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Effects of life-long exercise on circulating free fatty acids and muscle triglyceride content in ageing rats

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Abstract

Regular physical exercise has emerged, together with dietary restriction, as an effective intervention in delaying degenerative diseases and augmenting life span in rodents. The mechanisms involved remain largely unknown, although a beneficial influence on the age-related alteration of insulin sensitivity has been hypothesized. As muscle triglyceride (TG) accumulation is considered a reliable index of muscle insulin resistance, in this study we explored muscle TG content in 23-month-old male Sprague–Dawley rats subjected to life-long training. Plasma glucose, insulin, free fatty acid (FFA) and leptin levels were also measured. Both voluntary running in wheels (RW) and forced training in treadmill (TM) were studied. As RW rats weighed less than controls, a cohort of untrained animals, fed to pair weight (PW) with RW, was added to discriminate the effect of exercise from that of food restriction. Sedentary ad libitum fed rats served as controls. In 23-month-old RW rats, muscle TG content was reduced by 50% with respect to age-matched sedentary controls, while in TM group this reduction was smaller but still highly significant, and occurred independently on the changes in body fat mass. In both the trained rat groups, there was a significant decrease in circulating FFA levels and a trend to reduced insulin levels. In PW rats, muscle TG levels decreased similarly to RW rats, while plasma parameters were less modified. In particular, RW training was more effective than PW in preventing the age-related increase in circulating leptin levels. Our results suggest that voluntary exercise effectively counteracts the development of insulin resistance in the muscles of ageing rats as well as other related changes such as hyperlipacidaemia and compensatory hyperleptinaemia. Forced training or moderate food restriction appear slightly less effective than voluntary exercise in preventing age-dependent alterations in nutrient distribution and/or utilization.

Keywords: Ageing; Physical exercise; Insulin resistance; Muscle triglyceride content; Free fatty acids; Leptin

1. Introduction

Regular physical exercise during the whole life has emerged, together with dietary restriction, as an effective intervention in augmenting life span and delaying degenerative diseases in rodents (Holloszy, 1988; Holloszy and Schechtman, 1991; Blain et al., 2000). Epidemiological studies have shown that human life expectancy is also enhanced by physical activity through a substantial reduction of all-cause mortality (Paffenbarger et al., 1986; Blair et al., 1989) and several physiological functions likewise benefit from this life style (Blain et al., 2000). The mechanisms involved in the positive effects of physical fitness remain largely unknown, although a beneficial influence on the age-related development of insulin resistance has been suggested to play an important role (Holloszy, 1988; Blain et al., 2000). Since little is known about the effect of exercise on tissue lipid metabolism in ageing and since muscle triglyceride (TG) content has been recently suggested to be a reliable index

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of muscle insulin resistance (Storlien et al., 1991; Dobbins et al., 2001; Kraegen et al., 2001), in this study we investigated muscle TG content in ageing rats subjected to two different types of prolonged exercise training, as compared to age-matched sedentary or food-restricted animals. Plasma glucose, insulin and free fatty acid (FFA) levels were also measured in these rats, as well as leptin levels in some groups, to explore several major fuel supplies and regulating factors of glucose and lipid metabolism in tissues.

Thus, this study intended to test the hypothesis that the one or the other long-life physical exercise (or possibly both) could counteract age-dependent alterations in lipid metabolism, including triglyceride accumulation in muscle, to the same extent as dietary restriction, thereby contributing to a better preservation of overall insulin sensitivity.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats were kept at the Institute of Physiology at the University of Veterinary Medicine, Vienna, under continuous veterinary control and subjected to a 12-h light and dark cycle, with temperature being at 22 ± 1 °C and humidity 40–-50%. All the experiments were conducted according to the guidelines of the Austrian federal law and with the approval of the Austrian committee for animal experiments. Exercise training started at the age of 5 months until 23 months, the age at which the animals were sacrificed and used for the present study. Two established types of exercise training—i.e. voluntary running in wheels (RW) and forced training in a treadmill (TM)—were investigated. A group of sedentary ad libitum fed rats was used as control (SC). As it has been shown that a slight limitation of food intake is important to drive the animals to run (Holloszy and Schechtman, 1991), RW group received daily 92% of the amount of food consumed by sedentary controls (a standard procedure for this type of training). Because of the reduced body weight gain of RW rats with respect to the sedentary group, a cohort of untrained animals, fed to pair weight with RW (PW), was studied in parallel, to compare the effect of exercise with that of food restriction. In PW rats, the amount of food was adjusted individually on a weekly basis; from the age of 5 months on, this group received in the average 65–70% of the food consumed by the SC group. TM group had free access to food, as usually described in literature (Skalicky et al., 1996). The animals were fed Altromin rat pellets (1324 Forti Haltungsfutter) and had continuous availability of tap water in drinking bottles, changed twice a week. From the age of 5 months on, all the rats were housed one per cage (inside length × width × height $25 \times 19.5 \times 14$ cm).

2.2. Voluntary running in running wheels and training in treadmill

These two types of exercises have been described in detail previously (Narath et al., 2001). Briefly, the rats in the RW group had running wheels attached to their cages with electronic equipment, coupled to a PC, which recorded the running characteristics of the individual animals continuously. When young, i.e. in the first months after starting of exercise at the age of 5 months, these animals run about 17,500 m/week in the average and when approaching the age of 23 months they run about 7000 m/week in the average. The TM animals were trained in a treadmill for twice 20 min per day at a speed of 20 m/min, for 5 days a week, which amounts to 4000 m/week. TM rats were trained until the day before sacrifice; for RW rats, running wheels were not blocked until sacrifice since this would generate within hours a frustration in the animals (unpublished data).

2.3. Fat mass

Total body fat mass was measured by bioelectrical impedance analysis as described previously (Narath et al., 2001).

2.4. Plasma measurements

Blood was collected by heart puncture from overnight-fasted anaesthetized animals, at the same time of the day for all groups, in tubes coated with EDTA. Plasma was separated by centrifugation, divided into aliquots and stored at −20 °C. Plasma glucose and FFA levels were determined using commercially available kits (Sclavo Diagnostics, Siena, Italy, for glucose, linearity up to 600 mg/dl, detection limit 2 mg/dl; Wako Chemicals Gmbh, Neuss, Germany, for FFA, linearity up to 2.0 mmol/l, detection limit 0.05 mmol/l). Insulin was measured by radioimmunoassay (RIA) according to Herbert et al. (1965) using rat insulin as a standard. The sensitivity and coefficients of variation of the insulin RIA were as follows: lower detection limit 0.15 ng/ml, intra-assay variation 3%, inter-assay variation 10%. Plasma leptin was measured by RIA, using a LINCO RIA kit, specific for rat leptin (LINCO Research, INC., St Charles, MO).

2.5. Muscle triglyceride (TG) content

Muscle triglyceride content was determined by extracting total lipids from frozen tibialis anterior muscle samples with chloroform:methanol (2:1 vol/vol) as described by Folch et al. (1957), separating the chloroform and methanol–water phases twice, discarding the upper phase and finally evaporating the lower phase under N_2. After this step, samples were stored at −20 °C until assayed. For the assay, samples were resuspended in 100 µl chloroform; 30 µl were quickly transferred to glass tubes in duplicate

and air dried. The dry pellet was resuspended with 12 µl of Thesit detergent (Sigma-Aldrich). The standard curve (1–50 µg) was prepared using triolein (Sigma-Aldrich) diluted in chloroform:methanol (2:1), air dried and resuspended with 12 µl Thesit. Samples and standards resuspended in Thesit were air dried and after addition of 50 µl of bidistilled water were vortexed and incubated at 37 °C in a shaking water bath for 10 min. One milliliter of GPO Trinder reagent (Sigma-Aldrich) was added to the tubes, which were then gently mixed and incubated at 37 °C for 5 min. The absorbance of duplicate samples was read at 540 nm in a spectrophotometer. The range of linearity of this method based on GPO Trinder reagent is up to 1000 mg/dl and the detection limit is 1.0 mg/dl.

2.6. Statistical analysis

Data are given as means ± SEM. Stat-View software (SAS publishing) was used to evaluate statistical significance by factorial analysis of variance (ANOVA), followed by the Bonferroni/Dunn test as a method of post hoc analysis to assess two-by-two differences. A p value <0.05, at least, was considered as significant. Statistical analysis included determination of regression lines and corresponding correlation coefficients.

3. Results

3.1. Body weight and total fat mass

Fig. 1 shows the body weight (A) and the total fat mass (B) of the various 23-month-old rat groups used for the study: SC (sedentary controls), RW (voluntary running in wheels), PW (sedentary fed to pair weight with RW), and TM (forced training in treadmill). At 23 months of age, RW and PW rats had significant 26% reduction in body weight and 60% reduction in fat mass with respect to SC, whereas in the TM group reductions in body weight and fat mass did not attain statistical significance. It should be outlined that body weight and fat mass measurements were performed longitudinally in these animals since the age of 5 months and the time course of their changes has been reported previously (Narath et al., 2001).

3.2. Plasma glucose, insulin, free fatty acids and leptin

In Fig. 2, plasma glucose (panel A) and insulin (panel B) levels of the above mentioned rats are shown. Glycaemic levels, which were in general higher than expected, likely as a consequence of anaesthesia (Bailey and Flatt, 1980), were not significantly different among the various groups. Differences in insulinaemic levels were not statistically significant between groups, although there was a clear trend to reduction in RW and TM groups with respect to SC.

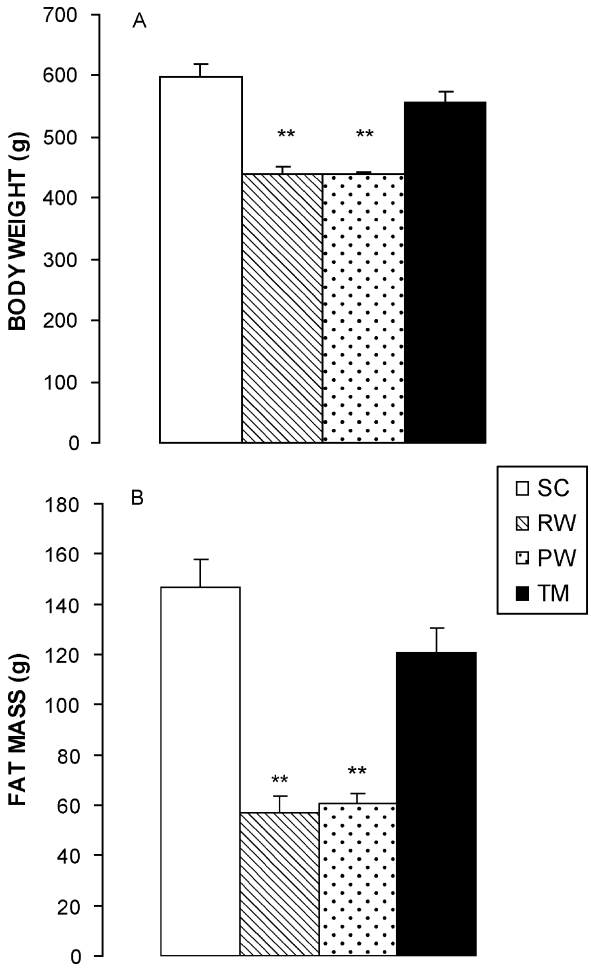

Fig. 1. Body weight (A) and fat mass (B) in 23-month-old Sprague–Dawley rats subjected to two types of exercise training or food restriction. Age-matched sedentary rats served as controls. Mean ± SEM (SC, $n=11$; RW, $n=11$; PW, $n=11$; TM, $n=7$). Statistical analysis: one-way ANOVA showed that differences among groups were highly significant for both body weight ($F=25.72$, $p<0.01$) and fat mass ($F=31.40$, $p<0.01$). **$p<0.01$ vs. SC (Bonferroni/Dunn test). SC, sedentary controls; RW, voluntary running in wheels; PW, fed to pair weight with RW; TM, forced training in treadmill. □, S rats; ▨, RW rats; ⁘, PW rats; ■, TM rats.

Plasma FFA values (Fig. 3) were significantly reduced not only in RW, but also in TM rats as compared to sedentary controls, while in PW animals the reduction did not achieve statistical significance.

Plasma leptin concentrations were also determined in SC, RW and PW groups (Fig. 4). Interestingly, the elevated leptin values (7.0 ± 0.7 ng/ml) observed in ageing sedentary fed ad libitum rats were reduced not only in the food-restricted group (2.9 ± 1.2 ng/ml, $p<0.05$) but also, and more markedly (1.0 ± 0.2 ng/ml, $p<0.01$), in the rats trained by voluntary exercise. For comparison, circulating leptin levels of 2–3-month-old untrained Sprague–Dawley rats, measured in the same assay (1.9 ± 0.5 ng/ml), were significantly lower than those of sedentary controls (not shown).

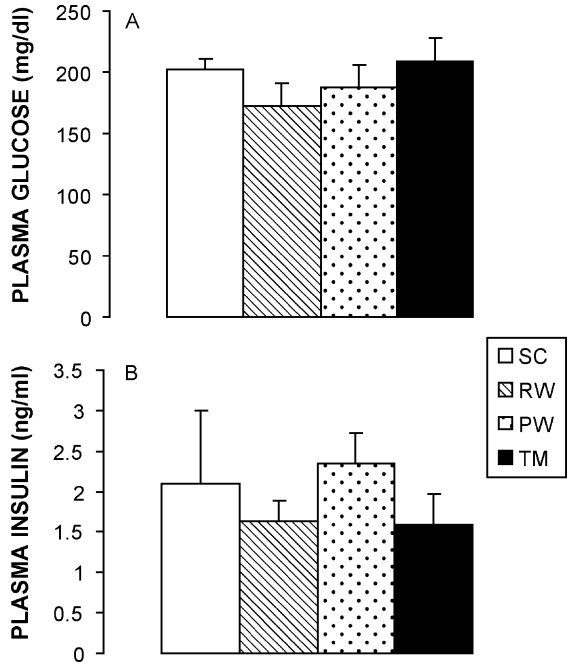

Fig. 2. Plasma glucose (A) and insulin (B) levels in trained, food-restricted and sedentary 23-month-old rats. Mean ± SEM (SC, $n=11$; RW, $n=10$; PW, $n=11$; TM, $n=7$). Statistical analysis: one-way ANOVA showed that differences among groups were not significant for both plasma glucose ($F=1.43$, NS) and plasma insulin ($F=1.25$, NS). Abbreviations like in the legend of Fig. 1. □, S rats; ▨, RW rats; ⁘, PW rats; ■, TM rats.

3.3. Muscle triglycerides content

TG content was evaluated in the tibialis anterior muscle (Fig. 5) and resulted to be significantly reduced by more than 50% in both RW and PW rats with respect to sedentary controls (in SC rats, muscle TG were 8.3 ± 1.4 mg/g tissue).

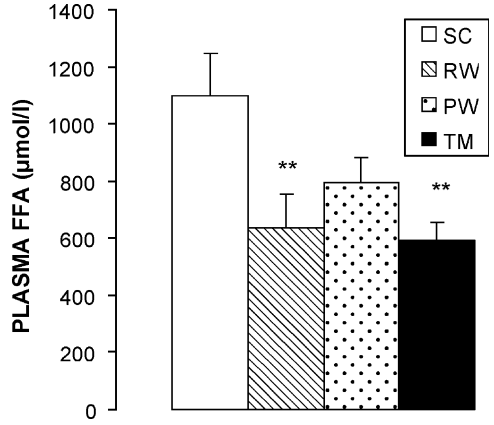

Fig. 3. Plasma free fatty acids (FFA) levels in trained, food-restricted and sedentary 23-month-old rats. Mean ± SEM (SC, $n=7$; RW, $n=8$; PW, $n=7$; TM, $n=7$). Statistical analysis: one-way ANOVA showed that differences among groups were significant ($F=4.48$, $p<0.02$). $^{**}p<0.01$ vs. SC (Bonferroni/Dunn test). Abbreviations like in the legend of Fig. 1. □, S rats; ▨, RW rats; ⁘, PW rats; ■, TM rats.

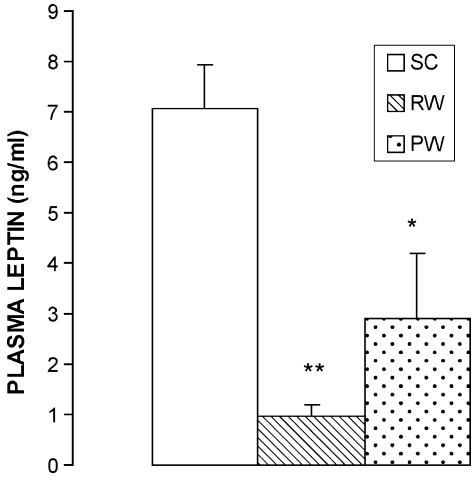

Fig. 4. Plasma leptin concentrations in voluntary running in wheels (RW), food-restricted (PW) or sedentary (SC) 23-month-old Sprague ± Dawley rats. Mean ± SEM ($n=5$–6 rats per group). $^{*}p<0.05$, $^{**}p<0.01$ vs. SC (Student's t test). □, S rats; ▨, RW rats; ⁘, PW rats.

A significant reduction in TG content (by approximately 45%) was also observed in TM rats. In young rats (5-month-old), TG content of tibialis anterior (2.2 ± 0.3 mg/g tissue) was significantly lower than in all the ageing groups (not shown).

3.4. Correlation between fat mass and plasma FFA levels or muscle TG content

As shown in Fig. 6, circulating FFA (panel A) as well as muscle TG content (panel B) of all experimental groups showed significant positive correlations with fat mass ($p<0.02$, at least).

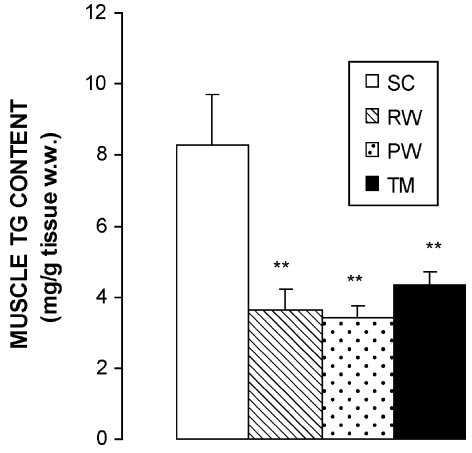

Fig. 5. Muscle triglyceride (TG) content in trained, food-restricted and sedentary 23-month-old rats. TG were assayed in the tibialis anterior muscle. Mean ± SEM (SC, $n=9$; RW, $n=9$; PW, $n=8$; TM, $n=6$). Statistical analysis: one-way ANOVA showed that differences among groups were highly significant ($F=6.123$, $p<0.01$). $^{**}p<0.01$ vs. SC (Bonferroni/Dunn test). Abbreviations like in the legend of Fig. 1. □, S rats; ▨, RW rats; ⁘, PW rats; ■, TM rats.

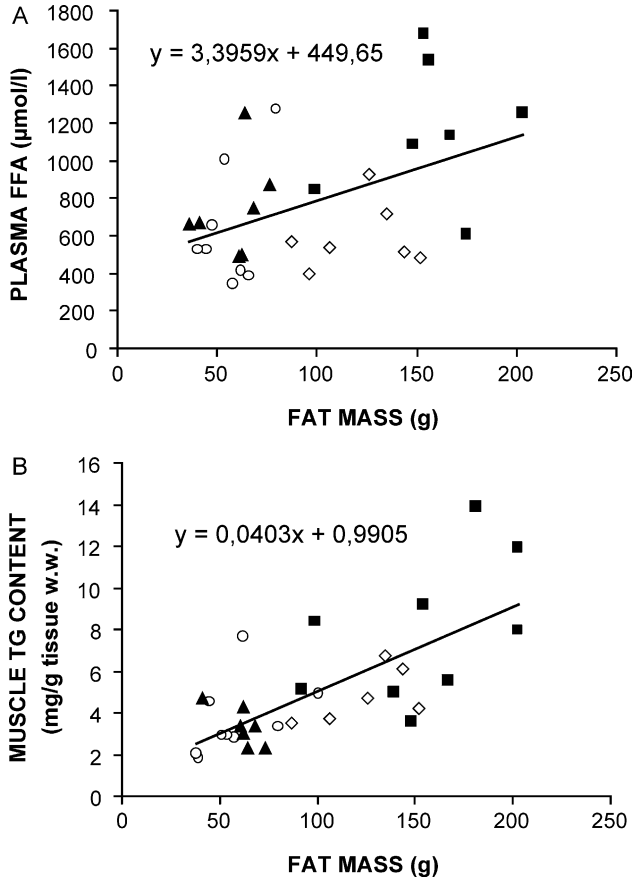

Fig. 6. Correlations between plasma FFA levels (A) or muscle TG content (B) and fat mass in 23-month-old rats of all experimental groups. A. linear regression, $r=0.455$, $n=29$, $p<0.02$; B. linear regression, $r=0.710$, $n=32$, $p<0.01$.

4. Discussion

The primary finding of this study was the evidence that two different types of life-long physical training, i.e. voluntary and forced training, markedly counteracted the age-dependent increase in muscle TG levels of sedentary rats, voluntary running appearing slightly more effective than forced exercise. In untrained food-restricted rats, muscle TG content was as low as in voluntary running animals. It should be remembered that skeletal muscles are responsible for the majority of whole body insulin-mediated glucose disposal and that TG accumulation in this tissue has been convincingly associated with global insulin resistance both in rodents (Storlien et al., 1991; Dobbins et al., 2001; Koyama et al., 1997) and in humans (Pan et al., 1997; Boden et al., 2001). Actually, clinical studies pointed out that insulin resistance correlates with muscle lipid content better than with BMI or percent body fat (Pan et al., 1997; Krssak et al., 1999) and that improvement of insulin action in weight loss is concomitantly associated to reduction in muscle TG content (Greco et al., 2002; Gray et al., 2002). The mechanism by which an excess of TG in skeletal muscle may play a role in inducing or aggravating insulin resistance is still uncertain, but it is likely that activation of muscle endogenous lipolysis is involved (Langfort et al., 2000), resulting in increased diacylglycerol and/or LCFA-CoA concentrations which can alter insulin signalling pathways (Griffin et al., 1999; Schmitz-Peiffer, 2000). It should also be taken into account that muscle TG accumulation is probably a consequence of a chronically increased availability of circulating FFA derived from abundant fat stores. Indeed, both plasma FFA levels and muscle TG content were positively correlated with fat mass in our experimental rat groups (Fig. 6). In trained animals, subjected to either voluntary or forced exercise, lipacidemia was significantly lower than in sedentary groups, while in food-restricted rats it remained at intermediate levels. Suppression of circulating FFA in trained animals may be related to an improved antilipolytic insulin action, as it has been reported previously after an 8-week period of exercise training (Lavoie et al., 1993). This is also supported by the observation that in trained animals plasma insulin levels were the lowest, although variations between groups were rather small.

While there is consensus about the benefits of physical activity on insulin sensitivity (Kraegen et al., 2001; Kitakoshi et al., 2001; Christ et al., 2002; Ross et al., 2000), the effects of exercise on muscular TG content are still controversial, since either an increase (Straczkowski et al., 2001) or a decrease (Sakamoto et al., 2000) or no change (Oakes et al., 1997) of intramuscular TG with training were reported. These discrepancies are most likely related to the occurrence of different variables in different studies, e.g. type and/or duration of exercise, muscle type, age and/or gender of the animals or humans, interval between the end of exercise and sampling. We should also underline that high muscle triglyceride levels were detected in endurance-trained athletes who were markedly insulin sensitive (Hoppeler et al., 1999). However, this observation is not necessarily in contrast with the stated association between muscle TG availability and insulin resistance, since the capacity for efficient lipid utilization might change according to the degree of fitness and the intensity of exercise (Goodpaster et al., 2001; Horowitz and Klein, 2000; Jeukendrup, 2002). Although differences in lipid metabolism in resting skeletal muscles of different fiber types have been reported (Dyck et al., 1997), a prolonged exercise usually leads, at least in humans, to enhanced lipid utilization in both muscles predominantly composed of slow-twitch oxidative or type 1 fibers and muscles predominantly composed of fast-twitch glycolytic or type 2B fibers (Krssak et al., 2000; Schmitt et al., 2003). The rat tibialis anterior muscle that we analysed is mainly composed of type 2B fibers (Polla et al., 2001), as assessed on the basis of the criterion of myosin heavy chain isoforms (Schiaffino and Reggiani, 1996), although a significant percentage of intermediate fibers (fast oxidative-glycolytic or type 2A fibers) is present in its deep part (Polla et al., 2001).

Thus, our results indicate that after a life-long physical training, and in particular after voluntary exercise, a 50% reduction of muscle TG content occurs, as compared to age-matched sedentary animals. In food-restricted rats, a similar reduction in muscular TG is also observed, compatible with the known protective effect of dietary intervention against the age-related development of insulin resistance in rodents (Davidson et al., 2002; Barzilai et al., 1998) and in primates as well (Bodkin et al., 1995). According to recent studies, even moderate levels of dietary restriction may favourably affect animals' ageing rate and survival (Duffy et al., 2001). Thus, we cannot rule out that the small food restriction (less than 10%) applied to RW rats to drive them to run, might have contributed to the metabolic effects observed in this group. On the other hand, it should be stressed that in the muscles of TM rats, a substantial reduction in TG content also occurred, despite that these rats were not food-restricted, and their fat mass was much less reduced than that of RW or PW groups. This observation suggests that both types of physical activity may favour optimal utilization of nutrient fuels by muscle independently on food restriction and effects on fat mass.

In order to provide a clue to the relevance of the above metabolic data with the ageing process, we should mention that the increase in thermal stability of tail tendon collagen in 23-month-old sedentary rats, taken as a reliable biomarker of aging, was retarded in RW and PW rats to the same extent and in TM rats to a less pronounced extent (Viidik and Skalicky, 2003). Looking at the survival at 23 months, RW group was the best (100% survival versus 84% of PW or S group and 69% of TM group); in this latter group, half of the deaths were caused by hypophysis adenomas or other adenomas before the age of 23 months (Narath et al., 2001).

Another major outcome of our work is the striking effect exerted by voluntary exercise on plasma leptin levels in ageing rats, which were reduced dramatically with respect to age-matched sedentary controls. Dietary restriction in PW group was also able to reduce circulating leptin, but to a lesser extent. As leptinaemia is highly correlated with body fat mass (Maffei et al., 1995; Reseland et al., 2001; Ishii et al., 2001), such a reduction could be related to the reduced fat mass observed in both RW and PW groups. Nevertheless, other factors should also play a role, since in the case we calculate leptin levels standardized per percentage of body fat, then the effect of food-restriction in PW group is not significantly different from sedentary group (which is compatible with the concept that reduction of circulating leptin is dependent on reduction in fat mass), whereas the effect of voluntary exercise remains strongly significant with respect to both sedentary and food-restricted groups (not shown). That exercise might exert leptin-lowering effects beyond those expected due to changes in fat mass has been also reported in middle age patients with type 2 diabetes (Ishii et al., 2001) or metabolic syndrome (Mooradian and Chehade, 2000) subjected to long-term exercise training.

It is well recognized that aging rats show elevated plasma leptin concentrations without concomitant significant changes in food intake, fat mass or adipose tissue distribution, so that they are considered leptin resistant (Ma et al., 2002; Fernandez-Galaz et al., 2002). Whereas food restriction has been reported to prevent the development of the age-related resistance to leptin (Ma et al., 2002; Kohrt et al., 1996), at our knowledge the issue of the effects of chronic exercise on circulating leptin concentrations in ageing animals or humans was not addressed so far, with the exception of a report on a 25% reduction of leptinaemia in older women after 1 year training (Hickey and Calsbeek, 2001). From our data, we cannot provide much insight into the mechanisms, but we can just speculate that exercise, by determining changes in nutrients fluxes, hormone levels and energy expenditure, might well contribute to the regulation of plasma leptin levels and presumably leptin action, as pointed out by Hickey and Calsbeek (2001).

In conclusion, our results indicate that voluntary exercise effectively counteracts the accumulation of triglycerides in the muscles of ageing rats as well as other related changes such as hyperlipacidaemia and compensatory hyperleptinaemia, suggesting that this intervention can limit the development of insulin and leptin resistance during ageing. Both forced training and moderate food restriction appear to be also effective, perhaps to a slightly smaller extent than voluntary exercise, in preventing the effects of age-dependent alterations in nutrient distribution and/or utilization.

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