Interleukin-18 activates skeletal muscle AMPK and reduces weight gain and insulin resistance in mice

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Abstract

Circulating interleukin (IL)-18 is elevated in obesity, but paradoxically causes hypophagia. We hypothesized that IL-18 may attenuate high fat diet induced insulin resistance by activating AMP activated protein kinase (AMPK). We studied mice with a global deletion of the α isoform of the IL-18 receptor (IL-18Rα/0), fed a standard chow or high fat diet (HFD). We next performed gain of function experiments in skeletal muscle, in vitro, ex vivo and in vivo. We show that IL-18 is implicated in metabolic homeostasis, inflammation and insulin resistance via mechanisms involving the activation of AMPK in skeletal muscle. IL-18Rα/0 mice display increased weight gain, and ectopic lipid deposition, inflammation and reduced AMPK signaling in skeletal muscle. Treating myotubes or skeletal muscle strips with IL-18 activated AMPK and increased fat oxidation. Moreover, in vivo electroporation of IL-18 into skeletal muscle activated AMPK and concomitantly inhibited high fat diet-induced weight gain. In summary IL-18 enhances AMPK signaling and lipid oxidation in skeletal muscle implicating IL-18 in metabolic homeostasis.
INTRODUCTION

The cytokine interleukin (IL)-18 was identified approximately 15 years ago as a co-factor that, together with IL-12, stimulates production of interferon (IFN)-γ (1). This ~18 kDa cytokine, which has structural similarities to the IL-1 cytokine family, is widely expressed in many mammalian cells/tissues including liver, adipose tissue, skeletal muscle, pancreas, brain and endothelium (2). IL-18 is best known for its role in inflammation, whereby pro-inflammatory stimuli such as lipopolysaccharide, Fas ligand and tumor necrosis factor (TNF)-α leads to caspase-1 mediated cleavage of pro-IL-18 into mature IL-18. IL-18 can then signal via a heterodimer of the transmembrane IL-18 receptors (α and β), and via a toll like receptor signaling cascade, ultimately leading to the activation of nuclear factor κB (NFκB) and subsequent regulation of gene transcription (3). While this is the most characterized signaling pathway for this cytokine, it is worth noting that IL-18 has also been implicated in mitogen activated protein kinase (MAPK), phosphatidylinositol-2 kinase (PI3-K) and Signal transducer and activator of transcription (STAT) 3 signaling (4), which are all implicated in energy metabolism.

It is now well accepted that obesity results in a state of low grade chronic inflammation (5) and, therefore, it is not surprising that circulating IL-18 levels are elevated in human obesity (6) and in patients with type 2 diabetes (7). Somewhat paradoxically, work from two separate groups have reported that mice with a global deletion of IL-18 become obese and insulin resistant, while exogenous administration of recombinant IL-18 rescued this phenotype (8;9). These previous studies ascribed the mechanism of action of IL-18 in modulating nutrient homeostasis to be exclusively via neuronal control of food intake (8; 9). However, in the earlier study
the authors demonstrated that the IL-18−/− mice displayed decreased peripheral insulin sensitivity and that IL-18 signals via STAT3 in the liver, raising the possibility that IL-18 may play a role in peripheral energy metabolism, because STAT3 plays a major role in maintaining metabolic homeostasis in the liver (10). In addition, we (11; 12) and others (13; 14) have shown that cytokines that result in activation of STAT3 via transmembrane receptor signaling, can activate the AMP-activated kinase (AMPK) signaling pathway to enhance fat oxidation in skeletal muscle, thereby attenuating high fat diet induced insulin resistance. Together, these previous studies raise the possibility that IL-18 may attenuate high fat diet-induced insulin resistance via affecting metabolic processes, such as activation of AMPK, in skeletal muscle.

This is important from a therapeutic viewpoint since drugs that effectively modulate food intake via targeting the central nervous system have, to date, proven unsuccessful due to side effects associated with activation of the lateral hypothalamus [for review see(15)]. In the present study, we tested the hypothesis that IL-18 signaling can modulate nutrient homeostasis via mechanisms associated with peripheral energy metabolism. We show that IL-18 activates AMPK and increases lipid oxidation in skeletal muscle implicating IL-18 in diet-induced obesity and insulin resistance.

Methods

Animal experimental protocols For the diet intervention study twelve week-old Il18R−/− (backcrossed 11 generations to C57BL/6J) and wild-type C57BL/6J mice were obtained from Charles River Laboratories (L'Arbresle, France). Subsequent to this initial cohort, we then performed experiments in Il18R−/− and wild-type mice obtained from heterozygous mating. Eight week old C57BL/6J mice (inbred, Herlev, Denmark)
were used for a DNA electroporation study, and 13 week old C57/BL6 mice (inbred, Melbourne, Australia) were used for \textit{ex vivo} experiments. All experiments were approved by The Animal Experiments Inspectorate in Denmark and/or the BakerIDI Alfred Medical Research and Education Precinct (AMREP) Animal Ethic Committee in accordance with the National Health and Medical Research Council of Australia Guidelines on Animal Experimentation. Mice were maintained on a 12-h light, 12-h dark cycle on a standard rodent chow diet (27\%, 13\%, and 60\% kcal from protein, fat, and carbohydrate, respectively) or a HFD composed of 60\% calories from Fat (Research Diet 12492, 20\%, 60\%, and 20\% kcal from protein, fat, and carbohydrate, respectively) for 18 weeks (diet-induced obesity study) or 4 weeks (\textit{in vivo} electroporation study).

\textit{In vivo} electroporation experiments were performed as previously described (16). Briefly, the regulatory plasmids pTet-On was both obtained from Clontech (Palo Alto, CA, USA). As IL-18 lacks a typical signal sequence, the V-J2-C region of murine immunoglobulin k-chain was cloned upstream of the mature IL-18 sequence. The Tibialis Anterior muscle of each mouse was directly injected with 20 \mu l plasmid solution (0.5 \mu g/\mu l) and electric pulsing was applied using 4 mm plate electrodes and an electric field of 1 high voltage pulse (100 \mu s, 800 V/cm) and 1 low voltage pulse (400 ms, 100 V/cm) (17). Induction of gene expression was obtained by administering drinking water consisting of distilled water containing 0.2 mg/ml of doxycycline (doxycycline hyclate, Sigma-Aldrich, Denmark) (18).

For \textit{ex vivo} experiments, mice were fed a standard chow diet and drinking water, available \textit{ad libitum}. To examine palmitate metabolism, mice were first anesthetized
and *soleus* muscles were carefully dissected into longitudinal strips from tendon to tendon. Strips were removed and $[^{14}C]$palmitate oxidation was analyzed as previously described (19). The dose of 100 ng/mL IL-18 was used and PBS served as placebo. The strips from the same animal were used for both IL-18 and PBS.

**Skeletal muscle cell culture** To examine whether IL-18 affected phosphorylation (Thr$^{172}$) of AMPK and ACCβ (Ser$^{79}$), fully fused L6 myotubes were treated with recombinant rat IL-18 (MBL-Woburn, MA, USA). Cells were treated with IL-18 for 10, 30 min and 60 min at doses of 1 and 10 ng/mL.

**Analysis of body composition** We measured body composition analysis in IL18R$^{-/-}$ by DEXA using the Lunar PIXImus Mouse Densitometer (GE Medical Systems, WI, USA) and in IL-18 electroporated mice by using a Lunar Prodigy scanner with a small animal software application (GE Healthcare Systems, WI, USA). Animals were anaesthetized by i.p. injection of Hypnorm (0.4 ml/kg; Janssen, Saunderton, UK) and Dormicum (2 mg/kg; Roche, Basel, Switzerland) and laid flat on the scanning platform on their ventral side. To confirm DEXA fat mass determination, mice were killed and two intra-abdominal fat pads (gonadal, retroperitoneal) and one subcutaneous fat pad (inguinal) were dissected and weighed.

**Insulin tolerance tests** We performed insulin tolerance tests in 7.5 month-old male mice, 4 h after removal of food. Blood samples were obtained by tail cut and analyzed for glucose content using a glucometer (Accu-chek Compact plus) immediately before
and at 15, 30, 45, 60, 90, and 120 min after an intraperitoneal injection of insulin (0.75 U/kg, Actrapid, Novo Nordisk, DK).

**Indirect calorimetry** Mice were placed in a sixteen-chamber indirect calorimetry system (TSE Systems, Bad Homburg, Germany) cages for 10 d; the first 5 d was considered the acclimation phase and data were analyzed only for the last final days. After 3 d the mice were fasted for 24 h. Oxygen consumption rate (VO$_2$: ml/h/kg), respiratory exchange ratio (RER), and activity (beam breaks) were measured using the system. Mice had free access to food and water while in the chambers. Food intake was measured for the duration of data collection while mice underwent the indirect calorimetry measurements.

**Plasma parameters analysis** We collected blood samples from the tail vein of the mice into EDTA tubes and were immediately spun at 6,000g for 10 min at 4°C and plasma was removed. Plasma was stored at –80°C until analysis. Plasma insulin was determined by ELISA (Crystal Chem.). Blood glucose was measured by glucometer (Accu-chek Compact Plus). Adiponectin concentration was measured by RIA kit. Leptin, MCP-1, PAI-1, IL-6, and TNF-α were measured by using a Lincoplex mouse serum adipokine panel (Linco).

**Insulin Signaling Tissue Collection** Animals were anesthetized with an injection of Hypnorm (0.4 ml/kg; Janssen, Saunderton, UK) and Dormicum (2 mg/kg; Roche, Basel, Switzerland) and the gastrocnemius muscle as well as the right lobe of the liver (after a ligature around the blood vessel) were removed and stored in liquid nitrogen until further processing. This was followed by injection of insulin (1.5 U/kg LBM)
into the abdominal aorta and removal of the contralateral \textit{gastrocnemius} and liver lobe occurred 2 min after injection. Samples were stored in liquid nitrogen until further processing.

\textit{RNA extraction and Real time quantitative PCR.} Mouse tissue was isolated, frozen in liquid nitrogen or in dry ice and absolute alcohol and stored at \(-80^\circ \text{C}\) until extraction. Total RNA was isolated from adipose tissue with TriZol (Life Technology), as described by the manufacturer. Five-hundred nanograms of RNA were reverse transcribed to cDNA with the use of random hexamers (Applied Biosystems, Taqman™ reverse transcription reagents). Real-time PCR was performed on an ABI PRISM 7900 sequence detector or 7500 fast sequence detector (Applied Biosystems). Each assay included (in triplicate): a cDNA standard curve of five serial dilution points (ranging from 1 to 0.01), a no-template control, a no-reverse transcriptase control, and 7.5 ng (0.375 ng for 18S rRNA) of each sample cDNA. For 18S rRNA, SREBP1c, FAS, HADB, PEPCK, G-6Pase and CPT1 the amplification mixtures were prepared with 2 X Taqman Universal PCR master mix. All assay reagents were from Applied Biosystems. Primers and Taqman probes were designed for SREBP1c and FAS using a mouse specific database (ensemble.com) and Primer Express (Applied Biosystems) and primers for CPT-1 and HADB using the free program “primer3”. The sequences to amplify a fragment of SREBP1c was FP: 5' GACCACGGAGCCATGGAT 3', RP: 5' GGCCCAGGAAGTCACTGT 3' and TaqMan probe: 5' ACATTTGAAGACATGCTCCAGCTCATCAACA 3', a fragment of FAS FP: 5' ATCCTGGAACGAGAACGATCT 3', RP: 5' GGACTTGGGGGCTGTCGTGTCA 3' and TaqMan probe: 5' CACGCTCGGAACTTCAGGAAATGT 3', a fragment of HAD FP:
GTGGAGAAGACCTGAGCTA and RP: GCAAATCGGTCTTGTCTAGT, a fragment of PEPCK FP: GGCGGAGCATATGCT and RP: CCACAGGCACTAGGGAAGGC, a fragment of G6-Pase FP: TCAACCTCGTCTTTCAAGTGGATT and RP: GCTGTAGTAGTCGGTGTCCAGGA and of CPTI FP: GTTCGCTTTCTCAAGGTCTGG and RP: AAGAAAGCAGCAGCAGTTCCAT. Oligo’s for SREBP1, FAS were obtained from TaqCopenhagen (Copenhagen, Denmark) for CPT1, HAD from DNA technology (Aarhus, Denmark) and for PEPCK and G6Pase from Geneworks (South Australia, Australia). The 18S rRNA content was determined using a pre-developed assay reagent (Applied Biosystems). The relative concentrations of measured mRNA’s were determined by plotting the threshold cycle (Ct) versus the log of the serial dilution points, and the relative expression of the gene of interest was determined after normalization to 18S, which was unaffected by genotype and diet.

**AMPK-activity and lipid measurements**  AMPK activity was assayed in frozen in the tibialis anterior muscle homogenized in lysis buffer as described previously (12). Briefly, muscle lysate containing 200 µg protein was immunoprecipitated with antibody specific to the α2 or α1 catalytic subunit of AMPK and protein A/G agarose beads. Beads were washed five times, and the activity of the immobilized enzyme was assayed based on the phosphorylation of “SAMS” peptide (0.2 mmol/l) by 0.2 mmol/l ATP (containing 2 µCi [γ-32P] ATP) in the presence and absence of 0.2 mmol/l AMP. Label incorporation into the SAMS peptide was measured on a Racbeta 1214 scintillation counter. For measurement of intramuscular triglycerides, freeze dried muscle samples were dissected free of visible connective tissue and blood. Lipid was
extracted by a Folch extraction, the triacylglycerol was saponified in an ethanol / KOH solution at 60°C, and glycerol content was determined fluorometrically as described previously (12).

Protein analysis Tissue lysates (40 µg) were solubilized in Laemmli sample buffer and boiled for 5 min, resolved by SDS-PAGE on 10 % polyacrylamide gels, transferred (semi-dry) to nitrocellulose membrane, blocked with 5% BSA and immunoblotted with primary antibodies (2.5% BSA) overnight. After incubation in horse radish peroxidase conjugated secondary antibody (2.5% BSA)(Amersham Bioscience), the immunoreactive proteins were detected with enhanced chemiluminescence (Amersham Bioscience) and quantified by densitometry (ChemiDoc XRS). Membranes were stripped, washed and re-probed for total protein content or housekeeping protein where appropriate. Total AKT was run on a separate gel and was not stripped. Samples from mice fed a chow or a HFD were run on separate gels. The antibodies used for detection of total AKT and beta-actin and phosphorylation of JNK1/2, AKT, AMPKalpha and ACCβ were purchased from Cell Signalling. The alpha-tubulin antibody was obtained from Sigma Aldrich.

Statistical analysis All analyses were performed using SAS 9.1. We performed comparison between two groups using unpaired Student’s t-test and one-way ANOVA followed the Tukeys post hoc test. Time-series data were analyzed with PROC MIXED. P<0.05 were considered as significant. When appropriate values were logarithmic transformed to ensure normality and equal variance.
RESULTS

IL-18 Receptor deficient mice are prone to weight gain that is not associated with hyperphagia To evaluate the role of IL-18 signaling in the etiology of body weight homeostasis, we first performed loss of function experiments by phenotyping mice with a global deletion of the α isoform of the IL-18 receptor (IL-18R\(^{-/-}\)). Consistent with one previous study (8), IL-18R\(^{-/-}\) mice become heavier than their wild-type counterparts (CON) at ~ 6 months of age when fed a regular chow diet (Fig. 1a).

The increase in body mass was due to an increase in adiposity, since we observed differences in percent fat (Fig. 1b), but not in percent fat free mass (data not shown). This increase in adiposity was due to increases in visceral, but not subcutaneous fat mass, since both epididymal and retroperitoneal, but not inguinal fat pad masses were higher in IL-18R\(^{-/-}\) mice relative to CON (Fig. 1c). While both IL-18R\(^{-/-}\) and CON mice markedly increased body mass and fat pad mass when fed a high fat diet (HFD) for 16 wk, the differences observed when comparing genotypes on a regular chow diet was not evident when animals were fed a HFD (Fig. 1a-d). As discussed, previous studies have demonstrated that IL-18\(^{-/-}\) mice are prone to weight gain due to hyperphagia (8; 9). While the earlier study reported weight gain and insulin resistance in IL-18R\(^{-/-}\) mice, they did not make reference to altered feeding behaviour in these animals (8). To determine whether the increase in adiposity observed in IL-18R\(^{-/-}\) mice was due an increase in food intake and/or a decrease in energy expenditure, we next performed whole body indirect calorimetry experiments. We observed no difference in food intake (Fig. 1e), whole body oxygen consumption (Fig. 1f) or activity (data not shown) measured over 24 h when comparing IL-18R\(^{-/-}\) mice with CON irrespective of diet. As expected, consumption of the HFD decreased whole
body RER, indicative of an increase in whole body fat oxidation. When mice were provided with HFD ad libitum, average RER (over a 72 h period) was not different when comparing IL-18R⁺⁻ mice with CON irrespective of diet (Fig. 1h). Of note, however, when mice were fasted and refed at the cessation of the 72 h period, IL-18R⁻/⁻ mice displayed a significantly higher RER in both the fasted and refed condition, relative to CON (Fig. 1h).

**IL-18 Receptor deficient mice are insulin resistant**

We next examined whether IL-18R⁺⁻ mice were insulin resistant. At 3 month of age, and before the IL-18R⁺⁻ mice became obese on a chow diet, there were no differences in insulin resistance as measured by an intraperitoneal tolerance test (ITT) (Data not shown). However, with age and irrespective of diet, IL-18R⁻/⁻ mice displayed whole body insulin resistance as measured by both fasting hyperinsulinemia (Fig. 2a) and impaired glucose clearance during an ITT (Fig. 2b, Supplementary Fig. 1). We next examined insulin signaling in both muscle and liver by analyzing the phosphorylation of Akt (Ser⁴⁷³) before and 2 min after a bolus dose of insulin. While insulin increased p-Akt in the skeletal muscle (Fig. 2c) and liver (Fig. 2e) of CON animal fed a chow diet, this effect was markedly blunted in IL-18R⁺⁻ mice (Fig. 2c,e). No differences in p-Akt in either skeletal muscle (Fig. 2d) or liver (Fig. 2f) were observed when comparing IL-18R⁻/⁻ with CON mice when fed a HFD.

**IL-18 Receptor deficient mice store excess lipid in skeletal muscle and have inflamed livers and skeletal muscle**

Excess adiposity is often associated with ectopic lipid storage in metabolic tissues such as liver and skeletal muscle which can mediate insulin resistance either directly or via the upregulation of serine threonine kinases
such as c-jun terminal kinase (JNK) and inhibitor of κB kinase (IKK) (5). Accordingly, we next measured intramyocellular and intrahepatic lipid content and the phosphorylation of JNK (Thr$^{183}$/Tyr$^{185}$) and IKKαβ (Ser$^{180}$/Ser$^{181}$) in these tissues. Irrespective of diet, triacylglycerol (TAG) content was higher in the skeletal muscles of IL-18R$^{-/-}$ relative to CON mice (Fig. 3a). This was associated with an elevated JNK (Fig. 3b), but not IKK (Supplementary Fig. 2) phosphorylation. Conversely, we did not observe any differences in intrahepatic TAG concentration when comparing IL-18R$^{-/-}$ with CON mice irrespective of diet (Fig. 3c). Notwithstanding, phosphorylation of JNK (Fig. 3d) and the mRNA expression of key fatty acid synthesis transcription factors/enzymes sterol-regulatory-element-binding protein-1c (SREBP1c) and fatty acid synthase (FAS) (Fig. 3e,f) were elevated in the liver when comparing IL-18R$^{-/-}$ with CON mice when animals were fed a chow and a high fat diet. However, no differences were observed in the key gluconeogenic enzymes Phosphoenolpyruvate carboxykinase (PEPCK) or glucose-6 phosphate dehydrogenase (G6Pase) when comparing IL-18R$^{-/-}$ with CON mice irrespective of diet (Supplementary Figure 3). Given that neither hepatosteatosis, nor the expression of key enzymes involved in regulating hepatic glucose production, were different when comparing IL-18R$^{-/-}$ with CON mice, it is unlikely that changes in liver insulin sensitivity were responsible for the reduced whole body insulin sensitivity, observed in IL-18R$^{-/-}$, although this possibility cannot be entirely ruled out.

**IL-18R$^{-/-}$ mice have reduced AMPK signaling in metabolic tissues** Since IL-18R$^{-/-}$ mice are prone to weight gain on a normal diet and ectopic lipid storage independent of feeding, we next examined whether pathways associated with lipid oxidation were impaired. One major pathway that regulates fatty acid oxidation is AMPK. AMPK
phosphorylates acetyl CoA carboxylase β (ACCβ) resulting in inhibition of ACC activity, which in turn leads to a decrease in malonyl CoA content, relieving inhibition of carnitine palmitoyl transferase 1 (CPT-1) and increasing fatty acid oxidation. No significant differences between phenotypes were observed when measuring the phosphorylation of AMPK (Thr172) (data not shown). However, the phosphorylation of ACCβ (Ser218), a downstream marker of AMPK activity, was reduced in the skeletal muscle, liver and adipose tissue of IL-18R−/− when fed a chow diet (Fig.4a-c). In addition, this effect was maintained in skeletal muscle (Fig. 4a) and liver (Fig. b), but not adipose tissue (Fig. 4c) when mice were fed the HFD.

Exogenous IL-18 treatment increases AMPK signaling and fat oxidation in skeletal muscle in vitro and ex vivo Since we observed that IL-18R−/− mice become obese, store more lipid in skeletal muscle and have defective ACCβ phosphorylation in this organ, we next performed in vitro and ex vivo experiments in muscle cells and whole muscle strips to confirm the role of IL-18 on AMPK signaling and fat oxidation in this important metabolic tissue. In initial experiments, we demonstrated that as little as 1.0 ng/ml of recombinant (r) IL-18 protein was sufficient to phosphorylate both AMPK (Thr172) (Fig. 5a) and ACCβ (Ser218) (Fig. 5b) in L6 myotubes. We next performed experiments in isolated intact soleus muscle as previously reported (16; 20)(21). Treating these muscles with 100 ng/mL rIL-18 was sufficient to increase both palmitate oxidation (Fig. 5c) and AMPK phosphorylation (Thr172) (Fig. 5d).

Ectopic expression of IL-18 in a single Tibialis Anterior muscle is sufficient to protect against excess adipose tissue storage in mice fed a high fat diet To determine whether the results obtained in muscle cell and ex vivo muscle strips were also prevalent in
in vivo, we utilized the *in vivo* electroporation technique to overexpress IL-18 cDNA in the tibialis anterior (TA) muscles of C57Bl/6 mice that were placed on a HFD diet for 4 wk. Using this technique, we have previously observed a transfection efficiency of ~60%, as measured by the electroporation of a GFP construct as a control (21). IL-18 protein expression in the TA was increased 30-40 fold above basal when compared with mice where the TA was electroporated with an empty vector (Sham) (Fig. 6a). No difference in body weight was observed when comparing IL-18 with Sham electroporated mice (Fig. 6b). In accordance with this observation, adiposity was reduced when comparing IL-18 with Sham electroporated mice at 4 wk (Fig. 6c,d, Supplementary Fig. 4). However, this reduction in adiposity was insufficient to result in increased whole body insulin sensitivity or glucose tolerance as measured by ITT and GTT (Supplementary Fig. 5).

**Ectopic expression of IL-18 in a single Tibialis Anterior muscle increases AMPK signaling and markers of lipid oxidation in this organ** We next measured AMPK signaling and markers of lipid oxidation in IL-18 and Sham electroporated muscles. Both AMPK activity (Fig. 7a) and ACCβ phosphorylation (Ser^{218}) (Fig. 7b) were markedly elevated in IL-18 compared with Sham electroporated muscles. In addition, we also observed increased mRNA abundance of β-hydroxyacyl-CoA-dehydrogenase (β-HAD), a key enzyme involved in mitochondrial function (Fig. 7c) and carnitine palmitoyltransferase 1 (CPT1) (Fig. 7d), the enzyme that controls the transfer of long chain fatty acyl CoA into mitochondria and enhances rates of fatty acid oxidation in the IL-18 electroporated mice relative to Sham mice. It is now well known that skeletal muscle can act as an endocrine organ, producing “myokines” to result in tissue cross talk (22). To examine whether IL-18 could act as a myokine when
overexpressed in skeletal muscle, we examined circulating levels of IL-18 and markers of insulin sensitivity, fat oxidation and inflammation in other tissues such as the liver and adipose. Despite the increase in intramuscular IL-18 expression with electroporation, plasma IL-18 was not elevated in the IL-18 electroporated mice relative to Sham (Supplementary Fig. 5). It was not surprising, therefore, that pAkt, pAMPK, pACC and pJNK were not altered in the liver and/or adipose tissue of IL-18 electroporated mice relative to Sham mice (Supplementary Figs 5,6). Together, these data provide evidence that IL-18 can activate AMPK in skeletal muscle in vivo.

**DISCUSSION**

Interleukin-18 signaling has been implicated in the aetiology of metabolic homeostasis and insulin resistance, however, studies in IL-18 deficient mice have suggested that IL-18 is required to prevent hyperphagia (8; 9). In the current study, rather than use a model of genetic deletion of IL-18, we initially studied mice that harbor a global deletion of the functional IL-18R. We show that IL-18 receptor deficient mice are prone to weight gain on a chow diet and develop insulin resistance, a phenotype which is associated with ectopic skeletal muscle lipid expression, inflammation and reduced AMPK signaling.

The IL-18R−/− mice displayed significantly increased body weight and increased fat pad mass after ~26 weeks of age. While others have shown that IL-18 deficient mice display hyperphagia (8; 9) we did not observe this in the IL-18R−/− mice. We were careful to monitor food intake in both their normal habitat and during metabolic caging, so we are confident that the food intake between the IL-18R−/− and CON mice were comparable. Given the identical oxygen consumption between the IL-18R−/− and
CON mice, why would the IL-18R\textsuperscript{−/−} mice gain more weight if both energy input and expenditure were comparable? It should be noted that the difference in body weight when comparing the IL-18R\textsuperscript{−/−} and CON mice was \( \sim 10\% \) by the end of the study (Fig. 1a), while the overall difference in percent body fat was \( \sim 4\% \) (Fig. 1b). This would equate to an approximate increase of 0.15 g fat or 0.70 kJ energy per day. The daily energy expenditure of a mouse has been estimated to be 42 kJ per day (23). Consequently, less than a 2% difference in daily energy expenditure would be sufficient to result in the increased body weight, but unlikely to be detected with available techniques, such as metabolic caging. Similar problems have been encountered in other mouse models of obesity (23; 24), and work from our group has also recently observed such an anomaly (25). Notwithstanding this apparent anomaly, it appears based on the data we have reported, that the IL-18R\textsuperscript{−/−} mice gain weight and store excess lipid in skeletal muscle which results in whole body and skeletal muscle insulin resistance.

It has been suggested that IL-18R might also be activated by other ligands than IL-18 (26; 27) and this, potentially, could explain why hyperphagia was previously observed in IL-18 deficient mice (8; 9) but not in IL-18R\textsuperscript{−/−} mice in the present study. Interestingly, when mice fed a high fat diet were fasted and refed, IL-18R\textsuperscript{−/−} mice displayed a significantly higher RER in the refed condition (Fig. 1h). This suggests that mice lacking the IL-18 receptor cannot oxidize lipid and rely on utilization of carbohydrate as a preferred energy substrate during refeeding. This is supported by our gain of function data, as IL-18 increased fat oxidation. Interestingly, Zorilla \textit{et al} (9) observed that mice with IL-18 deficiency were not hyperphagic when fed a high
fat diet and suggested that they differentially process carbohydrate- vs. lipid-rich diets or differentially use these macronutrients as fuel. Our study supports this hypothesis.

By performing whole body insulin tolerance tests, we cannot ascertain whether the whole body insulin resistance observed in the IL-18R−/− mice relative to control mice, was due to insulin resistance in skeletal muscle, liver or a combination of the two. However, several lines of evidence suggest that the defect in the IL-18R−/− mice was primarily in the skeletal muscle and this is the rationale for choosing to study this organ in depth. Firstly, it is well acknowledged that ectopic lipid expression is a primary mechanism leading to insulin resistance (28). While intramuscular triglycerides were elevated in the IL-18R−/− mice relative to control mice, no such increase was observed in the liver (Fig. 3). Secondly, the mRNA expression of key gluconeogenic enzymes PEPCK and G6Pase were not different in the liver of the IL-18R−/− mice. Thirdly, even though the IL-18R−/− mice were insulin resistant on a HFD relative to control mice, there was no evidence of decreased p-Akt (Fig 2f), or the mRNA expression of SREBP1 or FAS (Fig. 3e,f) in the liver. Together, these data suggest that skeletal muscle was the origin of the primary defect, although we acknowledge that the effects of IL-18R deficiency on the liver cannot be completely discounted as we did not directly measure insulin sensitivity in this organ.

Of note was our observation that p-JNK was markedly elevated in the livers of the IL-18R−/− mice relative to control mice despite equivalent lipid content when animals were fed a chow diet (Fig. 3). While this may seem counterintuitive, this observation is not novel. Indeed, we have previously observed such a phenomenon in IL-6 deficient mice (25). A potential mechanism has recently been proposed by Flavell and
colleagues who demonstrated that NLRP6 and NLRP3 inflammasomes and, importantly, IL-18 negatively regulate NAFLD/NASH progression, as well as multiple aspects of metabolic syndrome via modulation of the gut microbiota, not necessarily related to hepatosteatosis (29). Although speculative, the increased inflammation observed in the IL-18R°/° mice in the presence of relatively normal lipid levels may be related to such a mechanism.

Based on both our loss of function and gain of function models, IL-18 signaling is implicated in fatty acid oxidation rates in skeletal muscle as a result of activation of AMPK. As discussed, AMPK phosphorylates ACCβ resulting in inhibition of ACC activity, which in turn leads to a decrease in malonyl CoA content, relieving inhibition of carnitine palmitoyl transferase 1 (CPT-1) and increasing fatty acid oxidation. The phosphorylation of ACCβ was reduced in the skeletal muscle, liver and adipose tissue of IL-18R°/° (Fig. 4). Moreover, when cultured skeletal muscle cells or isolated skeletal muscle strips were treated with IL-18, phosphorylation of AMPK, and/or ACC was increased and, in the case of intact ex vivo treated skeletal muscle, this increase was associated with enhanced fatty acid oxidation. Finally, when IL-18 was overexpressed in skeletal muscle in vivo, AMPK activity and ACC phosphorylation were increased, not only in the electroporated muscle (Fig. 7a,b) Taken together, the data provide evidence that implicates IL-18 in the activation of AMPK.

It is now well known that many cytokines, including leptin, adiponectin, ghrelin, interleukin-6 and ciliary neurotrophic factor can activate AMPK (30; 31), but this is the first report that IL-18 can act as an AMPK agonist. This observation, however, is
consistent with these previous studies, since IL-18 can act as an activator of STAT3 (4). Work from our group has previously shown that members of the IL-6 family of cytokines, which potently activate STAT3, also enhance fat oxidation via AMPK (11; 12). Importantly, when mice that harbor a truncation of the C-terminal domain that eliminates these tyrosine residues on the gp130 receptor (gp130<sup>ΔSTAT</sup> mice) are treated with CNTF, the phosphorylation of STAT3 is abolished, as too is the activation of AMPK (12).

Although feeding mice a high fat diet did not result in differences in body weight, fat mass or insulin signaling in skeletal muscle and liver when comparing the genotypes, the IL-18R<sup>−/−</sup> mice nevertheless displayed elevated fasting insulin levels and impaired insulin tolerance as measured by an ITT (Fig. 2). Although speculative, this may be due to the fact that under high fat fed conditions, the activation of the AMPK pathway remained impaired in the IL-18R<sup>−/−</sup> mice, at least in skeletal muscle and liver (Fig. 4), leading to elevated lipid levels in skeletal muscle of the IL-18R<sup>−/−</sup> mice under high fat fed conditions (Fig. 3).

In summary, we have identified that IL-18 can activate AMPK. Moreover, mice that harbor a genetic deletion of a functional IL-18R are prone to weight gain and develop insulin resistance and inflammation in important metabolic tissues such as skeletal muscle and liver. Our data, therefore, adds IL-18 to a growing list of catabolic, pro-inflammatory cytokines that paradoxically are required to maintain pathways important for fatty acid oxidation, and thus prevent insulin resistance.
Author Contributions

BL, BKP and MAF designed research; BL, VBM, CB, PH, TLA, MJW, CRB, EE, CR, OM, SS, JA, HP, SD, TJA, ANM performed and/or analysed research; JH contributed new reagents/analytical tools; BL, and MAF wrote the paper and all authors contributed to the writing of the final submitted version.

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Figure 1. Whole body phenotype of IL-18R−/− mice. (A) Growth curves during chow and high fat diet (CON-chow: n=21; CON-HFD: n=12; IL-18R−/−-chow: n=14; IL-18R−/−-HFD: n=14), (B) fat percentage during chow and high fat diet (CON-chow: n=19; CON-HFD: n=19; IL-18R−/−-chow: n=12; IL-18R−/−-HFD: n=11), (C) Inguinal, gonadal, and retroperitoneal fat pads during chow diet (CON=13; IL-18R−/−: n=7), (D) Inguinal, gonadal, and retroperitoneal fat pads during high fat diet (CON=11; IL-18R−/−: n=10). E-F) Average 24 h food intake (E) and VO2 (F) during 2 days of chow and high fat diet and G-F) respiratory exchange ratio (RER) during 3 days of ad libitum feeding, 24 hr fasting, and 24 hr refeeding of chow (G) and HFD (F) obtained from control (CON; black bars) and IL-18R−/− (grey bars) male mice at 7 months of age mice (CON-chow: n=7; CON-HFD: n=8; IL-18R−/−-chow: n=8; IL-18R−/−-HFD: n=6). Results are presented as mean ± SEM . *P <0.05 vs. controls.

Figure 2. IL-18R−/− mice exhibit insulin resistance when fed a chow diet and a high-fat-diet. (A) Plasma insulin (CON-chow: n=7; CON-HFD: n=8; IL-18R−/−-chow: n=8; IL-18R−/−-HFD: n=6), (B) insulin tolerance with AUC inserted (CON-chow: n=12; CON-HFD: n=12; IL-18R−/−-chow: n=14; IL-18R−/−-HFD: n=12) (For the actual data, see Supplementary Fig. 1) (C-F) total and phosphorylated (Ser473) during chow diet (C) (n=4-6) and high fat diet (D) (n=8-9) in muscle, total and phosphorylated (Ser473) during chow diet (E) (n=5-6) and high fat diet (F) (n=7-9) in liver. Total AKT was run separately and was not stripped. The signaling data were obtained in littermate controls. Samples from chow diet and HFD were run on separate gels. Results are presented as mean ± SEM . *P <0.05; **P <0.01, ***P <0.001 vs. chow (A) or vs basal (C-F), # P <0.05, ## P <0.01 vs control (A, C), $ interaction between genotype and diet p=0.06 (E).
Figure 3. **IL-18R<sup>−/−</sup> mice develop hyperlipidemia and inflammation on a chow diet but not on a high-fat-diet.** (A) Triglycerides in muscle  CON-chow: n=17; CON-HFD: n=9; IL-18R<sup>−/−</sup>-chow: n=8; IL-18R<sup>−/−</sup>-HFD: n=5), (B) representative immunoblot and densitometric quantification of JNK1/2 phosphorylation in soleus muscle (n=7-8), (C) triglycerides in liver (n=5-8), (D) representative immunoblot and densitometric quantification of JNK1/2 phosphorylation in liver (n=6-8), (E) mRNA levels of SREBP1c in liver (n=9-19), (F) mRNA levels of FAS in liver (n=9-19) during chow and high fat diet obtained from control (CON; black bars) and IL-18R<sup>−/−</sup> (grey bars) male mice at 7 months of age. Results are presented as mean±SEM, *P <0.05 vs. control, # P <0.05 vs. chow diet.

Figure 4. **IL-18R<sup>−/−</sup> mice exhibit blunted phosphorylation of ACCβ in muscle, liver, and adipose tissue.** (A) Representative immunoblot and densitometric quantification of ACCβ phosphorylation in soleus muscle (n=7-8), (B) representative immunoblot and densitometric quantification of ACCβ phosphorylation in liver (n=5-8), (C) representative immunoblot and densitometric quantification of ACCβ phosphorylation in adipose tissue (n=6-10) during chow and high fat diet obtained from control (CON; black bars) and IL-18R<sup>−/−</sup> (grey bars) male mice at 7 months of age. Results are presented as mean±SEM, *P <0.05 vs. control, # P <0.05 vs. chow diet.

Figure 5. **IL-18 increases fatty-acid oxidation and AMPK signaling in skeletal muscle in vitro and ex vivo.** (A) Representative immunoblot and densitometric quantification of AMPK Thr<sup>172</sup> in L6 cells stimulated with 1 ng/ml and 10 ng/ml IL-
18 for 30 min (n=9/dose), (B) representative immunoblot and densitometric quantification of ACCβ phosphorylation in L6 cells stimulated with 1 ng/ml and 10 ng/ml IL-18 for 30 min (n=9/dose), (C) fatty-acid oxidation in isolated soleus muscle strips in the presence of IL-18 (100 ng/mL) or PBS (UT) (n=10/group), (D) representative immunoblot and densitometric quantification of AMPK Thr172 phosphorylation in isolated soleus muscle strips in the presence of IL-18 (100 ng/mL) or PBS (UT) (n=6/group). Results are presented as mean±SEM, *P <0.05; **P <0.01 vs. Control (UT),

**Figure 6.** Mice over-expressing IL-18 in skeletal muscle are protected from Diet-induced obesity. (A) Densitometric quantification of IL-18 protein in the tibialis anterior muscle after DNA electrotransfer of 1 µg, 10 µg, and 20 µg plasmid (n=6/dose), (B) growth curves (n=6-10), (C) fat percentage (n=6-10), and (D) image of mice over-expressing IL-18 in to the tibialis anterior muscle (black bars) after 4 weeks of a high fat diet compared to mice over-expressing an empty vector (white bars). Results are presented as mean±SEM, *P <0.05 vs. empty vector.

**Figure 7.** Mice over-expressing IL-18 in skeletal muscle have enhanced AMPK signaling. (A) AMPKα1 activity (n=6/group), (B) representative immunoblot and densitometric quantification of ACCβ phosphorylation (n=6/group), (C) mRNA levels of β-HAD (n=8/group), and (D) mRNA levels of CPT-1β (n=7/group) in the muscle overexpressing IL-18 (black bars) compared to a muscle electroporated with an empty vector (white bars). RB = Reactive brown was used as loading control. Results are presented as mean±SEM, *P <0.05 vs. empty vector.
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Figure 1

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Figure 2

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Figure 3

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Figure 4
Figure 5

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Supplementary Figure 1. IL-18R<sup>-/-</sup> male mice<sup>-/-</sup> (grey bars) have decreased insulin sensitivity on a chow and a high fat diet compared to control (CON; black bars) at 7 months of age. Results are presented as mean±SEM. (n=6-9 per group)
**Supplementary Figure 2.** IL-18R⁻/⁻ mice have no change in phosphorylation of IKK in muscle. Representative immunoblot of IKK phosphorylation in soleus muscle (A) during chow and high fat diet obtained from control (CON; black bars) and IL-18R⁻/⁻ (grey bars) male mice at 7 months of age. Results are presented as mean±SEM, n = 4 animals per group.
Supplementary Figure 3. (A) Phosphoenolpyruvate carboxykinase (PEPCK) and (B) Glucose-6-phosphate dehydrogenase (G6Pase) in livers from IL-18R−/− male mice (grey bars) and control mice (CON; black bars) fed a chow or high fat diet (HFD). Results are presented as mean±SEM (n=6-8 mice per group).
Supplementary Figure 4. Mice electroporated with IL-18 had a lower visceral (A) and subcutaneous (B) fat mass weight compared to sham electroporated mice. Results are presented as mean±SEM, $n = 7$ for animals per group. ** $P < 0.01$ vs. Empty vector.
Supplementary Figure 5. Circulating plasma IL-18 (A), blood glucose during an insulin tolerance (B) and glucose tolerance (C) in mice transfected with IL-18 vector (gray) or empty vector (black) in the Tibialis Anterior. Results are presented as mean±SEM. (n=6-8 mice per group)
Supplementary Figure 6. Representative immunoblot and quantification of Akt phosphorylation (A) and AMPK and ACC phosphorylation (B) in the adipose tissue of mice transfected with IL-18 vector (gray) or empty vector (black) in the Tibialis Anterior. Results are presented as mean±SEM. (n=7-8 per group)
Supplementary Figure 7. Representative immunoblot and quantification of Akt phosphorylation (A) and JNK phosphorylation (B) in the liver of mice transfected with IL-18 vector (gray) or empty vector (black) in the Tibialis Anterior. Results are presented as mean±SEM. (n=7-8 per group)