Insulin resistance for glucose metabolism in disused soleus muscle of mice

MICHAEL J. SEIDER, WILLIAM F. NICHOLSON, AND FRANK W. BOOTH
Department of Physiology and Cell Biology, University of Texas Medical School, Houston, Texas 77030

SEIDER, MICHAEL J., WILLIAM F. NICHOLSON, AND FRANK W. BOOTH. *Insulin resistance for glucose metabolism in disused soleus muscle of mice.* Am. J. Physiol. 242 (Endocrinol. Metab. 5): E00–E00, 1982.—Our hypothesis was that insulin resistance for carbohydrate metabolism develops after a single day of muscular disuse. The immobilization of the mouse hindlimb for 24 h was used to produce muscular disuse (*group C*). As food intake was voluntarily decreased during the immobilization, two additional groups were used: *group A* was untreated and ate ad libitum, whereas *group B* was anesthetized with *group C* and was fed amounts of food similar to those eaten by *group C*. Because *groups B* and *C* differed only by limb immobilization, *group B* was used as the reference group. When insulin was present in the incubation media, the rates of 2-deoxyglucose uptake and glycogen synthesis were always significantly decreased in soleus muscles from *group C* (anesthetized, ate less, immobilized) as compared to *group B* (anesthetized, pair-fed food that *group C* ate). Significant interaction between the factors of insulin and muscular disuse for the rates of 2-deoxyglucose uptake and glycogen synthesis support the concept that disuse of skeletal muscle attenuates insulin action. These observations are a direct demonstration of a decrease in insulin responsiveness in skeletal muscle as the result of a single day of muscular inactivity. Because plasma insulin concentrations were significantly lower in *groups B* and *C* than in the untreated group, it seems likely that the development of insulin resistance in disused skeletal muscle is independent of plasma insulin levels.

limb immobilization; muscle atrophy; skeletal muscle; insulin responsiveness; corticosterone

INSULIN SENSITIVITY for glucose uptake decreases across the forearm of healthy people after a few days of continuous bed rest (21, 22). Indeed, one textbook of internal medicine offers the advice that physicians should be aware that false positive tests for glucose intolerance and diabetes can occur in humans just because of bed rest (34). The molecular bases of these observations are unknown. Because a direct measurement of insulin resistance in disused skeletal muscle has not previously been

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MATERIALS AND METHODS

Animal care and experimental groups. Male, outbred, albino mice, weighing 20–25 g, were obtained from Texas Inbreed Mouse Co. (Houston). They were housed in a constant temperature (22°C) animal room with a 12:12-h light:dark cycle for at least 1 wk prior to usage. They received food (Purina mouse chow) and water ad libitum prior to the experiment.

On the day that the experiment started, mice were randomly divided into three groups, and their body weights were measured. The three experimental groups were: *A*, untreated; *B*, anesthetized and food-restricted (fed an amount of food equivalent to that of *group C*);

and *C*, anesthetized while undergoing limb immobilization and given food and water ad libitum (Fig. 1). Further descriptions of the treatments that some of these groups received follow. *Groups B* and *C* were anesthetized (50 mg sodium pentobarbital/kg body wt) 1 day before removal of their soleus muscle. *Group C* had both of its hindlimbs immobilized with plaster of paris so that feet were in plantar flexion while the mice were anesthetized 1 day before removal of the soleus muscle. The immobilization procedure was a modification of that previously described for rats (6). Thus, the hindlimbs of *group C* were immobilized for 24 h. Pilot experiments established the amount of food eaten by *group C* during the 1st day of limb immobilization. This amount of food was given to *group B* on the day before removal of their soleus muscles.

In vitro incubation. Mice were anesthetized with sodium pentobarbital (50 mg/kg body wt ip). Plaster casts, if present, were removed and the soleus muscles dissected. The proximal tendon of the muscle was isolated and ligated with 6–0 silk. The muscle was then removed and quickly placed into a tissue bath (37°C) containing oxygenated Krebs-Ringer bicarbonate buffer (pH, 7.4) and 5 mM glucose and 1% fatty-acid-free bovine serum albumin. While in this bath, the muscle was placed on a Dow Corning Silastic strip by placing 27-gauge needles through the Achilles and proximal tendons of the soleus muscle. The muscle was stretched to its in vivo resting length when pinned to the Silastic block, and a gap of approximately of 3 mm was left between the block and muscle. The block with its pinned muscle was then transferred from the tissue bath to a plastic test tube for preincubation as described for each specific experiment.

Assay procedures. The same muscle was used to measure glucose oxidation and glycogen synthesis. For glucose oxidation, muscles pinned to Silastic blocks were preincubated for 30 min at 37°C in 2.5 ml of Krebs-Ringer bicarbonate buffer (pH, 7.4) containing 5 mM glucose, 1% fatty-acid-free bovine serum albumin, and insulin as indicated. The tubes containing muscles and medium were placed into a gyrotory shaker bath and were continuously gassed with O_2:CO_2 (19:1). After preincubation, muscles with their Silastic blocks were transferred to a second set of plastic tubes containing the incubation medium. This medium was identical to the preincubation medium except that 0.2 μCi [^{14}C]glucose (uniformly labeled, New England Nuclear) per milliliter of medium was included for the measurement of glucose oxidation and glycogen synthesis rates. Before the addition of the muscle to the incubation tubes, tubes were continuously gassed with O_2:CO_2 (19:1) for 1 h followed by an additional minute of gassing after the addition of muscle and before sealing the tube with a rubber stopper for the duration of the subsequent 1-h incubation. Following the

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Muscles in which glucose oxidation was measured were also utilized for assessment of glycogen synthesis. The formation of glycogen from radiolabeled glucose was determined using modifications of the methods of Stauffacher and Renold (33) and Cuendet et al. (10). After the muscles had been removed from the incubation medium, they were placed in preweighed tubes containing 0.5 ml of 1 N NaOH and 10 mg of carrier glycogen. Tubes were then reweighed and placed in boiling water for 10 min. A 50-μl aliquot of the resulting hydrolysate was removed for protein determination by the method of Bradford (7). Then 1.6 ml of absolute ethanol were added to the

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remaining hydrolysate, and glycogen was allowed to precipitate overnight at −80°C. The glycogen precipitate was then washed twice with 1 ml of 66% ethanol. The washed precipitate was dissolved in 0.5 ml water and added to Aquasol (New England Nuclear) for liquid scintillation counting. Correction of counts per minute to disintegrations per minute was made via a quench curve.

Procedures for the measurement of 2-deoxyglucose uptake into soleus muscles differed from the previous measurements. The preincubation medium consisted of Krebs-Ringer bicarbonate buffer (pH, 7.4) with 2 mM pyruvate and insulin as indicated. In addition to these modifications, the incubation medium contained 5 mM 2-deoxyglucose, 0.5 μCi 2-[^{3}H]deoxy-D-glucose per milliliter of medium, and 0.1 μCi [^{14}C]inulin per milliliter of medium. Experimental procedures performed during preincubation and incubation were identical to that described above except that the duration of incubation was 15 min with continuous gassing with O_2:CO_2 (19:1) during this time. Following incubation, muscles were removed, blotted, and then placed in preweighed tubes containing 0.5 ml of 1 N NaOH and reweighed. Muscles were then hydrolyzed in the NaOH, and a sample of the hydrolysate was counted. Extracellular amounts of 2-[^{3}H]deoxy-D-glucose were estimated from inulin-accessible spaces, and intracellular 2-[^{3}H]deoxy-D-glucose was obtained by subtracting the extracellular amount from the total in the muscle.

For the determination of whole muscle ATP and glycogen levels, soleus muscles were quick-frozen with liquid nitrogen-cooled Wollenberger tongs. Glycogen and ATP were determined with the fluorometric procedure of Lowry (24). The concentration of ATP in the mouse soleus muscle was not significantly changed from in vivo levels by 90 min of incubation in vitro. There was 25.5 ± 1.9 nmol ATP/mg protein in soleus muscles frozen in liquid nitrogen-cooled Wollenberger tongs immediately after the removal of the muscle from the anesthetized mouse, and there was 26.5 ± 3.3 nmol ATP/mg protein in soleus muscles after 90 min of in vitro incubation. This demonstrated the viability of these muscles during incubation.

Blood samples for the measurement of plasma glucose and immunoreactive insulin (IRI) were obtained from anesthetized mice via cardiac puncture into heparinized syringes. These mice were not the same animals used for muscle studies. Blood was immediately centrifuged and the plasma stored at −80°C until assayed. Plasma IRI measurements were made with an insulin radioimmunoassay kit (Amersham) using human insulin as standard. Plasma glucose was determined with a colorimetric procedure (29).

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Mice for the glucocorticoid study were brought daily to the laboratory and were handled for a period of 10 days to acclimate them. One-half of the mice had hindlimbs immobilized the day before decapitation, and the remainder were controls. Following decapitation, blood was collected in a heparinized tube, centrifuged, and then frozen until assayed. Plasma from two mice were pooled for a single observation. Plasma corticosterone was measured by the fluorometric procedure of Silber et al. (32).

Statistical analysis was performed with an unpaired Student's *t* test or with factorial analysis of variance (ANOVA). Tukey's least-significant difference and Fisher's *F* tests were used to identify significant differences among groups and treatments. A probability level of 0.05 was designated as significantly different.

RESULTS

Following the treatment period, there were no significant differences among *groups A*, *B*, and *C* for the wet weight and the total protein content of the soleus muscle (Table 1). Food intake and body weight, however, were significantly decreased in *group B* (anesthetized with food restriction) as compared to the untreated group (*group A*) (Table 1). Adding the treatment of limb immobilization contributed no further significant difference in either food intake or final body weight. Muscle

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Table 1

glycogen was significantly decreased in *group B* (Table 1). Food restriction is known to decrease muscle glycogen (19) so that the difference between *group A* (untreated) and *group B* (anesthetized, food-restricted) is not surprising. However, *groups B* and *C* ate similar amounts of food on the treatment day; yet, *group C* had approximately 2 times greater concentration of muscle glycogen than did *group B* (Table 1). Because the only treatment difference between these groups was limb immobilization, the reduction in the usage of the soleus muscle by limb immobilization may have resulted in a decreased rate of glycogenolysis in *group C*. Values for body weight, muscle wet weight, muscle total protein content, and muscle glycogen concentration of the untreated group are similar to that observed by others (19).

Significant hypoinsulinemia was observed in *groups B* and *C* with respect to *group A* (Fig. 2). There were, however, no significant differences between *groups B* and *C* in plasma IRI. Food restriction has been shown to produce hypoinsulinemia in mice (19). Because we decided to measure carbohydrate metabolism in muscles from *groups A–C* at the same time, the quantity of food given *group B* was based on a pilot experiment during which food intake was measured during the 1st day of hindlimb immobilization and was shown to be 1.2 g/day. However, mice whose muscles were used in measurements of carbohydrate metabolism in *group C* ate one-third less than those mice in the pilot experiment. To test whether this difference in food intake would effect plasma IRI, a third set of mice was used. No significant difference existed in plasma IRI between the groups of mice eating either 1.2 or 0.8 g/day during the 1st day of hindlimb immobilization (Fig. 2). These values also did not differ from a group of mice, not immobilized, but eating 1.2 g of food (Fig. 2). Thus, the difference in food intake from 1.2 to 0.8 g was not sufficient to cause a further significant change in plasma IRI. Values for plasma IRI in mice are similar to published values (14, 19, 25).

Plasma corticosterone was significantly increased ($P < 0.05$) after 1 day of hindlimb immobilization (30.8 ± 3.0 μg corticosterone/dl plasma) as compared to control values (21.2 ± 2.2). There were five to six observations per mean. Values for plasma glucose in *group B* (63.4 ± 7.8 mg glucose/dl) were not significantly different from either *group A* (72.9 ± 11.5) or *group C* (51.2 ± 5.7). There were three to seven observations per mean.

Significant differences among *groups A*, *B*, and *C* existed for 2-deoxyglucose uptake, glycogen synthesis, and glucose oxidation (as shown by the *F* value for the factor of muscular usage in the factorial ANOVA, Table 2). To determine which of these means were different,

Fig 2

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Significant differences among *groups A*, *B*, and *C* existed for 2-deoxyglucose uptake, glycogen synthesis, and glucose oxidation (as shown by the *F* value for the factor of muscular usage in the factorial ANOVA, Table 2). To determine which of these means were different, Tukey's multiple-range test was used. Treatment with sodium pentabarbital and food restriction for only 1 day prior to measurement resulted in some significant differences in carbohydrate metabolism. 2-Deoxyglucose uptake rate at 0.5 nM insulin in the incubation medium was significantly increased in *group B* versus *group A*, as was glycogen synthesis rate at 5 and 10 nM medium insulin (Figs. 3 and 4). Glucose oxidation rate at 5 nM medium insulin was decreased in *group B* versus *group A* (Fig. 5). Because the only treatment difference between *groups B* and *C* was the existence of hindlimb immobilization in *group C*, *group B* was chosen as the reference group for *group C* when determining whether or not statistical differences existed solely as a result of the treatment of hindlimb immobilization.

In the absence of insulin, there were no significant differences between *groups B* and *C* in any indices of carbohydrate metabolism utilized (Figs. 3–5). In the presence of all concentrations of insulin, however, 2-deoxyglucose uptake and glycogen synthesis were significantly depressed in *group C* versus group B (Figs. 3 and 4). This finding suggests decreased responsiveness and sensitivity to insulin in the soleus muscle as a consequence of limb immobilization. On the other hand, there was no significant difference in glucose oxidation in soleus muscle between *groups B* and *C*.

Table 2

Figs 3 & 4

Fig 5

The ANOVA test also indicated that the levels of 2-deoxyglucose uptake and glycogen synthesis were dependent on insulin, but that the amount of glucose oxidation was independent of the level of insulin. In addition, significant interaction existed between the factors of insulin and muscular usage for the processes of 2-deoxyglucose uptake and glycogen synthesis rates (Table 1). Thus, muscular disuse attenuated the effect of varied concentrations of insulin on 2-deoxyglucose uptake and glycogen synthesis rates. No significant interaction between these factors existed for glucose oxidation rate. The rates of 2-deoxyglucose uptake, glycogen synthesis, and glucose oxidation under control conditions observed in our present study are similar to previously published data (10, 20).

DISCUSSION

Insulin sensitivity and responsiveness for indices of glucose metabolism are decreased in the soleus muscle of mice whose hindlimbs had been immobilized during the previous day. Compared to previous reports, this change is noteworthy both in the degree of lost insulin responsiveness and in the rapidity of this loss. First, the loss of insulin responsiveness is greater in muscles from immobilized limbs. For example, indices of glucose metabolism in the soleus muscle of obese animals do not show the degree of loss in insulin sensitivity that was observed in the soleus muscles of immobilized limbs. Insulin responsiveness for 2-deoxyglucose uptake is 60–100% of normal values in the soleus muscle in vitro from obese animals (9, 10, 11, 15). In these same studies, the insulin responsiveness was 30–60% of normal for glycogen synthesis and 40–65% of normal for glucose oxidation in soleus muscles from obese animals. In contrast, there was essentially no insulin responsiveness of soleus muscles from immobilized mouse limbs in the present study. Second, the onset of the loss of insulin sensitivity and responsiveness is more rapid in soleus muscles from immobilized limbs than from limbs of starved or obese mice (15, 19). For example, insulin sensitivity and responsiveness for glucose oxidation in the soleus muscle increase after 1 day of fasting, but insulin sensitivity and responsiveness for glucose uptake and glycogen synthesis rates did not increase until the 2nd day of fasting (19). On the other hand, a marked insulin resistance for 2-deoxyglucose uptake and glycogen synthesis was present in the soleus muscle after 1 day of muscular disuse.

These observations on the more rapid and greater loss of insulin responsiveness in soleus muscles from immobilized limbs as compared to obese animals suggest that some process associated with limb immobilization is very powerful in modulating the sensitivity of the muscle to

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These observations on the more rapid and greater loss of insulin responsiveness in soleus muscles from immobilized limbs as compared to obese animals suggest that some process associated with limb immobilization is very powerful in modulating the sensitivity of the muscle to insulin. Glucose intolerance associated with insulin resistance exists in a number of clinical states, such as obesity, nonketotic diabetes mellitus, chronic renal failure, and states of adrenal corticoid excess (30). Hyperinsulinemia and/or increased blood glucocorticoids play roles in insulin resistance in certain of the above clinical conditions (30).

Hypoinsulinemia for a period of days in starved or streptozotocin diabetic mice is associated with an increased sensitivity to insulin by the soleus muscle (19). However, hypoinsulinemia is associated with a decreased, rather than an increased, sensitivity to insulin in soleus muscles from immobilized limbs. In contrast to the association of hyperinsulinemia and insulin resistance in the soleus muscle of obese mice (9–11, 15, 20, 27), insulin resistance in the soleus muscle after a day of disuse is not associated with hyperinsulinemia. Thus, insulin resistance in the disused soleus muscle is not a result of hyperinsulinemia and probably is not a result of insulin-receptor down-regulation, an event that is associated with hyperinsulinemia (16). On the other hand, levels of plasma corticosterone were most likely high throughout the 1 day of limb immobilization. We observed an increased plasma corticosterone level after 1 day of limb immobilization, which confirms a similar observation made by Ganong et al. (13) in dogs during

limb immobilization. An increase in plasma corticosteroids might therefore result in insulin resistance as corticosteroids decrease the ability of skeletal muscles to oxidize glucose (18, 31). Part of this action may be caused by a decreased affinity for insulin by insulin receptors of skeletal muscle because a similar postulate has been shown for hepatocytes and adipocytes (17, 28).

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FIG. 1. Treatments given to the 3 groups of mice used for measurements of carbohydrate metabolism in soleus muscle.

FIG. 2. Plasma immunoreactive insulin (IRI) was measured in 4 groups of mice. These groups were: *1*) controls who daily ate 4.1 g of food ad libitum ($n = 12$), *2*) anesthetized 1 day before and restricted to 1.2 g of food during next day ($n = 10$), *3*) anesthetized while hindlimbs were immobilized 1 day before and restricted to 1.2 g of food during next day ($n = 5$), and *4*) anesthetized while hindlimbs were immobilized 1 day before and eating 0.8 g of food during next day ($n = 12$). $^*P < 0.05$, treatment group vs. control group. No significant differences existed among treatment groups. A least-significant-difference test was used to establish where significant differences existed among means after a significant F value was obtained from a one-way ANOVA. Values are means ± SE; n = number of observations. Each observation is pooled blood from 2 mice.

FIG. 3. 2-Deoxyglucose uptake into mouse soleus muscle as a function of insulin concentrations in incubation medium. With Turkey's multiple-range test, a $P < 0.05$ within a single insulin concentration is indicated by * between ○ (*group B*) and ● (*group C*), † between ▲ (*group A*) and ●, and ‡§ between ▲ and ○. Values are means ± SE.

FIG. 4. Glycogen synthesis rates in mouse soleus muscle as a function of insulin concentrations in incubation medium. A $P < 0.05$ within a single insulin concentration is indicated by * between ○ (*group B*) and ● (*group C*) and ‡§ between ▲ (*group A*) and ○. Values are means ± SE.

FIG. 5. Glucose oxidation rates in mouse soleus muscle as a function of insulin concentrations in incubation medium. A $P < 0.05$ within a single insulin concentration is indicated by † between ▲ (*group A*) and ● (*group C*) and ‡§ between ▲ and ○ (*group B*). Values are means ± SE.

Previous reports showed a decreased uptake of glucose across the forearms of healthy people during continuous bed rest (21, 22). In these studies, muscle, skin, and bone were perfused. The existence of insulin resistance in skeletal muscle as a result of disuse was inferred. Our present data suggest a direct ~~insulin~~ insulin resistance in skeletal muscle as a consequence of disuse. The usage of isolated muscles in vitro has the advantage of testing for the direct involvement of skeletal muscle in insulin resistance. This methodology also has potential limitations. One disadvantage is diffusion limitations. Muscles in vitro must rely on the diffusion of substrates and hormones from the external medium instead of on delivery under pressure from capillaries. Utilization of the mouse soleus muscle minimizes this limitation for the following reasons. First, diffusion limitations for glucose uptake (8) and for glucose oxidation and glycogen synthesis (9) do not occur until the wet weight of the soleus muscle exceeds 30 mg (9). Soleus muscles were one-third this weight in the present study. Second, the concentration of ATP in soleus muscles did not differ between in vivo values and levels found at the end of the in vitro incubation period. This suggests that there was no major diffusion limitation for oxygen in isolated muscles of the present study.

The results of the present study on muscular disuse, as well as the results of earlier studies on muscular activity, suggest the following concept: there is a direct relationship between the quantity of contractile activity and the insulin responsiveness of the skeletal muscle. After 1 day of limb immobilization, physiological and pharmacological concentrations of insulin are not effective in stimulating carbohydrate metabolism in the disused soleus muscle. In contrast, lower than physiological concentrations of insulin in the plasma or perfusate are effective in stimulating glucose uptake into contracting skeletal muscle (1, 2). Although numerous studies have shown a decreased glucose tolerance in healthy humans after chronic bed rest (5, 12, 21, 22), an increased insulin sensitivity for glucose removal during glucose tolerance has been shown after physical training in humans (4, 23) and in animals (3, 26). Thus, responsiveness of the muscle to insulin is directly related to its immediate history of usage.

In summary, our present results provide the first direct evidence of insulin resistance in skeletal muscle that has undergone a previous period of reduced muscle usage. Furthermore, this lack of responsiveness to insulin developed in 1 day and in the presence of hypoinsulinemia.

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In summary, our present results provide the first direct evidence of insulin resistance in skeletal muscle that has undergone a previous period of reduced muscle usage. Furthermore, this lack of responsiveness to insulin developed in 1 day and in the presence of hypoinsulinemia. Future studies that utilize the model of hindlimb immobilization to determine the cause(s) of these changes now seem to be justified.

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TABLE 1. *Some characteristics of mice after various treatments*

	Group A	Group B	Group C
Body wt, g			
Before treatments	31 ± 1 (18)	31 ± 1 (24)	31 ± 1 (24)
One day later	30 ± 1* (16)	27 ± 1 (21)	28 ± 1 (20)
Food intake, g/day	5.0 ± 0.6† (16)	1.2 ± 0.4 (21)	0.8 ± 0.2 (20)
Soleus wet wt, mg	9.8 ± 0.5 (18)	9.4 ± 0.3 (42)	10.1 ± 0.3 (42)
Soleus protein content, mg protein/soleus muscle	1.52 ± 0.05 (18)	1.58 ± 0.05 (42)	1.66 ± 0.06 (42)
Soleus glycogen conc., μmol/g wet wt	22.6 ± 1.0† (17)	11.6 ± 2.0 (8)	23.4‡ ± 3.0 (8)

Values are means ± SE; numbers in parentheses are numbers of the observations. *Group A*: untreated; *group B*: anesthetized, food-restricted; *group C*: anesthetized, ate less, hindlimbs immobilized for 1 day. See METHODS for description of treatments. Statistical analysis was with an unpaired Student's *t* test. * $P < 0.005$ vs. *group B*: † $P < 0.001$ vs. *group B*; ‡ $P < 0.01$ vs. *group B*.

TABLE 2. *F values for factorial ANOVA for 2-deoxyglucose uptake, glycogen synthesis, and glucose oxidation*

Treatments	F values		
	2-Deoxyglucose	Glycogen synthesis	Glucose oxidation
Insulin	7.42*	8.49*	1.97
Muscle usage	13.35*	43.41*	40.90*
Insulin × muscle usage	3.00†	2.97‡	1.98

A 3 × 3 factorial ANOVA was used for 2-deoxyglucose, whereas a 3 × 5 factorial ANOVA was separately employed for glycogen synthesis and glucose oxidation. Application of Fisher's *F* test to *F* values from the factorial ANOVA demonstrated that significant differences among group means for 2-deoxyglucose and for glycogen synthesis occur because of variations in insulin dosage alone and that significant differences among group means occur for all three indices of glucose metabolism because of variation in usage amone. The *F* test also indicated that some of the significance among group means is due to interaction between the treatments of insulin muscle usage. In other words, the effects elicited by administration of these two treatments simultaneously attenuated the effect of insulin alone. * $P < 0.005$; † $P < 0.05$; ‡ $P < 0.01$.

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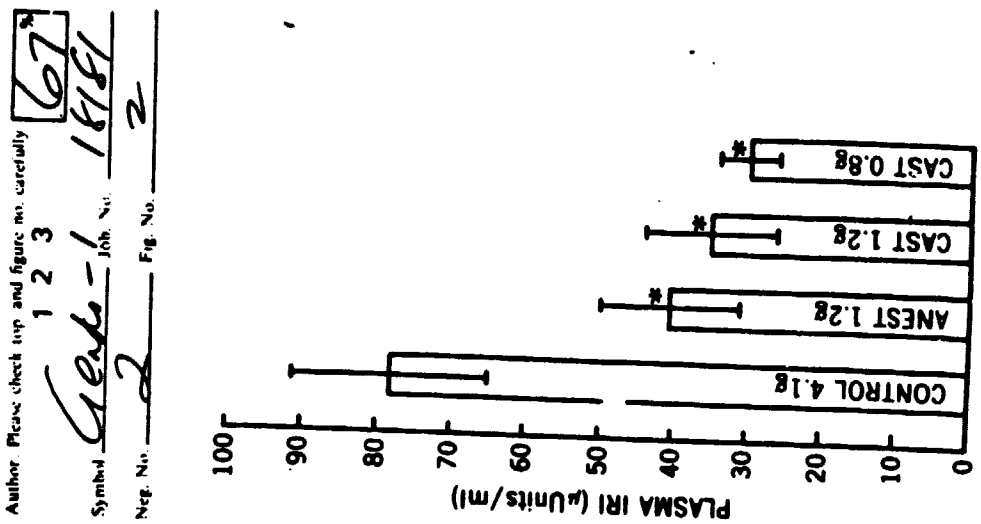
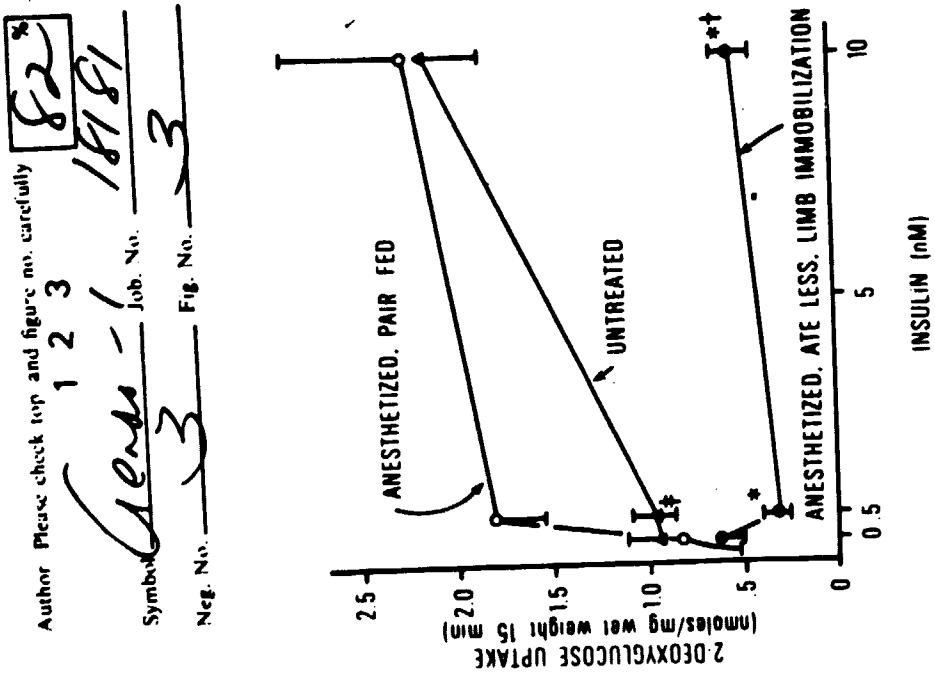
Treatment	Group A	Group B	Group C
Anesthesia	▬▬	Anesthetized 1 day prior	Anesthetized 1 day prior
Food restriction	▬▬	Food restricted 1 day prior	Ate less 1 day prior
Limb immobilization	▬▬	▬▬	Limb immobilization 1 day prior





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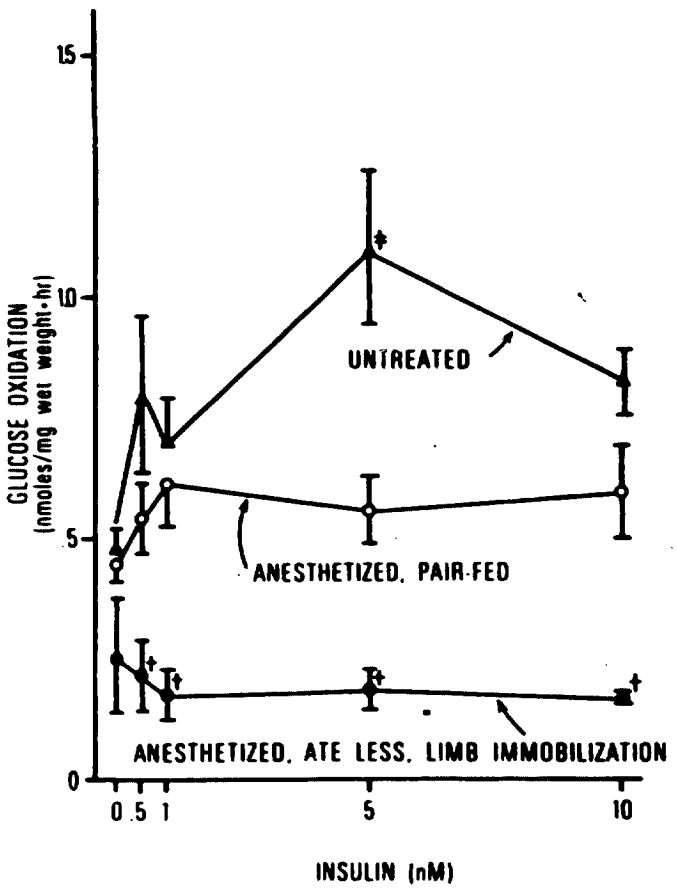

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