The Increase in Intramyocellular Lipid Content Is a Very Early Response to Training

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The present study investigated the influences of a 2-wk training program on intramyocellular lipid (IMCL) content, IMCL decrease during exercise, fat oxidation, and insulin sensitivity.

Nine untrained men (age, 23.3 ± 3.2 yr; body mass index, 22.6 ± 2.6 kg/m^2; maximal power output, 3.8 ± 0.6 W/kg body weight) trained for 2 wk. Before and after training, subjects cycled for 3 h while substrate oxidation was measured. IMCL content in the vastus lateralis muscle was determined before and after cycling by proton magnetic resonance spectroscopy. Before and after training, insulin sensitivity was assessed by an insulin tolerance test.

The training period resulted in a significant increase in IMCL content by 42 ± 14%. IMCL content decreased significantly during cycling. However, 2 wk of training were not sufficient to achieve increases in fat oxidation and/or use of IMCL during exercise. All markers used to test insulin sensitivity point toward improved insulin sensitivity, albeit not significant. We conclude that the increase in IMCL content is a very early response to training, preceding significant changes in insulin sensitivity. The results suggest that the presence of triglycerides alone does not necessarily have detrimental effects on insulin sensitivity. We confirm earlier reports that IMCL contributes to the energy used during prolonged submaximal exercise. (J Clin Endocrinol Metab 88: 1610–1616, 2003)

CHANGES IN LIFESTYLE over the last few decades have resulted in a dramatic increase in the prevalence of obesity and, consequently, type 2 diabetes worldwide (1). Type 2 diabetes is characterized by perturbations in glucose homeostasis due to decreased insulin sensitivity. Next to disturbances in glucose metabolism, fat metabolism in type 2 diabetes is deranged as well (2). An impairment in fat oxidation (3) and accumulation of im lipids (4) are found in type 2 diabetes. Moreover, strong positive correlations between insulin resistance and IMCL have been reported (5, 6). In this context, it is interesting to note that increased IMCL levels are reported in insulin-resistant offspring of type 2 diabetic subjects when compared with matched, insulin-sensitive offspring (7), suggesting that a high IMCL content might be involved in the development of insulin resistance.

Strikingly, endurance-trained athletes also show increased IMCL levels (8) while being very insulin sensitive. Training-induced improvements in insulin sensitivity have been observed within 1 wk after the onset of training in healthy sedentary individuals (9–12), whereas little is known about the time span needed to increase im lipid content with training. Therefore, the first aim of this study was to examine whether insulin sensitivity and IMCL content [determined by ^{1}H-magnetic resonance spectroscopy (^{1}H-MRS)] are changed in response to a short-term training program.

It is well known that training increases fat oxidation during exercise. We recently showed, using stable isotopes, that the increase in fat oxidation after a 3-month training program was completely accounted for by an increase in oxidation of im triglycerides and/or very low density lipoproteins (13). This suggests that training induces a shift of the source of lipids used for oxidation toward increased use of im triglycerides and/or very low density lipoproteins. However, with the stable isotope technique, it cannot be deduced whether the oxidation of specifically IMCL is increased. Nevertheless, other techniques indicate that trained subjects rely more on IMCL oxidation during exercise than untrained subjects (8, 14). Therefore, the second aim of the study was to examine with ^{1}H-MRS whether a short-term training program also increases the use of IMCL during exercise. ^{1}H-MRS facilitates quantification of IMCL content noninvasively and repeatedly in the same position in the muscle (15) and provides data from a relatively large tissue volume compared with conventional needle muscle biopsies.

Subjects and Methods

Subjects

Nine young, untrained, and healthy male subjects [age, 23.3 ± 3.2 yr; maximal power output (Wmax), 3.8 ± 0.6 W/kg] participated in this study. Subjects were recruited by advertisement and were excluded from participation if they had performed aerobic exercise competitively for more than 3 months or were still exercising on a regular basis. The

study was approved by the institutional Medical Ethics Committee. Subjects gave their written informed consent after the nature of the procedure was explained.

Experimental protocol

One week before the experimental trial, an incremental test to determine Wmax was performed; the test was repeated in the week after the experimental trial.

The setup of the trial was as follows (Fig. 1). Days 1–3 formed a standardization period, during which a standardized diet was provided and subjects refrained from exercise. In the afternoon of d 3, a 3-h cycling test was performed; subjects cycled at 40% of their predetermined Wmax while substrate oxidation was measured by indirect calorimetry, and blood samples were taken every hour. Before and about 15 min after the cycling test, IMCL content in the vastus lateralis muscle was quantified by ^{1}H-MRS. Subjects then participated in a daily training program with alternating days of either 2 h of endurance training or 45 min of interval training. Resting days were incorporated on d 4 and 10. Days 15–17 were used as a standardization period, with the same standardization diet as d 1–3 and with subjects refraining from exercise. In the afternoon of d 17, subjects again cycled for 3 h at 40% Wmax, with substrate oxidation being measured and blood samples being taken; again, IMCL content in the vastus lateralis muscle was quantified by ^{1}H-MRS before and after exercise. Insulin sensitivity was assessed by an insulin tolerance test (ITT) in the mornings of d 2 and 16, after an overnight fast. To ensure that training-induced alterations in insulin sensitivity were examined rather than the remnant effect of the final training session, the ITT was planned in the morning of d 14, so that there were 48 h between the last training session and the ITT. The ITT began with sampling of 10 ml of blood to obtain fasting values of blood parameters.

Diets

The macronutrient composition of the diets was such that 30% of the energy was consumed as fat, 55% as carbohydrate, and 15% as protein.

Subjects were given a fixed amount of food (1.65 × basal metabolic rate, based on Harris and Benedict equations; Ref. 16) and were asked to eat all of the food provided and nothing else. The diet consisted of breakfast, lunch, ready to use dinner, and snacks.

Procedures

Body composition. Whole body density was determined by underwater weighing in the morning in the fasted state. Body weight was measured with a digital balance with an accuracy of 0.01 kg (August Sauter GmbH, Albstadt-Ebingen, Germany, type E1200). Lung volume was measured simultaneously with the helium dilution technique using a spirometer (Volugraph 2000, Mijnhardt, Mannheim, Germany). Percentage body fat was calculated using the equations of Siri (17).

ITT. ITTs were performed as previously described (18) in the fasted state in the morning of d 2 and 16. Briefly, the fall in plasma glucose in response to the injection of 0.1 U/kg body weight human insulin (Actrapid, Novo Nordisk A/S, Bagsvaerd, Denmark) was monitored every

2 min during 16 min. Insulin was injected via a catheter in the antecubital vein, while arterialized blood samples were obtained via a catheter inserted into a dorsal vein of the contra lateral hand, which was warmed in a heated air box at 60 C. Because no changes were noted within 4 min after administration of insulin, the first 4 min were not considered in the analysis. Linear regression was used to fit a line through the plasma glucose values between 4 and 16 min. The time until half of the glucose had disappeared ($t_{1/2}$) was calculated and taken as an indicator of insulin resistance. Therefore, insulin-sensitive subjects will have a low $t_{1/2}$, whereas insulin-resistant subjects will have a high $t_{1/2}$.

Three-hour cycling test. The exercise test consisted of 3 h of cycling on an electromagnetically braked ergometer at an intensity of 40% of the predetermined Wmax. During the exercise test, substrate oxidation was measured every 15 min using indirect calorimetry (Oxycon-β, Mijnhardt), and blood samples were collected every 60 min. To facilitate the completion of the exercise test, subjects received two 125-ml servings of a maltodextrine drink (Extran Fresh Citrus: 131 kJ, 31 kcal, 7.9 g carbohydrates, 0 g proteins and lipids per 100 ml) during the second half of the test (at t = 90 min and 135 min.). Subjects were allowed to drink water *ad libitum* during the whole test. Fat and carbohydrate oxidation were calculated on the basis of the respiratory quotient values according to Peronnet and Massicotte (19).

Training program. From d 5–14, subjects reported to the laboratory daily to follow supervised training sessions on cycle ergometers, except for the resting day on d 10. Training consisted of alternating days of interval and endurance training and always started with 7.5 min of warming up at 40% Wmax and ended with 7.5 min of cooling down at 40% Wmax. On d 5, 7, 9, 11 and 13, cycling interval training was performed, which consisted of 45 min of alternating 3-min intervals at 70% Wmax and 35% Wmax. At d 6, 8, 12, and 14, exercise consisted of 35 min at 55% Wmax, followed by 35 min at 50% Wmax, and 35 min at 45% Wmax, for a total duration of 120 min.

MRS measurement. Image-guided localized single voxel ^{1}H-MRS was performed in the vastus lateralis muscle before and immediately after the 3-h cycling test on the test days at the beginning and at the end of the training period. The measurements were performed on a 1.5 T whole body scanner (Intera, Philips Medical Systems, Best, The Netherlands) with a flexible surface coil wrapped around the upper leg. To ensure that the fibers of the vastus lateralis muscle ran essentially parallel to the external magnetic field, the leg was placed at an angle of about 30° from the parallel position.

In every subject, voxels were carefully placed at the same position for all four measurements. Care was taken to avoid vascular structures and adipose tissue deposits within the voxel. To reproduce the same voxel position, the longitudinal distance of the voxel from the intercondylar eminence of the knee joint was determined in a coronal image of the upper leg. T2 weighted fast spin-echo MR images consisting of 5 transversal slices were acquired at this position (slice thickness, 5 mm; repetition time/echo time, 2000/85msec; echo train length, 12; field-of-view, 210 mm; and matrix size, 256 × 256). The patterns of the fat distribution were used to verify the longitudinal position and as landmarks to reproduce the voxel position in the transversal plane.
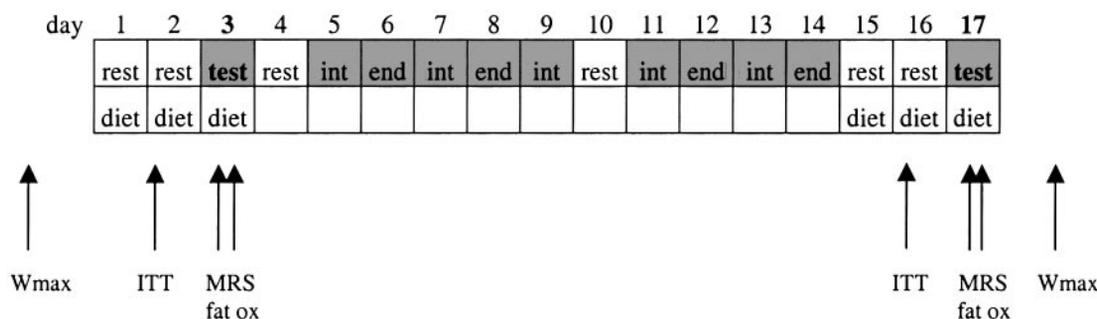

day	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
	rest	rest	**test**	rest	int	end	int	end	int	rest	int	end	int	end	rest	rest	**test**
	diet	diet	diet												diet	diet	diet

FIG. 1. Scheme of experimental setup. *White blocks* indicate days without physical exercise, and *gray blocks* indicate days with physical exercise. The physical exercise consisted of a 3-h cycling test (*test*) of endurance training (*end*) or interval training (*int*). The first 3 d before a test day consisted of a standardization period with standardized diet. MRS, Quantification of IMCL content by ^{1}H-MRS; fat ox, measurement of fat oxidation during the cycling test by indirect calorimetry.

^{1}H-MRS spectra from the regions of interest were acquired using a point-resolved spectroscopy sequence (PRESS) with the following acquisition parameters: repetition time/echo time, 3000/25 msec, 16 phase cycles, 128 averages, 1024 data points over 1000 Hz spectral width. The voxel volume was $12 \times 11 \times 18 \text{ mm}^3$. The water signal was suppressed using chemically selective saturation (CHESS). The unsuppressed water signal was subsequently measured in the same voxel under the same shimming conditions and was used as a reference signal.

Post processing. The spectra were fitted in the time domain using a nonlinear least-squares algorithm (AMARES; Ref. 20) in the Java-based magnetic resonance user interface (jMRUI) software package (21). Eight peaks were fitted in total, namely two peaks for total creatine and trimethylammonium compounds (TMA), three peaks for extramyocellular lipids (EMCL), and three peaks for IMCL (Fig. 2). Prior knowledge (20) of the relative peak positions (22) and area ratios (personal communication) as determined by Boesch *et al.* were used as a constraint. The line width of the CH_2 peak of IMCL was fixed relative to the line width of the unsuppressed water peak (measured in the same voxel) to increase the accuracy and reliability of the fitting procedure. The line widths of total creatine and TMA compounds were constrained to be equal. All other line widths were unconstrained. IMCL peaks and EMCL peaks were fitted with a Gaussian curve, whereas total creatine, TMA, and water peaks were fitted with a Lorenztian curve. The signals were corrected for T1 and T2 relaxation using the T1 and T2 relaxation times as determined by Schick *et al.* (23). The corrected area of the CH_2 peak of IMCL was expressed relative to the area of the water peak.

The reproducibility of the quantification of IMCL in the vastus lateralis muscle was determined in the setting of another study by measuring one subject several times, with the subject leaving the scanner table between measurements. From five corresponding pairs of measurements, we calculated an average coefficient of variation of 6.0% ± 8.2% (mean ± SD; our unpublished results). If total creatine was used as a reference instead of water, the coefficient of variation was larger, namely 11.5% ± 11.9% (mean ± SD).

Analysis

Blood analyses. For determination of free fatty acids (FFA), glycerol, and glucose, blood was collected in tubes containing 30 μl of 0.2 M EDTA. Plasma was immediately centrifuged at high speed, frozen in liquid nitrogen, and stored at −80 C for later analyses. Insulin concentrations were measured using RIA (Linco Research, Inc., St. Charles, MO). FFA were determined using the Wako Nefa C testkit (Wako Pure Chemical Industries Ltd., Neuss, Germany).

Plasma glucose was determined using the hexokinase method (LaRoche, Basel, Switzerland), and glycerol was determined using the GPO-trinder (Sigma, St. Louis, MO).
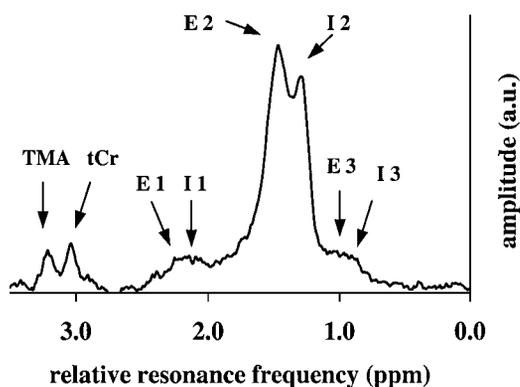
FIG. 2. Typical spectrum of the vastus lateralis muscle. TMA, Trimethyl ammonium compounds; tCr, total creatine; E1, broad peak of various, chemically different methylene protons of EMCL (22); E2, methylene protons of EMCL; E3, methyl protons of EMCL; I1, broad peak of various, chemically different methylene protons of IMCL (22); I2, methylene protons of IMCL; I3, methyl protons of IMCL.

Statistics. Characteristics of subjects and the coefficient of variation of the measurement are reported as mean ± SD, and results are reported as mean ± SEM. Statistical analyses were performed with SPSS for Windows 10.0.0 software (SPSS, Inc., Chicago, IL). Differences before and after training were detected with paired Student's *t* tests. The blood parameters during exercise were analyzed with a general linear model repeated measures analyses. If the repeated measures analyses detected significant differences, *post hoc t* tests were performed. *Asterisks* in the figures indicate significant differences according to the *t* tests. Results were considered significant if *P* values were less than 0.05.

Results

Maximal performance

Maximal performance did not change significantly due to the training program (272.8 ± 14.0 W *vs.* 281.7 ± 13.4 W before and after training, respectively).

Substrate oxidation

Fat oxidation gradually increased during the 3-h cycling test (Fig. 3A). During the first 90 min (before the carbohy-
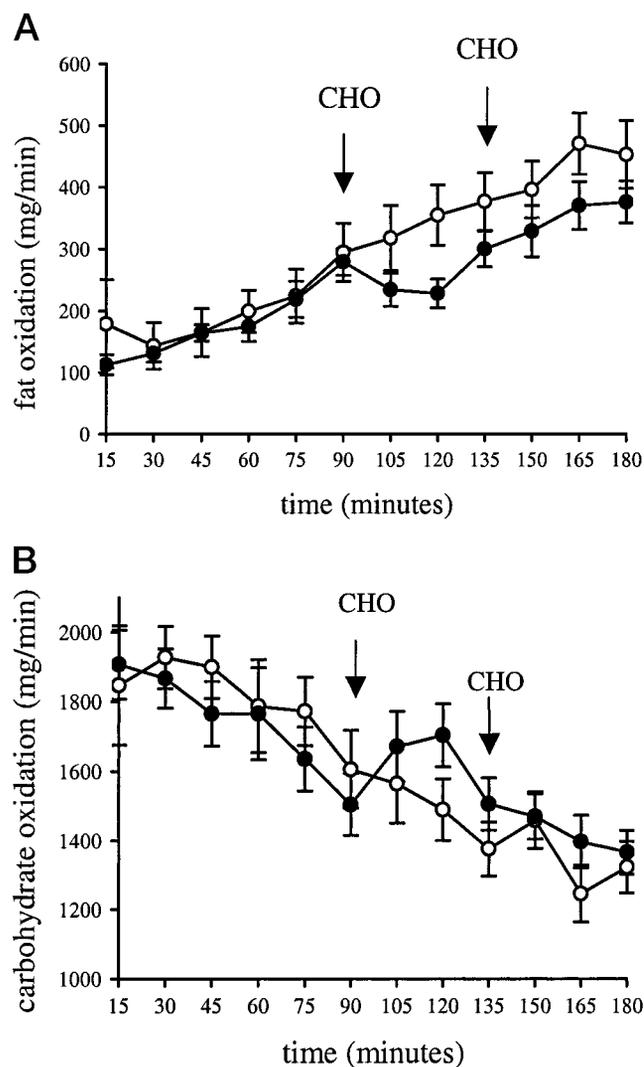
FIG. 3. Fat oxidation (A) and carbohydrate oxidation (B) were measured every 15 min by indirect calorimetry during the 3-h cycling test. *Open circles*, before training; *filled circles*, after training. *Arrows* indicate the gavage of a carbohydrate (CHO) drink.

drate drink), fat oxidation before and after training did not differ significantly. After training, a decrease in fat oxidation was observed at t = 90 min. This coincided with the administration of a carbohydrate drink. As a consequence, a significantly lower fat oxidation was observed after training compared with before training in the second 90 min of the test. The average fat oxidation over the entire 180 min of the test was not significantly different after training.

Reciprocal effects were observed for carbohydrate oxidation (Fig. 3B). During the first 90 min (before the carbohydrate drink), carbohydrate oxidation before and after training did not differ significantly. When the carbohydrate drink was administered, carbohydrate oxidation increased after training but not before training. As a consequence, a significantly higher carbohydrate oxidation was observed after training compared with before training in the second 90 min of the test. The average carbohydrate oxidation over the entire 180 min of the test was not significantly different after training.

Blood parameters

There were no significant differences before and after training in the fasting plasma values of insulin [7.46 ± 1.10 *vs.* 7.44 ± 1.12 μU/ml (mU/liter)], FFA (355.28 ± 48.34 μmol/liter *vs.* 349.11 ± 46.62 μmol/liter), and glycerol (67.99 ± 6.03 μmol/liter *vs.* 71.00 ± 6.05 μmol/liter). However, there was a tendency of decreased fasting plasma glucose after training (5.23 ± 0.08 mmol/liter *vs.* 5.07 ± 0.11 mmol/liter; $P = 0.062$).

During exercise, there was a significant effect of time for insulin, with values decreasing with time ($P < 0.001$), but no treatment (training) or treatment × time interaction effect (Fig. 4A). There was a significant effect of time ($P < 0.001$), treatment, and time × treatment interaction for FFA plasma values, with values increasing with time (Fig. 4B). After training, values were lower than before training in the second half of the cycling test. For glycerol plasma concentrations, there was a time effect ($P < 0.001$), a treatment effect, and a time × treatment interaction effect. Glycerol concentrations increased gradually during the test. In the second half of the exercise test, concentrations were lower after training compared with before training (Fig. 4C). There was a significant effect of time for glucose plasma values, with values decreasing with time, a tendency for a treatment effect with levels being higher after training ($P = 0.07$), and no treatment × time interaction effect (Fig. 4D).

ITT

After training, there was a tendency for the plasma glucose to drop faster in response to insulin. The $t_{1/2}$ of glucose disappearance tended to be shorter after training compared with before training (15.4 ± 1.4 min *vs.* 14.1 ± 1.1 min; $P = 0.12$).

IMCL content

Spectra from identical voxel positions at all four points in time were available from seven subjects. Before training, preexercise IMCL content was 0.35 ± 0.02% of the water reference signal. Training increased the preexercise IMCL
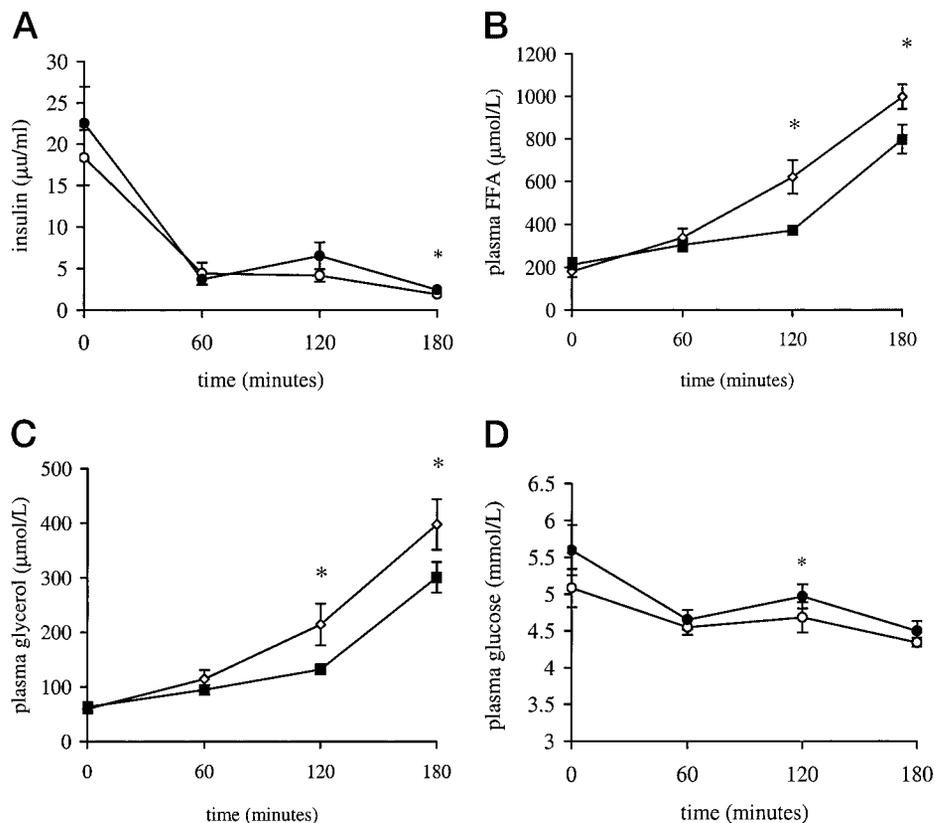

FIG. 4. Blood plasma parameters during the 3-h cycling test. *Open symbols*, before training; *filled symbols*, after training. *, Significant differences between pre- and posttraining values. A, Insulin plasma concentrations; B, FFA plasma concentrations; C, glycerol plasma concentrations; D, glucose plasma concentrations.
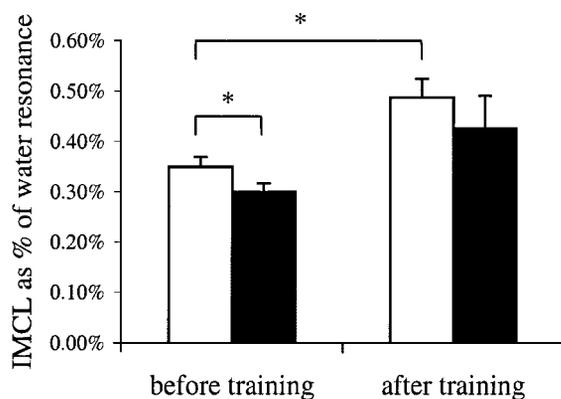
FIG. 5. IMCL content, expressed as percentage of the water resonance. *White bars*, before cycling test; *black bars*, after cycling test. *, Significant differences.

content significantly by 42 ± 14% (Fig. 5). Before training, the 3-h cycling test decreased the IMCL signal by 14 ± 3% ($P <$ 0.01). After training, the decrease in IMCL content during the 3-h cycling test was similar (14% ± 9%), but this decrease did not reach significance (Fig. 5). If total creatine was used as reference instead of water, similar results were obtained: a significant increase in IMCL with training, a significant depletion during cycling before training, and a (nonsignificant) depletion during cycling after training.

Insulin sensitivity did not correlate with IMCL content in this small group, either before or after training.

Discussion

The most important finding of the present study is that the IMCL content is increased significantly after a training period of as short as 2 wk. During this period, no significant changes in fat oxidation or insulin sensitivity were detected. From this, we conclude that the increase in IMCL content is among the earliest responses to training. Furthermore, the results of the present study suggest that the presence of triglycerides alone in myocytes does not have detrimental effects on insulin sensitivity. Also, we confirm earlier reports that IMCL contribute to the energy used during prolonged submaximal exercise.

It is generally accepted that training improves insulin sensitivity, and it has previously been shown that training can increase insulin sensitivity after a period of training as short as 7 d (9–12), although it cannot be excluded that some remnant effect of the last exercise bout was detected in these studies. In the present study, insulin sensitivity was not yet significantly increased. This lack of increase can be due to different indicators of insulin sensitivity used or to different training protocols used (18). We chose to use the ITT, which measures the hypoglycemic action of injected insulin, because it is a short test that has recently been shown to be sensitive enough to detect differences in the improvement in insulin sensitivity induced by different training programs (18). Although the increase in insulin sensitivity as quantified by the ITT was not significant ($P = 0.12$), we did find some indication of increased insulin action. There was a tendency for fasted glucose levels to be lower by 3% ($P = 0.062$), whereas insulin levels were unchanged. Furthermore, an oral

carbohydrate load, administered in the second half of the exercise test to facilitate the completion of the test, led to an increase in carbohydrate oxidation observed solely after training. In accordance with this, the carbohydrate load resulted in reduced lipolysis and fat oxidation after training in the second part of the test. These changes could indicate improved glucose absorption and disposal. It has been described that exogenous carbohydrate oxidation is higher in trained subjects (24) and that a training intervention of 12 wk can stimulate oxidation of exogenous glucose (25); this could indicate an increased insulin sensitivity. Furthermore, short-term training has been reported to increase GLUT-4 content (26) and hexokinase activity (27, 28), indicating improved glucose disposal capacity. Alternatively, changes in perfusion of splanchnic system occur with training (29) and may induce increased absorption of glucose and contribute to the increased oxidation after an oral glucose load. Future studies should address the mechanism of increased glucose oxidation after an oral glucose load in trained *vs.* untrained subjects in more detail.

The positive correlations of IMCL content with insulin resistance that were reported in different populations (5, 6) and the finding that the increase in IMCL content precedes the development of diabetes (7, 30) suggest that im lipids might be involved in the development of insulin resistance. However, the finding that IMCL content is also increased in highly trained, insulin-sensitive athletes (8, 31, 32) and that their IMCL contents are actually very similar to those reported in diabetic subjects is paradoxical and favors a more complex explanation (33). In the present study, we find an increase in IMCL, which is not coupled to a diminished insulin sensitivity; rather, we find indications of improved insulin sensitivity. Therefore, the results of the present study suggest that it is unlikely that the presence of triglycerides alone in myocytes is leading to a decrease in insulin sensitivity. Also, other authors have suggested that an interference of lipid degradation-derived intermediates such as long chain acyl-coenzyme A and diacylglycerol with insulin signaling (34–37) would be more likely than the triglycerides themselves to be responsible for disturbed insulin action.

We showed earlier that the increase in fat oxidation during exercise due to long-term training can be completely accounted for by increased oxidation of triglyceride-derived fatty acids (13). Therefore, and also based on earlier results from biochemical analyses of biopsies, it has been suggested that training might lead to an increased oxidation of im lipids during exercise (14). Others, however, found no changes in im lipid oxidation with training (38). In the current study, we confirm earlier results that showed that IMCL decrease during exercise (15, 39). However, the decrease in IMCL after 3 h of exercise was similar before and after training, suggesting that the use of IMCL was not altered with training. However, it is important to note that total fat oxidation over the 3 h was not changed after training. Furthermore, we cannot exclude that the improved use of carbohydrate after training, which reduced fat oxidation in the second part of the exercise test, could have interfered with a decrease of IMCL content after training.

There has been some concern about using the water signal as a reference after exercise because water and electrolyte

shifts are possible. When using total creatine instead of water as internal standard in the current study, we found no statistical difference between the results using the two methods. In both cases, we found a significant IMCL depletion before training, a nonsignificant depletion after training, and significant training-induced increase in baseline IMCL. We chose to use the water peak as internal reference, because the use of the small peak of total creatine introduced some additional variability so that the average coefficient of variation of repeated measurements increased from an average of 6% with water to 11.5% with total creatine. Because no trends in water signal or the water to total creatine signal ratio were found before *vs.* after exercise, we feel that it is justified to use the water signal as the internal standard in the present study.

It has been shown that training can lead to an increase in fat oxidation after 7 d of endurance training (40). The finding that fat oxidation during the first 90 min (before any carbohydrate was administered) did not increase with the current protocol could be due to the nature of the training protocol, which consisted of endurance as well as high intensity components. It is assumed that the current exercise protocol was too short to produce increases in fat oxidation in untrained subjects. In conclusion, we showed in the present study that in untrained subjects, IMCL content was increased with training before an adaptation in fat oxidation occurred and that the increase in IMCL content was not coupled to a decrease in insulin sensitivity. Rather, we find indications of improved insulin sensitivity. These results suggest that the presence of IMCL alone does not hamper insulin sensitivity. Instead, the data are in line with models that suggest a more indirect relationship between IMCL content and insulin resistance, *e.g.* models that suggest that accumulation of intermediates of fat oxidation might interfere with insulin signaling (34–37). More studies are needed to investigate these mechanisms in detail.

Acknowledgments

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