EFFECTS OF HYPOBARIC HYPOXIA ON SKELETAL MUSCLE ENZYMES AND GLUT-4 CONTENT IN DEVELOPING AND MATURE ANIMALS

by

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Effects of Hypobaric Hypoxia on Skeletal Muscle Enzymes + Glut-4 content in Developing and Mature Animals

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Abstract

The adaptations to chronic hypobaric hypoxia have been well characterized at the cardiovascular, hematological and respiratory level. Alterations in skeletal muscle, however, are less well defined, and often confounded by the changes in physical activity and diet associated with altitude exposure. An increased reliance on the catabolism of glucose following adaptation to altitude has been suggested, based on the high yield of ATP per mole oxygen with this fuel. The rate limiting step for glucose utilization is its transport into skeletal muscle. Since the tissue content of the insulin regulatable glucose transporter (Glut-4) is highly correlated to transport capacity, it was of interest to determine if an up regulation of content occurs in response to hypobaric hypoxia. In addition, several investigators have demonstrated a positive relationship between Glut-4 content and skeletal muscle oxidative capacity, highlighting the possibility of co-regulation of these factors in response to hypobaric hypoxia. Further, as protein synthetic capacities decline with maturity, the adaptation capacity of young and mature animals may differ.

Mature (6 month) and developing (3 week) female Sprague Dawley rats were group housed and randomly assigned to either control, dietary restricted control or hypobaric hypoxia groups for either one or four weeks (n=6 per group per age). After a four day adjustment to a 12 hour light dark cycle in all groups, the altitude groups were acclimated over four days to a final pressure equivalent to 16,000 ft (400 mmHg) within a vacuum chamber designed and constructed for this experiment (R. Dill, V. Stobbs, and W.S. Parkhouse).

The maximal activities of key enzymes of aerobic/anaerobic glycolysis and the Krebs cycle were then assessed in soleus and plantaris muscle. Analysis revealed no significant differences in the maximal activities of these enzymes or in any system relationships (enzyme activity ratios) as a result of hypobaric hypoxia or dietary restriction. Higher activities of several enzymes were found in the developing animals compared to group matched mature animals. Thus, it appears that skeletal muscle metabolic capacity was unaffected by four weeks exposure to hypobaric hypoxia.

Analysis of Glut-4 protein content, however, revealed a significant elevation in both the soleus (~37 percent) and the plantaris (~26 percent) muscles of young, four week altitude exposed animals when expressed per mg of muscle. Glut-4 content observed, in these instances, was directly attributable to an increase in total protein content, as no differences were observed in glut-4 content per ug protein. No differences were detected in Glut 4 content between experimental conditions in the muscles of mature animals. Soleus muscle samples were obtained after one week of altitude exposure to determine the relative rate of adaptation. This analysis revealed a significant increase (~25 percent) in Glut-4 content per mg tissue and per ug protein, and suggested that adaptation is fairly rapid. It also appears that metabolic capacity and Glut 4 content are not co-regulated in response to sedentary exposure to hypobaric hypoxia.

Acknowledgments

I would like to thank my friends, family, and lab mates for their support, through laughter, understanding and distraction, throughout my graduate work. I would also like to acknowledge Wade for trying to understand me, and creating the role that I needed for the end. For those who gave me their time and knowledge, Dr. S. Chadan, Dr. P. Willis, and Dr. L. Megeney, I wish continual growth, and give a sincere promise to provide as willingly to the neophytes of the future.

Dedications

To the rats;

withdrawn from the race,

because they are ugly and can not scream in english.

Table of Contents

List of Tables

List of Figures

Introduction

The capacity of mammals to perform and adapt aerobically at altitude has been the subject of extensive research. As a perturbation to whole organism metabolic profile and as a trigger for the adaptation of various organ and tissue systems, both acute and chronic hypobaric hypoxia have been the source of many ongoing debates and unanswered questions. What is known, is that sea level mammals, of various species, display a lower capacity for aerobic exercise at moderate altitude (15000ft; 4500m; 450 Torr; 400 mmHg) as a result of a decrease in the ambient oxygen tension (Sutton et al. 1988). Further, native altitude species (Hochachka et al, 1991) and chronically altitude exposed sea level mammals (Matheson et al. 1991; Horstman, Weiskoph and Jackson 1980; and Maher, Jones and Hartley 1974) display a higher work capacity and endurance time at altitude.

The means through which these chronic changes in exercise performance are accomplished, whether genetic or acclamatory, has not been fully elucidated. There are many potentially adaptable tissues in the pathway of oxygen from the air to its reduction within mitochondria. One tissue which may contribute to these changes in exercise performance following chronic altitude exposure is skeletal muscle. This tissue displays a high degree of plasticity, as reflected by changes in metabolic capacity with development, adaptation to disease and exercise training. The degree to which skeletal

muscle metabolic capacity adapts to short term chronic altitude exposure is relatively unknown. Many previous studies have been confounded by altitude-induced changes in physical activity and diet. An alteration in skeletal muscle metabolic provision of ATP with lower ambient oxygen tension is likely, and may be necessary to increase functional capacity at altitude.

Skeletal Muscle Metabolism

General

The energy required for skeletal muscle contraction is provided through the catabolism of lipids, carbohydrates, proteins and phosphocreatine for the production of adenosine triphosphate (ATP). Phosphocreatine, converted by creatine phosphokinase (CPK), is the first energy pool used to buffer ATP supply, and is used to support short burst type activity (Hochachka 1994). Thereafter, carbohydrates are broken down via the enzymes of glycolysis. They enter glycolysis from endogenous stores of glycogen through the activation of phosphorylase, or, alternatively, are transported into the cell via glucose specific transporter proteins, with subsequent phosphorylation by hexokinase (HK). The end product of glycolysis, pyruvate, can then be reduced to lactate (via lactate dehydrogenase (LDH)) anaerobically yielding 2 ATP from glucose and 3 ATP from glycogen. Alternatively, the three carbons

of pyruvate can be fully oxidized in the mitochondria via the Krebs cycle and the Electron Transport Chain (ETC) to yield 36 (glucose) or 37 (glycogen) ATP. The reducing equivalent NADH must be transferred into the mitochondria via shuttle mechanisms (involving Malate Dehydrogenase or alpha glycerophosphate) in order to maintain redox balance within the cytosol.

Free fatty acids (FFA) and glycerol make up the predominant forms of oxidizable lipids within the body. These are stored as triglycerides within muscle and adipose tissue. Triglycerides are broken down within the cell by triglyceride lipase, activated by adrenergic agonists. The entry of FFA into the mitochondria, through the aid of carnitine palmitoyl transferase (1 and 2), allows its breakdown into two carbon units (acetyl Co-A) through the B-oxidation cycle. These units are then incorporated into the Krebs cycle and the ETC for complete oxidation. The yield from the FFA palmitate (16 carbon unit) is 129 ATP, and three to four fold greater than the per carbon yield from carbohydrate.

A preference for the use of exogenous stores of glucose (liver) and FFA (adipose) during submaximal exercise prevents the depletion of endogenous stores, which signals muscle fatigue, and can be found best exploited in endurance adapted species and athletes (Weber 1989).

These pathways are all utilized to some extent during rest and maximal activation, with predominance being altered by the demand of the system. Anaerobic glycolysis is utilized for high power output due to its capacity to supply ATP rapidly. However, the low yield of ATP and the build up of the end products H+ and lactate make this pathway unfavorable except for the highest work rate activity. Aerobic glycolysis and FFA oxidation are both utilized during submaximal work, glycolysis yielding significantly more ATP per mole of oxygen utilized (10 percent theoretically, 25-40 percent empirically, due to FFA oxygen wasting) (Hochachka 1985).

Control / Supply and Demand Coupling

It can be said that the demand for muscle activity, as initiated by central nervous system stimulation of acetylcholine receptors at the motor end plate, is the ultimate determiner of ATP requirement within contracting skeletal muscle (Hochachka 1994). The resulting modulation of specific pathway flux is then determined by a combination of redox balance (NADH/NAD+), phoshorylation potential [ATP, ADP, AMP, IMP, Pi, Pcr, Cr], and the influence of regulatory hormones (Hochachka 1994).

The adrenergic induced release of phosphorylated free alpha-GTP, along with the calcium influx of contraction, initiates the activation of phosphorylase, and the breakdown of glycogen (Hochachka 1994). Flux through the pathway

is then determined by the isozyme properties of the regulatory enzymes phosphorylase, PFK and PK, which are in turn modified by the concentration of the adenylates (ie. ATP, AMP) and substrates (ie. F26P2, carnitine) (Hochachka 1994).

Regulation also exists in the competition between pathways for substrate. The predominance of phosphocreatine production is ensured by the low Km of CPK for ADP (Hochachka 1994). With prolonged demand, when phosphagen stores run out, an increase in ADP concentration activates the enzymes of glycolysis (Hochachka 1994). Higher levels of regulation can also be conferred by changes in isozyme content. In the competition for pyruvate a shift in the isozyme towards heart type (as seen with endurance adaptation) results in a predominance of lactate reduction to pyruvate, and the complete oxidation of carbohydrates (Hochachka 1994).

Acute aerobic flux, at the level of the ETS, is modified by metabolites and the availability of O2 (Hochachka 1994). Analysis of isolated dog gracilis demonstrated that oxygen availability may modify demand, and thereby alter ATP turnover requirements (Arthur et al. 1992). Balance between pathways, therefore, is also regulated by oxygen availability, though perhaps indirectly (Arthur et al. 1992). When the demand for ATP goes beyond the capacity of aerobic systems to supply it, alterations in energetic state ([ADP]) result in increases in anaerobic glycolysis (Hochachka 1994).

The maintenance of high ATPase activity without perturbation of the adenylate pool, and the inherent modification of flux capacity, is termed supply demand coupling (Hochachka 1994). A tightly coupled, or highly efficient, tissue is characterized by low changes in [ATP] (Hochachka 1994). The percent imbalance of a system has been defined as

change [ATP] * 100 / total ATP turnover

Endurance adapted species and individuals have high mitochondrial volume per gram muscle, increasing the capacity for aerobic flux. This confers a decrease in the required flux per ETS unit in order to maintain a given ATP demand per gram of muscle. Each ETS unit, therefore, operates at a lower [ADP] and [Pi] providing increased aerobic ATP output with lower perturbations of adenylate balance (Hochachka 1994).

It is obvious that the goal of homeostatic organisms is to maintain the optimal levels of intermediates to facilitate efficient function. The characteristics involved in these processes are genetically alterable and adaptable. This is evident with species differences as well as training and environmental response, and is extremely important in the long term maintenance of homeostasis.

Analysis of metabolic activity

The analysis and comparison of skeletal muscle metabolic potential within and between species has been extensive. Several and varied tools for the analysis of metabolic activation have been established. While the in vivo use of radiolabeled substrates and Nuclear Magnetic Resonance (NMR) imaging are possibly the most direct indicators of fuel utilization, they are not always the most practical methods of analysis. In vitro enzyme activity has often been used to delineate metabolic pathway flux capacity (Newsholme and Crabtree 1986). A novel approach to the illustration of maximal pathway flux and, therefore, fuel choice, within, and between, skeletal muscle fiber types has been the comparison of the maximal in vitro activity of representative enzymes from the various pathways of ATP production (Pette and Hoffer 1980). Through the analysis of several species and fiber types, these investigators established that the absolute activity of several enzymes within a pathway exist in constant relative proportions (Pette and Hoffer 1980), and that the absolute levels of their activities represent the capacity of flux for that pathway (Pette and Hoffer 1980). Further, extensive comparisons of the activity of selected representatives of these groups illustrated that system relationships exist within the fiber that indicate its metabolic organization (Pette and Hoffer 1980).

The organization of skeletal muscles at the extremes of adaptation have been investigated in this manner. Muscles adapted for predominantly glucose oxidation (hummingbird flight muscle), and muscles of human endurance athletes, display a high pyruvate kinase to lactate dehydrogenase (PK/LDH) ratio and malate dehydrogenase (MDH) and citrate synthase (CS) to LDH ratio (Hochachka et al. 1983; Hochachka 1992b). In contrast, the predominantly free fatty acid (FFA) utilization in heart is illustrated by a relatively low PK/LDH ratio (Hochachka et al. 1992). This organization indicates a high capacity for pyruvate production, with a low reduction potential. Further, the high level of MDH indicates a competitive advantage of this enzyme for the transfer of the reducing equivalent NADH into the mitochondria, and the complete oxidation of carbohydrates. The use of these illustrative examples will aid in the investigation of the adaptations occurring with altitude exposure.

A living example: Altitude populations

Investigation of native altitude populations has illustrated several genetic or developmental adaptations (Hochachka et al. 1991) that lend themselves to higher work capacity at altitude. Adenylate pool analysis of Andean natives during exercise at sea level and altitude indicates that the adaptation phenotype of these altitude dwellers is unique (Matheson et al. 1991). The

metabolic coupling exhibited in the Quechuas (Peruvian Andes) was similar to that found in highly adapted endurance athletes and much tighter than for power trained athletes who displayed a similar VO2 max (Matheson et al. 1991). Although the absolute work capacity was not elevated and only a moderately high aerobic capacity was found, these people displayed an extremely reduced anaerobic capacity (Matheson et al., 1991), as evident by low production of lactate for a given work rate (Hochachka et al. 1991). In addition, these altitude subjects had PK and MDH to LDH ratios approaching the high levels of endurance athletes (Hochachka 1992a,b; Hochachka 1992). A Respiratory Quotient (RQ) near unity during submaximal exercise (Hochachka et al. 1991), is further indicative of a preference for the efficient aerobic catabolism of carbohydrates. That these changes were also observed in native altitude animal species (Hochachka 1985) demonstrates the appropriateness of these adaptations to the imposed environment.

Adaptation to Chronic Hypobaric Hypoxia

The analysis of sea level mammals following adaptation to chronic exposure to moderate hypobaric hypoxia (4,300M) indicates that the adaptations occurring are similar to those found in native populations. A comparison of altitude natives and acclimated lowlanders during exercise found no differences in the work efficiencies and VO2 max responses between these groups (Favier et al 1995a).

There is consistent account in the literature of an increase in work capacity and endurance time following acclimation (Matheson et al. 1991; Horstman, Weiskopf and Jackson 1980; Maher, Jones, and Hartley 1974). These changes in capacity occur without a significant change in VO2 max (Matheson et al. 1991) and a lowering in blood lactate (Edwards 1936; Brooks et al 1991b); Green et al. 1992), as a result of decreased muscle lactate release (Bender et al. 1989). After chronic altitude exposure, rest and moderate exercise are associated with increased utilization of exogenous glucose (Brooks et al. 1991b) and a sparing of endogenous glycogen (Green et al. 1992; Young et al. 1982). Response to exercise also elicited lower [ADP], and higher ATP/ADP ratios indicative of an increased tightness of coupling within adapted skeletal muscle (Green et al, 1992).

Absolute enzyme activity has been investigated in sea level and adapted mammals by various groups. An increase in oxidative enzyme content (CS, HAD) and a decrease in anaerobic (LDH) activity was found within rat fast twitch muscle adapted to training at elevation (Bigard et al. (1991b). An increase in glycolytic capacity (HK and LDH activity) in fast and slow twitch muscle with sedentary exposure was also noted (Bigard et al. 1991b). A similar enhancement of training adaptation at altitude has been observed in humans (Terrados 1992). In addition, a moderate altitude (1750 m) training study on rats (11 weeks) revealed increased oxidative capacity over sea level controls,

that was subsequently matched by a two week acclimation of the sea level group (Terblanche et al. 1984).

In contrast to the above data on oxidative capacity, human high altitude studies, at various work rates, have illustrated a decline in oxidative enzyme activity following acclimation (Cerretelli et al. 1990; Howald et al. 1990). These investigators (Cerretelli et al. 1990) also revealed an increase in glycolytic capacity (PFK activity), but this finding was not supported in a subsequent study at moderate altitude (Green et al. (1992).

Morphological enhancements with altitude training have also been noted. Increases in skeletal muscle capillarization (Bigard et al. 1991a; Fisher, Schrader and Klitzman 1992) and hematocrit (Fisher, Schrader and Klitzman 1992; Snyder, Wilcox and Burnham 1985) with aerobic training in hypoxia, are thought to increase oxygen delivery at various partial pressures (Fisher, Schrader and Klitzman 1992). Sedentary adaptation, however, resulted in no alteration in capillary to fiber ratio (Sillau and Banchero 1977), but illustrated a decrease in fiber cross sectional area (FCSA) (effectively decreasing the diffusion distance) and an elevation in hematocrit (Snyder, Wilcox and Burnham 1985; Bigard et al. 1991a); Fisher, Schrader and Klitzman 1992).

Altered Carbohydrate Utilization

There is much inconsistency in the data obtained to date on the direction and degree of alterations to chronic hypoxia. A theory, put forth by Peter Hochachka in various publications (Hochachka et al. 1983, Hochachka 1985;), postulates that an increased reliance on glucose oxidation would both decrease the competitive flux to lactate (lactate paradox) as well as incur an increased yield of ATP/mole of oxygen taken up (VO2 Max paradox). There exists a clear line of evidence within the literature which supports this possibility. Much of this evidence comes from studies involving exercise training at various levels of hypoxia. Firstly, an increased oxidative capacity (Bigard et al. 1991b) and an increase in the stability of the adenylate pools during exercise (Green et al. 1992) indicate that there is tighter coupling in adapted individuals. An exercise RQ near unity in altitude populations supports this contention and indicates that carbohydrates are being utilized to a greater extent (Hochachka et al. 1991). This is also evident during exercise in adapted individuals, as there is an increased reliance on exogenous glucose (Brooks et al. 1991a). A decreased muscle release of lactate (Bender et al. 1989) and an endogenous glycogen sparing effect (Young et al. 1984; Green et al. 1992) during adapted exercise at altitude further indicate that this change may be supported by exogenous glucose flux (Brooks et al. 1991a).

Research into the potential for metabolic adaptation within skeletal muscle have indicated that metabolic organization (Pette and Hoffer 1980; Etgen et al. 1993b), and glucose utilization (Henriksen et al. 1990; Kern et al. 1990) are adaptable under the stress of exercise and disease. This illustrates the potential for muscle to adapt to the stress of hypobaric hypoxia.

An increased dependence on the oxidation of exogenous glucose may require an enhancement of the capacity for its transport into the cell (Brooks et al. 1991b), as this has been shown to be the rate limiting step for its utilization (Weber 1988; Furler et al. 1991; Ren et al. 1993). This has been demonstrated most recently through the use of dual tracers, in which uptakes of radiolableled 2-deoxy-D-glucose and 3-0-methylglucose were compared (Furler et al. 1991).

Glucose Transport

The facilitated diffusion of glucose between the interstitium and the cytosol of mammalian tissue is accomplished by the Glut family of transporters (Km 5-10 mM) They are thought to function via the alternating conformation model, allowing bi-directional flow (Mueckler 1994). There are six known members of this family, Glut 1-5, and Glut 7, that have cell and tissue specific expression and regulation (Mueckler 1995). These transporters share 65 % homology which is contained mostly within the twelve membrane spanning

sequences (Klip and Paquet 1990). Both the amino and carboxy termini of these transporters are intracellular. Difference between transporters are evident in the exposed regions, facilitating tissue specific regulation.

Of importance in this discussion of skeletal muscle adaptation are Glut 1 (45-50,000 Kd) and Glut 4 (43,000 Kd). Glut 1 is found in endothelium, brain, fetal tissues, and skeletal muscle (Mueckler 1995). About 5% of skeletal muscle transporter content is contributed by Glut 1, which is found in the plasma membrane, and contributes to basal transport (James et al. 1988; Klip and Paquet 1990; Mueckler 1995). The insulin regulatable transporter, Glut 4, is found in skeletal muscle and adipose tissue (Mueckler 1995). It accounts for the remainder of skeletal muscle content, and is found within intracellular vesicles, and the plasma membrane (Klip and Paquet 1990). Acute enrichment of the plasma membrane content, through the translocation of the vesicles to the plasma membrane, results in a 2 to 20 fold enhancement of transport (Klip and Paquet 1990, Slot et al. 1991). This elevation, required to deal with altered metabolic states, can be triggered by insulin binding, acute muscle hypoxia, contraction and cAMP (James et al. 1988; Slot et al. 1991; Marrette et al. 1992; Mueckler 1994). The mechanisms involved in the regulation of translocation to/from the intracellular pool have not been fully elucidated. Recent work indicates that Glut 4 is involved in a constitutive cycle between the Golgi associated vesicle pools and plasma membrane, with second messengers altering the rates of cycling (Mueckler 1994). Separate

studies have found regions in both the carboxy (Verhey et al. 1993) and amino (Piper et al. 1993) terminal regions of Glut 4 that are sufficient and necessary for sequestration in host cell lines (Mueckler 1994). The apparent discrepancy in these findings has yet to be resolved.

The positive correlation evident between the total cellular content of GLUT 4 transporter protein and the degree of response to acute stimulation provides investigators with a means to analyze the potential for exogenous glucose utilization (Henriksen et al. 1990; Kern et al. 1990; Marette et al. 1992; Ren, Semenkovich and Holloszy 1993). When transporter capacity has been analyzed in this way, a close relationship to oxidative capacity within the skeletal muscle has been observed (James et al. 1989). This is illustrated with fiber type analysis (James et al. 1989; Richardson et al. 1991) and training adaptation (Ploug et al. 1990; Richardson et al. 1991; Marette et al. 1992 ; Etgen et al. 1993b). That these capacities, oxidation and glucose transport, are co-regulated has been postulated based on muscle response to creatine analog (B-gunidinopropionic acid) administration (Ren, Semenkovich and Holloszy 1993). The potential for adaptation seems to be greatest in muscles with predominantly fast twitch fibers, and may be the result of a shift towards type II a fibers(Rodnick et al. 1992; Etgen et al. 1993a,b). The differences in response of fiber types to perturbation evident with exercise (Etgen et al. 1993a) demonstrates the need to analyze muscles of various fiber composition in an investigation of altitude adaptation.

The Sprague Dawley Rat Model

The animal model chosen for this thesis was the female Sprague Dawley rat. This choice was made for handling and housing reasons, as well as ease of comparison to the literature. The muscles we chose to analyse, the soleus (slow oxidative) and the plantaris (mixed fast glycolytic and fast oxidative glycolytic), represent two major metabolic classification of skeletal muscle. Fiber type changes in these muscles have been demonstrated with development over the first year of life (Maltin et al. 1989). An increase in the predominance of slow oxidative fibers is evident in the soleus, and a slight shift in predominance to fast oxidative glycolytic is evident in the plantaris (Maltin et al. 1989). While these changes are applicable to all experimental groups, they may be important in the discussion of observed alterations over the experimental period.

In addition to the metabolic changes occurring with development, alterations in glucose transporter compliment are evident over the rats first year of life. The expression of Glut 1 (mRNA and protein) in pooled hind limb musclulature is seen to decline from 100 to 5 % of fetal values within about two weeks of birth (one week prior to weaning) (Santalucia et al. 1992). The concomitant increase is the percentage of Glut 4 (pooled hind limb) follows a parallel time course (Santalucia et al. 1992). In addition, the capacity for

glucose uptake and the sensitivity to insulin changes during this time period. A decrease in hindlimb glucose uptake between 4 and 20 week old animals, as well as a significant differences in both basal and insulin stimulated uptake between 3, 5 and 16 week animals is evident (Goodman et al. 1983). Further, Glut 4 content has been shown to decline between 1 and 10 months in several skeletal muscles (excluding soleus) (Gulve et al. 1993; Cartee 1994) which illustrates that many variables may be at work over the developmental periods chosen for this experiment.

Dietary Influence

A reduction in food intake, weight loss, and/or reduced growth rate are observed frequently with ascent to moderate or high altitude and with acclimation to simulated hypobaric hypoxia (Sillau and Banchero 1977; Rose et al. 1988; Hoppeler et al. 1990; Bigard et al. 1991b). The source of this weight loss is not clear. A human study attributed a 1.6 percent decrease in body mass following 40 days at extreme hypobaric hypoxia (50 percent reduction in diet) to fat-free mass (Rose et al. 1988). Relative lean body mass, however, was unchanged in rats subjected to 14 weeks at moderate altitude (Bigard et al. 1991b).

The influence of chronic hypoxia in rats on weight gain seems to be transitory. Reduction in weight gain in both trained and sedentary animals

was confined to the first three weeks of a fourteen week experiment (Bigard et al. 1991b), and the first week of a six week experiment, both at moderate altitude (Sillau and Banchero, 1977).

Chronic nutritional deprivation has been studied in animal models, and has been shown to result in alterations in skeletal muscle mass (Boreham et al. 1988). Increases in connective tissue content and declines in muscle protein have also been observed, without changing fiber complement (Boreham et al. 1988). Dietary restriction is associated with binge ingestion followed by a majority of the day without access to food. This 'piecemeal, type feeding results in acute declines in protein synthesis, and a concomitant loss in muscle protein content (Boreham et al. 1988 ; El Haj et al. 1986). While these observations are valuable for our discussion, the severe dietary restriction in these studies (50-60 percent ad libitum), reduces their applicability to the situation at altitude (75-80 percent ad libitum) (Bigard et al. 1991b; personal observations).

Increased glucose uptake in controlled hind limb preparations, similar to that observed with exercise training, have been shown with moderate dietary restriction (85 percent ad libitum) (Ivy et al. 1991). Recently, however, the fasting glucose uptake following a food restriction protocol was seen not to be altered by the observed declines in insulin, glucagon and circulating insulin (Rao 1995). These investigators hypothesize an alteration in insulin

sensitivity and whole body glucose balance in response to diet restriction (Rao 1995). This indicates that the changes observed previously (Ivy et al. 1991) may have been confounded by imposing a standardized protocol on an altered homeostatic system. Investigation of muscle compliment of Glut 4 with dietary restriction indicated that increases in insulin stimulated glucose uptake in muscle was not due to changes in Glut 4 content, as no change in mRNA was noted (Wake wt al. 1991).

Objectives

It was the goal of this thesis to gain further understanding of mammalian adaptation to chronic hypobaric hypoxia. Specifically, the investigation of glucose metabolism, based on the quantification of metabolic enzyme activity and glucose transport capacity. This contributes to the delineation of the paradoxes of reduced lactate production and VO2 max that occur with adaptation to hypobaric hypoxia. In addition, a greater understanding of the control, regulation and plasticity of glucose uptake and metabolism, will aid the goal of combating disorders, such as diabetes mellitis, which involve the control of glucose utilization.

Hypothesis

It was hypothesized that adaptation to chronic hypobaric hypoxia would result in an increased dependence on aerobic exogenous glucose utilization by skeletal muscle, and that this would be reflected by :

1. an enhanced glucose transporter 4 content in skeletal muscle,
2. a shift in the enzymatic profile of the tissue to favour aerobic glycolysis, and,
3. a greater degree of adaptation in predominantly fast twitch muscle.

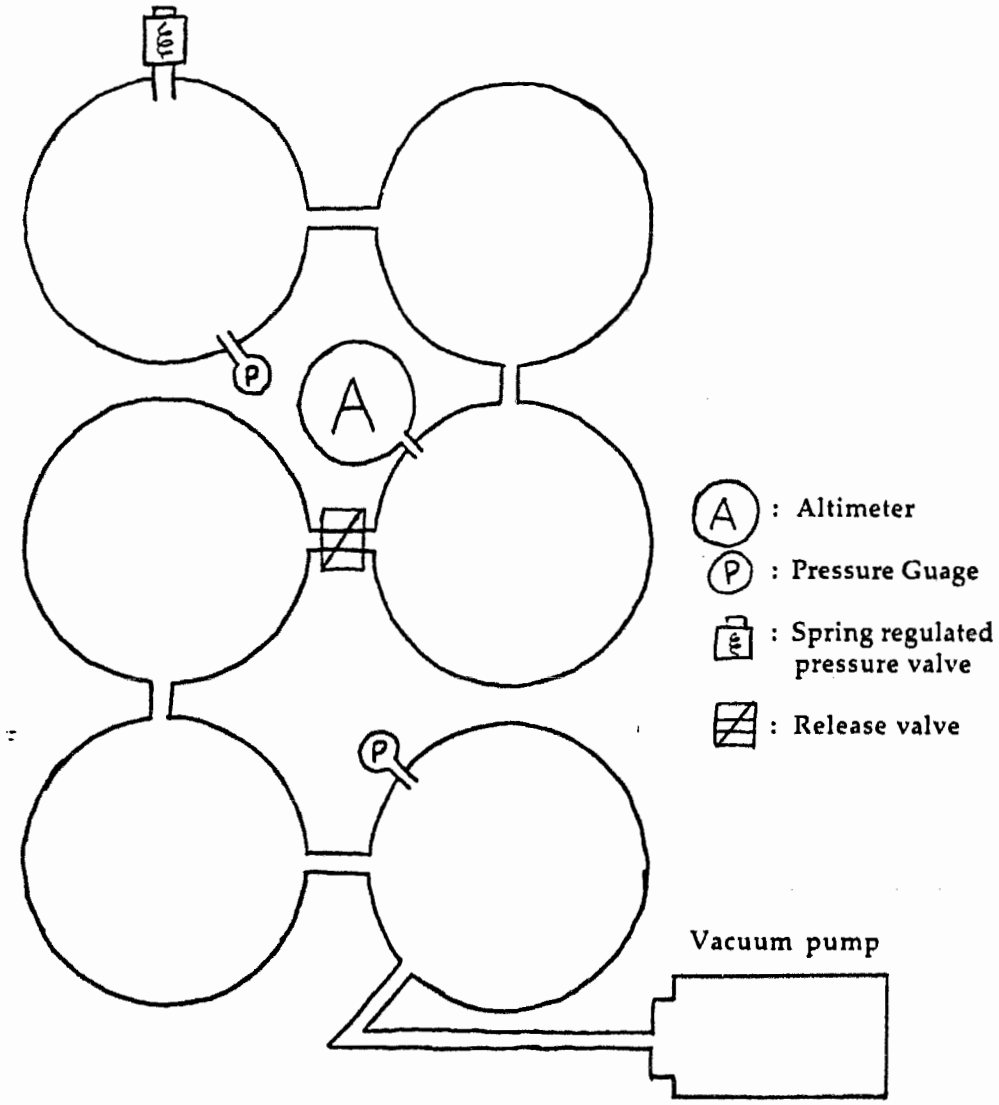
Methodology

Animal Treatment

Female Sprague Dawley rats (21 days and 6 months of age), supplied by Charles River, were randomly assigned to control (C), hypobaric hypoxic (H) or restricted diet (R) groups of n=12/age. Animals were maintained in our animal care facility for four days prior to initiating experiments. All animals were group housed, controls and hypoxic groups were fed ad libitum. Restricted dietary and control groups remained at sea level (Simon Fraser University Animal Care Facility, ~1000 ft). The hypoxic group was exposed to hypobaric hypoxia simulated within a vacuum chamber (designed and constructed by R. P. Dill, V. Stobbs and W. S. Parkhouse) (Fig. 1) with the use of a 3/4 Horse Power TaskAir rotary air pump. Altitude, was determined by an United Instruments altimeter (calibrated from 1,000 to 20,000 ft.). Hypobaric hypoxic animals were brought to 10,000 feet (525 Torr) for two days, then 12,000 ft. (495 Torr) for one day, prior to being maintained at 16,000 feet (405 Torr) for four weeks. Animals were returned to sea level once a day for ~20 minutes to change water and bedding and to provide food. A restricted diet group was also kept at sea level, food intake for these animals was controlled in order to mimic any potential weight changes of the altitude group (Figs. 5 and 6). Half the animals from each group (n=6) were sacrificed after 1 week, and the remainder (n=6) were sacrificed after 4 weeks. Animals

Figure 1 :

Schematic of Hypobaric Chamber
(Front view only)
Each Chamber is 3 ft. long and 21 ins. in diameter.



were anaesthetized using a 75 mg/kg intraperitoneal injection of somnitol (MTC Pharmaceuticals). Muscles were excised, immediately frozen in liquid nitrogen and stored at -80 °C until analysis. Sacrifice was by cardioectomy.

Enzymatic Analysis

The maximal activity of representative enzymes of aerobic / anaerobic glycolysis and the citric acid cycle were investigated to delineate muscle metabolic profile. The activity of the glycolytic enzymes, lactate dehydrogenase (LDH), pyruvate kinase (PK) and hexokinase (HK) were determined in 20 mg samples of plantaris and soleus muscle. Tissue was weighed frozen, and homogenized 3 times for 15 sec (Ultra Turax TP 18/10 ST at 70 percent of maximum on a Tekmar TR-10) in 60 volumes of 50mM Imidazole Chloride (I-Cl, pH 7.4), 1 mM EDTA, 0.1 percent Triton X-100, and 5 mM DTT. Samples were then spun down at 10,000g for two minutes (Hermle Z360 K centrifuge) and the supernatant decanted. Homogenates were kept on ice, maximum 10 minutes, until enzymatic analysis was performed.

Citrate synthase (CS) and malate dehydrogenase (MDH) activities, were obtained from 20 mg samples of soleus and plantaris muscle, homogenized 3 times for 15 sec (Ultra Turax TP 18/10 ST at 70 percent of maximum on a Tekmar TR-10) in 60 volumes of 50 mM I-Cl (pH 7.4), 1 mM EDTA, and 0.1 percent Triton X-100. Intermittent sonication was performed 3 times for 20

seconds using a Sonics and Materials Inc. Vibra cell at 70 percent of maximum. Homogenates were kept on ice, maximum ten minutes, until enzymatic analysis could be performed.

All assays were performed at 25 °C and 340 nm (unless otherwise indicated) with a Lambda 2 Perkin Elmer UV/Vis spectrometer equipped with constant temperature water circulator. 20 ul of sample (100 ul for HK) was added for each reaction in a 1.5 ml cuvette. Lactate Dehydrogenase activity was determined in a solution of 50 mM I-Cl (pH 7.4), 0.15 mM NADH, 5 mM DTT and 4 mM pyruvate (omitted for control). Hexokinase activity was determined in a solution of 50 mM I-Cl (pH 7.4), 1mM ATP, 0.5 mM NADP+, 0.5 units/ml Glucose-6-phosphate dehydrogenase, 5 mM MgCl2 , and 5 mM glucose (omitted for control). Pyruvate Kinase activity was determined in a solution of 50 mM I-Cl (pH 7.4), 10 mM MgCl2, 100 mM KCl, 5 mM ADP, 0.15 mM NADH, 0.02 mM fructose-1,6-bisphosphate, 0.6 units/ml LDH, and 5 mM phosphoenolpyruvate (omitted for control). Malate Dehydrogenase activity was determined in a solution of 50 mM I-Cl (pH 7.4), 0.15 mM NADH, 5 mM DTT and 10 mM Oxaloacetate (omitted for control). Citrate synthase activity was determined at 412 nm in a solution of 50 mM Tris-Cl (pH 8.0), 0.3 M Acetyl CoA, 0.1 mM DTNB, and 0.5 mM oxaloacetate (omitted for control).

Enzyme activity was then determined from the maximum slope of the absorbance curve (corrected for background) using the following formula.

activity = max. slope/sec * 1/E (L/(umol * cm)) * Vt (ul) / Vs (ul) * dilution factor (L/g) * 60 sec/min * 1 / 1cm (light path)

E = 1/6.22 L/(umol * cm)

E = 1/13.6 L/(umol * cm) for CS only

Vt = cuvette volume (approximately 1000 ul)

Vs = sample volume (20 ul, 100ul for HK)

ACTIVITY = umol/(g * min) wet weight

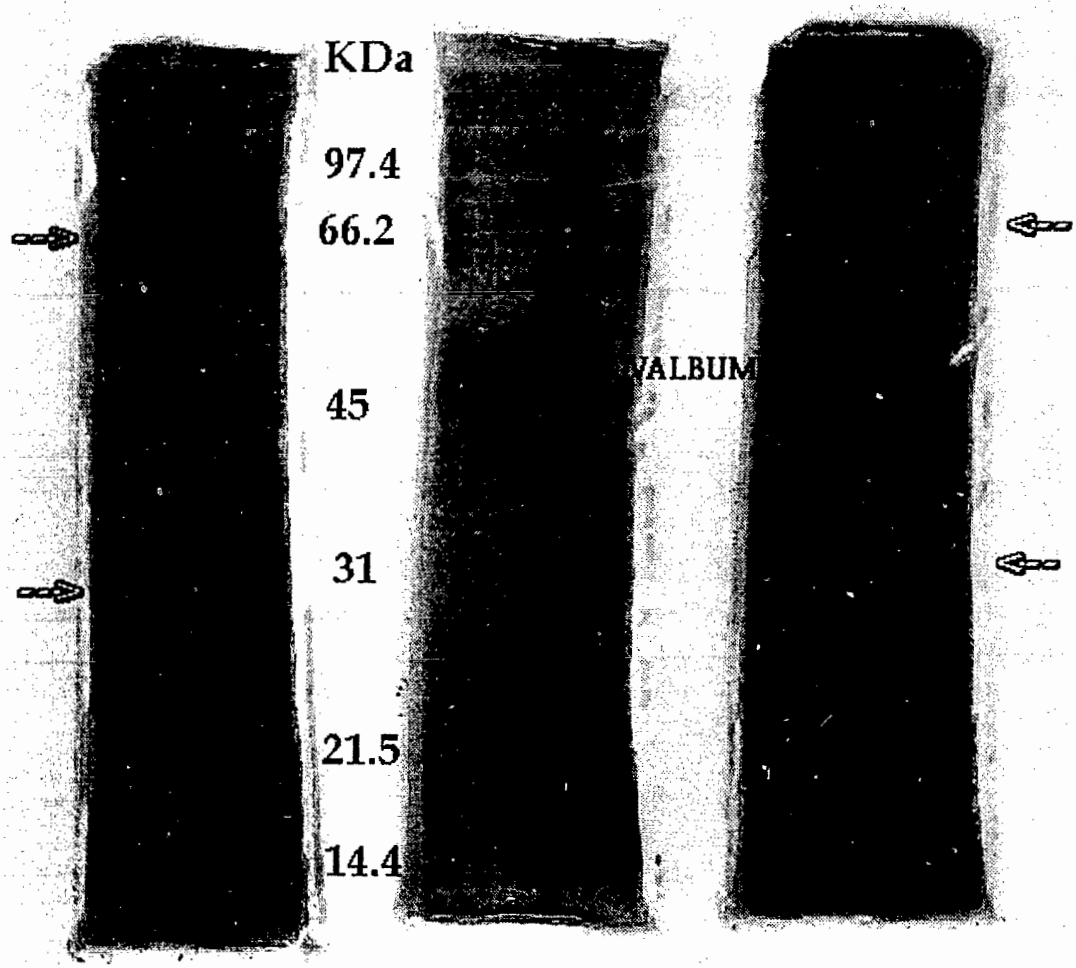
Glut 4 Transporter Content

Excised plantaris and soleus muscles (150-400mg) from old and young animals of each experimental group were homogenized with an Ultra Turax TP 18/10 ST at 70 percent of maximum (Tekmar TR-10) for 15 seconds on ice in 10 volumes of Hepes (20 mM), EDTA (1 mM), and Sucrose (250 mM). Samples were then spun for 10 minutes at 10,000 g in a Hermle Z360 K centrifuge. Protein content for the supernatant was determined with a Bradford micro assay at 595 nm using bovine serum albumin (BSA; 0-12 ug/ml) as standards with a Toshiba T3200 and a Molecular Devices Thermomax microplate reader (courtesy of Dr. G. Tibbits, Dept. of Kinesiology, SFU).

The volume of supernatant equivalent to 75 ug of protein was then boiled for four minutes in Laemmli buffer (60 mM Tris; 2 percent SDS; 10 percent Glycerol; 5percent ß-Mercaptoethanol) and loaded into a 1.5 mm protean II 12 percent polyacrylamide gel and separated for 12 hours at 10 mA per gel and maximum voltage using a Biorad 1000/500 power supply. A section of each gel containing molecular weight markers was cut out and stained in order to determine the location of the 45 Kd marker (Fig. 2). 3.5 cm sections (~31 Kd to 66 Kd) of each gel were then cut to allow simultaneous transfer of separate gels onto one membrane. Protein transfer to PVDF membrane (Biorad) was accomplished with a Hoeffer Scientific Intstruments hsi ps1500 power supply set to maximum for 3 hours. GLUT 4 content was determined by Enhanced Chemiluminescence (ECL, Amersham) with the aid of a polyclonal Rabbit anti-insulin regulatable glucose transporter antibody (East Acres Biologicals) specific for the carboxy terminus 12 amino acids of GLUT 4. Membranes were incubated overnight in a 1:1000 dilution of primary antibody in tris buffered saline-tween 20 (TBS-T), 1 percent BSA, 0.5 percent sodium azide. Membranes were then washed in TBS-T and incubated in a 1:10,000 dilution of HRP conjugated Goat anti-rabbit IgG (Santa Cruz Biotechnology) in TBS-T and 1 percent BSA for one and one-half hours. Final washing was performed in TBS-T and TBS, prior to a one minute bath in a 1:1 enhanced chemiluminesence (ECL) solution (Amersham). One to three minute exposure to ECL hyperfilm (Amersham) was followed by one minute

Figure 2 :

Location of molecular weight markers and
cut sites (66 and 31 KDa)
on stained portions of three gels ; used for the simultaneous transfer
of Glut 4 protein (45 KDa) to one blot.



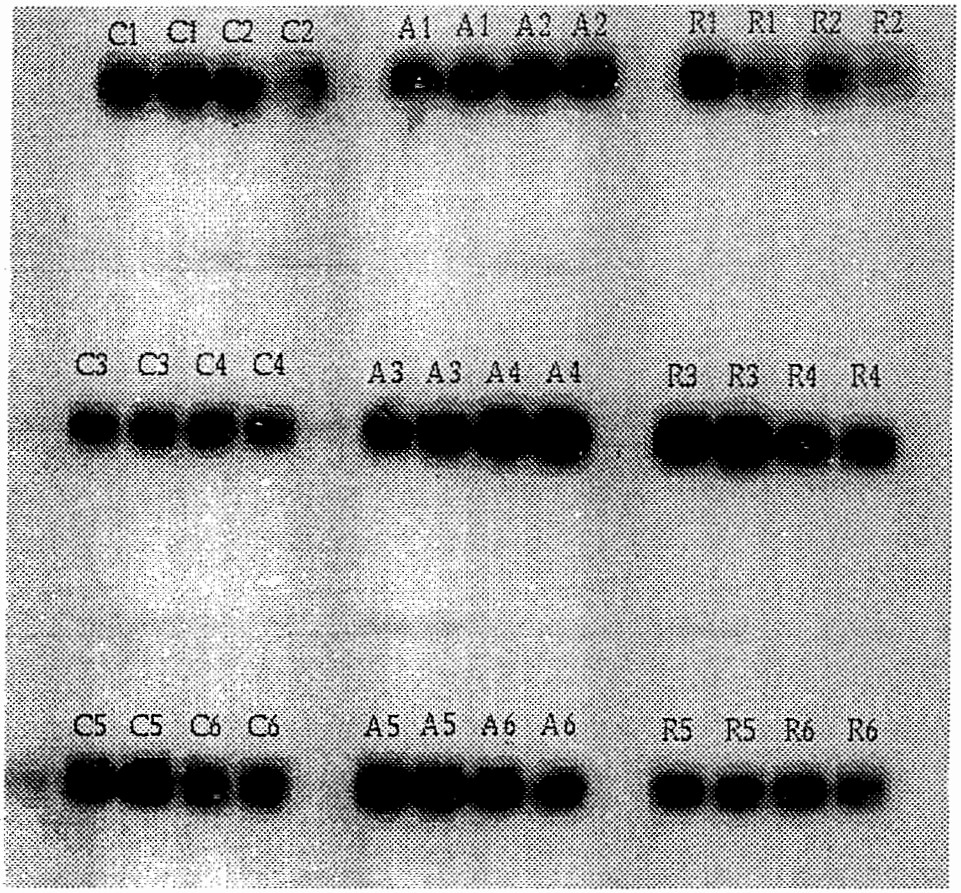
development (Kodak). Films were then analysed using Desk Scan II on a Power Macintosh with an HP ScanJet plus scanner. Each film contained all control, hypoxic and restricted diet protein separations from 35-65 Kd for a comparison group (Fig. 3) (ie. mature soleus after one month). Optical density of the bands was determined using NIH image 1.52. Final data was expressed in arbitrary optical density units per 75 ug total protein, allowing only intrafilm comparisons. Glut-4 content was also expressed relative to total mg tissue by comparison of protein content and homogenization volume. Control groups were run again on separate gels and transfered together for analysis on one film as described above. An anlaysis of linearity over a far greater range than data was obtained is provided in Figure 4.

Data Analysis

Data was expressed as a mean (n=6) $\pm$ standard error. Student T-tests were performed on Microsoft excel to determine significance at the level of $P<.05$.

Figure 3 :

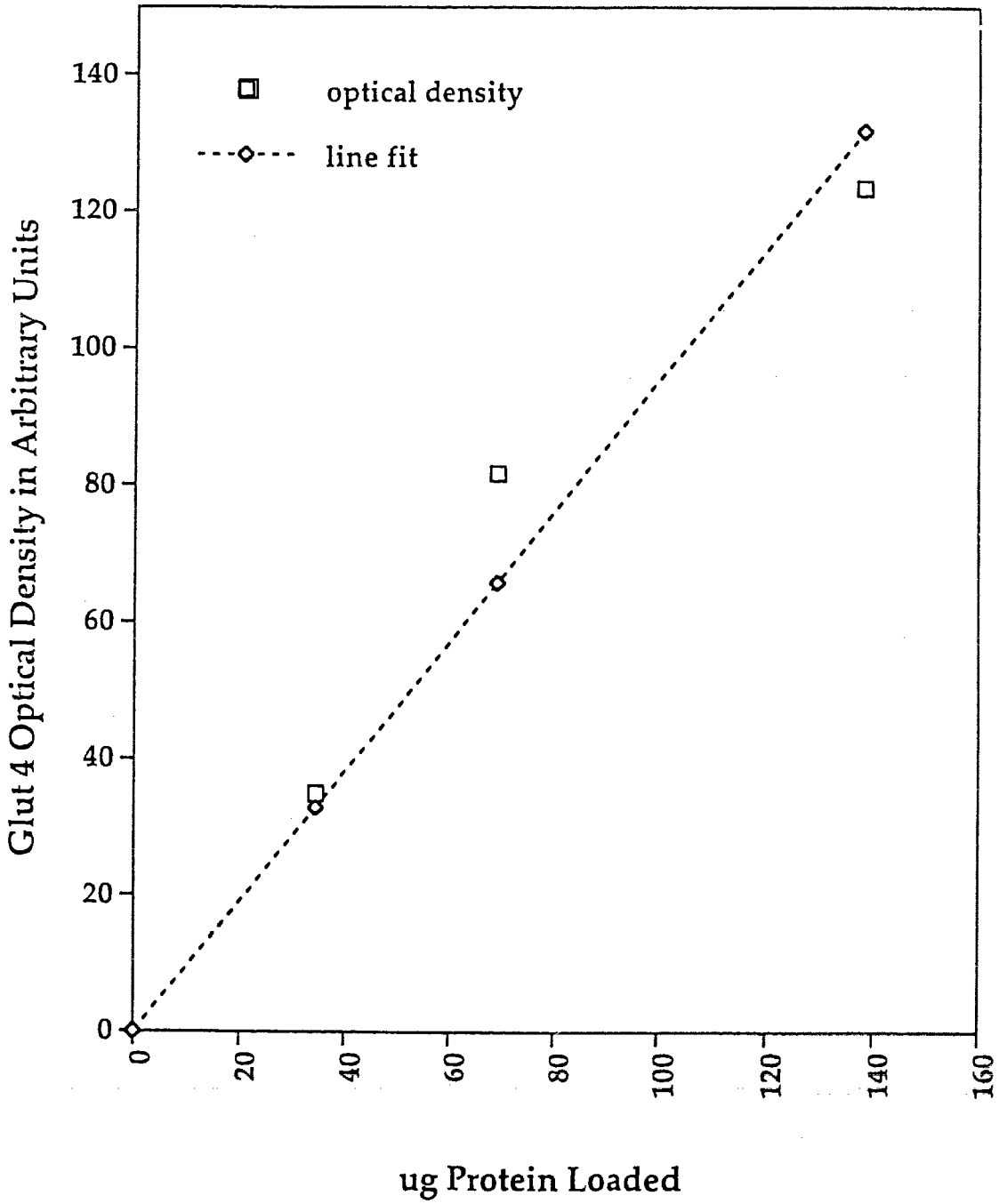
Representative Film for Glut 4 content analysis



C indicates control sample; A indicates hypobaric hypoxia sample; R indicates restricted dietary control sample. n = 6 per experimental group, per gel, all samples were run in duplicate.

Figure 4 :

Linearity Analysis:
Glut 4 Optical Density
vs.
ug Protein



Results

Body and Muscle Composition

Body weight, soleus and plantaris muscle mass and protein content were analyzed in all experimental groups, and a summary of the data is presented in Table 1. As illustrated (Fig. 5), the weight gain, and rate of growth in our developing animals was not significantly different between the altitude, restricted dietary control and control groups. When muscle weights were compared for these developing animals, no differences were observed in the soleus between groups after one week (H-54 $\pm$ 2 mg; R-55 $\pm$ 3 mg; C-52 $\pm$ 2 mg) or one month of exposure (Table 1). In the plantaris, however, significant declines from control were observed in both the altitude and restricted dietary groups (Table 1).

Protein yield (ug/mg tissue) (Table 1; Fig. 7), indicated significant protein enrichment per mg in both the soleus and plantaris muscles of the young hypoxic group compared to both the control and restricted dietary groups (Table 1; Fig. 7). However, no differences in protein yields were observed in the soleus muscle exposed to hypoxia for one week (H-87 $\pm$ 3 ug/mg; R-86 $\pm$ 6 ug/mg; C-89 $\pm$ 4 ug/mg).

TABLE 1:

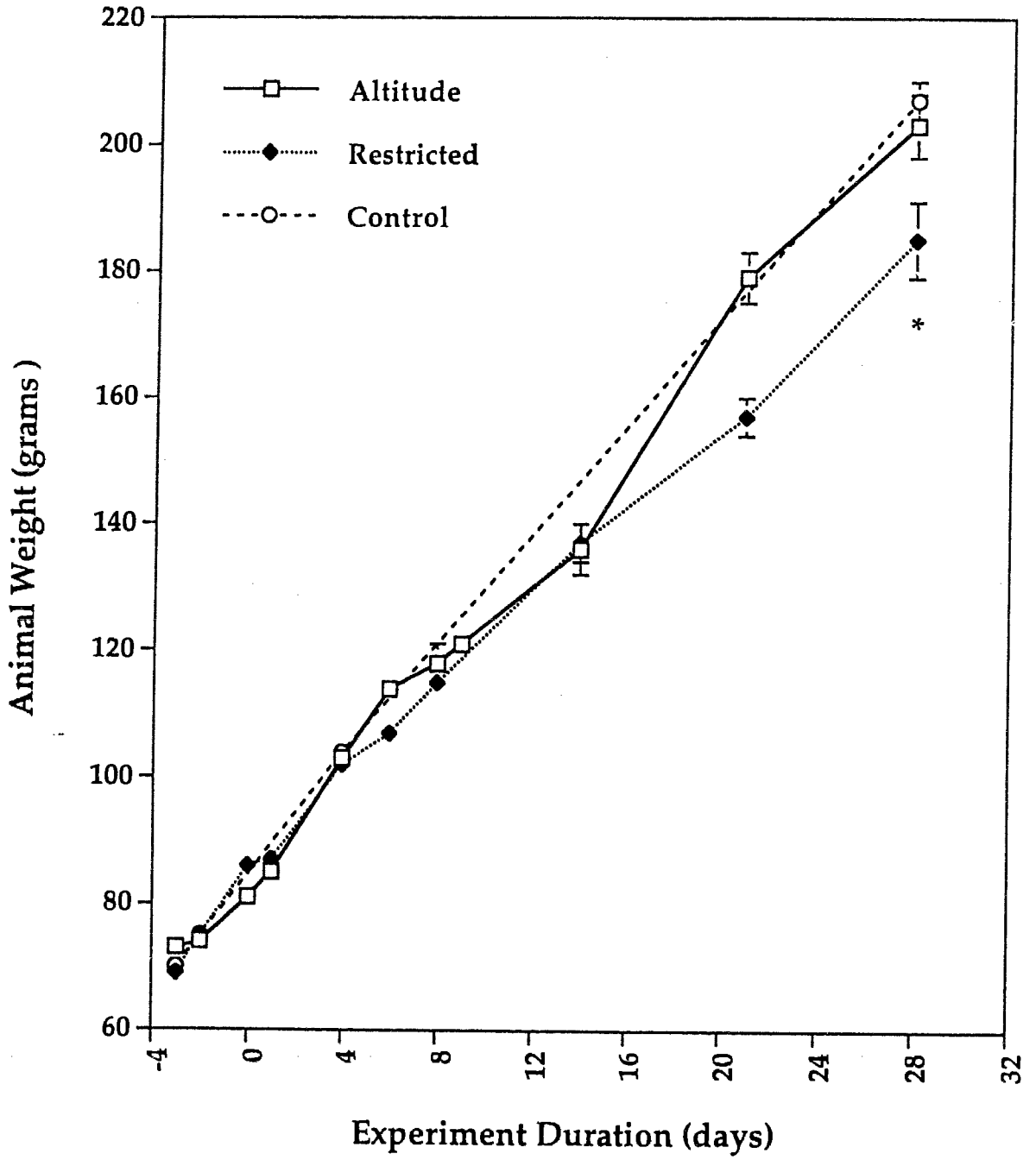
DEVELOPING ANIMAL MUSCLE AND BODY WEIGHTS AT SACRIFICE: CHANGES WITH EXPERIMENTAL CONDITION

	Animal Wt. (g)	Soleus Wt. (mg)	Soleus Protein Yield (ug / mg)	Plantaris Wt. (mg)	Plantaris Protein Yield (ug / mg)
altitude	203 ± 5 #	94 ± 5	89 ± 5 *#^	155 ± 12 *	89 ± 3 *#^
restricted	185 ± 6 *	90 ± 5	76 ± 3	162 ± 7 *	76 ± 3 ^
control	207 ± 4	99 ± 4	69 ± 3	182 ± 8	74 ± 7 ^

Animal physical data. * denotes significant difference from control; # denotes significant difference from restricted; ^ denotes significant difference between developing and mature animals within experimental group ($p < .05$).

Figure 5 :

Developing Animal Weights (grams)
vs.
Experiment Duration (Days)



Animal weights (grams) expressed as mean (n=6) with standard error. Experiment duration commences with first day at 15500 ft. * denotes significant difference from control ($p < .05$).

TABLE 2 :

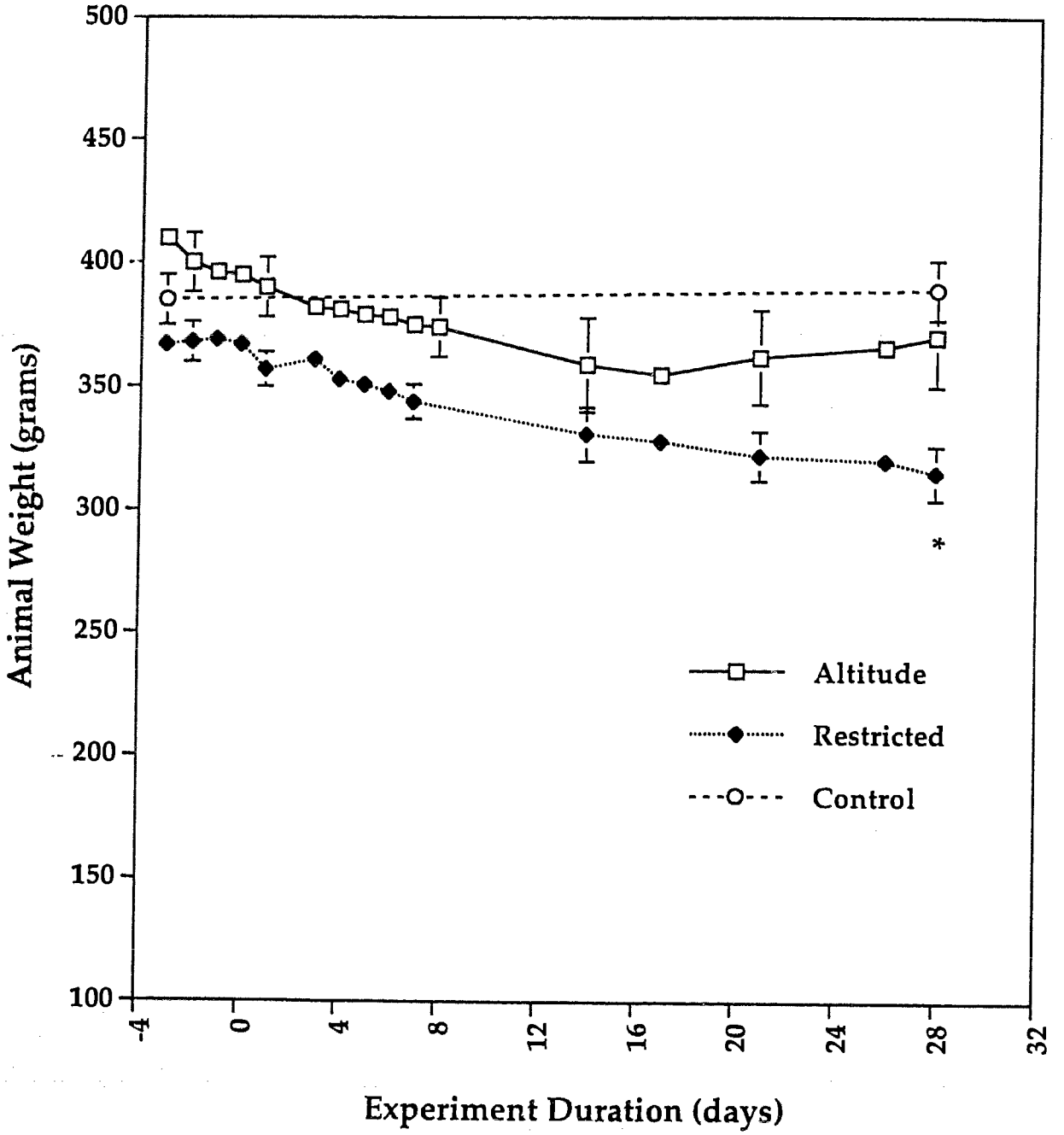
MATURE ANIMAL MUSCLE AND BODY WEIGHTS AT SACRIFICE :
CHANGES WITH EXPERIMENTAL CONDITION

	Animal Wt. (g)	Soleus Wt. (mg)	Soleus Protein Yield (ug / mg)	Plantaris Wt. (mg)	Plantaris Protein Yield (ug / mg)
altitude	370 ± 20 #	183 ± 7	67 ± 4 *	295 ± 16	113 ± 5
restricted	315 ± 11 *	167 ± 8 *	70 ± 4	310 ± 19	116 ± 3
control	389 ± 12	195 ± 9	79 ± 5	325 ± 19	113 ± 6

Animal physical data. * denotes significant difference from control; # denotes significant difference from restricted; ^ denotes significant difference between developing and mature animals within experimental group ($p < .05$).

Figure 6 :

Mature Animal Weights (grams)
vs.
Experiment Duration (Days)



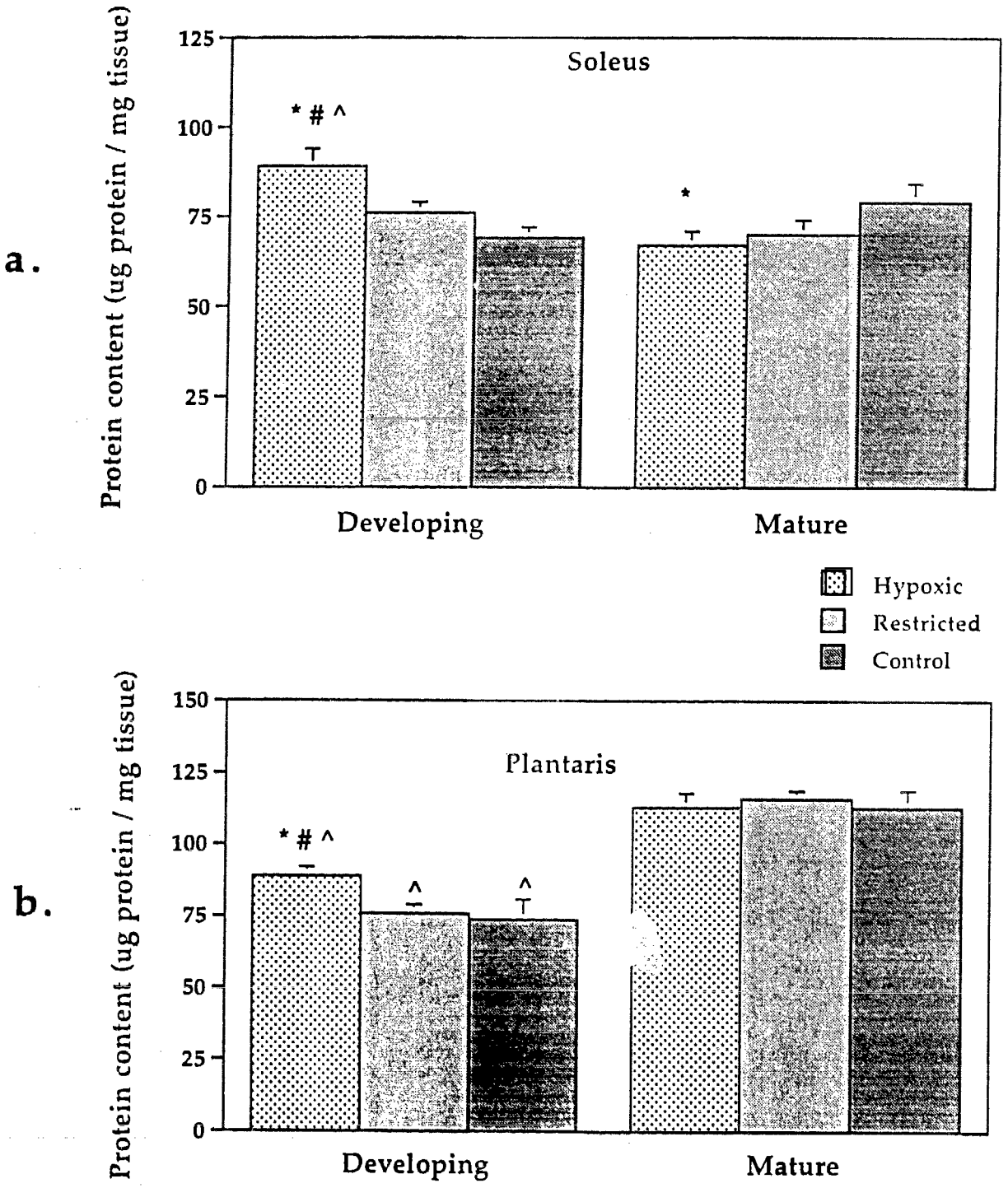
Animal weights (grams) expressed as mean (n=6) with standard error. Experiment duration commences with first day at 15500 ft. * denotes significant difference from control ($p < .05$).

The body weight of the old animals changed significantly with the experimental protocol (Fig. 6; Table 2). No significant differences between groups were noted for the plantaris muscle weights (Table 2). However, a significantly lower soleus muscle weight was found for the restricted dietary group when compared to the control soleus (Table 2). The soleus muscle protein content of mature hypoxic animals was significantly lower than the controls (Table 2; Fig. 7), which is in direct contrast to the young data (Table 1; Fig. 7). Plantaris protein content was unaltered by the experimental protocol in the mature animals (Table 2; Fig. 7).

The protein content was found to be higher in hypoxic developing soleus than in the hypoxic mature soleus (Tables 1 and 2; Fig. 7). In contrast, mature plantaris had a higher protein content than developing plantaris in all experimental groups (Tables 1 and 2; Fig. 7).

Figure 7 :

Protein content in soleus and plantaris muscles of control, restricted diet and hypoxic developing and mature animals.



Protein content (ug protein/mg tissue) mean (n=6) with standard error. * denotes significant difference from control; # denotes significant difference from restricted; ^ denotes significant difference from mature ($p < .05$).

Glucose Transporter 4 Content

Soleus and plantaris glucose transporter contents are summarized in Tables 3 and 4. Comparison of developing and mature muscle Glut 4 contents are illustrated in Figs. 8 and 9 for control animals. A lower Glut 4 content per ug protein is evident in the mature soleus and the plantaris muscles compared with the developing muscles. When Glut 4 content was expressed per mg tissue, the mature and developing muscle contents did not differ in the soleus (Fig. 8). In contrast, an increase in Glut 4 content per mg tissue was observed in mature relative to developing plantaris muscles (Fig. 9). This increase was due to the increase in protein content observed in the mature plantaris groups (Fig. 7). No differences were noted between the soleus and plantaris Glut 4 content per ug protein within age groups (Fig. 10). The Glut 4 content per mg tissue was higher in the plantaris than the soleus for the mature animals (Fig. 11).

Glut 4 content for the one week experiment are summarized in Table 3. Elevated Glut 4 content per ug protein was seen after one week in the soleus muscles of the hypoxic group (Table 3). Soleus muscle content (per mg tissue) of Glut 4 in the hypoxic group was also elevated over controls after one week (Table 3). This illustrated that up regulation is occurring rapidly in response to hypobaric hypoxia.

The effects one month of experimental condition on soleus and plantaris muscle Glut 4 contents are summarized in Tables 3 and 4, respectively, and illustrated in Figs. 12 and 13. Analysis of Glut 4 content per ug protein illustrated no enrichment or decline from control under any condition in the one month experiment for soleus and plantaris muscle (Fig. 12). An up regulation of Glut 4 content per mg tissue was observed in the developing animals for both the soleus and plantaris muscles after four weeks exposure to hypobaric hypoxia when compared to control and restricted dietary control groups (Fig. 13). No differences between the restricted dietary control and the control groups were observed for Glut 4 in any muscles in the one month experiment (Tables 3 and 4; Figs. 12 and 13).

TABLE 3 :

SOLEUS MUSCLE GLUT 4 CONTENT FOR CONTROL,
RESTRICTED DIETARY AND HYPOXIC DEVELOPING AND MATURE ANIMALS.

	ALTITUDE		RESTRICTED		CONTROL	
	OD/ug protein	OD/mg tissue	OD/ug protein	OD/mg tissue	OD/ug protein	OD/mg tissue
Mature	28551 ± 1084	25628 ± 1865	27671 ± 434	25746 ± 1518	27902 ± 355	29396 ± 1760
Developing	25708 ± 590	30449 + 1548 *#	23895 ± 949	24207 ± 1180	24107 ± 1307	22166 ± 1749
Developing (one week)	7212 + 281 *	33367 ± 2108 *	6654 ± 511	30268 ± 2799	5586 ± 472	26415 ± 2376

Data shown as mean (n=6) in optical density units (OD) with standard error. * Indicates significant difference from control, # Indicates significant difference from restricted ($P < .05$).

TABLE 4 :

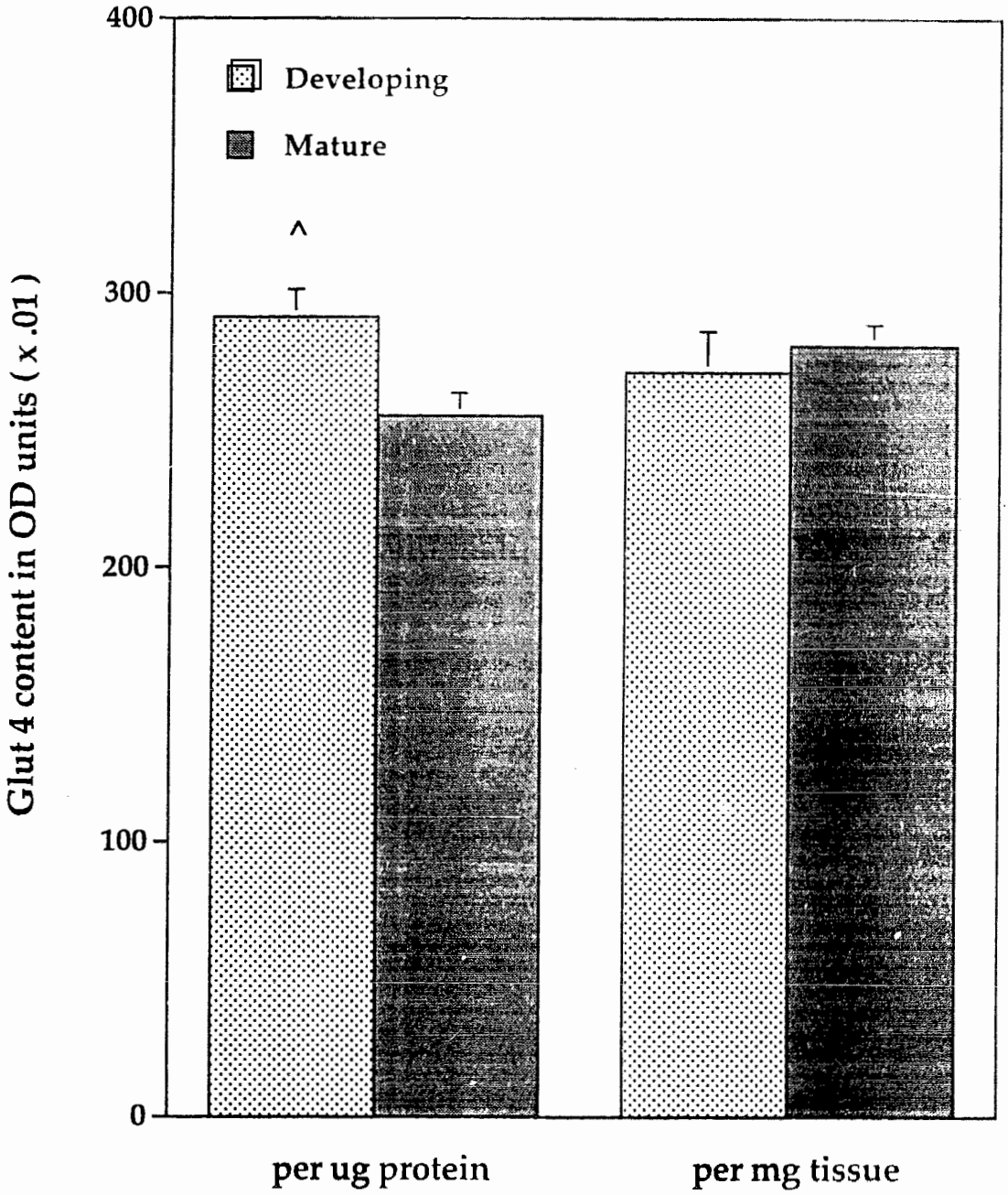
PLANTARIS MUSCLE GLUT 4 CONTENT FOR CONTROL,
RESTRICTED DIETARY AND HYPOXIC DEVELOPING AND MATURE ANIMALS.

	ALTITUDE		RESTRICTED		CONTROL	
	OD/ug protein	OD/mg tissue	OD/ug protein	OD/mg tissue	OD/ug protein	OD/mg tissue
Mature	10627 ± 136	16055 ± 806	10061 ± 468	15479 ± 564	10755 ± 384	16275 ± 1083
Developing	27058 ± 421	31904 ± 963 *#	27050 ± 669	27380 ± 1299	26724 ± 821	26342 ± 2109

Data shown as mean (n=6) in optical density units (OD) with standard error. * Indicates significant difference from control, # Indicates significant difference from restricted ($P < .05$).

Figure 8 :

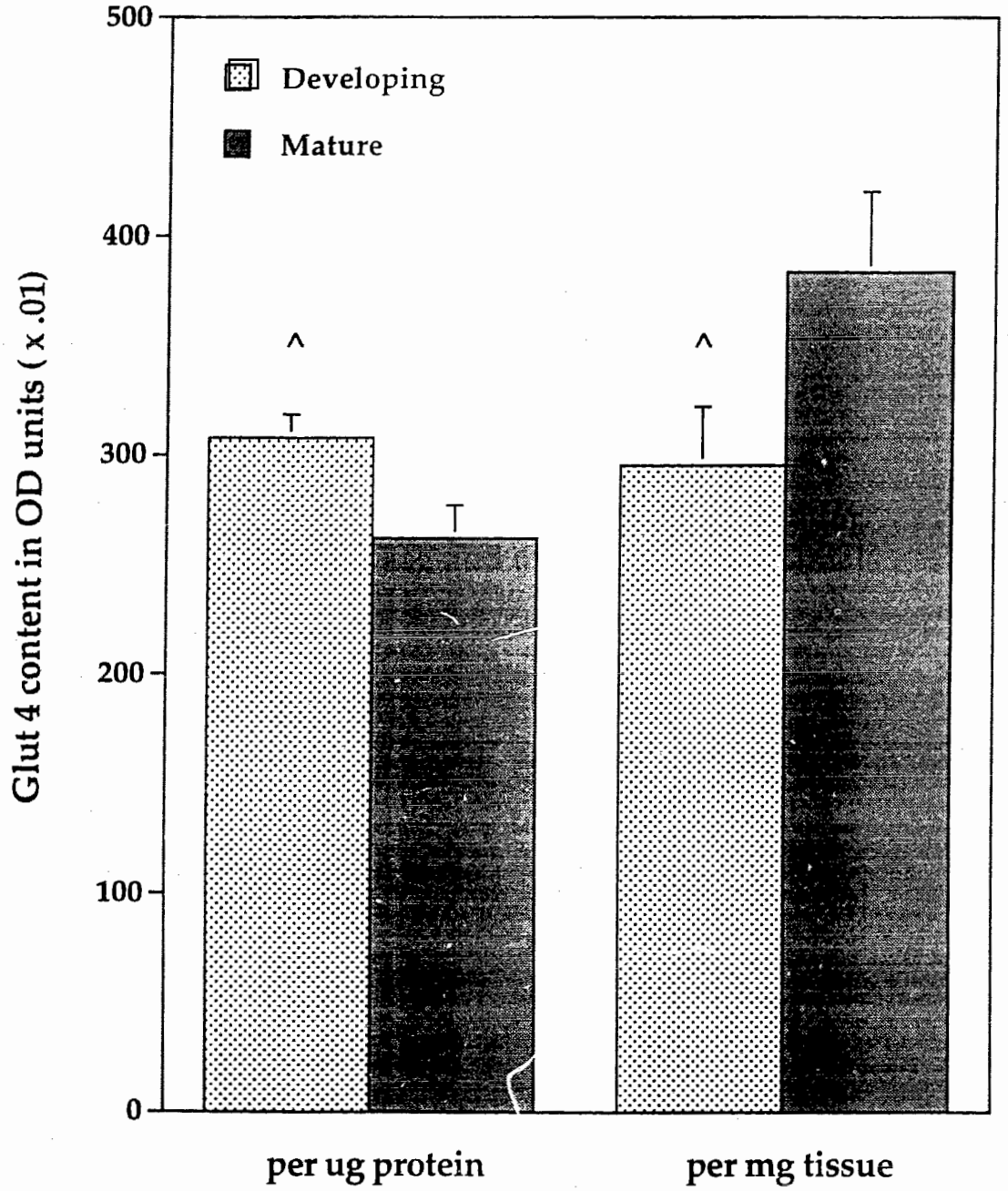
Glut 4 content in soleus muscle of developing and mature control animals.



Glut 4 content in optical density units (OD) per ug protein and per mg tissue. Data is expressed as mean (n=6) with standard error. ^ denotes significant difference from mature ($p < .05$).

Figure 9 :

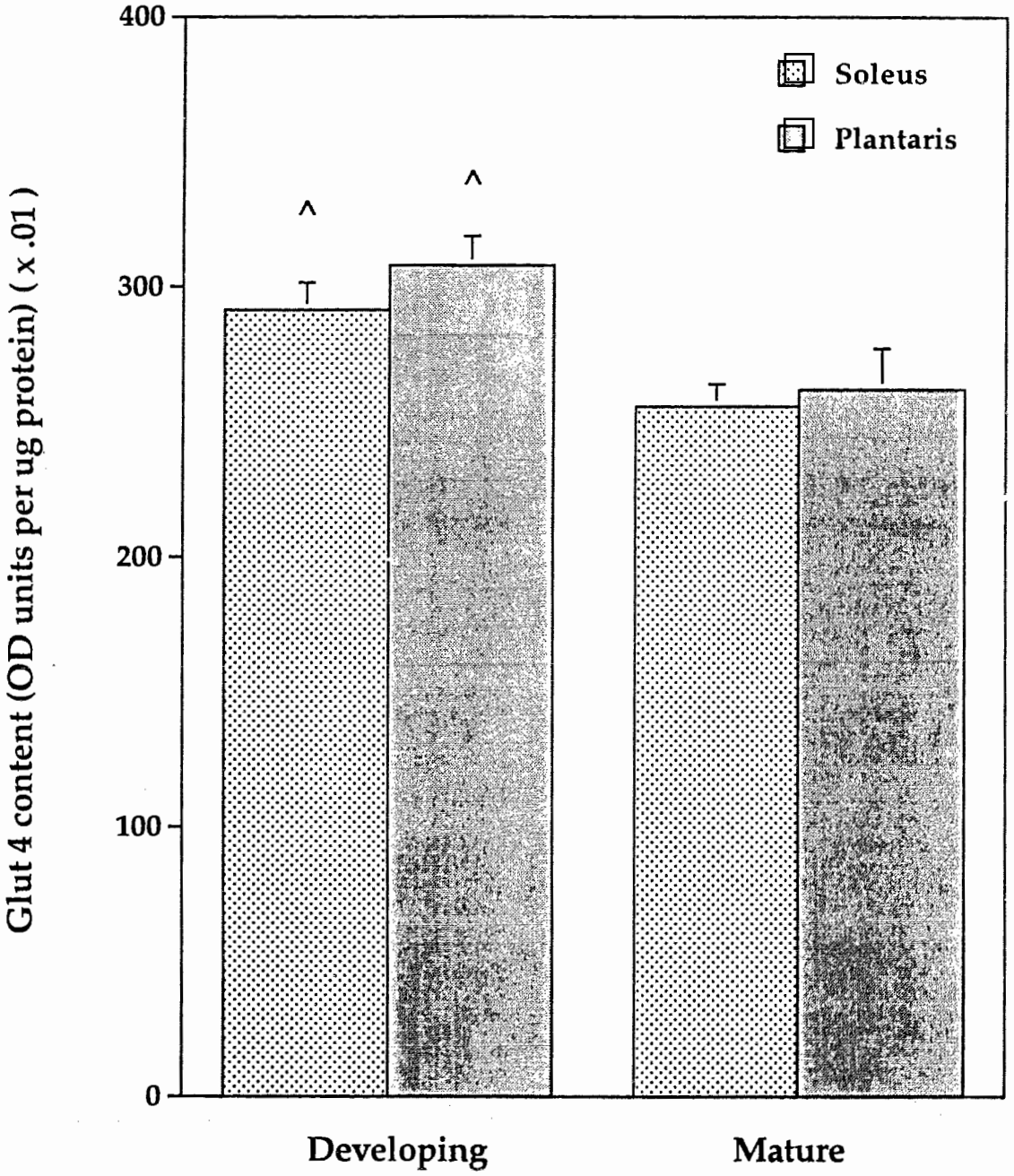
Glut 4 content in plantaris muscle for developing and mature control animals



Glut 4 content in optical density units (OD) per ug protein and per mg tissue. Data is expressed as mean (n=6) with standard error. ^ denotes significant difference from mature ($p < .05$).

Figure 10 :

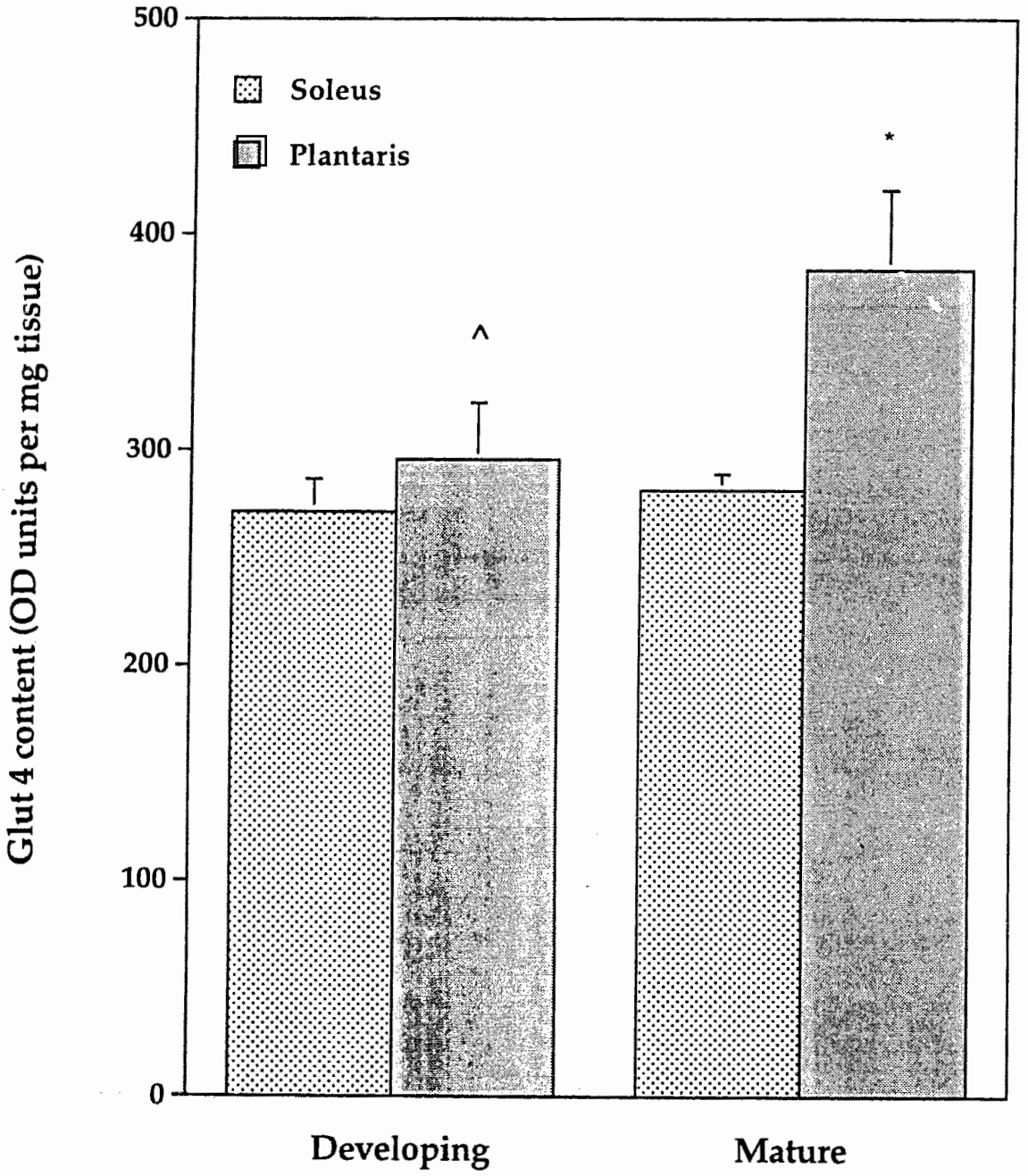
Glut 4 content per ug protein for control soleus and plantaris muscle



Glut 4 content in optical density units (OD) per ug protein. Data is expressed as mean (n=6) with standard error. ^ denotes significant difference from mature ($p < .05$).

Figure 11 :

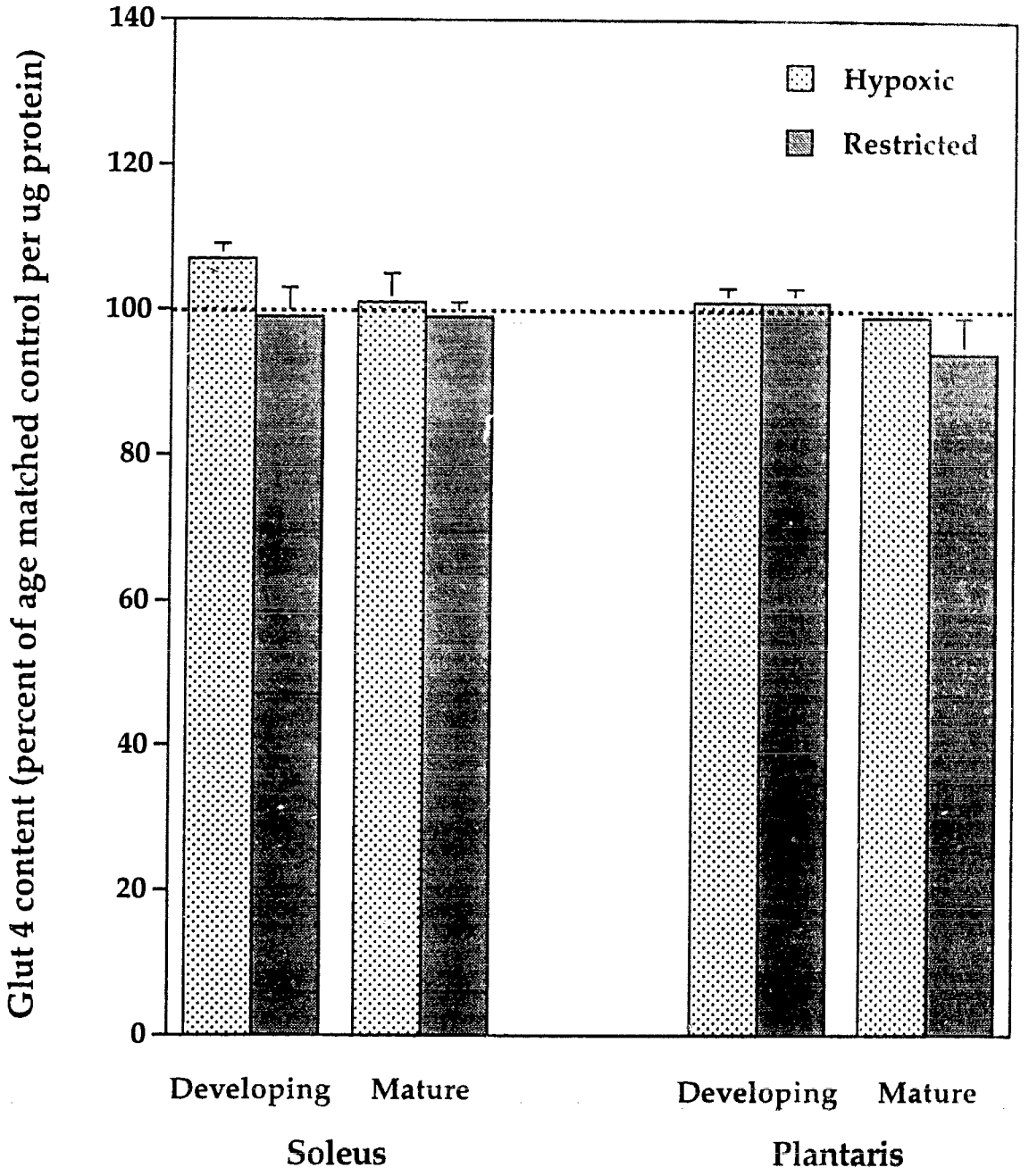
Glut 4 content per mg tissue for control soleus and plantaris muscle



Glut 4 content in optical density units (OD) per ug protein. Data is expressed as mean (n=6) with standard error. ^ denotes significant difference from mature; * denotes significant difference from age matched soleus muscle ($p < .05$).

Figure 12 :

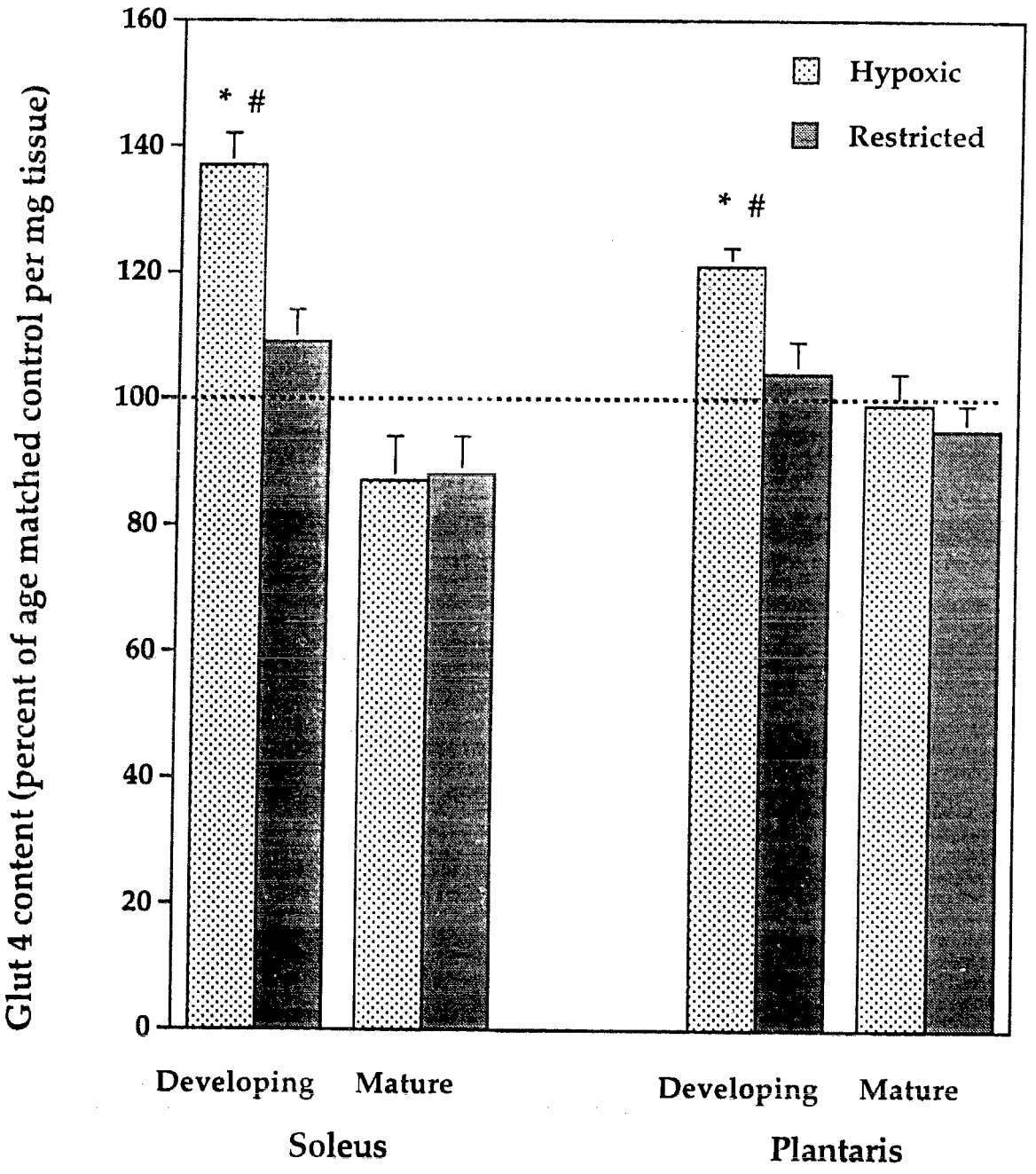
Effects of one month of Hypobaric Hypoxia or Dietary Restriction on Soleus and Plantaris Glut 4 Content Expressed as a Percent of Control Value per ug Protein



Glut 4 expressed as percent of age matched control value per ug protein. * denotes significant difference from conotrol; # denotes significant difference from restricted; ^denotes significant difference from mature ($p < .05$).

Figure 13 :

Effects of one month of Hypobaric Hypoxia or Dietary Restriction on Soleus and Plantaris Glut 4 Content Expressed as a Percent of Control Value per mg Tissue



Glut 4 expressed as percent of age matched control value per mg tissue. * denotes significant difference from control; # denotes significant difference from restricted ($p < .05$).

Enzyme Activity

Analysis of enzyme activity was performed for several representative enzymes of the major carbohydrate metabolic pathways, these were expressed in umol/g/min and are summarized in Tables 5 and 6. The first (hexokinase, HK) and last (pyruvate kinase, PK) non equilibrium enzymes of glycolysis, as well as lactate dehydrogenase were analyzed to indicate both the aerobic and anaerobic potential of the muscles analyzed. The activities of citrate synthase (CS) and malate dehydrogenase (MDH) of the Krebs cycle were also investigated to determine muscle aerobic potential, and to illustrate cytosol and mitochondrial competition for reducing equivalents. No significant differences were found for any enzyme within the plantaris or soleus muscles between any of the experimental protocols (Tables 5 and 6).

Comparisons over development revealed several differences in enzymatic activity (umol/g/min). The hexokinase activity of the developing hypobaric hypoxic plantaris and soleus were found to be higher than the mature (Tables 5 and 6). Further, all citrate synthase activities were found to be higher in the group matched developing animals (Tables 5 and 6), and pyruvate kinase was higher in the soleus of developing animals for all groups (Table 5).

TABLE 5 :

SOLEUS MUSCLE MAXIMAL ENZYME ACTIVITIES (umol/(g * min))
FOR MATURE AND DEVELOPING ANIMALS

ENZYME	ALTITUDE		RESTRICTED		CONTROL	
	DEVELOPING	MATURE	DEVELOPING	MATURE	DEVELOPING	MATURE
umol/(g*min)						
LDH	88 ± 8.6	79 ± 8.1	84 ± 5.5	96 ± 10.9	77 ± 7.6	82 ± 9.5
PK	61 ± 6.4 ^	38 ± 3.6	61 ± 5.5 ^	45 ± 4.8	50 ± 3.5 ^	39 ± 4.7
HK	.82 ± .09 ^	.49 ± .08	.7 ± .08	.59 ± .07	.82 ± .13	.66 ± .11
CS	20 ± 1.3 ^	13 ± 1.5	26 ± 2.0 ^	13 ± 1.3	23 ± 2.0 ^	16 ± 1.7
MDH	56 ± 8.6	49 ± 5.4	56 ± 3.6	53 ± 5.5	59 ± 2.4	56 ± 7.9
PK/LDH	0.7	0.5	0.7	0.5	0.6	0.5
CS/LDH	0.2	0.2	0.3	0.1	0.3	0.2
MDH/LDH	0.6	0.6	0.7	0.6	0.8	0.7

Data expressed as umol/(g * min) wet weight, mean of n=6, expressed with standord error of the mean.
^ denotes difference between developing and mature animals within experimental condition ($p < .05$).

TABLE 6 :

PLANTARIS MUSCLE MAXIMAL ENZYME ACTIVITIES (umol/(g * min))
FOR MATURE AND DEVELOPING ANIMALS

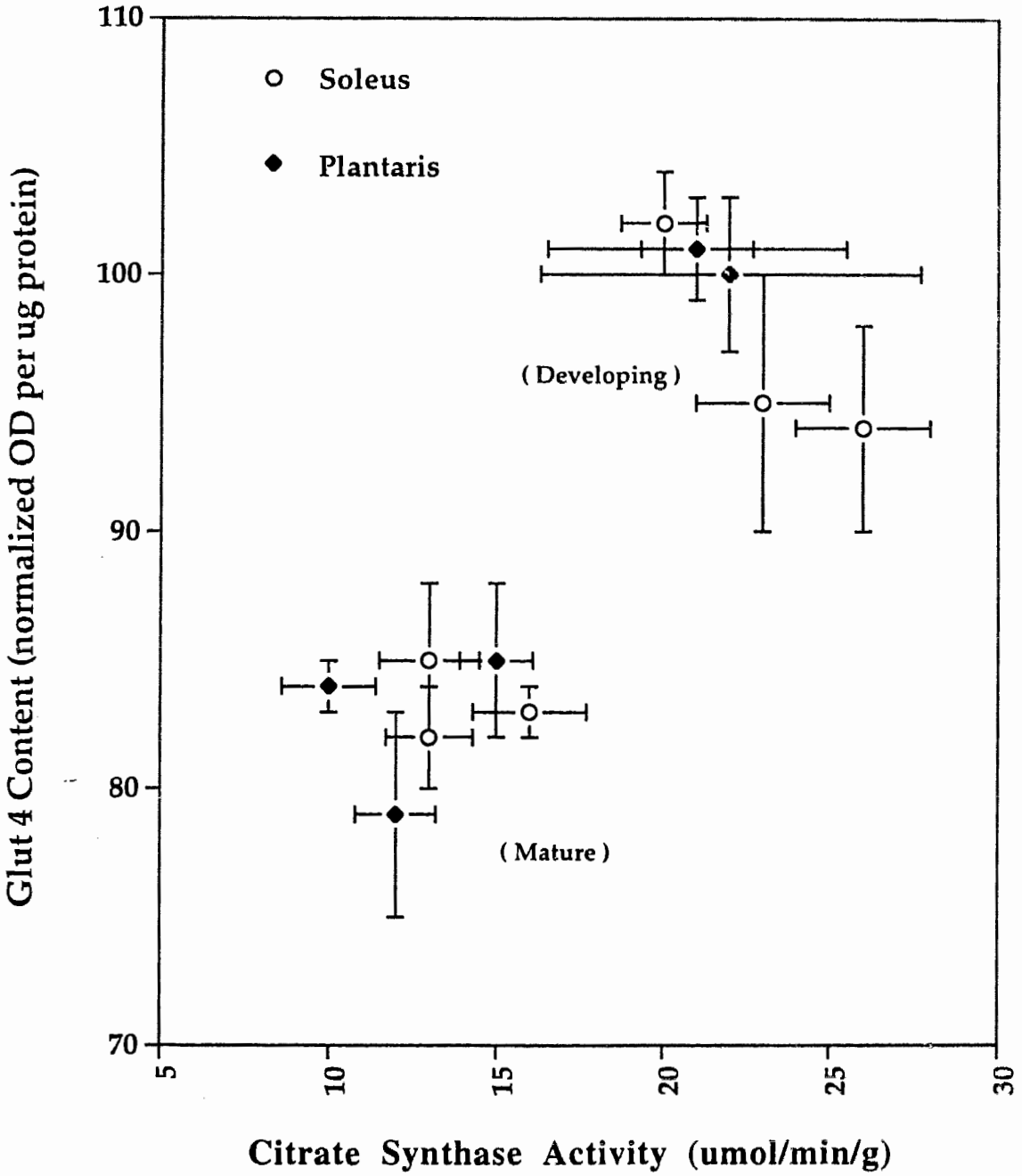
ENZYME	ALTITUDE		RESTRICTED		CONTROL	
	DEVELOPING	MATURE	DEVELOPING	MATURE	DEVELOPING	MATURE
umol/(g*min)						
LDH	358 ± 22	329 ± 29	339 ± 23	397 ± 35	315 + 8	394 ± 43
PK	241 ± 16	212 ± 10	234 ± 12	236 ± 5	248 ± 28	238 ± 29
HK	.74 ± .08 ^	.49 ± .08	0.6 ± .1	.61 ± .1	0.71 ± .1	.64 ± .1
CS	21 ± 4.5 ^	10. ± 1.4	21 ± 1.7 ^	12 ± 1.2	22 ± 5.7 ^	15 ± 1.1
MDH	80 ± 24	49 ± 1.4	69 ± 13	73 ± 21	57 ± 4.6	87 ± 15
PK/LDH	0.7	0.6	0.7	0.6	0.8	0.6
CS/LDH	0.06	0.03	0.06	0.03	0.07	0.04
MDH/LDH	0.2	0.1	0.2	0.2	0.2	0.2

Data expressed as umol/(g * min) wet weight, mean of n=6, expressed with standord error of the mean.
^ denotes difference between developing and mature animals within experimental condition ($p < .05$).

The average Glut 4 content and citrate synthase activity for muscles of each group were compared to illustrate their relationship (Figs. 14 and 15). Differences between the developing and mature tissue are evident with the comparison to Glut 4 content per ug protein, illustrating that these components are generaly related (Fig. 14). These differences are less clear when the comparison is made for Glut 4 content per mg tissue, due to the high protein content in the mature plantaris (Fig. 15). No differences between experimental conditions or between soleus and plantaris are evident within an age group (Figs. 14 and 15).

Figure 14 :

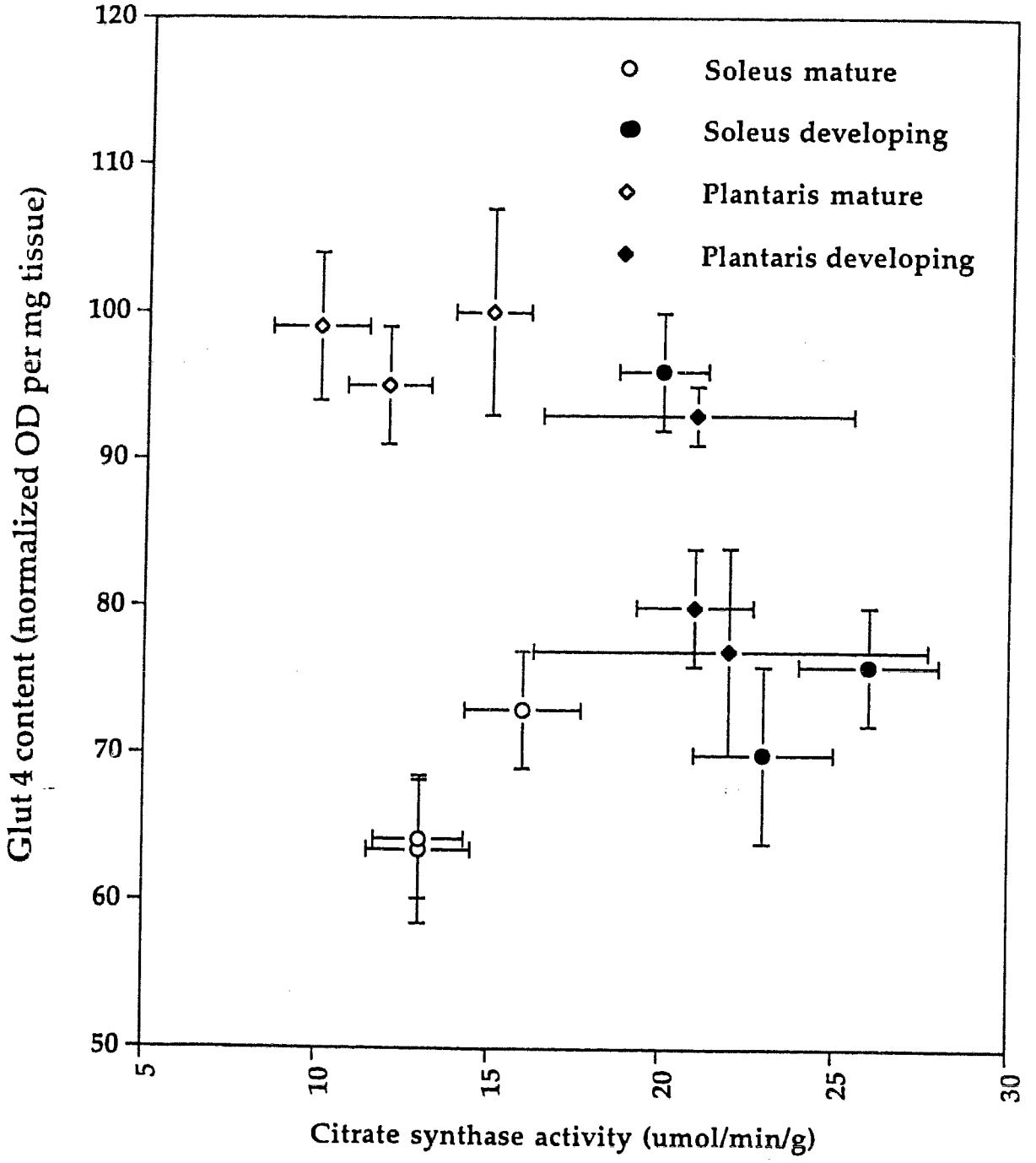
Relationship between citrate synthase activity and Glut 4 content per ug protein for all experimental groups.



Data expressed as mean (n=6) with standard error. Citrate Synthase activity is in umol/min/g wet weight. Glut 4 content was normalized to developing plantaris control from optical density units per ug protein.

Figure 15 :

Relationship between citrate synthase activity and Glut 4 content per mg protein for all experimental groups.



Data expressed as mean (n=6) with standard error. Citrate synthase activity is in umol/min/g wet weight. Glut 4 content was normalized to mature plantaris control from optical density units per mg tissue.

Discussion

General

This study of one week and one month adaptations in skeletal muscle to hypobaric hypoxia and dietary restriction illustrated several significant findings. An increase in Glut 4 content was evident in both the soleus and plantaris muscles of developing animals in response to hypobaric hypoxia. This change was found to be rapid, as one week of hypobaric hypoxia increased Glut 4 content in the soleus muscle of developing animals. This increase indicates that the capacity of these muscle to transport glucose into the muscle has increased, as transporter content is directly related to stimulated transport capacity (Henriksen et al. 1990; Kern et al. 1990). The pertubations of dietary restriction and hypobaric hypoxia over the experimental time frame did not alter the maximal enzyme activities in the soleus or plantaris muscles of developing or mature animals.

Body Weight and Muscle Composition

One month exposure to hypobaric hypoxia did not result in a significant alteration in body weight from controls in our developing animals (Fig. 5). The influence of hypobaric hypoxia on body weight in previous studies has been attributed to many confounding factors that are encountered with

acclimatization to altitude. These include, loss of appetite, alterations in diet, water loss, cold stress, comfort and the increased physical demand of climbing (Kayser 1992). It is important to note that many of the studies illustrating severe decreases in diet in humans (50 % sea level ad libitum) involved extreme altitude (> 20,000 ft) and environmental situations (cold, dryness). In contrast, the changes in body weight, which have been shown with moderate simulated altitude, have been found to occur only during the initial exposure to hypobaric hypoxia (Sillau and Banchero 1977; Bigard et al. 1991a,b). In this study, the initial stress of hypoxia was reduced through a stepwise decrease in the ambient pressure over several days. Further, the animals in the previous studies (Sillau and Banchero 1977; Bigard et al. 1991a,b) were older (~175-180 g) than our developing animals (~70 g) at the start of exposure.

In the developing animals restriction of diet for one month lowered body weight compared the control and the hypobaric hypoxic groups. The attenuated weight gain in this group should accentuate any changes in the investigated parameters that are attributable to the dietary reduction often observed at altitude.

A lower plantaris weight was found in developing animals following one month in hypobaric hypoxia (Table 1). This was not observed with hypobaric hypoxia in the developing soleus muscle (Table 1). Previous studies have concluded that there was not an alteration in lean body mass (muscle to body

weight ratio) with fourteen weeks at moderate altitude in rats (Bigard et al. 1991b). This conclusion, however, was established from comparisons in older animals, of total hind limb musculature to body weight and not for individual muscles (Bigard et al 1991b).

Mature animals (6 months of age, ~375 g) exposed to hypobaric hypoxia had a significant decrease in body weight compared to their group starting weight, within two weeks (Fig. 5). Body weight was seen to level off and even begin to approach control values after three weeks (Fig. 5). No significant differences in individual muscle mass were observed in the mature animals with one month of hypobaric hypoxia (Table 2). A trend in muscle weight, corresponding to the changes in body weight, however, was evident (Table 2). The difference in body weight response between the mature and developing animals may illustrate the overall adaptation potential at different stages of development.

Glucose Transporter Content

Comparisons between young and old control tissue illustrated a higher content of Glut 4 in the developing animal soleus and plantaris muscles when analysed per ug protein (Figs. 8 and 9). These findings are constistent with data illustrating lower rates of glucose uptake in EDL and soleus over this developmental period, under basal (0 insulin) and maximally stimulated

(200 uU/ml insulin) conditions (Goodman et al. 1983). When compared per mg tissue however, the developing and mature soleus were found not to differ (Fig. 8). The plantaris demonstrated a significantly higher Glut 4 content per mg tissue in mature compared to developing animals (Fig. 9). The protein content of the mature plantaris (~115 ug/mg) was found to be substantially higher than that in all other tissues (~70 ug/mg) analysed in the one month experiment (Table 2). This large difference in protein content with development is completely responsible for the discrepancy in results with expression per ug protein and per mg tissue for the plantaris. This difference in the plantaris does not influence comparisons between experimental conditions.

Comparison between soleus and plantaris controls exhibited no difference in the developing or mature animals per ug protein (Fig. 10). A higher content was expected in the soleus (James et al. 1988), though comparable Glut 4 levels have been found in the plantaris following exercise training (Etgen et al. 1993a). A significantly higher Glut 4 content was found in control developing animals soleus muscle after the one month experiment (10233 $\pm$ 319 od per ug protein) when compared with the one week experiment (8490 $\pm$ 195 od per ug protien). Glucose uptake has been shown to be slightly higher in both the unstimulated and maximally stimulated states at five weeks over eight weeks with hind limb perfusion in rats (Goodman et al 1983). However, the stimulation over basal activity was far greater in the eight week old animals

(+ 488 percent) compared to the five week old animals (+270 percent). This illustrates the developmental replacement of Glut 1 (basal transport) with Glut 4 that occurs during this time (Santalucia et al. 1992), and indicates that a rise in Glut 4 is expected.

One week exposure to moderate hypobaric hypoxia resulted in an enrichment in developing animal soleus Glut 4 content per ug protein (Table 3). The difference observed over one week indicates that a rapid increase in Glut 4 is occurring with exposure to simulated altitude. The capacity to uptake exogenous glucose is higher in these adapted animals, as muscle Glut 4 content is directly related to transport capacity under stimulated conditions (Henriksen et al. 1990; Kern et al. 1990). This increase was also observed per mg tissue, indicating that total muscle capacity for glucose uptake is also increased with one week at simulated altitude. This rapid chronic adaptation is consistent with previous findings in which a two fold increase in Glut 4 mRNA and protein content were noted after two days involving prolonged exercise training (Ren et al. 1994).

With one month of exposure to simulated hypobaric hypoxia an increase in developing soleus and plantaris (Fig. 13) Glut 4 content per mg muscle was observed. Comparison of glut 4 content per ug protein, however, did not illuminate any differences between experimental conditions (Fig. 12). As

with the one week data, we can conclude that simulated altitude results in an increase in whole muscle capacity for glucose uptake.

It is interesting, that the observed change in Glut 4 enrichment (Glut 4/ug protein) with one week of hypobaric hypoxia was lost with a further three weeks of exposure. It has been noted, that the body weight changes in response to altitude occur during initial exposure and reflect a severe pertubation to animal physiology, and a subsequent adaptation (Bigard et al. 1991b) It can be surmised from our data that the initial Glut 4 enrichment observed is a fast response to simulated altitude. Changes in older animals muscle capillarity and fiber compliment have also been studied over relatively longer periods (six and fourteen weeks) (Sillau and Banchero 1977; Bigard et al. 1993 a,b). These longer term adaptations may alter muscle level pO2, thereby removing the initial stimulus for Glut 4 adaptation. Muscle morphometric data is not available for this age group of animals with hypobaric hypoxia.

The observation that total muscle compliment of Glut 4 remains elevated with a further three weeks of experiment condition presents its own conundrum. This effect is attributed to a higher muscle protein content found in the hypoxic soleus and plantaris after one month. This sedentary adaptation in muscle protein compliment is not seen at one week. An increase in total protein compliment seems, therefore, to be an adaptation

temporaly behind the increase in Glut 4. A potential masking of the Glut 4 adaptation at the enrichment level may be illustrated by this comparison.

We predicted that the fast-twitch plantaris, would demonstrate a greater response to hypoxia based on muscle stimulation data (Etgen et al. 1993b). In that study, a higher degree of adaptation was observed in fast twitch muscle (extensor digitorum longus, EDL) due to its higher relative capacity for increase in aerobic components (oxidative enzymes, Glut 4) (Etgen et al. 1993b). This was not observed in the current study, however, as soleus muscle increases in Glut 4 content were relatively larger (Fig. 13). In our pilot work, we studied the EDL and found a greater change than was observed in the soleus (appendix one). This discrepency may be due to the higher content of fast glycolytic (FG) fibers in the EDL than the plantaris, as much of the adaptation in the work of Etgen and colleagues (1993b) was attributed to shifts from FG to fast oxidative glycolytic (FOG) fiber characteristics. Previous work at altitude has illustrated fast glycolytic fiber interconversion in fast-twitch muscles towards FOG with sedentary exposure (Sillau and Banchero 1977; Bigard et al. 1991b) and towards FG with mild exercise (Bigard et al. 1991b). Trained and sedentary exposure to altitude illustrated no changes in soleus fiber compliment under any condition (Bigard et al. 1991b). Though an increase (39 percent) in HK activity was noted with fourteen week sedentary altitude exposure in the soleus (Bigard et al. 1991b). These expectations for soleus and plantaris changes were based on data from older animals in all

cases. The changes in soleus muscle over the developmental period in question are quite profound, with increased compliment of slow fibers (Maltin et al. 1991) and a reduction in overall glucose transport capacity (Goodman et al. 1983). The current results reflect the plasticity of this tissue at this time, and are quite unique, given the exposure period for previous data.

Dietary restriction did not result in any significant changes in Glut 4 content in any tissue analyzed in the one month experiment, when observed per ug protein or per mg tissue (Figs. 12 and 13). Non significant increases in content (per ug protein and per mg tissue) were noted after one week of dietary restriction in the developing animals. While these may have contributed to the results observed with hypoxia, the absence of weight change in either group makes this unlikely. These results indicate that the previously observed increases in glucose transport capacity with dietary restriction are not applicable to simulated hypobaric hypoxia. Further, the changes in investigated parameters were not due to the anorexia often observed with chronic hypobaric hypoxia. This data confirms previous investigators observations of no change in Glut 4 mRNA with dietary restriction (Wake et al. 1991).

Metabolic Enzyme Activity

Our investigation of sedentary exposure to hypobaric hypoxia did not demonstrate any significant alterations in any maximal enzyme activities between experimental groups. A decrease in activity of several enzymes with development were evident. HK and PK activity declined in both the soleus and plantaris. Investigators have found increases in glycolytic capacity in sedentary hypoxic exposed animals (HK and LDH in soleus and plantaris), and increases in oxidative capacity with hypoxia and training (CS and HAD in EDL and plantaris (Bigard et al, 1991b). The adaptation evident above occurred over a significantly longer experimental duration (fourteen weeks) (Bigard et al. 1993b). It may be that the stimulus inducing Glut 4 alterations would result in enzymatic adaptation over a longer study period. Chronic low frequency fast twitch muscle stimulation results in increased Glut 4 content and CS activity (Etgen et al. 1993b). The time course for the change in CS was significantly longer in this experiment (Etgen et al. 1993b). As described earlier, the rate limiting step in the utilization of exogenous glucose is its transport into muscle (Weber 1988; Furler et al. 1991; Ren et al. 1993). We would expect, rationally, that this would be the first component to adapt as it would be most effective in alleviating an imposed demand. Using system relationship theory, however, we would expect the metabolic profile within the muscle to adapt along with Glut 4 capacity.

The metabolic system relationships within soleus and plantaris muscle did not differ between the experimental groups (Tables 5 and 6). The ratios observed fall within expected ranges (Hochachka et al. 1982; Hochachka 1985; Suarez, Brown and Hochachka 1986) in all cases. This was not surprising, as no significant alteration in the activity of any one enzyme was evident between experimental groups (Tables 5 and 6). The increased oxidative capacity for a given VO2 max. in altitude populations is the result of a lifetime at altitude, as well as selection over several generations. The unique metabolic system relationships observed in these populations may provide them with higher efficiency of energy provision in muscle. Adaptation in this direction was not evident over four weeks of simulated exposure in sea level animals.

Glut 4 and Oxidative Capacity

The comparison between oxidative capacity (citrate synthase (CS) activity) and Glut 4 content in our study presented some interesting findings. A separation in grouping between the developing and the mature tissue was clearly evident, and reflected the lower CS activity and Glut 4 content that we found in the mature soleus and plantaris muscles. These changes were expected, based on the observed declines in glucose uptake and HK activity in EDL muscle over this time period (Goodman et al. 1983). This should not be

mistaken for the documented changes with senescence, as the mature animals in this experiment were seven months of age.

The expected changes in oxidative capacity with Glut 4 content were not observed with one week or one month exposure to hypoxia or dietary restriction. The coordinate regulation of Glut 4 mRNA and HK-II mRNA over the first sixty days of development has been demonstrated (Postic et al. 1994). Further, with creatine depletion induced by six weeks of B-guanidinopropionic acid ingestion, Glut 4 expression and oxidative capacity (CS and HK activity), were seen to be co-regulated (Ren, Semenkovich and Holloszy 1993). That this did not occur with our observed increase in Glut 4 content per mg may be a result of the lower percent (~30 % vs. ~50 %) increase observed with hypoxic adaptation.

The soleus and plantaris muscles illustrated similar profiles of CS activity and Glut 4 content, with no differences evident between the muscles within a given age group (Fig. 14 and 15). The expected positive relationship between these two indicators of oxidative capacity was observed (Fig. 14), though changes with experimental condition in Glut 4 content were not mirrored in CS activity. The variability in time course in system adaptation to hypobaric hypoxia is evident with the difference observed between one and four weeks in Glut 4 content. As discussed above, an increased duration may have affected enzyme activity and, therefore, this relationship. Though large

increases in HK and Glut 4 were observed after only two days of prolonged exercise training (Ren et al. 1994).

Conclusions

Glucose transporter four content in skeletal muscle was seen to rise in response to one week and one month of hypobaric hypoxia in developing animals. It was concluded that this change was not due to the anorexic effect often observed with altitude acclimatization. This adaptation was found to be fairly rapid, taking place following only one week of exposure. The proposed co-regulation of muscle enzyme activity through an increase in the capacity to oxidize glucose was not observed. An increased reliance on exogenous glucose as an adaptation to chronic hypobaric hypoxia cannot be concluded from this data as all expected parameters were not up regulated, and enzymatic profile was to be the indicator of fuel preference.

Future Directions

Whether an alteration in glucose utilization is occurring as a response to hypobaric hypoxia requires further research. An investigation of insulin receptor number in the tissue from this experiment is currently underway, and will illustrate whether changes at the stimulus level are occuring. This is

important, as the changes in transport rate (Ivy et al. 1991) with restriction of diet were found not to be due to an upregulation of Glut 4 mRNA (Wake et al. 1991). Further, whether altitude populations and species display high relative muscle Glut 4 content, concomitant with the known changes in glucose utilization and oxidative capacity is not known. Hypobaric hypoxia has been shown to increase glycolysis and glucose transport in rat brain (Harik et al. 1995). This adaptation is due to changes in brain content of Glut 1, the only glucose transporter evident in brain tissue. While the content of skeletal muscle Glut 1 is minimal (James et al. 1988, Mueckler 1995), an increase is possible, which is especially relevant over the developmental period investigated (Santalucia et al. 1992). The most striking results from this study are the differences in adaptation between the mature and developing animals. The rapid time course of the adaptations in the developing animals, as well as the change between one week and one month of exposure to hyobaric hypoxia are also interesting. The plasticity of this developing tissue is very evident. Work on muscle cell cultures indicates that an increase in Glut 1 is induced rapidly with hypobaric hypoxia. An investigation of longer term adaptation, begining earlier in life (perinatal) seems to be the next step.

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Appendix One

Pilot Study

Methods

GLUT-4 Determinations

Muscles for the study of glucose transporter 4 content were chosen in order to provide a wide range of fiber type compositions. The use of the soleus 84%(type 1)-16%(type IIa)-0%(type IIb) and the extensor digitorum longus (EDL) 3-57-40% (Henriksen et al, 1990) enabled the representation of each fiber type predominantly. The Glut-4 content of several muscles will be analyzed to give an indication of chronic adaptation by the methodology obtained from L. Megeney of the U. of Waterloo. (Megeney et al. 1993), with some modifications. The muscles will be obtained following anesthetic administration as described above, and kept frozen at -80 C until homogenization can be performed.

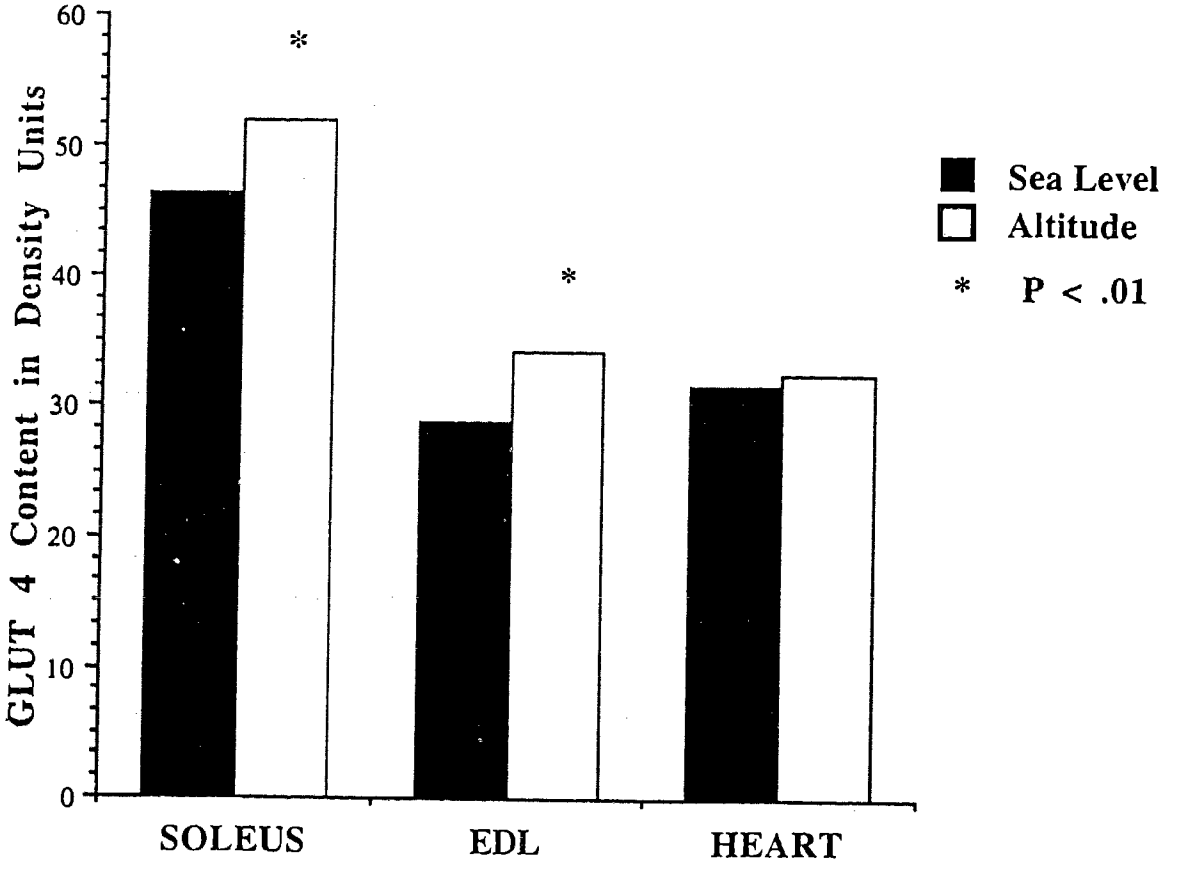
Homogenization of ~ 200 mg of sample will be performed using a polytron homogenizer at a setting of 7 for 15 seconds. To obtain 200 mg of some muscles pooling from the same animal group will be necessary. Homogenization will occur in Pilch buffer (Megeney et al. 1993). This buffer containing 25 mM Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (ph 7.4)), 25 mM benzamidine, 4mM EDTA, 0.5 mM Phenylmethylsulfonyl flouride and 1µM each of pepstatin, leupeptin and

aprotinin. To isolate total cellular protein from the homogenate, centrifugation using a Beckman 40 Ti rotor (130,000 g , 4 C, 1 hour). The pellet was then resuspended through homogenization in 360 µl (180 µl) pilch buffer. 40 µl (20 µl) of 10% Triton is then added prior to placing the resuspension on ice for 1.5 hours to solubilize membrane bound protein. Centrifugation is then repeated, and the protein content of the resulting supernatant analyzed by the Lowry method (Lowry et al, 1951). Bovine serum albumin suspended in pilch buffer, is used as protein standards. Content is then analyzed using a Perkin Elmer UV/VIS Spectrophotometer (750 nm).

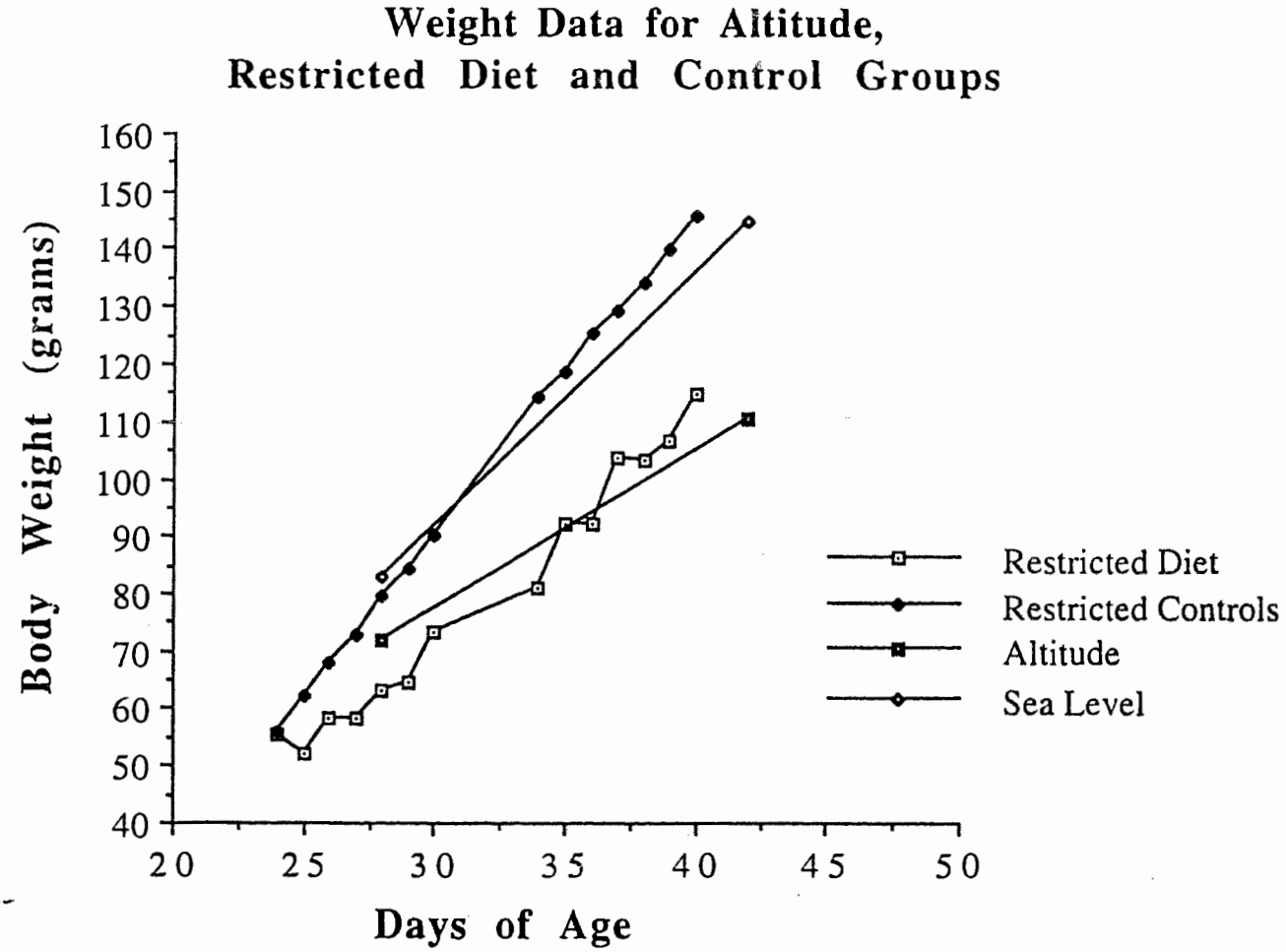
The samples are then analyzed for GLUT 4 content by western dot blot analysis. 30 µg of protein from each sample is diluted 1:4 with sample buffer containing Tris, SDS, glycerol, DTT and bromophenol blue. Samples are separated on an 12% SDS polyacrylimide gel for 45 minutes and then transferred to an immobolin membrane (millipore) by electromembrane transfer for 1 hour, at 4 C. Glut 4 content is then analyzed using a polyclonal antibody specific to Glut 4 (East Acres Biological) and an amplified Alkaline Phosphatase detection protocol (Biorad). The density of the blots is then determined using Desk scan II and Image 1.47 computer analysis procedures. Glut 4 content is expressed in density units relative to control values which are run in parallel on the same gel.

Pilot Study

GLUT 4 Content in Soleus, EDL and Heart for Control and Acclimatized Animals



Pilot Study


Weight Data for Altitude,
Restricted Diet and Control Groups
Body Weight (grams)
160
150
140
130
120
110
100
90
80
70
60
50
40
20
25
30
35
40
45
50
Days of Age
Restricted Diet
Restricted Controls
Altitude
Sea Level

APPENDIX TWO :
MUSCLE CONTENT OF GLUCOSE TRANSPORTER FOUR
(GLUT4) PROTEIN BY WESTERN BLOT ANALYSIS

SOLEUS

Group	ALTITUDE		RESTRICTED		CONTROL	
	OD/ug protein	OD/mg tissue	OD/ug protein	OD/mg tissue	OD/ug protein	OD/mg tissue
Mature	102 +/- 4	87 +/- 7	99 +/- 2	88 +/- 6	100 +/- 1	100 +/- 6
Young	107 +/- 2	137 *# +/- 5	99 +/- 4	109 +/- 5	100 +/- 5	100 +/- 8
Young one week	129 * +/- 4	126 * +/- 6	119 +/- 8	115 +/- 9	100 +/- 8	100 +/- 9

PLANTARIS

Group	ALTITUDE		RESTRICTED		CONTROL	
	OD/ug protein	OD/mg tissue	OD/ug protein	OD/mg tissue	OD/ug protein	OD/mg tissue
Mature	99 +/- 1	99 +/- 5	94 +/- 5	95 +/- 4	100 +/- 4	100 +/- 7
Young	101 +/- 2	121 *# +/- 3	101 +/- 2	104 +/- 5	100 +/- 3	100 +/- 9

Data shown as mean (n=6) of optical density (OD) with standard error. * Indicates difference from control, level of signigficance P< .05 using student T-Test. # Indicates difference from restricted, level of significance P< .05 using student T-Test.