Mechanism of Muscle Hypertrophy and Glucose Utilization during Exercise

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1994

CONTENTS

ABBREVIATIONS

ACTH:	adrecocorticotropic hormone
+dF/dt:	maximal rates of force development
-dF/dt:	maximal rates of force relaxation
CT:	twitch contraction time
EDL:	extensor difitorum longus
EMD:	electromechanical delay time
FG:	fast-twitch-glycolytic
FOG:	fast-twitch-oxidative
GIR:	glucose infusion rate
Po:	maximal isometric tetanic force
Rg':	glucose metabolic index
RT:	relaxation time
SO:	slow-twitch-oxidative

GENERAL INTRODUCTION

In recent Japan, many people are interested in the effects of exercise in order to maintain health because many of them are short of exercise. It is known that enormous of energy is consumed and that metabolic state of animals is drastically changed during exercise. Health depends on the equilibrium between energy intake and exercise. However, the mechanisms how exercise effects on animal health are not obvious. Though there are many researches intending the improvement of exercise ability, few studies have been done to aim at clarifying the role of exercise in maintaining health. These days more people will suffer from lack of exercise and overintake of energy. If the mechanisms how exercise effects on human health can be clarified, it will be theoretical background for using exercise to maintain and advance human health. The author selects exercise-induced muscle hypertrophy and glucose utilization during physical inactivity as the exercise status to analyze. Because exercise consists of various complicating factors, it is important to simplify in the case of analysis. In this point of view, the author develops the simple models that simulate exercise status mentioned above. The effects that caused by exercise are evaluated by the index that properly expresses healthy status.

It has been known for over 50 years that androgen such as testosterone can increase muscle protein synthesis and promote muscle development when administered to hypogonadal or castrated men (Wilson and Griffin). The role of androgen in exercise-induced muscle hypertrophy, however, is obscure. Because androgen is an equally circulating humoral factor, the hypertrophy occurred only in exercised muscle cannot be explained by increase in androgen concentration. Therefore the author pays attention to the regulation of sensitivity to androgen, i.e., changes in the androgen receptor in the muscle and investigates its contribution.

It is known that glucose tolerance is impaired by physical inactivity. Because skeletal muscle is the major site of insulin-mediated glucose transport (DeFronzo et al.), the decrease in sensitivity or responsiveness of glucose uptake to insulin in muscle is related to muscle disuse (Fell et al., Seider et al., Smith et al), and the state of physical fitness plays a role in regulation of the in vivo insulin action (Fell et al., Heath et al., James et al 1983,1984,1985). In this thesis, the author uses a physical inactivity model to investigate the insulin action and post insulin receptor action under reduced-activity conditions.

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CHAPTER I MECHANISM OF SKELETAL MUSCLE HYPERTROPHY INDUCED BY EXERCISE

1. Development of the animal model to simulate skeletal muscle hypertrophy that occurs in humans in response to exercise

Muscle performance is strictly regulated by physical activities. The performance is improved by exercise training and deteriorated by inactivity. One of the most familiar examples is the increase in force output and the hypertrophy against continuous load. This phenomenon is observed only in the loaded muscles and called exercise-induced muscle hypertrophy (or work-induced muscle hypertrophy). Muscle hypertrophy is good for health because it causes a rise in basal metabolic rate. This means that the body becomes to spend much energy and that easy to avoid obesity. In this respect, appropriate mass of muscle is needed even for modernized people not for physical labor but for health.

Although the biochemical adaptations of skeletal muscle to endurance training have been studied intensively (Holloszy and Coyle, 1984), the adaptations to heavy-resistance training have received much less investigative attention (Tesch, 1987). One of the reasons for this imbalance in research between the two areas is that the steady-state nature of endurance activities makes it relatively easy to design and conduct experiments to investigate exercise of this type (Timson, 1990).

However, it is inevitable to construct the animal model of heavy-resistance training to study exercise-induced muscle hypertrophy. In order to simulate muscle hypertrophy that occurs in humans in response to exercise, the author first developed animal model that rats were subjected to electrical stimulation in their gastrocnemius muscles. The propriety and feature of this model were elucidated.

Methods.

Electrical stimulation of rat muscles.

Rats were anesthetized with pentobarbital 24 hr after the final electrical stimulation, and then needle type electrodes, which were attached to an electrical stimulator (Nihon Kohden, Inc., Osaka, Japan, Type SEN-2101), were inserted into both gastrocnemius muscles of each rat. The tibia and foot of one leg were fixed at right-angles to each other with a knee-clamp and foot strap, and electrically stimulated. The contralateral leg of each rat was not fixed nor stimulated as a control. Rectangular pulses of 2 ms duration, 10 V at 100 Hz, were delivered to induce tetanic contraction. These stimulation pulses were given for 2 s, followed by a 5 s rest interval. One set consisted of 10 such stimuli. Three sets at 5 min. intervals were given to rats per day. The stimulus voltage was gradually increased to produce full tetanic contraction.

Experimental design.

Male Sprague-Dawley rats (9-week-old; Kitayama Co., Japan) were divided into 4 groups. The

gastrocnemius muscle of one leg of each rat was electrically stimulated every 2 days for 3(n=31), 5(n=31), 13(n=31) and 27(n=34) days. 3 day stimulation was carried out as follows; the rats were stimulated on the 1st and 3rd days; 5 day stimulation, stimulated on the 1st, 3rd and 5th days; 13 day, stimulated on the 1st to 13th day every two days; 27 day, stimulated on the 1st to 27th day every two days. The other leg was not stimulated and used as a control. The rats were sacrificed 24 hr after the final electrical stimulation, and muscles were carefully dissected. The muscles of 3rd day group, 5th day group and 13th day group were weighed, then a part of muscles (n=5~6) were subjected to the protein and water content assay. The 27th day group was used to determine muscle mass and a part of the rats (n=6) was subjected to determination of electromechanical properties and histochemical analysis. The ages of all rats on the day of sacrifice were adjusted to be 13-weeks-old. Therefore, the stimulation was started 4, 6, 14, and 28 days before sacrifice, respectively. Muscle wet weights were determined, and the degree of muscle enlargement was calculated as a percentage of the contralateral muscle weight.

Measurement of electromechanical properties.

Electromechanical properties of the stimulated leg muscle were measured according to the methods described Moritani et al. (1987). Each gastrocnemius muscle was adjusted to the optimal length at which maximal twitch tension was elicited. An isometric twitch contraction was elicited by means of a supramaximal square wave pulse of constant voltage with a duration of 0.2 ms (SEN 6100; Nihon Kohden, Japan). The electromechanical properties, determined after muscles had been allowed to acclimatize to the bath for 6 ~ 12 min., included the twitch contraction time (CT), relaxation time (RT), maximal isometric tetanic force (Po), maximal rates of force development (+dF/dt) and relaxation (-dF/dt), and electromechanical delay time (EMD) (Moritani et al., 1987). The EMD was measured as the time difference between the onset of the evoked mass action potential (M-wave) and that of the forces. The onsets of the M-wave and the force curve were defined as the points in time at which the EMG and force signal voltages become higher than 1% of the maximal values from the first 5 ms baseline voltage.

Histochemical analysis.

Histochemical analysis was performed as described previously (Itoh et al., 1990). Serial cross sections were stained for myosin ATPase (Davies and Gunn, 1972; Padykula and Herman, 1955), with pre-incubation at various pHs to permit classification of the fibers into fast-twitch-glycolytic (FG), fast-twitch-oxidative (FOG) and slow-twitch-oxidative (SO) ones in the entire cross sections. For each cross section examined, the total number of each fiber type was determined. At the same time, sections were divided into seven regions (Fig. 2a), about 90 fibers being sampled at random in each region to determine the areas of FG, FOG and SO fibers. Comparisons were made between corresponding regions of stimulated and control leg muscles.

Protein Assay

Whole muscle protein contents were determined using the Lowry method (1951). Whole

muscles were hydrolyzed in 1N NaOH overnight. They were subsequently heated in boiling water for 15 min. and thoroughly solubilized.

Statistical analysis.

A one-way analysis of variance was used to compare muscle mass and fiber area. Following significant analysis of variance results, comparisons of individual means were evaluated with the Fisher's LSD test as a post hoc procedure. Wilcoxon signed-rank tests were performed to compare the changes of fiber percentage and electromechanical properties between control and stimulated muscles. Statistical significance was set at the 0.05 level. Data in the text and figures are expressed as means ± SEM.

Results.

Changes of the muscle mass and histochemistry.

The stimulated gastrocnemius muscles significantly increased in wet mass by about 2.5%, after the 3rd day stimulation, 4.4% after the 5th day stimulation, and 5.9% after the 13th day stimulation,

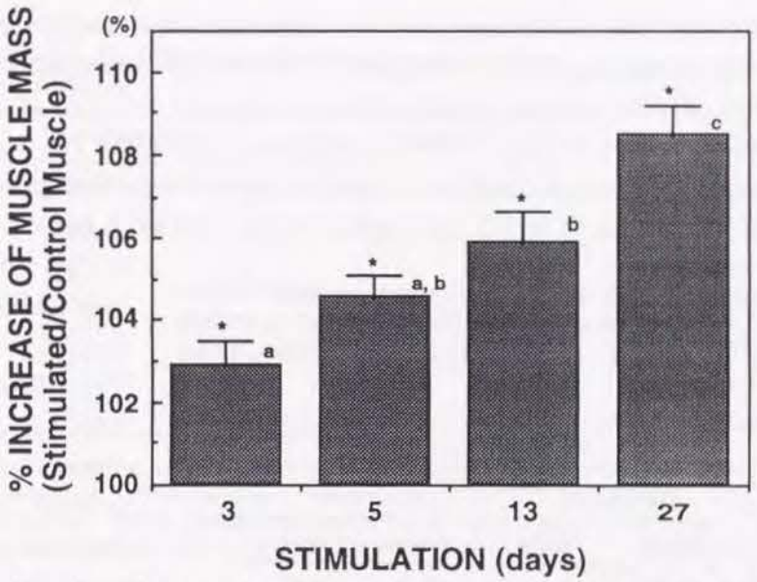

Fig. 1. Increase in gastrocnemius muscle mass during electrical stimulation of rats. Electrical stimulation was performed for one leg of each rat every 2 day, the other leg not being stimulated as a control. Muscle hypertrophy was determined by comparing the weights of the stimulated gastrocnemius muscle to those of each controls. The values are means ± SEM for 31 (3rd day), 31 (5th day), 31 (13th day) and 34 rats (27th day), respectively. * shows significant difference of stimulated muscle mass from contralateral muscle mass. Means not sharing a common alpabetic letter are significantly different.

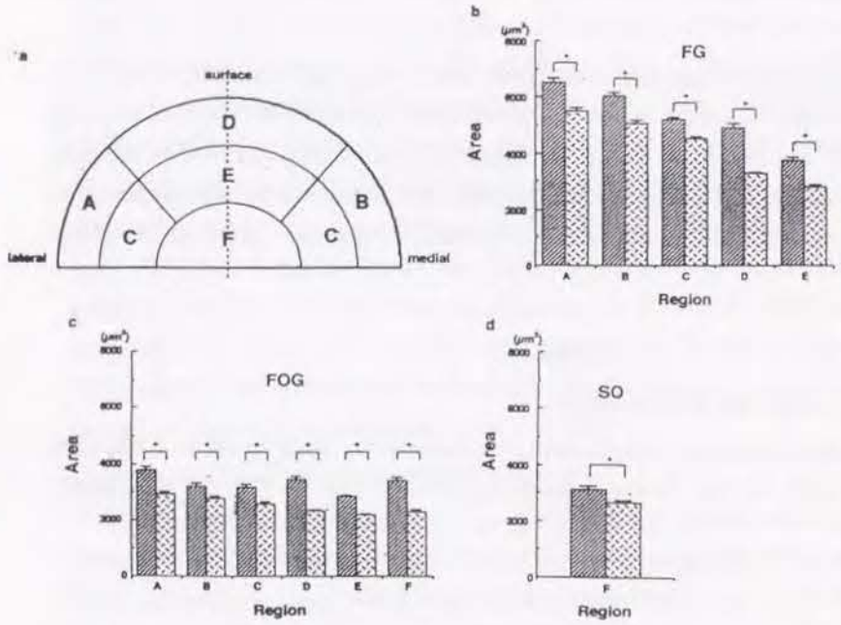

Fig. 2. Changes in mean fibre area of the gastrocnemius muscle in electrically stimulated and control legs. Muscle cross-sections were divided into seven regions as illustrated in (a). About 90 fibres in each region were sampled at random, and the areas of *FG* (b), *FOG* (c) and *SO* (d) fibres determined. Comparisons were made between the fibres in corresponding regions of stimulated and control muscles. *Each column* shows the mean and SEM for 60 to 140 fibres. * Significant difference. Significances among the non-corresponding regions are omitted for clarity; for other definitions see Table 1. ▧ Stimulated; ⊡ control

and finally reached 8.3% after the 27th day stimulation, when compared to the contralateral control legs ($p<0.001$, Fig. 1). Accompanying the muscle mass increase, the percentage of stimulated muscle protein contents also increased significantly when compared with those of contralateral muscles (3rd days: 1.4 ± 0.1, 5th days: 5.5 ± 1.0, 13th days: 6.8 ± 0.5%). The water contents between the stimulated and control muscles were not significantly different throughout the experiments (3rd days: 76.1 ± 0.6 vs. 75.4 ± 0.2, 5th days: 76.3 ±0.2 vs. 76.0 ± 0.2, 13th days: 76.5 ± 0.2 vs. 76.2 ± 0.1%, Stimulated muscle vs. control muscle, respectively). These data suggested that the muscle mass increases observed in the present experiment were due to muscle hypertrophy. After 4 weeks, stimulated gastrocnemius muscles showed a 15 to 50% increase in area in each region of a cross section (Fig. 2). The gastrocnemius muscles of the stimulated and control legs were divided into 7 regions as shown in Fig. 2, and the areas of the fibers in each region were compared. All fibers, i.e., FG, FOG and SO, in the stimulated legs' muscles showed increases in area in every region in which we carried out fiber sampling. The distribution of FOG fibers in the stimulated muscles increased and that of FG fibers concomitantly decreased, as compared to in the control leg muscles (Table 1). The FOG fibers in the stimulated leg muscle increased to 50.6% and the FG fibers decreased to 42.0%. Though the total fiber numbers in stimulated muscles were slightly but significantly decreased when compared with those in contralateral muscles (29707 ± 223 vs. 32379 ± 1231), the precise mechanisms of these changes in fiber distribution were not understood.

Table 1. Comparison of the percentages of fast twitch glucolytic (FG), fast twitch oxidative (FOG) and slow twitch oxidative (SO) fibres in control and stimulated gastrocnemius muscle

Fiber type	Fiber percentage			
	Stimulated		Control	
	Mean	SEM	Mean	SEM
FG	42.0*	2.1	50.7	2.1
FOG	50.6*	2.0	40.8	1.8
SO	7.5	0.3	8.5	0.4

Samples were obtained from 27-day stimulated rats (n=6). The counting methods are described in the Methods section.

Electromechanical changes.

Electromechanical parameters were measured during isometric twitch contraction at the optimal muscle length and with supramaximal stimulation. Typical differences in the parameters between stimulated and control leg muscles obtained from rats stimulated for 4 weeks are summarized in Table 2 The stimulated muscles showed marked increases in Po (3000 ± 392 vs. 2340 ± 293 mN, $p<0.1$) and dF/dt (98.4 ± 13.4 vs. 75.2 ± 7.17 mN ms-1, $p<0.1$), when compared to the control muscles. -dF/dt, CT (time to peak force), EMD (electromechanical delay time) and RT (relaxation time) of the muscles were not significantly different in the control vs. stimulated legs.

Table 2. Electromechanical properties of gastrocnemius muscles on supermaximal siometric twitch contraction

	Stimulated		Control	
Po	3000.8 ± 391.7*		2343.8 ± 293.3	
dF/dt	98.4 ± 13.4*		75.2 ± 7.2	
-dF/dt	46.9 ± 5.4		40.3 ± 3.1	
EMD	9.1 ± 0.5		10.0 ± 0.8	
CT	44.9 ± 1.6		45.2 ± 1.5	
RT	43.3 ± 1.0		43.2 ± 4.2	

Po, Maximal isometric tetanic force; dF/dt, maximal rate of force development; -dF/dt, maximal rate of relaxation; CT, time to peak force, EMD, electromechanical delay time, RT, relaxation time. Values are means ± SEM. * Significant difference

The fatigability of the muscle, as determined by means of the relative force decline from the 50% of Po stimulation protocol, was also affected by 4 weeks' stimulation. It was found that the stimulated muscles maintained significantly greater relative force than the control muscles at the end of 2 min. fatiguing intermittent contractions. Figure 3 shows the typical fatigability of the stimulated and control leg muscles.

Discussion

Human regimens for exercise training for the muscle hypertrophy generally consist of intermittent bouts of low frequency repetitions with high loads and long recovery periods between training

bouts (McDonagh and Davies, 1984). The present animal stimulating model resembles the training regimens commonly employed in human resistance training programs. For muscle enlargement in human weight-training programs, for example, a 23% increase in elbow flexor muscle after 100 days of isometric training (Ikei and Fukunaga, 1970) and an 8% increase after 8 weeks of isotonic strength training (McDonagh and Davies, 1984) has been reported. Several animal models of exercise-induced muscle hypertrophy have been reported to study the mechanisms of skeletal muscle enlargement that take place during strength training in human. Wong and Booth (1988) reported muscle enlargement in rats with a well devised weight-lifting model in 16 weeks and demonstrated an 18% increase in gastrocnemius muscle. Yarasheski et al. (1990) trained young rats to climb a 40-cm vertical incline with load. This program resulted in a 7.8% increase in rectus femoris muscle weight after an 8-wk training session. With the present model, the gastrocnemius enlargement reached 8.3% after 4 weeks' stimulation, suggesting reasonable muscle growth for this model of human resistant training. The stimulation of muscle for 4 weeks induced marked morphological and histochemical changes. The stimulated muscle showed a significant larger cross section than that in the case of a control. A significant increase in the maximal isometric tetanic force (Po) in the stimulated muscle corresponded to the increase in the cross section of the muscle. An increase in dF/dt means improvement of the rate of force development, probably due to improvement of the rate of signal propagation from nerves to the muscle. We found a higher percentage of FOG fibers, with a concomitant decrease in FG fiber, as compared to in the control muscle. This may be due to transformation in FG and SO fibers to FOG, but we could not rule out the mechanisms that a simultaneous reduction in the number of SO or FG fibers and de novo synthesis of FOG fibers, since the total number of fibers was not constant. Many investigators have been reported that high intensity training results in a greater hypertrophy of fast-twitch fibers (Costill et al. 1979; Green et al. 1979; Sale et al. 1987; Schantz et al. 1983). Yarasheski et al. (1990) described that a heavy-resistance training leads to an increase in type IIb fiber area. The increase in numbers of FOG fiber in the present result was rather unexpected because our experimental model was designed as a model for high intensity

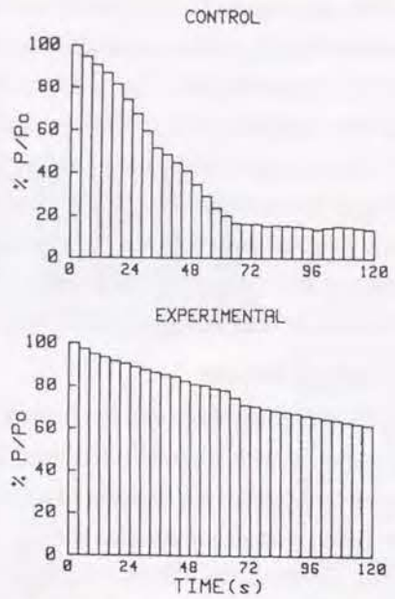

Fig. 3. Force-fatigue curves obtained for the gastrocnemius muscles. *Each column* shows the mean for six muscles for both groups (SEM are omitted for clarity). The data reflected the time-course of the decrease in the relative ratio of the contraction force (P) to the maximal isometric tetanic force (P_o), according to Moritani et al. (1987)

resistant training. We could not explain the reason why the FOG fiber increased. Further investigations are required. The fatigability of the stimulated muscle was well-consistent with the histochemical observations. The stimulated muscle showed improved fatigue resistance capacity, and it retained significantly greater relative force than the control muscle at the end of 2 min. fatiguing intermittent contractions.

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2 Physiological importance of androgen in exercise-induced muscle hypertrophy

It has long been recognized that androgens have anabolic activities in the body (Wilson and Griffin, 1980). Though exogenous androgen demonstrates its anabolic effect through the androgen receptor, the contribution of an endogenous androgen to exercise-induced muscle hypertrophy has not been elucidated. Despite widespread use of androgens by athletes and body-builders, because ot the presumption that muscle mass increases with androgens (Ryan, 1981), most studies in humans did not have controlled exercise condition. In this chapter, the author studied significance of androgen in the model described in chapter 1 applying female rats supplemented testosterone and male rats administered androgen receptor antagonist. Because female rats have very low concentration of androgen, testosterone supplement could suggest the effect of androgen on exercise-induced muscle hypertrophy in this model. In skeletal muscles, the presence of androgen receptors was demonstrated by means of ligand binding assay (Tremblay et al. 1977; Michel and Baulieu 1980), immunohistochemistry (Takeda et al. 1990) and RNA blot hybridization (Shan et al 1990). The antagonist used in this study competitively inhibits androgen binding to its receptor. If hypertrophy in the stimulated muscle is suppressed, it would also suggest the effect of androgen on exercise-induced muscle hypertrophy.

Materials and Methods.

Chemicals

Testosterone and dexamethasone were products of Wako Pure Chemical Industries (Tokyo, Japan). Oxendolone (16β-ethyl-17β-hydroxy-4-estren-3-one) was provided by Takeda Chemical Industries, Ltd. (Osaka, Japan). Copolyl (L-lactic acid/glycolic acid) was obtained from Kokusan Chemical Works (Tokyo, Japan). The [17α-^{3}H]methyltrienolone (322×10^{10} Bq/mmol) was obtained from DuPont/NEN Research Products (Boston, USA). Norit EXW charcoal was obtained from Nacalai Tesque (Kyoto, Japan). All other chemicals were purchased from Wako Pure Chemical Industries or Nacalai Tesque.

Effect of testosterone on exercise-induced muscle hypetrophy in female rats

Female Sprague-Dawley rats (8-week-old; Kitayama Co.; Kyoto, Japan) were divided into Testosterone group (n=8) and Vehicle group (n=7). Testosterone administration was carried out by implantation of the sustained release pellets. The testosterone pellets were consisted of 50 mg testosterone and 50 mg Copolyl (L-lactic acid/glycolic acid), while vehicle pellets were 100 mg copolymer only. Pellets were prepared according to the manufacturer's instruction. Rats were subcutaneously implanted with a pellet in their backs for 3 weeks. After the 1 week recovery period from the pellet implantation, electrical stimulation of muscle was started. Electrical stimulation was carried out on both groups of rats as described above. In addition to muscles and other organs, blood was collected at the end of experiment, and plasma testosterone was determined by radioimmunoassay (Ketsueki Kenkyusho, Kyoto, Japan).

Effect of androgen receptor antagonist on exercise-induced muscle hypertrophy in male rats.

Before animal experiments, the ability of oxendolone to inhibit the binding of methyltrienolone to the cytosolic fraction of rat gastrocnemius muscle was confirmed. Preparations of the muscle cytosol fraction and binding assay for cytosol androgen receptors were performed as previously described (Hickson et al. 1983). A series of molar excess of oxendolone was added into the cytosol fraction containing a 5×10^{-10} M methyltrienolone and the degrees of competitive inhibition of oxendolone against methyltrienolone were measured.

Male Sprague-Dawley rats (8-week-old; Kitayama Co., Japan) were divided into two groups. One group of animals was administered with 50 mg of oxendolone dissolved in 1 ml of vehicle (Ethanol:sesame oil = 2:8) orally every 2 days for 2 weeks (Antagonist group; n=9). The other group of rats was given an equal volume of vehicle as a placebo (Vehicle group; n=5). Administrations were performed 2 hours before electrical stimulation. Electrical stimulation was carried out on both groups of rats as described above.

Statistical analysis

Student's unpaired t-test was used to compare means of muscle mass between a treatment and a corresponding vehicle group. The same analysis was adopted with respect to other organ's mass and the degree of muscle enlargement. The difference of mass between electrically stimulated and contralateral gastrocnemius muscle in a rat was compared by Student's paired t-test. Statistical significance was set at the $p < 0.1$. A statistical analysis program, InStat (GraphPad, San Diego, U.S.A.) was used to perform these analyses and calculate p values.

Results.

Effect of testosterone on exercise-induced muscle hypetrophy in female rats

The body mass of the Testosterone group was significantly heavier than that of the Vehicle group (Fig. 1). The plasma testosterone concentration in the Testosterone group was 31.8 ± 15.9 ng·ml^{-1} (mean ± SEM). That in the Vehicle group was however, below the detection limit (0.1 ng·ml^{-1}). In the Vehicle group, no significant increase in the stimulated muscle was observed when compared with contralateral control muscle. On the other hand, the wet mass in the stimulated gastrocnemius in the Testosterone group was significantly increased (Fig. 2). The difficulty in inducing exercise-induced hypertrophy in female animals seems to be attributed primarily to a shortage of androgen. Since exercise-induced hypertrophy occurred only in the stimulated muscle in the presence of testosterone, the same mechanism as in male rats might function in female rats.

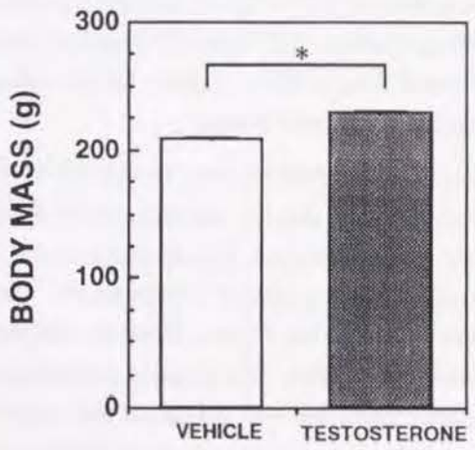

Fig. 2 Effect of testosterone on Body mass of female rats
Vertical bars indicate SEM. * shows significant difference between the Testosterone and the Vehicle group (p=0.007)

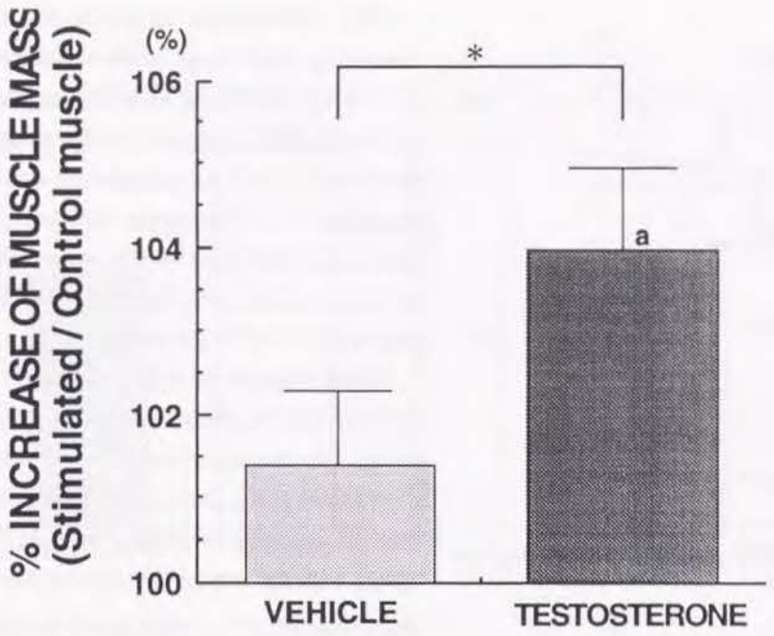

Fig. 2 Effect of testosterone on skeletal muscle hypertrophy by electrical stimulation in female rats.
The muscle hypertrophy was determined by dividing the mass of stimulated gastrocnemius muscle by that of contralateral control muscle.
Vertical bars indicate SEM; * shows significant difference between the Testosterone and the Vehicle group (p=0.069). The letter a shows significant difference between stimulated and contralateral control muscle mass in the same group (p=0.005)

Effect of androgen receptor antagonist on exercise-induced muscle hypertrophy in male rats

Oxendolone (Fig. 3) is an androgen receptor antagonist (Sudo et al. 1979a,b). We examined the binding of oxendolone to muscle androgen receptor. Figure 4 shows that a large excess of oxendolone inhibited the binding of [^{3}H]methyltrienolone to muscle, as well as to prostate androgen receptors.

Administration of oxendolone decreased the wet mass of the prostate, a target organ of androgen, by about 30 % (Fig. 5). This suggested that oxendolone was absorbed in an intact, functional form and sufficiently antagonized the testosterone receptor.

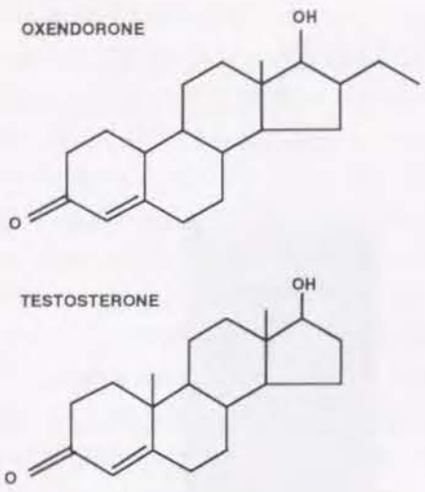

Fig. 1 Structure of oxendolone and testosterone

There were tendencies, but no significant differences in body mass between the two groups (389.74 ± 7.74 g for the Vehicle group and 411.52 ± 9.66 g for the Antagonist group; means ± SEM, respectively). Because oxendolone was originally developed to cure prostatic hypertrophy and to have little effect on body mass (Sudo et al. 1979a), it did not severely interfere with the body mass gain in any of the groups.

Figure 6 shows the degree of muscle hypertrophy resulting from electrical stimulation in each group. The stimulated gastrocnemius muscles of the Vehicle group showed a significantly heavier than the contralateral control muscle. This value agrees with the results described in section 1. On

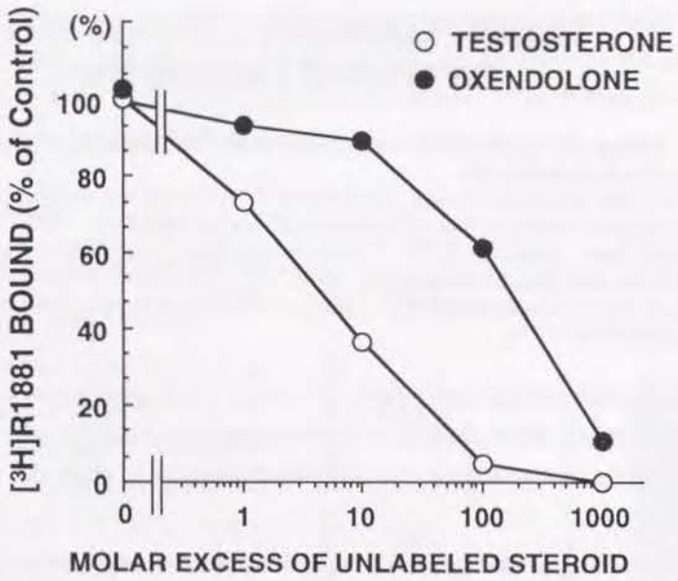

Fig. 4 Ability of testosterone and oxendolone to inhibit the binding of [^{3}H]methyltrienolone to the cytosol fraction of rat gastrocnemius muscle.

A series of molar excess of oxendolone was added into the cytosol fraction containing a 5 x 10^{-10}M methyltrienolone and the degrees of competitive inhibition of oxendolone against methyltrienolone were measured. A large excess of oxendolone could inhibit the binding of [^{3}H]methyltrienolone to muscle androgen receptor as well as to prostate androgen receptor.

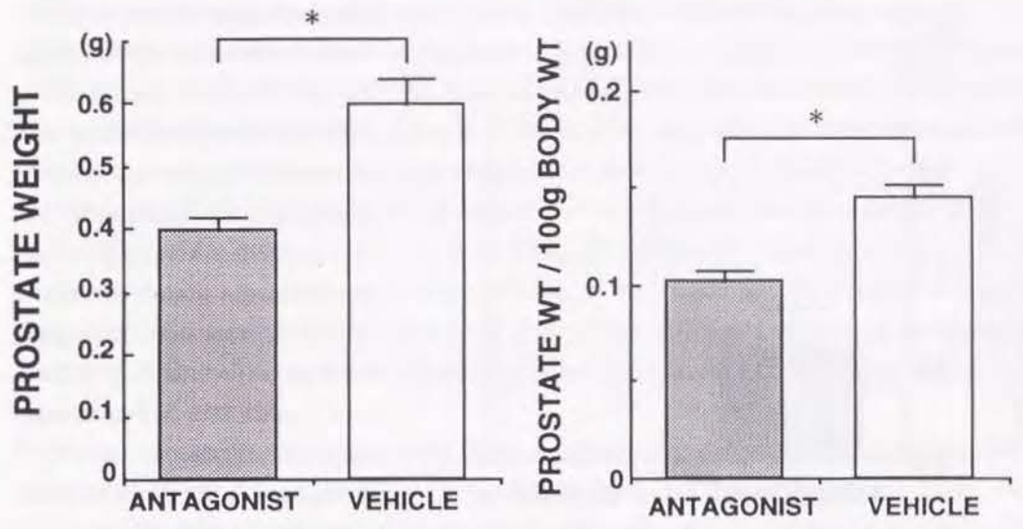

Fig. 5 Effect of oxendolone on rat prostate mass.

Antagonist group of rats were administered 50 mg of oxendolone dissolved in 1 ml vehicle (Ethanol:sesame oil = 2:8) orally every 2 days for 2 weeks. Vehicle group of rats were given equal volume of vehicle as a placebo. The prostate mass of antagonist-treated group were significantly lower than that of the Vehicle group when compared by both wet mass base (left) and body mass base (right).Values were means ± SEM. * shows significant difference; p value of prostate mass case = 0.0001 and prostate mass / body mass case =0.0012

the other hand, in the Antagonist group, the mean increase in muscle mass was 2.3%, which is significantly low compared with that of the vehicle group. When expressed as g·100 g^{-1} body mass, no significant difference was seen between the control muscle mass of the Antagonist group and that of the Vehicle group. This means oxendolone has no suppressive effect on muscle growth under sedentary conditions and inhibited only exercise-induced muscle hypertrophy.

Discussion.

The anabolic effect of androgen is well known. There remains however, the question as to whether the pathway driven by this hormone has a physiological contribution to exercise-induced skeletal muscle hypertrophy.

In the female rat, no significant increase in the stimulated muscle was observed when compared with the unstimulated control muscle in the identical rat. On the other hand, with testosterone treatment the wet mass in stimulated gastrocnemius was significantly increased. The presence of androgen receptor in skeletal muscles was also reported in female rats (Michel and Baulieu 1980; Dahlberg et al. 1981). Therefore, this may indicate the physiological contribution of androgen to exercise-induced skeletal muscle hypertrophy. Because the exercise condition in this study was well-controlled, the role of androgen on exercise-induced muscle hypertrophy was clearly demon-

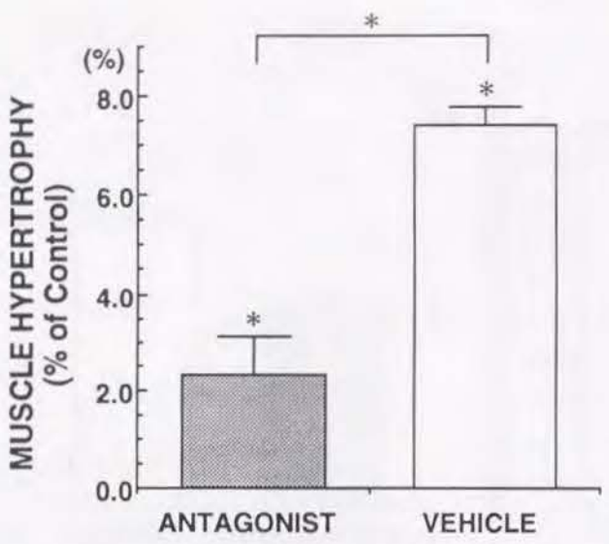

Fig. 6 Effect of oxendolone on skeletal muscle hypertrophy by electrical stimulation in male rat.

The muscle hypertrophy was determined by dividing the mass of stimulated gastrocnemius muscle by that of contralateral control muscle. Vertical bars indicate SEM; * shows significant difference between the Antagonist and the Vehicle group. (p=0.00055). The letter a shows significant difference between stimulated and contralateral control muscle mass in the same group. p value of the Antagonist group case = 0.0247 and the Vehicle group case = 0.0096

strated. The reason why exercise-induced hypertrophy is deficient in female animals might be primarily attributed to the lack of androgen (Dohler and Wuttke 1975). The fact that exercise-induced hypertrophy occurred in the presence of testosterone only in stimulated muscle suggests that the same mechanism may function in both male and female rats. When muscle mass was expressed as g·100 g^{-1} body mass, the unstimulated control mass of the Testosterone group was significantly smaller than that of the Vehicle group. Because testosterone administration decreased the proportion of unstimulated control muscle to the body mass, the effect of testosterone did not seem to be specific to skeletal muscles. A decrease in the ratio of muscle to body mass in female rats treated with anabolic steroids has also been observed (Tsika et al. 1987).

In the androgen antagonist experiment we used oxendolone that directly inhibits in a competitive manner, the binding of 5α-dihydrotestosterone to the cytosol androgen receptor in the rat prostate (Sudo et al. 1979b). The decrease in wet mass of prostate (Fig. 3) suggested that oxendolone was absorbed as the intact and functional form and that it antagonized the androgen receptor.

Recently, some investigators have suggested that the androgen receptor does not occur as a unique form (Govindan 1990; van Laar et al. 1990). There may be several subtypes of androgen receptors as well as other steroid hormone receptors and their densities on organs would account for the different response to androgen. Liao et al. (1973), Krieg et al. (1978), and Saartok et al. (1984) demonstrated that the binding properties of muscle androgen receptor were different from those of the target organ, such as the prostate and seminal vesicle.

We therefore examined the binding of oxendolone to muscle androgen receptors. Figure 2 shows that a large excess of oxendolone inhibited the binding of [^{3}H]methyltrienolone to muscle,

as well as to prostate androgen receptors.

There were no significant differences in the body mass between the Antagonist and Vehicle groups. Because oxendolone was originally developed to cure prostatic hypertrophy and scarcely affected body mass (Sudo et al. 1979a), it did not severely interfere with the body mass gain under our conditions. In addition to this property of oxendolone, there might be compensational effects by other factors that cooperate for body development under sedentary conditions.

Although the muscle mass of the stimulated gastrocnemius was heavier than that of the contralateral muscle in both the Vehicle and the Antagonist group, the degree of hypertrophy in the Antagonist group was significantly lower than that in the Vehicle group. This indicates that the blockade of androgen receptor by oxendolone in the muscle efficiently suppressed the hypertrophy caused by exercise. The signal that is generated by exercise seemed to be interrupted by this androgen antagonist.

When expressed as a ratio to body mass, no significant difference was seen between the unstimulated control muscle mass of the Antagonist group and that of the Vehicle group, that is, oxendolone seems not to affect the muscle growth under sedentary conditions but suppress only the muscle hypertrophy induced by exercise load.

Oxendolone could not completely inhibit the exercise-induced hypertrophy. This was partly because of the difficulty of completely blocking the receptors. However, the androgen pathway does not seem to be unique for exercise-induced muscle hypertrophy; that is, the contribution of other growth factors can not be ruled out. For example, Tollefsen and colleagues (1989) have reported that differentiating myoblasts produce and autocrine/paracrine IGF-I. In this experiment, however, 70% of the muscle hypertrophy were suppressed by oxendolone, compared with that of the Vehicle group. Therefore the contribution of the androgen-driven pathway to exercise-induced hypertrophy is important.

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3. Rapid increase in the number of androgen receptors following electrical stimulation of the rat muscle

It is obvious that muscle hypertrophy is mainly induced in loaded muscle. Humoral factors are irrelevant as to the triggering of hypertrophy, because their circulating levels are equal in the loaded muscle and resting muscle. This suggests that local factors are predominant for the muscle hypertrophy. From this viewpoint, many local factors and mechanisms have been proposed for the acceleration of muscle growth during exercise. It is most likely that many factors cooperatively affect muscle growth, suggesting the existence of many local factors. Some investigators (Darr and Schultz, 1987; Schultz, 1989; Yamada et al., 1989) have advocated that the proliferation of satellite cells in muscle caused by local growth factors may increase the muscle mass. Humoral factors themselves are not such local factors as mentioned above, but receptors of growth-promoting factors, which may locally increase on muscle loading, seem likely candidated for these local factors. Androgens, whose anabolic activities in muscle have been recognized for a long time, may be one of the most possible groups of growth-promoting factors, and their receptors have been demonstrated in skeletal muscle (Tremblay et al., 1977; Michel and Baulieu, 1980; Mainwaring and Mangan, 1973; Takeda et al., 1990). An androgen receptor is a transcription factor that belongs to a superfamily of nuclear receptors whose function is dependent on the binding of cognate steroid hormones. The high capacity of androgen binding to the cytosol of skeletal muscle was reported in compensatory hypertrophy of plantaris muscles, the synergistic gastrocnemius muscles being surgically removed for 31 - 51 days (Hickson et al., 1983). The results, however, failed to show directly that the increase in the number of androgen binding sites caused muscle hypertrophy. The time-course of cytosol androgen receptor number changes on loading of muscle may clarify that the increased number of androgen receptors was the cause for muscle hypertrophy or resulted in the hypertrophy. In this chapter, the author used an exercise model in rats involving electrical stimulation. Electrical stimulation of the gastrocnemius muscle induced a rapid change in the cytosol androgen receptor number, which occurred as an early event for the muscle hypertrophy.

Materials and Methods.

Materials.

Unlabeled testosterone, 5α-dihydrotestosterone and dexamethasone were products of Wako Pure Chemical Industries (Tokyo, Japan). Norit EXW charcoal was obtained from Nacalai Tesque (Kyoto, Japan). [17α–^{3}H]Methyltrienolone (87 Ci/mmol) was obtained from DuPont/NEN Research Products (Boston, USA). All other chemicals were purchased from Wako Pure Chemical Industries (Osaka Japan) or Nacalai Tesque (Kyoto, Japan).

Experimental design.

Male SD rats (9-week-old; Kitayama Co., Japan) were divided into 3 groups. The gastrocnemius muscle of one leg of each rat was electrically stimulated every two days for 3(n=31), 5(n=31) and 13(n=31) days. The procedure of electrical stimulation was performed as described in section 1. Three day stimulation was carried out as follows; the rats were stimulated on the 1st and 3rd day; 5 day stimulation, stimulated on the 1st, 3rd and 5th day; 13 day, stimulated on the 1st to 13th day every two days. The other leg was not stimulated and used as a control. The rats were sacrificed 24 hr after the final electrical stimulation, and muscles were carefully dissected. The muscles of 3rd day group, 5th day group and 13th day group were weighed, then a part of muscles (n=25~26) were used to determine androgen receptor content, and the remainder (n=5~6) were subjected to the protein and water content assay.

The ages of all rats on the day of sacrifice were adjusted to be 13-weeks-old. Therefore, the stimulation was started 4, 6 and 14 days before sacrifice, respectively. Muscle wet weights were determined, and the degree of muscle enlargement was calculated as a percentage of the contralateral muscle weight.

Preparation of the muscle cytosol fraction.

All procedures were carried out at 4 °C. The gastrocnemius muscles of both legs were taken and used for preparing the muscle cytosol fraction, according to the method described by Hickson et al. (1983). Briefly, two days after the final set, the gastrocnemius muscles were rapidly removed, minced, and then homogenized in 4 vol. of Tris-EDTA buffer (0.05 M Tris, 1.5 mM EDTA, pH 7.4) and 10 mM sodium molybdate with a Bio-Mixer (Nihon seiki Co., Japan) for 30 sec at setting 45. The homogenate was ultracentrifuged at 100,000 × g for 75 min. and then the supernatant was used for the binding assay. Cytosol protein was determined by the method of Lowry et al. (1951), using bovine serum albumin as a standard. Contamination by steroid hormone binding proteins from nuclei was minimal, as judged from the contamination by DNA during fractionation. Only 15% of the total DNA was detected in the cytosol fraction (data not shown).

Binding assay for cytosol androgen receptors.

The [^{3}H]methyltrienolone binding assay for the determination of cytosol androgen receptors was carried out by the method of Hickson et al. (1983). Briefly, 0.02 ml of [^{3}H]methyltrienolone at increasing concentrations, from 5×10^{-10}M to 1.5×10^{-8}M, in the presence of 0.02 ml of a 1000-fold molar excess of dexamethasone, to mask the glucocorticoid receptors, was incubated with 0.5 ml of cytosol (approx. 7 to 10 mg protein) at 4 °C for 20h for complete exchange with endogenous ligand. Free [^{3}H]methyltrienolone was removed on dextran-coated charcoal. Specific binding was determined as the difference from with a 1000-fold excess of unlabeled testosterone. The data were analyzed by Scatchard analysis. [^{3}H]Methyltrienolone binding concentration was calculated by dividing total binding sites in whole muscle by whole muscle protein contents, and then expressed as the percentage increase compared to the contralateral control.

Results.

[^{3}H]methyltrienolone binding in cytosol of muscle.

5α-Dihydrotestosterone, is reported to be active in binding to the androgen receptors in sexual glands (Mainwaring and Mangan, 1973). However, Krieg et al. (1978) reported that in muscle, testosterone showed higher affinity for the androgen receptors than 5a-dihydrotestosterone. As shown in Fig. 1, the displacement of [^{3}H]methyltrienolone by a large excess of testosterone was much greater than that by 5α-dihydrotestosterone, suggesting that the addition of a large amount of testosterone will give a precise value for the non-specific binding.

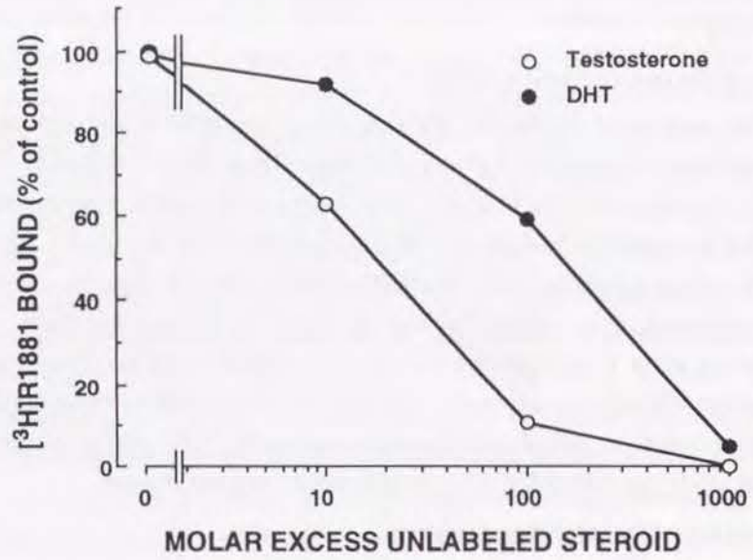

Fig.1 Competing activities of graded dose of testosterone or dihydrotestosterone for the binding of [^{3}H]methyltrienolone (0.7nM) in gastrocnemius muscle cytosol of adult male rats.

The peak decrease of [^{3}H]methyltrienolone biding by 1000-fold excess of unlabeled testosterone was taken as 100%. Each value was the Mean of 4 determinations. SEMs were omitted for calrity.

Figure 2 demonstrates that the [^{3}H]methyltrienolone binding (binding sites per mg of muscle protein) markedly increased in the stimulated leg muscles after the 3rd day stimulation, being 25% higher than that in the control legs. This level was kept throughout the experimental period. This increase was not due to an increase in muscle mass because the increase in [^{3}H]methyltrienolone binding was much greater than that in muscle mass. From the profiles of the increases in [^{3}H]methyltrienolone binding and the muscle protein content during the stimulation, it was suggested that the increase in the [^{3}H]methyltrienolone binding preceded the muscle hypertrophy. The receptor dissociation constants (Kd) were within the range of 0.3 to 0.4 nM in all muscles, regardless of whether they came from the stimulated or the control leg. Typical profiles obtained on Scatchard analysis after 5 days' stimulation are shown in Fig. 3, the parallel lines for the data for the stimulated muscle and the control muscle, indicating that the Kds were not altered by the muscle hypertrophy.

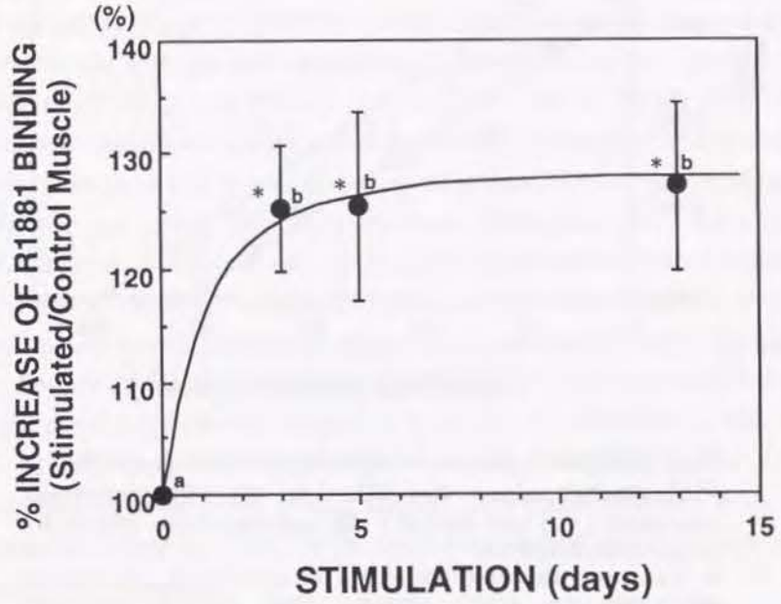

Fig 2 Effect of electrical stimulation on the [^{3}H]methyltrienolone binding to rat gastrocnemius muscles.

[^{3}H]methyltrienolone binding was expressed as percentage increase of control for each point on a single muscle basis. Values are means ± SEM of at least 4 experiments. *; $p<0.05$, **; $p<0.02$

Discussion.

Androgen binding sites in the cytosol fraction rapidly increased during electrical stimulation of the muscle. The 3rd day stimulation (1st and 3rd day stimulation followed by a 24 hr rest) induced a 25% increase in the androgen receptor number per mg total protein in the stimulated muscle, compared to in the contralateral control leg muscle. This increase slowed down after the 5th day stimulation and thereafter the stimulated leg muscles exhibited higher androgen receptor levels than the control leg muscles. The increases in muscle mass and protein content showed completely different profiles from this rapid increase in the androgen receptors, i.e., the muscle weights increased with a gentle slope throughout the experimental period. It seemed likely that the muscle androgen receptors increased ahead of muscle mass growth, and were maintained at a high level during the increase in muscle mass. A simple increase in the number of receptors and/or their

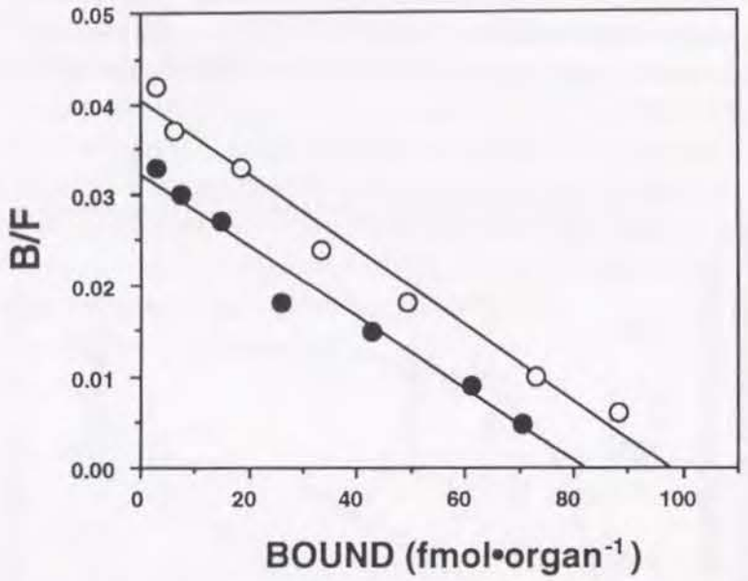

Fig 3 Scatchard plot of the specific equilibrium binding of [^{3}H]methyltrienolone for 20hr at 4°C to electrically stimulated (○) and control (●) gastrocnemius muscle fro 5 days stimulated rat.
In both cases data were corrected for non-specific binding by subtracting the values obtained from parallel incubation containing a 1000-fold excess unlabeled testosterone.

affinity for ligands leads to an increase in the ligand action at low ligand concentrations. This would result in a leftward shift of the dose-response curve, which is regarded as an increase in ligand sensitivity. It is not difficult to find such instances. An increase in insulin binding sites after prolonged exercise training enhances insulin sensitivity of the organ, that is, a lower concentration of insulin than that before exercise training can induce the same insulin action (LeBlanc et al., 1979; Soman et al., 1979; Kahn, 1980). The up regulation and down regulation of many kinds of ligand-receptor interactions have indicated that changes in the number of ligand binding sites are important for cells to regulate locally the sensitivity of receptor binding to a ligand in the cells. A rapid increase in androgen receptors in the cytosol is likely to enhance the sensitivity of muscle to circulating androgen. Consequently it would contribute at least in part to the following muscle hypertrophy. Furthermore, an increased androgen receptor level would also highly contribute to maintenance of the muscle mass. This is consistent with a previous observation that the number of skeletal muscle androgen receptors was high after 31-51 days compensatory hypertrophy of plantaris muscles following surgical removal of the synergistic gastrocnemius muscles (Hickson et al., 1983).

The specificity of the receptor is important for steroid hormones, since the molecular structures

of steroid hormones are very similar. It has been reported that the receptor specificities of steroid hormones are rather broad and cross-bindings exist (Ho-Kim et al., 1981; Danhaive and Rousseau, 1986). The deduced amino acid sequence of the DNA-binding domain of the cDNA for human androgen receptors closely resembles the amino acid sequence of the human progesterone, mineralocorticoid and glucocorticoid receptors (Chang et al., 1988; Tilley et al., 1989). In the present experiment, the glucocorticoid receptor was blocked by the addition of a large excess amount of dexamethasone to the androgen binding medium. [^{3}H]methyltrienolone, which is an analog of androgen, was used in the present binding assay because it does not bind to serum proteins, possible contaminants from blood trapped within the muscle. Androgens were reported to bind to glucocorticoid receptors as well as androgen receptors in the muscle cytosol (Ho-Kim et al., 1981; Danhaive and Rousseau, 1986). Thus, methyltrienolone binding was carried out in the presence of a 1000-fold molar excess of dexamethasone to mask the glucocorticoid receptors throughout the present study. Specific binding was obtained by subtraction of the nonspecific binding from the total binding. The non-specific binding was obtained by the addition of a 1000-fold molar excess of testosterone. 5α-Dihydrotestosterone is thought to be an active form in metabolism in the accessory sexual glands (Mainwaring and Mangan, 1973; Bruchovsky and Wilson, 1968; Anderson and Liao, 1968), although there is little information on muscle. A large amount of testosterone replaced [^{3}H]methyltrienolone in the cytosol fraction more completely than in the case of 5α-dihydrotestosterone, indicating clearly that the data we obtained on specific binding with testosterone were much more acceptable.

The distribution of androgen binding proteins in cells and the translocation of the complex of androgen and its receptor through the nuclear pore have not been well elucidated. The interaction of the glucocorticoid receptor with heat shock protein hsp90 was reported as a model of ligand-mediated receptor transformation and translocation (Catelli et al., 1985; Pratt et al., 1989). The authors suggested that, in intact cells, ligand-dependent dissociation of the MW. 90,000 heat shock protein permits the steroid receptors to proceed, through some ordered mechanism, to their affinity sites of action within the nucleus (Pratt et al., 1989). Also, the highly conserved amino acid sequences of the steroid-binding domains of the steroid receptors within each steroid receptor class (Tilley et al., 1989) suggested that this mechanism would be conserved among receptors for different steroid classes. The cytosol androgen receptors in the present study may have been associated with such heat shock proteins, since the 10 mM sodium molybdate in the muscle homogenizing buffer can stabilize the binding between receptors and hsp90. The concentration of hsp90 is thought to be very sufficient for trapping the steroid receptors and less specific to each receptor (Pratt et al., 1989), so that the concentration of the heat shock protein may not have been a limiting factor for the androgen effect in the present study. Moreover, contamination by androgen binding proteins derived from the nucleus during preparation of the cytosol fraction seemed minimal, since DNA that leaked into the cytosol fraction amounted to less than 15% of the total DNA.

In conclusion, the rapid increase in androgen receptors in the cytosol fraction may contribute as

a local factor, to muscle hypertrophy. However, the androgen receptor is one of the receptors of growth promoting factors. Other receptors of growth factors, as well as androgen, insulin, growth hormone, IGF-1, FGF for example, are likely to be involved in the induction and maintenance of muscle hypertrophy. Further studies on changes in such receptor numbers and the affinity to a ligand during muscle loading are required.

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CHAPTER II MECHANISM OF THE CHANGES IN MUSCLE GLUCOSE UTILIZATION IN RESPONSE TO PHYSICAL ACTIVITIES

1. Decrease in muscle glucose transporter number in chronic physical inactivity in rats

Increase in the number of patients with type II diabetes (non-insulin-dependent diabetes mellitus), which includes both decreased insulin sensitivity and decreased insulin responsiveness, may be related in part to reduced physical activity. Because skeletal muscle is the major site of insulin-mediated glucose transport (DeFronzo et al., 1981), the decrease in sensitivity or responsiveness of glucose uptake to insulin in muscle is related muscle disuse (Fell et al.,1985; Seider et al., 1982; Smith and Lawrence, 1985), and the state of physical fitness plays a role in regulation of the in vivo insulin action (Fell et al.,1985; Heath et al, 1983; James et al, 1983; James et al, 1984; James et al.,1985). In the present study, we used a physical inactivity model to investigate the insulin action and post insulin receptor action under reduced-activity conditions. The effect of reduced activity of rodent hindlegs on muscle glucose metabolism has been studied using denervation (Smith and Lawrence, 1985), limb immobilization (Fittz et al., 1986; Seider et al., 1982), limb suspension (Bonen et al., 1988; Hauschka et al., 1987; Henriksen et al., 1986), and whole body suspension (Fell et al.,1985). These treatments generally reduce the ability of insulin to stimulate glucose accumulation and glucose uptake, although the published results indicated that the degree of muscle disuse and the response to insulin varied widely among the experiments, a post insulin receptor defect may cause the changes in glucose metabolism in the reduced-activity models. Smith and Lawrence (1985) reported that the loss of ability of insulin to stimulate glycogen synthesis after denervation was the result of a post insulin receptor defect, since denerved and control muscles bound the same amount of insulin over a wide range of insulin concentrations, and Scatchard analysis indicated that denervation changes neither the receptor number nor affinity. However, some investigators (Bonen et al., 1988; Henriksen et al., 1986) reported that, after hindlimb suspension, marked increases in insulin binding and glucose metabolism occurred in soleus muscle.

A simple decrease in the number of insulin receptors and/or their affinity for insulin would lead to a decrease in the insulin action at low insulin concentrations, with a normal response at high concentrations. This would result in a rightward shift of the dose-response curve, which is regarded as a decrease in insulin sensitivity. A defect in a postbinding step of insulin action, which is mainly due to a decrease in the number of glucose transporters (Kahn and Cushman, 1985; Simpson and Cushman, 1986) and decrease in the efficiency of translocation of glucose transporters, should be caused by a decreased insulin effect at all insulin concentrations, and this is defined as decrease in insulin responsiveness.

One aim of the present study is to characterize our physical inactivity animal model for humans showing reduced physical activity, the whole body insulin action with our model being compared

with that of other models. Another aim is to determine the changes in the muscle glucose transporter number in isolated plasma and microsomal membranes to elucidate the effect of reduced activity on post insulin receptor events.

Materials and Methods

Materials.

Cytochalasin B, cytochalasin E and bovine insulin were obtained from Sigma. [^{3}H]cytochalasin B (sp act 13.5 Ci/mmol) was purchased form NEN Research Products. Other chemicals were purchased from Wako Pure Chemicals (Japan) unless stated otherwise.

Animals.

Male Wistar rats weighing 170-200 g were used in all the experiments. The rats were fed a stock diet (type MF; Oriental, Japan) ad libitum, allowed free access to water and housed in a constant-temperature (22 ± 0.5°C) and constant-humidity (50%) animals room with a 12:12 h light-dark cycle. They were randomly assigned to control and experimental groups. The experimental group was placed for 2wk in Ballman-type mild-restraint cages modified as described by Green and Nasset (1983). Food intake by the rats was measured for the first 3 days of the 2nd week. After 2wk of feeding, each group was randomly divided into tow subgroups, one for euglycemic clamp experiments and the other for the cytochalasin B binding assay. Control rats were maintained in standard size cages (38 × 26 × 18 cm, 3 rats/cage) under the same conditions.

Determination of movement of rats under physical inactivity conditions.

A red luminous diode (3 mm diam x 4 mm; Sato Parts, Japan) with a button battery (12 mm diam × 2 mm, 3 V; National, Japan) was attached to the back of a rat that was placed in either a restraint cage or a standard cage. The movement of the rat for every 5 min. was traced as a bright line, created by the luminous diode, on a Polaroid 667 film (Polaroid, 8.5 × 10.8 cm, ISO 3000) mounted in a Polaroid MP-4 Land camera system (Polaroid). Experiments were carried out in a completely dark room between 1900 and 2300 h. The rats were previously accustomed to the surroundings for several days.

Determination of adrenocorticotropin levels in physical inactivity.

Plasma adrecocorticotropic hormone (ACTH) levels in physically inactive rats were determined at 0, 3, and 7 days after the start of the experiment. Blood was collected at 0900 h, and plasma ACTH was determined by radioimmunoassay using ^{125}I-labeled ACTH (Amersham).

Euglycemic clamp experiments.

The animals were deprived of food for 6-10 h overnight before the euglycemic clamp study. The rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (5 mg·100 g body wt^{-1}·h^{-1}) and then surgically prepared under anesthetized conditions for the euglycemic clamp study, as described by Kraegen et al. (1983). An intravenous Silastic catheter (Dow Corning) was inserted into the left jugular vein (0.02 in. ID × 0.031 in. OD) and filled with heparin-saline (50 IU

heparin/ml 0.9% saline). Two other catheters were fitted with 23-gauge needles, and the needles were inserted into the left femoral vein and the posterior vena cava, respectively. The femoral catheter was filled with insulin and the posterior catheter with 10% (wt/vol) glucose. The jugular catheter was used for blood sampling, the femoral catheter for intravenous insulin infusion, and the posterior catheter for intravenous glucose infusion. All experiments were conducted at least 30 min after surgery to allow the high blood glucose level caused by the anesthesia and surgery to return to the basal fasting level. Bovine insulin (Sigma) was infused at a constant rate (1.4, 3.6, or 14 mU $\cdot kg^{-1} \cdot min^{-1}$) via the femoral catheter for 2 h using a Familic-100N microinfusion pump (Japan Spectroscopic). Blood glucose was maintained at the basal fasting level by estimating the blood glucose concentration at regular intervals (7.5 min) in 25-μl samples taken from the jugular catheter and then accordingly adjusting the rate of infusion of a 10% glucose solution via the posterior catheter. Glucose was infused using a Terfusion syringe pump STC-521 (Termo, Japan). The glucose infusion rate (GIR) during the 2nd h of the clamp (GIR_{60-120}) experiment was taken as the steady-state net whole body glucose disposal rate. Blood samples (1.0 ml) were obtained for insulin determination in all cases at the end of each experiment. Every 7.5 min, the blood glucose concentrations in samples were measured with a Wako glucose C test kit (Wako Pure Chemicals); this assay kit is based on an enzymatic method. One mole of D-glucose is defined as 1 mol of hydrogen peroxide produced from D-glucose by glucose oxidase.

At the completion of the experiments, the following tissues and hindlimb muscles were rapidly removed and weighed: liver, kidney, heart, epididymal fat pad, and gastrocnemius and quadriceps muscles.

Glucose uptake in individual tissues in vivo.

2-Deoxy-D-[^{3}H]glucose incorporation was determined under conditions based on methods by Kraegen et al. (1985) and James et al. (1985). Briefly, 2-deoxy-D-[2,6-^{3}H]glucose (20 μCi) and D-[U-^{14}C]sucrose (4 μCi; Amersham) were administered together as an intravenous bolus via the venous cannula at 60 min after the start of the glucose clamp in the presence of 14 mU/Kg insulin. Blood samples (200 μl) for determination of plasma glucose concentrations and plasma tracer concentrations were obtained at 5, 7, 9, 12, and 15 min after bolus administration. Immediately after the final blood sampling at 15 min, gastrocnemius and quadriceps muscles ere rapidly removed and frozen using alumina tongs precooled in liquid nitrogen. An estimate of tissue glucose uptake [defined as the glucose metabolic index× (Rg')] was calculated as described by Kraegen et al. (1985).

Isolation of microsomal and plasma membrane fractions.

For the total cytochalasin B binding assay, microsomal and plasma membrane fractions were obtained from the rat muscles. Rats maintained in the standard size cages and in the mild-restraint cages were deprived of food overnight and then anesthetized with pentobarbital. the gastrocnemius and quadriceps muscles were quickly excised and homogenized for separations of the membrane fractions. On the other hand, for the translocatable cytochalasin B binding assay, 33 mU $\cdot kg^{-1} \cdot min^{-1}$ insulin (maximal dose) were administered to rats intravenously for 40 min with 10 mg $\cdot kg^{-1} \cdot min^{-1}$ glucose. Immediately after the insulin perfusion, the rats were killed and plasma membranes were isolated.

The isolation of microsomal and plasma membrane fractions was carried out by procedures based on the methods described elsewhere (Fushiki et al., 1989). Muscle tissue (4-6 g) was minced, followed by disruption with a Bio-mixer (Nihonseiki, Japan) for 30 s at setting 45 in an ice-cold buffer containing 250 mM sucrose, 20 mM N-2-hydroxyethylpiperazine-N'-2-ethansulfonic acid (HEPES), 10^{-3} U/l aprotinin, 0.1 mg/ml bacitracin, and 2mM phenylmethylsufonyl fluoride (PMSF), pH 7.4. After the disruption, further homogenization was performed by five strokes with a Teflon pestle homogenizer. The volume of the homogenate was adjusted to 20 ml with a homogenization buffer, and the 2 ml of 3 M KCl containing 250 mM sodium pyrophosphate were added. Immediately after the addition of the KCl medium, the homogenate was vigorously mixed and centrifuged at 100,000 g for 1 h with a Hitachi ultracentrifuge (model 55P-72) in a type RP30-2 angle rotor with 10 ml of homogenization buffer followed by the addition of 0.05 ml of 1 mg/ml deoxyribonuclease (DNase) (55 U/mg protein) and incubation at 30°C for 30 min. After the incubation, 10 ml of ice-cold homogenizaion buffere were added and the mixture was then centrifuged at 750 g for 15 min. The supernatant was collected carefully and then centrifuged at 100,000 g for 1 h, and the pellet was resuspended in 6 ml of 45 % sucrose. A discontinuous sucrose gradient was constructed on top of the resuspended crude membrane fraction by carefully layering 6 ml of 40%, 12 ml of 38%, 6 ml of 36%, 12 ml of 32%, 6 ml of 30%, 6 ml of 27%, and 6 ml of 12% sucrose successively over it. The sucrose density gradient was centrifuged at 100,000 g for 16 h in a Hitachi RPS-25-2 swinging-bucket rotor. After the centrifugation, the fractions containing the intracellular and plasma membrane fraction was diluted in 0.9% saline and then centrifuged at 100,000 g for 1 h. The pellets were frozen and stored in a freezer at -70°C until used for the enzyme and cytochalasin B binding assays.

Marker enzyme assays.

γ-Glutamyltransferase was measured by the method described previously (Fushiki et al., 1989). The membrane preparation (0.05 ml) was added to 0.5 ml of a solution containing 3.5 mM L-g-glutamyl-*p*-nitroanilide, 20 mM N-glycylgycine, and 100 mM tris (hydroxymethyl)aminomethane hydrochloride (Tris·HCl; pH8.2). The reaction was performed at 37°C and terminated by adding 0.5 ml of 1.7 N acetic aced. After centrifugation, the absorbance of the supernatant was read at 410 nm.

Microsomal phosphatase was reported by Klip and Walker (1983) to be an appropriate marker for the intracellular membrane fraction, which contains intracellular glucose transporters. The enzyme activity was assayed as previously described (Dohm and Newsholm, 1983). The membrane preparation (0.05 ml) was added to 0.05 ml of a solution containing 10 mM sodium glucose 6-phosphate, 8.75 mM histidine (pH6.5), and 0.5 mM Na3-EDTA (pH 8.0). The reaction was performed at 37°C and terminated by adding 2.5 ml of 8% trichloroacetic acid. After ice-cold

centrifugation for 2 min at 10,000 g, 2 ml of the supernatant were added to 0.5 ml of 2.5% ammonium molybdate in 5 N H_2SO_4 and 0.2 ml of a solution containing 1.85 mg/ml 1-amino-2-naphthol-4-sulfonic acid and 11.1 mg/ml $NaHSO_3$ followed by incubation at room temperature for 20 min. Then the absorbance at 660 nm was read.

The protein concentration was determined with a Protein assay kit (Bio-Rad), with bovine serum albumin as the standard.

Cytochalasin B binding.

Fifty microliters of a membrane suspension in a binding buffer [(in mM): 1 $CaCl_2$, 1 $MgCl_2$, and 5 HEPES, pH7.5] were added to 50 µl of binding buffer containing 600 mM of either D-glucose or D-mannitol followed by incubation with 30 µl of the same buffer containing 0.1 µM [^{3}H]cytochalasin B, 5 µM cytochalasin E, and various mounts of unlabeled cytochalasin B (3-3,000 pmol in the assay tube) for 10 min at room temperature. The reaction was stopped by adding 1 ml of an ice-cold stop solution (150 mM NaCl and 10 mM Tris·HCl, pH 7.5), and samples were then immediately filtered (within 2 s) through GE/C Whatman filters as reported by Klip et al (1987, 1983). These were rapidly washed once with 200 µl of ice-cold stop solution. The filters were transferred into scintillation vials and counted in 6 ml of Aquasol II (Du Pont) with a liquid scintillation counter. Binding at each concentration of cytochalasin B was examined in triplicate in the presence of D-glucose or D-mannitol. The binding in the presence of 500 mM D-glucose was subtracted from that in the presence of 500 mM D-mannitol. The data were analyzed by Scatchard analysis.

Insulin assay.

Blood insulin was assayed by a radio-immunoassay using a RIA insulin assay kit (Dinabot, Japan).

Statistics.

The results are expressed as means ± SEM. For statistical comparisons between groups, Student's *t* test was used.

Results

Figure 1 shows pictures of typical movements of rats in 15 min (5 min × 3) in a dark room at night (1900 to 2300 h). The total movement in the restraint cages in 2 h, determined by tracing a bright line on a film as described under Materials and Methods, was only 40 cm in the physically inactive rats, whereas it was 2,400 cm in the rats placed in standard size cages (controls). This suggested that physical activity was limited throughout the experiments, although rats could voluntarily retract their legs. Table 1 shows the mean body weight, mean food intake, and organ weights for each group. The food intake was not significantly affected by physical inactivity. The average body weighed gain was slightly lower in the physically inactive rats, but the difference was not significant. The weight-to-body weight ratios in the cases of the liver, kidney, heart, and epididymal fat pad did not differ between the physically inactive and control animals. Only the gastrocnemius and quadriceps weight-to-body weight ratios were slightly but significantly ($P < 0.05$) decreased in the physically inactive rats compared with the control rats.

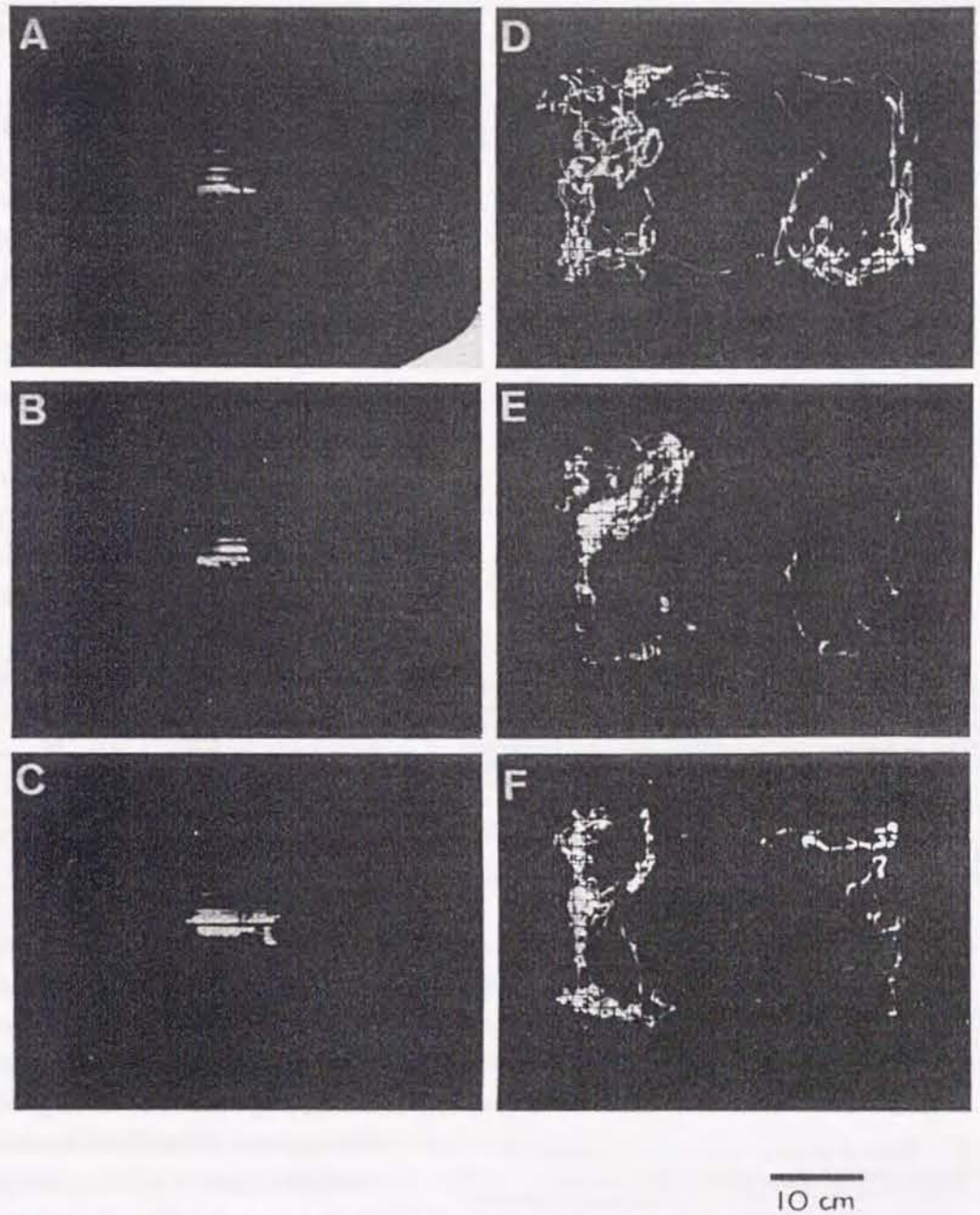

FIG. 1. Movement of rats in a restraint cage and in a control cage. A luminous diode with a small button battery was attached on the back of a rat that was placed in a control cage (*A, B, C*) or a restraint cage (*D, E, F*). Each movement of the rat was traced as a bright line caused by the luminous diode on a film mounted in a Polaroid MP-4 Land camera system. Typical patterns of movement in 15 min (5 min × 3) are shown. Experiments were carried out in a completely dark room between 1900 and 2300 h. Rats had previously been accustomed to the surroundings for several days.

TABLE 1. *Effect of physical inactivity on body weight gain, food intake, and organ weight of rats*

Condition	Body Wt Gain, g	Food Intake, g	Liver	Kidney	Heart	Epidymal Fat Pad	Gastrocnemius Muscle	Quadriceps Muscle
Physical inactivity	55.8±4.3	28.9±0.6	3.20±0.04	0.90±0.01	0.40±0.01	1.05±0.05	1.01±0.02*	1.28±0.04*
Control	62.2±4.3	27.7±2.0	3.29±0.04	0.89±0.02	0.39±0.01	1.00±0.02	1.08±0.01*	1.40±0.03*

Values are means ± SE for at least 10 rats. Food intake was measured for 3 days, i.e., from 7th to 9th day of experiment. Organ weights are means of weight-to-body weight ratio. * Significantly different ($P < 0.05$) when physical inactivity was compared with control.

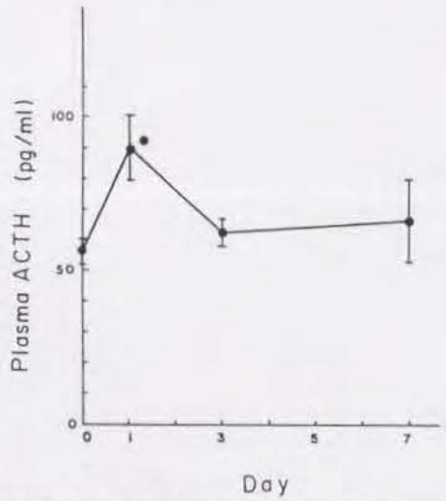

FIG. 2. Plasma ACTH under physically inactive condition. Rats underwent physical restraint for 0, 3, and 7 days. Each rat was killed by decapitation at 0900 h, and blood was collected. Plasma ACTH was determined by radioimmunoassay. Values are means ± SE for 7 rats. * Significantly different against unrestrained rats (*day 0*) ($P < 0.05$).

TABLE 2. *Effect of physical inactivity on glucose disposal rates in euglycemic clamp experiments*

Condition	Insulin Infusion Rate, $mU \cdot kg^{-1} \cdot min^{-1}$		
	1.4	3.6	14.0
	Glucose infusion rate, mg glucose $\cdot kg^{-1} \cdot min^{-1}$		
Physical inactivity	1.34±0.20	11.8±1.0	15.4±2.4*
Control	0.92±0.21	13.8±0.5	20.8±1.4*

Values are means ± SE for 6–10 rats. * Significantly different ($P < 0.05$) when physical inactivity was compared with control.

TABLE 3. *Effect of physical inactivity on 2-deoxy-D-[^{3}H]glucose uptake into muscles in vivo*

Condition	Glucose Metabolic Index, $\mu g \cdot mg^{-1} \cdot min^{-1}$	
	Gastrocnemius muscle	Quadriceps muscle
Physical inactivity	7.62±0.93*	11.57±3.46†
Control	12.99±1.84*	15.41±4.45†

Values are means ± SE for at least 5 rats. Glucose metabolic index = blood glucose concentration × rate constant of 2-deoxy-D-[^{3}H]glucose incorporation into each muscle. Details are as described by Kraegen et al. (25). * Significantly different ($P < 0.05$). † Probably significantly different ($P < 0.1$).

Plasma ACTH increase only on the 1st day of physical inactivity and returned to the normal level by the 3rd day of physical inactivity (Fig. 2)

Euglycemic clamp experiments were carried out on anesthetized rats according to the method described by Kraegen et al. (1983). The steady-state exogenous glucose infusion rate, estimated as the GIR60-120 required to maintain euglycemia, was significantly lower with the maximal inulin infusion rate of 14 $mU \cdot kg^{-1} \cdot min^{-1}$ ($P < 0.05$) in the physically inactive rats. However, with lower insulin infusion rates, 1.4 and 3.6 $mU \cdot kg^{-1} \cdot min^{-1}$, the differences in the steady-state glucose infusion rate were very small and not statistically significant (Table 2).

Rg' under the glucose-clamp condition was measured by means of 2-deoxy-D-[^{3}H]glucose incorporation into each muscle at 14 mU/kg insulin infusion. Table 3 indicated that a markedly smaller Rg' was obtained in gastrocnemius muscle of physically inactive rats than of controls ($P < 0.05$). Similar difference was observed in the case of quadriceps muscles ($P < 0.10$).

The glucose transporter numbers in the plasma and intracellular membranes purified from a mixture of gastrocnemius and quadriceps muscle tissues were determined. The plasma membrane

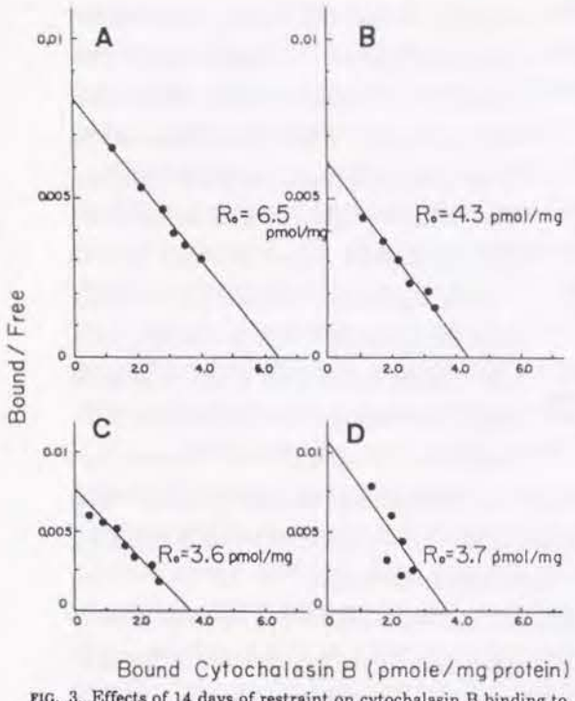

FIG. 3. Effects of 14 days of restraint on cytochalasin B binding to plasma and intracellular (microsomal) membrane fractions. Plasma and intracellular membrane fractions were isolated from rats maintained in physical inactivity and standard size cages (controls), and then cytochalasin B binding was assayed. *A*: plasma membranes from muscles of control rats; *B*: plasma membranes from physically inactive rat muscles; *C*: intracellular membranes from control rats; *D*: intracellular membranes from physically inactive rats. Each point for plasma membranes is mean for at least 5 membrane preparations. Points for intracellular membranes are means for at least 3 membrane preparations. Cytochalasin B binding was assayed in presence of 500 mM D-mannitol or 500 mM D-glucose. Binding data are presented as a Scatchard plot. Nonspecific binding was corrected for by subtracting D-glucose-inhibitable binding.

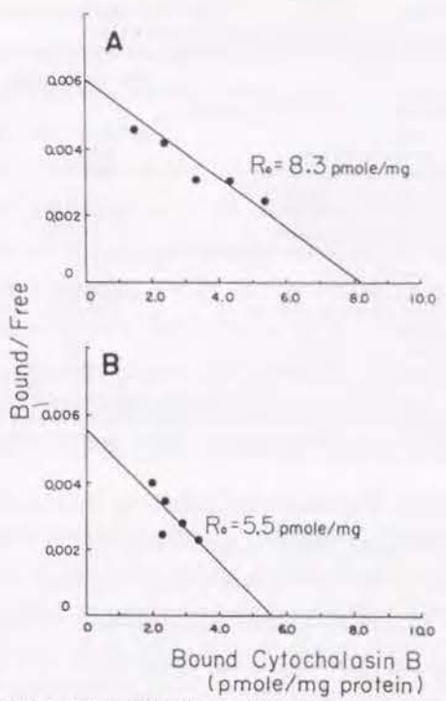

FIG. 4. Cytochalasin B binding to plasma membranes at maximally effective dose of insulin perfusion. Rats were perfused with 33 $mU \cdot kg^{-1} \cdot min^{-1}$ insulin for 40 min with 10 $mg \cdot kg^{-1} \cdot min^{-1}$ D-glucose. Immediately after perfusion, plasma membranes were isolated from gastrocnemius and quadriceps muscles isolated from control (*A*) and physically inactive (*B*) rats. Cytochalasin B binding was assayed as described in Fig. 3. Each point is mean for at least 4 membrane preparations.

marker, g-glutamyltransferase, was obtained as a tight white band in the 25% sucrose fraction. There was minimal contamination by the intracellular membrane marker enzyme of this fraction. The intracellular membrane marker occurred in two quite broad bands, corresponding to 30 and 35% sucrose fractions, respectively. The higher density fraction was highly contaminated by mitochondria and was therefore not used in these experiments.

The Scatchard plots for the D-glucose-inhibitable cytochalasin B binding sites of the plasma and intracellular membranes were shown in Fig. 3. It is clear from these plots that the restraint conditions shifted the line to the left but did not affect the slope for the plasma membrane fraction. The Maximal number of cytochalasin B binding sites on plotting decreased from 6.5 to 4.3 pmol/mg protein with restraint. This suggested that the number of glucose transporters decreased in the plasma membrane fraction. On the other hand, in the intracellular membrane, there was no obvious change in the concentration of glucose transporters due to the physical inactivity (Fig. 3).

Insulin and even muscle contraction cause the translocation of glucose transporters from intra-

TABLE 4. *Membrane distribution of glucose transporters in muscle of rats in physical inactivity*

	Physical Inactivity	Control
Membrane protein yield, μg protein/g muscle		
Plasma membrane	288±78	308±98
Intracellular membrane	349±63*	563±72*
Yield of marker enzymes, %		
γ-Glutamyltransferase	3.23±0.97	2.96±0.38
Microsome phosphatase	1.43±0.32	1.50±0.22
Total isolated glucose transporters†, pmol/g tissue		
Plasma membranes	1.23±0.22*	2.00±0.28*
Intracellular membranes	1.26±0.19*	2.02±0.21*
K_d, nM		
Plasma membranes	182	159
Intracellular membranes	180	129

Values are means ± SE. * Significant difference ($P < 0.05$) between physical inactivity and control. † Binding sites/g tissue were calculated by multiplying binding site/mg protein by membrane protein yield.

cellular microsomes to the plasma membrane (Dohm et al., 1986; Green and Nasset, 1983; Kraegen et al., 1983). thus the distribution of glucose transporters in each membrane must be quite variable. The "effective" glucose transporter number is obtained only form that on the plasma membrane isolated immediately after the maximal effect of insulin perfusion. Figure 4 shows the effective glucose transporter number on the plasma membrane after 33 $mU \cdot kg^{-1} \cdot min^{-1}$ insulin perfusion. Maximal cytochalasin B binding sites increased in concentration with the maximal-dose insulin perfusion as previously reported. The effective binding sites amounted to 8.3 pmol/mg protein for control rats and 5.5 pmol/mg protein for physically inactive rats.

Membrane protein yield (mg membrane protein/g tissue) and total isolated cytochalasin B binding site (pmol/g tissue), based on protein yield, of each membrane were summarized in Table 4. There was no significant difference in the average recovery of g-glutamyltransferase or that of microsomal phosphatase between the two groups. However, protein yields of intracellular membranes in physically inactive rats were significantly lower than in control rats. Total isolated cytochalasin B binding site on plasma membranes and intracellular membranes based on membrane protein yields were also lower in the physically inactive rats.

Discussion

The restraint cage used in the present experiments was a Ballman-type cage modified for minimum restraint by Green and Nasset (1983). This cage is made of 10 parallel stainless steel bars, which form a circle that surrounds the rat gently, and two plastic boards, which support the ends of the bars and can be used to adjust the seize. Rats usually hang their legs down through the bars, the body weight being supported by the bottom bars of the cage. As shown in Fig. 1, the rats placed in the restraint cage were limited as to movement, with decreased mechanical loading and decreased motor activity. However, the restraint cage allows rats to voluntarily retract their legs and support their body weight with them when they want to obtain food and water.

Green and Nasset (1983) have used this cage to maintain rats in studies on pancreatic exocrine. The data of Green and Nasset (1983) and others (Miyasaka et al., 1989) who used this cage showed that the secretion of gastrointestinal hormones and pancreatic exocrine and its nervous regulation were normal during prolonged restraint in the cage, suggesting that physical inactivity with this cage did not affect endocrine, exocrine, or nervous exocrine control.

The physically inactive rats showed a tentative increase of plasma ACTH level and a return to

normal level by the 3rd day. This finding is consistent with the observation by Popovic et al. (1982), who found that the plasma levels of prolactin, corticosterone, and ACTH increased only in the first few days of hindlimb suspension. We therefore assume that the stress provoked by the present model may not exceed that of the hindlimb suspension model.

Also the muscle atrophy was less than that observed with other models. Although these models of physical inactivity primarily affect slow-twitch muscle (Fittz et al., 1986), only 7 and 9% decreases in the muscle-to-body weight ratio were observed in the gastrocnemius and quadriceps muscles, respectively, in these experiments. Hindlimb suspension for 28 days was reported to lower the whole muscle-to-body weight ratio by 66% in young rats (Bonen et al., 1988). Fourteen days of hindlimb suspension brought about extensor digitorum longus (EDL) atrophy of ~15% (Fittz et al., 1986). Seven days of whole body suspension resulted in a decrease in the gastrocnemius muscle of ~50% (Fell et al., 1985). Compared with these data, the present model is notable for its mild effects as to body weight and muscle atrophy.

There was considerable heterogeneity in the glucose metabolic rate in response to physical exercise among different muscles despite the similarity of the basal values. James et al. (1985) reported that the glucose metabolic rate increased to 32-fold the basal level in soleus, 42-fold in red gastrocnemius, 5-fold in white gastrocnemius, and only 2-fold in EDL muscle during moderate exercise, because the EDL was not recruited much during exercise. In the present experiment we isolated plasma and microsomal membranes from whole gastrocnemius and whole quadriceps muscles, which are the biggest muscles, and since both muscles consist of a large percentage of fast-twitch fibers and a small percentage of slow-twitch fibers, the sum of their responses express mainly the response of fast-twitch muscles to insulin stimulation. The soleus is a simpler muscle and probably shows more obvious responses, but it is too small to determine the glucose transporter number in an individual rat.

Euglycemic clamp experiments indicated a decrease in whole body glucose uptake in maximal doses of insulin under the present conditions of physical inactivity. Porcine insulin infusion at 3.6 $mU \cdot kg^{-1} \cdot min^{-1}$ brought about nearly 50% maximal glucose disposal, whereas 14 $mU \cdot kg^{-1} \cdot min^{-1}$ was nearly the maximally effective dose in control rats (James et al., 1983). James et al. (1984) reported that $GIR_{60\text{-}120}$ values for 14 and 72 $mU \cdot kg^{-1} \cdot min^{-1}$ infusion were similar (26.1 vs. 28.2 $mg \cdot kg^{-1} \cdot min^{-1}$). The $GIR_{60\text{-}120}$ values in control rats on 3.6- and 14-mU insulin perfusion were in agreement with those reported by James et al. (1984, 1985). The steady-state glucose infusion rate on 14-mU insulin perfusion, nearly the maximal effective dose, was markedly low in the physically inactive rats, suggesting at least a postinsulin receptor defect (Kahn, 1980; Olefsky et al., 1982). However, we could not rule out other unidentified effects occurring at the same time, because we could not observe any reduction in the amount of glucose disposal at the submaximal dose of insulin. We also did not observe a decrease in insulin sensitivity; however, insufficient data were obtained in the present study to determine whether insulin sensitivity is unchanged. Further investigation is required on glucose uptake on low-level insulin perfusion.

The results obtained for the glucose transporter number supported a postreceptor defect. Glucose transport occurs through a process of facilitated transport mediated by a specific glucose transport protein in the plasma membrane. When glucose uptake is stimulated by insulin, the number of glucose transporters in the plasma membrane increases because of translocation of the transporters from an intracellular pool (Cushman and Wardzala, 1980; Karnieli et al., 1981; Kono et al., 1982; Suzuki and Kono, 1980). In the present study, the number of cytochalasin B binding sites on the plasma membrane decreased under 14 days of physical inactivity. Because cytochalasin B is a competitive inhibitor for glucose transport, it was postulated that the above binding would reflect the number of glucose transporters (Wardzala et al., 1978). Cytochalasin B binding sites on the intracellular membrane fraction (pmol/mg protein) did not show a marked difference between the two groups. On the other hand, total isolated cytochalasin B binding sites on the intracellular membranes based on membrane protein yield (pmol/g tissue) showed significant decrease with physical inactivity. This may be due in part to the loss on intracellular membrane protein induced by physical inactivity. Although we do not have an explanation for this, a similar loss in intracellular membrane protein was reported in response to exercise (Fushiki et al., 1989).

Horuk et al. (1986) suggested that, in the intracellular pool in adipocytes, there may be unrecruitable glucose transporters that may be ineffective for glucose uptake. Ramlal et al. (1988) also reported two kinds of intracellular pools of the glucose transporter in L6 muscle cells. These findings suggested that the total number of glucose transporters estimated from those on the plasma and microsome membranes does not always reflect the effective glucose transporters. Insulin-dependent glucose transporters for glucose uptake may be found on the plasma membrane after full translocation on maximal insulin stimulation. Glucose transporters on the plasma membrane after 33 $mU \cdot kg^{-1} \cdot min^{-1}$ insulin perfusion were markedly increased in number, resulting in insulin-induced translocation of transporters. The number of plasma membrane glucose transporters after insulin-induced translocation was much smaller in the physically inactive animals than in controls. The reduced glucose transporter number in the muscle can be linked to overall decrease in whole body glucose uptake in the physically inactive animals, because skeletal muscle is the major site of insulin-mediated glucose transport, accounting for ~90% of the insulin-stimulated glucose removal from the blood (DeFronzo et al., 1981; Klip and Walker, 1983).

Our data were consistent with those from other studies reporting decreased glucose transporters in diabetic skeletal muscle. Ramlal et al. (1989) reported that the number of glucose transporters was decreased in skeletal muscle of streptozotocin-treated mildly diabetic rats. The decrease was more pronounced in plasma membranes than in the intracellular membranes.

In conclusion, the decreased glucose uptake at the maximal dose of insulin after exposure to 14 days of physical inactivity may in part be explained by the decrease in the effective glucose transporter number in muscle.

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2. Effects of chronic hypoxia on the whole-body insulin action in rats

Chronic hypoxia has been shoen to induce cellular adaptations, i.e., increased capillary densities and myoglobin contents of heart and skeletal muscle (Cassin et al. 1971; Tureck et al. 1972). Cartee et al. (1991) reported that actue hypoxia caused a progressive, cytochalasin B inhibitable increase in the rate of 3-O-methylglucose transport in rat epitorchlearis muscles to approximately sixfold above the basal level. They also suggested that hypoxia and exercise-stimulated glucose transport occur through the same mechanism, because the maximal effects of hypoxia and exercise on glucose transort were not additive. Chronic exercise markedly increases the whole-body insulin sensitivity of glucose utilization (James et al. 1984). If acute hypoxia and acute exercise cause an increase in glucose utilization via the same mechanism, chronic hypoxia would be expected to have the same effect as chronic exercise. This could be measured by the euglycemic glucose clamp technique.

The present study was undertaken to determine whether chronic hypobaric hypoxia increase whole-body insulin action on clucose utilization. It is hypothesized that chronic hypbaric hypoxia, like chronic exercise (James et al. 1984), causes an increase in insulin sensitivity to glucose utilization.

Methods

Exprimental design

Male Sprague-Dawley rats (21 days old, initial body weight 68 ± 4 g) were randomly divided into two groups: sea-level control (C) and hypobaric hypoxia (H) groups. Each group of rats was housed separately in 36 × 36 × 48 cm cages at an ambient temperature of 22 ± 2°C, and were provided with food and water ad libitum. The H rats were kept in a hypobaric hypoxia chamber maintained at a simulated altitude of 3000 m (67.8 kPa) for 1 week, and than at an altitude of 4000 m (61.1 kPa). The airflow through thechamber was regualted at 20 L/min. After 10 weeks of experimental treatment, the whole-body glucose disposal was determined by the euglycemic clamp technique. Care and treatment of experimental animals conformed with Kyoto Univesity guidelines for the ethical treatment of laboratory animals.

Euglycemic clamp experiments

The euglycemic clamp technique was carried out according to the method described by Kraegen et al. (1983), with minor modifications as described elsewhere (Fushiki et al. 1991). The animals were deprived of food for 6-10 h overnight before the euglycemic clamp study. The rats ere anethetized with an intraperitonial injection of pentobarbital sodium (5 mg·100 g^{-1} body weight·h^{-1}) and surgically prepared for the euglycemic clamp study. An intravenous Silastic catheter (Dow Corinig) was inserted into the left jugular vein (0.02 in. i.d. × 0.037 in. o.d. (1 in. = 25.4 mm)) and filled with heparin-saline (50 IU heparin/mL 0.9% saline). Two other catheters were fitted with 23G needles, and the needles were inserted into the lift femoral vein and the posterior vena cava,

respectivily. The femoral catheter was filled with insulin and the posterior one with 10% (w/v) glucose. The jugular catheter was used for blood sampling, the femoral one for intravenous insulin iinfusion, and the posterior one for intravenous glucose infusion. All experiments were conducted at least 30 min after surgery to allow the high blood glucose level due to the anesthesia and surgery to return th the basal fasting level. Bovine insulin (Sigma Chemical Co., St. Louis, Mo.) was infused for 2 h, using Familic-100N microinfuion pump (Japan Spectroscopic Co., Japan). Blood glucose was maintained at the basal fasting level by estimating, at regular intervals (7.5 min), the plasma glucose concentration in 25-μL samples taken from the jugular catheter (glucose C-test Wako kit; Wako Pure Chemicals Co., Japan). The rate of infusion of a 10% glucose solution was then adjusted accordingly via the posterior catheter. Glucose was infused using a Terfusion syringe pump, STC-521 (Termo, Japan. The glucose infusion rate (GIR) during the 2nd h of the clamp experiment (GIR_{60-120}) was taken as the steady-state net whole-body glucose disposal rate. At completion of the experments, 1 mL of blood was collected for the insulin radioimmunoassay (RIA), and then the following tissues and hind-limb muscles were rapidly removed and weighed: epididymal fat pads and gastrocnemius, quadriceps, soleus, and extensor difitorum longus (EDL) muscles.

Statistics

Differentces between H and C groups were evaluated by the Student's t-test. Probability levels of < 0.05 were considered significant.

Results and Discussion.

At the end of the experimental period, an adptive increase in erythrocyte number in the blood was observed in group H. The mean body weights of groups H and C were 358 ± 16 and 435 ± 21 g, respectively ($p < 0.01$). the mean wet weights of the gastrocnemius and EDL muscles in group H were significantly less than in group C ($p < 0.05$). The weights of the quadriceps and soleus muscles were not significantly different. The epididymal fat pad weight was markedly less in group H than group C ($p < 0.01$). these changes of the muscle and fat pads, however, did not have a significant influence on the insulin sensitivity to glucose transport (Table 2). A euglycemic clamp study was carried out with 3.6 and 14 mU·kg^{-1}·min^{-1} bovine insulin infusion, which were the insulin cencentrations for giving approximately50% of the maximal response and a nearly maximal effect on glucose uptake, respectively. The GIR (mg·kg^{-1}·min^{-1}) plateaued at 45-60 min, without evidence of a further increase in glucose requirement. For the first 30 min of the measurement period, the blood glucose level decreased and then plateaued in both

TABLE 1. Effects of hypoxia on body, muscle, and fat pad weight (grams)

	Control	Hypoxia
Body weight	435±24	353±19**
Gastrocnemius	5.04±0.44	4.37±0.50*
Quadriceps	6.28±0.52	6.16±0.72 ns
Soleus	0.182±0.021	0.165±0.018 ns
EDL	0.225±0.020	0.178±0.11*
Epididymal fat pads	7.65±2.21	4.83±0.63*

NOTE: Values are means ± SEM for six rats. ns, no significant difference; *, $p < 0.05$, and **, $p < 0.01$, versus control rats.

TABLE 2. Effect of hypoxia on glucose transport activity with insulin infusion

	14 mU insulin · kg^{-1} · min^{-1}		3.6 mU insulin · kg^{-1} · min^{-1}	
	Control	Hypoxia	Control	Hypoxia
GIR_{60-120} (mg · kg^{-1} · min^{-1})	19.86±3.19	19.18±3.95 ns	12.65±2.59	13.92±3.01 ns
Fasting plasma glucose (mmol/L)	7.65±0.56	7.71±1.32 ns	7.47±0.89	7.88±1.05 ns
Steady-state plasma glocuse (mmol/L)	6.67±1.00	7.00±0.78 ns	6.94±0.83	7.06±0.94 ns
Steady-state plasma insulin (mU/L)	642±45	711±70 ns	129±3	141±42 ns

NOTE: GIR_{60-120} is the steady-state exogenous glucose disposal rate estimated as the mean glucose infusion rate between 60 and 120 min required to maintain the euglycemia with 3.6 and 14 mU · kg^{-1} · min^{-1} insulin infusion. Fasting plasma glucose was determined in blood collected before insulin infusion. Steady-state plasma glucose was measured in blood obtained immediately after the experiment; plasma insulin was measured by RIA in the same blood. ns, no significant difference between control and hypoxic rats (n = 4–6 in each group). Data are means ± SD.

groups. The steady-state glucose infusion rates at 3.6 and 14 mU·kg^{-1}·min^{-1} insulin infusion were the same in groups C and H (Table 2). The GIR_{60-12} values were highly consistent with our previous data, i.e., the mean glucose infusion rates on 3.6 and 14 mU·kg^{-1}·min^{-1} insulin infusion in normal control rats were 13.8 and 20.8 mg·kg^{-1}·min^{-1}, respectively (Fushiki et al. 1991). These results suggested that chronic hypobaric hypoxia did not increase the whole-body insulin action, in contrast to the results of James et al. (1984), who showed that chronic exercise increased insulin sensitivity to glucose transport. We cannot explain the discrepancy. Both acute exercise and cute hypoxia increase glucose transport in skeletal muscles (Cartee et al. 1991). Chronic exercise causes an adaptive increase in insulin action (James et al. 1984). Friedman et al. (1990) showed an adaptive increase in glucose transporter protein GLUT-4, which results in an increase ininsulin responsiveness (Kern et al. 1990). However, the chronic hypobaric-hypoxic stimuli did not increase insulin action in the present insulin action. Alternatively chronic hypobaric hypoxia and hypoxia by perfusion may be com;etely different models. Further sutdies are required on the mechanisms underlying the regulation of insulin receportor and glucose transporter expression.

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SUMMARY

CHAPTER I

1. The animal model of exercise-induced skeletal muscle hypertrophy was developed with rats. The gastrocnemius muscle of one leg, which was selected at random for each rat, was stimulated with needle type electrodes, i.e., for 2 s with 10V at 100 Hz, with 5 s intervals and contralateral leg was not stimulated as a control. One set consisted of 10 such stimuli, followed by 5 min. rest. Three sets every 2 day caused statistically significant increase in muscle mass, the increase being about 2.5% after the 3rd day stimulation, 4.4% after the 5th day, 5.9% after the 13th day and 8.3% after the 27th day compared with each control muscle ($p<0.001$ in each case). The protein contents also increased, but the water contents did not change. Four week-stimulation induced an increase in the area of the cross section of the muscle fibers to about 30% larger than that of control muscles, though the total muscle fiber numbers were slightly, but significantly, reduced. Electromechanical properties supported the development of the muscle with the stimulation, because the maximal isometric tetanic force and peak twitch force markedly increased in the stimulated muscle.

2. The physiological importance of androgen in exercise-induced muscle hypertrophy was investigated in rats. Along with training rat gastrocnemius muscles by electrical stimulation every other day for 2 weeks, male rats were administered with the androgen receptor antagonist, oxendolone. The androgen receptor antagonist effectively decreased the wet mass of prostate, an androgen target organ, and did not significantly affect body mass. The increase in muscle mass induced by electrical stimulation was effectively suppressed by the androgen receptor blockade. The mean degree of muscle hypertrophy in the antagonist-treated group was significantly lower than that in the vehicle group (102.30% vs. 107.41%, respectively. $p=0.006$).

The same stimulation in female rats failed to cause significant muscle hypertrophy. The hypertrophy was observed only in the stimulated muscle when female rats were treated with testosterone. This indicated that the mechanism of exercise-induced hypertrophy via androgen pathway was conserved in female animals. These results suggest that the androgen pathway has a significant effect in exercise-induced muscle hypertrophy.

3. The changes in the number of androgen receptors in rat gastrocnemius muscle on muscle contraction caused by electrical stimulation were investigated. The androgen receptors in the muscle cytosol fraction were determined by means of a binding assay involving [^{3}H]methyltrienolone, which is an analog of testosterone, the number having rapidly increased in the stimulated leg, when compared with that in the control leg, by about 25% after the 3rd day. The increase then slowed down, being plateau after the 5th day stimulation. The receptor dissociation constants for [^{3}H]methyltrienolone remained unchanged, i.e., approximately 0.3 to 0.4 nM throughout the experimental period. These findings suggested that a rapid increase in the number of androgen receptors occurred as an early event for a practical increase in muscle mass, and thus it

may contribute in part to the triggering of muscle hypertrophy by enhancing the muscle sensitivity to androgen.

CHAPTER II

1. Whole body insulin action on glucose uptake and muscle glucose transporter number of rats subjected to 14 days of physical inactivity conditions was examined. Unlike other suspension and denervation models of muscle disuse, this physical inactivity model allows voluntary contractile activity with minimal stress. Minimal depression of body weight gain and significant depression of gastrocnemius muscle growth were observed compared with that of control rats after 14 days of physical inactivity. The whole body insulin sensitivity and responsiveness were determined by the euglycemic clamp technique, with 1.4, 3.6, and 14 mU insulin·kg^{-1}·min^{-1} perfusion and 2-deoxy-D-[^{3}H]glucose incorporation. The rates of glucose disposal were the same in the restrained rats as in the controls with the 1.4 and 3.6 mU insulin perfusion; however, glucose disposal significantly decreased with 14 mU insulin perfusion. 2-deoxy-D-[^{3}H]glucose uptake into the gastrocnemius muscle was higher in the control rats than in the physically inactive rats. Glucose transporters in the gastrocnemius quadriceps muscles, measured by means of the D-glucose-inhibitable cytochalasin B binding assay, were significantly decreased in number in the physically inactive rats. These findings suggest that the decrease in whole body glucose uptake might in part be explained by the decreases in the total glucose transporter number in muscles.

2. The effect of chronic hypoxia on the whole-body insulin action in rats was investigated. Rats were kept in a hypobaric hypoxia chanber maintained at a simulated altitude of 4000 m for 10 weeks.At the end of the experimental period, the mean body weight of the pypoxic rat was significantly lower than that of the control rats. The muscle weight to body ratio of the quadriceps muscle in hypoxic rats was larger thanthat in control rats, but those of the gastrocnemius , soleus, and extensor digitorum longus muscles did not differ between the control and hypoxic rats. Onthe other hand, the epididymal fat pads of hypoxic rats were marked ly smaller than those of the control rats. The results of a euglycemic clamp experiment with infusions of 14 and 3.6 mU insulin·kg^{-1}·min^{-1} indicated that the steady-state glucose infusion rate was not statistically different between hypoxic and control rats. It is suggested that chronic hypoxia did not influence the whole-body insulin action on glucose transport activity.

ACKNOWLEDGMENTS

The author is deeply grateful to Dr. Etsuro Sugimoto, Professor of Nutritional Chemistry, Department of Food Science and Technology, Faculty of Agriculture, Kyoto University, for his kind guidance, continuous encouragement and generous support throughout this study.

The author would like to express his sincere gratitude to Dr. Kazuo Iwai, Professor emeritus of Kyoto University, for his advice and encouragement.

The author wishes to express his grateful acknowledgment to Dr. Tohru Fushiki, Associate Professor of the Laboratory for his valuable suggestion, discussion and kind encouragement in carrying out this study.

The author would like to thank Dr. Teruo Kawada and the late Dr. Masako Higuchi, the colleagues of the Laboratory for useful discussion and warm encouragement.

The author also acknowledges the members of Laboratory of Applied Physiology, College of Liberal Arts and Science, Kyoto University: to Dr. Toshio Moritani for his useful discussion and contribution to developing the animal model and for electromechanical analysis of muscles, and to Drs. Kazuo Itoh and Chiyoko Hirofuji for their collaboration in developing the model and for their skillful analysis in muscle histochemistry.

The author is greatly indebted to Ms. Tomomi Ishihara, formerly Kano, Messrs. Sho Yamasaki, Yoshimune Okada, Hiroya Ueminami and Akihiro Sakai, the members of the same research group, for their experimental work and valuable discussion.

Thanks are due to all the members of the Laboratory of Nutritional Chemistry, Kyoto University, for their helpful support and assistance.

Finally, the author thanks his family for their encouragement and support.

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