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Carbohydrate metabolism and the beta-adrenergic system in disuse muscle atrophy

Kirby, Christopher Robin, Ph.D.

The University of Arizona, 1990

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CARBOHYDRATE METABOLISM AND THE BETA-ADRENERGIC SYSTEM IN DISUSE MUSCLE ATROPHY

by

Christopher Robin Kirby

A Dissertation Submitted to the Faculty of the

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For the Degree of

DOCTOR OF PHILOSOPHY

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1990

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GRADUATE COLLEGE

As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Christopher Robin Kirby

entitled CARBOHYDRATE METABOLISM AND THE BETA-ADRENERGIC SYSTEM IN DISUSE MUSCLE ATROPHY

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

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I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Marc E. Tischler — 9/21/90

Dissertation Director
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Finally, I acknowledge the Lord God for all that I am and ever hope to be ... I owe it all to Thee.

DEDICATION

This dissertation is dedicated to my loving family and the glory of the Lord.

"Trust in the Lord with all your heart
and lean not on your own understanding;
In all your ways acknowledge him
and he will make your paths straight."

Proverbs 3 : 5-6

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ABSTRACT

Hindlimb unweighting by tail-cast suspension markedly alters the carbohydrate metabolism of rat soleus muscle. Due to reduced contractile activity, muscle glycogen concentrations increase dramatically. When normal weight-bearing function is restored (reloading), a triphasic response characterizes the return of glycogen to control levels. Between 15 min and 2 h of reloading, muscle glycogen concentrations decrease as a consequence of increased fractional activity of glycogen phosphorylase. From 2 to 4 h, phosphorylase activity declined and an elevated activity of glycogen synthase led to increased glycogen levels. Further increases of glycogen up to 24 h did not correlate with enzyme activities, thereby suggesting a transient uncoupling of the inverse relationship between glycogen concentrations and synthase activity. Between 24 and 72 h of reloading, glycogen decreased to control values, possibly initiated by high phosphorylase activity at 24 h. Further studies concerning the ß-adrenergic response of carbohydrate metabolism showed that isoproterenol inhibition of glucose uptake and the mechanism by which isoproterenol inhibits skeletal muscle glucose uptake were similar in unweighted and weight-bearing soleus muscle. In contrast, isoproterenol effects on glycogen metabolism were increased in unweighted, but not denervated, soleus. Increased response of cAMP accumulation to isoproterenol but not to forskolin, which directly activates adenylate cyclase, suggested a receptor-mediated alteration in ß-adrenergic response. Greater ß-adrenergic binding capacity per milligram muscle in unweighted soleus confirmed this hypothesis. Since the number of ß-receptors in the muscle did not change following unweighting, this suggests that increased receptor concentration in unweighted muscle is due to a preferential loss of structural proteins and not receptor up-regulation. Conversely, denervation did not alter the number of receptors per milligram muscle, but reduced the

total number of receptors in the muscle. These findings support a parallel loss of receptor and non-receptor protein during denervation. Since membrane receptors are degraded in lysosomes, contrasting ß-adrenergic responses and binding capacities provided a novel means for showing marked differences in lysosomal proteolysis between unweighted and denervated muscle. Results from these studies indicate that while both unweighting and denervation induce muscle atrophy, mechanisms of proteolysis and hormonal responses in these two models of reduced use are markedly different.

CHAPTER 1

CARBOHYDRATE AND PROTEIN METABOLISM IN SKELETAL MUSCLE: REGULATION BY ACTIVITY AND HORMONES

Over the past several decades the technical capability for prolonged space flight has increased dramatically. Present plans for a permanently manned space station (Task Group on Life Sciences, 1988), as well as future goals to sustain crews during missions to Mars, will continue this trend. While the technology for transportation and life-support during such extended missions has advanced rapidly, research addressing the mechanisms governing physiological adaptation to microgravity is relatively sparse. Concern over the compromised physical work capacity of individuals exposed to microgravity for long durations has led to close scrutiny of the feasability of man's continued physical presence in space. Thus, economic considerations make the optimization of physical performance imperative, while questions concerning the chronic effects of exposure(s) to microgravity and man's ability to readapt to earth's gravity provide the scientific impetus to investigate these phenomena. Also of intrinsic merit is the opportunity to examine the contribution of gravity to biological development via the microgravity conditions of space.

Two areas in which considerable progress has been made are in the understanding of alterations in carbohydrate and protein metabolism consequent to skeletal muscle unweighting. Enhanced glucose uptake, greater glycogen concentrations, and augmented insulin sensitivity (Henriksen, Tischler, and Johnson, 1986; Bonen, Elder, and Tan, 1988) have all been associated with the metabolic adaptation of skeletal muscle to unweighting. Muscle atrophy resulting from unweighting has been associated with marked alterations in the rates of protein synthesis and degradation (Thomason, Biggs, and Booth, 1989;

Tischler et al., 1990). In addition, recent evidence for a sparing of membrane proteins (e.g., the insulin receptor) during atrophy due to unweighting suggests a relationship between changes in carbohydrate and protein metabolism in unweighted skeletal muscle (Henriksen, Tischler, and Johnson, 1986).

In light of catecholamine control of carbohydrate metabolism and the possibility that the ß-adrenergic receptor may be spared during unweighting atrophy, the next logical step in defining the mechanisms that regulate muscle metabolism during unweighting was investigation of adrenergic influence on unweighted skeletal muscle. Therefore, the goal of this investigation was to extend the knowledge of altered carbohydrate metabolism in unweighted muscle to include responses to the ß-adrenergic agonist isoproterenol. In addition, this study attempted to further establish a relationship between changes in carbohydrate and protein metabolism in non-weight bearing skeletal muscle.

Regulation of Carbohydrate Metabolism in Normal Skeletal Muscle

The uptake, storage, and utilization of glucose are regulated by both the hormonal and activity state of normal skeletal muscle. Hormonal control is achieved primarily through the antagonistic influences of insulin and catecholamines. Contractile activity may influence glucose metabolism independently or in concert with changes in the hormonal milieu. Since the regulation of skeletal muscle carbohydrate metabolism is complex, the following overview will examine separately the mechanisms by which glucose uptake and glycogen metabolism are controlled.

Glucose uptake by skeletal muscle (Fig. 1) is governed by two distinct processes, transport and phosphorylation. Although there is currently no consensus as to which process is rate-limiting (Kubo and Foley, 1986; Zeil, Venkatesan, and Davidson, 1988), it must be emphasized that both processes may separately regulate glucose uptake in muscle.

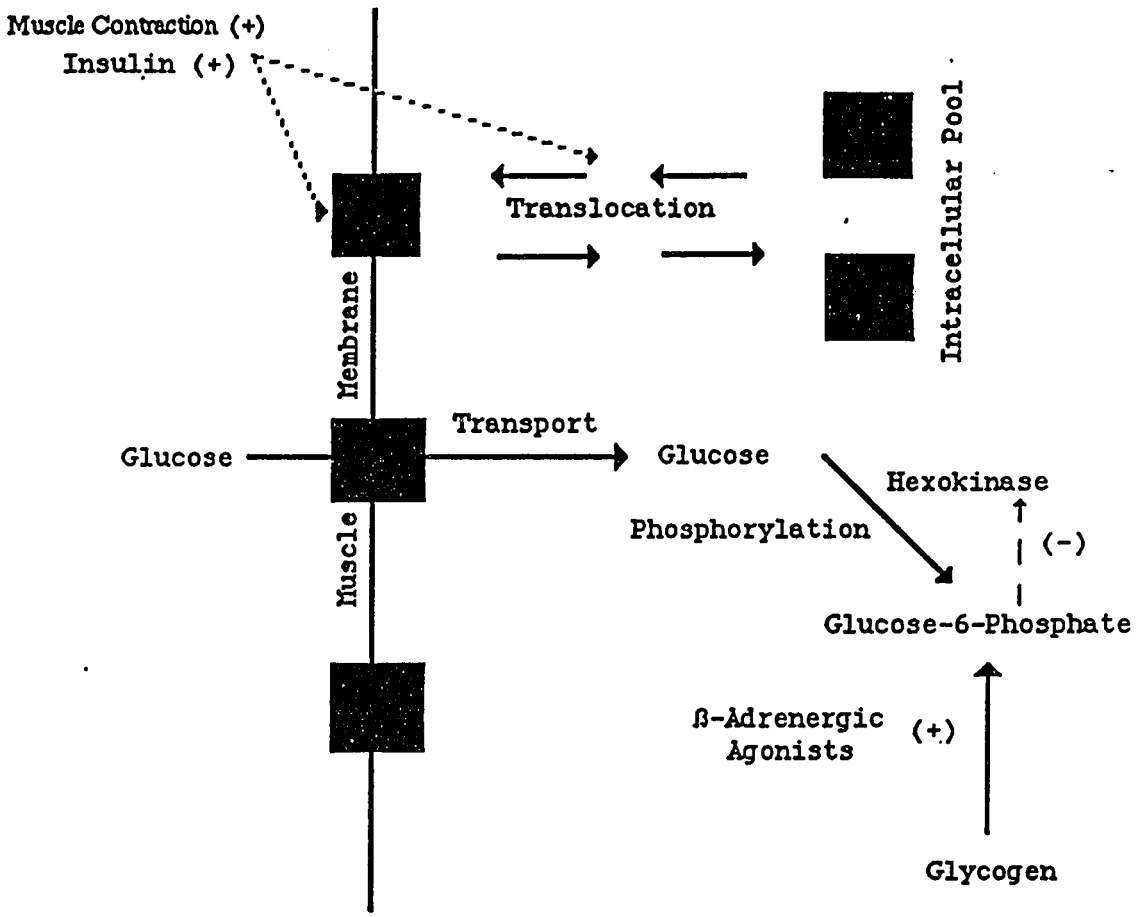

Fig. 1. Glucose Uptake in Skeletal Muscle.

Glucose uptake by skeletal muscle is determined by the rates of glucose transport and phosphorylation. These two processes are controlled by hormones and contractile activity. Insulin and contractile activity enhance glucose uptake by stimulating the translocation of glucose transporter proteins from an intracellular pool to the muscle membrane and by increasing the activity of transporters in the membrane. ß-adrenergic agonists antagonize insulin-stimulated glucose uptake by indirectly reducing the rate of glucose phosphorylation.

Phosphorylation of glucose by hexokinase maintains a concentration gradient for its diffusion into the muscle by preventing the accumulation of intracellular glucose. Glucose transport occurs via a carrier-mediated, facilitated diffusion mechanism (Baly and Horuk, 1988). The carrier-protein, known as the glucose transporter, is saturable, stereospecific, competitively inhibited and is thought to exist in two distinct, yet interchangeable fractions (Wardzala and Jeanrenaud, 1981; Sternlicht, Barnard, and Grimditch, 1988; Hirschman et al., 1990).

Insulin stimulation of muscle glucose uptake results from the translocation and insertion of glucose transporters from an "intracellular pool" (microsomal fraction) to the muscle membrane (Klip et al., 1987; Hirschman et al., 1990). Insulin increases the maximal transport (Vmax) of glucose uptake by increasing both the number of glucose carriers in the membrane and by increasing the activity of the transporter proteins in the membrane (Sternlicht, Barnard, and Grimditch, 1988). Insulin alters neither the affinity (Km) of the transporter for glucose, nor the rate of phosphorylation (Baly and Horuk, 1988). The intracellular signal that results from insulin binding to its receptor is presently unknown. However, there is evidence supporting the autophosphorylation of insulin receptor tyrosine kinase residues and subsequent activation of intrinsic receptor tyrosyl phosphotransferase activity which may be essential for many kinds of insulin action (Rosen, 1989).

Whether there is a direct effect of ß-adrenergic agonists (e.g., epinephrine and isoproterenol) on skeletal muscle glucose uptake is unclear. Investigations have demonstrated ß-adrenergic stimulation (Chiasson et al., 1981; Richter, Ruderman, and Galbo, 1982) and inhibition (Young et al., 1985; Sloan, Sawh, and Bihler, 1978) of basal glucose transport. Although the mechanism for this response has not been elucidated, the fact that these effects can be abolished by treatment with ß-adrenergic antagonists suggests

hormone action via the ß-adrenergic receptor (Sloan, Sawh, and Bihler, 1978; Chiasson et al., 1981; Young et al., 1985).

Under normal physiological, conditions ß-adrenergic agonists do not act independently, but rather, they serve to antagonize insulin effects on muscle carbohydrate metabolism. After binding to a receptor at the muscle membrane, ß-adrenergic agonists stimulate adenylate cyclase activity (Reddy, Oliver, and Engel, 1979) and thereby increase the cAMP concentration (Chasiotis, 1985). Through a cascade of well-defined events (Fig. 2) (Bowman and Nott, 1969), these responses ultimately stimulate glycogenolysis in the muscle (Drummond, Harwood, and Powell, 1969).

Treatment of muscle with ß-adrenergic agonists significantly attenuates insulin-stimulated glucose uptake (Chiasson et al., 1981; Challiss et al., 1986; James, Burleigh, and Kraegen, 1986). However, insulin-stimulated glucose transport, as determined by 3-O-methylglucose uptake, is not affected by ß-agonist treatment. In addition, the rate of glucose phosphorylation is significantly reduced and glucose-6-phosphate concentrations are increased during simultaneous incubation with insulin and ß-agonists. These results support the hypothesis that ß-adrenergic stimulation of glycogenolysis results in decreased hexokinase activity via the accumulation of glucose-6-phosphate, its allosteric inhibitor (Chiasson et al., 1981; Challiss et al., 1986).

Contractile activity enhances skeletal muscle glucose uptake in a non-insulin dependent manner (Constable et al., 1988). As in the insulin-stimulated muscle, increases in the Vmax of glucose uptake are associated with translocation of glucose transporters to the plasma membrane (Hirschman et al., 1988; Goodyear et al., 1990). Since contractile activity produces a two-fold increase in membrane transporters but a seven-fold increase in glucose transport, this suggests a contribution of enhanced intrinsic activity of the glucose transporter to the faster Vmax (Fushiki et al., 1989). While glucose uptake responses to

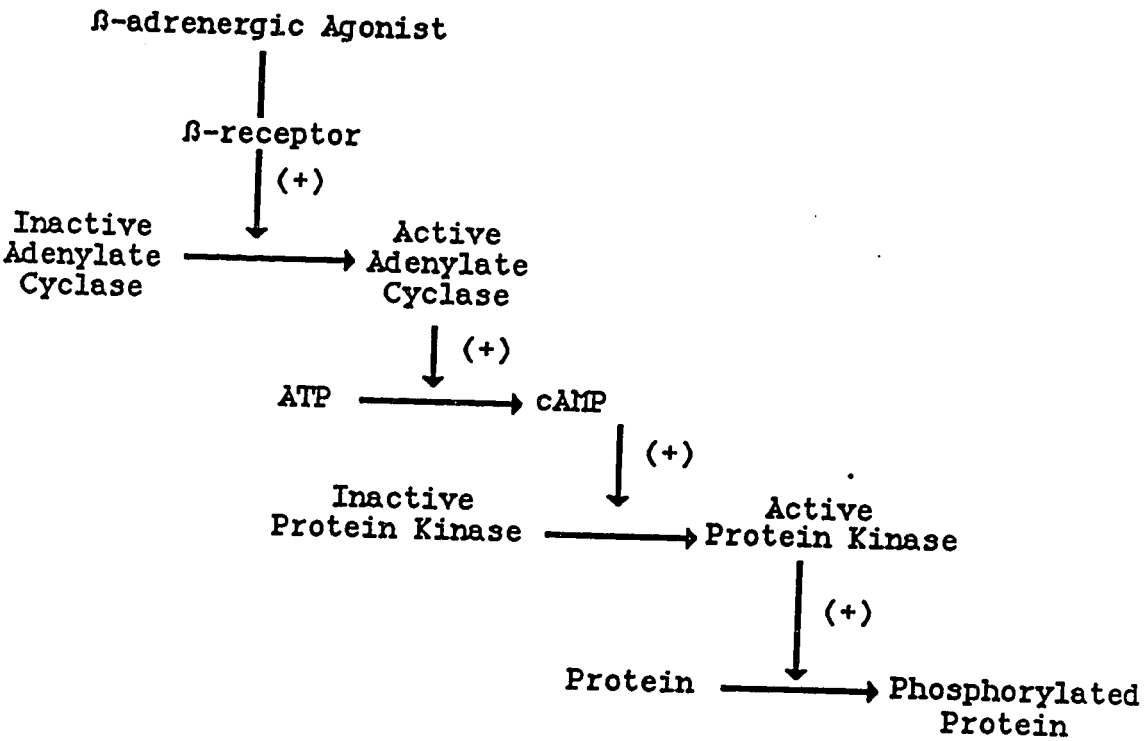

Fig. 2. ß-Adrenergic Cascade.

insulin and contractile activity appear to be similar, the fact that their effects are additive may argue for two distinct pathways of activation (Wallberg-Henriksson et al., 1988). Recent indirect evidence supports some as yet undefined role of calcium in the activation of glucose transport by insulin and contraction (Henriksen, Rodnick, and Hollozsy, 1989; Henriksen et al., 1989).

Regulation of skeletal muscle glycogen metabolism is achieved via alterations in the activities of glycogen synthase (Fig. 3) and glycogen phosphorylase (Fig. 4), two enzymes controlled by phosphorylation (Fischer, Heilmeyer, and Haschke, 1971). Both enzymes and their respective regulatory elements are thought to exist as a glycogen-protein-sarcoplasmic reticulum complex within the muscle cell (Meyer et al., 1970). Glycogen synthase, which catalyzes glycogen formation, exists in a non-phosphorylated I-form and a phosphorylated D-form (Friedman and Larner, 1963). The two forms of the enzyme are interconvertible (Villar-Palasi and Larner, 1960). The I-form is independent of glucose-6-phosphate for its activity, while the D-form requires glucose-6-phosphate for activity (Rosell-Perez and Larner, 1964). The activity ratio of glycogen synthase, expressed as the ratio of activity in the absence of glucose-6-phosphate to the activity in the presence of this allosteric activator, represents the degree to which the enzyme is phosphorylated (Villar-Palasi and Larner, 1961). In a similar fashion, glycogen phosphorylase, which catalyzes glycogen degradation, exists in two interconvertible forms , a and b. Phosphorylation of glycogen phosphorylase b, which depends upon AMP for its activity, produces the AMP-independent a-form of the enzyme (Krebs and Fisher, 1956). The activity ratio of this enzyme is described by the percentage of glycogen phosphorylase in the a-form; that is, the ratio of the activity in the absence of AMP to the activity in the presence of the allosteric activator.

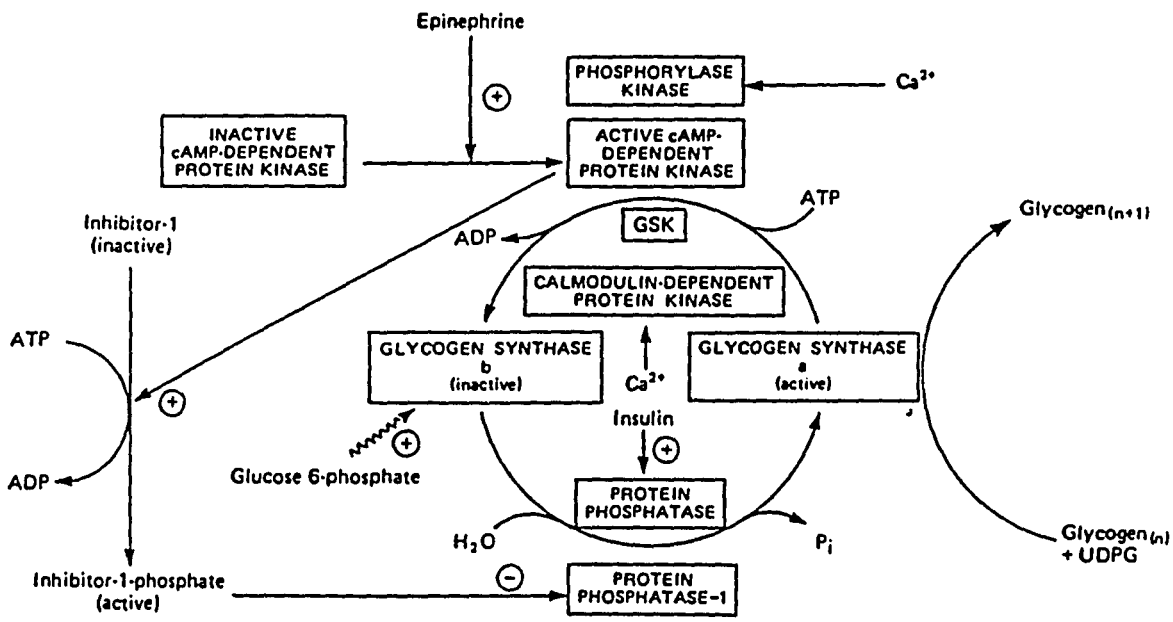

Fig. 3 Control of Glycogen Synthase.

(Modified from Mayes, 1985)

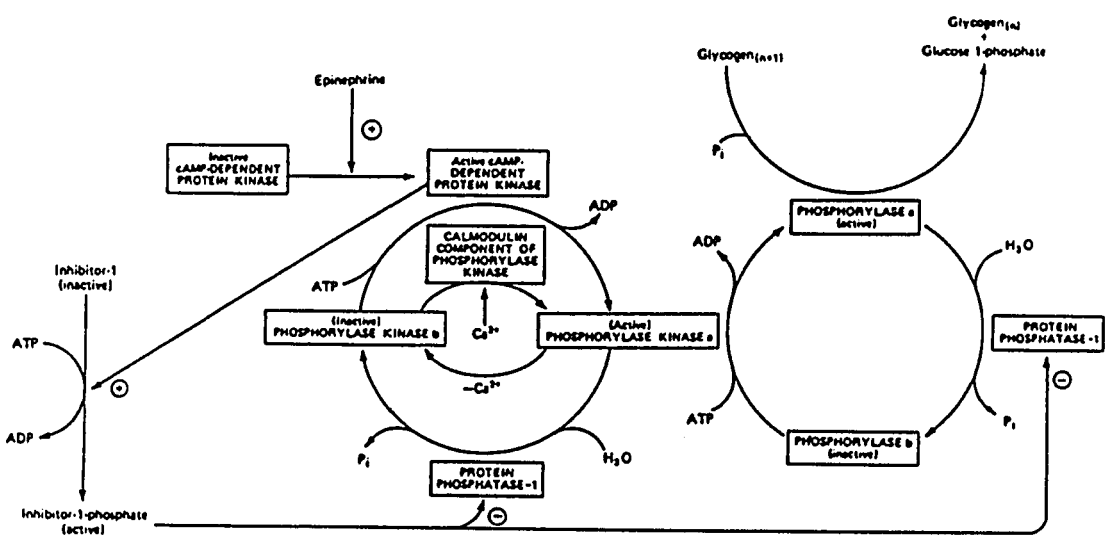

Fig. 4 Control of Glycogen Phosphorylase.

(Modified from Mayes, 1985)

As is the case for the regulation of glucose uptake, glycogen metabolism is controlled via hormonal influences, contractile activity, and allosteric effects. In order to prevent "futile cycling", the activities of glycogen synthase and phosphorylase are tightly counter-regulated in normal skeletal muscle. For example, in the case of net glycogenesis, increases in the activity of glycogen synthase are complimented by decreases in glycogen phosphorylase activity (Zhang et al., 1989).

Hormonal regulation of skeletal muscle glycogen metabolism is under the antagonistic influences of insulin and ß-adrenergic agonists (Cohen, 1985). Insulin, which promotes glycogen storage, dephosphorylates glycogen synthase, thereby increasing its activity ratio (Uhing, Shikama, and Exton, 1981; Yki-Jarvinen, 1987). While the mechanism of insulin stimulation of glycogen synthase is not yet known, a recent report hypothesizes a role for increased protein phosphatase activity (Zhang et al., 1989). In contrast, Okubo et al. (1988) reported no direct relationship between insulin stimulation of glycogen synthase activity and increased phosphatase activity. These results suggest that glycogen synthase activity is regulated through changes in the concentration of glucose-6-phosphate. This concept is supported by insulin-stimulated reductions in the $A_{0.5}$ of glycogen synthase for glucose-6-phosphate (Chiasson, 1980; Uhing, Shikama, and Exton, 1981). These alterations have not been associated with changes in the concentration of cAMP or cAMP-dependent protein kinase activity (Chiasson, 1980).

Several investigations have failed to detect insulin reduction of glycogen phosphorylase activity (Yki-Jarvinen, 1987; Le Marchand-Brustel and Freychet, 1981; Chiasson, 1980). However, others have reported insulin-stimulated reductions in glycogen phosphorylase activity ratios (Shikama, Chiasson, and Exton, 1981; Zhang et al., 1989) that were associated with dephosphorylation of glycogen phosphorylase and phosphorylase kinase (Zhang et al., 1989). In addition, the dephosphorylation of glycogen

phosphorylase was twice as great as that seen for glycogen synthase. This result agrees with the three-fold insulin stimulation of glycogenesis and seven-fold inhibition of glycogenolysis reported by Challiss et al. (1987). These authors hypothesized that insulin reduces the glycogen phosphorylase activity ratio via enhanced protein phosphatase-1 activity. Zhang et al. (1989) also suggest augmented phosphatase activity during insulin inhibition of glycogen phosphorylase. This hypothesis is supported by recent evidence that insulin activates Type I phosphatase *in vitro* (Lawrence et al., 1988) and stimulates dephosphorylation of inhibitor-1 (Nemenoff, Blackshear, and Avruch, 1983). Alternately, insulin may diminish phosphorylase b activity by increasing the cellular concentrations of glucose-6-phosphate (Fischer, Heilmeyer, and Haschke, 1971). This idea is in accord with the results of Okubo et al. (1988, see above) in supporting insulin regulation of glycogen metabolism via alterations in glucose-6-phosphate concentrations. Although the complete mechanism by which insulin regulates glycogen metabolism (i.e., the activities of glycogen synthase and phosphorylase) remains uncertain, these studies suggest two potential counter-regulatory pathways for insulin stimulation of glycogen synthase and inhibition of glycogen phosphorylase.

While insulin stimulates net glycogenesis, counter-regulation by ß-adrenergic agonists results in muscle glycogen breakdown. Mediation of skeletal muscle ß-adrenergic responses through the cAMP cascade is demonstrated by significant increases in adenylate cyclase activity (Reddy, Oliver, and Engel, 1979), cAMP concentrations (Dietz et al., 1980; Shikama, Chiasson, and Exton, 1981; Chasiotis, 1985), and cAMP-dependent protein kinase activity (Dietz et al., 1980; Shikama, Chiasson, and Exton, 1981) following treatment of skeletal muscle with ß-agonists. Increased phosphorylation of phosphorylase kinase (Zhang et al., 1989) and glycogen synthase (Challiss et al., 1987) leads to marked reductions in glycogen synthase activity ratios (Dietz et al., 1980; Shikama, Chiasson, and

Exton, 1981; Le Marchand-Brustel and Freychet, 1981). These changes along with ß-agonist-induced increases in the Ka of glycogen synthase D for glucose-6-phosphate (Dietz et al., 1980; Shikama, Chiasson, and Exton, 1981) attenuate muscle glycogenesis (Shikama, Chiasson, and Exton, 1981; Challiss et al., 1987). Conversely, increased phosphorylation of phosphorylase kinase and glycogen phosphorylase (Zhang et al., 1989) results in marked stimulation of glycogen phosphorylase activity (Dietz et al., 1980; Shikama, Chiasson, and Exton, 1981; Le Marchand-Brustel and Freychet, 1981; Chasiotis, 1985; Zhang et al., 1989). The net effect of these changes is an enhanced rate of glycogen breakdown (Chasiotis, 1985; James, Burleigh, and Kraegen, 1986; Challiss et al., 1987). As was noted for insulin effects on glycogen metabolism, ß-adrenergic agonists produce significantly greater effects on glycogenolysis than glycogenesis (Dietz et al., 1980; Shikama, Chiasson, and Exton, 1981; Challiss et al., 1987). This suggests that hormonal regulation of glycogen metabolism is achieved primarily through changes in the rate of degradation.

Most reports to date support no inhibitory influence of insulin on the ß-adrenergic effects detailed above (Shikama, Chiasson, and Exton, 1981; Zhang et al., 1989). Conversely, ß-adrenergic agonists markedly reduce the effects of insulin on glycogen accumulation. While the mechanisms by which ß-agonists inhibit the insulin response of glycogen metabolism are currently unknown, reversal of this inhibition by ß-adrenergic antagonists supports the concept that the effects are mediated via the ß-adrenergic receptor (James, Burleigh, and Kraegen, 1986).

Like the effects of ß-agonists, contractile activity stimulates net glycogen breakdown. However, a different mechanism is responsible for the glycogenolysis due to contractile activity. In contracting muscle, the release of calcium from the sarcoplasmic reticulum activates calmodulin-dependent phosphorylase kinase (Brostrom, Hunkeler, and

Krebs, 1971). The concentration of calcium (1 μM) required for muscle contraction (Portzell, Caldwell, and Ruegg, 1964) is very near the Km for this enzyme (Heilmeyer et al., 1970). Thus, increased intracellular calcium concentrations stimulate the conversion of glycogen phosphorylase b to a, glycogen synthase I to D, and net glycogenolysis. There is also recent evidence supporting potentiated insulin (Challiss et al., 1987) and adrenergic (Jansson, 1986) response of glycogen metabolism following acute contractile activity.

Non-hormonal control of glycogen metabolism is demonstrated by the inverse relationship between the activity of glycogen synthase and cellular glycogen concentrations (Danforth, 1965). Glycogen inhibition of synthase activity appears to be mediated through reductions in glycogen synthase phosphatase activity (Villar-Palasi, 1969). This inverse relationship has also been demonstrated in muscle partially depleted of glycogen by exercise (Conlee et al., 1978). However, accumulation of glycogen above pre-exercise levels (glycogen supercompensation) has been associated with a transient uncoupling of the inhibitory effect of glycogen on synthase activity (Kochan et al., 1979). This phenomenon may also be associated with a maintenance of post-exercise increases in insulin sensitivity (Garetto et al., 1984) or a transient change in the phosphorylation state of the enzyme, rendering it more sensitive to glucose-6-phosphate without altering the activity ratio (Kochan et al., 1979). One could also hypothesize a combined mechanism in which enhanced insulin sensitivity provides both more substrate for glycogenesis and an increase in the cellular concentrations of glucose-6-phosphate to activate glycogen synthase-D and the proposed hyper-sensitive transient form of the enzyme.

Summary. Glucose uptake by skeletal muscle (Fig. 1) is governed by two distinct processes, transport and phosphorylation. Insulin stimulates the transport of glucose into the muscle by increasing both the number and intrinsic activity of transporters in the plasma membrane (Sternlicht, Barnard, and Grimditch, 1988). In contrast, ß-

adrenergic agonists inhibit insulin-stimulated muscle glucose uptake by diminishing the rate of glucose phosphorylation (Challis et al., 1986). Like insulin, muscle contraction enhances glucose uptake by increasing the number and intrinsic activity of membrane transporters (Goodyear et al., 1990). However, the additive effects of insulin and contractile activity argue for two distinct pathways of activation (Wallberg-Henriksson et al., 1988).

Skeletal muscle glycogen metabolism is regulated by the activities of glycogen synthase and phosphorylase (Fischer, Heilmeyer, and Haschke, 1971). The activity of these enzymes is controlled by their degree of phosphorylation and the intracellular concentration of their allosteric effectors (Krebs and Fischer, 1956; Rosell-Perez and Larner, 1964; Fischer, Heilmeyer, and Haschke, 1971). Alterations in the degree of enzyme phosphorlation occur in response to the antagonistic influences of insulin and ß-adrenergic agonists (Cohen, 1985). Insulin promotes glycogen storage by increasing the activity of glycogen synthase and decreasing the activity of phosphorylase (Zhang et al., 1989). Insulin stimulation of protein phosphatase activity (Figs. 3 and 4) is thought to be the mechanism responsible for these alterations in enzyme activity (Lawrence et al., 1988; Zhang et al., 1989). In contrast to insulin-stimulated glycogenesis, ß-adrenergic agonists promote muscle glycogenolysis. ß-agonists stimulated glycogen breakdown results from increased phosphorylation of glycogen synthase and phosphorylase due to enhanced activity of cAMP dependent protein kinase (Dietz et al., 1980). The phosphorylation state of glycogen synthase and phosphorylase may also be altered by contractile activity. Net glycogenolysis during muscle contraction is achieved via activation of Ca^{++}-dependent protein kinase (Figs. 3 and 4; Brostrom, Hunkeler, and Krebs, 1971). Non-hormonal control of glycogen metabolism can be achieved through alterations in muscle glucose-6-phosphate and AMP concentrations. These metabolites serve as allosteric effectors for

glycogen synthase and phosphorylase, respectively (Figs. 3 and 4; Krebs and Fischer, 1956; Rosell-Perez and Larner, 1964; Fischer). In addition, an inverse relationship between the activity of glycogen synthase and muscle glycogen concentrations has been demonstrated (Danforth, 1965).

Carbohydrate Metabolism during Reduced Use of Skeletal Muscle

To contrast the influence of increased contractile activity (as detailed above), the following discussion will focus on the response of skeletal muscle carbohydrate metabolism to reduced use (Table 1). Although there are several models of reduced use, the three in which carbohydrate metabolism has been studied most extensively are immobilization, denervation, and unweighting (reduced load-bearing).

Immobilization, by limb-casting or joint-pinning, produces significant alterations in muscle carbohydrate metabolism. The direction and magnitude of these changes are dependent upon the length at which the muscle is fixed (i.e., relative to normal resting length). Fixing the muscle at a length less than or equal to resting length results in reduced use atrophy and has been the focus of investigations described below.

Following either acute (0-24 h) or chronic immobilization (≥7 d) basal muscle glucose uptake is unaltered in animals (Seider, Nicholson, and Booth, 1982) and humans (Richter et al., 1989). However, insulin stimulation of glucose uptake is significantly reduced after only 6 h (Nicholson, Watson, and Booth, 1984). A decreased insulin sensitivity is suggested by the fact that the reduction in glucose uptake is detectable at submaximal insulin concentrations (Kahn, 1980). The lack of alteration in insulin binding capacity further supports the concept of a post-receptor-mediated insulin resistance following immobilization (Nicholson, Watson, and Booth, 1984). Insulin resistance is also demonstrated for glycogen metabolism. Reductions in insulin-stimulated glycogen

Table 1. Time Course of Alterations in Carbohydrate Metabolism in Immobilized, Denervated, or Unweighted Skeletal Muscle.

Parameter	Day	Immobilized	Denervated	Unweighted
Glucose Uptake (Basal)	1	NC	D	D
	3	ND	I or D	NC
	7	NC	I or D	NC
Glucose Uptake (Insulin-stimulated)	1	D	D	NC
	3	ND	D	I
	7	D	D	I
Glycogen Synthesis (Insulin-stimulated)	1	D	D	NC
	3	ND	D	I
	7	ND	D	I
Glycogen Concentration	1	I	I	I
	3	ND	D	I
	7	D	D	I
Insulin Binding	1	NC	NC	ND
	3	ND	NC	ND
	7	ND	ND	I

I, increased relative to control; D, decreased relative to control; NC, no change relative to control; ND, not determined.

synthesis are associated with decreased glycogen synthase activity (Nicholson, Watson, and Booth, 1984). Although acute reductions in contractile activity result in elevated glycogen concentrations after 1 d (Seider, Nicholson, and Booth, 1982), prolonged immobilization is associated with reduced glycogen levels (Ward et al., 1986).

Reduced use by denervation (i.e., cutting the nerve supply to the muscle) results in alterations of carbohydrate metabolism that are similar to those observed in immobilized muscle. As in immobilization, insulin-stimulated glucose uptake is significantly attenuated after only 6 h (Burant et al., 1984). This reduction in insulin response has also been observed at 24 h (Burant et al., 1984; Smith and Lawrence, 1984; Sowell, Robinson, and Buse, 1988) and 3 to 7 d following denervation (Forsayeth and Gould, 1982; Smith and Lawrence, 1984; Davis and Karl, 1988). In contrast to the absence of changes in basal glucose uptake in immobilized muscle, both reductions (Burant et al., 1984; Shoji, 1986; Sowell, Robinson, and Buse, 1988) and increases in basal glucose uptake have been reported in denervated muscle (Smith and Lawrence, 1984; Davis and Karl, 1988).

Also in agreement with results from immobilized muscle, glycogen concentrations are increased after 24 h of denervation (Morruzi and Bergamini, 1983; Burant et al., 1984). From 3 d and beyond muscle glycogen concentrations are significantly reduced (Turner and Manchester, 1971; Davis and Karl, 1988). This reduction in glycogen concentration has been associated with alterations in the activities of both glycogen synthase and phosphorylase. Increased degradation of glycogen phosphorylase (Butler, Cookson, and Beynon, 1985) leads to a reduction in total enzyme activity (Turner and Manchester, 1971). However, the activity ratio of glycogen phosphorylase is actually increased following denervation (Turner and Manchester, 1971). Complementing this enhanced glycogenolysis, basal and insulin-stimulated glycogenesis are reduced (Canal and Frattola, 1968; Morruzi and Bergamini, 1983; Burant et al., 1984; Smith and Lawrence, 1984;

Smith and Lawrence, 1985; Davis and Karl, 1988; Smith, Roach, and Lawrence, 1988). Reduced glycogen synthase activity ratios have been attributed to increased glycogen synthase kinase activity (Bergamini and De Paoli, 1984), and an inability of insulin to promote dephosphorylation of glycogen synthase (Morruzi and Bergamini, 1983; Davis and Karl, 1988; Smith, Roach, and Lawrence, 1988). In addition, the Ka of glycogen synthase for glucose-6-phosphate may be reduced (Smith and Lawrence, 1984, 1985).

Like immobilized muscle, the capacity to bind insulin is not altered in muscle denervated for up to 3 d (Forsayeth and Gould, 1982; Smith and Lawrence, 1984; Burant et al., 1984; Sowell, Robinson, and Buse, 1988). Combined with reduced insulin responses associated with denervation, these results support a post-receptor mediated insulin resistance. Recent evidence of similar insulin stimulation of insulin receptor autophosphorylation and tyrosyl kinase activity in control and denervated muscle supports the concept of a post-receptor defect for insulin resistance (Burant, Treutelaar, and Buse, 1986).

Unweighting of skeletal muscle differs from immobilization in that unweighted muscles may move freely throughout their range of motion. In addition, unweighting differs from denervation in that the nerve supply to the muscle remains intact. Although the contribution of these differences to alterations in carbohydrate metabolism during reduced use are not fully understood, the response of skeletal muscle to unweighting is significantly different from the general insulin resistant state of immobilized and denervated muscles. In the absence of insulin, glucose uptake by the unweighted soleus is significantly reduced after 24 h (Henriksen and Tischler, 1988b), but returns to control following 3 or 6 d of unweighting (Henriksen, Tischler, and Johnson, 1986; Henriksen and Tischler, 1988a,

b). In contrast, while insulin stimulation of glucose uptake is similar to control at 24 h (Henriksen and Tischler, 1988a), it is significantly enhanced at 3, 6 and 28 d of unweighting (Henriksen, Tischler, and Johnson, 1986; Bonen, Elder, and Tan, 1988; Henriksen and Tischler, 1988 a, b). The increased response of glucose uptake at submaximal insulin concentrations and the significant increases in insulin binding capacity support an enhanced insulin sensitivity of glucose uptake following soleus unweighting (Henriksen, Tischler, and Johnson, 1986; Bonen, Elder, and Tan, 1988). There is also one report of reduced insulin sensitivity of glucose uptake in perfused hindlimbs of harness suspended rats (Fell, Steffen, and Musacchia, 1985). However, the insulin response of glucose uptake was only measured at two insulin concentrations, making the determination of alterations in sensitivity impossible (Kahn, 1980). In addition, Fell and colleagues have recently presented results supporting an increased insulin sensitivity in soleus muscles from harness suspended rats (Koebel et al., 1990). In contrast to the marked response of soleus muscle to unweighting by suspension, neither basal nor insulin-stimulated glucose uptake is altered in the extensor digitorum longus, a muscle that does not atrophy during suspension (Henriksen, Tischler, and Johnson, 1986; Henriksen and Tischler, 1988 a,b).

Upon entering the muscle cell, glucose can be metabolized via glycolysis, incorporated into glycogen, or utilized in the pentose phosphate pathway. Unweighting appears to influence both the storage of glucose as glycogen and the utilization of glucose in the glycolytic pathway. During the initial 24 h of unweighting, basal glucose oxidation and glucose incorporation into glycogen are significantly reduced (Henriksen and Tischler, 1988b). In contrast, similar rates of basal glucose oxidation, glucose incorporation into glycogen and glycolysis are seen at 3 and 6 d of unweighting (Henriksen, Tischler, and Johnson, 1986; Henriksen and Tischler, 1988a). The early reduction in basal glucose metabolism and subsequent return to control levels probably results from the transient

reduction in basal glucose uptake observed during the initial 24 h of unweighting (Henriksen and Tischler, 1988b). Similar to the insulin response of glucose uptake, insulin stimulation of glucose oxidation and glucose incorporation into glycogen does not differ from control after 24 h of unweighting. However, insulin stimulation of glucose oxidation, glucose incorporation into glycogen and glycolysis are significantly enhanced following 3, 6, and 28 d of unweighting (Henriksen, Tischler, and Johnson, 1986; Bonen, Elder, and Tan, 1988; Henriksen and Tischler, 1988a, b). These increased insulin responses can be attributed to the enhanced insulin sensitivity of glucose uptake observed following unweighting for 3 d or longer (Henriksen, Tischler, and Johnson, 1986; Bonen, Elder, and Tan, 1988; Henriksen and Tischler, 1988a, b).

Increased glycogen concentrations have been demonstrated in soleus muscle following unweighting for as little as 12 h (Henriksen and Tischler, 1988a). These increases are maintained for up to 7 d of unweighting (Fell, Steffen, and Musacchia, 1985). Investigation of the regulation of glycogen metabolism during unweighting has demonstrated significant alterations in the activities of glycogen synthase and phosphorylase. Reductions in glycogen phosphorylase activity are seen as early as 4 h, while glycogen synthase activity is decreased 12 h after unweighting commences. In addition, the measured rate of glycogen degradation is diminished more than is the rate of glycogen synthesis in the 24 h unweighted soleus (Henriksen and Tischler, 1988a). This finding supports the idea that increased glycogen concentration is due to reduced glycogenolysis, which precedes the attenuation of glycogenesis.

Increased muscle glycogen concentrations in unweighted-diabetic animals indicates that alterations in glycogen metabolism are not insulin-mediated (Henriksen and Tischler, 1988a). Animals made diabetic by streptozotosin treatment do not produce endogenous insulin. When these animals are suspended, muscle glycogen concentrations increased

whether or not exogenous insulin was provided. The fact that both glycogen synthase and phosphorylase decrease does not support ß-adrenergic regulation of enzyme activity. Combined, these results do not support a hormonal mechanism for the increased glycogen concentration in unweighted muscle. They do, however, suggest a possible role for altered contractile activity in regulating glycogenolysis. This hypothesis is supported by the finding that when muscle tension is maintained by passive stretch during unweighting, glycogen concentrations and the activity ratios of glycogen synthase and phosphorylase are similar to control (Henriksen and Tischler, 1988a).

Although insulin action is not required for increased glycogen concentrations in unweighted muscle, insulin stimulation of glycogen metabolism is significantly enhanced following unweighting (Henriksen, Tischler, and Johnson, 1986; Bonen, Elder, and Tan, 1988). An indirect mechanism for this increased insulin response is supported by the finding that while insulin enhances the rate of glucose incorporation into glycogen more in unweighted than control muscle, its ability to increase the activity ratio of glycogen synthase is not different from control at 1, 3, or 6 d of unweighting (Henriksen, Tischler, and Johnson, 1986; Henriksen and Tischler, 1988a). The fact that insulin stimulation of glucose-6-phosphate production is enhanced after unweighting could explain the faster *in vitro* rate of insulin-stimulated glycogen synthesis (Henriksen, Tischler, and Johnson, 1986; Henriksen and Tischler, 1988a). In the presence of glucose-6-phosphate, the dependent form of glycogen synthase is activated (Roach, 1981). Therefore, even with a lower glycogen synthase activity ratio, the unweighted muscle could synthesize more glycogen.

Summary. Reduced use by joint immobilization or denervation produces similar alterations of skeletal muscle carbohydrate metabolism. In both models, insulin resistance of glucose uptake and glycogen synthesis are evident after only a few hours (Burant et al.,

1984; Nicholson, Watson, and Booth, 1984). A post-receptor defect is suggested by the lack of alteration in insulin binding capacity in either model (Burant et al., 1984; Nicholson, Watson, and Booth, 1984). While muscle glycogen concentrations increase initially (i.e., ≤ 24 h) (Sieder, Nicholson, and Booth, 1982; Burant et al., 1984), continued immobilization or denervation is associated with reduced glycogen levels (Ward et al., 1986; Davis and Karl, 1988). One notable difference between these models of reduced use is the absence of alterations in basal glucose uptake in immobilized muscle (Sieder, Nicholson, and Booth, 1982) as compared to reports of increased or decreased basal glucose uptake in denervated muscle (Burant et al., 1984; Smith and Lawrence, 1984).

Reduced use by removal of weight-bearing function also produces marked alterations of skeletal muscle carbohydrate metabolism. However, these changes generally contrast those observed for immobilized or denervated muscle. In contrast to the insulin resistant state of immobilized or denervated muscle, insulin stimulation of glucose and glycogen metabolism is normal up to 3 d of unweighting and significantly enhanced thereafter (Henriksen, Tischler, and Johnson, 1986; Bonen, Elder, and Tan, 1988; Henriksen and Tischler, 1988a,b). This greater insulin sensitivity has been associated with increased insulin binding capacity in unweighted muscle. Muscle glycogen concentrations are increased at 12 h of unweighting and remain elevated during more prolonged periods of unweighting (Henriksen, Tischler, and Johnson, 1986; Bonen, Elder, and Tan, 1988). This increase is due to reduced contractile activity and is not mediated by insulin (Henriksen and Tischler, 1988a). Since similar changes are not observed in extensor digitorum longus muscle, which does not atrophy during suspension, these responses are not likely the consequence of systemic alterations (Henriksen and Tischler, 1988b; Henriksen, Tischler, and Johnson, 1986).

Relationship between Alterations in Protein and Carbohydrate Metabolism during Reduced use of Skeletal Muscle

Considerable evidence, both direct and indirect, has recently accumulated which supports a relationship between alterations in protein and carbohydrate metabolism during reduced use atrophy (i.e., the loss of muscle protein). This evidence has been used to construct a model (Fig. 5) that predicts how differences in the mechanism of proteolysis between unweighting and denervation may be linked to alterations in carbohydrate metabolism that are characteristic to each model. This model hypothesizes that atrophy in denervated muscle results from increases in both lysosomal and cytoplasmic proteolysis, while atrophy due to unweighting is associated primarily with cytoplasmic proteolysis. Membrane proteins (e.g., the insulin receptor) are degraded primarily via lysosomal proteases (Hare, 1988; 1990). Therefore, model-dependent differences in the sites of proteolysis are demonstrated by the relative concentration of insulin receptors associated with the membranes of denervated and unweighted muscles as compared to control. The following discussion focuses on results that support this hypothesis.

Muscle atrophy consequent to unweighting has been associated with a rapid reduction in protein synthesis followed by a slower transient increase in protein degradation (Thomason, Biggs, and Booth, 1989; Tischler et al., 1990). Enhanced protein degradation occurs primarily in the myofibrillar fraction (Jaspers and Tischler, 1986, 1988; Thomason, Biggs, and Booth, 1989), while sarcoplasmic protein content remains relatively constant. Sarcoplasmic proteins (e.g., membrane proteins) are thought to be degraded via a lysosomal pathway (Hare, 1988; 1990), while initial myofibrillar proteolysis occurs via cytosolic processes (Mayer and Doherty, 1986). These results provide indirect evidence supporting the idea that proteolysis during unweighting is due primarily to enhanced cytosolic protein degradation.

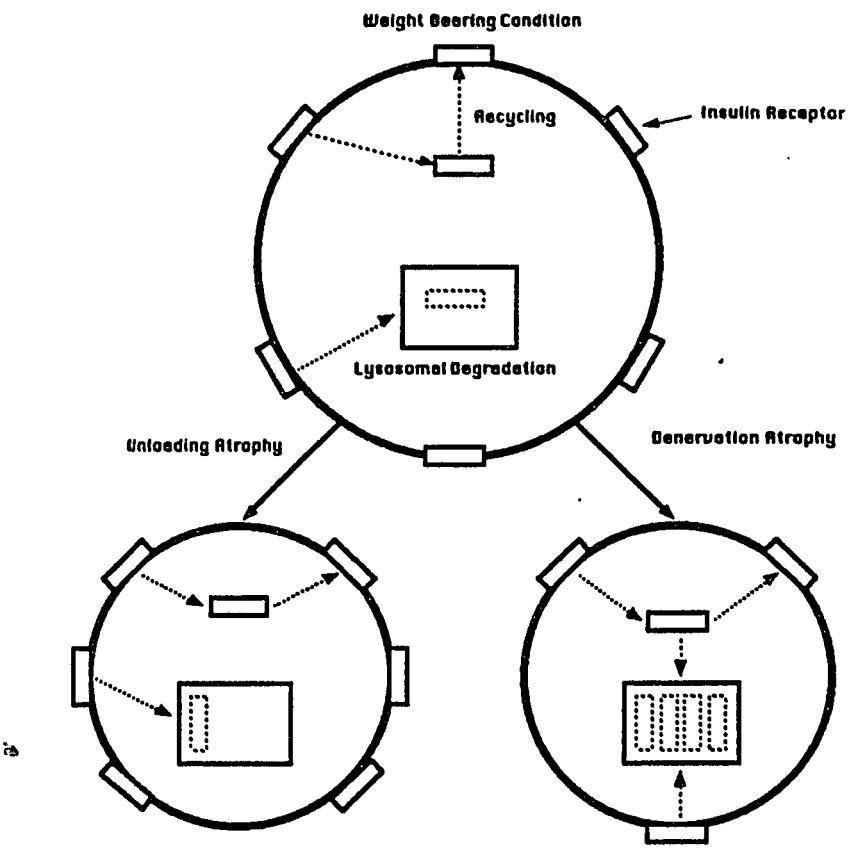

Fig. 5 Proposed Model of Differences in Insulin Receptor Turnover between Unweighted and Denervated Muscle.

Investigations demonstrating increased insulin binding capacity in unweighted muscles (Henriksen, Tischler, and Johnson, 1986; Bonen, Elder, and Tan, 1988) provide additional support for this model (Fig. 5) in that the insulin receptor appears to be "spared" during unweighting atrophy. The total number of receptors remains constant while muscle mass is lost. Since protein synthesis declines with unweighting (Tischler et al., 1990), the increased concentration of receptors is not likely due to increased synthesis of receptor protein. Alternatively, reduced plasma insulin levels in unweighted rats may produce a receptor up-regulation (Bonen, Elder, and Tan, 1988). The fact that enhanced insulin binding capacity was commensurate with the increased insulin response of glucose uptake, glucose oxidation, lactate and pyruvate release, and glycogen synthesis (Henriksen, Tischler, and Johnson, 1986; Bonen, Elder, and Tan, 1988) suggests a correlation between alterations in the metabolism of proteins and carbohydrates in unweighted skeletal muscle. In addition, the time course for the onset of detectable muscle atrophy and enhanced insulin sensitivity of carbohydrate metabolism following unweighting are virtually identical (Henriksen and Tischler, 1988a).

As in unweighting, denervation atrophy is associated with diminished protein synthesis and enhanced protein degradation. However, proteolysis in denervated muscle involves degradation of both myofibrillar and sarcoplasmic proteins (Goldberg, 1969). No alteration in insulin binding capacity (Burant et al. 1984; Smith and Lawrence, 1985) or a decrease in the number of high affinity insulin receptors (Donaldson, Evans, and Harrison, 1986) have been reported for denervated muscle. These results are in accord with commensurate degradation of both myofibrillar and sarcoplasmic proteins in that the amount of receptor protein declines in parallel with the loss of muscle mass (Fig. 5). While the insulin binding capacity has no relation to the insulin-resistant state of denervated

muscle, these results do support the hypothesis that lysosmal proteolysis may be more important in denervation than unweighting atrophy.

A recent investigation using both *in vitro* and *in vivo* measurements of protein turnover focused on the possibility of different sites of accelerated proteolysis in these two models (Tischler et al., 1990). To test the hypothesis that enhanced lysosomal proteolysis accounts for greater *in vitro* protein degradation in denervated as compared to unweighted muscle, incubations were performed using three agents known to inhibit lysosomal proteolysis. Chloroquine, methylamine, and leucine methylester abolished the difference of *in vitro* proteolysis between denervated and unweighted muscle. However, these lysosomotropic agents did not attenuate the greater proteolysis in unweighted muscle. The role of cytosolic proteolysis in unweighting and denervation atrophy was examined during incubations with mersalyl, an inhibitor of thiol proteases (e.g., calcium-activated proteases). While mersalyl only partially inhibited protein degradation in denervated muscle, it abolished the difference in proteolysis between unweighted and control muscle. In addition, reduction of intracellular calcium with TMB-8, which antagonizes calcium release from the sarcoplasmic reticulum (Malagodi and Chiu, 1974), almost completely attenuated the difference in proteolysis between unweighted and control muscle. Furthermore, increasing intracellular calcium concentrations with the calcium ionophore A23187 enhanced protein degradation to a greater extent in unweighted than in control muscle. These results suggest that cytosolic calcium-dependent proteolysis may play a primary role in accelerated protein degradation in unweighted muscle, while mostly lysosomal proteolysis contributes to enhanced protein degradation with denervation.

To determine the possible physiologic significance of the hypothesis, the effects of chloroquine and mersalyl were tested *in vivo*. Intramuscular injection of chloroquine did not affect the degree of muscle atrophy in unweighted muscle, while it prevented, over 24 h, the loss of protein by denervated muscle. In contrast, mersalyl injections not only terminated the loss of protein in the unweighted muscle, but led to a slight accretion of protein. Mersalyl did not prevent the loss of protein by denervated muscles. Neither chloroquine, mersalyl, nor saline injections influenced protein synthesis. Instead changes in protein content could be attributed solely to alterations in proteolysis. Thus, this unique comparison supports the model of different mechanisms of muscle proteolysis during unweighting subsequent to denervation or unweighting.

Summary. Muscle atrophy consequent to unweighting is associated with marked increases in cytosolic protein degradation (Jaspers and Tischler, 1986, 1988; Thomason, Biggs, and Booth, 1989). Denervation atrophy is a result of increases in both cytosolic and lysosomal proteolysis (Goldberg, 1969). Since membrane receptors are degraded primarily in lysosomes (Hare, 1988, 1990), differences in insulin receptor concentrations between unweighted and denervated muscles suggests differences in lysosomal proteolysis in these two models of reduced use. A recent study examined the mechanisms of proteolysis in unweighted and denervated muscle directly (Tischler et al., 1990). Inhibitors of lysosomal proteases abolished the difference of *in vitro* proteolysis between denervated and unweighted muscle, but did not attenuate the enhanced proteolysis in unweighted muscle. In addition, inhibitors of cytosolic proteolysis partially inhibited the increased protein degradation in denervated muscle and abolished the difference in proteolysis between unweighted and control muscle. Similar results were obtained when these agents were tested *in vivo*. These results suggest that cytosolic proteolysis may play a primary

role in accelerated protein degradation in unweighted muscle, while mostly lysosomal proteolysis contributes to enhanced protein degradation with denervation.

Specific Aims of the Project

A primary response of skeletal muscle to reduced use is an increase in the rate of protein degradation. The experimental evidence cited above suggests that the mechanisms responsible for protein degradation depend, in part, upon the reduced use model. For example, unweighting by tail-cast suspension causes preferential breakdown of myofibrillar proteins, whereas denervation is associated with loss of myofibrillar and sarcoplasmic proteins. These differences presumably reflect differential activities of lysosomal and cytosolic proteases. As membrane-bound hormone receptors are degraded primarily via lysosomal proteolysis, alterations in receptor-mediated metabolic responses may provide a means to investigate the process of protein catabolism in reduced use models. To date, evaluation of the model for differences in protein turnover and metabolic responses between different reduced use paradigms has included only studies of the insulin receptor. Owing to the significant role of catecholamines in the regulation of carbohydrate metabolism in skeletal muscle, investigations of the ß-adrenergic receptor, a membrane protein, would provide an additional approach for evaluating this model.

The hypothesis for this investigation states that:

Receptor-mediated stimulation of carbohydrate metabolism will be altered following reduced use and that resulting metabolic changes will be model dependent (i.e., unweighting versus denervation), due to different sites of protein degradation.

The specific aims of this project are:

1. To evaluate the response of carbohydrate metabolism in soleus muscle to a ß-adrenergic agonist, isoproterenol, following 3 d unweighting or denervation.

2. To determine the capacity of ß-adrenergic binding in soleus muscle following 3 d unweighting or denervation.

3. To identify possible post-receptor alterations in the cyclic adenosine 3',5' monophosphate (cAMP) cascade in soleus muscle following 3 d unweighting or denervation.

CHAPTER 2

GLYCOGEN SUPERCOMPENSATION IN RAT SOLEUS MUSCLE DURING RECOVERY FROM UNWEIGHTING

Abstract

This investigation examined the time course of glycogen changes in soleus muscle recovering from 3 days of unweighting by hindlimb suspension. Within 15 min and up to 2 h of reweighting, muscle glycogen decreased. Coincidentally, muscle glucose-6-phosphate and the fractional activity of glycogen phosphorylase, measured at the fresh muscle concentrations of AMP, increased. Increased fractional activity of glycogen synthase during this time was likely the result of greater glucose-6-phosphate and decreased glycogen. From 2 to 4 h of reweighting, when the synthase activity remained elevated and the phosphorylase activity declined, glycogen levels increased (glycogen supercompensation). A further increase of glycogen up to 24 h did not correlate with the enzyme activities. Between 24 and 72 h, glycogen decreased to control values, possibly initiated by high phosphorylase activity at 24 h. At 12 and 24 h of reweighting, the inverse relationship between glycogen concentration and the synthase activity ratio was lost, indicating that reweighting transiently uncoupled glycogen control of this enzyme. These data suggest that the activities of glycogen synthase and phosphorylase, when measured at physiological effector levels, likely provide the closest approximation to the actual enzyme activities *in vivo*. Measurements made in this way effectively explained the majority of the changes in the soleus glycogen content during recovery from unweighting.

Introduction

Removal of the normal weight-bearing function of the soleus muscle (unweighting) causes an increase in the concentration of glycogen, whether by tail-cast (Henriksen and Tischler, 1988a; Henriksen, Tischler, and Johnson, 1986), harness suspension (Fell, Steffen, and Musacchia, 1985), or exposure to microgravity (Baranski et al., 1979; Henriksen et al., 1985). The onset of this increase is extremely rapid, first becoming significant after 12 h and reaching a maximum at 24 h of unweighting (Henriksen and Tischler, 1988a). The concentration of glycogen remains at this elevated level during more prolonged periods of unweighting (Henriksen, Tischler, and Johnson, 1986). This altered turnover of glycogen is the direct result of changes in the intrinsic activity of the rate-limiting enzymes of glycogen metabolism, glycogen phosphorylase and glycogen synthase (Henriksen and Tischler, 1988a; Henriksen, Tischler, and Johnson, 1986).

Preliminary studies on the consequences of the restoration of weight-bearing (reweighting) to the 6-day unweighted soleus revealed the possibility of a rebound effect for glycogen, whereby the concentration of this polysaccharide rises above even the elevated level seen during unweighting (Henriksen et al., 1985). A similar effect, termed glycogen supercompensation, is observed in skeletal muscle during recovery from exercise of sufficient intensity to induce glycogen depletion (Garetto et al., 1984; Hultman, Bergstrom, and Roch-Norlund, 1972; Kochan et al., 1979; Richter et al., 1982a). However, the time course for this apparent glycogen supercompensation with reweighting and the underlying mechanism(s) for its appearance have yet to be more thoroughly investigated. The purpose of the present study was to characterize the events leading to the normalization of the altered glycogen metabolism in the soleus caused by short-term (3 d) unweighting. In addition, the relative importance of the factors regulating glycogen metabolism in skeletal muscle have been evaluated for various periods of reweighting.

Materials and Methods

Treatment of animals. Female rats (85 to 99 gm, 31 to 34 d old, Harlan Sprague-Dawley) maintained on food and water ad libitum were tail-casted using Hexcelite orthopedic tape and Dow Corning Silastic 382 medical-grade elastomer (Factor II, Lakeside, AZ) (Jaspers and Tischler, 1984). During the casting procedure, animals were tranquilized with an intramuscular injection of Innovar-Vet (10 µl/100 gm body weight; Pitman-Moore, Washington Crossing, NJ). To unweight the soleus, the animals were attached to an overhead runner, by paper clips embedded in the cast, in a head-down position so that the hindlimbs were suspended above the floor of the cage. After 3 days of suspension, animals were released from the apparatus and allowed to bear weight on their hindlimbs (reweighting) for the periods indicated.

Fresh-frozen tissue metabolites. To avoid any changes caused by diurnal variation, all animals were killed between 8 and 10 am by cervical dislocation. To accomplish this, restoration of weight-bearing commenced at appropriate intervals before this killing period. The muscles were quickly excised, weighed, and frozen in liquid nitrogen. The time required for dissection and weighing before freezing does not appear to affect tissue metabolite levels (Henriksen and Tischler, 1988a; Henriksen, Tischler, and Johnson, 1986). For analysis of glycogen (Hassid and Abraham, 1957), the muscle was dissolved by heating in 1 ml of 5 N KOH. Glycogen was purified by ethanol precipitation and then hydrolyzed to glucose by heating for 3 h at 100°C in 2 N HCl. After cooling, the sample was neutralized to pH 6-8 with 4 M NaOH-0.1 M triethanolamine HCl, and assayed spectrophotometrically for glucose (Bergmeyer et al., 1981). For analysis of glucose-6-phosphate (G-6-P) and AMP, frozen muscles were homogenized in 1.5 ml of 0.2 M $HClO_4$. The homogenates were centrifuged for 10 min at 5,000 g, and an aliquot (1 ml) of the supernatant was neutralized to pH 6.5-7.5 with 2.5 M KOH-0.1 M piperazine-

N,N'-bis(2-ethane-sulfonic acid) (PIPES). The tissue extract was centrifuged at 15,000 g for 10 min to remove the perchlorate precipitate and assayed fluorometrically for G-6-P (Lang and Michal, 1981) and AMP (Williamson and Corkey, 1969).

Glycogen synthase and glycogen phosphorylase activities. Glycogen synthase was assayed essentially as described by Thomas, Schlender, and Larner (1968). Muscles were homogenized in a Duall tube containing 2 ml of ice-cold 50 mM tris(hydroxymethyl)aminomethane (Tris) buffer (pH 7.8), 100 mM KF, 10 mM EDTA, 2 mM ethylene glycol-bis(ß-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), and 2 mM KH_2P0_4 at 4°C. Homogenates were centrifuged at 15,000 g for 15 min. An aliquot (30 µl) of the supernatant was added to 60 µl of 50 mM Tris buffer (pH 7.8), 20 mM EDTA, 25 mM KF, 10 mg/ml rabbit liver glycogen, and 5 mM UDP-[U-^{14}C]-glucose (150 µCi/mmol), and incubated for 20 min at 30°C without or with 5 mM G-6-P (basal or total activity) or at muscle G-6-P concentrations (fractional activity). A 60 µl aliquout was placed on a 2.4 cm filter paper and [U-^{14}C]-glycogen was separated from [U-^{14}C]-glucose by precipitation with 95% ethanol. The fractional activity ratio was calculated as the ratio between the fractional and total activities, whereas the basal activity ratio was calculated as the ratio between basal and total activities.

Glycogen phosphorylase was assayed by modifying the method of Gilboe, Larson, and Nuttal (1972). Muscles were homogenized in 1.5 ml of ice-cold 25 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer (pH 6.1), 50 mM KF, 10 mM EDTA, and 60 mM 2-mercaptoethanol. An aliquot (1 ml) of this homogenate was treated with 1 mg of activated charcoal for 10 min, and then was centrifuged at 15,000 g for 10 min. An aliquot (30 µl) of the supernatant was added to 60 µl of 200 mM KF, 10 mg/ml rabbit liver glycogen, and 100 mM D-[U-^{14}C]-glucose-l-phosphate (2.55 µCi/mmol) and incubated for 20 min at 30°C without AMP (basal activity) or with AMP at 2 mM (total activity) or at

its measured muscle concentration (fractional activity). Precipitation of labelled glycogen and caculation of fractional and basal activity ratios were as for glycogen synthase.

Data presentation and analysis. Data are presented as the means ± SE for the number of determinations indicated in each table or figure. Significance of differences was tested by factorial analysis of variance (ANOVA) with a post hoc Scheffe' F test.

Materials. Radiolabelled compounds from ICN Radioisotopes Division (Irvine, CA) included D-[U-^{14}C]glucose-l-phosphate and UDP-[U-^{14}C]glucose. All enzymes and biochemicals were obtained from Sigma (St. Louis, MO).

Results

Glycogen concentration. The time course for changes in the concentration of glycogen in the soleus was followed during recovery from 72 h of unweighting (Fig. 6). Within the first 15 min, the concentration had already decreased by 21% ($p<0.005$). This net loss of glycogen continued at a slower rate through the next 105 min of reweighting, until the concentration of glycogen was 44% less than in the unweighted soleus and was significantly less ($p<0.05$) than the value for the weight-bearing control. Within only 15 min of reweighting, G-6-P, a product of glycogen degradation, showed a 107% increase above the concentration in the unweighted muscle (Table 2), in accord with the loss of glycogen during this period. The further loss in glycogen up to 2 h caused no additional rise in G-6-P.

Between 2 and 12 h of recovery, a reversal of this net glycogen breakdown was observed (Fig. 6). After 4 h of reweighting, the concentration of glycogen was 67% greater than the value after 2 h and had also increased above control. This net glycogenesis continued through 12 h of recovery, at which time the concentration of glycogen was 77% greater than that of the weight-bearing muscle. Glycogen did not increase significantly

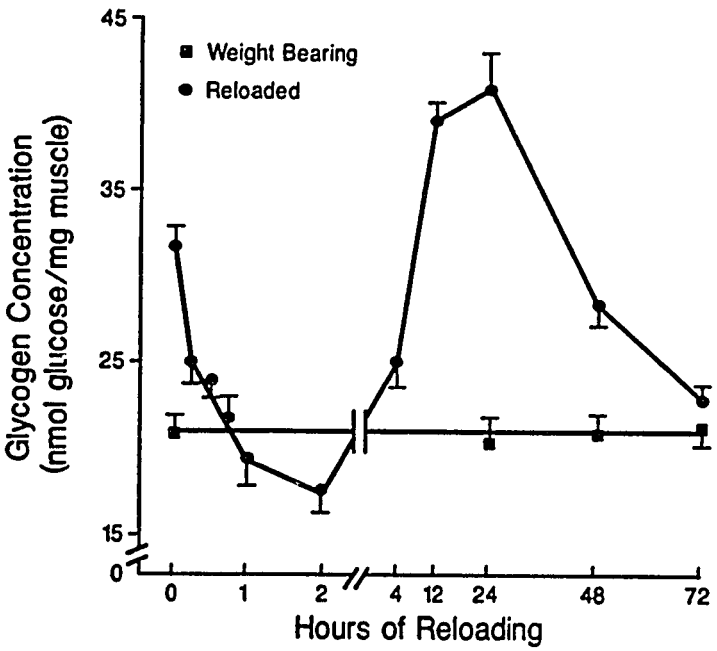

Fig. 6. Time Course of Glycogen Concentration in Soleus During Recovery from Unweighting.

After unweighting for 72 h, animals were allowed to bear weight on all 4 limbs. At indicated time points, glycogen was isolated from soleus muscle and assayed as described in MATERIALS AND METHODS. Values are means ± SE for 5 or 10 animals/group. (Modified from Henriksen, 1987).

between 12 and 24 h. During part of this period (at 4 and at 12 h), the concentration of G-6-P decreased coincident with the net glycogenesis (Fig. 6,Table 2). At 24 and 48 h, the concentration of G-6-P rose again, possibly as a consequence of the onset of the decline in glycogen. Both glycogen and G-6-P returned to control values by 72 h of reweighting.

Activities of glycogen synthase and glycogen phosphorylase during recovery. To investigate the underlying mechanism(s) for these changes in the concentration of glycogen, a parallel time course study was undertaken for basal activity ratios (see text below), fractional activity ratios (Table 2), and fractional activities (Fig. 7) of glycogen phosphorylase and glycogen synthase. As defined here, the basal activity ratios are the activity ratios typically reported by other investigators, and they indicate the amount of phosphorylated (active) glycogen phosphorylase and of dephosphorylated (active) glycogen synthase. To more accurately estimate the extent of *in vivo* activity, we have also measured the activities of these enzymes in the presence of the physiological concentration of its primary allosteric activator, defined here as fractional activity with the portion of the total being the fractional activity ratio. The total activities of glycogen synthase and glycogen phosphorylase did not differ significantly from control (3.5 ± 0.1 and 8.5 ± 0.6 nmol min^{-1} x mg $muscle^{-1}$, respectively) during either unweighting or reweighting. Within 15 min of recovery, the basal activity ratio (-AMP/+2 mM AMP) of glycogen phosphorylase rose 58% to 0.38 ± 0.03 from the diminished value (0.24 ± 0.02) caused by unweighting. This value at 15 min of recovery was significantly greater ($P<0.05$,+23%) than that of the weight-bearing control muscle (0.31 ± 0.02). Thereafter, the basal activity ratio for this enzyme returned to, and remained at, control levels. After 15 min of reweighting, the basal activity ratio of glycogen synthase (-G-6-P/+5 mM G-6-P) (0.18 ± 0.01) was 125% greater than the greatly diminished ratio in the unweighted muscle (0.08 ± 0.01). This rise continued up to 2 h, at which time the activity ratio for this enzyme

Table 2. Fresh Muscle G-6-P and AMP and Fractional Activity Ratios of Glycogen Synthase and Phosphorylase in Recovery of Unweighted Soleus.

Recovery, h	G-6-P, pmol/mg muscle	AMP, pmol/mg muscle	Fractional Activity Ratio	
			Glycogen Synthase	Glycogen Phosphorylase
Control	161±5	35.9±1.3	0.54±0.01	0.41±0.03
0	145±9	35.8±1.3	0.25±0.04	0.38±0.02
0.25	240±13*	36.1±0.5	0.40±0.03*	0.55±0.03*
0.50	209±17*	37.4±1.1	0.48±0.04*	0.48±0.04
0.75	200±6*	37.2±0.8	0.41±0.02*	0.45±0.03
1	215±7*	39.2±1.4	0.48±0.03*	0.46±0.04
2	204±11*	47.1±1.3*	0.53±0.02*	0.41±0.03
4	136±12	46.8±1.0*	0.64±0.04*	0.42±0.03
12	139±6	32.6±0.7	0.48±0.03*	0.54±0.04*
24	179±7*	35.0±1.4	0.45±0.07	0.60±0.04*
48	200±16*	36.1±1.1	0.55±0.02*	0.48±0.04
72	148±4*	35.5±1.4	0.50±0.04*	0.40±0.02

Values are means ± SE for muscles from 5 animals [glucose-6-phosphate (G-6-P) and AMP] and for muscles from 10 animals (fractional activity ratio). Fractional activity ratio was calculated as the ratio between enzyme activity in presence of muscle concentration of effector (fractional activity; see Fig. 7) and a maximal concentration of effector (5 mM G-6-P for synthase; 2 mM AMP for phosphorylase). * $P<0.05$ vs. no recovery (0 h).

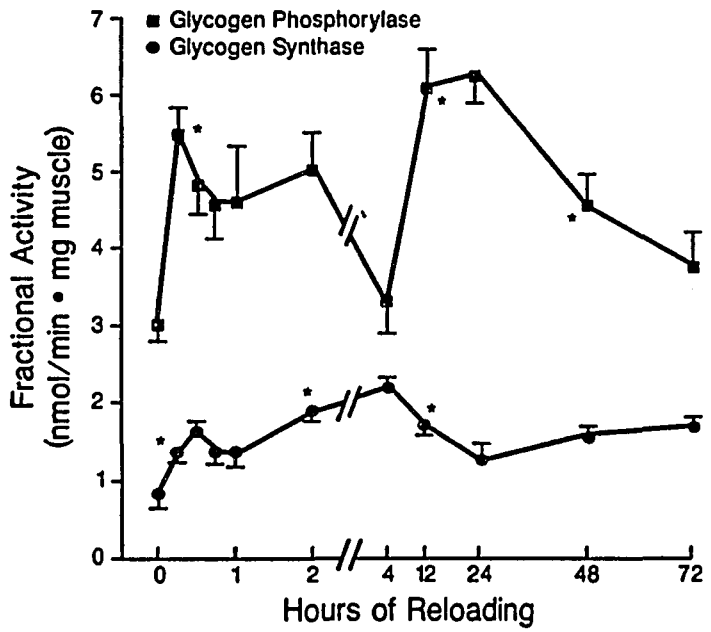

Fig. 7. Time Course of Fractional Activities of Glycogen Synthase and Glycogen Phosphorylase during Recovery from Unweighting.

At times indicated, in parallel to Fig. 6, fractional activities of glycogen synthase and glycogen phosphorylase were determined in the presence of glucose-6-phosphate (G-6-P) and AMP, respectively, at calculated fresh muscle concentrations. Fresh muscle concentrations were estimated from measured picomoles per milligram muscle given in Table 2 and an intracellular muscle volume of 0.8 µl/mg muscle (unpublished observation). Values are means ± SE for 10 animals/group. Weight-bearing control values for fractional activities of glycogen synthase and glycogen phosphorylase are 1.9±0.2 and 3.5±0.3 nmol x min^{-1} x mg $muscle^{-1}$. * $P<0.05$ vs. preceding time point.

(0.39 ± 0.03) was significantly greater ($P<0.05$, 44%) than that of controls (0.27 ± 0.01). At 4 h of reweighting, the basal activity ratio of glycogen synthase returned to the control value and remained at that ratio with further reweighting.

Because changes in the sensitivity of enzymes such as glycogen synthase to their activators may take place without any alteration in basal activity ratio (Roach and Larner, 1977), the fractional activities and fractional activity ratios for glycogen synthase and glycogen phosphorylase were determined using the fresh muscle concentration of the respective allosteric activator (Table 2). The fractional activity ratio of glycogen phosphorylase increased significantly (45%) at 15 min of reweighting before stabilizing near control values (Table 2). However, at 12 and 24 h of recovery, the fractional activity ratio of this enzyme once again increased above control, coincident with the period of diminished glycogenesis (12-24 h) and glycogen decline (after 24 h). Thereafter, the fractional activity ratio of glycogen phosphorylase returned to the control value.

The fractional activity ratio of glycogen synthase (Table 2), like its basal activity ratio, showed a rapid initial increase on reweighting. However, unlike the basal activity ratio, the fractional activity ratio of this enzyme at 4 h had increased above control, despite the decline in G-6-P, suggesting possibly an increased sensitivity to this effector. The increased fractional activity ratio at 2 and 4 h coincided with the net glycogenesis between 2 and 12 h (Fig. 6). Thereafter, the fractional activity ratio of glycogen synthase returned to control levels during the period of net glycogenolysis.

To attempt to get a more accurate approximation of relative changes of glycogen phosphorylase and glycogen synthase activities *in vivo*, we based the activities on the concentration of allosteric activator found in the muscle at each time point. After 15 min of reweighting, the fractional activity of glycogen phosphorylase increased sharply, most likely accounting for the observed decrease in glycogen concentration, and then stabilized at

a slightly lower level through 2 h. At the same time, the fractional activity of glycogen synthase increased significantly. At 4 h, the lower fractional activity of glycogen phosphorylase and the further increase in the fractional activity of glycogen synthase coincided with the period of net glycogenesis. Between 12 and 24 h when the concentration of glycogen plateaued, there was a marked increase in the fractional activity of glycogen phosphorylase coupled with a decrease in the fractional activity of glycogen synthase. From 24 to 72 h, the fractional activity of glycogen phosphorylase returned to the control value.

Discussion

Relationship between glycogen and glycogen phosphorylase during initial (≤2 h) reweighting. The decline of glycogen in the soleus, especially during the first 15 min of reweighting (Fig. 6) was probably the result of the activation of glycogen phosphorylase (Table 2, Fig. 7). Contraction of skeletal muscle, which is reduced significantly by acute unweighting (Alford et al., 1987), causes the release of Ca^{2+} from the sarcoplasmic reticulum, and this cation in turn can stimulate phosphorylase kinase to convert phosphorylase b to a (Brostrom, Hunkeler, and Krebs, 1971; Gross and Mayer, 1974). Increases of cytosolic Ca^{2+} levels, consequent to the resumption of contractile activity during rewieghting, might also contribute to the enhanced glycogenolysis. However, it is not yet possible to measure only cytosolic calcium in whole muscles.

The increased activity of glycogen phosphorylase reverses during prolonged contractile activity (Conlee et al., 1979; Rennie et al., 1982; Richter et al., 1982b). A similar observation was made in the present study during reweighting of the soleus regardless of how the data for glycogen phosphorylase were expressed (Table 2, Fig. 7).

Gross and Mayer (1974) have postulated that during glycogen breakdown there is a release and activation of phosphorylase phosphatase from the glycogen-protein-sarcoplasmic reticulum complex (Entman et al., 1980; Meyer et al., 1970). Glycogen depletion alone, however, cannot explain this blunting phenomenon (Constable, Favier, and Holloszy, 1986). Despite the continued contractile activity of the soleus in the weight-bearing state, the fractional activity of this enzyme stabilized from 30 min to 2 h at a value slightly less than the elevated values seen at 15 min. Coincident with this reduction in the fractional activity of glycogen phosphorylase was a slower rate of loss of glycogen from 30 to 120 min than during the first 15 min of reweighting (Fig. 6). The accumulation of G-6-P during this period (Table 2) also may have contributed to this diminution in glycogenolysis, because this metabolite can inhibit phosphorylase b activity (Fischer, Heilmayer, and Haschke, 1971; Morgan and Parmeggiani, 1964). Although adenosine 3', 5'-cyclic monophosphate (cAMP) and cAMP-dependent protein kinase can regulate glycogenolysis in skeletal muscle via ß-adrenergic activation (Dietz et al., 1980), the concomitant increase in the activity ratio of glycogen synthase within 15 min of reweighting (see Results) suggests that catecholamines are not likely the primary mediators of the alterations in enzyme acitivities during reweighting.

Relationship between glycogen and glycogen synthase during reweighting. An inverse relationship between the concentration of glycogen and the activity ratio of glycogen synthase has been described in skeletal muscle (Danforth, 1965), probably resulting from the effect of glycogen on glycogen synthase phosphatase (Villar-Palasi, 1969). This relationship is also seen in the unweighted soleus (Henriksen and Tischler, 1988a; Henriksen, Tischler, and Johnson, 1986). Interestingly, the regression curve for this relationship from our data (Fig. 8) is nearly identical with that derived from soleus muscle of rats during short-term (<4 h) recovery from exhaustive swimming

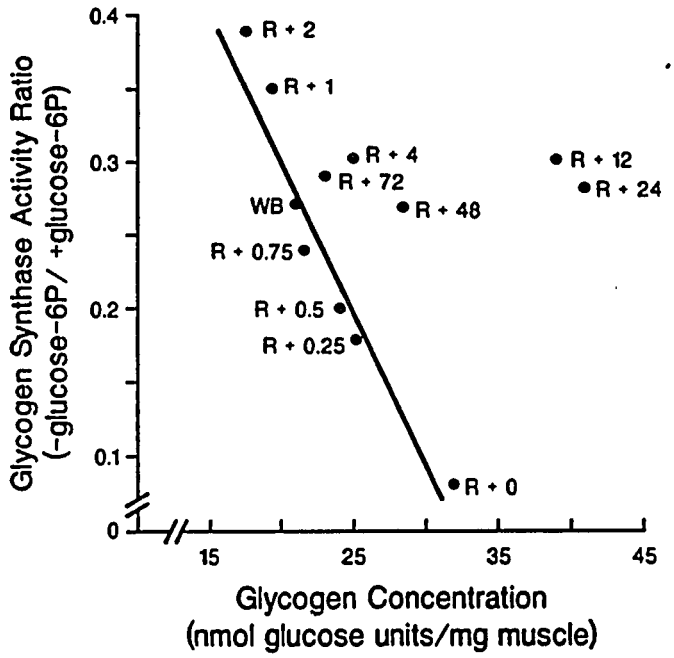

Fig. 8. Relationship between Concentration of Glycogen and Activity Ratio of Glycogen Synthase in Unweighted and Reweighted Soleus Muscle.

Data from 1- to 6-day unweighted and control soleus muscles (Henriksen and Tischler, 1988a; Henriksen et al., 1985).were used to plot regression line. Equation for this line is $y=-0.021x + 0.715$ ($r=-0.943, P<0.01$). Data points are for tail-casted, weight-bearing control (WB) or reweighted (R + reweighting time, in h) muscles. (Modified from Henriksen, 1987).

exercise (Conlee et al., 1978). In the present study, this association between glycogen concentration and the basal activity ratio of glycogen synthase was observed through 4 h and then again at 72 h of reweighting. At 12 and 24 h when glycogen was elevated significantly, there was no corresponding decrease in the basal activity ratio of this enzyme. Therefore, it appears that reweighting causes a transient uncoupling of the effect of glycogen on the activity ratio of glycogen synthase. A similar observation has been made in skeletal muscle that has undergone glycogen supercompensation after exercise-induced glycogen depletion (Garetto et al., 1984; Hultman, Bergstrom, and Roch-Norlund, 1972; Kochan et al., 1979; Richter et al., 1982a). The underlying causes for this phenomenon remain to be elucidated, but may be related to a transient change in the phosphorylation state of the enzyme rendering it more sensitive to G-6-P without any change in the basal activity ratio (Roach and Larner, 1977). This may explain the increase in fractional activity ratio (Table l) and fractional activity (Fig. 7) of glycogen synthase during the initial period of net glycogenesis (at 4 h).

Glycogen supercompensation with reweighting. The accumulation of glycogen between 2 and 12 h of reweighting (Fig. 6) probably resulted from the combined effects of several factors. First, between 2 and 4 h, the initial period of net glycogenesis, the fractional activity of glycogen synthase increased, whereas that of glycogen phosphorylase decreased markedly (Fig. 7). Related to this, the continued higher concentration of G-6-P at the onset of this period (2 h) (Table l) probably stimulated the dependent form of glycogen synthase to enhance glycogenesis (Roach, 1981). Kochan et al. (1979) have reported the appearance of intermediate forms of glycogen synthase, in muscle recovering from exhaustive exercise, that show enhanced sensitivity to G-6-P without any detectable change in activity ratio. Such an effect could explain at 4 h the fractional activity ratio of glycogen synthase increasing despite a fall in G-6-P and no

change in the basal activity ratio of this enzyme. Finally, the enhanced uptake of glucose in the presence of insulin by the reweighted muscle (Henriksen and Tischler, 1988b) could promote glycogen synthesis by increasing the substrate pool for this process. In support of this latter suggestion, the reloaded soleus from rats made diabetic using streptozocin has a much reduced capacity for insulin-stimulated glucose transport (Henriksen and Tischler, 1988a) and at the same time does not undergo glycogen supercompensation (Henriksen, 1987).

Phases of glycogen recovery from unweighting. The results of this study indicate that recovery of glycogen occurs in three distinct phases. The first phase, from 0 to 2 h, is characterized by a net loss of glycogen that is related to activation of glycogen phosphorylase possibly by muscle contraction against a load. In the second phase, which lasts from 2 to 24 h, there is an accumulation of glycogen above both control and unweighted values. The net glycogenesis from 2 to 4 h may in part result from increases in insulin-stimulated glucose transport, the intracellular concentration of G-6-P, and the fractional activity of glycogen synthase. However, no explanation is clear for the period from 4 to 24 h. The final phase, from 24 to 72 h, represents the return of glycogen to normal levels. At the onset of this period, the high fractional activity of glycogen phosphorylase may explain this response.

Although the regulation of glycogen metabolism during reweighting displays some striking similarities to that seen following acute, intense muscle contraction, further research will be needed to consider the potential role of other activators such as calcium and inhibitors such as glucose and ATP. Also difficult to explain is the continued accumulation of glycogen up to 12 h and the plateau between 12 and 24 h. At these times, the fractional activity of glycogen phosphorylase is elevated markedly whereas the measured activity of glycogen synthase has fallen, yet glycogen levels are maintained. Although these

conditions can explain the subsequent decline in glycogen after 24 h, they are inconsistent with the glycogen data between 4 and 24 h. As noted above, this period of time, in particular, is associated with no correlation between glycogen concentration and the glycogen synthase activity ratio. Clearly there must be other factors *in vivo* that control these events at this time other than those studied here. For instance, protein kinases other than cAMP dependent are capable of phosphorylating glycogen synthase (e.g., calmodulin-dependent protein kinase, protein kinase C; Ahmad et al., 1984). Another potential problem is that analysis of glycogen phosphorylase activity is conducted in the opposite direction of its normal flux. Possibly under certain conditions, glycogen phosphorylase activity measured *in vitro* does not give a reliable estimate of the *in vivo* activity.

Conclusions. When normal weight-bearing function is restored in previously unweighted soleus, a triphasic response characterizes the return of muscle glycogen to control levels. Data present here suggests that the activities of glycogen synthase and phosphorylase, when measured at physiological effector levels, likely provide the closest approximation to the actual enzyme activities *in vivo*. Thus, measurements made in this way effectively explain the majority of the changes in the soleus glycogen content during recovery from unweighting.

CHAPTER 3

ß-ADRENERGIC EFFECTS ON CARBOHYDRATE METABOLISM IN THE UNWEIGHTED RAT SOLEUS

Abstract

The effects of insulin on carbohydrate metabolism in atrophied rat soleus muscle are increased following unweighting by tail-cast suspension. This work has been extended by testing the effect of unweighting on the response of carbohydrate metabolism to isoproterenol, a ß-adrenergic agonist. Isoproterenol promoted glycogen degradation more in the unweighted than in weight-bearing soleus, but showed no differences in the extensor digitorum longus, which is unaffected by hindlimb unweighting. In soleus muscles depleted of glycogen, to avoid varied inhibitory effects of glycogen on glycogen synthesis, isoproterenol inhibited glycogen synthesis more so in the unweighted muscle. Isoproterenol did not have a greater inhibitory effect on net uptake of 2-deoxy-[^{3}H]-glucose by the unweighted muscle. Measurements of intracellular 2-deoxy-[^{3}H]-glucose-6-phosphate and 3-O-[^{3}H]methylglucose, which cannot be phosphorylated, showed that isoproterenol inhibited glucose phosphorylation but not transport. This effect could be explained by an increase of glucose-6-phosphate, an inhibitor of hexokinase. At 100 μU insulin/ml, but not at a lower amount (10 μU insulin/ml), isoproterenol inhibited hexose phosphorylation more in the control than in the unweighted muscle. This result may be explained by greater insulin antagonism in the unweighted muscle owing to increased insulin sensitivity. However, insulin antagonism of isoproterenol stimulation of glycogenolysis or inhibition of glycogenesis was not altered by unweighting. Therefore, for some aspects of carbohydrate metabolism the unweighted muscle has an increased

response to ß-adrenergic activation, just as this muscle shows increased response to insulin.

Introduction

Short term unweighting (<10 days) by hindlimb suspension or spaceflight produces significant metabolic changes in rat soleus muscle. Specific alterations in carbohydrate metabolism following unweighting include increased glycogen (Henriksen, Kirby, and Tischler, 1989; Henriksen and Tischler, 1988a; Henriksen, Tischler, and Johnson, 1986), enhanced insulin-stimulated glucose uptake (Bonen, Elder, and Tan, 1988; Henriksen and Tischler, 1988a; Henriksen, Tischler, and Johnson, 1986), and reduced rates of glycogenesis and glycogenolysis (Henriksen, Kirby, and Tischler, 1989; Henriksen and Tischler, 1988a). While these responses have been attributed to reduced contractile activity and/or increased insulin sensitivity (Bonen, Elder, and Tan, 1988; Henriksen, Tischler, and Johnson, 1986), no previous studies have investigated the counter-regulatory effects of ß-adrenergic hormones on carbohydrate metabolism in the unweighted soleus muscle.

Several reports have substantiated the ability of ß-adrenergic agonists to influence carbohydrate metabolism in skeletal muscle. Such effects include increased activity of glycogen phosphorylase and reduced activity of glycogen synthase mediated by cAMP-dependent activation of protein kinase (Drummond, Harwood, and Powell, 1969). Synthase activity is diminished both by an increase in the extent of phosphorylation and the Ka for glucose-6-phosphate, an allosteric activator (Dietz et al., 1980). Combined, these effects cause a loss of glycogen through dose-dependent stimulation of glycogenolysis and inhibition of glycogenesis (Challiss, Crabtree, and Newsholme, 1987).

In addition to their direct effects on glycogen metabolism, ß-adrenergic agonists may antagonize insulin action on carbohydrate metabolism. Isoproterenol reduces insulin-

stimulated glucose uptake presumably by decreasing glucose phosphorylation via glucose-6-phosphate inhibition of hexokinase (Challiss et al., 1986; Chiasson et al., 1981). While insulin does not inhibit the action of epinephrine on glycogen synthase, glycogen phosphorylase, or cAMP-dependent protein kinase activities (Shikama, Chiasson, and Exton, 1981), ß-adrenergic agonists may reduce the ability of insulin to stimulate glucose phosphorylation and glycogen synthesis (Challiss et al., 1986).

In light of enhanced insulin effects on carbohydrate metabolism following unweighting, we considered whether ß-adrenergic influence and the interaction between insulin and a ß-adrenergic agonist might be altered. The purpose of this invesigation was to test the effects of isoproterenol, a ß-adrenergic agonist, on the regulation of glycogen metabolism and glucose uptake in unweighted soleus muscle at low and high insulin. Utilizing two insulin concentrations at which differences in sensitivity were either not significant or not detectable, enabled us to identify those responses which were insulin-dependent, isoproterenol-dependent, or co-dependent.

Methods

Treatment of animals. Female Sprague-Dawley rats (final body weight of 70-80 gm, 28 to 30 d old, SASCO, Omaha, NE) were maintained on food and water ad libitum. Tail-casting was performed as detailed in Chapter 2. Animals were tail-cast suspended or remained weight-bearing for 3 d prior to use in experiments. Food consumption is not altered by this model of hindlimb unweighting (Jaspers and Tischler, 1984).

Glucose-6-phosphate measurement. Animals were killed by cervical dislocation and muscles were quickly excised and weighed. Muscles were preincubated for 30 min in 3 ml of Krebs-Ringer bicarbonate buffer (37°C, pH 7.4) containing 2 mM

pyruvate, 1.5% fatty acid-free bovine serum albumin (BSA), 100 μU insulin/ml, and equilibrated with 95% O_2-5% CO_2. Subsequently muscles were incubated for 15 min in fresh buffer containing 1 mM 2-deoxyglucose, substituted for the pyruvate, with or without 1 μM isoproterenol. Incubations were terminated by removing muscles and freezing them in liquid nitrogen. Muscles were then homogenized in 1.5 ml of 0.2 N perchloric acid. The homogenates were centrifuged for 10 min at 5,000 g and an aliquot (1 ml) of the supernatant was neutralized to pH 6.5 to 7.5 with 2.5 N KOH, 0.1 M Pipes [piperazine-N,N-bis(2-ethanesulfonic acid)]. The tissue extract was centrifuged at 15,000 g for 10 min to remove the perchlorate precipitate and assayed fluorometrically for glucose-6-phosphate (Lang and Michal, 1981).

Measurements of 2-deoxyglucose uptake and phosphorylation. Muscles were preincubated as above except that insulin concentrations varied as indicated in the legends. Muscles were then incubated for 15 min in fresh buffer containing 2-deoxy-[1,2-^{3}H]D-glucose (1 mM, 1 mCi/mmol; ICN, Irvine, CA), substituted for the pyruvate, [carboxyl-^{14}C]inulin (60 nCi/ml; ICN), to correct for ^{3}H in the extracellular space, and isoproterenol at the concentrations indicated in the legends. The incubations were terminated by rinsing the muscle for 10 s in ice-cold 0.9% saline, placing muscles in tubes containing 1 ml deionized water, and heating in boiling water for 10 min (Burant et al., 1984). Aliquots (0.25 ml) of the extract were counted in 5 ml aqueous counting scintillant (ACS, Amersham, Arlington Heights, IL). To determine the 2-deoxy- [^{3}H]-glucose-6-phosphate content, a 0.5 ml sample was placed on a 1 ml Dowex-1-Cl^- ion-exchange column. 2-deoxy-[^{3}H]-glucose was eluted first with 20 ml of deionized water and discarded and then 2-deoxy-[^{3}H]-glucose-6-phosphate was eluted with 6 ml of 0.2 N formic acid, 0.5 N ammonium acetate. Samples (0.5 ml) of the eluant were analyzed for radioactivity in 5 ml ACS by dual label counting.

Measurement of 3-O-methylglucose uptake. Muscles were treated exactly as for measuring the uptake of 2-deoxyglucose except that 3-0-methyl-D-[1-^{3}H] glucose (1 mM, 1 mCi/mmol; Amersham) was provided in the incubation medium. Incubations were terminated by blotting the muscles quickly and solubilizing them in 0.5 ml of 0.5 N NaOH. Samples were analyzed as above.

Measurement of glycogen metabolism. The simultaneous rates of glycogen synthesis and glycogenolysis were calculated from the rates of [^{14}C]glucose incorporation into glycogen, total lactate production, and [^{14}C]lactate production using the method of Challiss, Crabtree, and Newsholme (1987) with slight modifications. Muscles were preincubated for 30 min in 3 ml of Krebs-Ringer bicarbonate buffer (37°C, pH 7.4) containing 1.5% BSA, 5 mM glucose, 5 mM succinate, 4 mM pyruvate, 4 mM glutamate, and insulin at the concentrations indicated in the legends. Muscles were then incubated for 1 h in fresh buffer containing 1.5% BSA, [U-^{14}C]D-glucose (5 mM, 0.2 mCi/mmol; ICN), insulin and isoproterenol at concentrations indicated in the legends. The incubation was terminated by removing the muscle and adding 0.5 ml of 10% perchloric acid to 2.5 ml of the medium. The acid-treated medium was centrifuged for 10 min at 10,000 g and then neutralized with 2.5 N KOH, 0.1 M Pipes. Incorporation of [^{14}C]glucose into glycogen was estimated by measuring the total [^{14}C] in glycogen isolated from the muscle (Hassid and Abraham, 1957). Total lactate released into the medium was determined spectrophotometrically (Gutmann and Wahlfeld, 1981). [^{14}C]Lactate in the medium was measured following ion-exchange chromatography by applying 2 ml to a 5 ml Dowex-2-formate column (Hammerstedt, 1980). Glucose was eluted with 20 ml of deionized water, followed by 7 ml of 0.5 N formic acid to elute the [^{14}C]lactate. A sample (0.5 ml) of this fraction was counted in 5 ml of ACS.

Because only a small quantity (0.3%) of the total radioactivity applied to the column was found in the [^{14}C]lactate, even a slight contamination of this fraction by [^{14}C]glucose would produce a large error in measuring [^{14}C]lactate. To correct for any contamination, the amount of [^{14}C]glucose (18.7% ± 0.9% of [^{14}C]lactate cpm) in the lactate fraction was estimated as follows. A 1 ml sample of the lactate fraction was neutralized with 5 N KOH, 0.1 M Pipes. Samples were then assayed fluorometrically for glucose (Lang and Michal, 1981) and [^{14}C]glucose was estimated based on its specific radioactivity in the original medium. Radioactivity in lactate in the eluant was then calculated as (total cpm) - (cpm in glucose).

The rates of flux from hexose monophosphate to glycogen (B), glycogen to hexose monophosphate (A), and glucose to hexose monophosphate (C) were calculated as follows:

$$B = (E \times D)/F$$

$$C = (D + B) \times F/D$$

$$A = B + D - C$$

Where D = total lactate formation, E = [^{14}C]glucose incorporation into glycogen, and F = [^{14}C]lactate formation. All data are expressed as nmol glucosyl units/mg muscle/h.

Data presentation and analysis. Data are the means ± SE for the number of muscles indicated in each table or figure. Significance of differences between muscles of unweighted and weight-bearing animals was tested by the unpaired Student's t-test or factorial analysis of variance (ANOVA) with a post hoc Scheffe F-test. Percent effects of isoproterenol treatment between unweighted and weight-bearing muscles were analyzed using the Mann-Whitney U test.

Results

Muscle and Body Weights. The initial body weight of control animals was approximately 15 gm less than that of tail-suspended animals. Body weight gains of 12.3 ± 0.6 gm (weight-bearing) and 2.3 ± 0.5 gm (suspended) resulted in similar final body weights for weight-bearing (72.8 ± 0.7 gm) and suspended (78.5 ± 1.0 gm) animals. Soleus weights were also similar for weight-bearing (28.3 ± 0.4 mg) and suspended (24.8 ± 0.3 mg) animals.

Isoproterenol effects on glucose uptake and phosphorylation. In accord with prior findings (Henriksen and Tischler, 1988a), unweighting for 3 days enhanced the effect of insulin on 2-deoxy[^{3}H]-glucose uptake when the hormone was increased from 10 to 100 μU/ml (Table 3). This enhanced response was evident whether or not isoproterenol was present. Isoproterenol (1 μM) reduced net uptake of 2-deoxyglucose in both unweighted and weight-bearing muscles. At the lower concentration of insulin (10 μU/ml), this effect was similar in both muscles. However, when insulin was increased to 100 μU/ml, at which a difference in response to insulin was detected (Table 3), isoproterenol decreased total uptake of 2-deoxyglucose more in the weight-bearing muscle (Table 3).

To examine the mechanism by which isoproterenol inhibits net uptake of 2-deoxy-[^{3}H]-glucose, the formation of 2-deoxy-[^{3}H]-glucose-6-phosphate was measured and was used to calculate the accumulation of intracellular 2-deoxyglucose (Table 3). Isoproterenol decreased in parallel the total uptake and the phosphorylation of 2-deoxyglucose at both concentrations of insulin. Since accumulation of non-phosphorylated 2-deoxyglucose was either unaffected or increased, the inhibitory effect of isoproterenol on total uptake of 2-deoxyglucose must be a consequence of reduced glucose phosphorylation.

Table 3. Effect of Isoproterenol on 2-deoxy[1,2-^{3}H] Glucose Metabolism.

Condition	Isoproterenol	Insulin μunit/ml	Accumulation pmol x mg muscle^{-1}		
			Total	2-Deoxyglucose-6-phosphate	2-Deoxy glucose
Weight-bearing	-	10	324±17	249±21	75±15
	+	10	260±17	198±17	62±13
			(-20%)*	(-20%)*	
Unweighted	-	10	327±49	298±40	28±13
	+	10	259±17	236±41	30±37
			(-21%)*	(-21%)*	
Weight-bearing	-	100	764±63	707±59	57±11
	+	100	493±54	378±41	115±18
			(-35%)*	(-47%)*	
Unweighted	-	100	1078±68	972±58	107±15
	+	100	841±37	723±40	118±10
			(-22%)*¥	(-20%)*¥	

Contralateral muscles were incubated 15 min in the absence or presence of 1 μM isoproterenol with the amount of insulin indicated. Muscle 2-deoxyglucose contents were calculated as the difference between total and the phosphorylated form. Values are means ± SE for 7-10 animals. * $P<0.05$ compared to no isoproterenol. ¥ $P<0.05$ compared to weight-bearing muscles at the same concentration of insulin.

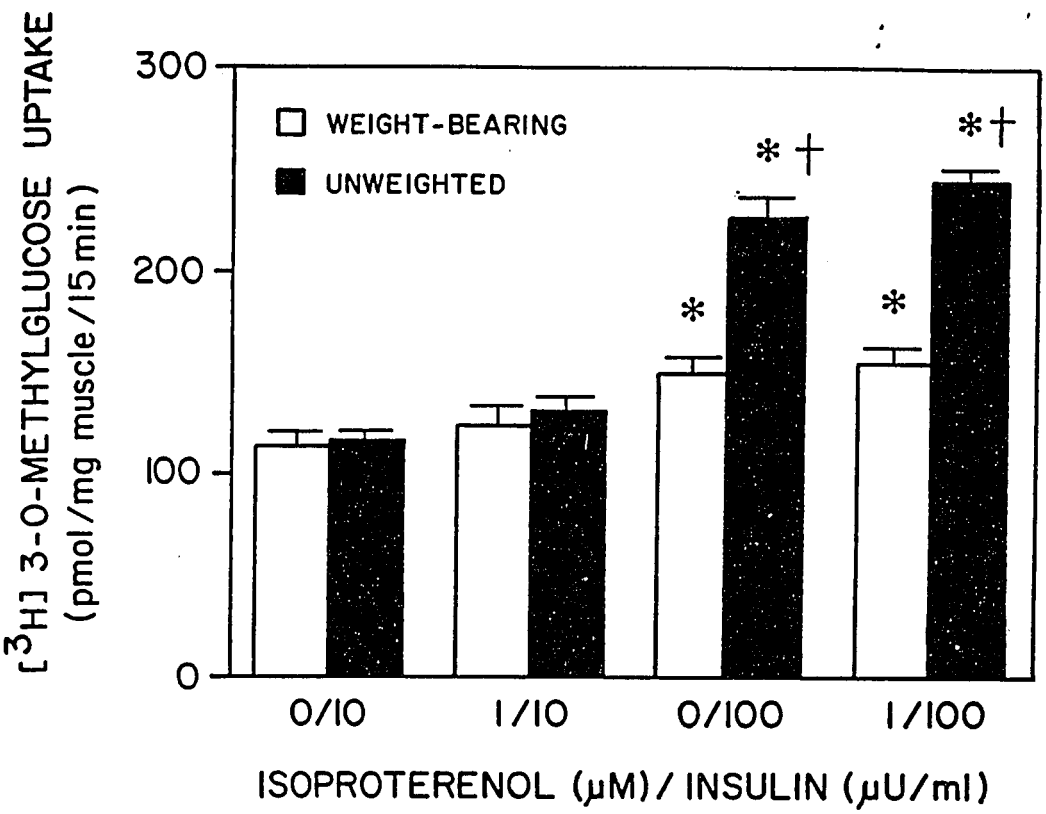

Fig. 9. Effect of Isoproterenol on the Uptake of 3-O-[^{3}H]Methylglucose.

Values are means ± SE for muscles from 10 animals. * $P<0.05$ compared to 10 μU insulin/ml. † $P<0.05$ compared to weight-bearing.

To further demonstrate that isoproterenol does not affect glucose transport, we measured the uptake of 3-O-[^{3}H]methylglucose, which is not phosphorylated (Fig. 9). At both concentrations of insulin tested, isoproterenol did not affect the uptake of 3-O-methylglucose. In accord with our studies of 2-deoxyglucose uptake (Henriksen and Tischler, 1988a; Henriksen and Tischler, 1988b; Table 3), insulin increased the influx of 3-O-methylglucose more so in the unweighted than in the weight-bearing muscle. These results and those which follow showed significant differences whether expressed per milligram muscle or per whole muscle. To test the idea that increased concentrations of glucose-6-phosphate are responsible for the diminution of total 2-deoxyglucose uptake, we assayed glucose-6-phosphate in unweighted and weight-bearing muscles following incubation with insulin (100 μU/ml), in the absence or presence of isoproterenol (Fig. 10). Isoproterenol increased glucose-6-phosphate concentrations in both conditions though the increase was greater in the weight-bearing than in the unweighted muscle.

Isoproterenol effects on glycogen metabolism. Isoproterenol, in the presence of insulin (100 μU/ml), increased the rate of total lactate formation and decreased the incorporation of [^{14}C]glucose into glycogen and lactate (Table 4). Most of these effects were only significant at 1 μM isoproterenol in both weight-bearing and unweighted muscles. The effect of isoproterenol was only greater for total lactate formation in the unweighted muscle. Increasing the concentration of isoproterenol to 10 μM did not further stimulate lactate formation in either condition (not shown). As at lower concentrations of isoproterenol, lactate formation remained greater in unweighted than in weight-bearing muscle. The greater formation of [^{14}C]lactate from [^{14}C]glucose in the unweighted muscle reflects the enhanced sensitivity to insulin of glucose uptake under these conditions.

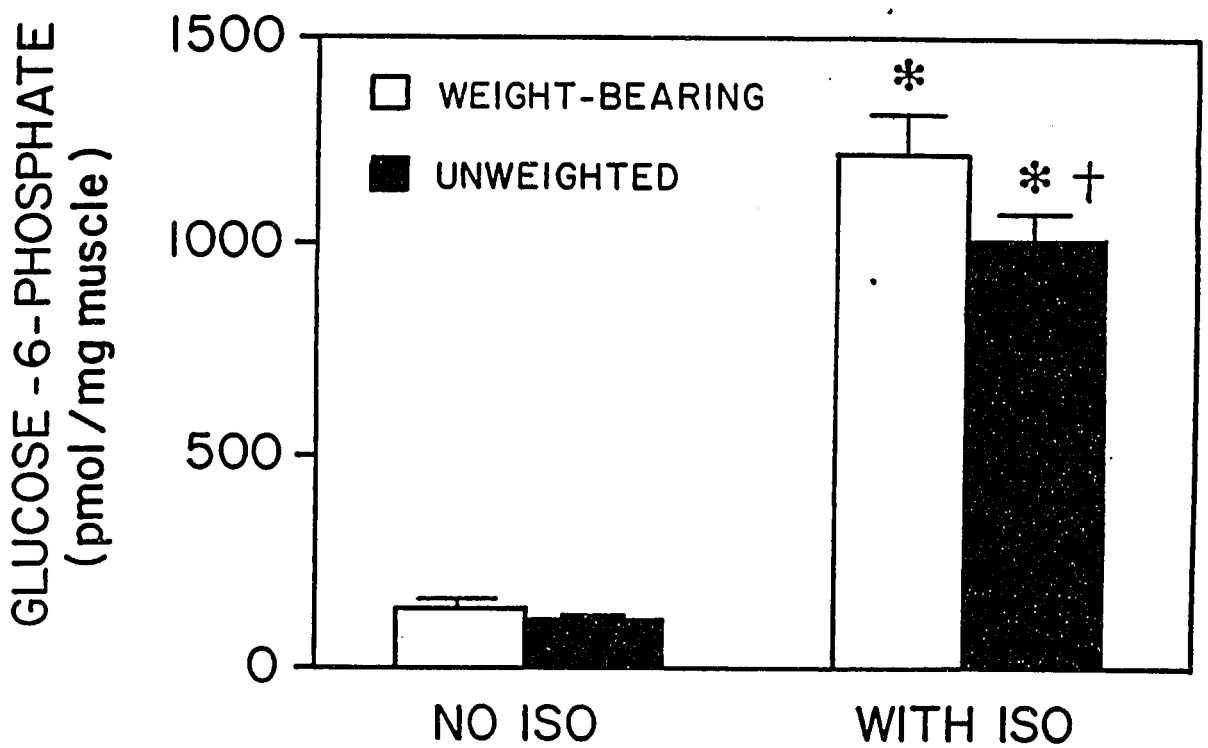

Fig. 10. Effect of Isoproterenol on Glucose-6-phosphate Concentrations.

Glucose-6-phosphate concentrations at 100 μU insulin/ml in the absence or presence of 1 μM isoproterenol (ISO). Values are means ± SE for muscles from 10 animals. * $P<0.05$ compared to no isoproterenol. † $P<0.05$ compared to weight-bearing.

Table 4. Effects of Isoproterenol on the Metabolism of [^{14}C]Glucose and Glycogen.

Endproduct Formed	Condition	Formation, nmol x mg muscle $^{-1}$ x h^{-1}				
		No ISO	0.01 μM	0.1 μM	1.0 μM	(%)
Lactate	Weight-bearing	12.6±0.3	13.1±0.6	14.3±0.7	16.0±0.7*	(+27±4)
	Unweighted	16.1±0.7¥	14.8±0.7	18.0±0.7¥	22.9±0.6*	(+42±4)¥
[^{14}C]Lactate	Weight-bearing	5.0±0.2	4.4±0.3	4.7±0.3	4.0±0.2*	(-24±3)
	Unweighted	6.6±0.3¥	6.1±0.6	6.2±0.5	5.1±0.6*¥	(-22±4)
[^{14}C]Glycogen	Weight-bearing	2.0±0.1	1.8±0.2	0.8±0.1	0.17±0.01*	(-91±1)
	Unweighted	0.9±0.1¥	0.7±0.1¥	0.7±0.1	0.10±0.01*¥	(-89±1)

Muscles were incubated with 100 μU insulin/ml and the isoproterenol (ISO) concentration indicated above. Values are means ± SE for muscles from 13 to 34 animals. Numbers in parentheses are the percent effect of 1 μM isoproterenol. * $P<0.05$ compared to no isoproterenol. ¥ $P<0.05$ compared to weight-bearing.

To ascertain whether differential effects of isoproterenol might be due to factors other than unweighting, we measured lactate formation in the extensor digitorum longus muscle, which is not affected directly by unweighting (Henriksen, Tischler, and Johnson, 1986; Jaspers and Tischler, 1984). In the absence of isoproterenol, the rates of lactate formation in these muscles were 10.9 ± 0.7 nmol/mg/h in unweighted and 11.4 ± 0.4 nmol/mg/h in control muscle. Isoproterenol (1 μM) stimulated lactate production the same in these muscles. Therefore, in accord with previous results (Henriksen and Tischler, 1988b; Henriksen, Tischler, and Johnson, 1986), alterations in the hormonal responses of unweighted soleus muscles cannot be attributed to systemic factors.

The measurements of total lactate, [^{14}C]lactate and [^{14}C]glycogen were used to calculate fluxes in glycogen metabolism (Table 5). In agreement with the data for uptake of 2-deoxyglucose (Table 3), isoproterenol (1 μM) diminished the rate of glucose phosphorylation in unweighted and weight- bearing muscle. Isoproterenol also reduced the flux from hexose monophosphate to glycogen (glycogenesis) and increased the flux of glycogen to hexose monophosphate (glycogenolysis) in both conditions. The only significant alteration in these fluxes at lower isoproterenol concentrations occurred at 0.1 μM for glucose phosphorylation and glycogenesis in weight-bearing muscle. As predicted from the data for total lactate formation, glycogenolysis showed a greater response to isoproterenol in the unweighted than in the weight-bearing muscle. In contrast, isoproterenol had a smaller effect on glucose phosphorylation in the unweighted muscle just as for phosphorylation of 2-deoxyglucose (Table 3). The effect of isoproterenol on glycogen synthesis was slightly less in the unweighted muscle.

Since greater insulin sensitivity of unweighted muscle was apparently responsible for the difference in isoproterenol effects on glucose phosphorylation, we determined whether the ß-adrenergic effect on glycogen metabolism might differ at a lower

Table 5. Effects of Isoproterenol on the Calculated Rates of Glucose Phosphorylation, Glycogenesis, and Glycogenolysis.

Pathway	Condition	Pathway Flux, nmol glucose x mg muscle^{-1} x h^{-1}				
		No ISO	0.01 μM	0.1 μM	1.0 μM	(%)
Glucose Phosphorylation	Weight-bearing	7.0±0.2	6.2±0.5	5.5±0.3	4.1±0.2	(-41±2)
	Unweighted	7.5±0.3	6.7±0.6	6.9±0.6	5.2±0.6*¥	(-31±3)¥
HMP to glycogen	Weight-bearing	2.6±0.2	2.7±0.3	1.2±0.2*	0.35±0.03	(-87±1)
	Unweighted	1.2±0.1¥	1.2±0.5	0.9±0.2	0.24±0.03*	(-80±2)¥
Glycogen to HMP	Weight-bearing	2.0±0.4	3.1±0.4	2.9±0.4	4.2±0.3*	(+112±13)
	Unweighted	1.7±0.3	1.8±1.1	3.1±0.5	6.5±0.4*¥	(+274±22)

Data were calculated from the results in Table 3. Values are means ± SE. Numbers in parentheses are the percent effect of 1 μM isoproterenol (ISO). HMP is hexose monophosphate. * $P<0.05$ compared to no isoproterenol. ¥ $P<0.05$ compared to weight-bearing.

Table 6. Effects of Isoproterenol on the Formation of Lactate and [^{14}C]Glycogen at Lower Insulin.

Endproduct Formed	Condition	Formation, nmol x mg muscle $^{-1}$ x h^{-1}				
		No ISO	0.01 µM	0.1 µM	1.0 µM	(%)
Lactate	Weight-bearing	10.7±0.6	12.4±0.7	17.8±0.8	21.1±0.9*	(+97)
	Unweighted	10.1±0.6¥	11.6±1.5	19.6±1.4¥	24.8±1.1*	(+146)¥
[^{14}C]Glycogen	Weight-bearing	0.50±0.06	0.42±0.03	0.32±0.09	0.14±0.03*	(-72)
	Unweighted	0.30±0.03¥	0.15±0.05¥	0.22±0.09	0.10±0.01*¥	(-67)

Incubations were performed at 10 µU insulin/ml and the isoproterenol (ISO) concentration indicated above. Values are mean ± SE for muscles from 5 to 14 animals. Numbers in parentheses are the percent effects of 1 µM isoproterenol. * $P<0.05$ compared to no isoproterenol. ¥ $P<0.05$ compared to weight-bearing.

concentration of insulin (10 μU/ml) where the sensitivity to insulin appears similar (Table 6). Total lactate formation increased following treatment with isoproterenol (0.1 and 1 μM), but [^{14}C]glucose incorporation into glycogen was reduced only at the highest isoproterenol in both conditions. Comparison of percent effects showed in unweighted muscles a greater response of total lactate formation. Attenuation of [^{14}C]glycogen formation was similar for both muscles. These results were not different from data collected at 100 μU/ml insulin except that isoproterenol promoted lactate formation to a greater extent when less insulin was present.

It seemed contradictory that in the unweighted muscle responses of glycogen synthesis to isoproterenol were not commensurate with the enhanced response of glycogen degradation to this agonist since control of both processes is mediated through cAMP. We considered next whether this apparent contradiction might be a consequence, in the unweighted muscle, of greater amounts of glycogen (Henriksen, Kirby, and Tischler, 1989; Henriksen and Tischler, 1988a; Henriksen, Tischler, and Johnson, 1986), which inhibits glycogenesis (Villar-Palasi, 1969). Therefore, the effect of isoproterenol on glycogen synthesis was retested in muscles partially depleted of glycogen and incubated with only 10 μU insulin/ml. Pretreatment of muscles with 5 μM isoproterenol lowered glycogen to 10 nmol glucosyl units/mg in the unweighted muscle from 29 nmol/mg in non-depleted muscles. In the weight-bearing muscle, glycogen decreased to 3 nmol glucosyl units/mg from 22 nmol/mg (Henriksen, Tischler, and Johnson, 1986). The much higher rates of [^{14}C]glycogen formation in these muscles (Table 7) than in non-depleted muscles (Table 6) showed the success of glycogen depletion. Even though glycogen remained greater in the unweighted muscle following depletion, this difference was not sufficient to produce an inhibition of glycogenesis (Table 7, no ISO). Instead, with glycogen depletion, rates of glycogen synthesis became faster in the unweighted muscle as compared to weight-

Table 7. Effects of Isoproterenol on [^{14}C]Glycogen Formation in Muscle Partially-depleted of Glycogen.

Endproduct Formed	Condition	Formation, nmol x mg muscle $^{-1}$ x h^{-1}				
		No ISO	0.01 μM	0.1 μM	1.0 μM	(%)
[^{14}C]Glycogen	Weight-bearing	1.5±0.1	1.7±0.1	1.3±0.1	0.8±0.1*	(-48±7)
	Unweighted	3.6±0.4¥	3.0±0.4¥	3.4±0.5¥	1.0±0.1*¥	(-73±2)¥

Glycogen was partially depleted by first incubating muscles for 1 h with 5 μM isoproterenol (ISO). The usual 30 min preincubation period was then used to remove the isoproterenol prior to the final incubation. Final incubations for 1 h contained 10 μU insulin/ml and varied amounts of isoproterenol. Numbers in parentheses are the percent effects of 1 μM iso. Values are means ± SE for muscles from 5 to 14 animals. * $P<0.05$ compared to no iso. ¥ $P<0.05$ compared to weight-bearing.

bearing muscle. In addition, the response of this process to isoproterenol became much greater in the unweighted muscle (Table 7). Increasing the isoproterenol concentration to 10 μM further inhibited [^{14}C]glycogen formation in both unweighted and weight-bearing muscles and the percent inhibition remained greater in the unweighted muscle (not shown). These results suggest that 1 μM isoproterenol could maximally stimulate lactate production, but not maximally inhibit [^{14}C]glycogen formation.

Discussion

Influence of Body Weight Gain, Food Consumption, and Muscle Atrophy on Soleus Carbohydrate Metabolism. Although food consumption by suspended and weight-bearing animals is similar, body weight gain of suspended animals is depressed during the first few days of unweighting (Jaspers and Tischler, 1984). The difference in weight gain between conditions is likely explained instead by the diuresis (Deavers, Musacchia, and Meninger, 1980) and mild stress effects associated with suspension (Jaspers and Tischler, 1984). This difference in body weight gain likely did not influence insulin action independent of unweighting as previous reports (Henriksen and Tischler, 1988a; Henriksen and Tischler, 1988b) showed no change in insulin responses of soleus muscle unweighted for 24 h, the period in which weight gain is slowest (Jaspers and Tischler, 1984). In addition, the extensor digitorum longus, which does not atrophy during suspension, does not display altered insulin response of glucose metabolism following suspension (Henriksen and Tischler, 1988b; Henriksen, Tischler, and Johnson, 1986).

The expression of results per mass of muscle may be misleading when tissue is undergoing dynamic changes in mass (Henriksen and Tischler, 1988b; Henriksen, Tischler, and Johnson, 1986). Even so results in the present investigation are expressed

per unit muscle mass rather than per whole muscle. This form of data presentation was chosen to facilitate comparison with other investigations of the influence of ß-adrenergic agonists on carbohydrate metabolism (Challiss, Crabtree, and Newsholme, 1987; Challiss et al., 1986; Chiasson et al., 1981; Shikama, Chiasson, and Exton, 1981). As the interpretations presented here are based on the percent effects of isoproterenol, these results are the same whether or not data are normalized to muscle mass. Such relative comparisons of isoproterenol antagonism of insulin-stimulated carbohydrate metabolism seem most appropriate in light of the differences in insulin sensitivity between unweighted and weight-bearing soleus muscles (Bonen, Elder, and Tan, 1988; Henriksen, Tischler, and Johnson, 1986).

Glucose uptake and phosphorylation. Several studies demonstrated increased insulin response of glucose uptake in unweighted soleus (Bonen, Elder, and Tan, 1988; Henriksen and Tischler, 1988a; Henriksen, Tischler, and Johnson, 1986; Fig. 9). In contrast, Fell and co-workers reported no difference in insulin response of perfused hind limbs (Fell, Steffen, and Musacchia, 1985). However, more recently this group has reported greater insulin response in the soleus of perfused, unweighted hind limbs (Koebel, Steffen, and Fell, 1990). Isoproterenol antagonism of insulin-stimulated hexose uptake was not different following unweighting of soleus. Although isoproterenol inhibited total 2-deoxyglucose uptake less in unweighted soleus at 100 μU insulin/ml (Table 4), this difference can be attributed to the greater insulin sensitivity (Bonen, Elder, and Tan, 1988; Henriksen, Tischler, and Johnson, 1986;) and thus antagonism of isoproterenol effects on glucose uptake in unweighted muscle. Accordingly, the isoproterenol effect in these muscles was similar at an insulin concentration where differences in sensitivity are not detectable (Henriksen, Tischler, and Johnson, 1986; Table 3).

ß-Adrenergic agonists inhibit insulin-stimulated glucose uptake in skeletal muscle probably by decreasing phosphorylation rather than transport of glucose (Challiss et al., 1986; Chiasson et al., 1981). This effect is associated with increased formation of glucose-6-phosphate from glycogen and subsequent attenuation of hexokinase activity. Our results concur with these previous findings. First, isoproterenol reduced in parallel the total uptake of 2-deoxyglucose and the formation of 2-deoxyglucose-6-phosphate, but did not diminish the concentration of intracellular 2-deoxyglucose (Table 3). Second, isoproterenol did not reduce the uptake of 3-O-methylglucose. Third, isoproterenol inhibited phosphorylation of [^{14}C]glucose (Table 5). These effects on phosphorylation of 2-deoxyglucose and glucose were similar in magnitude under the same conditions (1 μM isoproterenol, 100 μU insulin/ml) (c.f. Tables 2 and 4). Finally, the elevation of glucose-6-phosphate by isoproterenol under these conditions was consistent with an allosteric inhibition of hexokinase, as the higher concentrations of glucose-6-phosphate in the control muscle (Fig. 10) coincided with a greater inhibition of 2-deoxyglucose-6-phosphate accumulation (Table 3).

The difference in the magnitude of insulin stimulation of 2-deoxyglucose and 3-O-methylglucose uptake does not agree with earlier reports (Challiss et al., 1986; Chiasson et al., 1981). The increase in 3-O-methylglucose transport (27%) in response to raising the insulin concentration (Fig. 9) compares favorably with the 29% increase reported by Challiss and co-workers (Challiss et al., 1986). However, the much larger increase in 2-deoxyglucose uptake exceeds that reported previously (Challiss et al., 1986; Henriksen, Tischler, and Johnson, 1986). We have no explanation for this difference in insulin stimulation of uptake of these two analogues.

Glycogen degradation. In accord with a smaller glycogen phosphorylase activity ratio (Henriksen and Tischler, 1988a), glycogenolysis tended to be lower in the

unweighted muscle when isoproterenol was absent or low (0.01 μM) (Table 5). High isoproterenol (1 μM) stimulated lactate formation (Table 4) and glycogen degradation (Table 5) in both unweighted and weight-bearing muscles. These responses are associated with increased production of cAMP and enhanced activities of cAMP dependent protein kinase and glycogen phosphorylase leading to increased formation of glucose-6-phosphate (Challiss, Crabtree, and Newsholme, 1987; Challiss et al., 1986; Danforth, 1965). A major goal of this study was to determine whether the ß-adrenergic response in unweighted muscle might be enhanced in parallel to the response of insulin (Bonen, Elder, and Tan, 1988; Henriksen and Tischler, 1988a; Henriksen, Tischler, and Johnson, 1986). The calculation of flux from glycogen to hexose monophosphate suggests that glycogenolysis in unweighted muscle is more responsive to ß-adrenergic activation.

Glycogen synthesis. The slower rate of glycogenesis with unweighting (Table 5), coincides with a lower activity ratio of glycogen synthase following 3 days of unweighting (Henriksen and Tischler, 1988a). Isoproterenol decreased [^{14}C]glycogen formation and glycogenesis in both unweighted and weight-bearing muscles (Tables 3, 4, 5). The percent reduction of [^{14}C]glycogen formation in weight-bearing muscle in this study (-87%, $P<0.05$) agreed with previous results (-79%; Challiss et al., 1986). Despite the greater response of glycogenolysis to isoproterenol in the unweighted muscle, we were unable to initially detect a greater response of glycogenesis. Elevated glycogen can inhibit its own production by decreasing the activity of glycogen synthase phosphorylase (Villar-Palasi, 1969). Indeed, a significant inverse relationship ($r = -0.943$, $P<0.001$) exists between increased glycogen concentrations and the reduced glycogen synthase activity ratio in unweighted soleus muscle (Henriksen, Kirby, and Tischler, 1989). Thus, depletion of glycogen led to more rapid formation of glycogen and the percent inhibition by isoproterenol increased in the unweighted muscle. Therefore, under these conditions

glycogenesis showed a greater response to isoproterenol in the unweighted than in the weight-bearing muscle, just as for glycogenolysis.

Even though we attempted to equally deplete both the unweighted and weight-bearing muscles of glycogen, the content remained greater in the unweighted soleus. Even so, the results support a greater ß-adrenergic response with unweighting. If isoproterenol inhibition of glycogen formation had been similar in both muscles, the rate of glycogen formation would have been greater in the weight-bearing muscle on the basis of a lower concentration of glycogen. However, despite the higher glycogen in the unweighted muscle, the rate of glycogen synthesis was lower in the presence of isoproterenol. Therefore, glycogen was depleted sufficiently so that the remaining difference in concentration was of little or no consequence to the conclusion that isoproterenol inibition of glycogen synthesis was enhanced in unweighted muscle.

Insulin antagonism of Isoproterenol effects on Carbohydrate Metabolism. Evidence for the antagonism of ß-agonist responses by insulin has been equivocal (Challiss et al., 1986; Shikama, Chiasson, and Exton, 1981; Zhang et al., 1989). Shikama, Chiasson, and Exton (1981) did not detect antagonism of epinephrine stimulation of cAMP-dependent protein kinase or glycogen phosphorylase activity ratios even at supra-physiologic insulin concentrations. In addition, while insulin seems to decrease the activity ratio of glycogen phosphorylase, it did not diminish the increased activity produced by epinephrine (Zhang et al., 1989). In contrast, the stimulation by isoproterenol of lactate formation was lowered by increasing the insulin concentration from 1 to 100 μU/ml (Challiss et al., 1986). In our study, although the increase in insulin diminished the percent stimulation by isoproterenol of total lactate formation (Tables 3, 5), this rise in insulin did not diminish isoproterenol inhibition of glucose uptake (Table 3). Therefore, insulin antagonism of isoproterenol stimulated lactate production must be mediated via

glycogen breakdown and not through alterations of glucose uptake. This hypothesis is in accord with the recent suggestion that insulin inhibits glycogen phosphorylase via enhanced protein phosphatase-1 activity (Challiss et al., 1987; Zhang et al., 1989)

Our results and those of Challiss and co-workers (1986) demonstrate insulin antagonism of isoproterenol-stimulated glycogenolysis, while measurement of the activity ratios of the associated glycogenolytic enzymes do not (Shikama, Chiasson, and Exton, 1981; Zhang et al., 1989). This contradiction may indicate that the activity ratio measures only the covalent modification of an enzyme, while *in vitro* glycogenolytic flux represents the complex interactions of covalent modification, allosteric effectors, and substrate concentrations.

A possible reason that insulin antagonizes isoproterenol in this study, but not epinephrine in other investigations (Shikama, Chiasson, and Exton, 1981; Zhang et al., 1989), could be inherent differences in characteristics of the agonist. Epinephrine is a non-specific adrenergic agonist, whereas isoproterenol is ß-specific. Isoproterenol was used in the present investigation to eliminate possible alpha receptor mediated effects on glucose uptake (Richter, Ruderman, and Galbo, 1982). Comparison of various studies show variability in the threshold for responses of both epinephrine and isoproterenol. In isolated muscles incubated with 1 mU insulin/ml, epinephrine responses of glycogen metabolism did not occur below 100 nM (Challiss, Crabtree, and Newsholme, 1987). In contrast, in hindlimb perfusion studies with 1 mU insulin/ml a threshold of only 10 nM epinephrine was required for significant changes in glycogen formation (Shikama, Chiasson, and Exton, 1981). *In vitro*, at 100 µU insulin/ml, isoproterenol effects on glycogen metabolism were observed at 10 nM (Challiss et al., 1986). In the present study, at 100 µU insulin/ml, significant isoproterenol response of glycogen metabolism was not detected below 100 nM. Differences in the threshold effects of these agonists may reflect a variety

of methodological differences between laboratories. Rat plasma concentrations of epinephrine have been reported to be 1 nM (James, Burleigh, and Kraegen, 1986). Even so, higher amounts clearly are required *in vitro* and *in situ* to produce minimal responses. Since isoproterenol is a synthetic agonist, it is therefore difficult to predict what amount would be relatively "physiological" *in vitro.*

Neither our results nor those of Challiss and co-workers (1986) demonstrate insulin antagonism of isoproterenol-inhibited [^{14}C]glycogen formation. Accordingly, insulin diminishes glycogenolysis more than it increases glycogenesis (Challiss, Crabtree, and Newsholme, 1987). These results may be explained by the finding that insulin stimulates dephosphorylation of glycogen phosphorylase to a greater extent than the dephosphorylation of glycogen synthase (Zhang et al., 1989). Increasing the insulin concentration does not attenuate isoproterenol inhibition of glucose phosphorylation in any condition (Challiss et al., 1986; Table 3). These observations support the idea that inhibition of hexokinase is not mediated through a direct ß-adrenergic effect. Instead, it represents the complex interactions of insulin and ß-adrenergic agonists on several pathways which can affect the concentration of glucose-6-phosphate, the principal regulator of hexokinase. Therefore, our results for isoproterenol inhibition of glucose phosphorylation do not reflect solely the response of muscle to this ß-agonist. Instead, differences in hexose phosphorylation in part reflect enhanced insulin sensitivity of glucose uptake in unweighted muscle.

CHAPTER 4

ß-ADRENERGIC RESPONSE AND BINDING CAPACITY OF UNWEIGHTED AND DENERVATED SOLEUS MUSCLE

Abstract

The demonstration of enhanced ß-adrenergic response of glycogen metabolism in rat soleus unweighted by tail-cast suspension (Chapter 3) is in accord with our previous observation of increased insulin sensitivity in unweighted soleus (Henriksen,Tischler, and Johnson, 1986). Increased insulin sensitivity in unweighted, but not denervated soleii (Smith and Lawrence, 1985), suggested that membrane receptors may be "spared" during unweighting atrophy. To further study the mechanisms of increased hormone response and protein degradation during muscle atrophy we measured receptor- and post-receptor-mediated stimulation of cAMP accumulation and ß-adrenergic binding capacity in 3 d unweighted or denervated soleus. Basal cAMP concentrations were lower in unweighted (255 ± 14 fmol/mg muscle) than in weight-bearing (348 ± 24) or denervated (330 ± 22) muscles. This response may reflect greater insulin stimulation of phosphodiesterase activity in accord with the enhanced insulin sensitivity of unweighted muscle. At all concentrations of isoproterenol, cAMP accumulation (pmol/muscle/10 min) was greater in unweighted than in weight-bearing or denervated muscles, which yielded similar values. Forskolin stimulated cAMP accumulation similarly in all conditions. ß-adrenergic binding capacity, estimated from [^{3}H] dihydroalprenolol saturation binding curves, was significantly greater in unweighted (B_{max} = 21.7 ± 3.4 fmol/mg muscle) as compared to weight-bearing (10.9 ± 0.8) or denervated (9.2 ± 3.3) muscles. In contrast, binding capacity per whole muscle was decreased in denervated (216 ± 79 fmol/muscle) relative to

both weight-bearing (467 ± 45) and unweighted (474 ±74) soleii. These results support an enhanced ß-adrenergic sensitivity in unweighted, but not denervated, soleus. Similar to the augmented insulin response of unweighted soleus, enhanced isoproterenol response of unweighted soleus can be attributed to increased binding capacity per milligram muscle. Combined with the absence of altered ß-adrenergic response or binding capacity in denervated soleii, these results support the hypothesis of differences in lysosomal proteolysis in these two models of muscle atrophy.

Introduction

Previous reports from our laboratory have demonstrated an enhanced insulin sensitivity of carbohydrate metabolism following six days of unweighting of the rat soleus muscle (Henriksen, Tischler, and Johnson, 1986). Increased insulin sensitivity was attributed to greater insulin binding capacity in the unweighted soleus. In contrast, denervation produced a marked insulin resistance of skeletal muscle carbohydrate metabolism (Smith and Lawrence, 1985; Burant, Truetelaar, and Buse, 1986), which is associated with a post-receptor defect (Burant, et al. 1984) and no alteration in insulin binding capacity (Smith and Lawrence, 1985). Since membrane receptors are degraded primarily via the lysosomal pathway (Hare, 1988 and 1990), our recent results demonstrating enhanced lysosomal proteolysis in denervated, but not unweighted soleus, could account for the contrasting effects of muscle atrophy on insulin binding capacity in these two models (Tischler, et al. 1990). To further study possible differences in protein degradation between unweighting and denervation atrophy, we have investigated ß-adrenergic responses of carbohydrate metabolism in an effort to examine the turnover of another membrane receptor. Initial experiments demonstrated a significantly greater isoproterenol response of glycogen metabolism in unweighted as compared to weight-

bearing soleus muscles (Chapter 3). It was, therefore, the purpose of this study to investigate the mechanism of enhanced isoproterenol response in unweighted soleus by examining both receptor and post-receptor stimulated cAMP accumulation. In addition, the hypothesis of model-dependent differences in membrane receptor degradation was further tested by comparing ß-adrenergic binding capacity in unweighted and denervated muscle.

Methods

Treatment of Animals. Female rats (29 to 32 d old, Sasco, Sprague-Dawley, Omaha, NE) were maintained on food and water ad libitum and tail-cast suspended as described in Chapter 2 or remained weight-bearing for 3 d.

Bilateral denervation of rat hindlimbs was performed under combined Innovar-Vet and ether anesthesia. The surgical area was alternately cleaned (3 times) with isopropyl alcohol and povidone/iodine scrub solution (Medline Industries, Mundelein, IL). A small incision was made in the skin on the posterior aspect of the thigh and the overlying muscle tissue was blunt dissected to reveal the sciatic nerve. A small piece (2-3 mm) of the nerve was removed to prevent nerve regeneration and the incision was closed with surgical wound clips. Hindlimbs were denervated for 3 d prior to excision of soleii for use in experiments.

cAMP Determinations. Animals were killed by cervical dislocation and soleii were dissected and weighed. Muscles were then pre-incubated in 3 ml of Krebs-Ringer bicarbonate solution (pH 7.4, 30°C) equilibrated with 95% O_2:5% CO_2 and containing 5 mM glucose, 5 mM succinate, 4 mM pyruvate, 4 mM glutamate, 1.5% bovine serum albumin, and 10 μU insulin/ml for one hour. Muscles were transferred to fresh Krebs-Ringer bicarbonate containing 5 mM glucose, 25 mM theophylline, 1.5% bovine serum albumin, 10 μU insulin/ml ± isoproterenol or forskolin (as indicated in the figures and

tables). Isoproterenol stimulates cAMP accumulation via the ß-adrenergic receptor, while forskolin promotes cAMP accumulation by stimulating adenylate cyclase directly (i.e., post-receptor). Following a 10 min incubation, muscles were blotted, frozen in liquid nitrogen, and homogenized in a Duall tube containing 0.5 ml acidic ethanol (1 ml 1N HCl/100 ml ethanol). Homogenates were transferred to eppendorf tubes and spun at 12,000 g for 15 min. The supernatant was saved and the pellet was washed with 0.5 ml of ethanol:water (2:1) and centrifuged for an additional 10 min. Supernatants were combined and evaporated to dryness under a stream of nitrogen at 55°C. The residue was dissolved in 0.1 to 2.0 ml 50 mM Tris (hydroxymethyl) aminoethane (Tris), 4 mM ethylenediamine-tetraacetic acid (EDTA) buffer (pH 7.5) and frozen at -20°C until assayed. The volume of buffer for dissolving the residue was selected such that a 50 µl aliquot of all samples fell within the range of maximum sensitivity (0.5 - 4.0 pmol) for the cAMP assay. cAMP was assayed with a commercial protein binding kit (Amersham, Arlington Heights, IL).

Membrane Preparations. Following dissection, muscles were weighed and immediately frozen in liquid nitrogen. Membranes were prepared as described by Vallieres, Cote, and Bukowiecki (1979). Muscles were minced in ice-cold buffer (50 mM Tris-HCl, pH 7.4) and homogenized in 20 volumes of the same buffer with a Polytron PT10 (Brinkmann Instruments, Westbury, NY) at a rheostat setting of 8 for two 30-s periods. The homogenate, after filtration through a nylon mesh (pore size 1 mm^2), was centrifuged at 2,000 g for 10 min. The supernatant was centrifuged at 40,000 g for 45 min and the final pellet was dispersed in a buffer containing 75 mM Tris-HCl and 15 mM $MgCl_2$, pH 7.4 by being passed (20X) rapidly through a 23-gauge hypodermic needle.

Binding Assays. Membrane suspensions, 100 µl, were incubated at 37°C with (-)[^{3}H] dihydroalprenolol (47.7 or 52.3 Ci/mmol, New England Nuclear, Boston, MA) (25-200 nM) in a final volume of 150 µl containing 50 mM Tris-HCl and 10 mM $MgCl_2$,

pH 7.4. Following 10 min the reaction was terminated by adding 5 ml of ice-cold 10 mM Tris-HCl, pH 7.4 and vacuum filtering through a Whatman GF/F glass fiber filter. The filter was immediately washed three times with 5 ml of ice-cold buffer, placed in a scintillation vial, covered with 5 ml Cytoscint (ICN, Irvine, CA) and allowed to sit overnight. Bound (-)[3H] dihydroalprenolol was determined by liquid scintillation counting and non-specific binding was defined by incubation of parallel tubes with 10 μM L-propranolol (Sigma, St. Louis, MO). Receptor densities (B_{max}) and apparent dissociation constants (Kd) were estimated by multiple iterative non-linear analysis of saturation binding data (Munson and Rodbard, 1980) using the computer program LIGAND (Elsevier-Biosoft, Cambridge, UK). Specific binding per milligram muscle or per muscle was calculated by determining the wet weight of tissue or number of muscles represented in the 100 μl membrane suspension used in the saturation binding experiments.

Data Analysis. Testing for significant differences ($P<0.05$) was done by factorial analysis of variance (ANOVA) with a post hoc Scheffe' F test.

Results

Muscle and Body Weights. Weights of soleus muscles used for cAMP determinations were similar in weight-bearing and denervated muscles, while soleii from unweighted animals were slightly, but significantly, smaller than both other groups (Table 8). Weight-bearing animals weighed approximately 15 gm less initially than unweighted and denervated animals, so that final muscle weights would be more closely matched for incubations. Similar final muscle weights eliminate the contribution of different *in vitro* diffusion distances as a possible reason for altered isoproterenol or forskolin responses of cAMP accumulation between conditions. Soleus atrophy in unweighted and denervated animals is suggested by their greater final body weights, yet similar or slightly lower

Table 8. Muscle and Body Weights of Animals Used for cAMP Determinations.

			Body Weight (gm)	
Condition	N	Soleus Weight (mg)	Initial	Final
Weight-bearing	47	29.2 ± 0.4	61.7 ± 1.0	76.2 ± 1.1
Unweighted	42	27.1 ± 0.4*¥	75.1 ± 1.1*	86.2 ± 1.1*¥
Denervated	42	28.6 ± 0.4	76.2 ± 1.0*	91.6 ± 1.0

Values are means ± SE, * P<0.05 compared to weight-bearing, ¥ P<0.05 compared to denervated.

muscle weights as compared to weight-bearing animals. Soleus muscles used for binding studies were significantly larger in weight-bearing (43.5 ± 0.6 mg) as compared to unweighted (21.8 ± 0.3 mg) and denervated (23.5 ± 0.3 mg) animals . Calculations of the soleus to body weight ratio supported marked atrophy in unweighted (0.299 mg/g) and denervated (0.282 mg/g) animals as compared to weight-bearing controls (0.376 mg/g).

cAMP Accumulation. To explore the mechanism of enhanced isoproterenol response in unweighted soleus, receptor-mediated (i.e., isoproterenol-stimulated) cAMP accumulation was measured. Following the incubation, the basal cAMP concentration was similar in weight-bearing and denervated muscles, but was significantly lower in unweighted soleus (Table 9). Since the unweighted soleus has an enhanced insulin sensitivity (Chapter 3; Henriksen, Tischler, and Johnson, 1986; Bonen, Elder, and Tan, 1988) this result may indicate a greater antagonistic effect by insulin on cAMP accumulation (i.e., prior to the addition of theophylline). Examination of possible differences in insulin response of cAMP accumulation will be the focus of future investigations.

In accord with an enhanced isoproterenol response of glycogen metabolism (Chapter 3), cAMP accumulation was markedly greater in unweighted as compared to weight-bearing or denervated muscles (Fig. 11). At the lowest isoproterenol concentration tested, stimulation of cAMP accumulation was 7-fold greater in unweighted soleus (Fig. 12). Since the ratio of cAMP accumulation in unweighted to weight-bearing muscle (Fig. 12) approached unity at increasing isoproterenol concentrations the data suggested increased sensitivity rather than responsiveness following soleus muscle unweighting.

As expected, cAMP concentrations following incubation were significantly greater in unweighted, as compared to weight-bearing and denervated soleii, at all isoproterenol concentrations (Table 9). These results show the accumulation of cAMP in the muscle.

Table 9. cAMP Concentrations after Incubation.

Additions	Weight-bearing	N	Unweighted	N	Denervated	N
None	0.35 ± .02	22	0.26 ± .01*	18	0.33 ± .02	16
Isoproterenol (μM)						
0.01	0.34 ± .04	8	0.48 ± .04*	9	0.32 ± .02	12
0.1	0.77 ± .08	6	1.49 ± .08*	6	0.52 ± .04	5
1	2.61 ± .34	11	4.68 ± .74*	10	2.49 ± .28	7
100	5.72 ± .53	8	7.60 ± .48*	8	5.11 ± .52	8
Forskolin (μM)						
100	3.07 ± .20	18	2.98 ± .16	13	2.57 ± .23	14
300	6.13 ± .43	9	5.24 ± .25	8	5.11 ± .31	9
500	5.49 ± .45	4	5.19 ± .27	4	6.70 ± .32	4

Muscles were incubated with 10 μU insulin/ml and the isoproterenol or forskolin concentration indicated above. Values are means ± SE (pmol/mg muscle). * $P<0.05$ compared to weight-bearing.

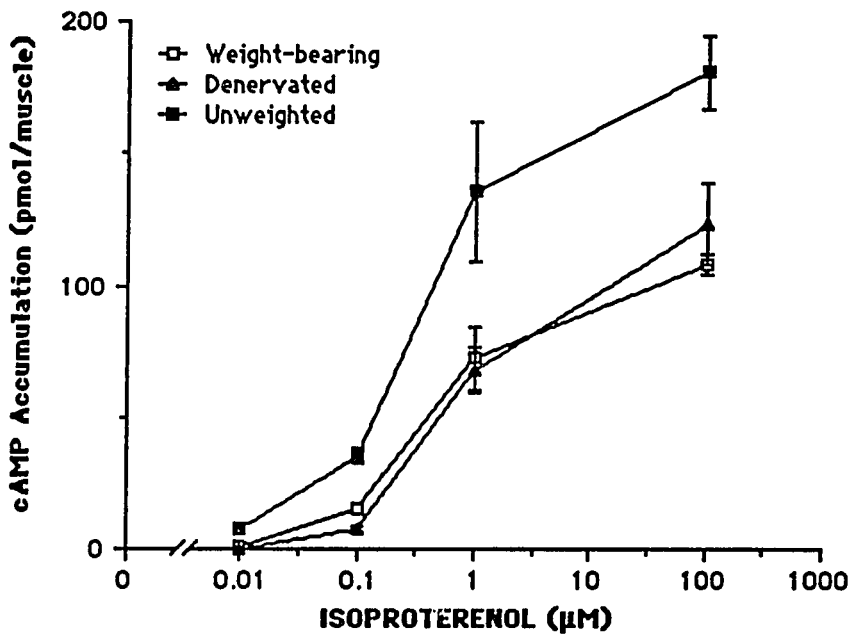

Fig. 11. Effect of Isoproterenol on cAMP Accumulation .

Three day weight-bearing, unweighted, or denervated muscles were incubated with 10 μU insulin/ml and the isoproterenol concentration indicated above. Values are means ± SE for 5-12 muscles.

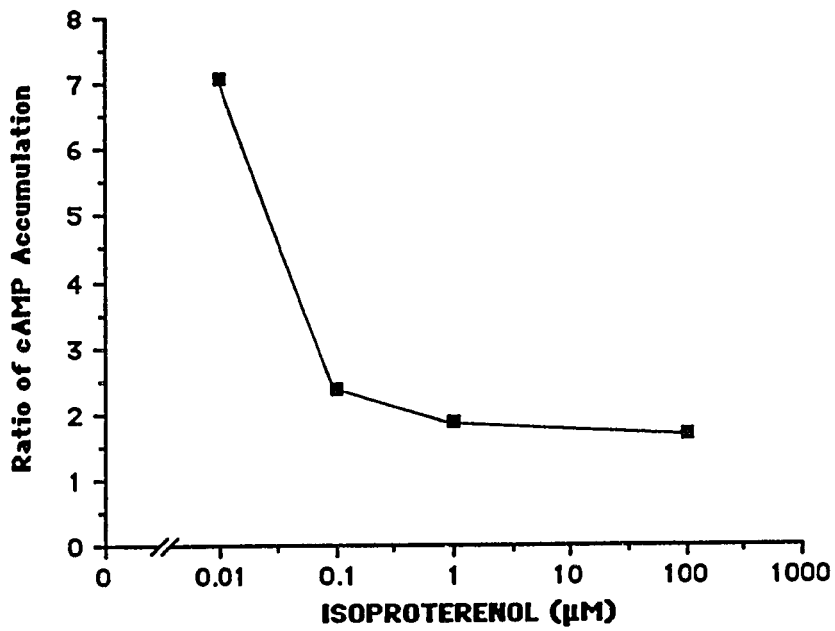

Fig. 12. Effect of Isoproterenol on the Ratio of cAMP Accumulation in Unweighted/Weight-bearing Muscles.

Data are calculated from Fig.11.

Differences in cAMP accumulation could not be attributed to differences in cAMP efflux between the three conditions, since cAMP in the incubation media was below the detectable (0.2 pmol) limit (not shown). Incubations with 1 mM isoproterenol were attempted, but extensive oxidation of isoproterenol under these conditions prohibited the accurate determination of cAMP accumulation. Further experiments are necessary to exclude the possibility of alterations in isoproterenol responsiveness.

It was possible that the greater effects of isoproterenol on glycogen (Chapter 3) and cAMP metabolism (Table 9, Figs. 11 and 12) in unweighted muscle could be attributed to alterations in adenylate cyclase activity. Therefore, we measured soleus cAMP concentrations following incubation with forskolin which activates the cyclase independent of the ß-adrenergic receptor. Forskolin treatment produced similar relative increases in cAMP concentrations (8-20 fold) in all conditions (Table 9). Combined with similar absolute cAMP concentrations following maximal forskolin stimulation, these results support comparable total adenylate cyclase activity in weight-bearing, unweighted, and denervated soleii. These results suggest that the site of enhanced isoproterenol response in the unweighted soleus is proximal to the adenylate cyclase catalytic subunit in the ß-adrenergic receptor-effector cascade.

ß-adrenergic Binding. The most effective means of distinguishing between altered hormone sensitivity and responsiveness is to measure the binding capacity of the membrane receptor. Therefore, [^{3}H] dihydroalprenolol saturation binding experiments were performed with plasma membrane preparations from soleus muscles. [^{3}H] dihydroalprenolol binding appeared saturable and could be inhibited by 10 µM L-propranolol (Fig. 13). Specific binding occurred in zone A (i.e., < 10% of the free radioligand was bound) and represented 60 ± 1% of total binding. Scatchard analysis (Scatchard, 1949) of binding data demonstrated similar receptor affinity (Kd) for [^{3}H]

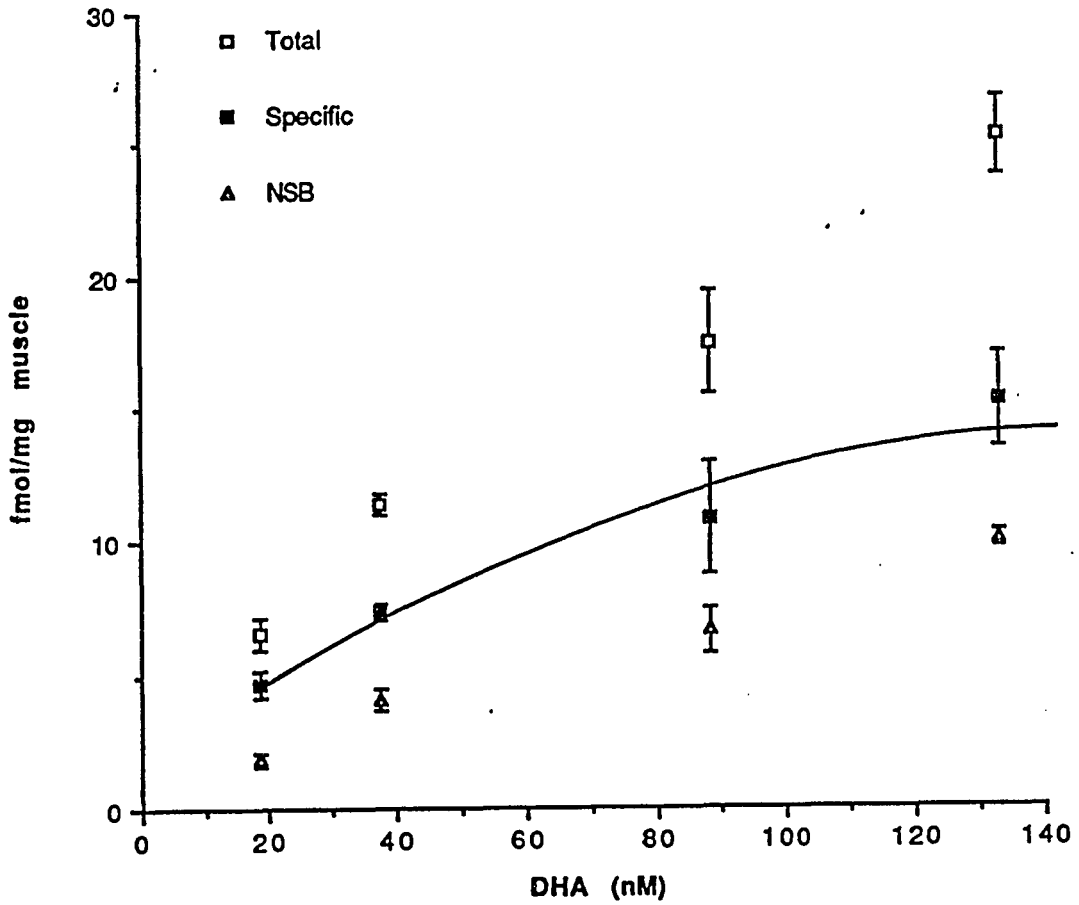

Fig. 13 Saturation Binding Curves for [^{3}H] Dihydroalprenolol Binding in Unweighted Soleus Plasma Membrane Preparations.

Representative binding curves for [^{3}H] Dihydroalprenolol (DHA) binding in unweighted soleus muscle membranes. Each point represents the mean ± SE of triplicate determinations from four experiments. The curve drawn is for Specific Binding data only. Specific Binding (fmol/mg muscle) = Total Binding - Nonspecific Binding (NSB- defined as [^{3}H] DHA binding in the presence of 10 μM L-propranolol).

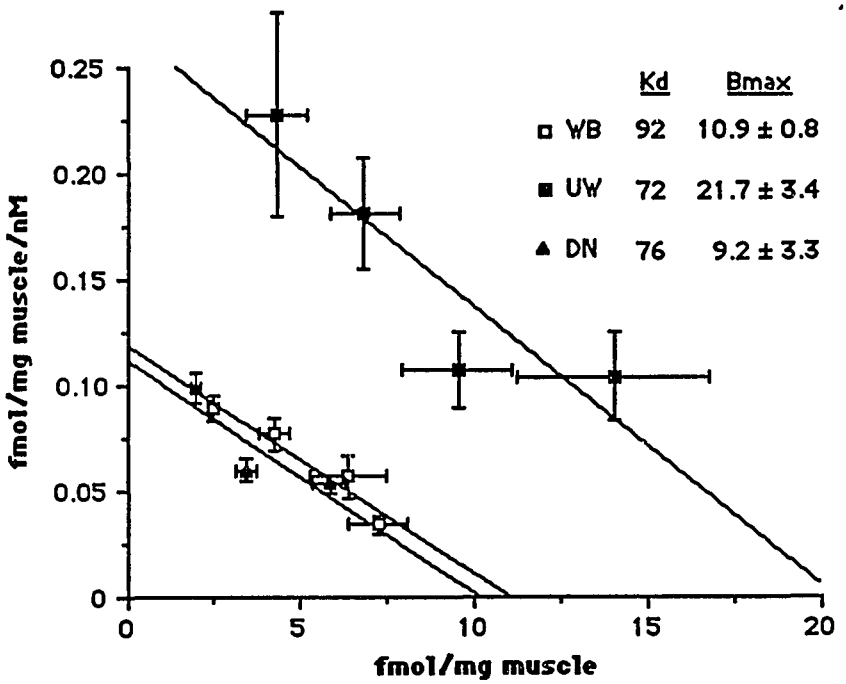

Fig. 14. Scatchard Analysis of [^{3}H]Dihydroalprenolol Binding.

Scatchard plot of saturation binding in muscle membranes from three day weight-bearing, unweighted, and denervated soleii. Estimates of binding affinity (Kd) and maximal binding capacity (B_{max}) calculated by non-linear regression. Each point represents the mean ± SE of triplicate determinations from four experiments.

dihydroalprenolol in the three conditions (Fig. 14). In contrast, maximal binding capacity per milligram muscle was markedly increased in the unweighted soleus as compared to the similar values estimated for weight-bearing and denervated muscles (Fig. 14). When expressed relative to the whole muscle, maximal binding capacity was lower in the denervated soleus (216 ± 79 fmol/muscle) than weight-bearing (467 ± 45) or unweighted (474 ± 74) muscles. These results indicate that the increase in ß-receptor number per milligram muscle in unweighted soleus must be mostly a consequence of muscle atrophy and not of an increase in the total receptor population. The reduction in ß-receptors per muscle following denervation suggests that loss of this membrane protein parallels the decreases in structural proteins with denervation atrophy.

Discussion

Receptor and Post-receptor Stimulation of cAMP Accumulation. We have previously demonstrated an enhanced isoproterenol response of glycogen synthesis and degradation in unweighted soleus (Chapter 3). In that study similar maximal rates of lactate production in weight-bearing and unweighted soleii suggested that the enhanced submaximal isoproterenol response of unweighted muscle was due to alterations in ß-adrenergic sensitivity. However, greater isoproterenol inhibition of glycogen formation in unweighted muscle at the highest concentration of agonist tested left open the possibility of altered hormone responsiveness. Changes in hormone effects can be characterized as alterations in sensitivity and/or responsiveness. Differences in sensitivity or responsiveness are thought to represent receptor and post-receptor modifications, respectively (Kahn, 1980). Thus investigation of receptor and post-receptor stimulation of the ß-adrenergic receptor-effector cascade aided us in identifying the mechanism of enhanced isoproterenol action in unweighted soleus, as discussed below.

In agreement with an enhanced isoproterenol response of glycogen metabolism, submaximal isoproterenol stimulation of cAMP accumulation was significantly greater in unweighted soleus. Furthermore, differences in the stimulation of cAMP accumulation between unweighted and weight-bearing soleii were detected at an isoproterenol concentration 100-fold lower than that observed to produce differences in glycogen metabolism (Chapter 3). This suggests that enhanced insulin sensitivity of glycogen metabolism in unweighted soleus (Henriksen, Tischler, and Johnson, 1986; Bonen, Elder, and Tan, 1988; Henriksen and Tischler, 1988a) may have masked differences in isoproterenol response between the two conditions. While our inability to demonstrate similar maximal isoproterenol response of cAMP accumulation in unweighted and weight-bearing muscles prohibits the definitive exclusion of altered responsiveness in unweighted soleus, the marked decline in the ratio of cAMP accumulation (unweighted : weight-bearing) with increasing isoproterenol suggests that cAMP accumulation is approaching a similar maximum in both conditions. In addition, similar forskolin-induced increases of cAMP concentration in unweighted and weight-bearing muscles eliminates alterations in responsiveness due to changes in adenylate cyclase activity as a potential explanation for the enhanced ß-adrenergic response.

These results cannot exclude alterations in G protein content, efficiency of receptor-G protein coupling, or other changes proximal to the adenylate cyclase catalytic subunit as possible contributors to enhanced isoproterenol response following soleus unweighting. Reports of decreased isoproterenol response of cardiac muscle (Davies, De Lean, and Lefkowitz, 1981; Phornchirasilp and Matangkasombut, 1982), with no alterations in ß-receptor density or affinity (Davies, De Lean, and Lefkowitz, 1981) in adrenalectomized animals, support a possible role for glucocorticoids in altered ß-adrenergic responses. In addition, the ability of dexamethasone treatment to reverse reductions in Gß-protein subunit

mRNA of adipocytes from adrenalectomized rats and to increase Gß-protein subunit mRNA in normal animals suggests the ability of glucocorticoids to modulate ß-adrenergic receptor-effector coupling (Ros et al., 1989). Several-fold increases of plasma glucocorticoids (Jaspers et al., 1989) and soleus glucocorticoid receptor levels (Steffen and Musacchia, 1987) during suspension may provide an explanation for our inability to detect similar maximal isoproterenol responses in unweighted and weight-bearing muscle. Such a mechanism would imply alterations in both sensitivity and responsiveness following unweighting (Kahn, 1980). Clearly, further studies are needed to evaluate this possibility.

In earlier studies of ß-adrenergic response of glycogen metabolism, incubations contained physiological insulin concentrations in order to assess potential differences in insulin-isoproterenol antagonism between conditions. Due to the marked insulin resistance of carbohydrate metabolism in denervated muscle (Smith and Lawrence, 1985), we were unable to determine if the ß-adrenergic response of denervated soleus was altered. However, results from this study clearly indicate that neither receptor- nor post-receptor mediated stimulation of cAMP accumulation is altered following three days of soleus denervation. These results agree with the recent demonstration of similar forskolin-stimulated adenylate cyclase activity and [^{3}H] forskolin binding in 10 d denervated gastrocnemius (Hashimoto et al. 1989). In contrast, a 50% decline in basal, catecholamine-, and fluoride-stimulated adenylate cyclase activity has been reported for mixed hindlimb muscle membranes following denervation for 5 d (Smith, Grefrath, and Appel, 1978). This decrease was attributed to reductions in the amount of enzyme per muscle. The discrepancy between results from this study and Hashimoto et al. (1989) as compared to those of Smith, Grefrath, and Appel (1978) is not clear. However, the absence of phosphodiesterase inhibitors in the latter study confounds the determination of differences in adenylate cyclase activity.

ß-adrenergic Binding Capacity in Atrophic Soleus. Results from isoproterenol effects on glycogen metabolism (Chapter 3) and cAMP accumulation (Figs. 11 and 12) suggest enhanced ß-adrenergic sensitivity in unweighted muscle. In accord with the concept that alteration in sensitivity is a receptor-mediated phenomenon (Kahn, 1980), increased ß-adrenergic binding capacity clearly confirms the interpretation of enhanced isoproterenol sensitivity in unweighted soleus. As with the parallel increased insulin binding capacity and sensitivity of unweighted soleus (Henriksen, Tischler, and Johnson, 1986; Bonen, Elder, and Tan, 1988), enhanced isoproterenol sensitivity in unweighted muscle can be attributed to increased ß-adrenergic receptor concentration. Possible mechanisms for the increase in ß-receptor density could include; 1) receptor up-regulation due to reduced circulating catecholamines (Stiles, Caron, and Lefkowitz, 1984), 2) alterations in hormones (e.g., glucocorticoids) demonstrated to "permissively" effect ß-receptor expression (Davies and Lefkowitz, 1984), and 3) a sparing of membrane receptors during unweighting atrophy (Henriksen, Tischler, and Johnson, 1986; Tischler, et al., 1990). Catecholamine-induced ß-receptor up-regulation seems unlikely as plasma epinephrine and norepinephrine levels are increased during the first several days of suspension (C. Stump, personal communication). If increased plasma glucocorticoids (Jaspers, et al., 1989) had induced ß-receptor expression during unweighting then we should have detected an increase in the total receptor population. These data cannot, however, exclude a role for glucocorticoids in the maintenance of ß-receptors during unweighting atrophy. In accord with previous data for insulin (Henriksen, Tischler, and Johnson, 1986), the similar number of ß-receptors per muscle in unweighted and weight-bearing soleii suggests that the increase in receptors per milligram of muscle is a result of preferential loss of structural proteins and not an up-regulation of the ß-receptor.

In agreement with similar responses of cAMP accumulation to isoproterenol and forskolin (Table 9 and Fig. 11), dihydroalprenolol binding capacity per milligram muscle is similar in denervated and weight-bearing soleus (Fig. 14). The significant reduction in total binding capacity (per muslce) with denervation suggests that receptor and non-receptor proteins are degraded proportionately, thereby preserving a receptor density comparable to weight-bearing muscle. This lack of alteration in ß-adrenergic binding capacity has been previously demonstrated for mixed hindlimb muscle membranes following a five day period of denervation (Smith, Grefrath, and Appel, 1978). In addition, similar ß-adrenergic binding capacity in weight-bearing and denervated soleus agrees with the similar insulin binding capacity in these two conditions (Smith and Lawrence, 1985). These results support the concept that although both unweighted and denervated muscles undergo atrophy, certain hormone responses and binding capacities are distinctly different in these two models of reduced use.

Mechanisms of Proteolysis in Unweighted and Denervated Soleus. One of the principal aims of this investigation was to further evaluate differences in proteolysis between unweighted and denervated soleii. Considerable evidence suggests that membrane proteins are degraded primarily through lysosomal proteolysis (Hare, 1988 and 1990). Thus, increased insulin receptor concentrations in unweighted soleus (Henriksen, Tischler, and Johnson, 1986), but not in denervated muscle (Smith and Lawrence, 1985) led to the hypothesis that lysosomal degradation of membrane proteins may play a greater role in denervation as compared to unweighting atrophy. Recent studies in which injection of the lysosomotropic agent chloroquine significantly diminished atrophy of the denervated soleus, but failed to diminish accelerated proteolysis in unweighting atrophy are in accord with this hypothesis (Tischler, et al. 1990). Likewise, the marked increase in ß-adrenergic receptor concentration in unweighted, but not denervated soleus, in

this study support the idea of differences in lysosomal proteolysis between the two models of reduced muscle use.

CHAPTER 5

SUMMARY: THE ß-ADRENERGIC SYSTEM, CARBOHYDRATE METABOLISM AND PROTEOLYSIS IN ATROPHIC SOLEUS

Hindlimb unweighting by tail-cast suspension markedly alters the carbohydrate metabolism of the rat soleus muscle. The atrophy and decreased contractile activity associated with unweighting produce dramatic increases in soleus glycogen concentration. Studies presented herein examined the time course of changes in muscle glycogen during recovery from unweighting. The principal aim of these experiments was to explore the mechanism governing the triphasic response of soleus glycogen concentrations during recovery. This was accomplished by measuring the activities of glycogen synthase and phosphorylase at their physiological effector concentrations (i.e., fractional enzyme activities). Other investigations were designed to evaluate the recent hypothesis of different mechanisms of proteolysis in unweighted and denervated skeletal muscle (Chapter 1, Fig. 5). These studies employed the novel approach of measuring hormone responses and binding capacities to detect possible differences in lysosomal proteolysis between these two models of reduced use. The following discussion focuses on the major findings of this research and potential directions for future study of muscle atrophy.

When normal weight-bearing function is restored (reloading) in previously unweighted soleus, a triphasic response characterizes the return of muscle glycogen to control levels. The initial phase of recovery (15 min to 2 h) is associated with elevated glycogenolysis, resulting in glycogen concentrations significantly lower than in unweighted or control muscle. Coincidentally, muscle glucose-6-phosphate and the fractional activity of glycogen phosphorylase, measured at the fresh muscle concentrations of AMP, are

increased. The fractional acitivity of glycogen synthase was also increased, likely as a consequence of greater glucose-6-phosphate, its allosteric effector, and reduced glycogen. During the second phase of recovery (2 to 4 h), the continued elevation of glycogen synthase activity, coupled with a decline in phosphorylase activity caused glycogen concentrations to increase above control. The further increase of glycogen concentrations between 4 and 24 h, in excess of the elevated levels in unweighted soleus, did not correlate with enzyme activities. Similar increases (termed glycogen supercompensation) seen in normal muscle following intense muscular activity have been associated with an uncoupling of the inverse relationship between glycogen concentration and synthase activity. In the final phase of recovery (24 to 72 h), the decrease of glycogen to control values was possibly initiated by high phosphorylase activity at 24 h. These findings suggest that measurement of enzyme activities at physiological effector levels probably provide the closest approximations of *in vivo* activity, and therefore, best explain the majority of changes in soleus glycogen content during reloading.

Although unweighted and denervated muscles undergo commensurate atrophy, the attendant alterations in the ß-adrenergic response of carbohydrate metabolism and ß-adrenergic binding capacity are generally different between the two models (Table 10). Isoproterenol responses of insulin-stimulated glucose transport and phosphorylation were unaffected by either unweighting or denervation. However, unweighting significantly enhanced isoproterenol effects on both glycogen synthesis and degradation. Increased receptor-stimulated (isoproterenol), but not post-receptor-stimulated (forskolin), cAMP production suggested enhanced ß-adrenergic sensitivity in unweighted soleus. In contrast, stimulation of cAMP production by isoproterenol or forskolin was not altered by denervation. The mechanism of differences in ß-adrenergic response was further examined by determining ß-adrenergic binding capacity in soleus plasma membranes from each

Table 10. Comparison of ß-adrenergic Responses and Binding Capacities in Unweighted and Denervated Soleus.

Parameter	Unweighted	Denervated
ISO inhibition of Glycogenesis	I	ND
ISO stimulation of Glycogenolysis	I	ND
ISO stimulation of cAMP Production	I	NC
FOR stimulation of cAMP Production	NC	NC
ß-adrenergic Binding Capacity per milligram muscle	I	NC
ß-adrenergic Binding Capacity per whole muscle	NC	D

The ß-adrenergic responses and binding capacities in 3 d unweighted and denervated soleus muscles, as compared to weight-bearing controls, are presented below. Results for ß-adrenergic responses are from *in vitro* incubations containing physiological concentrations of insulin and 1 µM isoproterenol (ISO) or 300 µM forskolin (FOR). ß-adrenergic binding capacities were estimated by non-linear analysis of [^{3}H]dihydroalprenolol saturation binding data. I, increased relative to control; D, decreased relative to control; NC, no change relative to control; ND, not determined.

condition. Unweighted soleus demonstrated a markedly greater ß-adrenergic binding capacity per milligram muscle, without alterations in receptor number per muscle. These results are in accord with the idea that membrane receptors are spared during unweighting atrophy (Chapter 1). Conversely, in denervated soleus, ß-adrenergic binding capacity per milligram muscle was unchanged, whereas binding per muscle was significantly reduced. These findings support similar degradation of receptor and non-receptor proteins during denervation atrophy. Since membrane receptors are degraded primarily in lysosomes, these results suggest a greater role for lysosomal proteolysis in denervation, as compared to unweighting atrophy. Thus, the contrasting ß-adrenergic responses and binding capacities of unweighted and denervated soleii agree with previous observations of different insulin responses and binding capacities in these two models of reduced use.

Recent evidence from this laboratory has demonstrated inhibition of proteolysis by *in vivo* injection of chloroquine, a lysosomotropic agent, in denervated but not unweighted soleus. In addition, mersalyl, an inhibitor of thiol-proteases (i.e., cytosolic proteases), was shown to abolish unweighting atrophy between 2 and 3 d. Combined with results from the present study and earlier investigations, these findings have led to an updated model of differences in proteolysis between unweighted and denervated muscle (Fig. 15). In this model, structural protein degradation by cytosolic proteases increases in both unweighting and denervation atrophy. In contrast, lysosomal proteolysis of membrane receptors is enhanced only in denervated muscle. Thus, inherent differences in proteolysis between these two conditions can be linked to alterations in receptor-mediated effects and ultimately to changes in metabolic response.

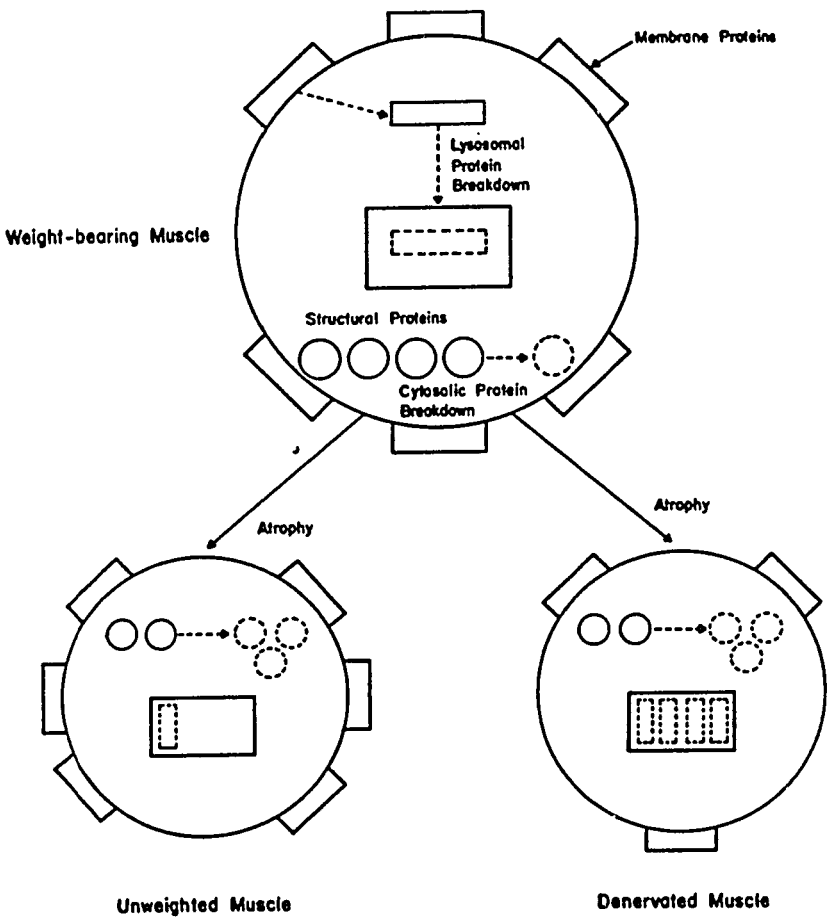

Fig. 15. Model for Accelerated Protein Breakdown in Muscle Atrophy.

As detailed above, the examination of accelerated protein degradation via study of differences in hormonal response provides a unique approach to the mechanisms of proteolysis characteristic of each model of atrophy. Since unweighting and denervation represent only two of several forms of muscle atrophy, future investigations of the type described in the preceding chapters could be utilized to examine mechanisms of proteolysis in immobilization, cancer cachexia, as well as other types of muscle atrophy. Further evaluation of the current model could be achieved by combining *in vivo* injections of lysosomotropic agents with determinations of binding capacity in unweighted and denervated muscles. The fate of receptor proteins could also be followed by utilizing a newly developed antibody to the ß-adrenergic receptor. Depending upon the size of ß-adrenergic receptor-peptide recognized and the minimum detectable quantity of peptide, this antibody may be useful in identifying proteolytic fragments of the the ß-adrenergic receptor in lysosomal fractions of muscle homongenates. In addition, since the ß-adrenergic receptor has recently been cloned, an antibody for small segments of this protein could be produced to identify ß-adrenergic receptor degradation products within atrophic muscle. Finally, the marked increase in the application of molecular biological techniques to the study of muscle atrophy makes it now feasible to examine alterations in the transcriptional and translational control of lysosomal protease expression during muscle atrophy. Thus, while the investigations described herein have contributed to the understanding of mechanisms of proteolysis in unweighted and denervated muscle, a great deal of research examining these and other types of muscle atrophy remains to be accomplished.

APPENDIX A

UNIVERSITY LABORATORY ANIMAL CARE COMMITTEE PROPOSAL APPROVAL

UNIVERSITY OF ARIZONA
Tucson, AZ 85721

VERIFICATION OF REVIEW OF ANIMAL CARE AND USE
BY THE INSTITUTIONAL COMMITTEE

PHS Assurance No. A-3248-01 - USDA No. 86-3

IACUC Control # 86-0273

Title: Adrenergic Influence on Carbohydrate Metabolism in Atrophic Muscle

Principal Investigator: Marc E. Tischler, Ph.D.

Department: Biochemistry

Submission Date: January 13, 1989

Agency: National Aeronautics and Space Administration

The University of Arizona Institutional Animal Care and Use Committee reviews all sections of proposals relating to animal care and use. The above-named proposal:

( ) Has been reviewed and approval withheld.

( ) Will be reviewed within the next 60 days and results of review submitted.

(XX) Has been reviewed and approved by IACUC on January 13, 1989
Revisions (if any), are listed below:
Continuation

Michael A. Cusanovich, Ph. D.
Vice President for Research

Date: JANUARY 13, 1989

APPENDIX B

IODOPINDOLOL BINDING

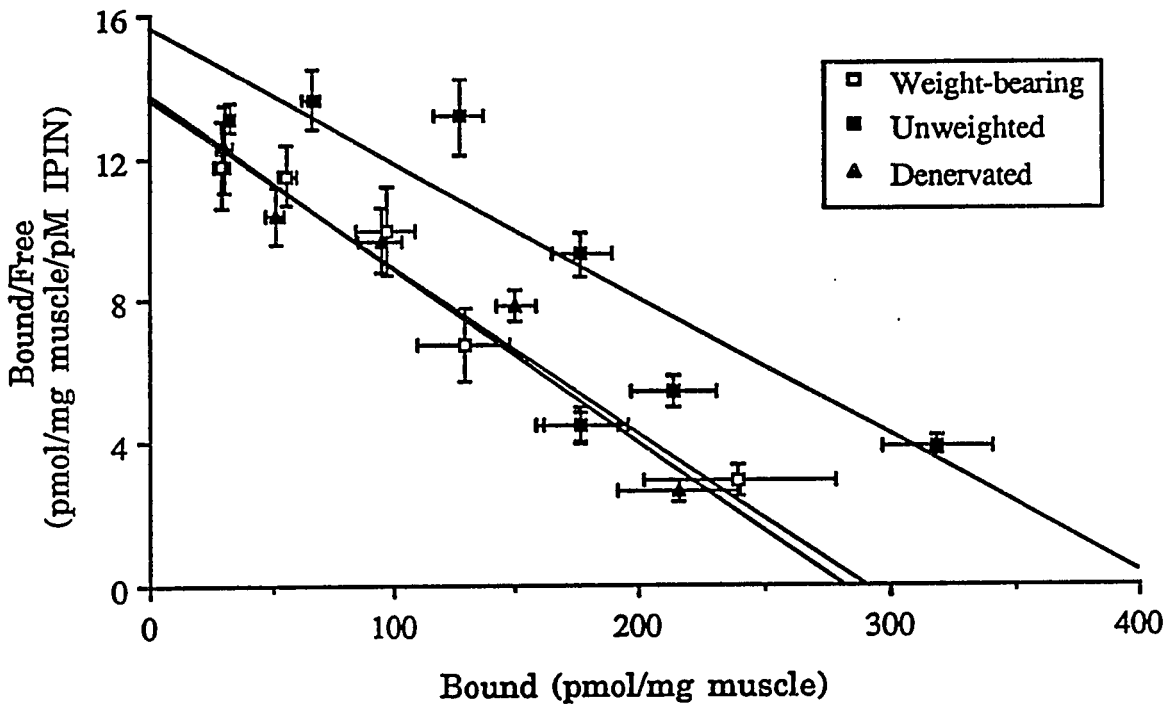

Figure 16. Scatchard Analysis of Iodopindolol Binding

Scatchard plot of saturation binding in muscle membranes from three day weight-bearing, unweighted, and denervated soleii. Estimates of binding affinity (Kd) and maximal binding capacity (B_{max}) calculated by non-linear regression. Each point represents the mean ± SE of triplicate determinations from three to four experiments.

Table 11. Soleus Iodopindolol Binding per Whole Muscle

	Weight-bearing	Unweighted	Denervated
Bmax (fmol/muscle)	11.2 ± 0.8	11.5 ± 1.2	7.8 ± 1.1*

Values are means ± SE for triplicate determinations in 3-4 preparations. * $P<0.05$ compared to weight-bearing.

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