Exercise Training Prevents Maturation-Induced Decreases in Insulin Receptor Substrate-1 and Phosphatidylinositol 3-Kinase in Rat Skeletal Muscle

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We have previously reported that exercise training prevents a maturation-induced decrease in insulin sensitivity and suggested that an improvement of insulin sensitivity by exercise training was attributable, in part, to an increase in insulin-sensitive GLUT-4 on the skeletal muscle plasma membrane. In this study, we examined the effects of maturation and exercise training on the gene expression and protein content of the components of post–insulin receptor signal transduction in rat skeletal muscle. Rats aged 3 weeks were sedentary or trained by voluntary running through 4 or 27 weeks of age, and then the rats in both the sedentary and trained groups were killed and the gastrocnemius muscle was immediately removed for analysis of mRNA and protein content. The concentration of mRNA and protein for insulin receptor substrate-1 (IRS-1) in sedentary rats significantly decreased with maturation (49% and 63%, respectively, at age 27 weeks *v* age 4 weeks), but in trained rats they did not decrease with maturation. Although the level of phosphatidylinositol 3-kinase (PI 3-kinase) mRNA in sedentary rats was not altered with maturation, PI 3-kinase protein in sedentary rats significantly decreased with maturation (73% at 27 weeks *v* 4 weeks). However, PI 3-kinase protein in trained rats did not decrease with maturation. These results suggest that the prevention of maturation-induced decreases in the protein content of IRS-1 and PI 3-kinase is involved in the mechanisms responsible for the improvement of insulin sensitivity by exercise training, and exercise training may affect transcriptional regulation of the IRS-1 gene and posttranscriptional regulation of PI 3-kinase expression.

A DECLINE in physical activity and changes in body composition with advancing age may contribute to the deterioration of glucose metabolism.[1] Previous studies have reported that skeletal muscle is the major site of insulin-mediated glucose disposal,[2] and the decline in glucose utilization in aged humans appears attributable to insulin resistance of skeletal muscle.[3] Such insulin resistance related to aging also has been demonstrated in rats.[4,5] Goodman et al[4] reported that glucose utilization in skeletal muscle was diminished progressively with aging in rats. In Wistar rats, sexual maturity occurs at 7 to 10 weeks of age.[6] During this period, insulin resistance develops.[4,5] A previous study suggested that aging-related insulin resistance in skeletal muscle is associated with an impairment of insulin signal transduction,[7] but little is known about the effect of maturation.

It is well established that exercise training prevents the development of insulin resistance.[8-10] We have reported that exercise training prevents the maturation-induced decrease in insulin sensitivity, and suggested that the improvement of insulin sensitivity by exercise training was, in part, attributable to an increase in insulin-sensitive GLUT-4 on the plasma membrane in rat skeletal muscle.[10] On the other hand, many studies were performed to elucidate the mechanisms responsible for exercise-induced stimulation of glucose uptake into skeletal muscle, and indicated that components such as the insulin receptor, insulin receptor substrate-1 (IRS-1), and phosphatidylinositol 3-kinase (PI 3-kinase) of the insulin signal transduction system are not involved in the mechanisms underlying glucose uptake due to an acute bout of exercise, suggesting that the underlying molecular mechanism for the acute bout of exercise is distinct from that for insulin.[8,9,11] Further studies are required to clarify the mechanisms for glucose uptake into skeletal muscle stimulated by acute exercise and for an improvement of insulin sensitivity by exercise training. Especially evidence for the latter is lacking because fewer studies have been performed.

It is important to clarify the mechanisms underlying the improvement of insulin sensitivity by training, because exercise training is generally recommended for the prevention and treatment of insulin resistance. There is a good possibility that maturation and aging might downregulate, and in contrast, exercise training might upregulate, the gene expression for some components of insulin signal transduction. This hypothesis is supported, in part, by the evidence that endurance training increases insulin receptor and IRS-1 mRNAs in rat skeletal muscle.[12] The present study was performed to obtain further evidence to demonstrate this hypothesis. We report here that the expression of IRS-1 and PI 3-kinase in rat skeletal muscle is affected by maturation and exercise training.

MATERIALS AND METHODS

Materials

Female Wistar rats aged 3 weeks were obtained from CLEA Japan (Tokyo, Japan). [γ-^{32}P]adenosine triphosphate (ATP) and [^{125}I]anti-rabbit immunoglobulin were purchased from Amersham Japan (Tokyo, Japan). All other reagents were of biochemical grade.

Animal Care and Experimental Design

The rats were fed laboratory chow (CE-2; CLEA Japan) and tap water ad libitum and were housed individually in an animal room. The room temperature was maintained at 23°C with a 12-hour light/dark cycle

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(light from 5 AM to 5 PM). All procedures involving animals were approved by the experimental animal care committee of Nagoya Institute of Technology.

The rats (aged 3 weeks) were randomly allocated to sedentary and exercise-trained groups. Rats in the exercise-trained group were housed in a cage with a running wheel (1.0 m per revolution) throughout the experiment, which permitted the animals to exercise voluntarily. Since we reported previously that the maturation-induced decrease in insulin sensitivity measured with the insulin clamp technique was clearly observed in rats at age 27 weeks compared with age 4 weeks,[10] rats aged 4 weeks and 27 weeks in both groups were killed and the muscle and blood were immediately removed. The exercise-trained rats were transferred to a regular cage (without the wheel) approximately 32 hours before death to minimize the effect of acute exercise. On the final day of the experiment, rats were anesthetized at 3 PM with sodium pentobarbital (60 mg intraperitoneal injection/kg body weight) under the fed condition, and the gastrocnemius muscles were removed and the blood was collected to prepare serum. The muscles were immediately freeze-clamped at liquid nitrogen temperature and stored at −80°C until analysis.

Blood Analyses

Blood glucose was determined using a glucose analyzer (model 23A; Yellow Springs Instruments, Yellow Springs, OH). Serum insulin was measured by radioimmunoassay.[13] Serum nonesterified fatty acids (NEFAs) were determined with a commercial kit (Wako Pure Chemical Industries, Osaka, Japan).

Reverse Transcription–Polymerase Chain Reaction

The relative mRNA abundance for the insulin receptor, IRS-1, and PI 3-kinase in the muscle were measured by a reverse transcription–polymerase chain reaction (RT-PCR) method.[14] Total RNA was extracted from skeletal muscle by a guanidinium thiocyanate water-saturated phenol extraction method.[15] First-strand cDNA synthesis was performed on 5 μg total RNA using oligo(dT) per the manufacturer's instructions (SuperScript Preamplification system for first-strand cDNA synthesis; GIBCO-BRL, Tokyo, Japan). The primers were 5′-CCTGATAACTGTCCAGAGAG-3′ and 5′-TCCGTTTGATGCTCAGAGAG-3′ for insulin receptor cDNA, 5′-GCCAATCTTCATCCAGTTGC-3′ and 5′-CATCGTGAAGAAGGCATAGG-3′ for IRS-1 cDNA, and 5′-CAGGATCAAGTTGTCAAAGAAGAT-3′ and 5′-TATGTATTCTTTGCTGTACCGCTC-3′ for PI 3-kinase cDNA. Before PCR amplification, the primers were labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Takara, Kyoto, Japan). The reactions were performed in a Takara PCR Thermal Cycler MP using the following conditions: denaturation at 94°C for 1 minute, except for an initial denaturation of 6 minutes, annealing at 58°C (for insulin receptor and IRS-1) or 62°C (for PI 3-kinase) for 2 minutes, and extension at 72°C for 3 minutes. PCRs were repeated 23 cycles for the insulin receptor, IRS-1, and PI 3-kinase. The number of cycles was confirmed to be within the linear range of amplification in the semilogarithmic graph (Fig 1). PCR amplification within the logarithmic phase is a prerequisite for the use of PCR in combination with a quantifiable detection system as reported previously.[16] PCR products (10 μL from 20 μL final reaction mixture) were analyzed by electrophoresis on 7.5% polyacrylamide gels. Radioactivity in the bands corresponding to each mRNA was quantified by a laser image analyzer (Fuji BAS1000; Fuji Film, Tokyo, Japan).

Western Blot Analysis

The protein concentration of insulin-signaling molecules in the muscle was measured by a Western blot method. The frozen samples of gastrocnemius muscle were homogenized using a Polytron homogenizer in solubilization buffer (20 mmol/L Tris hydrochloride, pH 7.6, 150 mmol/L NaCl, 1% Nonidet P-40, 100 mmol/L NaF, 10 mmol/L Na_3VO_4, 10 mmol/L EDTA, 1 mg/mL leupeptin, and 1 mg/mL aprotinin). The homogenate was kept on ice for 1 hour and centrifuged at 38,000 rpm at 4°C for 1 hour using a Hitachi RP40T rotor (Hitachi, Tokyo, Japan), and the supernatant was collected. Supernatant proteins (80 μg) in each sample were size-fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (6% or 10% acrylamide) according to the method of Laemmli.[17] After electrophoresis, the proteins were electrophoretically transferred onto an Immobilon polyvinylidene difluoride membrane (Millipore, Bedford, MA) in electrode buffer (10 mmol/L 3-cyclohexylaminopropanesulfonic acid and 10% methanol, pH 11, with NaOH) for 3 hours at 0.4 A. Nonspecific protein binding to the membrane was reduced by preincubating the membrane at 22°C for 1 hour in blocking buffer (5% bovine serum albumin, 20 mmol/L Tris hydrochloride, pH 7.5, and 0.5 mol/L NaCl). The membranes were then incubated with anti–insulin receptor β-subunit, anti–IRS-1, or anti–PI 3-kinase (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies overnight at 22°C. Bound antibodies were detected by incubation with [^{125}I]anti-rabbit Ig for 2 hours at 22°C. Radioactivity in the bands corresponding to each protein was quantified by the laser image analyzer.

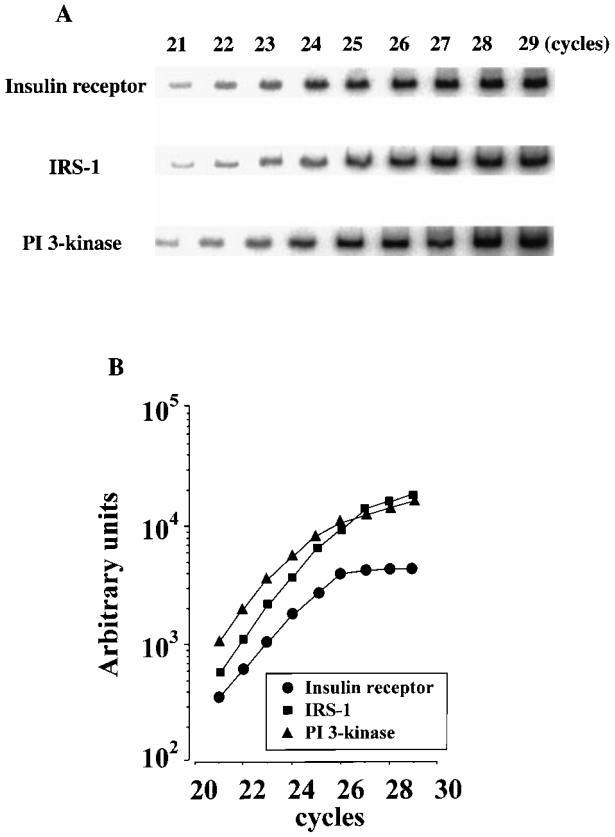

Fig 1. Quantitative analysis of insulin receptor, IRS-1, and PI 3-kinase mRNA levels by RT-PCR. Representative autoradiograms of PCR products amplified with an increasing number of PCR cycles (A). The intensity of each band was measured by a laser image analyzer and plotted on a logarithmic scale as a function of cycle number (B).

Statistical Analysis

The data are expressed as the mean ± SE. Comparisons between the 2 treatment groups (maturation and exercise training) were evaluated by a 2-way ANOVA, and significance was determined with the Bonferroni/Dunn test.

RESULTS

Rat Body Weight, Blood Glucose, Serum Insulin, and NEFAs

The body weight at 4 and 27 weeks of age was not different between trained and sedentary rats. In sedentary rats, blood glucose increased with maturation. On the other hand, blood glucose in trained rats did not increase with maturation. In both sedentary and trained rats, serum insulin increased with maturation. However, at 27 weeks of age, the serum insulin level was significantly lower in trained versus sedentary rats. Maturation did not affect serum NEFA concentrations in either trained or sedentary rats, but NEFA levels at both ages were significantly lower in trained versus sedentary rats (Table 1).

Effects of Exercise Training and Maturation on Insulin Receptor, IRS-1, and PI 3-Kinase mRNAs in Rat Skeletal Muscle

Maturation and exercise training did not affect the level of insulin receptor and PI 3-kinase mRNA abundance. On the other hand, IRS-1 mRNA abundance in sedentary rats significantly decreased with maturation (49% at age 27 weeks *v* age 4 weeks), but in trained rats it did not decrease with maturation and was significantly higher than the level in sedentary rats at 27 weeks (Fig 2).

Effects of Exercise Training and Maturation on Insulin Receptor, IRS-1, and PI 3-Kinase Proteins in Rat Skeletal Muscle

Maturation and exercise training did not affect the content of insulin receptor protein. IRS-1 protein in sedentary rats significantly decreased with maturation (63% at age 27 weeks *v* 4 weeks), but in trained rats it did not decrease with maturation. PI 3-kinase protein in sedentary rats significantly decreased with maturation (73% at age 27 weeks *v* 4 weeks), but in trained rats it did not decrease with maturation (Fig 3).

DISCUSSION

The present study demonstrates that exercise training prevents maturation-induced decreases in IRS-1 and PI 3-kinase proteins in rat skeletal muscle. We have previously reported that exercise training prevents a maturation-induced decrease in insulin sensitivity,[10] and suggested that the improvement of insulin sensitivity by exercise training is attributable, in part, to an increase in insulin-sensitive GLUT-4 on the skeletal muscle plasma membrane. Thus, exercise training may exert effects not only on GLUT-4 but also on IRS-1 and PI 3-kinase for the

Table 1. Body Weight, Blood Glucose, Serum Insulin, and NEFA

	4 Weeks		27 Weeks	
Parameter	Sedentary	Trained	Sedentary	Trained
Body weight (g)	121 ± 3	121 ± 1	287 ± 10	291 ± 9
Glucose (mg/dL)	160 ± 4	151 ± 2	177 ± 4†	157 ± 5*
Insulin (μU/mL)	2.7 ± 0.2	2.4 ± 0.2	15.9 ± 0.7†	12.6 ± 0.7*‡
NEFA (μmol/mL)	0.35 ± 0.02	0.27 ± 0.02*	0.35 ± 0.02	0.22 ± 0.03*

NOTE. Values are the mean ± SE for 7 rats.
*Significantly different *v* sedentary rats at same age ($P < .0083$).
†Significantly different *v* sedentary rats at 4 weeks ($P < .0083$).
‡Significantly different *v* trained rats at 4 weeks ($P < .0083$).

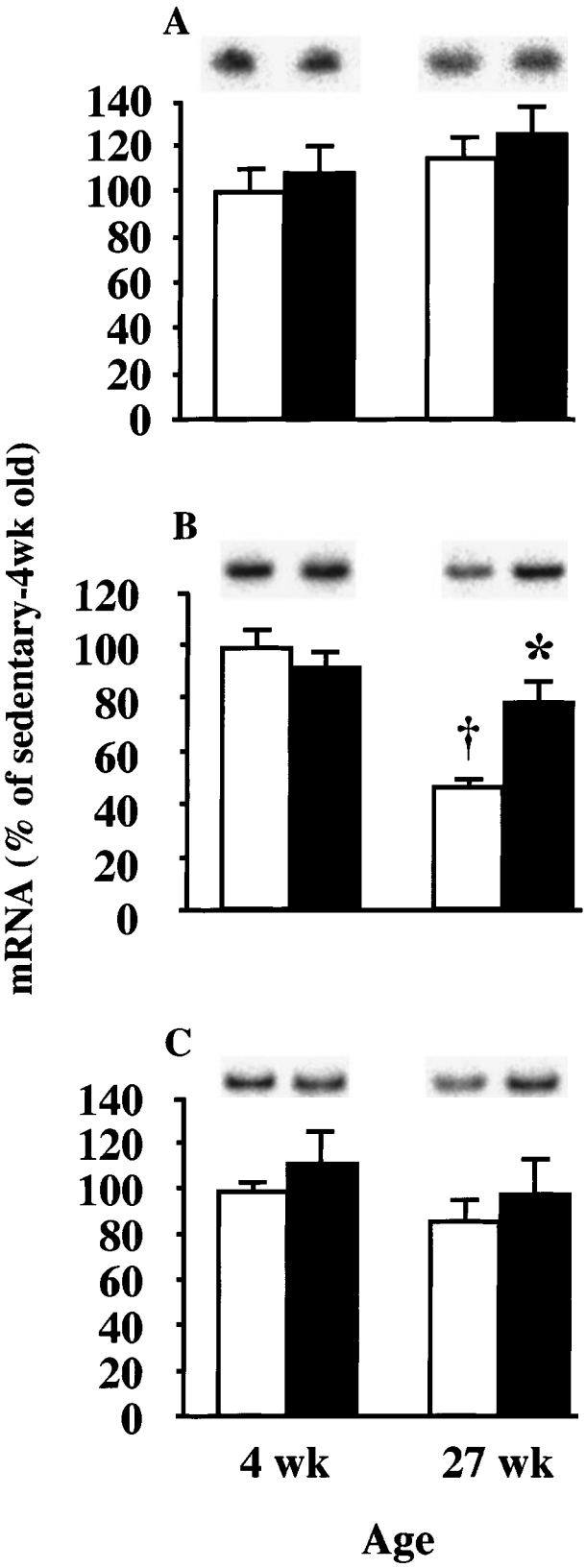

Fig 2. Insulin receptor (A), IRS-1 (B), and PI 3-kinase (C) mRNA levels in rat gastrocnemius muscle. Values are the mean ± SE for 7 rats. (□) Sedentary rats; (■) trained rats. *Significantly different *v* sedentary rats at 27 weeks ($P < .0083$). †Significantly different *v* sedentary rats at 4 weeks ($P < .0083$).

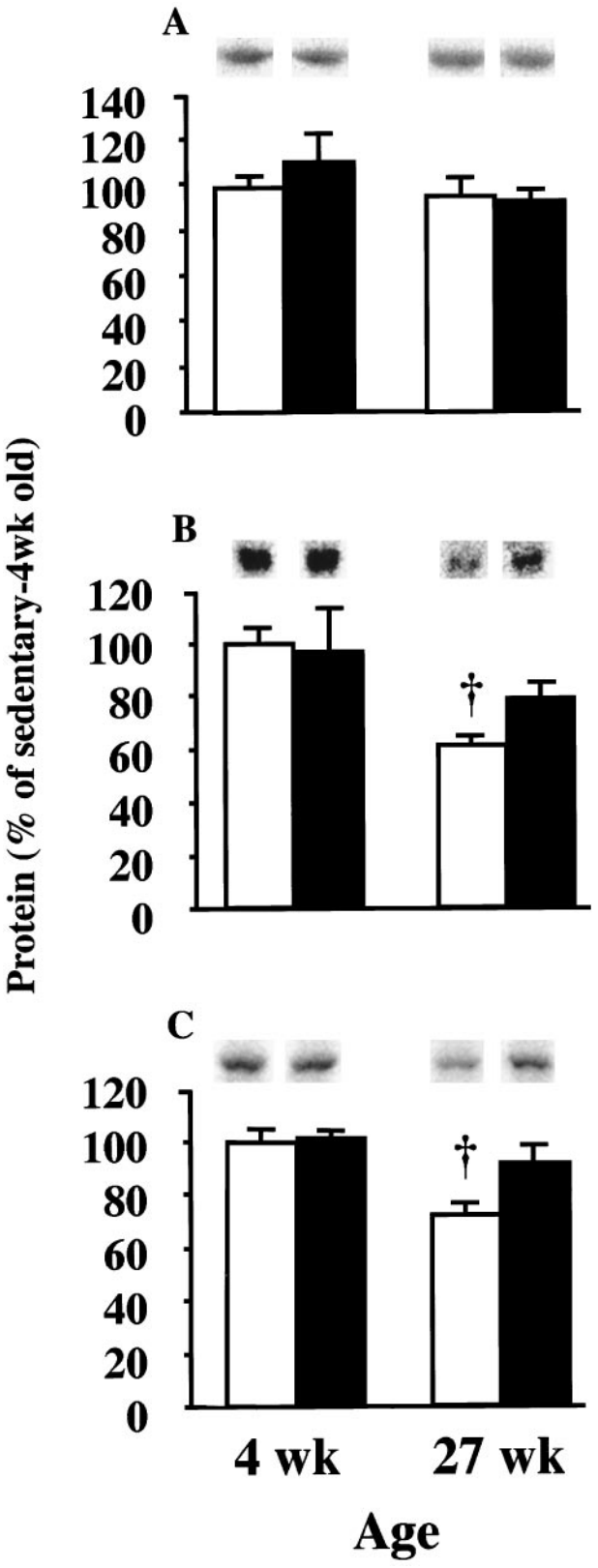

Fig 3. Insulin receptor (A), IRS-1 (B), and PI 3-kinase (C) protein levels in rat gastrocnemius muscle. Values are the mean ± SE for 7 rats. (□) Sedentary rats; (■) trained rats. †Significantly different *v* sedentary rats at 4 weeks ($P < .0083$).

prevention of the maturation-induced decrease in insulin sensitivity.

Many studies have shown that insulin sensitivity decreases with maturation.[4,5,18] Goodman et al[4] reported that the progressive decrease in both the basal rate of glucose utilization and the maximally insulin-stimulated glucose utilization occurred between 3 and 24 weeks of age in rats. Although we did not measure whole-body insulin sensitivity and muscle glucose utilization in the present study, we have reported previously[10] that insulin resistance developed with maturation in rats under the same conditions. Furthermore, in the present study, blood glucose increased with maturation in sedentary rats but not in trained rats. In both sedentary and trained rats, serum insulin increased with maturation, but the serum insulin level at 27 weeks of age was significantly lower in trained versus sedentary rats. These results suggest that insulin resistance developed in sedentary rats at 27 weeks of age and that the insulin resistance could be alleviated by exercise training.

The results for IRS-1 mRNA and protein in the present study appear consistent with the findings of Carvalho et al,[7] who reported that the IRS-1 protein and its phosphorylation in skeletal muscle of sedentary rats were decreased between 2 and 5 months. Reduced IRS-1 expression was also observed in Zucker fatty rats.[19] In addition, mice that are deficient in IRS-1 prepared by targeted gene-knockout exhibit hyperinsulinemia and glucose intolerance.[20-22] These findings suggest that the decreased expression of IRS-1 protein is involved in the development of insulin resistance. Since it has been reported that muscle contractile activity does not stimulate phosphorylation of IRS-1,[23,24] the upregulation of IRS-1 expression caused by exercise training might be important for the prevention of insulin resistance. On the other hand, Sun et al[25] have reported that the high insulin level promotes IRS-1 degradation through the proteasome pathway. Taking these findings into consideration, both the decreased expression of IRS-1 and the accelerated degradation of IRS-1 protein may be responsible for the decrease in IRS-1 protein in skeletal muscle of sedentary rats. In contrast to the sedentary rats, the level of IRS-1 protein in trained rats was not altered after maturation. This may be due to both a higher expression of IRS-1 and a lower concentration of serum insulin in trained rats versus sedentary rats.

Since it has been reported that IRS-1 plays a central role in insulin action for glucose transport in muscles,[20,22] we examined the effects of maturation and exercise training on IRS-1 expression in skeletal muscle. It is suggested that other IRSs are also involved in the mechanisms for the modulation of insulin sensitivity.[26] Further studies are required to elucidate the effects on the expression of other IRSs in skeletal muscle.

The activation of PI 3-kinase is required for insulin-stimulated glucose transport.[27,28] In the present study, PI 3-kinase protein, but not mRNA, decreased with maturation. Our results are consistent with the findings of Carvalho et al,[7] who reported that the activity and protein level of PI 3-kinase in rat skeletal muscle were decreased between 2 and 20 months of age and suggested that these decreases are responsible for the insulin resistance. On the other hand, exercise training prevented the decrease in PI 3-kinase protein with maturation in the present study, although PI 3-kinase is not involved in the mechanisms for contraction-induced glucose uptake.[29-31] The

mechanism for the maintenance of PI 3-kinase protein by exercise training is not clear, but it may be related with the findings of Zhou and Dohm[32] that the insulin-stimulated PI 3-kinase activity in rat skeletal muscle was enhanced by acute exercise.

In the present study, the expression of mRNA and protein for insulin receptor were not affected by maturation and exercise training. These results are consistent with the finding in previous studies that the protein level for the insulin receptor did not decrease with maturation.[7,33] However, there is a possibility that maturation might decrease insulin receptor function, such as tyrosine kinase activity.[33] The effects of maturation and exercise training on the insulin receptor should be more carefully examined, and this investigation is ongoing.

In conclusion, our results show that exercise training prevents maturation-induced decreases in proteins for IRS-1 and PI 3-kinase in skeletal muscle, suggesting that an improvement of insulin sensitivity by exercise training may be attributed, at least in part, to maintaining these proteins in the muscle, and exercise training may exert effects on transcription of the IRS-1 gene and posttranscriptional regulation of PI 3-kinase expression.

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