal signalling by the occupied receptor, as well as true post-receptor defects. In the case of denervated muscles, we and others (7,9,15) found no defect in insulin binding; the current work demonstrates that in addition, the tyrosyl-kinase activity of the β-subunit is normally stimulated by insulin, thus the severe insulin resistance which develops in muscle shortly after denervation appears to be a post-receptor defect. It is possible, however, that other, undiscovered properties of the receptor are impaired shortly after denervation. Whether the insulin unresponsiveness is due to alterations in membrane potential (13), defects in the generation of second messengers, development of a metabolic milieu which is unfavorable for the propagation of signals or alterations in the levels or properties of the proposed substrates for the insulin receptor kinase is not known. Further studies of this model may provide clues concerning the mechanism of insulin action and its post-receptor modulation.

In the course of these studies we developed techniques to assess the in vivo activation state of the insulin receptor in skeletal muscle. Rapid freezing of the excised muscles in liquid nitrogen, solubilizing and purifying the insulin receptors in the presence of phosphoprotein-phosphatase and kinase inhibitors (NaF, Na_4 P_2O_7 Na_2VO_4 and EDTA) allowed the demonstration of increased histone H_2b phosphorylation by WGA eluates from animals which had been treated in vivo with insulin. Two methods were used to clear the solution of EDTA and inorganic phosphate (which would chelate the Mn++ ions required for activation). In the first assay, $MgSO_4$ was added in sufficient quantities to remove the chelators. Using 5 μM ATP no stimulation of histone kinase activity was seen when insulin was added to the incubation medium prior to the initiation of the kinase reaction. The inability to activate the exogenous substrate kinase of the receptor with insulin at this low ATP concentration has been previously reported (25,35) and is attributed to the decreased phosphorylation of a critical site on the insulin receptor β-subunit (25,35). By raising the ATP concentration to 50 μM an increase in the histone kinase activity could be demonstrated in the WGA eluates in response to maximally stimulating insulin concentrations in vitro; as expected the increment caused by insulin in vitro was much greater using receptors prepared from controls, than from insulin treated rats. It is noteworthy that while the baseline histone phosphorylation occurred predominantly on serine residues, the increment in histone phosphorylation elicited by insulin in vivo or in vitro occurred exclusively on tyrosine.

In the second method the insulin receptors were further purified by binding them to insulin-agarose, thus removing them from the WGA eluate

containing Mn++ chelating anions as well as enzymes which may modify receptor-induced histone phosphorylation. Preliminary reports described a similar approach for assessing the activation state of the receptor in isolated cell systems (36,37). The results obtained with this method (Fig. 4) confirmed the preceding ones (Fig. 3). Insulin-agarose bound receptors prepared from insulin-treated rats demonstrated ~ 2-fold greater histone-H_2b phosphorylation, in the presence of 5 μM [γ-^{32}P]ATP than identically prepared receptors from saline treated controls. When both sets of insulin-agarose bound receptors were preincubated with unlabelled, 750 μM ATP, a concentration which fully activates the receptor tyrosyl-kinase (25 and our data, not presented), histone phosphorylation by receptors from saline treated controls was activated to the same level as that by receptors from rats treated in vivo with insulin. The insulin binding activity of the WGA eluates from insulin and saline treated rats was determined before incubation with insulin-agarose, and the beads were incubated with equal binding activities. In preliminary experiments insulin binding curves prepared from WGA eluates derived from insulin treated and saline treated rats revealed no difference in binding kinetics between the groups. Furthermore, addition of Mn++ and ATP to binding assays did not alter the apparent affinity of the insulin receptor. This confirms previous observations that insulin-induced receptor phosphorylation does not alter the affinity of insulin binding (38).

It is unlikely that our data were compromised by insulin carried over through the receptor purification procedure because a) when ^{125}I-insulin was included in muscle homogenates, with or without unlabeled insulin (10 μg/ml), less than 0.1% of the radioactivity was associated

with the WGA eluate and this was non-specifically bound, b) when 0.5 U/ml (20 μg/ml) insulin was included in the buffer in which muscles from saline treated controls were homogenized, no activation of the receptor tyrosyl-kinase was detected in assays of the WGA eluate. Similarly, inclusion of 40 mM ATP in the homogenization buffer did not affect the subsequent measurements of histone kinase activity in any group. WGA eluates prepared from skeletal muscle were devoid of detectable phosphoprotein phosphatase activity (not shown). ATPase activity was assayed under a number of experimental conditions, by measuring the radioactivity associated with $\gamma[^{32}P]$ATP and with inorganic phosphate, separated by HPLC (as described in Methods) after incubation of the receptor preparations with $\gamma[^{32}P]$ATP. Less than 10% of $\gamma[^{32}P]$ATP was degraded after 4 min. incubation in any histone kinase assay (or in assays of receptor autophosphorylation), and this measurement was not affected by previous treatments e.g. insulin or denervation. While significant serine-kinase activity was associated with the receptor preparations, even in the assay using insulin-agarose bound receptors, receptor-induced histone phosphorylation on serine residues was unchanged after treating rats with insulin in vivo or after activating the receptors in vitro. On the other hand, both maneuvers markedly stimulated the phosphorylation of histone H_2b on tyrosine residues (Fig. 5).

Since denervated muscles demonstrate decreased sensitivity as well as a decreased response to insulin (5-9) we injected high concentrations of the hormone to elicit maximal responses in both the denervated and innervated muscles. In innervated muscles both receptor activation and the biological response (assessed as glucose incorporation into glycogen) were essentially fully stimulated within 5 min of such

injections. In denervated muscles, identical activation of the receptor tyrosine kinase by insulin was not accompanied by the biological response. In experiments where receptor histone-kinase activity and stimulation of glycogen synthesis were measured in parallel 5 min. and 30 min. after the i.v. injection of insulin, the responses were essentially identical at the two time points, suggesting that phosphorylation of the tyrosine, which is critical for activation of the receptor-tyrosyl-kinase, occurs rapidly and persists in vivo. Dose response curves injecting lower doses of insulin have not yet been tested; they may be required for the quantitative assessment of receptor activation in different physiological and pathological conditions. While skeletal muscle is a major site of insulin action, similar measurements should be feasible in other tissues as well. The method for assessing the activation state of the insulin receptor in skeletal muscle in vivo is relatively rapid, easily applicable to various animal models of insulin resistance and in contrast to cell culture systems, the availability of tissue is not limiting. It should be useful in characterizing insulin resistant states, by identifying conditions which may be associated with defective activation of the β-subunit tyrosyl-kinase upon insulin binding in vivo.

Table 1

A. Autophosphorylation of the insulin receptor β-subunit in incubated solei. Groups of 6 hemisolei (from control and denervated hindlimbs) were preincubated for 1 hr in low phosphate DMEM, then for 3 hrs in this medium supplemented with [^{32}P]phosphate (0.15-0.25 mCi/ml), followed by 30 min incubation with and without insulin as described in Methods. Solei were immediately frozen and insulin receptors solubilized and partially purified by Method 2, and immunoprecipitated and processed as described in Fig. 2C. Values represent the mean ± S.E. for 5 experiments. Cpm incorporated into the β-subunit were normalized to the wet weight of the muscles prior to incubation. * $p < 0.05$ vs. no insulin, paired analysis.

B. Nucleotide concentration and specific activity in incubated solei. Solei from control and denervated hind limbs were incubated exactly as described in A. except the specific activity of the [^{32}P]phosphate was decreased to 1.5-2.5 μCi/ml. The muscles were frozen immediately following the 3 hr. incubation in DMEM and nucleotides extracted and separated as described in "Methods". The concentration of the nucleotides was normalized to the protein content of individual muscles. Means ± S.E. of 5 different experiments are shown, 6 control and denervated hemi-solei were assayed individually in each experiment.

Table 1

A. cpm incorporated into β subunit/100 mg wet weight

Insulin in Media	Control	Denervated
0	242 ± 32	252 ± 37
10 mU/ml	379 ± 45*	320 ± 25*

B. Nucleotide levels in soleus

	Concentration (nmoles/mg protein)		Specific activity (cpm/nmole)	
Nucleotide	Control	Denervated	Control	Denervated
ATP	22.1 ± 2.8	22.3 ± 1.9	629 ± 116	564 ± 106
ADP	3.9 ± 0.6	4.2 ± 0.6	244 ± 69	233 ± 69
GTP	4.4 ± 0.8	4.2 ± 0.6	107 ± 22	107 ± 25

Figure 1. Equilibrium Binding of A14[^{125}I]insulin to solubilized, WGA-purified insulin receptors. Aliquots of WGA eluate (7.5 μg of protein) from control and denervated skeletal muscle were incubated with tracer A14[^{125}I]insulin (specific activity 100-140 μCi/μg) and increasing concentrations of unlabeled insulin in a total volume of 200 μl buffer containing 50 mM Hepes, 100 mM NaCl, 0.05 mg/ml BSA, 0.1% Triton X100 and 0.075 M N-acetyl glucosamine, pH 7.8 for 16 hr. at 4°C. The receptor and bound insulin were precipitated by the sequential addition of 100 μl 0.3% bovine γ-globulin and 300 μl 25% polyethylene glycol. Non-specific binding was considered that which occurred in the presence of 10 μg/ml unlabeled insulin and was subtracted from each point. The main figure shows a Scatchard plot of data from control (0---0) or denervated muscle (x---x) derived receptors. Each point is the mean ± S.E. of three receptor preparations, each point assayed in triplicate. Inset shows displacement curve analysis of the same data.

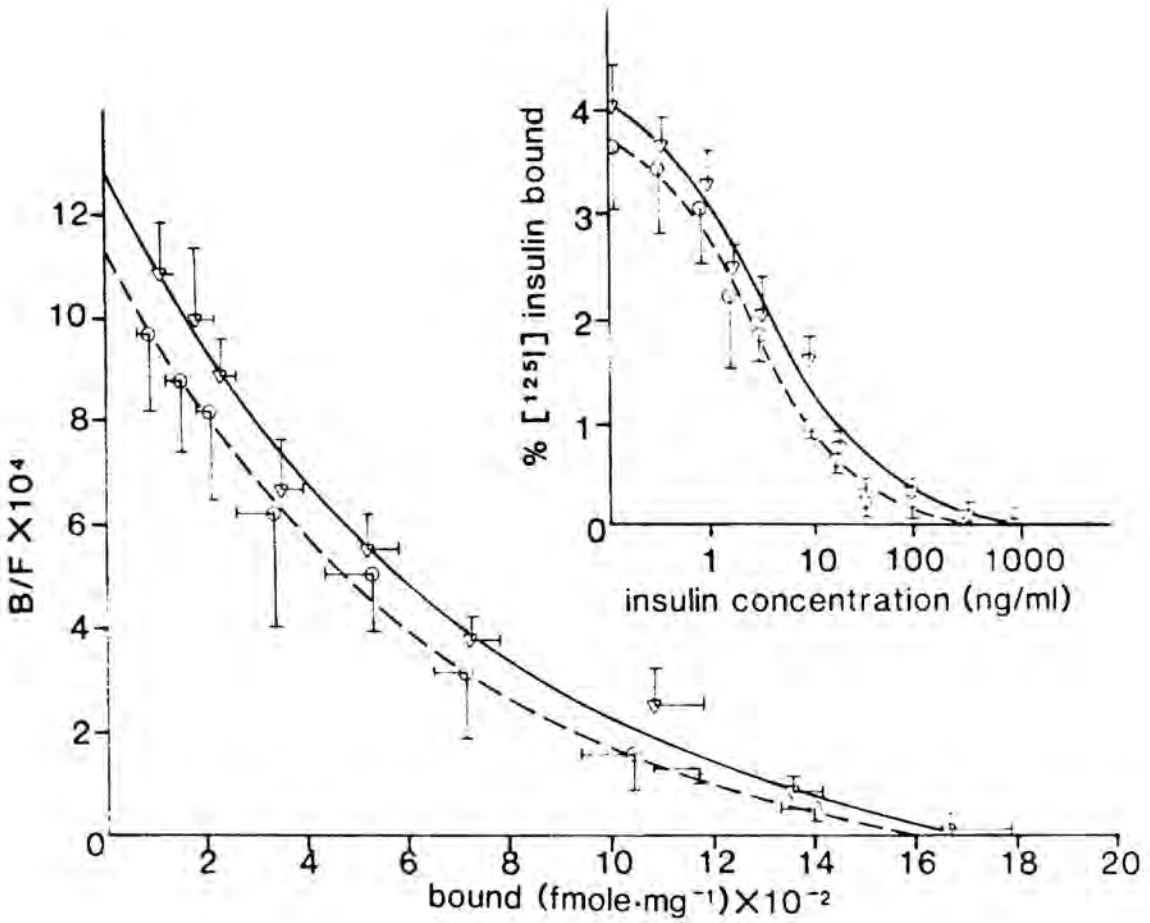

B/F X10⁴
bound (fmole·mg⁻¹)X10⁻²
% [¹²⁵I] insulin bound
insulin concentration (ng/ml)

Figure 2. A. Insulin induced autophosphorylation of the solubilized, WGA-purified insulin receptors. In the standard assay equal insulin binding activities of control and denervated derived WGA eluates were incubated in phosphorylation buffer without or with 10^{-9} or 10^{-7}M insulin for 1 hr. as described in "Methods". $\gamma[^{32}P]$ ATP (50 μM final concentration, 7.5 μCi/vial) was added and the autophosphorylation allowed to continue for 4 min. at 25°C. After electrophoresis and autoradiography the excised β-subunit was digested and the radioactivity quantitated by scintillation counting. [//], control derived receptors; [ ], denervated muscle derived receptors. Each value is the mean ± S.E. of 4 receptor preparations from control and denervated skeletal muscles respectively, each assayed in triplicate.

B. Phosphorylation of histone H_2b by the solubilized insulin receptor activated on insulin-agarose. Equal insulin binding activity of control and denervated derived wheat germ eluate (approx. 150 μl) were preincubated with 50 μl dithiothreitol-heat inactivated insulin agarose at 25°C for 1 hr and subsequently with 50 μl active insulin-agarose for 1 hr. at 25°C as described in "Methods". The suspension was washed and then incubated in the presence of 5 mM $MnCl_2$ and 750 μM unlabeled ATP for 20 min. at 25°C. After extensive washing the beads were resuspended in phosphorylation buffer with 1 mg/ml Histone H_2b. The kinase reaction was initiated by the addition of $\gamma[^{32}P]$ATP (5 μM final concentration) and incubated at 25°C for 4 min. The samples were electrophoresed and the histone bands localized by autoradiography, excised and the radioactivity determined by scintillation counting. [//], Histone phosphorylation by control or [ ], denervated derived receptors.

C. Immunoprecipitation of the autophosphorylated insulin receptor. WGA eluates were incubated without or with increasing concentrations of insulin as described in A. After autophosphorylation, anti-insulin receptor antibody (1:400 dilution) was added and incubated for 16 hr. at 4°C then for an additional 2 hr. with protein A bearing, formalin fixed S. aureus (final 1% solution). After washing, the solution was electrophoresed under reducing conditions and autoradiographed. Lanes A and B, control; C and D, denervated derived receptors, without (A and C) or with 10^{-7} M (B and D) insulin.

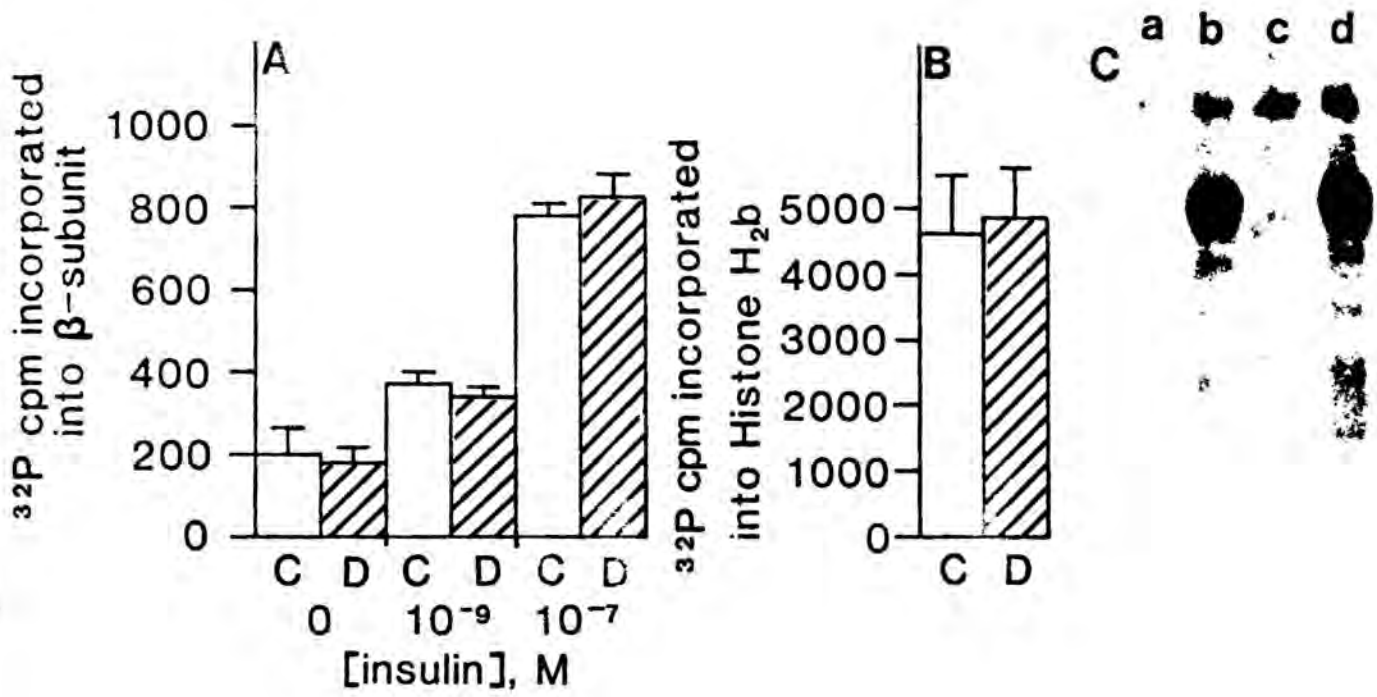

A
^{32}P cpm incorporated into β-subunit
1000
800
600
400
200
0
C D C D C D
0 10^{-9} 10^{-7}
[insulin], M
B
^{32}P cpm incorporated into Histone H_2b
5000
4000
3000
2000
1000
0
C D
C
a b c d

Figure 3. In vivo activation of the insulin receptor tyrosyl-kinase. Unilaterally denervated rats were anesthetized and injected i.v. with regular insulin (5U) or with normal saline. After 5 min. the hindlimb muscles innervated by the sciatic nerve were removed and immediately frozen in liquid nitrogen. The insulin receptors were solubilized and partially purified on WGA by Method 2 described in "Methods". The WGA eluate was incubated without or with $2X10^{-7}$ M insulin in phosphorylation buffer which was made 50 mM in $MgSO_4$ to precipitate the phosphatase inhibitors. The kinase reaction was initiated by adding either 5 or 50 μM $\gamma[^{32}P]ATP$ and terminated after 4 min. After electrophoresis and autoradiography, the histone bands were excised, digested and the radioactivity determined. The values plotted are the mean ± S.E. of duplicate determinations from four different control and denervated muscle receptor preparations. The schema for the different treatments is indicated below the histogram and above the histone H_2b containing area of a representative autoradiogram.

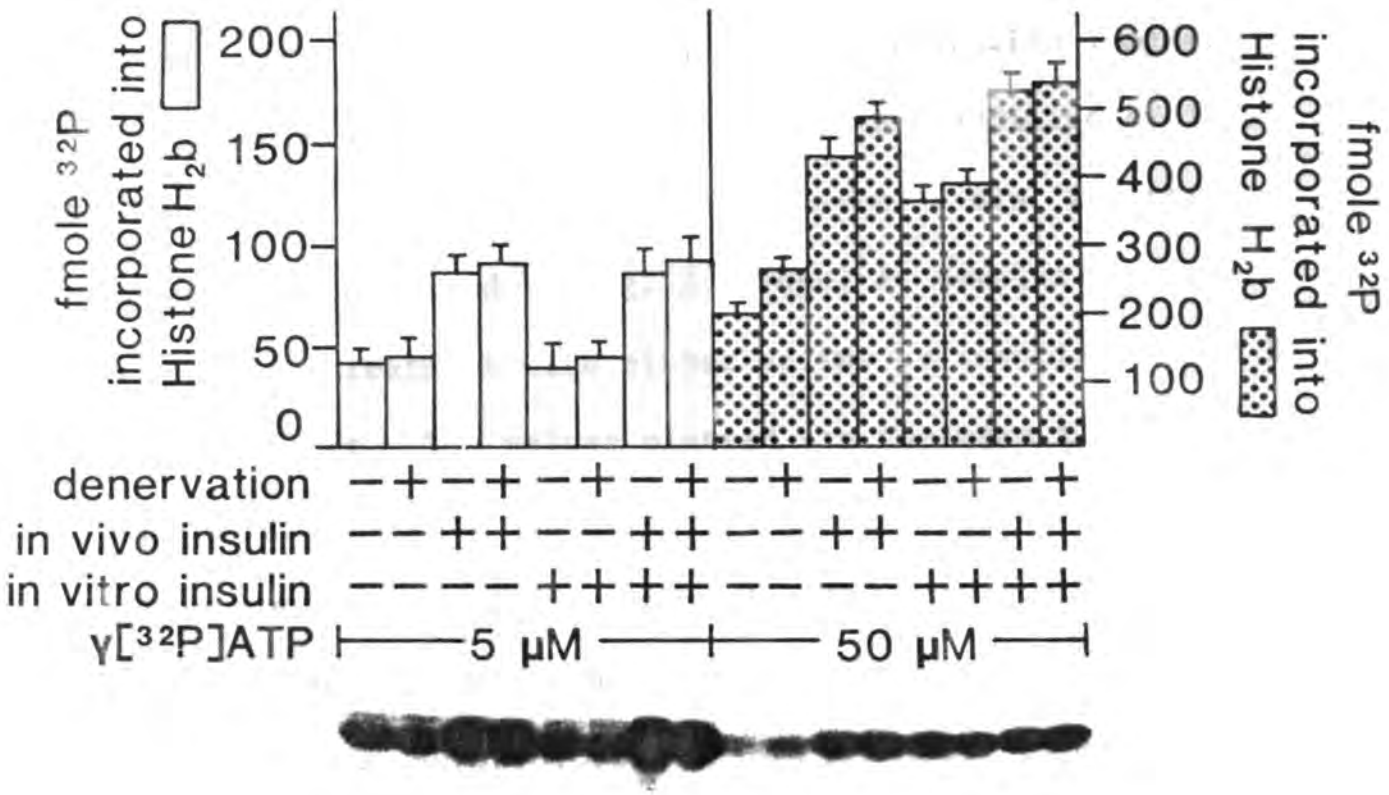
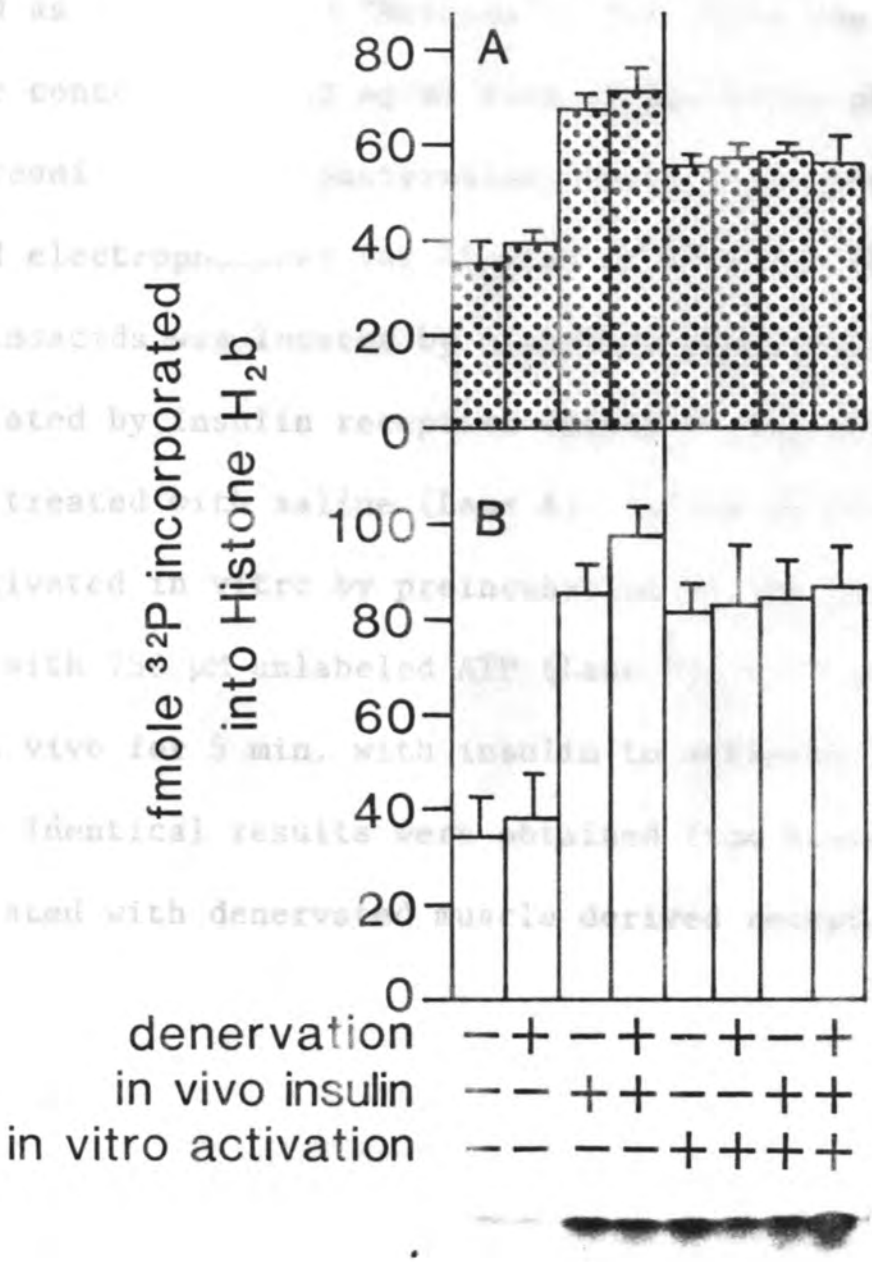

fmole ^{32}P incorporated into Histone H_2b
200
150
100
50
0
600
500
400
300
200
100
denervation − + − + − + − + − + − + − + − +
in vivo insulin − − + + − − + + − − + + − − + +
in vitro insulin − − − − + + + + − − − − + + + +
γ[^{32}P]ATP 5 μM 50 μM

Figure 4. In vivo activated insulin receptor kinase assayed on insulin-agarose. Treatment of rats and insulin receptor solubilization is described in Figure 3. WGA eluate from control and denervated skeletal muscle (approx. 150 µl, with equal insulin binding activity) was incubated with 50 µl insulin-agarose and the resin washed as described in "Methods". Part of the samples were allowed to autophosphorylate in the presence of 750 µM unlabeled ATP at 25°C for 20 min. The samples were resuspended in phosphorylation buffer with 1 mg/ml Histone H_2b and the kinase reaction initiated by the addition of 10 µl $\gamma[^{32}P]$ATP (5 µM final concentration), stopped after 4 min. and the samples processed as described in Fig. 3. Panel A: Samples obtained after 5 min. in vivo treatment with either saline or insulin. Panel B: 30 min. after treatment. The values plotted are the mean ± S.E. of duplicate determinations from 4-6 different control and denervated muscle receptor preparations. The schema for the different treatments is indicated below the histograms and above the histone H_2b containing area of a representative autoradiogram (5 min treatment experiment). The slightly lower histone phosphorylation in panel A was due to a lower concentration of insulin binding activity used in these experiments.


A
B
80
60
40
20
0
100
80
60
40
20
0
fmole ^{32}P incorporated into Histone H_2b
denervation − + − + − + − +
in vivo insulin − − + + − − + +
in vitro activation − − − − + + + +

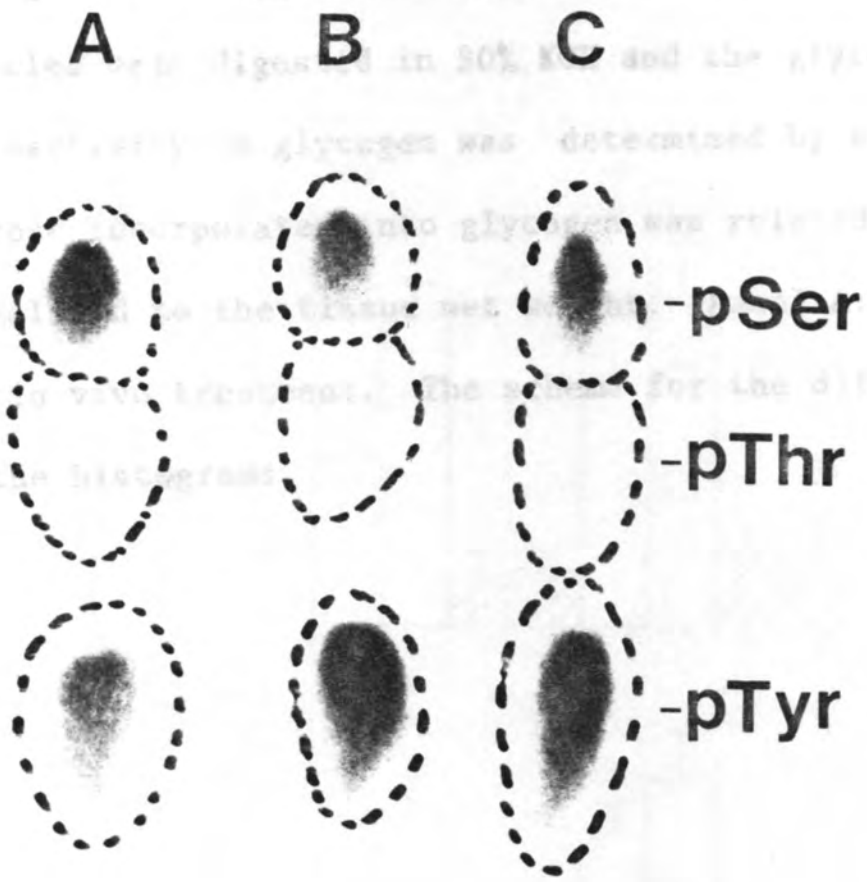
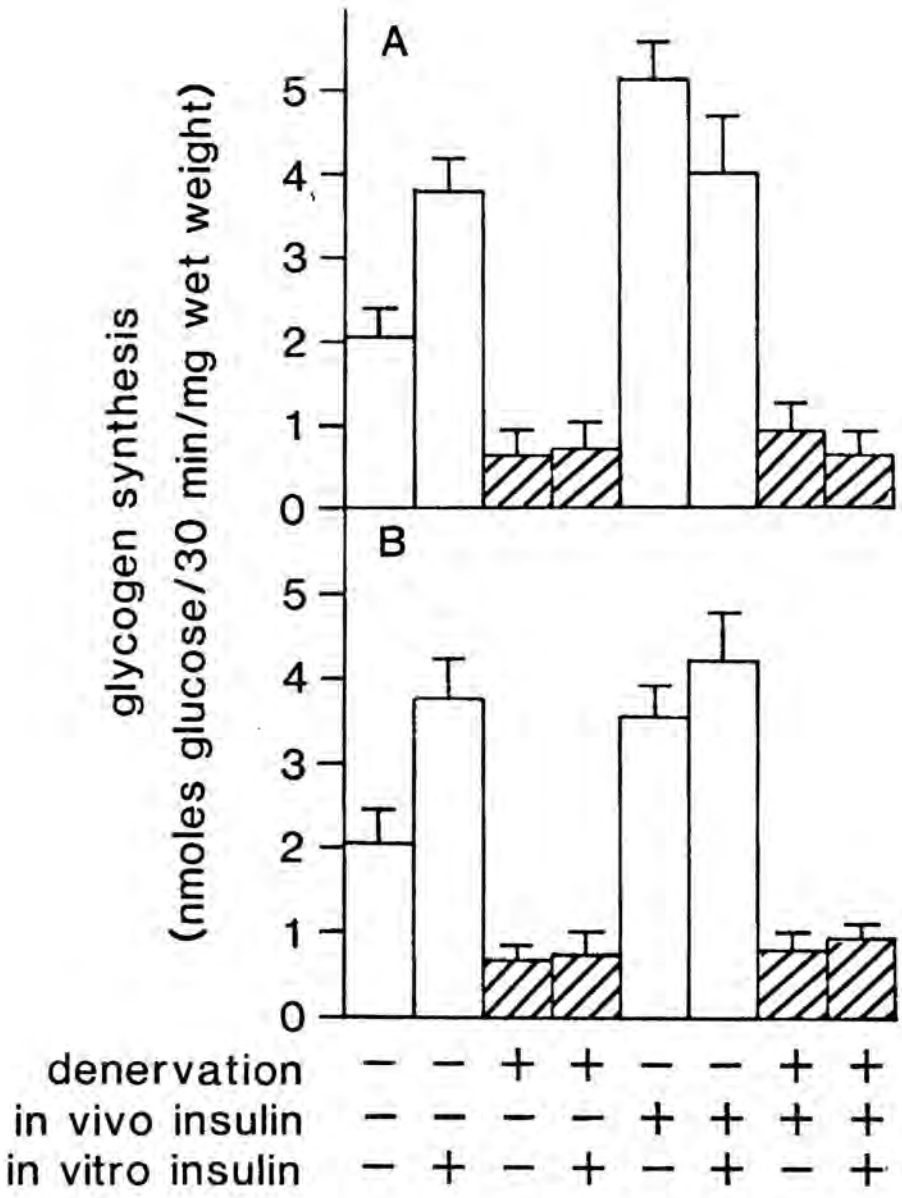

A
B
C
-pSer
-pThr
-pTyr

Figure 6. Glycogen synthesis by control and denervated solei. Rats were injected with saline or insulin as described in Figure 3 and after 5 or 30 min. the soleus muscles were removed and incubated for 30 min. in a balanced salt solution containing 5 mM [^{14}C]glucose (0.3 μCi/ml) without or with 10 mU/ml insulin. The muscles were digested in 30% KOH and the glycogen precipitated and washed. The radioactivity in glycogen was determined by scintillation counting and the nmol glucose incorporated into glycogen was related to the media specific activity and normalized to the tissue wet weight. Panel A: 5 min. in vivo treatment. Panel B: 30 min. in vivo treatment. The schema for the different treatments is indicated below the histograms.


A
B
glycogen synthesis
(nmoles glucose/30 min/mg wet weight)
0
1
2
3
4
5
denervation − − + + − − + +
in vivo insulin − − − − + + + +
in vitro insulin − + − + − + − +

REFERENCES

1. Richter, E.A., L.P. Garretto, M.N. Goodman, and N.B. Rudermann . "Muscle Glucose Metabolism Following Exercise in the Rat: Increased Sensitivity to Insulin." J. Clin. Invest. 69; 785-793, 1982.

2. Kochan, R.G., D.R. Lamb, E.M. Reimann, and K.K. Schlender, K.K. "Modified Assay to Detect Activation of Glycogen Synthase Following Exercise." Am. J. Physiol. 240; E197-E202, 1981.

3. Zorzano, A., T.W. Balon, L.P. Garetto, M.N. Goodman, and N.B. Ruderman. "Muscle α-aminoisobutyric Acid Transport after Exercise: Enhanced Stimulation by Insulin." Am. J. Physiol. 248; D546-E552, 1985.

4. Plough, T., H. Galbo, and E.A. Richter. "Increased Muscle Glucose Uptake during Contractions: No Need for Insulin." Am. J. Physiol. 247; E726-E731, 1984.

5. Buse, M.G., and J. Buse. "Glucose Uptake and Response to Insulin of the Isolated Rat Diaphragm. The Effect of Denervation." Diabetes 8; 218-225, 1959.

6. Buse, M.G. and J. Buse. "Effect of Denervation and Insulin on Penetration of D-xylose into Rat Hemidiaphragms." Diabetes 10; 134-141, 1961.

7. Forsayeth, J.R. and M.K. Gould. "Inhibition of Insulinstimulated Xylose Uptake in Denervated Rat Soleus Muscle: A Post-receptor Effect. Diabetologia 23; 511-516, 1982.

8. Smith, R.L. and J.C. Lawrence, J.C. "Insulin Action in Denervated Rat Hemidiaphragms." J. Biol. Chem. 259; 2201-2207, 1984.

9. Burant, C.F., Lemmon, S.K., Treutelaar, M.K. and Buse, M.G. "Insulin Resistance of Denervated Rat Muscle: A Model for Impaired Receptor-function Coupling." Am. J. PhysiolI. 247; E657-E666, 1984.

10. Booth, F.W. "Effect of Limb Immobilization on Skeletal Muscle." J. Appl. Physiol: Respirat. Environ. Exercise Physiol. 52; 1113-1118, 1982.

11. Hartzel, H.C. and P.M. Fambrough. "Acetylcholine Receptors: Distribution and Extrajunctional Density in Rat Diaphragms after Denervation, Correlation with Acetylcholine Sensitivity." J. Gen. Physiol. 60; 248-262, 1972.

12. Harris, J.B., and S. Thesleff. "Studies on Tetrodotoxin Resistant Action Potentials in Denervated Skeletal Muscle." Acta Physiol. Scand. 83; 382-388, 1971.

13. Axelsson, J. and S.J. Thesleff. "A Study of Supersensitivity in Denervated Mammalian Skeletal Muscle." J. Physiol. (Lond.) 147; 178-195, 1959.

14. Apple, S.H., C.G. Andrew, and R.R. Almon. "Phophatidyl Inositol Turnover of Membranes Following Denervation." J. Neurochem. 33; 1077-1080, 1974.

15. Smith, R.L. and J.C. Lawrence. "Insulin Action in Denervated Skeletal Muscle. Evidence that the Reduced Stimulation of Glycogen Synthesis Does Not Involve Insulin Binding." J. Biol. Chem. 260; 273-278, 1985.

16. Grunberger, G., Y. Zick, and P. Gorden. "Defect in Phosphorylation of Insulin Receptors on Cells from an Insulin-resistant Patient with Normal Insulin Binding." Science 223; 932-934, 1984.

17. Grigorescu, F., J.S. Flier, and C.R. Kahn. "Defect in Insulin Receptor Phosphorylation in Erythrocytes and Fibroblasts Associated with Severe Insulin Resistance." J. Biol. Chem. 259; 15003-15006, 1984.

18. Kadowaki, T., M. Kasuga, Y. Akanuma, O. Ezaki, and F. Takaku. "Decreased Autophosphorylation of the Insulin Receptor Kinase in Streptozotocin-diabetic Rats." J. Biol. Chem. 259; 14208-14216.

19. Czech, M.P. "New Perspectives on the Mechanism of Insulin Action." Rec. Prog. Hormone Res. 40; 347-377, 1984.

20. Kahn, C.R. "The Molecular Mechanism of Insulin Action." Ann. Rev. Med. 36; 429-451, 1985.

21. Taylor, S.E., J.A. Hedo, C.H. Underhill, M. Kasuga, M.J. Elders, and J. Roth. "Extreme Insulin-resistance in Association with Abnormally High Binding Affinity of Insulin Receptors from a Patient with Leprachanism: Evidence for a Defect Intrinsic to the Receptor." J. Clin. Endocrinol. Metab. 55; 1108-1113, 1982.

22. Bradford, M.M. "A Rapid and Sensitive Method for Quantitation of Microgram Quantities of Protein Utilizing the Principal of Insulin Binding. Biochem. 72; 248-254, 1976.

23. Burant, C.F., M.K. Treutelaar, G.C. Landreth, and M.G. Buse. "Insulin-resistance of Denervated Rat Skeletal Muscle: A Model for Impaired Receptor-function Coupling." Diabetes 33; 704-708, 1984.

24. Laemmli, V.K. "Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T_4." Nature (Lond.) 227; 571-578, 1970.

25. Yu, K-T. and M.P. Czech. J. Biol. Chem 259; 5277-5286, 1984.

26. Gey, G.O. M.K. and Gey. "The Maintenance of Human Normal and Tumor Cells in Continuous Culture." Am. J. Cancer 27; 45-76, 1936.

27. Lowry, O.H. J.V. and Passonneau. "ATP Extraction from Tissue by PCA." in A Flexible System of Enzymatic Analysis, pp 123-124.

28. Folley, L.S., S.D. Power, R.O. and Poyton. "Separation of

Nucleotides by Ion Pair, Reverse-phase High-performance Liquid Chromatography." J. Chromatography 281; 199-207, 1983.

29. Lowry, O.H., N.Y. Rosenbrough, N.Y., A.L. Farr, and R.J. Randall. "Protein Measurement with the Folin Phenol Reagent." J. Biol. Chem. 193; 265-275, 1951.

30. Cooper, J.A., B.M. Sefton, and T. Hunter. "Base Hydrolysis and Amino Acid Analysis for Phosphotyrosine in Proteins." Meth. Enzymol. 99; 387-403, ___.

31. Takayama, S., M.F. White, M.F., V. Lauris, V., C.R. "Phorbol Esters Modulate Insulin Receptor Phosphorylation and Insulin Action in Cultured Hepatoma Cells." Proc. Natl. Acad. Sci (USA) 81; 7797-7801, 1984.

32. Pang, D.F., B.R. Sharma, B.R., J.A. Shafer, M.F. White, M.F. and C.R. Kahn. "Predominance of Tyrosine Phosphorylation of Insulin Receptors during the Initial Response of Intact Cells to Insulin". J. Biol. Chem. 260; 7131-7136, 1985.

33. Flier, J.S. "Insulin Receptors and Insulin Resistance." Ann. Rev. Med. 34; 145-160, 1983.

34. LeMarchand-Brustel, Y., T. Gremeaux, R. Ballotte, and E. Van Obberghen. "Insulin Tyrosine Kinase is Defective in Skeletal Muscle of Insulin Resistant Obese Mice." Nature 315; 676-679, 1985.

35. White, M.F., H.V. Haring, H.V., M. Kasuga, and C.R. Kahn. "Kinetic Properties and Sites of Autophosphorylation of the Partially Purified Insulin Receptor from Hepatoma Cells." J. Biol. Chem. 259; 255-264, 1984.

36. Klein, H., and M. Kladde. (Diabetes Abstract). Diabetes 34 (Suppl. 1); 49A, 1985.

37. Yu, K-T. The Endocrine Society Program and Abstracts, 67th Annual Meeting, p. 6, 1985.

38. Rosen, O.M., R. Herrera, Y. Olowe, L.M. Petruzzelli, and M.H. Cobb. "Phosphorylation Activates the Insulin Receptor Tyrosine Protein Kinase." Proc. Natl. Acad. Sci. (USA). 80; 3237-3240, 1983.

CHAPTER 5: DIABETES INDUCED FUNCTIONAL AND STRUCTURAL CHANGES IN INSULIN RECEPTORS FROM RAT SKELETAL MUSCLE

Insulinopenic diabetes in man is associated with peripheral insulin resistance (1-4), which appears to be related to prior glycemic control (5). Using a closed-loop subcutaneous insulin infusion system, Mayfield et al (5) reported a direct correlation between elevated HbA_{Ic} levels and increased insulin requirements in Type I diabetic patients. Strict control of blood glucose levels increased the subjects' sensitivity to insulin (5,6,7). Insulin resistance is also seen in animals with experimentally induced insulinopenia. Reaven et al (8) observed insulin resistance in alloxan diabetic dogs when fasting plasma glucose levels exceeded 150 mg/dl while those with lower glycemia responded to insulin as the controls. In rodents, streptozotocin-induced diabetes results in a diminished response to insulin in isolated adipocytes (9,10,11), liver (12,13) as well as skeletal (14) and heart muscle (15), while insulin binding (e.g. the apparent number of insulin receptors) is increased (9,10,13). This has led to the suggestion that the insulin resistance associated with poorly-controlled Type I diabetes is primarily a post-receptor defect. Alternatively, it may represent a defect in signal transmission by the occupied insulin receptor.

The initial event in insulin action on target tissues is its binding to specific cell surface receptors. The insulin receptor is a

heterotetramer glycoprotein, consisting of two α and two β subunits. The α subunit with a MW_{app} ~ 135000 daltons appears to contain the insulin binding domain, while the β-subunit MW_{app} ~ 95000 daltons may be primarily involved in signal transmission. Insulin binding induces the autophosphorylation of the β subunit in both intact cells and in cell-free systems (16-23). Subsequent to this autophosphorylation, the insulin receptor acquires the ability to act as a kinase towards exogenous substrates (18,21,23). These phosphorylation events occur on tyrosine residues and have been suggested to trigger the phosphorylation-dephosphorylation cascade seen in cells upon exposure to insulin (16,20,22).

Since skeletal muscle is an important site of insulin action, we investigated the effect of streptozotocin-induced diabetes on the functional and structural properties of the solubilized rat skeletal muscle insulin receptor. We found that the insulin-stimulated autophosphorylation and exogenous substrate kinase activity of diabetic derived receptors was impaired. This alteration was reversed by in vivo insulin treatment. The functional changes were paralleled by the appearance of a subpopulation of insulin receptor β-subunits in diabetic skeletal muscle with a slower electrophoretic migration; the latter may reflect altered glycosylation of the β-subunit.

Materials: Streptozotocin was a gift from Dr. William Dulin, Upjohn Research Laboratories, Kalamazoo, MI. Polyclonal anti-insulin receptor antibody (A410) was a gift from Dr. Steven Jacobs (Wellcome Research Laboratories, Research Triangle Park, NC) and from Dr. C. Ronald Kahn (B9) (Joslin Research Laboratories, Boston, MA). Monocomponent, crystalline pork insulin was a gift from Dr. Ronald Chance,

Lilly Research Laboratories, Indianapolis, IN. Monoiodinated A14[^{125}I]insulin used in some studies was kindly provided by Dr. B. H. Frank, Lilly Research Laboratories. Wheat germ agglutinin was from Vector (Burlingame, CA); Aprotinin from FBA Pharmaceuticals (New York, NY); Bacitracin from Pfizer (New York ,NY). Electrophoresis reagents were from Serva (Heidelberg, Germany) and the apparatus from LKB (Houston, TX). Protein A-bearing S. Aureus (Pansorbin) and neuraminidase (Vibrio Cholera) were from Calbiochem (San Diego, CA). S. Aureus V8 protease and endoglycosidase H were from Miles (Elkhart, IN). ^{125}I-iodine was from New England Nuclear (Boston, MA). γ[^{32}P]ATP was prepared from ^{32}P orthophosphate (ICN, Cambridge, MA) in Gammaprep Vials (Promegabiotec, Madison, WI) as described by the manufacturer. CNBr-Sepharose-4B beads were from Pharmacia (Piscataway, NJ). Thin layer TLC plates, Polygram CEL300, were from Brinkmann Instruments (Westbury, NY). Autoradiography film, X-Omat AR was from Kodak (Rochester, NY). Lightening + intensifying screens were from Dupont (Wilmington, DE). All other reagents and chemicals were from Sigma (St. Louis, MO) unless otherwise specified.

Animals: Male Wistar rats (Charles River Breeding Co., N.J.) 100-140 g initial body weight were used in all experiments. They were allowed food and water ad libitum. Diabetes was induced by i.p. injection of streptozotocin in citrate buffer, pH 4.5, 130 mg/kg body weight, to overnight fasted animals. In insulin reversal experiments diabetic rats were initially treated with 2U regular and 2U protamine zinc insulin (PZI) s.c. at 1700 hr on the fifth day after streptozotocin and subsequently with 2U PZI at 0900 hr and 1700 hr. the next two days for a total of five treatments. Glycosuria was monitored by Tes-Tape (Lilly)

and was 4+ in untreated diabetic rats, and trace, or negative in insulin treated rats by the second day of treatment. Plasma glucose levels were determined with a Beckman Glucose Analyzer 2 on blood samples obtained at sacrifice. Diabetic rats with or without insulin treatment were studied on the 7th day after streptozotocin, and compared to control animals. Rats were killed by decapitation, the hindlimb muscles were rapidly removed and frozen in liquid nitrogen. Insulin receptors from control and experimental animal groups were always processed on the same day and analyzed in the same experiments.

Solubilization and partial insulin receptor purification were performed as previously described (23). Briefly, the frozen muscles, approx. 5g, were powdered and membrane components solubilized by homogenization as a frozen slurry in 4 vol. 25mM Hepes, pH 7.4, 5mM EDTA, 1 TIU/ml aprotinin, 2mM phenylmethyl sulfonylfluoride (PMSF) and 1% Triton X-100. The homogenate was centrifuged at 10,000 x g for 10 min and the supernate stirred at room temperature for 1 hr. The mixture was then centrifuged at 150,000xg for 90 min. The receptors were further purified by adsorption to and elution from Agarose-bound wheat germ agglutinin (WGA). Receptors were eluted from the WGA column with 25mM Hepes pH 7.4, 0.1% Triton X-100 containing 0.3 M N-acetylglucosamine (approx 1 ml/gm original tissue weight) and used directly or further purified by precipitation with polyclonal anti-insulin receptor antibody. In some preparations ^{125}I-insulin (~1.5 μCi) was added after homogenization of muscles with or without 50 μg/ml unlabeled insulin. Aliquots were taken at various times during the isolation procedure and ^{125}I-insulin specifically bound was determined by polyethylene glycol precipitation. Recovery and trichloroacetic acid (TCA) precipitability

of the ^{125}I-insulin were determined by counting aliquots in a γ-counter as described below.

Insulin Binding: WGA eluate (50 µl) was incubated with 10,000 cpm ^{125}I-insulin (sp. act. 125-175 µCi/µg, prepared by the lactoperoxidase method as described in 24) and increasing concentrations of unlabelled insulin for 16 hrs at 4°C in a buffer containing 25 mM Hepes, 0.1% Triton X-100, 150 mM NaCl, 0.1% bovine serum albumin and 100 U/ml Bacitracin as described by Taylor et al (25). The receptor and bound insulin were precipitated by the addition of 100 µl 0.3% bovine γ-globulin and 300 µl 25% polyethylene glycol and collected by centrifugation. The resulting pellet was washed with 300 µl 12.5% polyethylene glycol and counted in a Beckman Gamma 4000 γ-counter. Non-specific binding (10-15%) was estimated as that occurring in the presence of 10 µg/ml unlabelled insulin. Binding was related to the protein concentration in the WGA eluate estimated by the Bradford reaction (26).

Equalization of receptor number: In all experiments assessing functional and structural properties of solubilized insulin receptors derived from experimental and control animals, approximately equal insulin binding activity was used in comparing groups. Since no change in insulin binding affinity was apparent in receptors from diabetic animals (see results) equal numbers of insulin receptors were estimated as follows: ^{125}I-insulin binding per 50 µl WGA eluate was determined in the presence of 1, 5, 25, 100, 1000 and 10,000 ng/ml unlabelled insulin (the latter used as estimate of non-specific binding after overnight incubation at 4°C or 45 min at 25°C). Ratios of the specifically bound counts in the experimental group vs. the control were determined at each

insulin concentration, these ratios were averaged and the mean value used as an estimate of relative number of insulin receptors. Individual values were within 10-15% of this mean. Appropriate volumes of the WGA eluate were then used in subsequent experiments. Identical ratios were obtained after overnight or 45 min incubation with ^{125}I-insulin; the latter resulted in approximately 60-70% of the equilibrium binding obtained in the former. All dilutions of WGA elute were made with 25 mM Hepes pH 7.4, 0.1% Triton X-100. The presence of 0.3 M N-acetylglucosamine had no effect on the functional properties of the insulin receptor (18, and our data). Experimental groups were always compared to control insulin receptors prepared the same day from untreated animals.

Insulin specifically bound to receptors in the wheat germ eluate was linear between 12.5 and 75 µl of the eluate assayed in a constant volume ($r = 0.97$ and 0.96 for control and diabetic receptor preparations respectively). The ^{32}P incorporated into the β-subunit of the insulin receptor, after exposure to maximally stimulating insulin concentrations (10^{-7}), was also linear between 10 and 50 µl of the wheat germ eluate, ($r = 0.98$ and 0.97 for control and diabetic receptor preparations respectively). These values were derived from three different pairs of receptor preparations (diabetic and control), each point assayed in duplicate or triplicate.

Receptor autophosphorylation. Fifty µl of WGA eluate from control animals and appropriately diluted receptors from diabetic or insulin-treated diabetic animals were incubated in 25 mM Hepes pH 7.4, 0.1% Triton X-100, 5 mM $MnCl_2$, 0.05% bovine serum albumin (Buffer A) with increasing insulin concentrations in 75 µl final volume for 45 min at

25°C. The reaction mixtures were then cooled to 4°C and $\gamma[^{32}P]$ATP (10 µl ~ 15 µCi, 100 µM final concentration) was then added and after 30 min at 4°C the phosphorylation reaction was terminated by the addition of 28 µl 4X concentrated Laemmli's sample buffer, heated at 100°C for 3 min and the proteins separated by SDS-PAGE (see below). The β-subunit of the insulin receptor was localized by autoradiography, excized and digested in 1 ml 30% H_2O_2 at 60°C for 10 hrs and ^{32}P content determined by counting in 10 ml Scintiverse II (Fisher Scientific, Norcross, GA) in a Beckman LS7000 scintillation counter. Insulin degradation by the receptor preparation during incubation at 25°C in buffer A was estimated by TCA precipitability of added ^{125}I-insulin tracer; less than 2% of added insulin became TCA soluble after 2 hours of incubation.

Exogenous substrate kinase activity was determined using Angiotensin II as the phosphoacceptor, as described by Rosen et al. (18). Approximately 10-20 fmoles insulin binding activity was incubated with or without insulin at 25°C for 1 hr in 225 µl buffer A. Autophosphorylation was initiated by the addition of $\gamma[^{32}P]$ATP (50 µCi, 100 µM final concentration) and allowed to proceed for 30 min at 4°C. Aliquots (35 µl) were then added to 5 µl of buffer A or buffer A containing 2 mM angiotensin II and the phosphorylation carried out at 4°C for 5 min which was in the linear part of the velocity curve as determined in preliminary experiments. The reaction was stopped by the addition of 5 µl 1.5% BSA and 50 µl 5% TCA. After centrifugation, duplicate 40 µl aliquots were spotted onto phosphocellulose paper, washed and counted as described (18).

Kinase activity of insulin receptors bound to insulin-agarose. Control receptors (120 µl) and equal binding activity of diabetic

derived receptors were diluted to a final volume of 320 µl with buffer consisting of 50 mM Hepes, pH 7.4, 0.1% Triton X-100, 1 µg/ml leupeptin, and 2 mM PMSF (buffer B); 50 µl insulin-agarose beads or agarose-beads inactivated with hot dithiothreitol (27) were added and the mixture incubated for 1 hour at 25°C. After centrifugation the pellet was washed twice with 1 ml buffer B supplemented with 0.5 M NaCl and then with buffer B alone. The pellet was then resuspended in 75 µl 50 mM Hepes, pH 7.4, 0.1% Triton X100, 0.025% BSA, 5 mM $MnCl_2$ (phosphorylation buffer) supplemented with 750 µM ATP and incubated for 15 min at 25°C to induce maximal receptor autophosphorylation (27). The mixture was then diluted with 1 ml of salt supplemented buffer B, centrifuged, the pellet washed with 1 ml buffer B, and then resuspended in 75 µl phosphorylation buffer, supplemented with 1 mg/ml histone H_2b and 50 µM $\gamma[^{32}P]$ATP (2.5 µCi/vial) and incubated for 4 min at 25°C. The reaction was stopped by the addition of hot 4X Laemmli's sample buffer and the sample separated on SDS-PAGE and autoradiographed as described below.
The bands corresponding to Histone H_2b were excized and the ^{32}P quantitated as described above for the insulin receptor β-subunit.

ATPase activity: Equal binding activities of control and diabetic derived wheat germ eluates were incubated as described for the autophosphorylation assays except that the specific activity of the ATP was decreased (~ 6 µCi/ml, ATP final concentration 10-100 µM). At various time intervals (30 sec-120 min) the reaction was stopped by the addition of 65 µl of 3M perchloric acid and after 5 min equilibration at 4°C 200 µl of dH_2O were added and the protein removed by centrifugation. The supernatant (300 µl) was neutralized with 60 µl 2 M $KHCO_3$, clarified by centrifugation, supplemented with 2 µl of a 2 M solution of unlabeled

carrier ATP and 20 µl were analysed on a Perkin-Elmer High Performance Liquid Chromatograph using a 5 cm C_{18} reverse phase column. ATP was eluted isocratically in a buffer composed of 20 mM phosphoric acid (pH to 6.5 with triethylamine, 98%) and methanol (2%) at a flow rate of 2 ml/min (28). The first 1.5 min of the run (containing inorganic phosphate) and the ATP peak were collected separately and ^{32}P cpm quantitated by liquid scintillation counting. These assays were performed in parallel with assays of insulin-receptor kinase activities. All kinase assays reported here were carried out under conditions where ATP degradation was less than 10%.

Cross-linkage of solubilized insulin receptors with [^{125}I]insulin: Wheat germ eluates (1 ml) from control and diabetic derived muscle extracts were dialyzed against 25 mM Hepes pH 7.6 and concentrated approximately four-fold with Aquacide (Calbiochem). Equal binding activities were incubated for 15 hrs at 4°C with A14[^{125}I]insulin (0.5 µCi) with or without 10 µg/ml unlabeled insulin in 250 µl final volume, then disuccinimidyl suberate was added in DMSO to a final concentration of 2 mM at 4°C, for 15 min (29). After addition of 4X concentrated Laemmli's sample buffer the mixture was boiled for 3 min. Electrophoresis and autoradiography were performed as described below.

Immunoprecipitation and partial proteolytic digestion of phosphorylated insulin receptors. WGA eluate was incubated in the presence of 10^{-7}M insulin for 45 min at 25°C in Buffer A (100µl final volume) and γ [^{32}P]ATP (10µM, 10µCi) added for an additional 10 min. The reaction was stopped by addition of 20 µl 40 mM ATP, 10 mM Na_4PO_5, 2mM PMSF, in Buffer A. Anti-insulin receptor antibody (1:400 dilution) was added and incubated overnight at 4°C. Protein A bearing formalin-

fixed S. aureus was added and incubated for an additional 2 hrs and the precipitated complex washed three times with 25 mM Hepes, 0.1% Triton X-100. Partial proteolytic digestion of the immune complex was achieved by resuspending it in 50 mM sodium acetate pH 5 buffer (10 µl) containing 75 µg/ml V8 protease and incubating for 5 min at 37°C. The reaction was stopped either by the addition of an equal volume twice concentrated Laemmli's sample buffer or by the addition of 10 µl Protease Inhibitor Cocktail I (PIC I) and 5 µl PIC II (PIC I = 1.5 mg/ml Benzimidine, 1.5 mg/ml leupeptin, 1.5 mg/ml Benzoyl arginine ethyl ester in 10 TIU/ml aprotinin. PICII = 1 mg/ml PMSF and 1 mg/ml antipain in DMSO). The protease inhibited mixtures were then treated with glycosidases as described below.

Phosphoamino acid analysis: The phosphorylated, immunoprecipitated β-subunit was identified by autoradiography of the wet, unfixed gel after SDS-PAGE and solubilized by electroelution as follows: A 2x2cm gel slice was placed in a dialysis bag containing electrophoresis buffer (1.5M Tris, 58mM glycine, pH 8.8) and immobilized between the glass plates of the vertical gel electrophoresis apparatus, immersed in this buffer. One end of the sandwich was plugged with 15% acrylamide and a potential of 500 V was applied for 1 hour. The contents of the bag were removed, 30 µg bovine γ-globulin added and the protein precipitated by making the solution 20% in TCA. The precipitate was dissolved in 250 µl 6N HCl and hydrolyzed at 110°C for 60 min under N_2 atmosphere. The hydrolysate was adsorbed to 0.5 ml Dowex 1-X8, washed with 2 ml dH_2O and eluted with 1 ml 0.1N HCl (30). The lyophilized sample was redissolved in 5 µl of a mixed phosphoamino acid standard containing 0.33 mg/ml each of phosphoserine, phosphothreonine and phosphotyrosine and spotted on

thin cellulose TLC plates (0.1mm thickness). The plates were electrophoresed at 1000 V for 40 min in pyridine:acetic acid:H_2O (5:15:945) towards the anode (30). The phosphoamino acids were localized by ninhydrin staining and by autoradiography at -80° with intensifying screen.

Tryptic phosphopeptide mapping: Electroeluted receptors were precipitated at 4°C in 10% TCA with 30 µg carrier bovine γ-globulin and the precipitate extracted 3 times with 750 µl 1:1 ether: ethanol (31). The washed pellet was oxidized at 25°C for 2 hrs by the addition of 200 µl 9:1 formic acid:H_2O_2, diluted to 2 ml with water and lyophilized. The lyophilizate was resuspended in 0.3 ml 50 mM $(NH_4)_2CO_3$, pH 8.0, containing 75 µg/ml TPCK treated trypsin and incubated at 25°C for 12 hrs when an additional 40 µg trypsin was added and incubation continued for another 12 hrs. The mixture was brought to 3 ml with water and lyophilized three times, resuspending in 3 ml water each time (31). Phosphopeptides were separated in 2 dimensions on 20cm X 20cm X 0.1mm TLC plates by electrophoresis in 1% ammonium bicarbonate, pH 8.9, for 40 min towards the anode and then by ascending chromatography in pyridine: butanol:acetic acid:water (70:50:15:60) in the second dimension (32). Phosphopeptides were localized by autoradiography at -80°C for 4 with intensifying screens.

Treatment of immunoprecipitated receptors with Endoglycosidase H. Washed, immunoprecipitated receptors with or without prior partial protease digestion were made 0.1% in sodium dodecylsulfate and heated for 1 min in boiling water. After cooling, 10 µl PIC I and 5 µl PIC II were added, followed by 50 µl 30 mU/ml endoglycosidase H in 50 mM sodium acetate, pH 5.5, 10 mM $CaCl_2$, 0.01% bovine serum albumin and incubated for 4 hours at 37°C. The samples were boiled in Laemmli's sample buffer (4X concentrated) and separated on SDS-PAGE.

Neuraminidase: Immunoprecipitated receptors with or without prior partial V8 digestion were resuspended in 10 µl PIC I and 5 µl PICII, followed by the addition of 40 mU/ml neuraminidase in 50 mM sodium acetate, pH 5.0, 10 mM $CaCl_2$, 150 mM NaCl and incubated 4 hours at 37°C. As above the reaction was stopped by the addition of 4X Laemmli's sample buffer and proteins separated on SDS-PAGE. Methods for treatment of insulin receptors with glycosidases were modified from those described by Ronnett et al. (33).

Electrophoresis: SDS-PAGE was performed under reducing condition using the discontinuous buffer system of Laemmli (34) with a 7.5% polyacrylamide resolving gel for non-protease digested samples, a 9% polyacrylamide resolving gel for samples previously treated with V8 protease and a linear 6-18% polyacrylamide resolving gel for reactions containing Histone H_2b. A 4.5% polyacrylamide stacking gel was used for all samples. Samples were electrophoresed at 150-170 volts, constant voltage. Gels were stained, unless indicated, in 50% methanol, 10% acetic acid and 0.125% Coomassie blue R and destained in 30% methanol and 10% acetic acid. The gels were dried in vacuo and autoradiography performed with Kodak AR autoradiography film with or without Dupont Lightening + intensifying screens at -80°C.

Results

Animal condition: Rats rendered diabetic by i.p. injection of streptozotocin gained little or no weight during the subsequent 7 days. Final weights of the diabetic rats were 111 ± 4 gm (N = 23) vs 164 ± 4 gm (N = 15) for the controls. Insulin treatment (60 hrs) of diabetic rats resulted in rapid weight gain (final weight 145 ± 5 gms; N = 10). At the time of sacrifice the plasma glucose concentration of control

rats was 130 ± 4 mg/dl (N = 15), that of untreated diabetic rats was 510 ± 14 mg/dl (N = 17), and that of insulin treated diabetic rats was 94 ± 6 mg/dl; (N = 12).

Insulin Binding. Equilibrium binding of ^{125}I-insulin by the partially purified insulin receptors extracted from hind limb muscles of diabetic rats increased approximately two-fold per mg protein in the wheat germ eluate as compared to controls (Figure 1). When calculated per gm muscle the increase in receptor number was 60-70% (~ 100-125 pmoles insulin bound/gm in control vs. 180-250 pmoles bound/gm in the diabetic). Administration of insulin for 60 hours caused a reduction in insulin receptor number per mg protein and per gm tissue weight to nearly that of controls. The yield of glycoprotein mg/gm muscle from diabetic rats was ~ 25% less than that obtained from controls, 0.55 ± 0.06 vs. 0.72 ± 0.07 respectively, (N=10/group, $p < 0.01$, paired analysis). The increase in receptor number solubilized from muscles of diabetic rats occurred without an apparent change in the affinity of the insulin receptor. These values were obtained after incubation at 4°C for 16 hrs. Binding of ^{125}I-insulin to control and diabetic derived insulin receptors for 45 minutes at 25°C showed that 65-75% of equilibrium binding occurred in this time frame in both groups and the ratio of insulin bound by diabetic vs. control preparations was identical to that obtained with incubations for 15 hrs at 4°C. Since the kinetics of insulin binding were identical in diabetic and control animals, the tyrosine kinase activity of the solubilized insulin receptors was routinely studied after 45 min to 1 hr incubation with insulin at 25°C. Experiments performed following incubation with insulin for 16 hrs at 4°C yielded identical results to those shown below.

Insulin stimulated autophosphorylation: Insulin stimulates the autophosphorylation of the insulin receptor through an intramolecular event (20,35). As previously reported (23) when the WGA purified insulin receptors from rat skeletal muscles were incubated with $\gamma[^{32}P]$ATP, 5 mM $MnCl_2$ and increasing concentrations of insulin there was an insulin dose-dependent increase in the amount of ^{32}P incorporated into a ~97,000 dalton peptide when the reaction mixture was analysed by SDS-PAGE and autoradiography. This peptide was identified as the β-subunit of the insulin receptor by its apparent molecular weight, its ability to increase labelling after incubation with insulin, and its specific immunoprecipitability with anti-insulin receptor antibodies (see ref. 23).

When equal binding activities of control and diabetic derived receptors were allowed to autophosphorylate at 4°C for 30 min in the presence of 100 μM ATP, there was a significant decrease ($p < 0.05$) in the amount of ^{32}P incorporated into the β-subunit of the insulin receptor derived from diabetic animals incubated without insulin and at all insulin concentrations tested (Fig. 2). At maximally stimulating insulin concentrations, the mean autophosphorylation of control receptors was 40% greater than that of the diabetics, however the insulin stimulated autophosphorylation over baseline (Δ insulin) was only 20% greater in controls than in diabetics. Although the latter difference was not significant, as will be shown below, it resulted in marked impairment of insulin stimulated exogenous kinase activity. Half-maximal activation of autophosphorylation occurred at ~ 2×10^{-9} M insulin in controls and diabetics. When diabetic rats were treated with insulin to normalize their blood glucose levels, the insulin receptors derived

from these animals showed nearly identical autophosphorylation kinetics as controls (Fig. 2).

Exogenous substrate kinase activity: The insulin receptor displays exogenous substrate tyrosyl kinase activity that is activated by the autophosphorylation event (18,21). To investigate this property insulin receptors from control, diabetic or diabetic-treated animals were incubated for 1 hr at 25°C with the indicated concentrations of insulin and then allowed to autophosphorylate in the presence of 100 μM $\gamma[^{32}P]$ATP at 4°C for 30 min; exogenous kinase activtiy was then assessed for 5 min at 4°C using Angiotensin II as the phosphoacceptor. Little exogenous kinase activity was observed in any of the groups in the absence of added insulin. At 10^{-9} and 10^{-7} M insulin, a significant decrease in the exogenous substrate kinase activity was seen in the diabetic derived receptors ($p < 0.01$, Fig. 3). The ability of insulin to stimulate the exogenous kinase activity over basal was significantly less in diabetic derived receptors than in controls ($p < 0.01$). Treatment of diabetic rats with insulin restored the exogenous substrate kinase activity of the receptors toward normal (Fig. 3).

The decreased ability of diabetic derived receptors to phosphorylate exogenous substrates was not temperature dependent nor substrate specific, since similar decreases in the diabetic derived receptor kinase activity were observed at 25°C using histone H_2b as the phosphoacceptor. In these experiments the wheat germ eluates were preincubated with insulin coupled to agarose, the agarose-bound receptors were extensively washed and then allowed to fully autophosphorylate in the presence of 750 μM ATP. The exogenous kinase activity was then assessed using 50 μM $\gamma[^{32}P]$ATP. While equal insulin binding activities derived

from control and diabetic muscles were incubated with the beads, there was again a significant decrease in the amount of label incorporated into the histone by the diabetic derived receptors as compared to the controls (Fig. 4). When receptors were bound to insulin-agarose beads which had been inactivated with hot dithiothreitol (27), histone-H_2b phosphorylation was ~ 10% of that observed with fully activated agarose-bound receptors (data not shown).

A number of mechanisms may explain the decreased kinase activity of the diabetic derived receptor population, including errors in the estimation of insulin receptor number or contaminants in the wheat germ eluate altering insulin receptor phosphorylation. Insulin that was "carried over" in the preparation of the insulin receptor could lead to an underestimate of the true insulin receptor number, especially in the control derived preparations. This possibility was examined by the addition of ^{125}I-insulin to the muscle homogenate and following its recovery during different stages of purification. We found that $\leq$ 0.1% of the added radioactivity was recovered in the final wheat germ eluate and this was entirely nonspecific binding since an identical homogenate which was supplemented with 50 μg/ml unlabeled insulin showed identical recovery of ^{125}I-insulin. A second approach was to exhaustively dialyse the insulin receptors in the wheat germ eluate to remove insulin. Upon rebinding, identical ratios of estimated insulin receptor numbers from control and diabetic animals were obtained as those before dialysis. Furthermore, no change in basal or insulin stimulated autophosphorylation was observed in the dialysed receptors.

The alterations in insulin receptor kinase activities did not appear to be due to contaminating insulinases in the diabetic derived

receptors nor to contaminating phosphatases since less than 2% of added ^{125}I-insulin was degraded by the WGA eluates over a two hour period and no change in the amount of ^{32}P associated with the insulin receptor β-subunit or with Angiotensin II or histone H_2b were seen for 30 min after the addition of unlabeled ATP to previously phosphorylated insulin receptors and kinase substrates in either the control or diabetic derived preparations (data not shown). While significant ATPase activity was detected in the wheat germ eluates under some conditions, the results shown here were derived from assays, where less than 10% of γ[^{32}P]ATP was degraded.

Figure 5 shows the phosphoamino acid analysis of control and diabetic derived insulin receptor β-subunits which were maximally stimulated by insulin and then exposed to 10 μM γ[^{32}P]ATP for 10 min at 25°C. As with other insulin receptors phosphorylated in vitro (18,19) labeling occurred predominantly on tyrosine residues in both groups. Although a small amount of serine phosphorylation was observed, it contributed little to the total amount of phosphoamino acid present in either group.

Two dimensional phosphopeptide maps generated from trypsin digestion of the β-subunit of the insulin receptors derived from control and diabetic animals and autophosphorylated at 25°C are shown in Figure 6. Although slight variations occur in the relative labeling of the various phosphopeptides generated by this procedure, no consistent changes occurred in a number of maps analysed, to suggest that a site or sites are eliminated from the diabetic derived population that would account for the decrease in overall labeling.

Structure of the Insulin Receptor subunits. Incubation of wheat germ eluates from control and diabetic animals with A14 [^{125}I]insulin with subsequent addition of disuccinimidyl suberate specifically labeled a protein of MWapp 131kD (Fig 7), which corresponds to the approximate molecular weight of the α-subunit of the insulin receptor. No differences were observed in the electrophoretic migration of the insulin cross-linked α-subunit between control and diabetic derived insulin receptor populations. Furthermore, the amount of label bound per receptor was identical when diabetic or control derived wheat germ eluates were cross-linked with ^{125}I-insulin.

Exploiting the property of autophosphorylation to localize the β-subunit of the insulin receptor, we investigated the possibility that the functional alterations observed in the insulin receptor from diabetic animals could stem from an identifiable structural alteration in this subunit. Insulin receptors were phosphorylated, immunoprecipitated and separated under reducing conditions by SDS-PAGE. Autoradiography revealed that the β-subunit of the insulin receptor derived from diabetic animals included a component that showed a small but reproducible slowing in electrophoretic migration as compared to controls (Fig. 8 lanes A and B). This change in electrophoretic migration became more prominent after short proteolytic digestion of the phosphorylated, immunoprecipitated receptor with V8 protease (Fig. 8 lanes C and D). V8 protease treatment reproducibly generated 2-4 major phosphorylated species each of which showed this altered migration when derived from diabetic muscle.

The major phosphorylated peptides seen after V-8 digestion were shown to be derived from the β-subunit (as opposed to the variably

phosphorylated α subunit) in the following manner. Phosphorylated, immunoprecipitated insulin receptors were electrophoresed under reducing conditions and the unstained, unfixed gel autoradiographed at 4°C. The β-subunit was localized and excised, the gel slice placed in a dialysis bag and the phosphoprotein eluted as described in "Methods". The dialysed, lyophilized protein was then subjected to partial V8 digestion, equal numbers of counts from each treatment group (~4000 cpm) were loaded in each lane and reelectrophoresed. The autoradiograph, (Fig. 8, lanes F and G) shows the same major phosphorylated species as were generated by partial digestion of the immunoprecipitated intact receptor (lanes C and D) - with the fragments generated from diabetic derived β-subunits again showing a component with retarded mobility when compared to controls.

As shown in Figure 8, Lanes E and H, the diabetes associated alteration in electrophoretic mobility was reversed by 60 hrs of in vivo insulin treatment that normalized blood sugar. Lanes I and J show autoradiograms of control and diabetic derived insulin receptors that were immunoprecipitated and partially digested with V-8 protease. Brief autoradiography revealed the diabetic derived β-subunit (Lane J) to consist of two distinct populations, one that comigrates with the control receptor (Lane I) and one with retarded migration.

We next investigated the possibility that sugar side chains associated with this glycopeptide could be altered in the diabetic state. Considering the difficulty in metabolically labeling the sugar side chains and the minute amounts of insulin receptors available for chemical modification, we used enzymatic digestion of the insulin receptor with glycosidases in an attempt to characterize the structure of the

glycosyl residues. Incubation of the immunoprecipitated insulin receptor with endoglycosidase H caused an ~ 5kD decrease in MW_{app} in control and diabetic derived insulin receptors (Fig. 9a, Lanes A, B and E, F) but did not correct (and actually accentuated) the difference between the control and diabetic. Endoglycosidase H digestion of the immunoprecipitated receptor after partial V8 proteolysis was accomplished by adding an excess of protease inhibitors before the addition of the endoglycosidase. Again a decrease in MW_{app} of ~ 4-5 kD occurred in the major phosphopeptides generated from control and diabetic derived β-subunits and allowed the separation of the diabetic derived receptors into two distinct populations (Figure 9b, Lanes E & F), one that comigrates with the control and one with retarded electrophoretic migration. The decrease in MW_{app} of the β-subunit after endoglycosidase H treatment suggested that this glycoprotein contains 'high mannose' sugar side chains.

Neuraminidase is an exoglycosidase that removes terminal sialic acid residues from the sugar side chains of glycoproteins. When the immunoprecipitated insulin receptor was digested with this enzyme a decrease in MW_{app} of approximately 2 kD occurred in control lanes (Fig. 9a, lanes A and C). A similar decrease occurred in the diabetic derived insulin receptor (Fig. 9a, lanes B and D) and in addition there was an apparent normalization in the electrophoretic migration of this population as compared to control (Figure 9a, Lanes C and D). Neuraminidase treatment caused a slight decrease in MW_{app} of the major (~ 54 kD) phosphorylated peptide in the diabetic derived population (Fig. 9b, lanes B and D), and nearly normalized the migration of the diabetic derived phosphopeptides as compared to control (Figure 9b, lanes C and D).

However, double glycosidase digestion i.e. neuraminidase followed by endoglycosidase H showed that the second, slower migrating band in the diabetic derived preparation increased its electrophoretic migration but was not completely eliminated after neuraminidase treatment (Fig 10, demonstrating the main ~ 54 kD phosphopeptide generated by V8 protease digestion). This result raises the possibility that an additional component, possibly 'core' sugars, may contribute to the heterogeneity in electrophoretic migration of the diabetic derived β-subunit.

DISCUSSION

An increase in the number of insulin receptors associated with insulinopenia has been observed in adipocytes (9,10), and hepatocytes (13,36). In the present study the number of insulin receptors solubilized per gram hindlimb muscle was increased 60-70% after 7 days of diabetes, without an apparent change in affinity. Insulinopenic diabetes in rats has been reported to cause no change (10,36) or to increase the affinity of insulin receptors (10,37) in adipocytes and liver. Studies of insulin binding to intact, incubated soleus muscles from streptozotocin diabetic mice reported increased binding affinity without a change in receptor number (38). These discrepancies may reflect the difficulty in interpreting changes in insulin binding kinetics, especially when intact tissues are used, as well as possible differences between solubilized, total receptors assessed in the present study versus surface receptors on intact muscle cells studied in Ref.38. It should be noted that the increased receptor number observed in muscles after 7 days diabetes in this report reflects in part differences in growth rate and muscle weight between control and diabetic rats and the ~ 25% decrease in the amount of glycoprotein

eluted from the wheat germ affinity column in the isolation of insulin receptors from diabetic rats as compared to controls.

The decrease in the ability of the diabetic derived insulin receptors to incorporate ^{32}P from $\gamma[^{32}P]ATP$ into its β-subunit and into exogenous substrates (angiotensin II or histone H_2b) was a consistent finding and does not appear to reflect biased estimates of insulin receptor number nor contaminating insulinase, phosphatase or ATPase activities. Note that cross-linking experiments revealed identical labelling of α-subunits in diabetic and control receptor preparations (see Fig. 7). The decrease in autophosphorylation was presumably not due to a decrease in the number of β-subunits in the diabetic derived wheat germ eluate since similar binding kinetics were observed and removal of a significant proportion of the insulin receptor β-subunits would likely result in a noticeable change in receptor affinity. The impairment of insulin stimulated exogenous kinase activity of diabetic derived receptors (Fig. 3) was more evident than that of the insulin activated autophosphorylation (Fig. 2). Preliminary data (41) suggest that phosphorylation of different tyrosyl-sites on the β-subunit may not be equally important for the activation of the exogenous kinase.

Our results are in essential agreement with those reported by Kadowaki et al. (36) who showed impaired autophosphorylation of the insulin receptors derived from livers of streptozotocin-diabetic rats and by the recent report of decreased autophosphorylation in skeletal muscle from rats rendered diabetic by gold thioglucose treatment (39). The latter report suggests that the defect in the insulin receptor kinase activity is secondary to the diabetic state and not due to insulinopenia per se since the rats in ref. 39 were hyperinsulinemic.

However, Amatruda et al. isolated hepatic plasma membranes from diabetic rats and found no defect in insulin stimulated receptor autophosphorylation (40). Whether or not the insulin receptor population which manifests diabetes related structural and functional alterations is actually present on the plasmalemma or represents an intracellular species requires further investigation.

The phosphopeptides generated by treating the isolated β-subunit with TPCK treated trypsin appeared identical in the diabetic and control muscle derived receptors. We consistently generated 10 or more phosphopeptides from immunoprecipitated, electroeluted β-subunits even after digesting for 72 hours. Previous studies utilizing high performance liquid chromatography to separate the products of trypsin digestion reported the isolation of 3-5 phosphopeptides (27,35). Whether the larger number observed in our system is due to incomplete digestion or to receptor heterogeneity or to the higher sensitivity of autoradiography after 2-dimensional peptide separation is not clear. Visual analysis of these maps failed to reveal salient differences in the relative phosphorylation of these fragments between controls and diabetics. The receptors used in generating these maps were phosphorylated for 10 min at 25°C in order to maximize ^{32}P incorporation for visualization of phosphopeptides. However, a more detailed, quantitative examination of the phosphopeptides separated from receptors autophosphorylated at lower temperatures or for shorter time periods at 25°C may reveal differences between the two populations.

The presence of a subpopulation of insulin receptor β-subunits in diabetic rat skeletal muscle with retarded migration on SDS-PAGE suggests that diabetes affects the processing of the insulin receptor in

muscle. We were able to detect this subpopulation of receptors in some rats 24 hrs. after streptozotocin injection and in most animals by 3 days. In some animals this slower migrating species comprised up to 50% of the phosphorylated β-subunit band detected by autoradiography. It was difficult to discern this altered population consistently on a 7.5% polyacrylamide gel; however it became distinct after limited proteolytic digestion of the insulin receptor with V8 protease. This is likely due to the inverse logarithmic relationship between molecular weight and relative mobility on isocratic polyacrylamide gels resulting in improved resolution of smaller proteins, as well as the increased relative preponderance of the component with decreased mobility in the fragments. The changes in the insulin receptor β-subunit structure appear to be in response to insulinopenia and/or resulting changes in the metabolic milieu rather than to toxic effects of streptozotocin since the slower migrating component was reduced or eliminated by 60 hrs of insulin treatment which normalized blood sugar. It is interesting to note that approximately 10% of control animals studied demonstrated a small amount (most less than 10%) of a higher molecular weight subpopulation.

The glycoprotein nature of the insulin receptor has been demonstrated by direct labeling of the sugar moiety (33,42) and indirectly by its ability to bind to lectins (43). Ronnett et al. (33) observed that the insulin receptor is synthesized as a single polypeptide chain, which is cleaved and subsequently glycosylated on both the α and β subunits. These intermediates are sensitive to endoglycosidase H and neuraminidase resistant. Salzman et al. (44) and Herzberg et al. (45) reported that the mature α and β subunits from 3T3-L1 adipocytes and IM-9 lymphocytes contain both endoglycosidase H sensitive and insensitive sugar side

chains. The latter report also showed that endoglycosidase F could not remove all the sugar side chains from the β-subunit but treatment with a chemical deglycosylating agent resulted in an additional decrease in molecular weight suggesting the presence of O-linked side chains. The insulin receptor β-subunit isolated here from skeletal muscle of control or diabetic rats appeared to be sensitive to digestion by endoglycosidase H; on autoradiography the 95 kD band migrated at ~ 89 kD after endoglycosidase H treatment, suggesting that the β-subunit isolated from skeletal muscle also contains 'high mannose' oligosaccharide side chains. The peptides generated after V8 digestion, especially the prominent 54kD band was also endoglycosidase H sensitive. The diabetic derived β-subunit population separated into two distinct bands after V8 protease and endoglycosidase H treatment, one that comigrated with the control and one with ~ 1.5 kD higher MW_{app} (Fig. 8b). The further improvement in the resolution of the diabetes induced β-subunit populations after endoglycosidase H treatment likely reflects the elimination of oligosaccharides which are known to affect protein migration on SDS-PAGE (46). Treatment of the immunoprecipitated, insulin receptor populations with neuraminidase increased the migration of the diabetic derived β-subunit more than that of the control, especially that of the prominent 54 kD peptide fragment (Fig. 9). This suggested that the heterogeneity of the diabetic derived β-subunit was due in part to an increase in its sialic acid content. When V8 digested insulin receptors were treated with neuraminidase followed by endoglycosidase H, the slower migrating component of the ~ 54 kD fragment (seen in diabetic derived β-subunits) appeared to decrease in molecular weight while the 'control band' did not change its migratory

behavior (Fig. 10). This suggests that there may be an additional sugar side chain on some diabetic derived β-subunits or an alteration in the structure of one or more normally occuring oligosaccharide side chains. We encountered similar difficulties as Herzberg et al. (45) in attempting to completely remove the oligosaccharide side chains from the β-subunit with Endoglycosidase F or glycanase (broad spectrum N-linked glycosidases) suggesting that the β-subunit may have O-linked sugar side chains.

Streptozotocin diabetes results in decreased sialic acid and other carbohydrate content of major glycoproteins on hepatocyte membranes (47) as well as decreased activity of certain glycosyltransferase enzymes, without a detectable change in sialyltransferase activity (48). The insulin receptor however is spared from the diabetes associated decline in hepatic membrane glycoproteins, since hepatic insulin binding and receptor number appear to increase in insulinopenic diabetes (13,36). The mechanism for the selectivity in hepatic glycoprotein synthesis or degradation is not understood; to our knowledge there is no information concerning the effect of diabetes on glycoprotein turnover or sialyltransferase activity in muscle. We did however see in diabetic rats a decline in the amount of glycoprotein per gram muscle eluted from the wheat germ columns in the insulin receptor purification protocol.

In our hands, there appeared to be a relationship between the appearance of the β-subunit population with decreased electrophoretic mobility and changes in tyrosyl-kinase activity, whenever one phenomenon was detected, so was the other. It is tempting to speculate that the metabolic alterations associated with insulin deficiency result in conformational changes in the receptor that renders the coupling between

insulin binding and autophosphorylation less efficient. The precise relationship, if any, between the observed alteration in the kinase kinetics in the diabetic derived insulin receptor and the structural heterogeneity of the β-subunit remains to be defined. Yamaguchi et al. (49) recently reported that neuraminidase treatment of highly purified placental insulin receptors increased insulin binding and tyrosine kinase activity. This observation suggests that alterations in sugar side chains may indeed modulate insulin-receptor function.

The insulin resistance which accompanies poorly controlled insulinopenic diabetes involves both the liver and peripheral tissues; skeletal muscle has been implicated as the primary site (4). The work of Kadowaki in liver (36) and the present work in skeletal muscle suggest that diabetes induced changes in the β-subunit of the insulin receptor may play a role in this insulin resistance. In recent years there has been increasing awareness that insulin resistance associated with different pathological conditions is caused to variable degrees by a defect in the binding of insulin to its targets, or to defects which occur after insulin binding. The latter abnormalities are generally referred to as post-receptor defects, with the tacit implication that they are distal to the receptor, involve the intracellular metabolic milieu, second messengers or their intracellular targets. Our data as well as those of others (36,39), including a rare genetic defect of the insulin receptor (50,51) suggest that post-binding defects may include "intra-receptor" defects, resulting in defective signalling by the receptor in response to hormone binding.

Figure 1 Scatchard analysis of ^{125}I-insulin binding to solubilized, wheat germ agglutinin purified insulin receptors. Wheat germ agglutinin eluate (50 µl) was incubated for 16h at 4°C in a solution containing 25 mm Hepes, 0.1% Triton X-100, 150 mM NaCl, 0.1 mg/ml bovine serum albumin and 100 U/ml bacitracin with ^{125}I-insulin (10,000 cpm; 125-175 µCi/µg) and increasing concentrations of unlabeled insulin in a final volume of 200 µl. The receptor and bound insulin were precipitated by sequential addition of 100 µl 0.3% bovine γ-globulin and 300 µl 25% polyethyleneglycol. Non-specific binding was estimated as the radioactivity precipitated in the presence of 10,000 ng/ml unlabeled insulin. Specific binding was normalized to protein concentration in the wheat germ eluate estimated by the Bradford reaction (26). Control (0-0), diabetic (□-□) and diabetic-treated (Δ-Δ) muscle derived receptor population. Points are means of 3-5 determinations from two separate experiments. SEM of pmole bound/mg protein ranged from 7-17% of the mean.

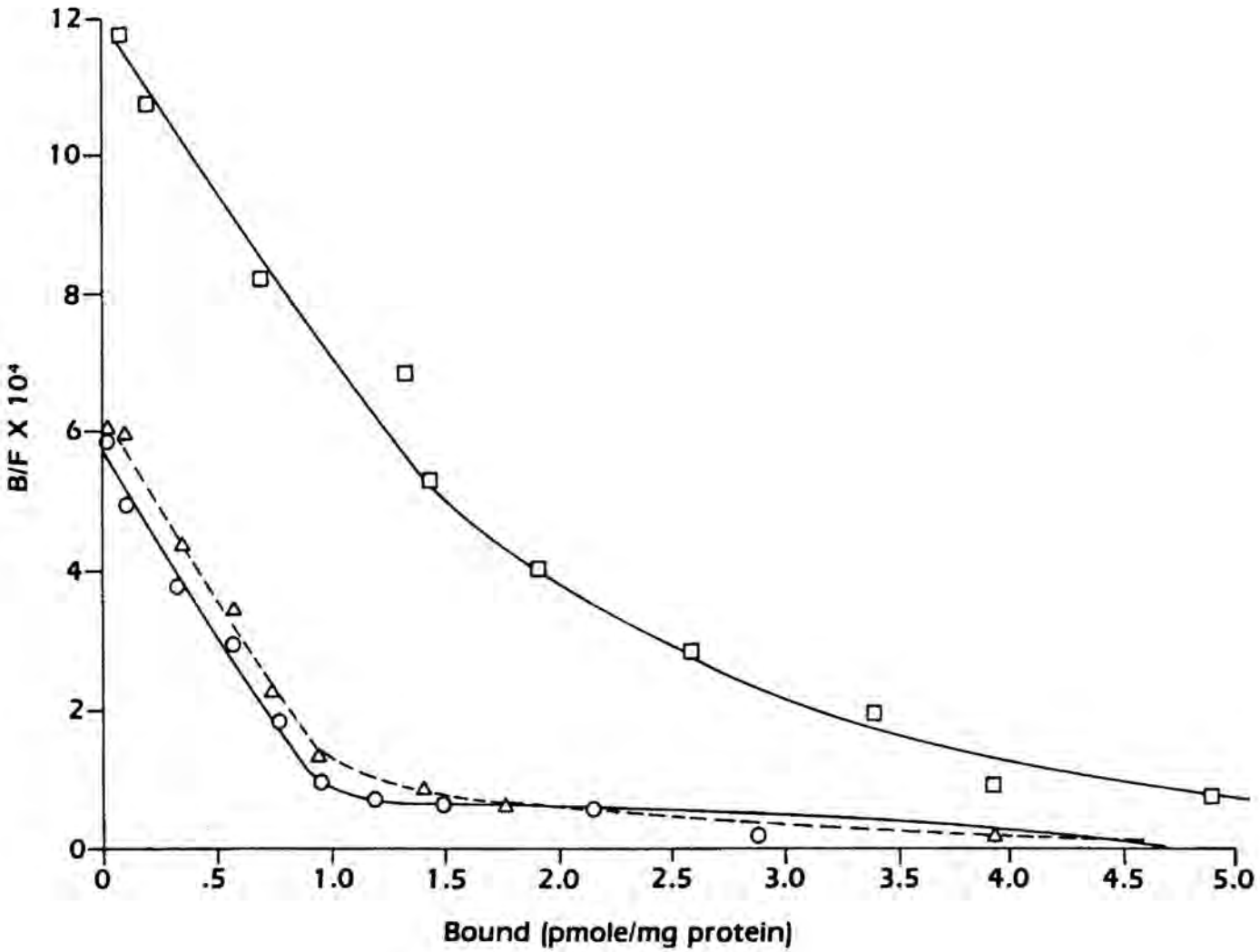

12
10
8
6
4
2
0
B/F X 10⁴
0
.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
Bound (pmole/mg protein)

Figure 2 Dose-response of insulin-stimulated autophosphorylation of the β-subunit of solubilized insulin receptors. Wheat germ eluate (50-70 µl) from control derived receptors (O-O) and equal binding activities from diabetic (□-□) and insulin treated diabetic rats (Δ-Δ) were incubated for 45 min in 25 mM Hepes, pH 7.4, 0.1% Triton X-100, 0.05% bovine serum albumin, 0.15-0.3M N-actylglucosamine and 5 mM $MnCl_2$ at 25°C without or in the presence of 10^{-10} to 10^{-6} M insulin in a final volume of 75 µl. After cooling the vials to 4°C, autophosphorylation was initiated by the addition of $\gamma[^{32}P]$ ATP (100 µM, 15 µCi/vial) and the reaction continued for 30 min at 4°C and terminated by the addition of 4X concentrated Laemmli's sample buffer containing 5 mM 2-mercaptoethanol and heated for 3 min at 100°C. The samples were analysed by 7.5% SDS-PAGE, the β-subunit was localized by autoradiography, excised, digested in 1 ml 30% H_2O_2 and counted in a liquid scintillation counter. Values are means for 5 separate preparations from control and diabetic rats respectively and 3 insulin treated diabetics; preparations were assayed in triplicate in each group. All points in the diabetic derived preparation are different from control, $p < 0.05$; SEM are 7-12% of the means.

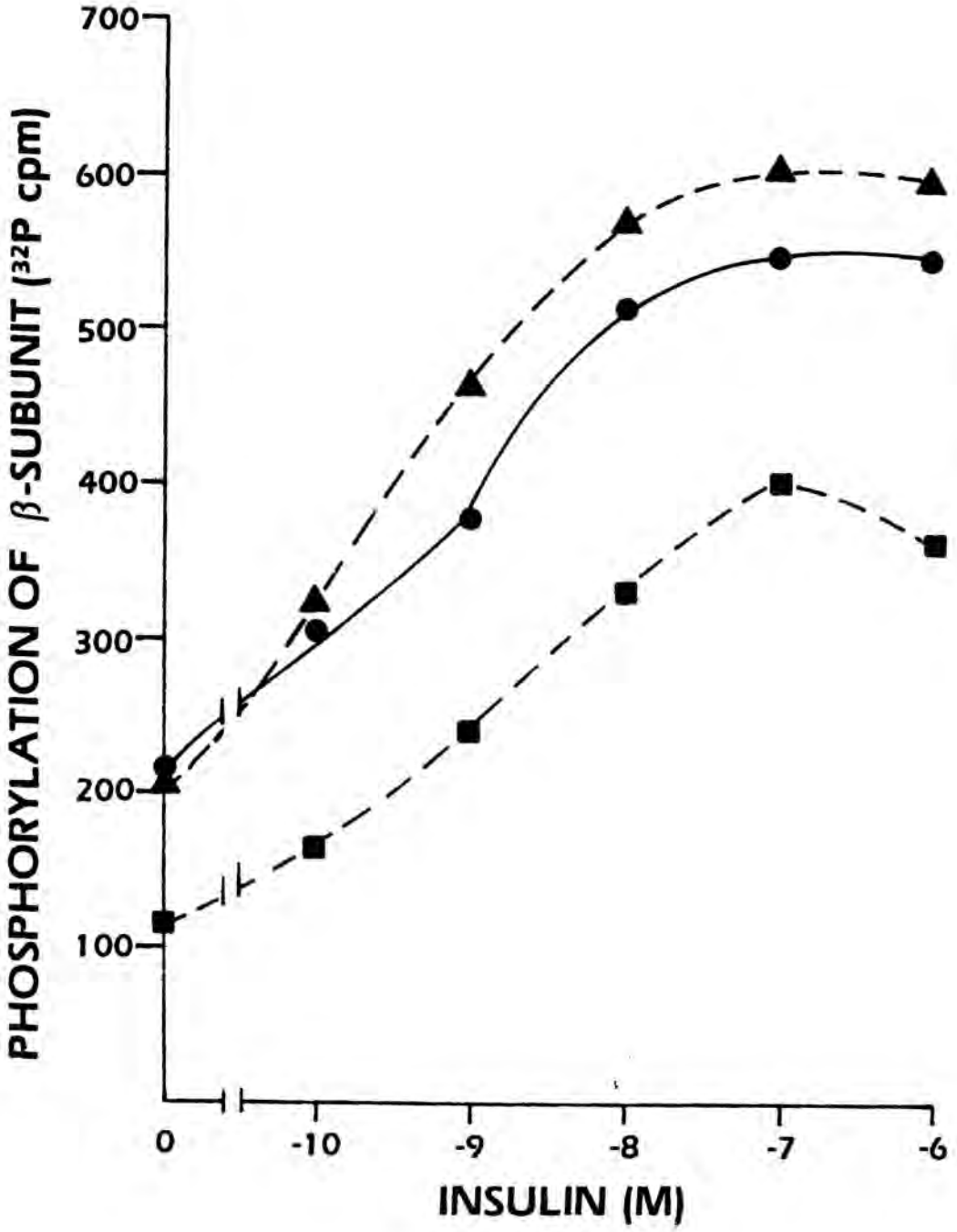

700
600
500
400
300
200
100
PHOSPHORYLATION OF β-SUBUNIT (32P cpm)
0
-10
-9
-8
-7
-6
INSULIN (M)

Figure 3 Angiotensin II phosphorylation by skeletal muscle derived insulin receptors. Wheat germ agglutinin eluates were preincubated with 0, 10^{-9} or 10^{-7} M insulin for 45 min at 25°C and then allowed to autophosphorylate for 30 min at 4°C in the presence of 100 μM $\gamma[^{32}P]$ATP. Aliquots (35 μl) were added to 5 μl of buffer or Angiotensin II (2 mM final conc.) and the reaction continued for an additional 5 min when 50 μl 5% TCA were added. After cooling on ice for 30 min the protein precipitate was removed by centrifugation and duplicate 40 μl aliquots were spotted onto phosphocellulose paper. After washing, the papers were counted in a liquid scintillation counter. Equal binding activities were assayed in each preparation. ( ), control; ( ), diabetic, ( ), diabetic-treated. Means ± S.E.M. for 3-5 different receptor preparations are shown, each assayed in triplicate. *, significantly different from control, $p < 0.05$.

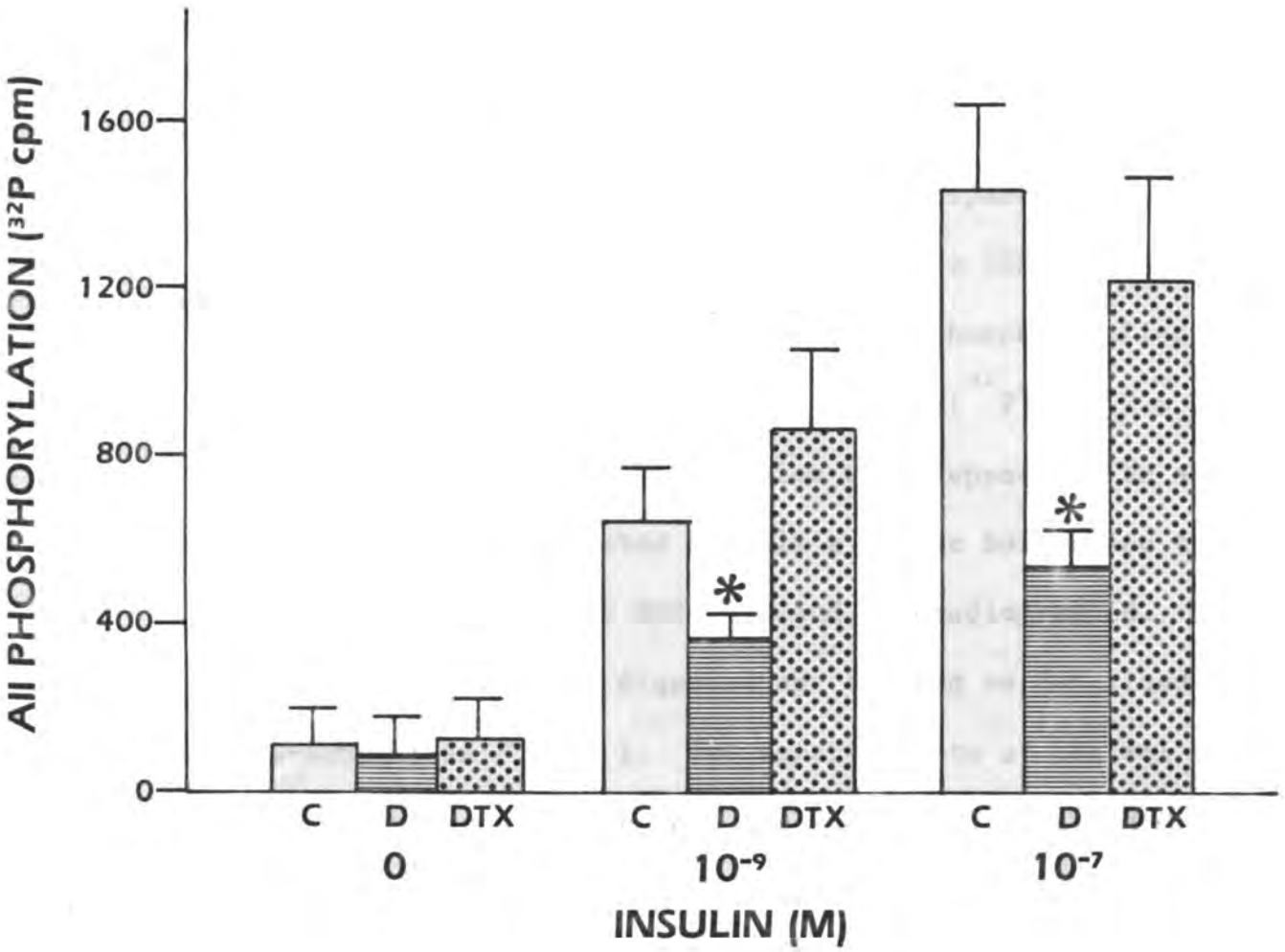

AII PHOSPHORYLATION (^{32}P cpm)
1600
1200
800
400
0
C
D
DTX
0
10^{-9}
10^{-7}
INSULIN (M)
*
*

Figure 4 Autoradiogram of Histone H_2b phosphorylated by skeletal muscle derived insulin receptors. Wheat germ eluates [illegible] µl from controls and equal binding activity from diabetics were incubated with insulin-agarose conjugates for 60 min at 25°C as described in "Methods". After washing, the insulin-agarose bound receptors were allowed to autophosphorylate in the presence of 750 µM ATP, 5 mM $MnCl_2$ and 0.025% BSA for 10 min at 25°C, then washed and incubated in phosphorylation buffer with 1 mg/ml of histone H_2b and 50 µM $\gamma[^{32}P]$ATP (2.5 µCi/vial) at 25°C for 4 min. The reaction was stopped by the addition of 4 times concentrated Laemmli's sample buffer and the samples separated by SDS-PAGE and autoradiographed. Histone bands were excised, digested and counted as described for the β-subunit in Figure 2. Two separate sets of control and diabetic derived receptors are shown. Lanes A and C: H_2b phosphorylated by control receptors; lanes B and D: H_2b phosphorylated by diabetic derived receptors. Arrow indicates the position of H_2b. Inset: Mean ± S.E.M. of ^{32}P cpm incorporated into histone-H_2b from 4 different control and 4 diabetic derived insulin receptor preparations (all with equal binding activity) each assayed in triplicate.

A B C D

cpm into Histone H_2b

Control: 1498± 28 (n=4)

Diabetic: 878± 37 (n=4)

Figure 5 Phosphoaminoacid analysis of the β-subunit of skeletal muscle insulin receptors. Wheat germ purified insulin receptors from control and diabetic animals were incubated with 10^{-7}M insulin as described in Figure 2. γ[^{32}P]ATP (25μM, 10 μCi/vial) was added for 10 min at 25°C and the reaction stopped with 20 μl 25 mM Hepes, 0.1% Triton, 40 mM ATP, 10 mM sodium pyrophosphate and 2 mM PMSF, then anti-insulin receptor antibodies (1:400 dilution) were added and samples incubated overnight at 4°C. The immune complex was precipitated by the addition of Pansorbin, washed 3 times and boiled in Laemmli's sample buffer. The phosphorylated β-subunit was eluted from the wet, unfixed gel as described in Methods, precipitated with 20% TCA and hydrolysed for 1h at 110°C in 6N HCl. The hydrolysate was adsorbed to Dowex 1-X8, the phosphoaminoacids eluted with 0.25N HCl and lyophilized. After addition of mixed phosphoaminoacid standards, the mixture was spotted on a TLC plate and electrophoresed for 45 min in pyridine: acetic acid: H_2O (5:15:950) pH 3.5. The phosphoaminoacid standards were localized by ninhydrin and ^{32}P by autoradiography. The autoradiogram presented shows control derived receptors (C) and diabetic derived receptors (D). The interrupted lines indicate the location of phosphoserine (P-ser), phosphothreonine (P-thr) and phosphotyrosine (P-tyr) standards. A small amount of phosphoserine was detected on the autoradiogram but did not reproduce on the photograph.

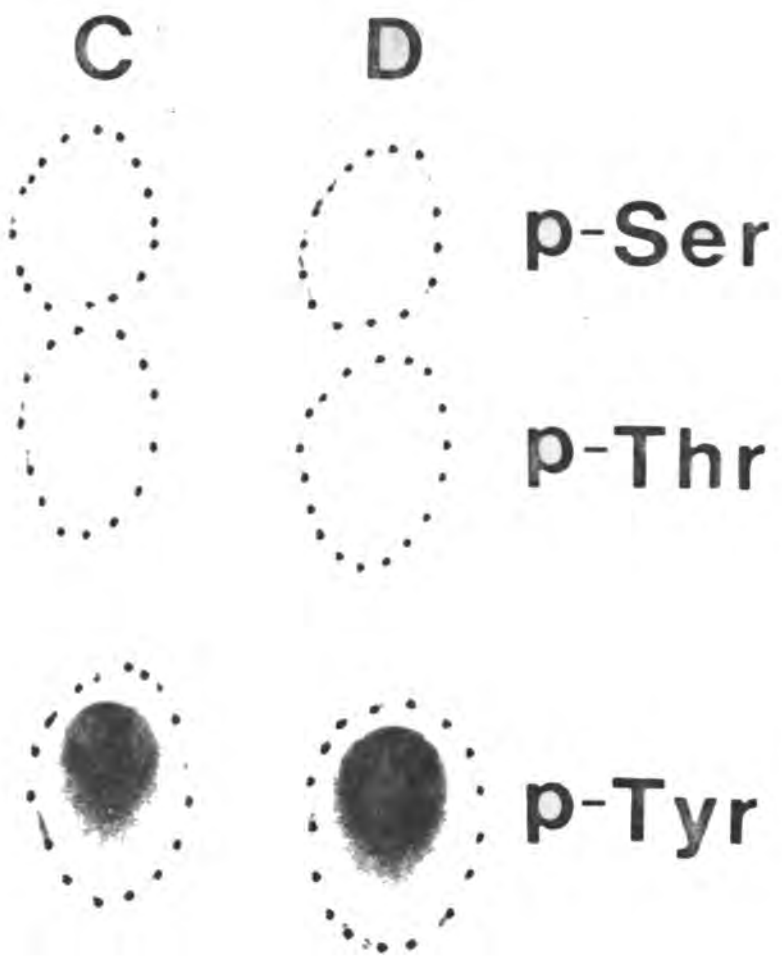

C
D
p-Ser
p-Thr
p-Tyr

Figure 6 Two-dimensional phosphopeptide analysis and autoradiography of insulin receptor β-subunit. Insulin receptors from control (A) and diabetic (B) animals were phosphorylated, immunoprecipitated, eluted and TCA precipitated as described in Figure 5. The precipitated protein was washed with ether/ethanol (1:1), oxidized with formic acid/H_2O_2 (9:1) and digested with TPCK treated trypsin in 50 mM $(NH_4)_2CO_3$ pH 8 for 24 hrs. After removal of $(NH_4)_2CO^3$ by lyophilization the hydrolysate was spotted on 20 x 20 cm TLC plates, electrophoresed for 40 min. in the first dimension in 1% $(NH_4)^2CO_3$ pH 8.9, and ascended chromatographically in the second dimension with N-butanol: pyridine: acetic acid: H_2O (30:15:7:37.5). The dried plate was then autoradiographed for 4 days at -80°C with intensifying screens.

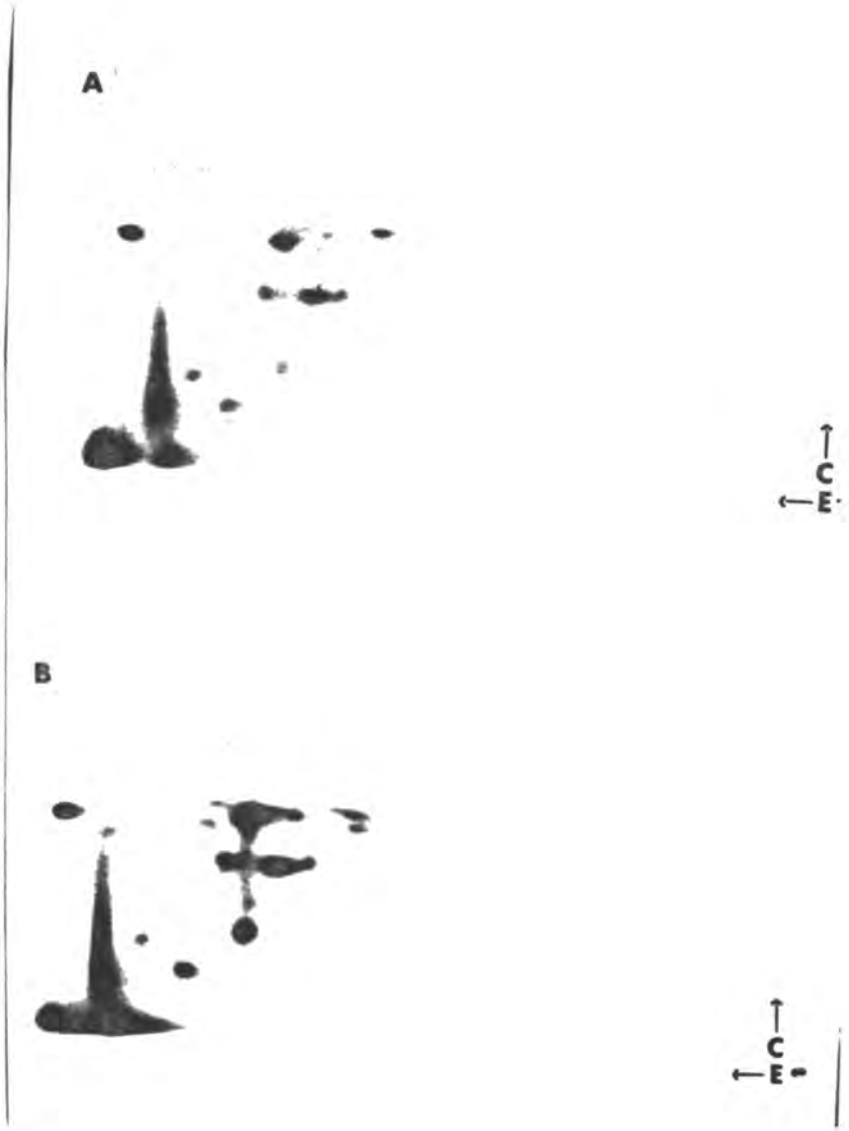

A
C
E
B
C
E

Figure 7 Structural analysis of the insulin receptor α-subunit. Control and diabetic derived insulin receptors were dialysed against 50 mM Hepes, pH 7.8, concentrated with "Aquacide" and equal binding activities incubated for 16 hrs with 0.5 μCi A14[^{125}I]insulin with or without 10 μg unlabeled insulin at 4°C. Disuccinimidyl suberate was added in DMSO to a final concentration of 2 mM and incubated for an additional 15 min when 4X concentrated Laemmli's sample buffer with 5 mM 2-mercaptoethanol were added. The samples were separated on 6% SDS-PAGE and the dried gel autoradiographed. Lane A, control derived receptors; lane B, diabetic derived receptors, lane C and D control and diabetic derived receptors crosslinked in the presence of 10 μg/ml unlabeled insulin.

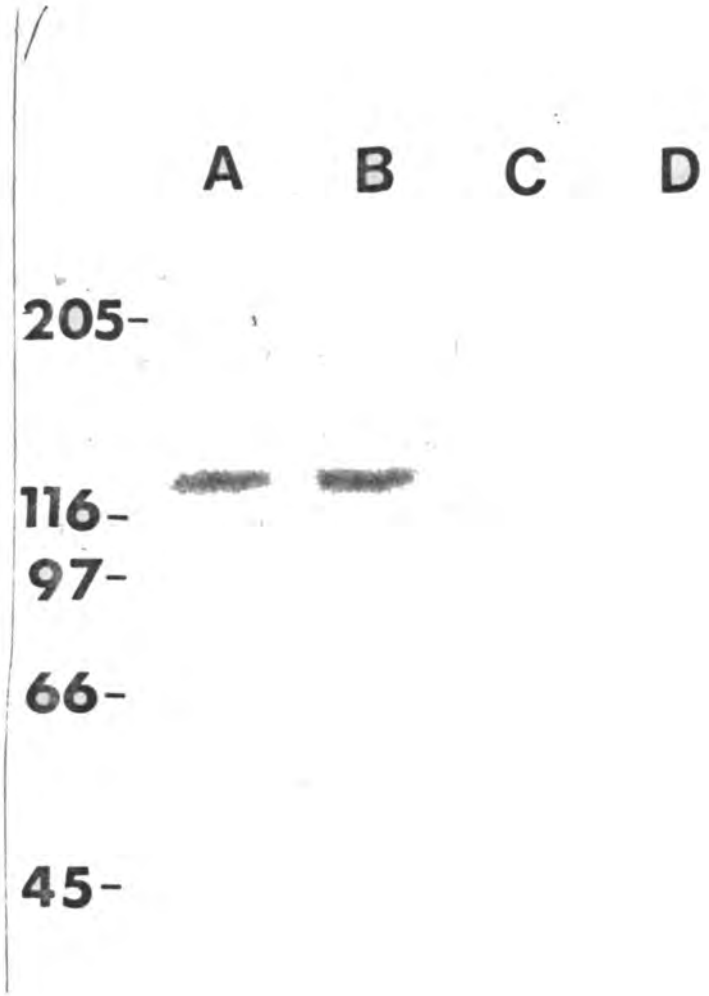

A
B
C
D
205-
116-
97-
66-
45-

Figure 8 Structural analysis of insulin receptor β-subunit. Wheat germ eluates with approximately equal insulin binding activities, prepared from muscles of control, diabetic and diabetic treated-rats were incubated with 10^{-7}M insulin for 45 min and autophosphorylated for 10 min in the presence of 10 μM γ[^{32}P]ATP, (~ 15 μCi/100 μl). Receptors were immunoprecipitated with anti-insulin receptor antibody and Pansorbin. The precipitates were either boiled in Laemmli's sample buffer and analyzed by 7.5% SDS-PAGE under reducing conditions or first digested for 5 min at 37°C in 50 mM sodium acetate buffer pH 5 with 75 μg/ml S. Aureus V8 protease and then analyzed by 9% SDS-PAGE under reducing conditions. Dried, fixed gels were autoradiographed with intensifying screens at -80°C for 12-24 hrs. Lane A represents control, B diabetic derived receptors without protease digestion. Lanes C, D, and E, represent receptors derived from control, diabetic and insulin-treated diabetic rats respectively, with prior V8 protease digestion. Lanes F, G and H represent β-subunits from control, diabetic and diabetic-treated respectively, that were isolated from a 7.5% gel by electroelution and subsequently digested with V8 as described in the text. Lane I represents control and J, diabetic derived receptors after V8 protease digestion, and short autoradiographic exposure.

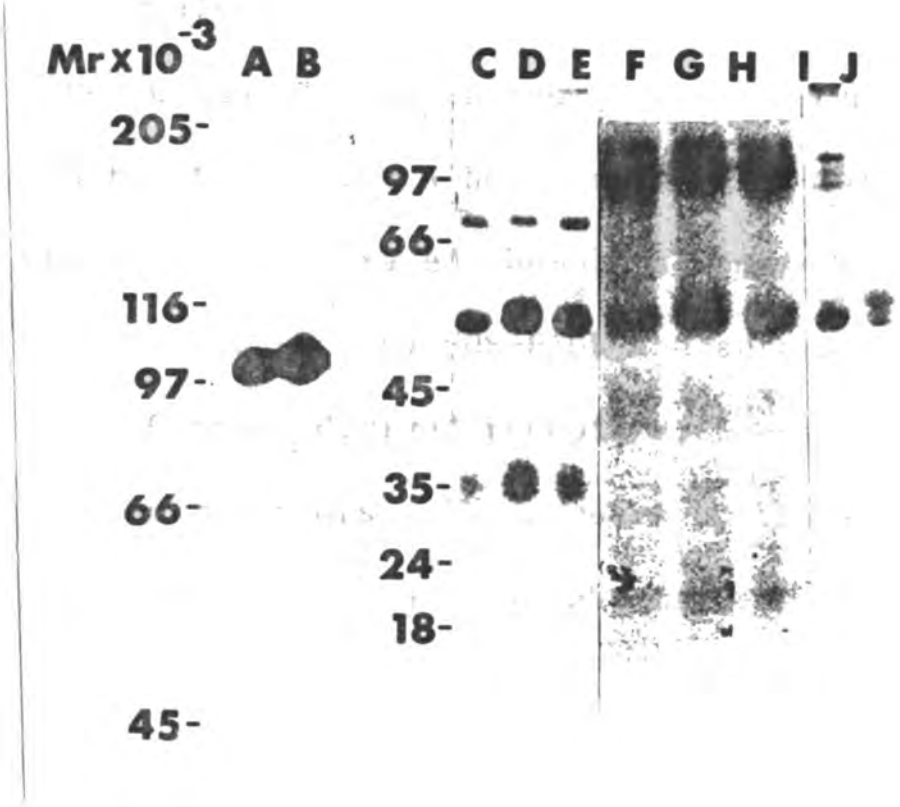

Mr x10-3
A B
C D E F G H I J
205-
116-
97-
66-
45-
97-
66-
45-
35-
24-
18-

Figure 9 Glycosidase digestion of phosphorylated, immunoprecipitated insulin receptors. Autophosphorylated insulin receptors were immunoprecipitated and incubated for [illegible] without (9a) or with (9b) V8 protease as described in Figure 8. After addition of protease inhibitors samples were incubated with buffer alone (Lanes A and B) or with neuraminidase (40 mU/ml, Lanes C and D) or with Endoglycosidase H (30 mU/ml, Lanes E and F) at 37°C for 4 hours as detailed in "Methods" after which 4X concentrated Laemmli's sample buffer was added, the samples analysed by SDS-PAGE and autoradiographed. Lanes A, C, E: Control derived receptors; Lanes B, D, F: Diabetic derived receptors. The numbers on the left indicate the positions of molecular weight markers.

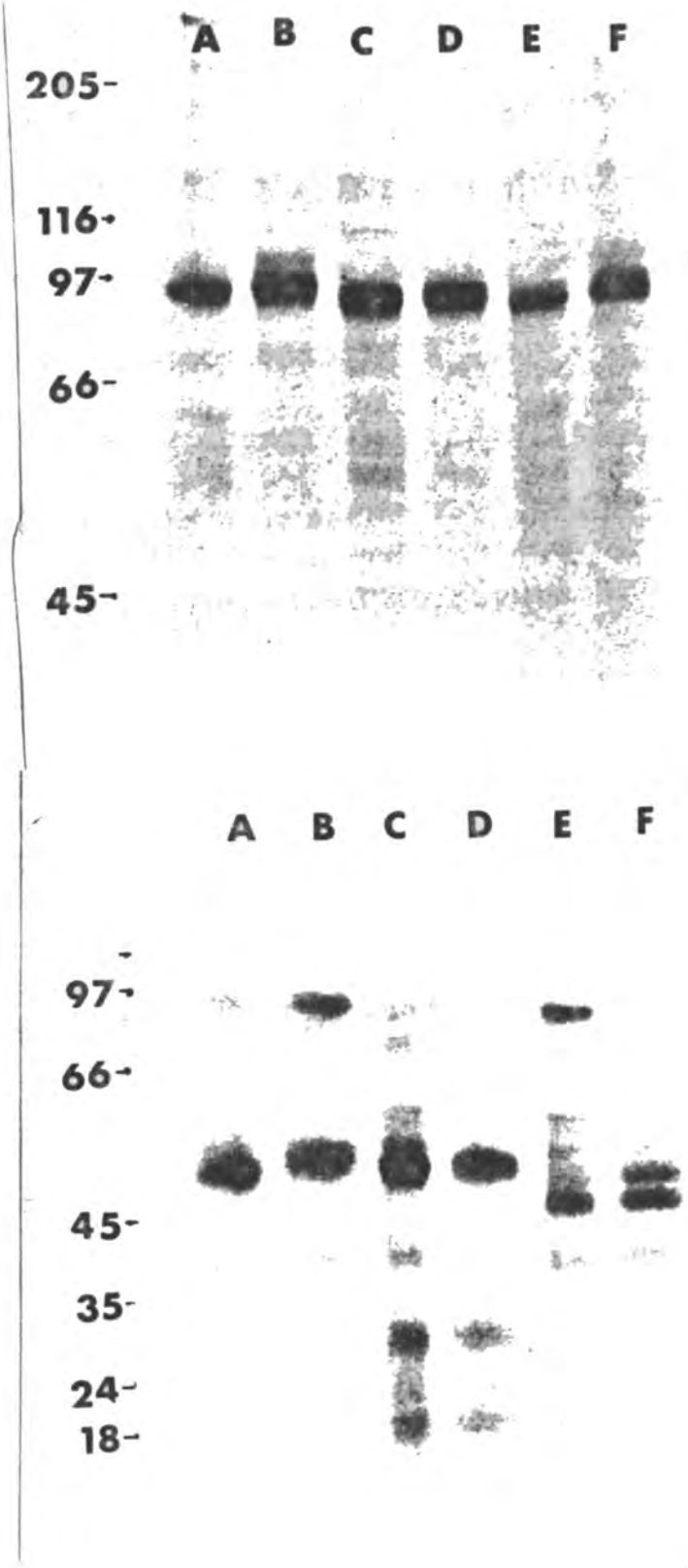
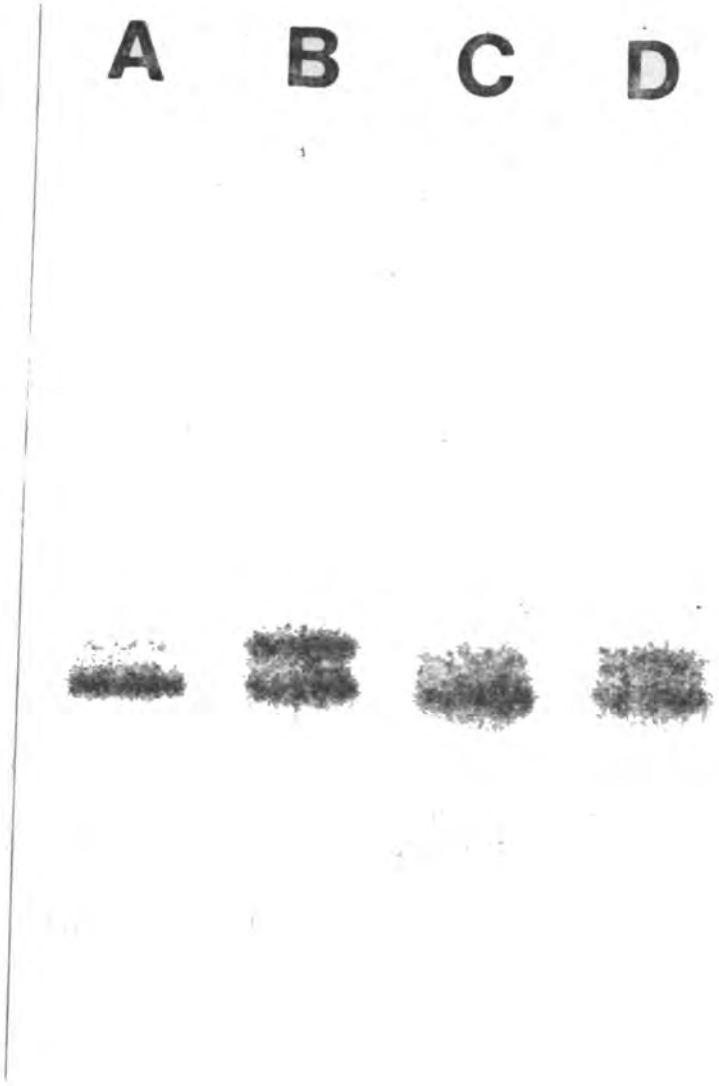

A B C D E F
205-
116-
97-
66-
45-
A B C D E F
97-
66-
45-
35-
24-
18-

Figure 10 Sequential neuraminidase and endoglycosidase H treatment of V8 digested insulin receptors. Insulin receptors were immunoprecipitated and digested with V8 protease as described in Figure 8. After addition of protease inhibitors and incubation with 40 mU/ml neuraminidase for 2 hrs at 37°C samples were placed in boiling water for 2 min. After cooling, endoglycosidase H (final concentration 30 mU/ml) was added in 4X concentrated endoglycosidase H buffer (1/3 vol.) and the sample incubated for an additional 2 hrs at 37°C, then processed as described in Figure 9. The major (MW_{app} 54 Kd) phosphopeptide generated by V8 protease digestion is shown. Lane A: Control, endoglycosidase H treated; Lane B: Diabetic, endoglycosidase H treated; Lane C: Control, treated sequentially with neuraminidase and endoglycosidase H; Lane D: Diabetic, sequentially treated with neuraminidase and endoglycosidase H.


A
B
C
D

REFERENCES

1. Himsworth, H.P. and R.B. Kerr. "Insulin-sensitive and Insulin Insensitive Types of Diabetes Mellitus." Clin. Sci. 4;119-152, 1939.

2. Martin, F.I.R. and A.E. Stocks. "Insulin-sensitivity and ^{131}I-metabolism in Juvenile-type Diabetes." Aust. Ann. Med. 16; 289-296, 1961.

3. Harano, Y., S. Ohgaku, H. Hidaka, K. Hareda, R.K. Kikkawa, Y. Shigeta and H. Abe. "Glucose, Insulin and Somatostatin Infusion for the Determination of Insulin Sensitivity." J. Clin. Endocrinol. Metab. 45;1124-1127, 1977.

4. DeFronzo, R.A., R. Hendler and D.Simonson. "Insulin-resistance is a Prominent Feature of Insulin-dependent Diabetes." Diabetes 31;795-801, 1983.

5. Mayfield, R.K., F.M. Sullivan, J.A. Colwell and H.J. Wohltmann. "Predicting Insulin Requirements for a Portable Insulin Pump Using the Biostator." Diabetes 32;908-914, 1983.

6. Foss, M.C., F.V. Vlachokosta, L.N. Cunningham and T.T. Aoki. "Restoration of Glucose Homeostasis in Insulin-dependent Diabetic Subjects: An Inducible Process." Diabetes 31;46-52, 1982.

7. Sherwin, R.S., W.V. Tamborlane, M. Genel and P. Felig. 1980. "Treatment of Kuvenile-onset Diabetes by Subcutaneous Infusion of Insulin with a Portable Pump." Diabetes Care 3;301-308, 1980.

8. Reaven, G.M., N.S. Sageman and R.S. Swenson. 1977. "Development of Insulin Resistance in Normal Dogs Following Alloxan-induced Insulin Deficiency." Diabetologia 13;459-462, 1977.

9. Kasuga, M. Y. Akanuma, Y, Iwamoto, K. Kosaka. "Insulin Binding and Glucose Metabolism in Adipocytes from Streptozotocin-Diabetic Rats. Am. J. Physiol. 235;E175-E182, 1978.

10. Kobayashi, M. and J.M. Olefsky. "Effects of Streptozotocin-Induced Diabetes on Insulin Binding, Glucose Transport, and IntraCellular Glucose Metabolism in Isolated Rat Adipocytes." Diabetes 28;87-95, 1979.

11. Kaenieki, E., P.J. Hissin, I.A. Simpson, L.B. Salans and S.W. Cushmann. "A Possible Mechanism of Insulin Resistance in the Rat Adipose Cell in Streptozotocin-induced Diabetes Mellitus." J. Clin. Invest. 68;811-814, 1981.

12. Haft, D.E. "Studies of the Metabolism of Livers of Normal and Alloxan-diabetic Rats Perfused with Insulin." Diabetes 17;244-250, 1968.

13. LeMarchand, Y., E.G. Loten, F. Assimacopoulos-Jeannet, M.E. Forgue, P. Freychet and B. Jeanrenaud. "Effects of Fasting and Streptozotocin on the Obese-hyperglycemic (ob/ob) Mouse." Diabetes 26;582-590, 1976.

14. Kipnis, D.M. and C.F. Cori. "Studies of Tissue Permeability. V. The Penetration and Phosphorylation of 2-deoxyglucose in the Rat Diaphragm." J. Biol. Chem. 234;171-177, 1959.

15. Morgan, H.E., E. Cadenas, D.M. Regen and C.R. Park. 1961. "Regulation of Glucose Uptake in Muscle. II. Rate Limiting Steps and Effects of Insulin and Anoxia in Heart Muscle from Diabetic Rats." J. Biol. Chem. 236;262-268, 1961.

16. Kasuga, M., F.A. Karlsson and C.R. Kahn. "Insulin Stimulates the Phosphorylation of the 95,000 Dalton Subunit of the Insulin Receptor." Science 215;185-187, 1982.

17. Kasuga, M., Y. Zick, D.C. Blithe, F.A. Karlsson, H. Haring and C.R. Kahn. "Insulin Stimulates the Phosphorylation of the β-subunit of the Insulin Receptor." J. Biol. Chem. 257;9891-9894, 1982.

18. Rosen, O.M., R. Herrera, Y. Olowe, L.M. Petruzzelli and M.H. Cobb. "Phosphorylation Activates the Insulin Receptor Tyrosine Protein Kinase." Proc. Natl. Acad. Sci. USA 80;3237-3240, 1983.

19. Avruch, J., R.A. Nemenoff, P.J. Blackshear, M.W. Pierce and R. Osathanondh. "Insulin Stimulates Tyrosine Phosphorylation of the Insulin Receptor in Detergent Extracts of Human Placental Membranes." J. Biol. Chem. 257;15162-15166, 1982.

20. Van Obberghen, E., B. Rossi, A. Kowalski, H. Gazzano, and C. Ponzio. "Receptor-mediated Phosphorylation of the Hepatic Insulin Receptor: Evidence that the MV 95000 Receptor Subunit is its own Kinase." Proc. Natl. Acad. Sci. USA 80;945-949, 1983.

21. Zick, Y., J. Wittaker and J. Roth. "The Insulin Receptor is Coupled to Tyrosine Kinase Activity." J. Biol. Chem. 259;3431-3434, 1983.

22. Kasuga, M., Y. Fijita-Yamaguchi, D.C. Glithe and C.R. Kahn. 1983. "Tyrosine-specific Proten Kinase Activity is Associated with the Purified Insulin Receptor." Proc. Natl. Acad. Sci. USA 80;2137-2141, 1983.

23. Burant, C.F., M.K. Treutelaar, G.E. Landreth and M.G. Buse. "Phosphorylation of the insulin receptor solubilized from rat Skeletal Muscle." Diabetes 33;704-708, 1984.

24. Galbraith, R.A., C. Wise and M.G. Buse. "Insulin Binding and Response in Erythroblastic Leukemic Cells." Diabetes 29;571-578, 1980.

25. Taylor, S.E., J.A. Hedo, L.H. Underhill, M. Kasuga, M.J. Elders, and J. Roth. "Extreme Insulin Resistance in Association with Abnormally High Binding Affinity of Insulin Receptors from a Patient with Leprechaunism: Evidence for a Defect Intrinsic to the Receptor." J. Clin. Endocrinol. Metab. 55;1108-1113, 1982.

26. Bradford, M.M. "A Rapid and Sensitive Method for Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-binding." Anal. Biochem. 72;248-254, 1976.

27. Yu, K-T and M.P. Czech. "Tyrosine Phosphorylation of the Insulin Receptor β-subunit Activates the Receptor-associated Tyrosine Kinase Activity." J. Biol. Chem. 259;5277-5286, 1984.

28. Folley, L.S., S.D. Poner and R.O. Poyton. "Separation of Nucleotides by Ion Pair, Reverse-phase High-performance Liquid Chromatography." J. Chromatography 281;199-207, 1983.

29. Pilch, P.F. and M.P. Czech. "Interaction of Crosslinking Agents with the Insulin Effector System of Isolated Fat Cells." J. Biol. Chem. 254;3375-3381, 1979.

30. Cooper, J.A., B.M. Sefton and T. Hunter. "Detection and Quantitation of Phosphotyrosine in Proteins." Meth. Enzymol. 99;387-403, 1983.

31. Beeman, K. and T. Hunter. "Characterization of Rous Sarcoma Virus Src Gene Products Synthesized in vitro." J. Virol. 28;551-566, 1978.

32. Carlin, C.R. and B.B. Knowles. "Biosynthesis of Epidermal Growth Factor Receptor in Human Epidermoid Carcinoma Derived A431 Cells." J. Biol. Chem. 259;7902-7908, 1984.

33. G.V. Ronnett, V.P. Knutson, R.A. Kohanski, T.C. Simpson and M.D. Lane. "Role of Glycosylation in the Processing of Newly Translated Insulin Proreceptor in 3T3-L1 Adipocytes." J. Biol. Chem. 259;4566-4575, 1984.

34. Laemmli, V.K. "Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. Nature (Lond.) 227;571-78, 1970.

35. White, M.F., H.U. Haring, M. Kasuga and C.R. Kahn. "Kinetic Properties and Sites of Autophosphorylation of the Partially Purified Insulin Receptor from Hepatoma Cells." J. Biol. Chem. 259;255-264, 1984.

36. Kadowaki, T., M. Kasuga, Y. Akanuma, O. Ezaki and F. Takaku. "Decreased Autophosphorylation of the Insulin Receptor Kinase in Streptozotocin-diabetic Rats." J. Biol. Chem. 259;14208-14216, 1984.

37. Okamoto, M., H. Kuzuya and H. Imura. "Increased Affinity of Insulin Receptors on Hepatocytes from Streptozotocin-induced Diabetic Rats." Endocrinol. Japon. 31;235-243, 1984.

38. LeMarchand-Brustel, Y. and P. Freychet. "Effect of Fasting and Streptozotocin Diabetes on Insulin Binding and Action in the Isolated Mouse Soleus Muscle." J. Clin. Inv. 64;1505-1515, 1979.

39. LeMarchand-Brustel, Y., T. Gremeaux, R. Ballotte and E. Van Obberghen. "Insulin Receptor Tyrosine Kinase is Defective in Skeletal Muscle of Insulin-resistant Obese Mice." Nature 315;676-679, 1985.

40. Amatruda, J.M. and A.M. Roncone. "Normal Hepatic Insulin Receptor Autophosphorylation in Non-ketotic Diabetes Mellitus." Biochem. Biophys. Res. Comm. 129;163-170, 1985.

41. Goren, H.J. and M.F. White. "Mapping Domains of the β-subunit of the Insulin Receptor" (Abstract). Diabetes 34 (Suppl 1):55A.

42. Hedo, J.A., M. Kasuga, G. VanObberghen, J. Roth and C.R. Kahn. "Direct Demonstration of Glycosylation of Insulin Receptor Subunits by Biosynthetic and External Labelling: Evidence for Heterogeneity." Proc. Natl. Acad. Sci. USA 78;4291-4795, 1981.

43. Hedo, J., L.C. Harrison and J. Roth. "Binding of Insulin Receptors to Lectins. Evidence for Common Carbohydrate Determinants on Several Membrane Receptors." Biochemistry 20;3385-3393, 1981.

44. Salzman, H., C.F. Wan, C.S. Rubin. "Biogenesis, Transit and Functional Properties of the Insulin Proreceptor and Modified Insulin Receptors in 3T3-Ll Adipocytes. Use of Monensin to Probe Proreceptor Cleavage and Generate Altered Receptor Subunits." Biochemistry 23; 6555-6565, 1984.

45. Herzberg, V.L., F. Grigorescu, A.S.B. Edge, R. Spiro and C.R. Kahn. "Characterization of Insulin Receptor Carbohydrate by Comparison of Chemical and Enzymatic Deglycosylation." Biochem. Biophys. Res. Comm. 129;789-796, 1985.

46. Bettelheim, F.A. "Molecular Weight Determination and Physical Properties." In: The Glycoconjugates, edited by M.I. Horowitz and W. Pigman. New York: Academic Press, 111-128, 1977.

47. Carter, J.R. and V. Chandramouli. "Cell Surface Changes in Diabetes." In: Diabetes Mellitus, edited by M. Brownlee. New York: Garland STPM press, 277-286, 1981.

48. Tepperman, H.M., J. DeWitt, and J. Tepperman. "The Effects of Streptozotocin Diabetes on the Activities of Rat Liver Glycosyltransferases." Diabetes 32;412-415, 1977.

49. Yamaguchi, Y.F., Y. Sato and S. Kathuria. "Removal of Sialic Acids from the Purified Insulin Receptor Results in Enhanced Insulin Binding and Kinase Activities." Biochem. Biophys. Res. Comm. 129;739-745, 1985.

50. Grunberger, G., Y. Zick, P. Gordon. "Defect in Phosphorylation of Insulin Receptors in Cells from an Insulin Resistant Patient with Normal Insulin Binding." Science 223;932-934, 1984.

51. Grigorescu, F., J.S. Flier and C.R. Kahn. "Defect in Insulin Receptor Phosphorylation in Erythrocytes and Fibroblasts Associated with Severe Insulin Resistance." J. Biol. Chem. 259;15003-15006, 1984.

CHAPTER 6: SUMMARY

In this dissertation I have attempted to define the role of the insulin receptor in muscle insulin resistance. As pointed out in the introductory chapter, the insulin receptor has two domains: an insulin binding domain and an insulin stimulatable kinase domain. It is the working hypothesis that these two domans work in concert to transduce insulin's signal from the outside of the cell to the interior. In Chapter II investigations were undertaken to define the insulin response in denervated skeletal muscle. It was found that after severing the sciatic nerve, the soleus showed a very rapid onset of decreased insulin responsiveness and sensitivity. In as little as 6 hours there was nearly a 50% decrease in the ability of the denervated soleus to incorporate glucose into glycogen. The insulin unresponsiveness was complete at 24 hours. This effect was not unique to the slow twitch, red muscle in that the predominantly white, fast twitch extensor digitorum longus was also resistant to insulin stimulation of glycogen synthesis after denervation. Other parameters of glucose metabolism were also affected. By 24 hours after denervation there was a decrease in glucose transport activity in the absence of insulin, as assessed by studies on 2-deoxy-glucose uptake experiments, as well as unresponsiveness to insulin stimulation of glucose transport. As expected, concordant with the decrease in glucose transport, the levels of glucose-6-phosphate fell

after denervation and did not increase after insulin treatment as was seen in the contralateral control muscles. This did not appear to be related to a defect in hexose kinase activity since in the 2-deoxyglucose experiments the percent of transported glucose that was in the 2-deoxyglucose state was not different between controls and denervated muscle.

Activation of glycogen synthase was defective 24 hours after severing the sciatic in both the soleus and EDL. It has been suggested that the ability to activate glycogen synthase is dependent on the levels of glycogen in the muscle and this may be true in the case of the denervated soleus in that there was a significant increase in the content of glycogen in solei after 24 hours denervation. However, there was no significant difference in the glycogen content of the EDL after denervation which suggests that the insulin unresponsiveness of the glycogen synthase system is due to factors other than an increase in glycogen stores secondary to inactivity.

Even with the severe insulin unresponsiveness of the denervated muscle, there was not a parallel change in the muscle's response to adrenergic agents. Addition of epinephrine to incubation media resulted in a decrease in glycogen synthesis as well as a decrease in the glycogen content of both control and denervated solei. No detectable differences in the sensitivity to the adrenergic agent between the groups was observed.

Despite the many reported changes in the membrane of muscles after denervation there was no alteration in insulin binding that could account for the insulin resistance that was observed in the studies of Chapter II. I was only able to demonstrate a statistically significant

change in insulin binding at low insulin concentrations using repeated determinations. This is unlikely to account for the insulin resistance since there was no difference in binding at high insulin concentrations while the denervated soleus was still unresponsive at supra-physiological concentrations of insulin. Since multiple metabolic pathways that are responsive to insulin are affected by denervation it appears that the defect of denervated skeletal muscle occurs at a proximal step in insulin action. This led us to speculate that the other functional end of the insulin receptor, that of the insulin-sensitive kinase activity, may be affected by denervation. Investigation of this possibility required the development of a technique for the isolation of functional insulin receptors from skeletal muscle.

The objective of Chapter III was to isolate and purify insulin receptors in sufficient quantities to allow the assessment of binding and enzymatic properties. The main difficulty to overcome was the resistance of muscle to homogenization which makes cellular fractionation difficult. Initial attempts at isolation of membrane fractions with subsequent solubilization of insulin receptors with non-ionic detergents proved unsuccessful. It was discovered that by freezing the tissue in liquid nitrogen, finaly powdering with a mortar and pestle and homogenizing in a buffer containing protease inhibitors and Triton X100, it was possible to obtain a solution that contained insulin receptors which gave typical curvilinear Scatchard binding curves. Further purification on agarose-bound wheat germ agglutinin purified the binding activity 10-fold and resulted in a solution that again showed specific insulin binding as well as an intact kinase activity.

The properties of the insulin receptor kinase were similar to those reported for other tissues. Incubation of the solubilized, wheat germ agglutinin purified fraction with [^{32}P]ATP resulted in the labeling of a ~ 95,000 dalton protein which was immunoprecipitable with anti-insulin receptor antibodies. Addition of insulin increased phosphorylation of this protein which identified it as the β-subunit of the insulin receptor. Addition of insulin to the wheat germ agglutinin eluate also increased the rate of phosphorylation of added exogenous substrates.

In Chapter III the feasibility of assessing the in situ phosphorylation of the insulin receptor in incubated solei was demonstrated. Incubation of the solei in inorganic [^{32}P]phosphate containing medium resulted in the equilibration of the ATP pools with labeled phosphate. After stimulation of the incubated muscles with insulin and subsequent homogenization in buffer containing phosphatase inhibitors, we could demonstrate the increase in labeling of the β-subunit of the insulin receptor. The methods developed in Chapter III were then used in studying the insulin receptor in denervated versus control muscles.

Equilibrium binding studies of the insulin receptor solubilized from 24 hour denervated skeletal muscle showed a slight but not significant decrease in the number of insulin receptors when normalized to the protein content of the final wheat germ eluate. This is consistent with the finding presented in Chapter II where binding of insulin to whole muscles showed only a small decrease after denervation. Likewise, the purified insulin receptors from denervated skeletal muscle showed no change in the ability to autophosphorylate in response to insulin nor was there a change in the exogenous substrate kinase activity of the fully activated insulin receptor. The activation of the

insulin receptor was accomplished by binding the receptors to an insoluble matrix of insulin agarose. The receptors were fully activated by an initial autophosphorylation in the presence of 750 μM ATP and, after washing, the exogenous kinase activity was assessed.

The assessment of in situ autophosphorylation of denervated skeletal muscle insulin receptors gave inconclusive results. The amount of autophosphorylation of the insulin receptor β-subunit that occurred in the presence of insulin was lower than that observed in controls. However, the decrease in autophosphorylation was paralleled by a decrease in the specific activity of the ATP pools attained just prior to insulin addition. It was of interest that there was no difference in the amount of radioactivity associated with the β-subunit in the absence of insulin despite the consistent finding of a decrease in ATP specific activity. This result suggests there may be an increase in the basal autophosphorylation of the receptor.

With the in situ phosphorylation results somewhat murky in their interpretation, we developed an alternate method for assessing the activation of the insulin receptor in the denervated versus the control muscle. An intravenous injection of saline or insulin and subsequent homogenization of the hind limb muscles in buffer containing phosphatase inhibitors allowed the demonstration of an increase in the kinase activity of the insulin receptors isolated from insulin treated rats. Two different methods were used to clear the solutions of phosphatase and kinase inhibitors with identical results obtained in each. Furthermore, the increase in the exogenous substrate kinase activity witnessed after insulin treatment was due to an increase in tyrosine phosphorylation, most likely due to insulin receptor associated tyrosine

kinase activity. In these experiments no difference was seen in the ability of the kinase activity from denervated skeletal muscle to be activated even though the ipsilateral soleus muscles from the same legs demonstrated resistance to both in vivo and in vitro insulin stimulation of glycogen synthesis.

The finding that the insulin receptors in denervated skeletal muscle can bind insulin and the intrinsic tyrosine kinase activity of the insulin receptors can be activated as well as controls demonstrates that the insulin resistance of denervated skeletal muscle is a true 'post receptor' defect. As such, the denervated skeletal muscle can serve as a model for insulin resistance. The exact cause of the insulin resistance of denervated skeletal muscle remains unsolved. It may be due to a change in the activity of the 'second messenger' of insulin or perhaps to a decrease in the sought substrate for the insulin receptor kinase, or may be related to the change in the membrane potential seen after denervation. Whatever the reason, finding the cause will likely give insight into the mechanism of insulin action.

In contrast to denervated skeletal muscle, there was a pronounced change in the insulin receptor derived from diabetic skeletal muscle. The binding domain of the insulin receptor did not appear to be altered after 7 days of insulinopenic diabetes induced in the rat by treatment with streptozotocin except that the number of insulin receptors increased nearly two fold when normalized to the protein in the wheat germ agglutinin eluate without a change in binding affinity. Cross-linking of the α-subunit showed no changes in the migration in the diabetic derived muscle receptor as compared to the control. When the

kinase activity of the diabetic derived insulin receptor was investigated a significant decrease in the ability to autophosphorylate was observed as well as a concommitant decrease in the exogenous substrate kinase activity when assessed by two different methods with two different phosphoacceptors. The kinetic changes that occurred could be reversed by treatment of the animals with insulin to normalize blood glucose levels.

In addition to the change in kinase activity of the β-subunit of the insulin receptor, we also discovered that 7 days insulinopenic diabetes also resulted in the appearance of a subpopulation of β-subunits that had a retarded migratory behavior on electrophoresis gels. This subpopulation could be eliminated by insulin treatment of the diabetic rat. Digestion of the immunoprecipitated insulin receptor with glycosidases increased the migration of the phosphorylated β-subunit. Treatment with endoglycosidase H enhanced the separation of the phosphorylated β-subunit that co-migrated with control and the one that appeared in the diabetic derived insulin receptor preparation. Neuraminidase, on the other hand, appeared to reduce or eliminate the differences in migration of the control and diabetic derived insulin receptor populations, suggesting that the differences in migration were due to an excessive sialidation of the β-subunit of the insulin receptor in diabetes. We were however unable to completely eliminate the second, slower migrating band of the diabetic β-subunit by sequential digestion with neuraminidase and endoglycosidase H, which suggests that other carbohydrate components contribute to the alteration in insulin receptor migratory behavior on electrophoresis gels. Whether this alteration in structural properties is the cause of the change in kinase activity

observed in the insulin receptor or is coincidental is unknown. It is interesting that both the structural change and the alteration in kinase activity are reversed by insulin treatment and when one is observed, so is the other.

In conclusion, the work presented in this dissertation does point out that some, but not all, insulin resistant states may show a concordant change in the properties of the insulin receptor. The exact role of the alteration in the kinase activity and structural changes play in the insulin resistance associated with insulinopenic diabetes awaits the direct demonstration of the role the kinase activity plays in initiating insulin's action in the cell. In the case of skeletal muscle, one must look past the receptor to discover the reason for the insulin resistance seen so soon after denervation.

experiments we established that 2-deoxyglucose uptake of muscles incubated with or without insulin was linear with time under these conditions.

The correlation coefficients (r) of 2-deoxyglucose uptake (0.1 mM) vs. time (10,15,20 and 30 min.) were 0.996 in the absence and in the presence of 1 mU/ml of insulin; with 5 mM 2-deoxyglucose the respective r values were 0.972 without and 0.989 with insulin, (n = 4 muscles/time point). With 5 mM 2-deoxyglucose in the presence of insulin initial rates of 2-deoxyglucose uptake decreased after 15 min., and only the first two time points were used in the calculation of the r value. In subsequent experiments, all incubations with 5 mM 2-deoxyglucose were limited to 15 min.

The degree of phosphorylation of intracellular 2-deoxyglucose was determined after separating 2-deoxyglucose from 2-deoxyglucose-6-PO_4 by chromatography on Dowex 1-X8 columns (11). It was assumed that [^{14}C]sucrose and extracellular free 2-deoxyglucose were distributed identically, and that 2-deoxyglucose-6-PO_4 was only found intracellularly.

Glucose-6-phosphate (G-6-P) content. Muscles were preincubated and incubated as described for the glycogen synthesis experiments except that [^{14}C]glucose was omitted. Immediately after incubation the muscles were frozen between two blocks of dry ice, placed into 1 ml of ice cold 6% perchloric acid and homogenized for 1 min. with an ice-cooled Polytron tissue homogenizer (Brinkman, Ill.). The precipitated protein was pelleted by centrifugation at 5000 x g for 15 min. The supernate was neutralized with $KHCO_3$ and G-6-P assayed by the method of Lowry and Passoneau (12) in an Aminco-Bowman spectrofluorometer.