IL-6 DEFICIENCY INCREASES FATTY ACID TRANSPORTERS AND INTRAMUSCULAR LIPID CONTENT IN RED BUT NOT WHITE SKELETAL MUSCLE

IL-6 is a biologically active substance which appears to be involved in regulating skeletal muscle lipid oxidation. Ablation of IL-6 (IL-6−/−) may therefore be expected to increase intracellular lipid accumulation, possibly via a concurrent increase in fatty acid transporters such as FAT/CD36 and FABPpm. This however may only occur in oxidative muscles which utilize fatty acids at a greater rate than glycolytic muscles. In the present study we examined the fatty acid transporter protein expression as well as the lipid content and profiles of free fatty acids (FFA), diacylglycerols (DGs) and triacylglycerols (TGs) in skeletal muscles of IL-6 deficient mice at 4 and 12 months of age. FAT/CD36 and FABPpm protein content was increased in red muscles in IL-6−/− mice compared to WT mice at 4 (RG) and 12 months (soleus and RG). Along with this, FFA, DG and TG concentrations were also increased in these red IL-6−/− muscles. In addition, the IL-6−/− genotype increased the saturated FA acid composition of the intramuscular TG fraction. In contrast, in white gastrocnemius muscle the IL-6−/− genotype has no effect on the expression of fatty acid transporters as well as the lipid content and composition at either 4 mo or 12 months of age. IL-6 ablation increases fatty acid transporter expression and intramuscular lipid accumulation, particularly the saturated fatty acids. These effects however were confined to oxidative muscles, as glycolytic muscles were not affected.

Key words: IL-6, FABPpm, FAT/CD36, lipids
INTRODUCTION

Recently, a number of cytokines have been implicated in regulating skeletal muscle fatty acid (LCFA) oxidation in skeletal muscles (for review: 1, 2). Among these cytokines only IL-6 is produced by skeletal muscle (2). Skeletal muscle contraction results in a rapid increase in IL-6 secretion, which is not attributable to muscle cell damage nor to macrophage infiltration (3). In isolated oxidative muscle (soleus), as well as, in cultured myotubes (4) it appears that IL-6 increases the capacity for LCFA oxidation, possibly by altering the rate of LCFA uptake into skeletal muscle (5). Together these data may lead to the hypothesis that IL-6 reconciles insulin-sensitizing effects of physical activity and one would expect protection from the development of diabetes. However, recent studies, in humans, provided an opposite associations. Specifically, it has been shown that increased serum IL-6 is positively correlated with obesity and insulin resistance (6, 7). These population-based studies strongly suggest a strong relationship between elevated serum IL-6 and whole body lipid metabolism in age-related diseases such as obesity and diabetes (for review: 8). It is tempting to speculate this as serum IL-6 levels tend to increase with ageing (for review: 9). However, whether IL-6 is involved in the deregulation of lipid metabolism is unclear. Initial studies, in rodents, had observed that IL-6−/− mice develop maturity-onset obesity (10). Nonetheless, in other studies these mature IL-6−/− mice exhibited normal lipid and carbohydrate metabolism, while elevated serum glucose concentrations were observed only after a GTT (11).

Disordered lipid metabolism of skeletal muscles increases with age, the accumulation of intramuscular lipids is associated with the onset of insulin resistance, such as occurs in obesity and type 2 diabetes (12, 13). Increases in intramuscular lipid accumulation have been associated with a reduced capacity in LCFA oxidation (14, 15) and the subsequent accumulation of lipids (16). However, this is not always the case, as in obese humans palmitate oxidation is not necessarily altered while triacylglycerols accumulation does occur (15, 17). Recently, the enhanced intramuscular lipid accumulation has been linked to the increased rate of fatty acid transport into skeletal muscles, in obese animals (18, 19) and diabetic animals (20), and in obese humans and in individuals with type 2 diabetes (17). This upregulation of fatty acid transport, in animals and in humans, has been attributed to an increase in fatty acid transporters, primarily FAT/CD36. Thus, an increased intramuscular lipid accumulation is not necessarily linked to a reduced rate of fatty acid oxidation, but can also be provoked by an increased rate of fatty acid transport into muscle.

FAT/CD36 and FABPpm are key proteins involved in the regulation of LCFA transport into skeletal muscles (21, 22). For both FAT/CD36 and FABPpm many studies have shown that, there is a good correlation between skeletal muscle expression of these transporters and the relative rates of LCFA transport into myocytes (for review: 22). In ZDF rats it appears that fatty acid transport and
Transporters are upregulated only in red but not white muscle as these animals transition from insulin resistance to type 2 diabetes (20). This has also been observed in muscles of high-fat fed rats (23). In addition, ablation of FAT/CD36 impairs LCFA metabolism primarily in red (oxidative) muscles, not in white (glycolytic) muscles (24). This suggests, as have others (25, 26), that disturbances in muscle lipid metabolism in models of insulin resistance are primarily evident in more oxidative (red) types of skeletal muscle.

The available literature indicates that i) IL-6 contributes to the regulation of LCFA oxidation in skeletal muscle, ii) LCFA metabolism appear to be dysregulated in IL-6−/− mice, particularly as they age, and iii) dysregulated LCFA metabolism primarily affects red, but not white skeletal muscle, particularly at the level of fatty acid transporters. Therefore, we examined in red (soleus and RG) and white muscles (WG) of young (4 months) and older (12 month) IL-6 deficient mice and wild type (WT) mice, a) the changes in skeletal muscle fatty acid transporter proteins, b) the changes in intramuscular lipid content, and c) the changes in the FA species profile in lipid pools.

MATERIALS AND METHODS

Materials

FAT/CD36 and FABPpm were detected using the ab 36977 antibody (Abcam) and FABPpm antisera (27), respectively. All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO). Female mice (C57B4/6J IL6−/−tm1Kopf) were bred on site and maintained at 22°C on a reverse light-dark cycle in approved animal holding facilities. They had unrestricted access to food and water. This study was approved by the local ethics committee on animal care.

Methods

Genotyping in IL-6−/− vs. wild type mice

Genomic DNA for genotyping was isolated from mouse tails using "Genomic mini" kit (A&A Biotechnology, Gdansk, Poland) according to the manufacturer's instructions. PCR was performed using DNA polymerase Taq "Marathon" (A&A Biotechnology, Gdansk, Poland) and custom made primers (F 5′-AAGTGCATCATCGTTGTTCATAC3′; R 5′-CCATCCAGTTGCCTTCTTG-3′). Twenty nine PCR cycles were performed under following conditions: 94°C 5 seconds, 94°C 20 seconds, 95°C 30 seconds, 72°C 2 min 50 seconds, 72°C 7 seconds, 10°C at the end of the procedure. Then DNA was separated by electrophoresis on the 1% agarose gel with ethidium bromide. Under these conditions material from wild-type animals yielded DNA fragments with size ca.900 bp, whereas DNA fragments from knock-out animals contained also a fragment of neomycin cassette and were size about 1400 bp.

The mice were killed by cervical dislocation. Blood was collected from mice at the time of death and serum levels of glucose (Sigma, St. Louis, MO) as well as fatty acid concentrations were determined using spectrophotometric procedures (Wako Chemicals, Richmond, VA). Samples of selected skeletal muscles (soleus, red and white sections of gastrocnemius) were taken. They were rapidly cleaned of any visible non-muscle tissue, frozen in liquid nitrogen and finely powdered. The
powder was transferred to a glass tube and lipids were extracted using the Folch et al. method (28) as modified according to van der Vusse et al. (29). Individual fatty acid methyl esters were identified and quantified according to the retention times of standards by gas liquid chromatography (Hewlett-Packard 5890 Series II gas chromatograph, HP-INNOWax capillary column) and these were subsequently summed to obtain total FFA, TG, DG and PL concentrations as described recently (30). We have also calculated the percentage of saturated fatty acids (SFA) indices in each lipid fraction examined in muscle samples.

**Western blotting**

The total protein expression of FAT/CD36 and FABPpm was determined in crude membranes of soleus, and the red and white section of the gastrocnemius muscle. Routine Western blotting procedures were used to detect proteins as described previously (18, 20). Briefly, proteins were separated using 10% SDS-polyacrylamide gel electrophoresis. Membranes were immunoblotted with primary antibodies: ab 36977 (FAT/CD36) and FABPpm antiserum. Protein content was determined with bicinchoninic acid method with BSA serving as a protein standard. Equal protein concentrations were loaded in each lane as also confirmed by Ponceau staining the blot membrane. Signals obtained by Western blotting were quantified by densitometry (Biorad, Poland). The protein expression (Optical Density Arbitrary Units) in control was set to 100 and the experimental groups were expressed relative to this control.

**Statistics**

All data are expressed as mean ± SEM. Statistical differences between groups were tested with analyses of variance to determine the statistical significance, and if variances were heterogeneous among groups (Levene’s test), Dunnett’s T3 test was used instead. Statistical significance was set at $P \leq 0.05$.

**RESULTS**

Genotyping confirmed deficiency of the IL-6 gene in the IL-6-/− mice (data not shown). IL-6-/− and wild type (WT) mice showed no differences in either body weight or food intake. Non-fasting glucose and serum free fatty acids concentrations were similar in IL-6-/− and WT mice at both 4 and 12 months of age (Table 1). Others have reported no difference in serum triglyceride concentration between IL-6-/− and WT mice (10, 11).

**Effects of age and IL-6-/− genotype on skeletal muscle fatty acid transporters expression (FAT/CD36, FABPpm)**

No difference was observed in either FAT/CD36 or FABPpm protein expressions in the soleus, and RG and WG muscles of WT mice at 4 and 12 months of age (Fig. 1). This lack of change over the course of 4-12 months in these transporters corresponds to observations in red rat muscle (31).

Red muscle: In IL-6 deficient mice, FAT/CD36 protein content was significantly increased in muscles of IL-6-/− mice compared to the WT mice at 4 months (+31%, $P < 0.05$, Fig. 1C), and at 12 mo (+40%, $P < 0.05$, Fig. 1D). A
Fig. 1. Representative Western blots showing the effects of IL-6 genotype on total expression of FAT/CD36 and FABPpm in soleus (A, B), RG-red gastrocnemius (C, D) and WG-white gastrocnemius (E, F) muscles at either 4 or 12 months of age. Crude membranes were prepared from muscles homogenates as described in Materials and Methods. Data are based on 10 independent determinations for each muscle.

* $P < 0.05$, IL-6$^{-/-}$ vs. WT in corresponding muscles
similar trend was observed in soleus muscle at 4 months (+10%) which became significant at 12 months (+18%, \( P < 0.05 \), Fig. 1B).

In soleus muscle from IL-6 deficient mice, FABPpm protein content was not increased either at 4 or 12 months of age (Fig. 1A, B). In IL-6\(^{-/-}\) RG muscles the changes in FABPpm were modestly increased, at 4 and 12 months (+13% and +12%, respectively, \( P < 0.05 \), Fig. 1C, D)

White muscle: In contrast to the oxidative soleus and RG muscles, no difference in FAT/CD36 or FABPpm protein content was observed in WG muscle at either 4 or 12 months of age in IL-6\(^{-/-}\) mice (Fig. 1E, F).

**Effects of age and IL-6\(^{-/-}\) genotype on the intramuscular lipid content (FFA, DG, TG, PL)**

Significant increase was observed in lipid fractions of DG and TG, in the soleus and RG but not in WG muscles of WT mice at 4 and 12 months of age (Fig. 2A, B, D). This intramuscular lipid accumulation over the course of 4-12 months in these fractions corresponds to the others observations (32).

Red muscle: At 4 months of age, the lipid fractions such as FFA, DG, TG were increased in the soleus muscle of IL-6\(^{-/-}\) mice compared to wild type mice (FFA: +21%, \( P < 0.05 \); DG: +16%, \( P < 0.05 \); TG: +51%, \( P < 0.05 \); Fig. 2A). Similarly, at 12 months of age IL-6 deficiency resulted in an increase in intramuscular lipid pools (FFA: +22%, \( P < 0.05 \); DG: +23%, \( P < 0.05 \); TG: +49%, \( P < 0.05 \); Fig. 2B). Phospholipids remained not altered at 4 and 12 months (Fig. 2A, B).

In IL-6\(^{-/-}\) mice the changes in intramuscular lipid fractions in RG muscles, just as in soleus muscle, were also increased at 4 months of age (FFA: +18%, \( P < 0.05 \); DG: +25%, \( P < 0.05 \); TG: +29%, \( P < 0.05 \); Fig. 2C), as well as at 12 months of age (FFA: +32%, \( P < 0.05 \); DG: 16%, \( P < 0.05 \), TG: +37%, \( P < 0.05 \), Fig. 2D) with no significant change in phospholipids (Fig. 2C, D).

**Table 1.** Body weight, non-fasting glucose (Glc) and serum non-esterified free fatty acids (FFA) concentrations.

<table>
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<tr>
<th></th>
<th>4 mo</th>
<th>12 mo</th>
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<tbody>
<tr>
<td></td>
<td>WT</td>
<td>IL6(^{-/-})</td>
</tr>
<tr>
<td>Glc (mmol/L)</td>
<td>6.7±0.34</td>
<td>7.2±0.78</td>
</tr>
<tr>
<td>FFA ((\mu)mol/L)</td>
<td>0.59±0.08</td>
<td>0.58±0.1</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>30.9±1.1</td>
<td>31.8±1.0</td>
</tr>
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Data represents mean ± SEM, \( n = 10 \) in each group.
Fig. 2. Effects of IL-6 genotype on lipid content in soleus (A, B), RG-red gastrocnemius (C, D) and WG-white gastrocnemius (E, F) muscles at either 4 or 12 months of age. Different lipid pools were extracted from muscle homogenates as described in Materials and Methods. Data are based on 10 independent determinations for each muscle (mean ± SEM).

FFA - free fatty acids,
DG - diacylglycerols,
PL - phospholipids,
TG - triacylglycerols,
*P < 0.05, IL-6−/− vs. WT in corresponding muscles
# P < 0.05, 4 mo vs. 12 moths in corresponding muscles of WT mice
White muscle: In contrast to the oxidative muscles, no significant changes in intramuscular lipid content were observed either at the 4 months of age or at the 12 months of age (Fig. 2E, F).

Fig. 3. Effects of IL-6 genotype on lipid composition in soleus (A, B), RG-red gastrocnemius (C, D) and WG-white gastrocnemius (E, F) muscles at either 4 or 12 months of age. Saturation status was recalculated as the % of SFA residues in each lipid fraction, as described in Materials and Methods. Data are based on 10 independent determinations for each muscle (mean ± SEM).

*P < 0.05, IL-6−/− vs. WT in corresponding muscles
Effects of age and IL-6\(^{-/-}\) genotype on the composition of skeletal muscle intracellular lipids

Fatty acid profiles were determined in all examined muscles in the FFA, DG, TG and PL fractions.

Red muscle: In the soleus and RG muscles from IL-6\(^{-/-}\) mice we observed no change in the percentage of SAT in FFA, DG and PL fractions compared to the respective wild type muscles (Fig. 3A-F), at either 4 or 12 months. However, in the TG pool there was a significant increase in the percentage of SAT species at 12 months of age in soleus (+28%, \(P < 0.05\), Fig. 3B) and RG muscles (+44%, \(P < 0.05\), Fig. 3D).

White muscle: In the WG muscles we did not observe any significant difference in the saturation profile in any of the lipid fractions examined in IL-6\(^{-/-}\) and WT mice at either 4 or 12 months (Fig. 3E-F).

DISCUSSION

The present study has shown that IL-6 deficiency increases a) skeletal muscle fatty acid transporter content (primarily FAT/CD36), b) intramuscular lipid accumulation (FFA, DG and TG), and c) the saturated fatty acid content in intramuscular TG. Remarkably these changes were confined to red skeletal muscle, as none of these changes were observed in white skeletal muscle.

FAT/CD36 and FABPpm expression correlate with the muscles oxidative potential, as skeletal muscles containing more oxidative fibers have higher rates of lipid fatty acid transport and esterification and oxidation, and they have a higher content of fatty acid transport proteins (e.g. red>white muscles) (21, 33). Several studies have also shown that red muscles are more susceptible than white muscles in metabolic changes during the development of insulin resistance and type 2 diabetes (20, 23). Our present study underscore these observations as we observed upregulation of FAT/CD36 and FABPpm proteins in red muscles but, not in white muscle of IL-6\(^{-/-}\) mice. The red muscle observed pattern of change in the expression of FAT/CD36 was also age-related, as we observed relative higher increase in the protein expression at 12 months of age in IL-6\(^{-/-}\) mice. Presumably, the increase in red muscle FAT/CD36 promotes the influx of fatty acids into these oxidative muscles, since the increased expression of fatty acid transport proteins leads to enhanced LCFA flux into myocytes (for review: 22).

There is a considerable amount of information indicating that high circulating IL-6 levels are associated with insulin resistance in humans (6) and that IL-6 may mediate several of the prodiabetic effects of palmitate-induced insulin resistance in C2C12 cells (34). Conversely, rodent studies reported that, IL-6 released from working skeletal muscles acts to sensitize myocytes for insulin action, by increasing LCFA oxidation in these muscles (5, 35). Further evidence showed both: 1) strong association between IL-6 levels and AMPK activation in
incubated rat muscles (36) and 2) diminished AMPK activity in IL-6 knockout mice (36). Collectively above mentioned data suggest that a lack of IL-6 (AMPK action) in myocytes might result in the predominance of LCFA esterification over LCFA oxidation. This seems to be the case in our study, which shows augmented intramuscular lipid accumulation (i.e. increased FFA, DG and TG lipid pools). Presumably, this intramuscular lipid accumulation contributes to the development of insulin resistance, as accumulation of these skeletal muscle lipid fractions is deleterious (17, 38, 39). There are now plausible mechanistic links between the development of insulin resistance and accumulation of DG and TG in skeletal muscle (14, 17). Accordingly in IL-6−/− mice features of type 2 diabetes were observed by 9 mo of age (10). Paradoxically, data obtained by Di Gregorio et al. (11) do not support this hypothesis, as in their study IL-6 deficient mice did not develop overt diabetes and obesity, and they proposed that IL-6 deficiency has no effect on lipid metabolism (11). While there is no obvious explanation for these differences in the studies of Wallenius et al. (10) and Di Gregorio et al. (11), our studies clearly indicate that LCFA transport and metabolism are markedly altered in IL-6−/− mice, supporting strongly the notion that these mice become insulin resistant.

In the present study we also examined the saturation status of FA’s present in specific intramuscular lipid fractions (FFA, DG, TG and PL). Unexpectedly, we found that the lack of the IL-6 gene results in an increase of saturated fatty acids species only in TG fraction, specifically in oxidative muscles (soleus and red gastrocnemius). This type of skeletal muscles (oxidative muscle fibers) have an enhanced susceptibility for LCFA induced insulin resistance (20, 23) however, it is unclear whether an increase in TG saturation status contributes to insulin resistance (40). A strong correlation has been observed between saturation of FA species in an intramyocellular lipid pools and the severity of insulin resistance (41, 42). It has been also shown that only saturated long-chain fatty acids block insulin signaling in C2C12 myotubes while promoting lipid accumulation (i.e. ceramide and DG accumulation) (41).

In present study we have provided data regarding the effects of IL-6 deficiency on fatty acid transporters and lipid profiles in skeletal muscle. We have demonstrated increments in the skeletal muscle expression of key fatty acid transport proteins FAT/CD36 and FABPpm but only in the oxidative muscles. Accordingly, in these muscles from IL-6−/− mice there was a significant accumulation of intramuscular lipids. IL-6 deficiency also increased the saturation profile of FA species in the red but not white muscles’ TG fraction.

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