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ARTICLE

Long-term treatment with interleukin-1β induces insulin resistance in murine and human adipocytes

C. Lagathu · L. Yvan-Charvet · J.-P. Bastard · M. Maachi · A. Quignard-Boulangé · J. Capeau · M. Caron

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Abstract
Aims/hypothesis Adipose tissue inflammation has recently been implicated in the pathogenesis of insulin resistance and is probably linked to high local levels of cytokines. IL1B, a proinflammatory cytokine, may participate in this alteration.
Materials and methods We evaluated the chronic effect (1–10 days) of IL1B (0.1–20 ng/ml) on insulin signalling in differentiating 3T3-F442A and differentiated 3T3-L1 murine adipocytes and in human adipocytes. We also assessed expression of the gene encoding IL1B in adipose tissue of wild-type and insulin-resistant mice (diet-induced and genetically obese *ob*/*ob* mice).
Results IL1B inhibited insulin-induced phosphorylation of the insulin receptor β subunit, insulin receptor substrate 1, Akt/protein kinase B and extracellular regulated kinase 1/2 in murine and human adipocytes. Accordingly, IL1B suppressed insulin-induced glucose transport and lipogenesis. Long-term treatment of adipose cells with IL1B decreased cellular lipid content. This could result from enhanced lipolysis and/or decreased expression of genes involved in lipid metabolism (acetyl-CoA carboxylase, fatty acid synthase). Down-regulation of peroxisome proliferating-activated receptor γ and CCAAT/enhancer-binding protein α in response to IL1B may have contributed to the altered phenotype of IL1B-treated adipocytes. Moreover, IL1B altered adipocyte differentiation status in long-term cultures. IL1B also decreased the production of adiponectin, an adipocyte-specific protein that plays a positive role in insulin sensitivity. Expression of the gene encoding IL1B was increased in epididymal adipose tissue of obese insulin-resistant mice.
Conclusions/interpretation IL1B is upregulated in adipose tissue of obese and insulin-resistant mouse models and may play an important role in the development of insulin resistance in murine and human adipose cells.

Keywords Adiponectin · Adipose tissue · Cytokine · Differentiation · Inflammation · Insulin signalling · Mouse

Abbreviations

ACAC	acetyl-CoA carboxylase
FABP4	adipocyte-specific fatty acid binding protein 4
C/EBP	CCAAT/enhancer-binding protein
ERK	extracellular regulated kinase
FASN	fatty acid synthase
HF	high fat
HOMA-IR	homeostasis model assessment–insulin resistance
IR	insulin receptor
IRS	insulin receptor substrate

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C. Lagathu · J.-P. Bastard · M. Maachi · J. Capeau · M. Caron (✉)
INSERM, U680, Université Pierre et Marie Curie (UPMC-Paris 6), Faculty of Medicine,
27 rue Chaligny,
75012 Paris, France
e-mail: caron@st-antoine.inserm.fr

J.-P. Bastard · M. Maachi · J. Capeau
Tenon Hospital, Department of Biochemistry and Hormonology,
Paris, France

L. Yvan-Charvet · A. Quignard-Boulangé
INSERM, U671, Université Pierre et Marie Curie (UPMC-Paris 6), Institut des Cordeliers,
Paris, France

LF	low fat
LPL	lipoprotein lipase
PKB	protein kinase B
PPAR	peroxisome proliferating-activated receptor
SLC2A4	solute carrier family 2 (facilitated glucose transporter), member 4 (previously known as GLUT4)
SREBP-1	sterol regulatory element-binding protein 1
WT	wild type

Introduction

Insulin resistance is a prominent feature of the metabolic syndrome, obesity and type 2 diabetes, and is a major risk for cardiovascular disease [1]. It has emerged that obesity and type 2 diabetes are associated with chronic inflammation in adipose tissue, which could play a role in insulin resistance. Increased expression in adipose tissue of key genes involved in inflammation pathways, such as those encoding cytokines and other macrophage-related factors, has been linked to obesity and insulin resistance in mouse and human studies [2–4]. Hence, some cytokines may play a key role in the pathogenesis of insulin resistance.

Many studies have provided clear evidence that circulating levels and adipose tissue expression of TNF-α and IL6 are elevated in obese subjects and subjects with type 2 diabetes [5–7]. Only a few studies have implicated IL1B in these diseases. Circulating levels of IL1B are correlated with the BMI of obese alcoholic subjects [8] and are increased in overweight and obese compared with lean subjects [9]. Moreover, individuals with a combined increase in IL1B and IL6 levels are at greater risk of developing type 2 diabetes than individuals with an increase in the IL6 level alone [10]. In adipose tissue from obese subjects, the total release of IL1B was comparable to that of TNF-α, and it originated from non-adipose cells [11]. Expression of *IL1B*, the human gene encoding IL1B, is increased in the visceral adipose tissue of obese subjects [12]. In addition, treatment of human adipose tissue explants with IL1B decreased the level of mRNA for adiponectin [13], an adipocyte-secreted protein that plays a positive role in insulin sensitivity [14, 15]. In adipose tissue and isolated adipocytes, IL1B has been shown to upregulate the release and expression of IL6 [16, 17]. IL1B could also play a role in the increased production of monocyte chemoattractant protein 1 [18, 19], a chemokine involved in the recruitment of macrophages to inflammation sites and the mRNA content of which is elevated in adipose tissue of obese subjects [20]. Thus, IL1B may have a permissive role in the IL6-mediated acute-phase response that precedes the onset of type 2 diabetes [5, 10]. IL1 receptor antagonist, which inhibits the binding of IL1A and IL1B to their receptors, is also overexpressed in the adipose tissue of mice with diet-induced and genetic obesity, as it is also in the subcutaneous adipose tissue of obese patients [12, 21, 22]. These increased levels of IL1 receptor antagonist in human obesity could contribute to the development of insulin resistance [22]. All these findings support the hypothesis that IL1 signalling pathways—more specifically, IL1B signalling in adipose tissue—may play an important role in obesity-linked insulin resistance.

IL1B, IL6 and TNF-α are produced mainly by macrophages activated during the inflammatory process [23, 24], but adipocytes and preadipocytes also produce these proinflammatory cytokines [25–27]. Many studies have shown that TNF-α and IL6 induce insulin resistance in adipocytes, including at the insulin receptor level [25, 28–30], but no data are available on the direct impact of IL1B on insulin signalling in adipocytes.

We examined whether long-term treatment of murine adipocytes (differentiating 3T3-F442A cells and differentiated 3T3-L1 adipocytes) and primary human adipocytes with IL1B (0.1–20 ng/ml) affected insulin signalling and insulin-dependent processes (glucose transport and lipogenesis). We then examined the intracellular lipid content and lipid metabolism of IL1B-treated cells. Finally, we investigated whether the expression of *Il1b* is increased in adipose tissue from mice with genetic (*ob*/*ob*) or diet-induced insulin resistance.

Materials and methods

Cell culture and treatment

Murine 3T3-F442A and 3T3-L1 preadipocytes were cultured and induced to differentiate as described previously [26]. Human preadipocytes (Zen-Bio, Research Triangle Park, NC, USA) were maintained in DMEM/Ham's F-12 with 10% fetal bovine serum in 5% CO_2 at 37°C. When the cells became 90% confluent, differentiation to adipocytes was induced using DMEM/F-12 containing 3% fetal bovine serum, 10 nmol/l insulin, 1 μmol/l dexamethasone, 0.20 mmol/l isobutylmethylxanthine (IBMX) and 10 μmol/l rosiglitazone. After 3 days, IBMX and rosiglitazone were removed from the medium. Recombinant mouse IL1B was purchased from R&D Systems (Minneapolis, MN, USA). The effect of IL1B (0.1–20 ng/ml) was tested in long-term experiments with differentiating 3T3-F442A cells (from day 0 [confluence] to day 8 of differentiation), differentiated 3T3-L1 adipocytes (from days 8 to 14 or 18 of differentiation) and differentiated human adipocytes (from days 22 to 28 of differentiation).

The cytotoxicity of IL1B was evaluated with the 3-[4,5-2-yl]-2,5 diphenyltetrazolium bromide (MTT) test on day 8 (3T3-F442A cells), days 14 and 18 (3T3-L1 cells) and day 28 (human adipocytes) of differentiation.

Western blotting

Cells were solubilised in Laemmli buffer with 100 mmol/l dithiothreitol. Cell lysates (10^4 cells) were subjected to SDS–PAGE and Western blotting with antibodies to sterol regulatory element-binding protein 1 (SREBP-1) (antibody K-10), CCAAT/enhancer-binding protein (C/EBPα) (C-19), peroxisome proliferating-activated receptor (PPAR)γ (H-100), C/EBPβ (C-19) and solute carrier family 2 (facilitated glucose transporter), member 4 (SLC2A4, previously known as GLUT4) (H-61), obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cell lysates were prepared at day 8 (3T3-F442A cells), 14 (3T3-L1 adipocytes) or 28 (human adipocytes) of differentiation, using cells cultured for 18 h in serum-free medium. Cells were stimulated for 10 min with 100 nmol/l insulin. Aliquots of cell lysates were immunoblotted with an anti-phosphotyrosine antibody (PY-99), with antibodies anti-phospho extracellular regulated kinase (ERK)1/2-tyr204 (E-4) (Santa Cruz Biotechnology) or anti-phospho-Akt-ser473 (catalogue no. 9271) (Cell Signaling Technology, Danvers, MA, USA). Protein expression was checked by using antibodies directed against insulin receptor (IR) β (C-19), insulin receptor substrate (IRS)-1 (C-20), ERK1/2 (C-16) (Santa Cruz Biotechnology) or Akt/protein kinase B (PKB) (catalogue no. 9272; Cell Signaling Technology). Immune complexes were visualised with a chemiluminescence method (ECL kit; Amersham Biosciences, Saclay, France). Protein expression, determined by Western blotting, was normalised to the cell number. The relative protein expression in each sample was quantified by chemiluminescence using a ChemiGenius2 image analyser and software (Ozyme, St Quentin en Yvelines, France).

Lipogenesis and glucose transport

Insulin-stimulated lipogenesis and glucose transport were studied on day 8 (3T3-F442A cells) or 14 (3T3-L1 adipocytes) of differentiation in cells cultured for 18 h in serum-free medium, as described previously [25]. Glucose transport results were expressed as pmol of 2-deoxy-glucose per 10 cells per 5 min ± SEM, and lipogenesis as pmol of glucose incorporated into lipids per 10^6 cells per h ± SEM.

Oil Red O lipid staining and lipolysis

On day 8 (3T3-F442A cells), 14 and 18 (3T3-L1 cells) or 28 (human adipocytes) of differentiation, lipid accumulation was assessed by lipid staining with Oil Red O. Staining was quantified at 520 nm after solubilisation in 10% SDS. Results are expressed as the percentage ± SEM of the untreated control value (100%). Glycerol release was assessed in 24-h culture supernatants by using the enzymatic BioAnalysis kit from Boehringer Mannheim (Darmstadt, Germany). Results were expressed as μg of glycerol per 10^6 cells per 24 h ± SEM.

Adiponectin measurements

Adiponectin concentrations were determined on days 8 (3T3-F442A cells), 14 (3T3-L1 cells) and 28 (human adipocytes) of differentiation in 24-h supernatants by using the ELISA murine kit from B-Bridge International (Sunnyvale, CA, USA) or the ELISA human kit from R&D Systems.

Animal study

Male C57BL/6 mice were fed from weaning to 13 weeks of age with either a low-fat diet (Wild-type [WT]-LF; 4% fat wt/wt) or a high-fat diet (WT-HF; 25% fat wt/wt), as described previously [31]. Thirteen-week-old C57BL/6 *ob*/*ob* male mice and littermate controls were purchased from Charles River Laboratories (Wilmington, MA, USA). All animal experiments were performed according to the French guidelines for care and use of experimental animals. Blood was collected into heparinised tubes by cardiac puncture. Fasting glucose was assayed with a glucometer (Roche Diagnostics, Meylan, France) and fasting insulin was determined by radioimmunoassay (CIS Biointernational, Gif sur Yvette, France). Insulin resistance was quantified in terms of homeostasis model assessment–insulin resistance (HOMA-IR) as fasting glucose (mmol/l) × fasting insulin (mU/l)/22.5.

RNA preparation and real-time RT-PCR

Total RNA was extracted from 3T3-F442A (day 8) and 3T3-L1 (day 14) cells and human adipocytes (day 28) and cDNA was synthesised as described previously [26]. Total RNA was isolated from mouse epididymal adipose tissue as described previously [32] and cDNA was synthesised from 1 μg of total RNA with Superscript reverse transcriptase (Invitrogen, Cergy-Pontoise, France). Real-time PCR was performed with the LightCycler system (Roche Diagnostics, Meylan, France) and the LightCycler SYBR green fluorophore. A list of primer sequences is given in the Electronic Supplementary Material (ESM). Results were expressed as percentage ± SEM of untreated control values (100%) normalised to 18S RNA expression.

Statistical analysis

Results are mean ± SEM of the indicated number of independent experiments. Statistical significance was determined with parametric (Student's *t*) and non-parametric (Mann–Whitney *U*) tests, as appropriate. The significance of correlations was determined by using the non-parametric Spearman's rank correlation test. The threshold of significance was set at $p=0.05$.

Results

IL1B induces insulin resistance in murine 3T3-F442A and 3T3-L1 adipocytes

We first examined whether long-term treatment with IL1B altered insulin signal transduction in differentiating 3T3-F442A (8 days of treatment) and differentiated 3T3-L1 adipocytes (6 days of treatment). Western blot analysis showed that IL1B (10 or 20 ng/ml) did not alter the expression of IRβ (Fig. 1a,b) or its major substrate, IRS-1 (Fig. 1c,d). However, IL1B inhibited acute activation by insulin of tyrosine phosphorylation of IRβ and IRS-1 in both cell lines, in a concentration-dependent manner (Fig. 1a–d). Furthermore, in differentiating and differentiated adipocytes, IL1B reduced by 40–75% the insulin-induced activation of Akt/PKB, a key enzyme of the insulin signalling pathway mainly involved in short-term metabolic responses (Fig. 1e,f); it also reduced by 45–75% the insulin-induced activation of ERK1/2, a mitogen-activated protein kinase that mediates part of the insulin response on transcription (Fig. 1g,h). Lower concentrations of IL1B (0.1 and 1 ng/ml) also inhibited acute insulin-induced phosphorylation of Akt/PKB (by 25 and 35%, respectively) and ERK1/2 (by 20 and 40%, respectively) in 3T3-L1 adipocytes. Shorter times of incubation with IL1B (24 h) also blunted insulin-induced phosphorylation of Akt/PKB (by 50, 60 and 80% at 1, 10 and 20 ng/ml, respectively) and ERK1/2 (by 30, 55 and 65% at 1, 10 and 20 ng/ml, respectively). These results indicated that IL1B can induce insulin resistance after incubation for 24 h and at low concentrations.

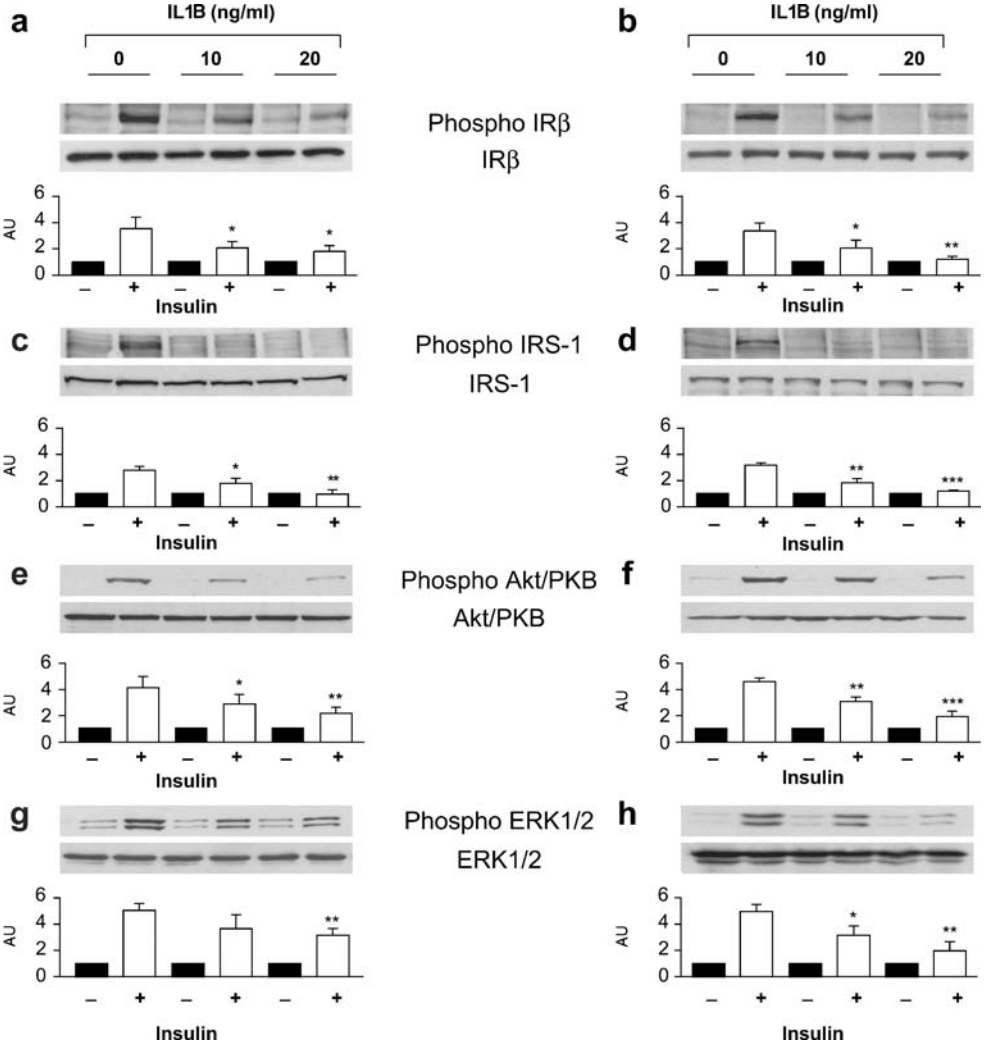

Fig. 1 IL1B alters insulin signalling in 3T3-F442A and 3T3-L1 adipocytes. Differentiating 3T3-F442A cells (from days 0 to 8) and fully differentiated 3T3-L1 adipocytes (from days 8 to 14) were treated with IL1B at the concentration indicated. On day 8 (3T3-F442A) (**a, c, e, g**) or 14 (3T3-L1) (**b, d, f, h**) of differentiation, cells were stimulated for 10 min with 100 nmol/l insulin. Proteins were extracted and analysed by immunoblotting. Representative blots are shown of **a**, **b** the insulin receptor β subunit (*IRβ*) and its insulin-stimulated tyrosine phosphorylation (*Phospho-IRβ*), **c**, **d** IRS-1 and its insulin-stimulated tyrosine phosphorylation (*Phospho-IRS-1*), **e**, **f** Akt/PKB and its phosphorylated form (*Phospho Akt/PKB*) and **g, h** ERK1/2 and its phosphorylated form (*Phospho ERK1/2*). Scanned data, expressed in arbitrary units (*AU*), are shown below and represent insulin-stimulated samples compared with their respective control sample (1.0) normalised to their protein level. Results are mean ± SEM of three or four experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$

We then examined the effect of IL1B on lipogenesis and glucose transport (Fig. 2). IL1B significantly altered basal glucose transport in differentiating 3T3-F442A cells but not in differentiated 3T3-L1 cells, and strongly inhibited insulin-stimulated glucose transport in both cell lines (by up to 86% in 3T3-F442A cells and up to 84% in 3T3-L1 cells) (Fig. 2a,b). IL1B had only a moderate effect on *Slc2a4* mRNA expression (Table 1) and no effect on SLC2A4 protein level (Fig. 2c,d), suggesting that IL1B altered the insulin-induced membrane translocation of SLC2A4. Moreover, IL1B did not modify the basal level of lipogenesis in 3T3-F442A or 3T3-L1 cells, but markedly reduced insulin-induced lipogenesis in both cell lines (by up to 96% in 3T3-F442A cells and up to 93% in 3T3-L1 cells) (Fig. 2e,f). This is in agreement with the decreased level of transcripts of the genes encoding lipogenic enzymes (fatty acid synthase [*Fasn*] and acetyl-CoA carboxylase [*Acac*]) in both cell lines treated by IL1B (Table 1).

IL1B reduces lipid content in murine differentiating 3T3-F442A and differentiated 3T3-L1 adipocytes

In 3T3-F442A cells, IL1B prevented the lipid accumulation that normally occurs during the differentiation process, as shown by a 30 and 50% decrease in Oil Red O lipid staining after 8 days of treatment at 10 and 20 ng/ml, respectively (Fig. 3a). The effect of IL1B was time-dependent in differentiated 3T3-L1 adipocytes. Six days of treatment of 3T3-L1 cells with IL1B did not significantly alter the lipid content (Fig. 3b). By contrast, the lipid content fell in 3T3-L1 cells treated for 10 days (by 43 and 60% at 10 and 20 ng/ml, respectively) (Fig. 3b). This was confirmed by the ability of IL1B to increase the basal rate of lipolysis (glycerol release) in 3T3-L1 adipocytes (2.2- to 2.8-fold after 6 days; 2.4- to 3.4-fold after 10 days at 10 and 20 ng/ml, respectively) (Fig. 3d). IL1B had no effect on the basal rate of lipolysis in 3T3-F442A differentiating adipocytes (Fig. 3c) or on the mRNA expression of hormone-sensitive lipase

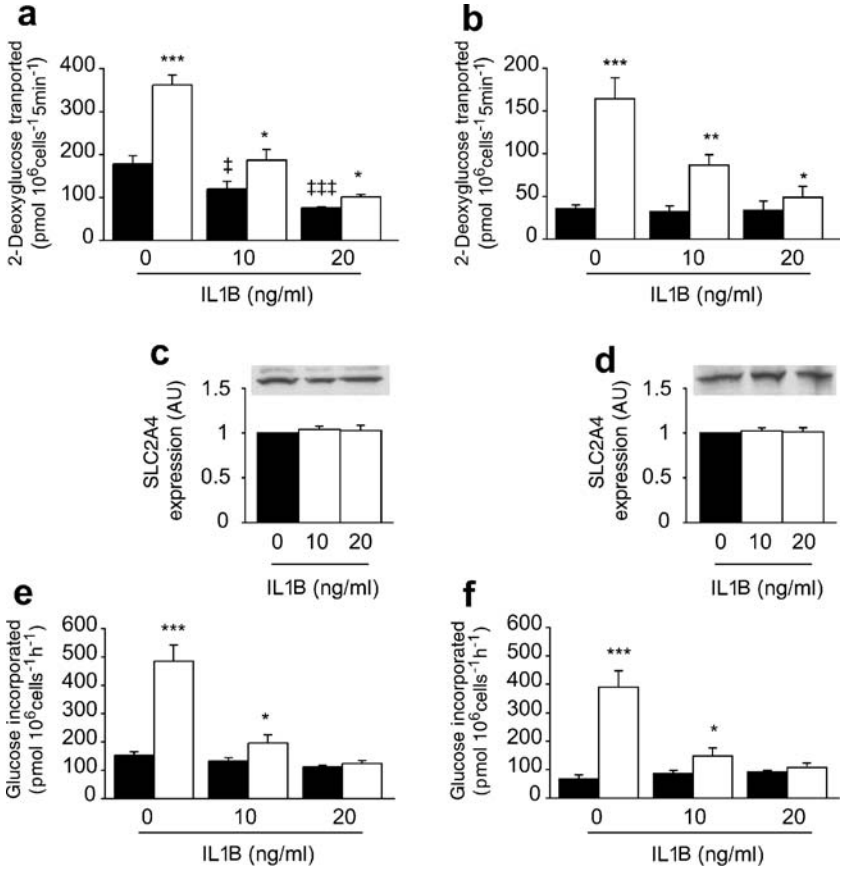

Fig. 2 IL1B alters insulin-stimulated lipogenesis and glucose transport in 3T3-F442A and 3T3-L1 adipocytes. Murine adipocytes were treated with IL1B as described (Fig. 1 legend). On day 8 (3T3-F442A) (**a**) or 14 (3T3-L1) (**b**) 2-deoxy-D-[^{14}C]-glucose transport was evaluated (see Materials and methods). Results ± SEM. On day 8 (3T3-F442A) (**c**) or 14 (3T3-L1) (**d**), proteins were extracted from IL1B-treated and untreated cells and analysed by immunoblotting; representative immunoblots of SLC2A4 and the scanned data (arbitrary units [*AU*]) of three experiments are also shown. On day 8 (3T3-F442A) (**e**) or 14 (3T3-L1) (**f**) of differentiation, [^{14}C]-glucose incorporation into lipids was evaluated (see Materials and methods). Results ± SEM. The experiments were repeated three times on triplicate samples. Insulin-stimulated values (*open bars*) vs basal values (*solid bars*): *p<0.05, **p<0.01, ***p<0.001. Control basal value vs IL1B-treated basal value: ‡p<0.05, ‡‡‡p<0.001

Table 1 Chronic effects of IL1B on the expression of adipogenic markers in 3T3-F442A cells and 3T3-L1 adipocytes

	3T3-F442A cells			3T3-L1 cells		
	IL1B			IL1B		
	None	10 ng/ml	20 ng/ml	None	10 ng/ml	20 ng/ml
Slc2a4	100	89.6±30.9	76.0±14.5[a]	100	91.0±9.3	78.3±15.3
Fasn	100	70.2±6.9[b]	51.8±8.1[c]	100	46.8±4.8[b]	51.5±6.5[c]
Acac	100	65.0±8.4[a]	53.6±14.2[a]	100	73.5±18.6	44.2±9.7[c]
Fabp4	100	84.7±6.5	59.0±4.1[b]	100	83.2±16.1	66.3±12.9[a]
Lpl	100	64.5±10.8[a]	48.8±13.7[a]	100	76.0±6.7[a]	65.8±7.3[b]

mRNA levels, normalised to 18S rRNA expression, were determined relative to untreated cells (100). Results are expressed as percentage ± SEM of control values in three experiments performed in triplicate

[a] $p<0.05$, [b] $p<0.01$, [c] $p<0.001$ vs untreated cells

(*Hsl*) in either differentiating or differentiated cells (data not shown). The expression of major lipid markers (*Fasn*, *Acac*, *Lpl* [lipoprotein lipase] and adipocyte-specific fatty acid binding protein [*Fabp4*]) was reduced in cells treated during differentiation (3T3-F442A) or after completion of differentiation (3T3-L1) (Table 1), which may contribute to the decreased lipid content observed in both cell lines.

As the lipid content and insulin sensitivity of adipocytes are closely related to the differentiation status of the cells, we examined the protein level of the main transcription factors involved in adipogenesis. In differentiating 3T3-F442A adipocytes, IL1B reduced PPARγ and C/EBPα levels and prevented the differentiation-associated fall in C/EBPβ, without affecting SREBP-1 (Fig. 4a). These results suggest that IL1B can partly alter the adipose cell differentiation programme. By contrast, differentiated 3T3-L1 adipocytes treated with IL1B for 6 days showed normal levels of these transcription factors (Fig. 4b). Ten days of

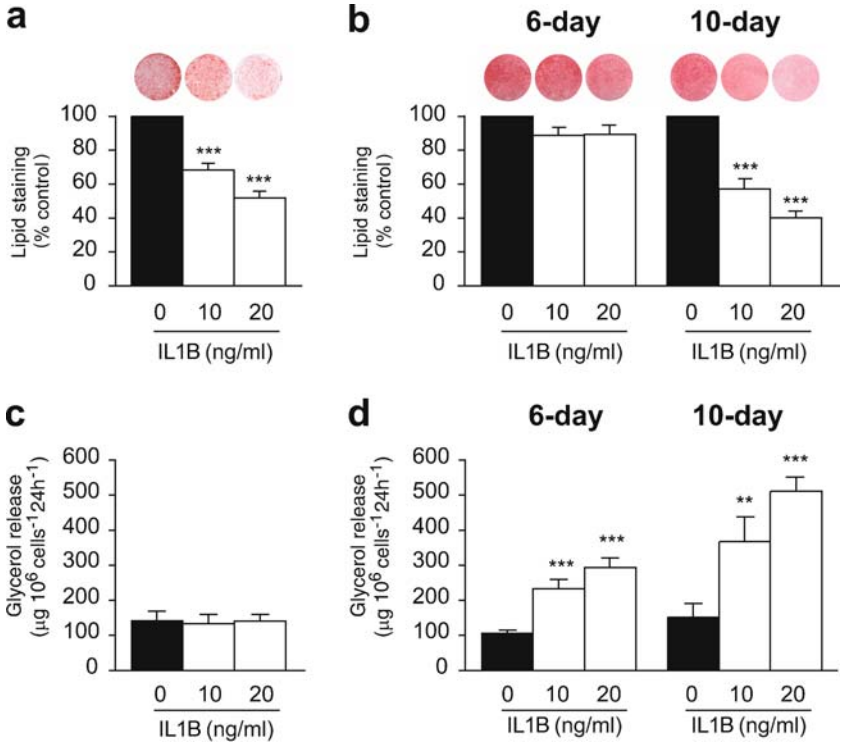

Fig. 3 IL1B alters the lipid status of 3T3-F442A and 3T3-L1 adipocytes. Murine adipocytes were treated with IL1B as described (legend Fig. 1). After 8 days (3T3-F442A) (**a**) or 6 or 10 days (3T3-L1) (**b**) of IL1B treatment, cells were fixed and stained with Oil Red O. Staining was quantified at 520 nm and expressed as the percentage of untreated controls ± SEM for three experiments performed in duplicate. After 8 days (3T3-F442A) (**c**) and 6 or 10 days (3T3-L1) (**d**) of IL1B treatment, the medium was replaced and, 24 h later, basal glycerol release was determined by using an enzymatic bio-analysis kit (see Materials and methods). Results ± SEM for three or four experiments performed in duplicate. $^{**}p<0.01$, $^{***}p<0.001$ vs control (*filled bars*)

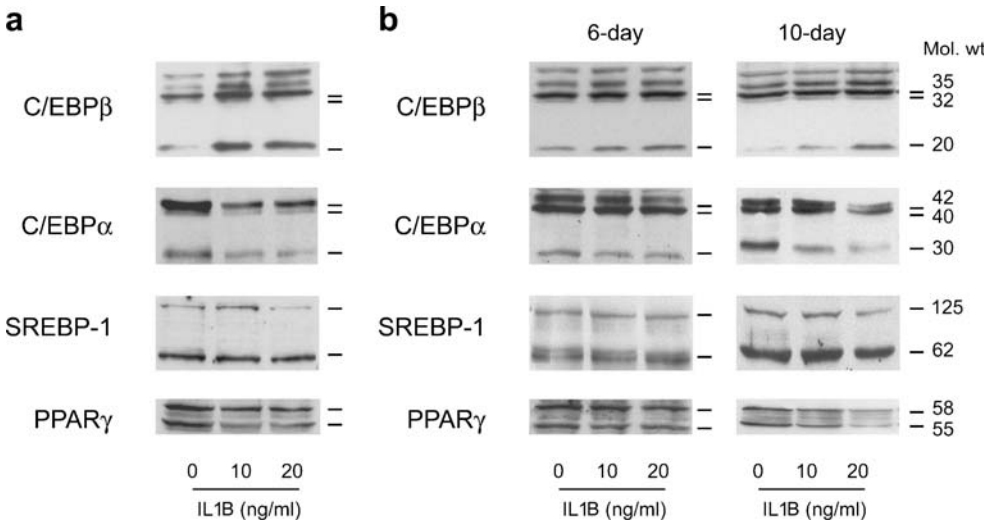

Fig. 4 IL1B decreases the level of adipogenic transcription factors in 3T3-F442A cells and 3T3-L1 adipocytes. Murine adipocytes were treated with IL1B (see Fig. 1). After 8 days (3T3-F442A) (**a**) or 6 or 10 days (3T3-L1) (**b**), IL1B-treated and untreated cells were solubilised and aliquots were immunoblotted with anti-C/EBPβ, C/EBPα, SREBP-1 and PPARγ antibodies. A representative immunoblot from three separate experiments is shown

treatment with IL1B reduced C/EBPα and PPARγ and enhanced C/EBPβ (Fig. 4b), in keeping with the altered lipid phenotype of 3T3-L1 cells. These results are consistent with the observed insulin-resistant state of 3T3-L1 adipocytes, as C/EBPα and PPARγ are involved both in the final stages of the differentiation process and in insulin sensitivity [33], and suggest that long-term treatment with IL1B partly alters the differentiation status of the cells.

IL1B induces insulin resistance and decreases lipid content in primary human adipocytes

We next investigated the effect of IL1B in differentiated primary human adipocytes. As shown in Fig. 5, IL1B treatment (from days 22 to 28) at different concentrations (0.1–20 ng/ml) did not alter the protein levels of the insulin signalling components IRβ, IRS-1, Akt/PKB and ERK1/2 in human adipocytes. However, IL1B at 1, 10 and 20 ng/ml blunted the ability of insulin to increase the tyrosine phosphorylation of IRβ (Fig. 5a) and IRS-1 (Fig. 5b) and decreased the insulin-induced activation of Akt/PKB (Fig. 5c) and ERK1/2 (Fig. 5d). As observed in the murine adipose cell lines, IL1B decreased the expression of the major transcription factors C/EBPα and PPARγ (at 10 and 20 ng/ml) and increased the level of the early transcription factor C/EBPβ (Fig. 6a) in human primary adipocytes. Chronic IL1B (20 ng/ml) treatment also decreased SREBP-1 and the mRNA expression of *SLC2A4* and of several proteins involved in lipid metabolism (*FASN*, *ACAC*, *FABP4* and *LPl*) (Table 2), showing that IL1B altered the lipid status of human adipocytes. Accordingly, IL1B decreased cellular lipid content evaluated by lipid staining with Oil Red O (by 35, 45 and 55% at 1, 10 and 20 ng/ml, respectively) (Fig. 6b). Thus, as in murine adipocytes, IL1B induced insulin resistance and altered lipid metabolism and the differentiation status in primary human adipocytes.

IL1B suppresses adiponectin production by murine and human adipocytes

As shown in Fig. 7, IL1B markedly reduced adiponectin secretion by differentiating 3T3-F442A and fully differentiated 3T3-L1 adipocytes (by 50–60 and 50–70%, respectively) (Fig. 7a,b). IL1B also dramatically decreased adiponectin secretion by human primary adipocytes by 80–90% (Fig. 7c), which reinforces the relevance of our results to the potential role of IL1B in insulin resistance in human pathophysiology. Accordingly, adiponectin expression fell by 75–85% in differentiating 3T3-F442A cells (Fig. 7d), by 45–65% in differentiated 3T3-L1 adipocytes (Fig. 7e), and by 60–80% in human primary adipocytes treated with IL1B (Fig. 7f).

Elevated IL1B expression in adipose tissue of insulin-resistant mice

To further study the involvement of IL1B in insulin resistance, including obesity-induced insulin resistance, we measured *Il1b* expression in epididymal adipose tissue of mice receiving a high-fat diet (WT-HF) and of genetically obese mice (*ob*/*ob*) [34]. After 12 weeks of high-fat feeding, WT-HF mice displayed increased epididymal fat pad weight (1.50-fold) and fasting plasma insulin level (1.85-fold) when compared with wild-type mice fed a low-fat diet (WT-LF) (Table 3). When compared with their wild-type littermates, *ob*/*ob* mice exhibited higher epididymal fat pad weight (10.5-fold) and fasting plasma insulin (13.1-fold). As expected, the HOMA-IR [35] index was higher in WT-HF than in WT-LF mice (38.9 vs 21.2) and was higher in *ob*/*ob* than in wild-type mice (231.0 vs 19.0)

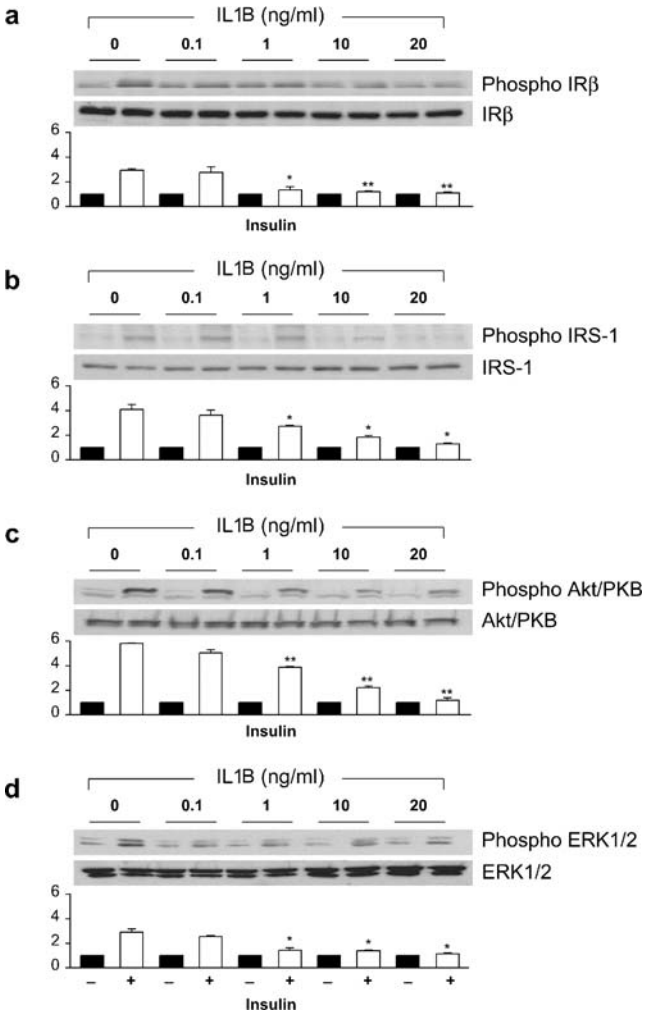

Fig. 5 IL1B alters insulin signalling in primary human adipocytes. Human adipocytes were treated with IL1B from days 22 to 28 at the concentration indicated. On day 28, cells were cultured for 18 h in serum-free medium and stimulated for 10 min with 100 nmol/l insulin. Proteins were extracted and analysed by immunoblotting. Representative blots are shown of (**a**) the insulin receptor β subunit (*IRβ*) and its insulin-stimulated tyrosine phosphorylation, (**b**) IRS-1 and its insulin-stimulated tyrosine phosphorylation, (**c**) Akt/PKB and its phosphorylated form, and (**d**) ERK1/2 and its phosphorylated form. Scanned data expressed in arbitrary units are shown below and represent insulin-stimulated samples compared with their respective control sample (1.0) normalised to their protein expression. Results are mean ± SEM for two or three experiments. *$p<0.05$, **$p<0.01$

(Table 3). Expression of the gene for IL1B in epididymal adipose tissue was increased 3.3-fold ($p=0.006$) in WT-HF vs WT-LF mice and 3.5-fold ($p=0.01$) in *ob*/*ob* vs wild-type mice (Fig. 8a). *Il1b* mRNA content correlated with the HOMA-IR index in WT-HF and WT-LF mice ($r=0.727$, $p=0.022$). Moreover, adiponectin mRNA expression was decreased in both WT-HF vs WT-LF mice (by 65%) and *ob*/*ob* vs wild-type mice (by 80%) (Fig. 8b). Adipose tissue expression of the lipogenic enzyme FASN was also decreased by 60 to 90% in both mouse models of insulin resistance, and LPL was decreased in *ob*/*ob* mice compared with WT littermates (Fig. 8c,d). Taken together, these results indicate that IL1B could contribute to the development of insulin resistance.

Discussion

Inflammation and macrophage infiltration of adipose tissue is suspected to play a key role in the pathogenesis of insulin resistance [3, 4]. Upon activation, macrophages secrete several proinflammatory cytokines, such as TNF-α, IL1B and IL6 [23, 24]. Adipose tissue inflammation is characterised by high secretion levels of IL6 and TNF-α that contribute to adipocyte insulin resistance [25, 28, 29, 36, 37]. However, the direct effect of IL1B in insulin resistance in adipose cells has never been studied. We therefore investigated the impact of long-term treatment (24 h and 6–8 days) with IL1B (from 0.1 to 20 ng/ml) on the development of insulin resistance in human adipocytes and measured *Il1b* mRNA expression in the adipose tissue of two insulin-resistant mouse models.

We found that IL1B induced insulin resistance in both differentiating and differentiated cultured adipocytes. Indeed, long-term treatment with IL1B during the differentiation programme (3T3-F442A cells for 8 days) or after completion of adipogenesis (3T3-L1 cells and human adipocytes for 6 days) induced cellular insulin resistance. IL1B acted at the early steps of insulin signalling by affecting the tyrosine phosphorylation of IRβ and its major substrate IRS-1 in all cell lines, in keeping with a recent study performed on a rat pancreatic cell line [38]. Moreover, insulin failed to activate Akt/PKB and ERK1/2, which is consistent with its negative effect on insulin-induced glucose transport and lipogenesis.

In a more acute condition (24 h of treatment), IL1B induced insulin resistance in 3T3-L1 differentiated cells, as shown by the marked inhibition of the insulin-induced phosphorylation of Akt/PKB and ERK1/2. IL-1α, like IL1B, can rapidly decrease IRS-1 insulin-induced tyrosine phosphorylation in 3T3-L1 differentiated adipocytes [39], suggesting that both IL-1α and IL-1β, which act through the same receptor, can trigger insulin resistance in cultured adipocytes.

IL1B altered the lipid content of both murine and primary human adipose cell lines. In 3T3-F442A cells, IL1B impeded lipid accumulation during the differentiation process, whereas in 3T3-L1 mature adipocytes the effect of IL1B on the cellular lipid content was dependent on the length of the treatment. Indeed, a 6-day treatment affected the 3T3-L1 cell lipid content only slightly, but a further 4-day treatment (10 days in total) induced a 60% fall in lipid content. This was probably due to IL1B increasing the basal rate of lipolysis and decreasing the level of mRNAs

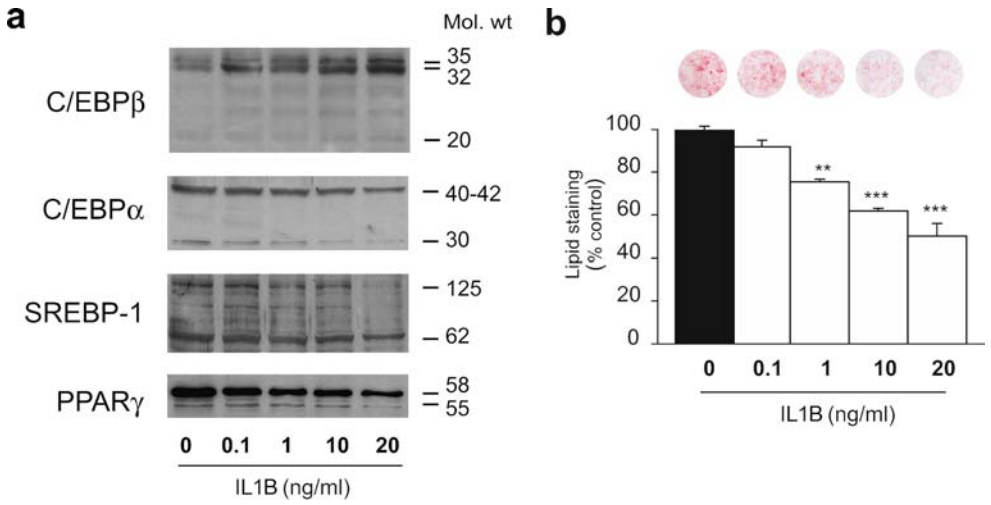

Fig. 6 IL1B decreases the level of adipogenic transcription factors and cellular lipid content in primary human adipocytes. Human adipocytes were treated with IL1B (see Fig. 5). **a** IL1B-treated and untreated adipocytes were solubilised and aliquots were immunoblotted with anti-C/EBPβ, C/EBPα, SREBP-1 and PPARγ antibodies. A representative immunoblot from three separate experiments is shown. **b** Cells were fixed and stained with Oil Red O. Staining was quantified at 520 nm and expressed as the percentage of untreated controls ± SEM for three experiments performed in duplicate. $^{**}p<0.01$, $^{***}p<0.001$ vs control (*filled bars*)

encoding lipogenic enzymes (*Fasn* and *Acac*), and of other genes encoding proteins involved in lipid metabolism (*Fabp4* and *Lpl*). Human primary adipocytes treated with IL1B also exhibited decreased lipid accumulation and expression of genes involved in lipid metabolism.

In 3T3-F442A differentiating adipocytes, IL1B impeded the differentiation programme, as shown by the decreased levels of PPARγ and C/EBPα and the increased level of C/EBPβ. In 3T3-L1 differentiated adipocytes, C/EBPα or PPARγ, two transcription factors that are involved in terminal differentiation, maintenance of the adipocyte phenotype, and insulin sensitivity [33, 40] were not affected by 6 days of treatment with IL1B, although the cells already displayed strong insulin resistance. The expression of adipogenic markers that increase with adipogenesis (*Fasn*, *Acac*, *Lpl* and *Fabp4*) was also affected after a 6-day treatment of 3T3-L1 cells with IL1B, while differentiation was unaltered. Thus, under these conditions, IL1B appears to act on the establishment and maintenance of insulin responsiveness rather than on the adipocyte differentiation programme itself. In contrast, 10 days of treatment with IL1B significantly reduced C/EBPα and PPARγ and increased C/EBPβ, indicating an alteration of the 3T3-L1 differentiation status. Similarly, in primary human adipocytes chronically treated (6 days) with IL1B at high concentrations (10 and 20 ng/ml), C/EBPβ increased and PPARγ, C/EBPα and SREBP-1 decreased, which suggests dedifferentiation of these adipocytes.

The circulating adiponectin level is an index of insulin sensitivity [14, 15]. Here we found that in both differentiating 3T3-F442A cells and fully differentiated 3T3-L1 adipocytes, and in human adipocytes, IL1B strongly attenuated adiponectin expression and secretion. Similarly it has been shown that TNF-α and IL6 strongly suppress

Table 2 Chronic effects of IL1B on the expression of adipogenic markers in primary human adipocytes

	Primary human adipocytes				
	IL1B (ng/ml)				
	None	0.1	1	10	20
SLC2A4	100	103.1±6.0	96.4±3.6	77.8±2.9[a]	56.7±4.9[a]
FASN	100	99.0±5.3	55.3±6.6[a]	36.4±2.1[a]	34.9±2.0[b]
ACAC	100	93.3±4.7	81.6±10.2	70.1±3.7[a]	65.0±3.4[b]
FABP4	100	102.2±5.3	91.2±8.1	46.7±1.6[b]	43.9±5.2[a]
LPL	100	52.1±4.4[a]	28.4±2.0[b]	20.2±6.9[a]	10.9±2.3[b]

mRNA levels, normalised to 18S rRNA expression, were determined relative to untreated cells (100). Results are expressed as percentage ± SEM of control values in three experiments performed in duplicate

[a] $p<0.01$, [b] $p<0.001$ vs untreated cells

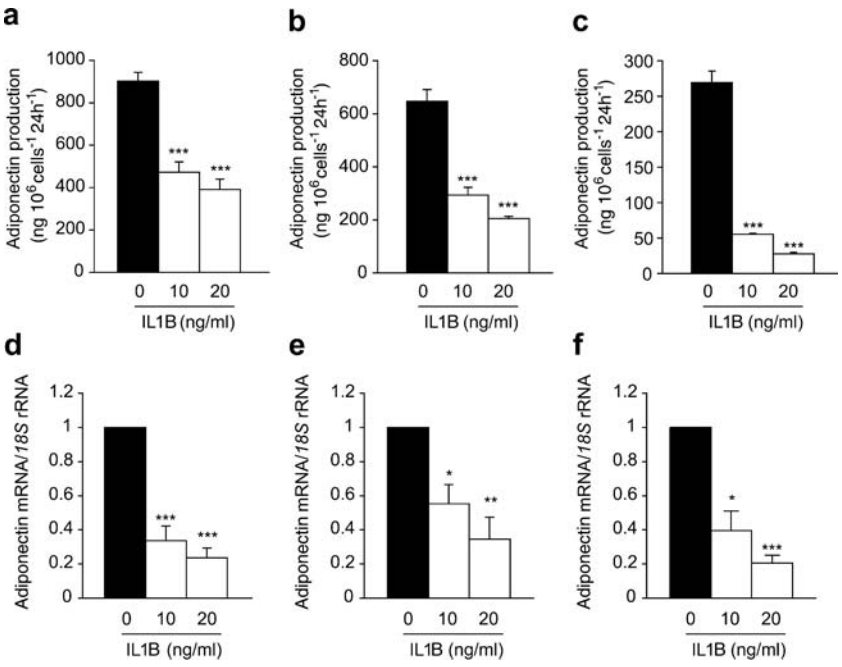

Fig. 7 IL1B decreases adiponectin secretion and expression in 3T3-F442A, 3T3-L1 and human primary adipocytes. Differentiating 3T3-F442A cells, fully differentiated 3T3-L1 adipocytes and differentiated human adipocytes were treated with IL1B. On day 8 (3T3-F442A) (**a**), 14 (3T3-L1) (**b**) or 28 (human adipocytes) (**c**) of differentiation, the medium was replaced and 24 h later the level of adiponectin in the cell medium was determined by ELISA. Results ± SEM of three experiments performed in duplicate. Total RNA was extracted from (**d**) 3T3-F442A (**e**) 3T3-L1 and (**f**) human adipocytes and adiponectin mRNA levels normalised to 18S rRNA expression were determined relative to untreated control cells. Results are percentage ± SEM of control values in three or four experiments performed in duplicate. *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs control (*filled bars*)

adiponectin production [41], consistent with the antagonism between the action of adiponectin and proinflammatory cytokines [42]. Since IL1B can increase IL6 secretion in cultured adipocytes [17], part of the IL1B effect could be mediated by upregulation of IL6 release. Our finding that IL1B is also a potent inhibitor of adiponectin production in murine and human adipose cells is in line with data showing that IL1B suppresses the mRNA expression of adiponectin in human adipose tissue explants [13].

Finally, our results obtained with insulin-resistant mouse models add further evidence for the role of IL1B as an important mediator of insulin resistance in vivo. Indeed, IL1B expression is increased three-fold in the epididymal adipose tissue of both genetic obese/insulin-resistant mice (*ob*/*ob*) and mice with diet-induced insulin resistance (WT-HF). These results are not in accordance with those found in a previous study that failed to find a significant increase of IL1B expression in epididymal adipose tissue of *ob*/*ob* mice [21]. The increased expression of IL1B in the adipose tissue of insulin-resistant mice reported in the present study is in agreement with the decreased mRNA expression of adiponectin, *Fasn* and *Lpl*. Moreover, IL1B expression in mouse adipose tissue was related to the degree of insulin resistance, as measured by the HOMA-IR index, supporting

Table 3 Metabolic parameters of insulin-resistant mouse models

	WT-LF (n=6)	WT-HF (n=6)	WT (n=7)	*ob/ob* (n=9)
Epididymal fat pad weight (g)	0.28±0.02	0.42±0.03[a]	0.45±0.8	2.83±0.19[b]
Fasting insulin (mU/l)	42.9±2.2	76.6±24.8[a]	38.9±11.1	510.3±169.9[b]
Fasting glucose (mmol/l)	11.1±0.7	11.29±0.3	11.0±0.8	11.0±0.9
HOMA-IR	21.2±1.8	38.9±10.9[a]	19.0±5.5	231.0±67.5[b]

All metabolic parameters were measured as indicated in Materials and methods. Insulin resistance was quantified by using the HOMA-IR in WT mice fed with a low-fat (LF) or a high-fat (HF) diet, and in *ob/ob* mice and their littermate controls

[a]$p<0.05$, [b]$p<0.001$

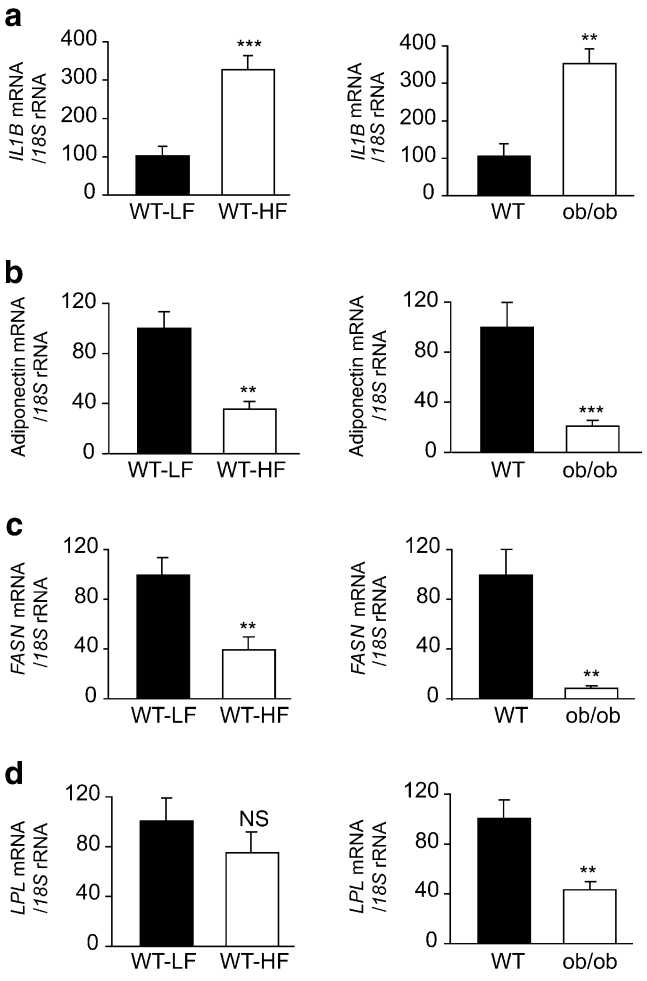

Fig. 8 Increased IL1B expression in epididymal adipose tissue from insulin-resistant mouse models. Total RNA was extracted and subjected to real-time PCR (see Materials and methods). **a** *Il1b*, **b** adiponectin, **c** *Fasn* and **d** *Lpl* mRNA levels were normalised to 18S rRNA expression and arbitrarily set at 100 for WT-LF and wild-type (*filled bars*) compared with WT-HF and *ob*/*ob* (*open bars*), respectively. *Error bars* represent SEM. $^{**}p<0.01$, $^{***}p<0.001$ vs control (*filled bars*)

a role for IL1B in the development of insulin resistance [10]. These results are in keeping with previous results showing a 2.1- and 3.8-fold increase in IL1B-converting enzyme expression in WT-HF and *ob*/*ob* mice, respectively [4]. IL1B-converting enzyme is necessary for the processing and subsequent secretion of bioactive IL1B [24]. However, the contribution of adipocytes to IL1B secretion by adipose tissue remains to be determined.

In conclusion, this study clearly indicates that IL1B induces insulin resistance in cultured murine and human adipocytes. Chronic exposure to IL1B inhibited insulin signal transduction in both differentiating and differentiated adipocytes. IL1B also altered the differentiation status of adipocytes. While we found that IL1B targeted IRβ and IRS-1 phosphorylation, the precise mechanism whereby IL1B triggers insulin resistance is unclear. IL1B, which is upregulated in adipose tissue of insulin-resistant mice, might be a key mediator of the adipose tissue inflammation that is associated with obesity and insulin resistance.

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