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ARTICLE

C3H/HeJ mice carrying a toll-like receptor 4 mutation are protected against the development of insulin resistance in white adipose tissue in response to a high-fat diet

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Abstract
Aims/hypothesis Inflammation is associated with obesity and has been implicated in the development of diabetes and atherosclerosis. During gram-negative bacterial infection, lipopolysaccharide causes an inflammatory reaction via toll-like receptor 4 (TLR4), which has an essential function in the induction of innate and adaptative immunity. Our aim was to determine what role TLR4 plays in the development of metabolic phenotypes during high-fat feeding.
Materials and methods We evaluated metabolic consequences of a high-fat diet in TLR4 mutant mice (*C3H/HeJ*) and their respective controls.
Results TLR4 inactivation reduced food intake without significant modification of body weight, but with higher epididymal adipose tissue mass and adipocyte hypertrophy. It also attenuated the inflammatory response and increased glucose transport and the expression levels of adiponectin and lipogenic markers in white adipose tissue. In addition, TLR4 inactivation blunted insulin resistance induced by lipopolysaccharide in differentiated adipocytes. Increased feeding efficiency in TLR4 mutant mice was associated with lower mass and lower expression of uncoupling protein 1 gene in brown adipose tissue. Finally, TLR4 inactivation slowed the development of hepatic steatosis, reducing the liver triacylglycerol content and also expression levels of lipogenic and fibrosis markers.
Conclusions/interpretation TLR4 influences white adipose tissue inflammation and insulin sensitivity, as well as liver fat storage, and is important in the regulation of metabolic phenotype during a fat-enriched diet.

Keywords Adipose tissue · High-fat diet · Insulin resistance · Lipid metabolism · Liver steatosis · Obesity · TLR4 · Toll-like receptor 4

Abbreviations

IL1RN	IL1 receptor antagonist
KRP	Krebs–Ringer phosphate buffer
LPS	lipopolysaccharide
PAI-1	plasminogen activator inhibitor-1
PPARG	peroxisome proliferator-activated receptor γ
TLR4	toll-like receptor 4
TNFRSF1B	TNF receptor superfamily, member 1b
WAT	white adipose tissue

Introduction

Evidence from human populations [1] and animal research [2] has established correlative as well as causative [3] links between chronic inflammation and insulin resistance during obesity. However, the fundamental mechanisms for activating inflammatory pathways in obesity are poorly under-

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stood. One of the commonest causes of inflammation is microbial infection. The host's acute-phase response to pathogens involves physiological changes including hypermetabolism, anorexia and body weight loss [4], but it is not known how this pathway intervenes in the chronic inflammatory state in obesity and in the control of insulin sensitivity.

Lipopolysaccharide (LPS) is continuously produced in the gut by the death of gram-negative bacteria and translocated into intestinal capillaries and the general circulation [5], where it can reach peripheral tissue such as adipose tissue and cause a local inflammatory reaction. Interestingly, LPS is found in the circulation of apparently healthy subjects [6, 7] and its activity may be associated with high-fat diet and dyslipidaemia. Infusion of triacylglycerol enhances LPS responses in humans, including the production of cytokines, suggesting that LPS can be locally released from lipoproteins in peripheral tissues through lipoprotein lipase action [8].

Portal endotoxin is involved in the progression of non-alcoholic steatohepatitis [9, 10], which mainly accounts for hepatic insulin resistance [11]. Genetically obese rodents quickly develop steatohepatitis after exposure to low doses of LPS [12], while polymyxin B diminishes hepatic steatosis during total parenteral nutrition in the rat by reducing caecal flora [13]. Moreover, endotoxins exert a strong effect on liver of mice fed a high-fat diet [14].

Interestingly, some groups [15] but not all [16] observed that toll-like receptor 4 (TLR4) 299Gly allele was associated with reduced C-reactive protein levels and a decreased risk of clinical diabetes, suggesting that TLR4 intervenes in the complications of obesity.

LPS in combination with CD14 serves as ligand for TLR4 [17]. TLR4 is produced by macrophages and Kupffer cells and recent data have demonstrated its presence in murine [18] and human adipocytes [19].

We hypothesised that the presence of non-functional TLR4 should modify the development of adipose tissue and liver steatosis during a high-fat diet. We studied the effects of an obesity- and insulin resistance-inducing high-fat diet in endotoxin-resistant *C3H/HeJ* mice ($Tlr4^{Lps\text{-}d}$), which carry a spontaneous mutation inactivating *Tlr4* [20], and in their respective *C3H/HeOuJ* control mice. Complementary experiments were performed in $Cd14^{-/-}$ mice.

Materials and methods

Mice and animal care Adult (12-week-old) male *C3H/HeJ* and *C3H/HeOuJ* mice, as well as $Cd14^{-/-}$ and C57Bl/6J control mice, were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and housed with a 12-h light–dark cycle. Mice had free access to a high-fat diet and water. The diet consisted of either a pelleted (42.7% carbohydrate, 42% fat, 15.3% protein, 18.8 kJ/g; TD88137; Harlan Teklad, Madison, WI, USA) or a liquid diet (47% carbohydrate, 35% fat, 18% protein, 4.186 kJ/ml; Lieber–De Carli Control diet; Bioserv Industries, Frenchtown, NJ, USA). Food intake was measured daily. The use of the liquid diet allowed the constitution of an additional group of pair-fed control *C3H/HeOuJ* or C57Bl/6J mice (pair-fed group), which were fed the same quantity of the diet as that consumed on the previous day by the *C3H/HeJ* or $Cd14^{-/-}$ mice. In addition, ten mice of *C3H* genotype were fed a standard chow diet (60% carbohydrate, 12% fat, 28% protein, 14.2 kJ/g; A03; UAR-SAFE, Epinay sur Orge, France). Body weights were measured weekly. Feeding efficiency was calculated by dividing body weight gain by energy intake over the diet period.

After a 6-h fast, mice were killed under anaesthesia with isoflurane inhalation. After blood collection from the retro-orbital sinus, plasma samples were stored at −20°C. Adipose tissue and liver were collected and weighed. In some experiments, mice were injected with LPS from *E. coli* serotype 026:B6 (100 mg/kg i.p; Sigma, St Louis, MO, USA). This study protocol was approved by the local ethics committee of the Faculté de Médecine of Marseille (authorisation number 52–23).

Biochemical and immunological analyses Plasma triacylglycerol and glucose were determined by routine chemical methods. Insulin levels of fasting mice were measured by radioimmunoassay (Linco Research, St Louis, MO, USA). Plasma ghrelin levels were assayed with a rat ghrelin enzyme immunoassay kit (Phoenix Peptide, Belmont, CA, USA). Leptin, TNF, TNF receptor superfamily, member 1b (TNFRSF1B, previously known as TNF receptor type 2 [TNFR2]) and IL6 were measured with ELISA (R&D systems, Abingdon, UK). Murine plasminogen activator inhibitor-1 (PAI-1, now known as serine [or cysteine] peptidase inhibitor, clade E, member 1 [SERPINE1]) was assessed with a home-made ELISA [21].

Tissue lipid analysis Lipids from white adipose tissue (WAT) and liver were extracted by the method of Folch et al. [22]. Triacylglycerol was measured using a triacylglycerol and free glycerol reagent (Sigma).

Total DNA content from adipose tissue WAT was incubated overnight in the presence of proteinase K (100 μg/ml). DNA was then purified using phenol/chloroform and quantified by measuring the absorbance at 260 nm.

Histological studies Pieces of WAT and liver were embedded into Tissue-Tek OCT (Miles, Elkhart, IL, USA). Cryosections were stained with Nuclear Fast Red. Images of adipocytes were acquired from a Leica DMRB light

microscope fitted with a colour CCD camera (Coolsnap; Princeton Instruments, Evry, France). About 300 cell surfaces per fat pad were measured at ×10 magnification using Scion imaging software (Scion Image, Frederick, MD, USA). Liver sections were stained with Oil Red O and observed under the same microscope at ×20 magnification.

Isolation and culture of peritoneal macrophages Peritoneal macrophages were prepared as described [13] and then incubated for 18 h with or without LPS from *E. coli* serotype 026:B6 (50 ng/ml, Sigma). Media were collected and stored at −80°C. Total cell proteins were assayed according to specifications of the bicinchoninic acid protein assay kit from Sigma.

In vivo lipolysis After a 6-h fast, five mice were injected intraperitoneally with (−)-isoproterenol hydrochloride (10 mg/kg; Sigma). Blood was collected before and 15 min after injection. Plasma NEFA concentrations were measured using NEFA C kit (Wako, Neuss, Germany). These mice were not used for further experiments.

Glucose turnover studies and in vivo glucose utilisation index in individual tissues These experiments were performed as described [23, 24]. For details, see Electronic supplementary material (ESM).

In vitro glucose uptake Primary adipocytes (10 days post-differentiation) [25] were cultured overnight in serum-free DMEM with low glucose (1 g/l). After Krebs–Ringer phosphate buffer (KRP) wash, cells were incubated at 37°C for 30 min in the presence or absence of 100 nmol/l of insulin in KRP containing 1% BSA. Then 18.5 kBq per well 2-deoxy[^{3}H]glucose (Amersham Biosciences, Orsay, France) was added and incubated for 10 min at 37°C. The cells were washed with cold KRP and solubilised in 0.1% SDS. The radioactivity of a 200-µl aliquot was determined in a scintillation counter. Glucose uptake was expressed as the degree of increase relative to that of basal *C3H/HeOuJ* cells.

Real-time quantitative PCR Total RNAs were prepared using Trizol reagent (Invitrogen, Cergy Pontoise, France). cDNA was synthesised using MMLV transcriptase (Invitrogen) from 1 µg of total RNA and stored at −80°C until use. PCRs were performed under standard conditions [26]. The relative amounts of each studied mRNA were normalised to acidic ribosomal phosphoprotein P0 (*Arbp*, also known as *36b4*) mRNA levels and expressed relatively to the mean value of the control mice group (User Bulletin no. 2; Applied Biosystems, Foster City, CA, USA). Primer express software (Applied Biosystems) was used to design primers (available upon request from tanti@unice.fr).

Data and statistics Data are expressed as mean±SEM. Differences between independent variables were tested using the Mann–Whitney test. Differences between paired variables were tested using the paired Wilcoxon test. The Kolmogorov–Smirnov Z test was used to test differences in adipocyte surface. A p value <0.05 was considered significant.

Results

C3H/HeJ mice do not respond to LPS To confirm the absence of functional TLR4 in *C3H/HeJ* mice, we studied the LPS responsiveness of their macrophages in vitro and whole-body LPS responsiveness in vivo. Comparable numbers of peritoneal macrophages were retrieved from the two genotypes and incubated for 18 h with or without LPS. Under basal conditions, the levels of IL6, PAI-1 and TNFRSF1B were strongly reduced in TLR4-deficient macrophages, whereas levels of TNF were similar (Table 1).

Table 1 Inflammatory marker production by peritoneal macrophages from *C3H/HeOuJ* and *C3H/HeJ* mice incubated with or without LPS stimulation for 18 h

	C3H/HeOuJ			*C3H/HeJ*		
	Without LPS	With LPS[a]	p value[b]	Without LPS	With LPS[a]	p value[b]
TNF (pg/mg protein)	951±251	9,058±1,781	<0.01	585±99	475±119	0.40
IL6 (pg/µg protein)	51±22	253±38	<0.01	3.2±0.3*	3.1±0.5	0.58
TNFRSF1B (pg/µg protein)	13.6±2.2	44.8±3.3	<0.01	6.0±1.1*	6.5±2.3	0.51
PAI-1 (pg/mg protein)	255±63	1,111±243	<0.01	56.6±6.6*	59.8±9.0	0.37

Results are expressed as mean±SEM (n=7–10)
[a] 50 ng/ml
[b] Wilcoxon matched pairs test without vs with LPS
*p<0.01 compared with *C3H/HeOuJ* without LPS

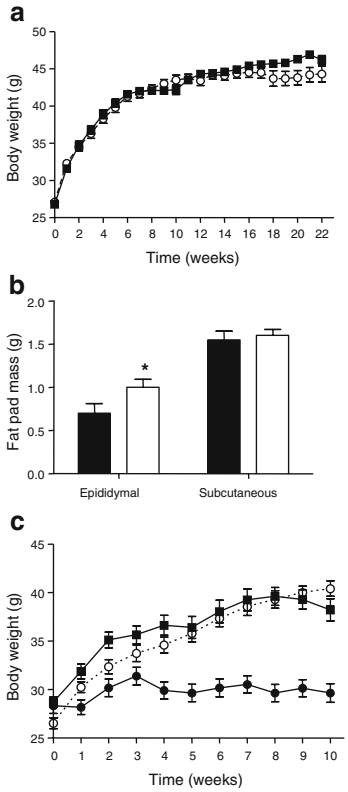

Fig. 1 **a** Growth curves of *C3H/HeJ* (*open circles*) and *C3H/HeOuJ* pair-fed mice (*closed squares*) given free access to a pelleted high-fat diet for 22 weeks. Data are mean±SEM of 20 mice. **b** Epididymal and subcutaneous adipose tissue masses of *C3H/HeJ* (*white bars*) and *C3H/HeOuJ* (*black bars*) mice given free access to the same diet for the same period. Data are mean±SEM of ten mice. *p=0.01 **c** Growth curves of *C3H/HeJ* (*open* circles), *C3H/HeOuJ* pair-fed (*closed circles*) and *C3H/HeOuJ* (*closed squares*) mice. Mice had free access to a liquid high-fat diet for 10 weeks. Data are mean±SEM of ten mice

After LPS stimulation all levels were significantly increased in *C3H/HeOuJ* macrophages, whereas they were not affected in *C3H/HeJ* macrophages.

Plasma TNF levels in *C3H/HeOuJ* mice increased at 1.5 h after LPS injection to 771±335 pg/ml (n=6), but remained undetectable in *C3H/HeJ* mice (n=5).

C3H/HeJ mice exhibit a higher feeding efficiency than control mice Consistent with previous reports [27], body mass was significantly lower in *C3H/HeJ* than in *C3H/HeOuJ* control mice after 10 weeks of standard chow diet (30.1±0.5 vs 33.0±1.0 g; p<0.01). However, weight gain was large in the *C3H/HeJ* and *C3H/HeOuJ* control mice that had free access to the high-fat diet (Fig. 1a). The weight of *C3H/HeJ* mice changed similarly to that of the control mice and body weights after 22 weeks of high-fat diet were not statistically different between the two groups (Table 2). While the weight of subcutaneous fat pads did not differ between the groups, *C3H/HeJ* mice had larger epididymal fat pads (Fig. 1b). In *C3H/HeJ* mice the total triacylglycerol content of epididymal tissue was increased by 15%, whereas the total DNA content was reduced by 23%. This led to a significantly higher triacylglycerol:DNA ratio in *C3H/HeJ* than in *C3H/HeOuJ* (fold increase: 3.1; n=10; p<0.05), indicating an increase in adipocyte size in *C3H/HeJ* mice. This was confirmed by histological analysis showing an increase in adipocyte surface in both epididymal (*C3H/HeJ* vs *C3H/HeOuJ*: 1,749±77 vs 1,432±44 μm^2; p<0.01) and subcutaneous (*C3H/HeJ* vs *C3H/HeOuJ*:1,152±31 vs 996±37 μm^2; p<0.01) tissues. Both groups developed hyperglycaemia, hyperinsulinaemia and hypertriacylglycerolaemia (Table 2), but showed no difference in leptin or ghrelin levels (Table 2).

To ensure that food intake did not differ between the groups, food consumption was summed over the diet period (Table 2). Unexpectedly, although the *C3H/HeJ* reached the same body weight, they consumed less energy than *C3H/HeOuJ* control mice. As a result, feeding efficiency was increased in mutant mice (Table 2).

C3H/HeJ mice exhibit a higher body weight than pair-fed C3H/HeOuJ control mice The difference in feeding efficiency between the groups prompted us to perform a pair-feeding experiment, in which *C3H/HeOuJ* were fed the same quantity of the diet *C3H/HeJ* had consumed on the previous day (pair-fed). To make the pairing as precise as possible, we used a high-fat liquid diet. The pair-fed *C3H/*

Table 2 Characteristics of *C3H/HeOuJ* and *C3H/HeJ* mice with free access to a pelleted high-fat diet for 22 weeks

	C3H/HeOuJ	*C3H/HeJ*	p value
Body weight (g)	46.8±0.4	44.6±0.6	0.12
Glucose (mmol/l)	12.6±1.6	12.0±2.3	0.68
Insulin (pmol/l)	663±69.7	383±35	<0.01
Leptin (ng/ml)	42.9±7.0	38.4±8.0	0.60
Ghrelin (ng/ml)	1.10±0.38	0.96±0.18	0.84
Triacylglycerol (mmol/l)	1.20±0.07	1.26±0.04	0.48
NEFA (mmol/l)	0.67±0.11	0.73±0.10	0.20
Cumulative energy intake (kJ/mouse)	12,060±184	9,222±92	<0.05
Feeding efficiency (g/100 kJ)[a]	0.33±0.007	0.44±0.026	<0.05

Data are mean±SEM. n=10 per group

[a] Feeding efficiency was calculated after 10 weeks of high-fat diet during the linear phase of the growth curve

Table 3 Fat distribution and food intake of *C3H/HeOuJ* and *C3H/HeJ* mice with free access to a liquid high-fat diet for 10 weeks

	C3H/HeOuJ	*p* value	*C3H/HeJ*	*C3H/HeOuJ* pair-fed	*p* value[a]
Body weight (g)	36.4±1.9	0.70	37.3±0.7	27.0±0.9	<0.001
Epididymal fat pad					
Weight (g)	1.23±0.11	<0.01	1.85±0.13	0.50±0.08	<0.001
% Body weight	3.4±0.7	<0.01	5.0±0.4	1.8±0.2	<0.001
Subcutaneous fat pad					
Weight (g)	0.82±0.09	0.32	0.96±0.08	0.25±0.05	<0.001
% Body weight	2.2±0.4	0.29	2.6±0.2	0.9±0.2	<0.001
Cumulative energy intake (kJ/mouse)	4,496±218	<0.05	3,830±88	3,784±105	NS
Feeding efficiency (g/100 kJ)	0.23±0.04	<0.05	0.35±0.02	0.03±0.04	<0.0001

Data are mean±SEM. $n=10$ per group
[a] For *C3H/HeJ* vs *C3H/HeOuJ* pair-fed

HeOuJ mice gained significantly less weight than *C3H/HeJ* mice (Fig. 1c), leading to a 12.4-fold greater feeding efficiency in *C3H/HeJ* (Table 3). The huge differences in weight gain were associated with alterations in adiposity. The epididymal and the subcutaneous fat pad masses in *C3H/HeJ* mice were markedly larger than in *C3H/HeOuJ* pair-fed mice (Table 3). As previously shown, *C3H/HeJ* with free access to food reached the same body weight as *C3H/HeOuJ* (Fig. 1c), but again *C3H/HeJ* consumed less energy and had significantly greater feeding efficiency than *C3H/HeOuJ* mice (Table 3). Hence, non-functional TLR4 promoted weight gain despite the lower food intake than in control mice. Taken together, these data suggest that TLR4 plays a role in regulating the equilibrium between food intake and energy expenditure. We then studied key regulators of weight gain. To avoid any bias due to body weight difference, we investigated mice that had had free access to food during 22 weeks and whose body weights were similar.

TLR4 inactivation does not increase whole-body insulin sensitivity after 22 weeks of high-fat diet To understand better why mice with non-functional TLR4 gained identical weight while ingesting less food, we investigated whether this was due to modifications of insulin sensitivity. After 22 weeks of high-fat diet, *C3H/HeJ* mice had similar glycaemia but lower insulinaemia after fasting (Table 2) than *C3H/HeOuJ* mice, although glycaemia was still higher than in *C3H/HeJ* mice fed normal chow (41.8±5.2 pmol/l; $n=10$). However, the glucose infusion rate needed to maintain stable glycaemia during the hyperinsulinaemic–euglycaemic clamps was not different, indicating that whole-body glucose uptake did not differ between the two genotypes (Fig. 2a).

TLR4 inactivation supports higher insulin sensitivity and lipogenesis in WAT after 22 weeks of high-fat diet but not in skeletal muscle We hypothesised that TLR4 inactivation could affect insulin sensitivity of insulin target tissues in

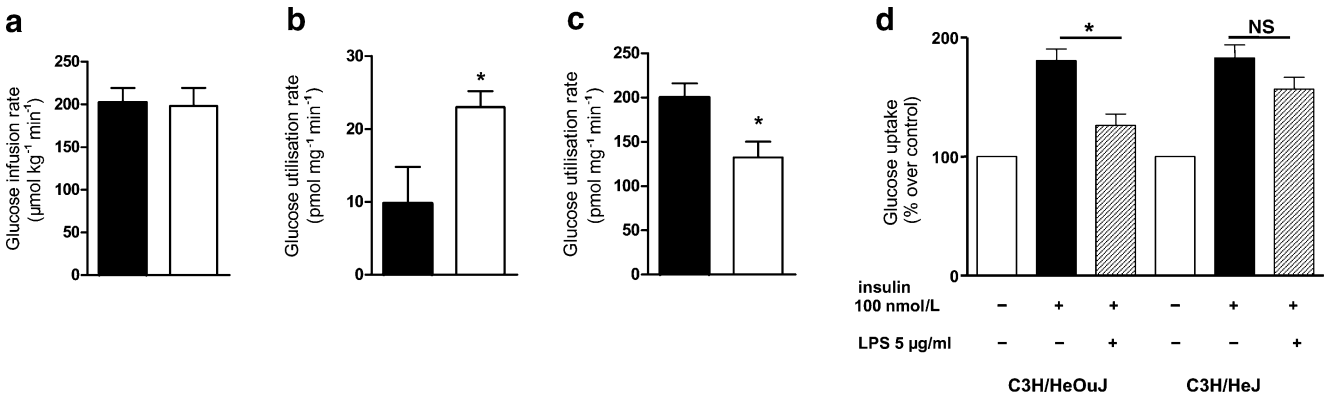

Fig. 2 Glucose metabolism assessment in *C3H/HeOuJ* and *C3H/HeJ* mice studied after 22 weeks of a pelleted high-fat diet. **a** Glucose infusion rate during a euglycaemic–hyperinsulinaemic clamp. **b** In vivo glucose clearance rate by subcutaneous adipose tissue and (**c**) vastus lateralis. *Black bars*, *C3H/HeOuJ*; *white bars*, *C3H/HeJ*. Data are mean±SEM of five mice. *$p<0.05$. **d** Effects of TLR4 activation by LPS treatment on glucose uptake in *C3H/HeOuJ* and *C3H/HeJ* primary adipocytes (day 10 of differentiation) in response to insulin stimulation. LPS was added 4 h prior to insulin. Data are mean±SEM of three independent experiments. *$p<0.05$

different ways. Indeed, it has recently been demonstrated that inflammation is regulated differently in adipose tissue than in muscle [28], as is insulin sensitivity [29]. Insulin-stimulated glucose utilisation was significantly higher in subcutaneous adipose tissue of *C3H/HeJ* than in that of *C3H/HeOuJ* mice (Fig. 2b). To further investigate the effects of TLR4 signalling on insulin action, we evaluated whether TLR4 activation by LPS could affect insulin-dependent glucose uptake by adipocytes. While insulin increased glucose uptake in adipocytes from both genotypes, LPS significantly decreased the ability of insulin to stimulate glucose uptake, this inhibitory effect being less pronounced in *C3H/HeJ* adipocytes (Fig. 2d). In contrast, the insulin-stimulated glucose utilisation rate in vastus lateralis was lower in *C3H/HeJ* mice than in *C3H/HeOuJ* mice (Fig. 2c). A trend towards a decrease, albeit not significant, was also observed in tibialis anterior (data not shown). Hence, these results demonstrate that obese TLR4-deficient mice have an adipose tissue-specific increase in insulin sensitivity.

Non-functional TLR4 does not modify isoproterenol-induced lipolysis after 22 weeks of high-fat diet Inflammation is known to increase adipocyte lipolysis [30]. We thus examined whether *C3H/HeJ* mice exhibited a lower lipolytic potential. Fasting plasma NEFA levels were not different between both strains (*C3H/HeOuJ*: 0.79±0.04 vs *C3H/HeJ*: 0.82±0.08 mmol/l). Isoproterenol administration led to a similar increase in plasma NEFA concentrations in the two groups (*C3H/HeOuJ*: 1.72±0.07 vs *C3H/HeJ*: 1.70±0.09 mmol/l).

TLR4 inactivation supports a lower adipose tissue inflammatory state and attenuates obesity-induced changes in adipose tissue gene expression Increased expression of genes that encode proinflammatory proteins has been observed in adipose tissue of obese animals [31]. In subcutaneous adipose tissue from *C3H/HeJ* mice, we observed a decrease in *Tnf* and *Il1b* mRNA levels (Fig. 3a). We also observed an increase in the mRNA level of interleukin 1 receptor antagonist (*Il1rn*, previously known as *Il1ra*) (Fig. 3a), recently shown to regulate adipogenesis, food intake and energy expenditure [32]. A similar tendency was noticed in epididymal adipose tissue (Fig. 3d). These differences were not due to a reduction in the number of tissue-infiltrated macrophages, since the constitutive macrophage marker, EGF-like module containing, mucin-like, hormone receptor-like sequence 1 (*Emr1*, previously known as *F4/80*), exhibited a higher expression level in *C3H/HeJ* than in *C3H/HeOuJ* subcutaneous WAT (Fig. 3a).

We then studied whether these changes in the inflammatory pattern of CH3/HeJ adipose tissue were associated with higher mRNA levels of genes involved in insulin action and sensitivity, glucose and triacylglycerol metabolism, and adipocyte differentiation. Adipose tissue of obese TLR4 mutant mice expressed higher levels of *Irs1*,

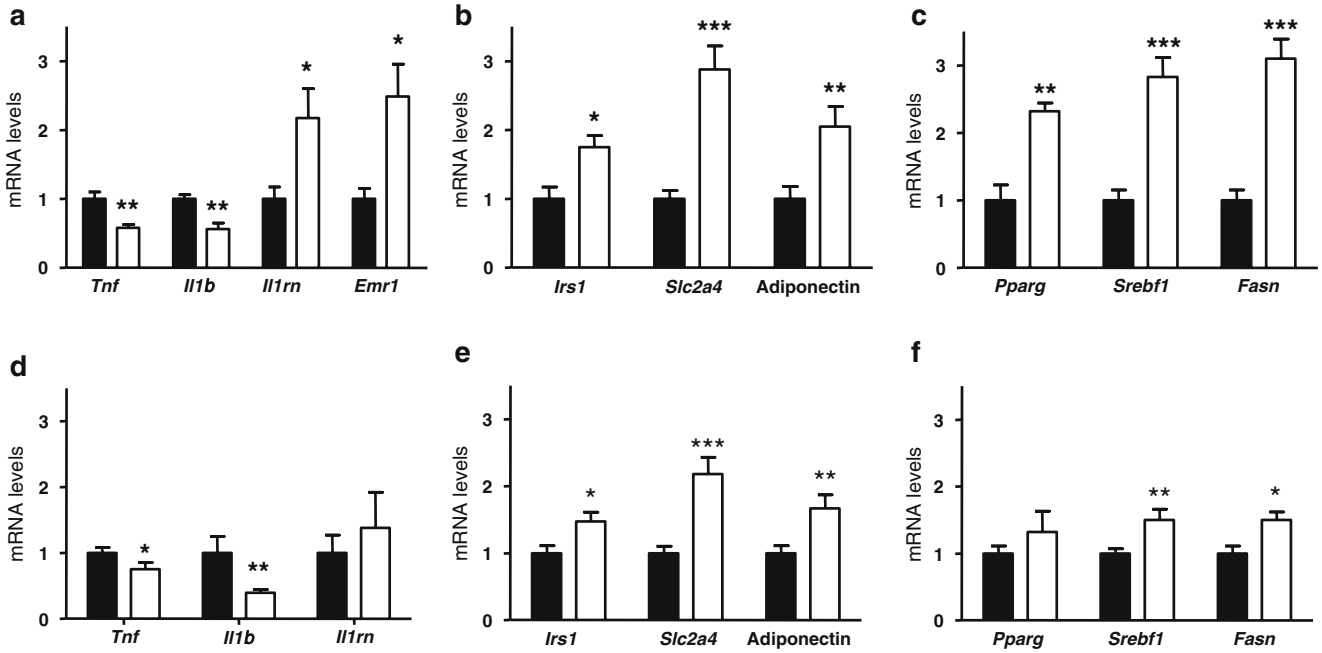

Fig. 3 Expression of genes encoding proteins involved in inflammation (**a**, **d**), insulin signalling (**b**, **e**) and lipogenesis (**c**, **f**) in subcutaneous (**a**–**c**) and epididymal (**d**–**f**) adipose tissue from *C3H/HeJ* (*white bars*) and *C3H/HeOuJ* (*black bars*) mice given free access to a pelleted high-fat diet for 22 weeks. mRNA levels were normalised by those of *Arbp* mRNA. Data are mean±SEM (n=8–10 mice). *p<0.05, **p<0.02, ***p<0.01

adiponectin and solute carrier family 2 (facilitated glucose transporter), member 4 (*Slc2a4*, previously known as *Glut4*) mRNA (Fig. 3b,e). The mRNA levels of two key lipogenic factors, sterol regulatory element binding factor 1 (*Srebf1*, previously known as *Srebp1c*) and fatty acid synthase (*Fasn,* previously known as *Fas*), were also higher (Fig. 3c,f). Further, peroxisome proliferator-activated receptor γ (*Pparg*) mRNA followed a similar pattern (Fig. 3c,f). These data suggest that the pro-inflammatory changes that occur in adipose tissue during the development of obesity are in part TLR4-dependent. The lower inflammatory state observed in *C3H/HeJ* adipose tissue may support the possibility of a higher insulin sensitivity.

TLR4 inactivation attenuates high-fat diet-induced liver steatosis To determine whether improved insulin sensitivity in adipose tissue of *C3H/HeJ* mice ameliorated hepatomegaly or reduced hepatic steatosis, we analysed the livers from *C3H/HeJ* and *C3H/HeOuJ* mice. Despite the presence of a similar degree of obesity, the liver mass of *C3H/HeJ* was lower than that of control mice (Table 4). The reduced liver size was associated with a 40% lower hepatic triacylglycerol content in *C3H/HeJ* than in *C3H/HeOuJ* mice, concomitant with lower hepatic levels of *Pparg* and *Fasn* mRNA (Fig. 4a,b) and lower histological steatosis (Fig. 4c). Similar results were obtained after 10 weeks of liquid high-fat diet (Table 4). The liver mass of *C3H/HeJ* fed a liquid high-fat diet was lower than that of control mice and did not differ from that of control mice fed a standard chow diet (1.21±0.06 g; n=10).

Significant fibrosis is associated with non-alcoholic steatohepatitis and has been considered a factor in prognosis [33]. We thus investigated mRNA levels of *Tgfb1* and *Pai-1*, key effectors of the activity of TGFB1. Both *Pai-1* and *Tgfb1* mRNA levels were lower in the liver of *C3H/HeJ* mice than in that of *C3H/HeOuJ* (Fig. 4b). These data argue for an effect of the TLR4 pathway on the evolution of fibrosis associated with fatty liver.

$Cd14^{-/-}$ mice develop a phenotype close to that of C3H/HeJ mice To confirm the contribution of the LPS pathway to the observed results, we completed our study by phenotyping *$Cd14^{-/-}$* mice and their controls after 10 weeks of free access to a high-fat diet. High-fat feeding for 10 weeks led to a significant weight gain in both *$Cd14^{-/-}$* and control mice. Similarly to *C3H/HeJ* mice, feeding efficiency was higher in *$Cd14^{-/-}$* mice than in controls given free access to food (data not shown). In subcutaneous adipose tissue, *Cd14* inactivation was associated with 5- to 25-fold higher mRNA levels of genes involved in insulin action (*Slc2a4, Irs1*, adiponectin) and lipogenesis (*Pparg, Fasn*) (ESM Fig. 1). Despite the same liver mass (C57B1/6J: 4.3±0.1, *$Cd14^{-/-}$*: 4.5±0.4% of body weight), the hepatic triacylglycerol content tended to be higher in C57Bl/6J than in *$Cd14^{-/-}$* mice (176±13 vs 139±11 mmol/g; p=0.08).

Discussion

Our data demonstrate that TLR4 exerts an important role on obesity-associated phenotypes. When mice were fed a high-fat diet, absence of TLR4 modulated the feeding behaviour and increased WAT insulin sensitivity and lipogenesis. Importantly, TLR4 mutant mice were protected from developing hepatic steatosis.

We found that TLR4 inactivation improved insulin-induced glucose uptake in WAT. This protection from high-fat diet-induced adipose tissue insulin resistance was associated with a failure of the high-fat diet to induce expression of inflammatory mediators in WAT. This finding was recently supported by a report that NEFA stimulate proinflammatory pathways and reduce insulin sensitivity in WAT through TLR4 activation [34]. The attenuated effect of LPS on insulin-induced glucose uptake in mutant adipocytes may reflect a direct contribution of TLR4 present in adipocytes. Accordingly, TLR4 activation by LPS in 3T3-L1 adipocytes prevented insulin-dependent glucose uptake [35].

Table 4 Effect of TLR4 deficiency on liver weight

	C3H/HeOuJ	*C3H/HeJ*	p value	*C3H/HeOuJ* pair-fed	p value[a]
Pelleted high-fat diet					
Liver weight (g)	3.39±0.27	2.3±0.21	<0.001		
Liver (% body weight)	7.22±0.34	5.11±0.24	<0.001		
Liquid high-fat diet					
Liver weight (g)	1.38±0.07	1.17±0.04	<0.05	0.99±0.03	<0.005
Liver (% body weight)	3.78±0.11	3.16±0.07	0.02	3.67±0.08	<0.003

Twelve-week-old male mice had free access to a pelleted high-fat diet during 22 weeks or a liquid high-fat diet during 10 weeks. n=10 in each group. Data are mean±SEM

[a] For *C3H/HeJ* vs *C3H/HeOuJ* pair-fed

Our data may also result from the inhibition of TLR4-mediated inflammatory pathways in macrophages, with a resultant decrease in stimulatory action on adipocytes. In support of this hypothesis, TLR4 protein levels were much lower in adipocytes than in macrophages [36]. Remarkably, the attenuation of inflammation was not accompanied by a reduction of macrophage infiltration into WAT. Indeed, we did not find a decrease in *Emr1* mRNA levels in WAT of mutant mice. Consistent with this, Suganami et al. [36] showed that co-culture of 3T3-L1 adipocytes and *C3H/HeJ* macrophages was associated with an attenuation of *Tnf* and *Il1B* mRNA expression, but also with a significant increase in mRNA expression levels of the powerful monocyte chemoattractant, monocyte chemoattractant protein 1.

TNF is known to reduce adipose tissue mass by decreasing PPARG production, stimulating lipolysis and repressing genes involved in the uptake and metabolism of lipids and glucose [37–41]. Considering all this, the higher insulin sensitivity that we observed in our study in WAT and that was associated with higher expression of genes mediating insulin sensitivity (*Irs1, Slc2a4*, adiponectin) and lipogenesis (*Srebf1*, *Fasn*, *Pparg*) may be secondary to the tissue TNF and IL1B depletion, and might contribute to the increase in adipocyte volume seen in *C3H/HeJ* mice despite lower food intake. This notion is consistent with our finding that insulin sensitivity was increased in WAT from *C3H/HeJ* mice during euglycaemic–hyperinsulinaemic clamp conditions. Greater expression of *Srebf1* and *Fasn* could be consecutive to the increased insulin sensitivity of the adipocytes, since insulin regulates the expression of both.

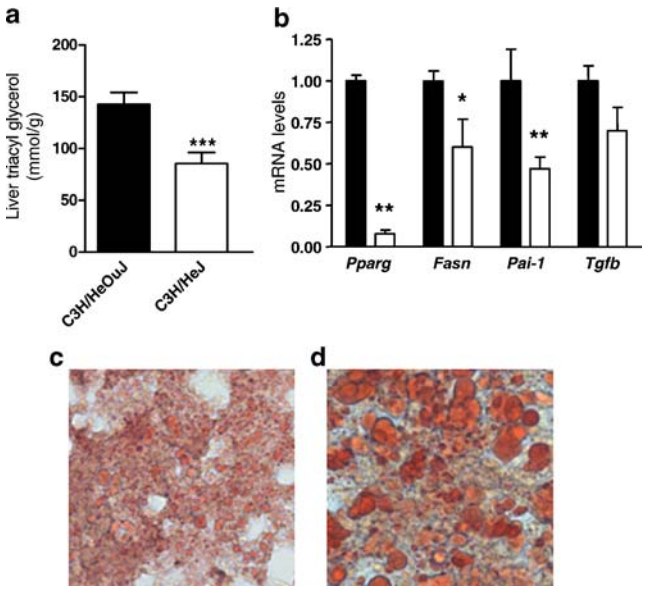

Fig. 4 **a** Levels of liver triacylglycerol content (n=10) and expression (**b**) of lipogenic and fibrotic markers in liver from *C3H/HeOuJ* (*black bars*) and *C3H/HeJ* (*white bars*) mice given free access to a pelleted high-fat diet for 22 weeks (n=5). mRNA levels were normalised by those of *Arbp* mRNA. Data are mean±SEM. *p<0.05, ** p<0.02, ***p<0.01. **c** Representative histological results of Oil Red O staining from *C3H/HeOuJ* (**c**) and *C3H/HeJ* (**d**) mice fed as above for 22 weeks

IL1RN is an anti-inflammatory cytokine [42]. Human and rodent WAT is a major source of IL1RN [43, 44]. *Il1rn*$^{-/-}$ mice, in which excess IL1 signalling may be induced, show a lean phenotype [45]. This phenotype has been associated with a defect in adipogenesis, a reduction of genes involved in lipogenesis, an increase in energy expenditure and a reduction in diurnal food intake [32]. The increase in IL1RN production in WAT may be directly involved in the phenotype obtained in TLR4 mutant mice, which exhibited both increased peripheral lipogenesis and lower thermogenesis. Note that in our study, *Il1rn* expression was increased in a situation where inflammation was dampened, suggesting that IL1RN may not only represent a counter-regulatory mechanism that limits inflammation, but may also be produced independently of an inflammatory process during fat accumulation.

We observed contrasting behaviour with regard to insulin sensitivity in skeletal muscle and adipose tissue. This could be related to the low inflammation level in skeletal muscle during obesity [28]. The skeletal muscle produces low levels of CD68 and monocyte chemoattractant protein 1 and neither pioglitazone nor metformin has any effect on production of these two [46]. Thus, a modification of peripheral glucose disposal under inflammation manipulation is more likely to be due to changes in adipose tissue and then to secondary effects on muscle. Another plausible explanation is that the ability of TLR4 deficiency to improve insulin resistance in adipose tissue and not muscle is regulated through PPARG , whose levels are up to 50-fold higher in adipose tissues than in skeletal muscles [47]. IL1RN may be involved in the described phenotype by virtue of its ability to reduce insulin sensitivity in muscle through a specific decrease in glucose uptake as recently described in rats [48]. Alternatively, it remains possible that plasma factor(s) dominantly affect insulin signalling in muscles by mechanisms that are independent of TLR4.

We found a reduction in total brown adipose tissue fat mass in *C3H/HeJ* associated with decreased *Ucp1* expression. This finding shows that TLR4 inactivation may play a role in brown adipose tissue development.

Our data provide evidence that TLR4 deficiency slows down non-alcoholic liver steatosis. This result should be seen together with those obtained in alcohol-induced steatosis. *C3H/HeJ* mice fed ethanol do not develop fatty liver but *C3H/HeOuJ* do. Chronic alcohol administration increases gut-derived endotoxin in the portal blood, partly due to gut leakiness [49], which activates Kupffer cells and

causes liver injury. Interestingly, we found that the protective effect of TLR4 inactivation was maintained throughout the diet period. Even after 22 weeks of high-fat diet the expression levels of key lipogenic markers still differed. *Tgfb1* and *Pai-1* expression levels in liver were decreased in *CH3/HeJ* mice, arguing for an effect of the TLR4 pathway, known as a key determinant of non-alcoholic steatohepatitis [50], on the evolution of hepatic fibrosis. Several studies have shown an inverse relationship between hepatic triacylglycerol stores and hepatic insulin sensitivity [11]. The observation that TLR4 mutant animals have a much lower insulin level at similar plasma glucose concentrations suggests that the liver responds more sensitively to insulin-induced inhibition of glucose output.

One limitation of our study could be the use of non-littermate controls. However, we obtained similar findings in *Cd14*-deficient mice, demonstrating that two approaches to blunting the LPS pathway lead to the same modifications: (1) in feeding behaviour; (2) in adipose tissue expression levels of genes involved in glucidolipidic metabolism; and (3) in the development of liver steatosis.

Our results indicate that, apart from an acute LPS challenge, TLR4 contributes to the production of key cytokines in WAT during obesity. They also highlight the contribution of the TLR4 pathway to the control of obesity and its related complications. We propose that interventions known to reduce endotoxin toxicity [51] or to modulate the TLR4 pathway may help control some key elements of the metabolic syndrome.

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Duality of interest The authors declare that they have no duality of interest.

References

1. Festa A, D'Agostino R Jr, Howard G et al (2000) Chronic subclinical inflammation as part of the insulin resistance syndrome: the Insulin Resistance Atherosclerosis Study (IRAS). Circulation 102:42–47
2. Wellen KE, Hotamisligil GS (2005) Inflammation, stress, and diabetes. J Clin Invest 115:1111–1119
3. Xu H, Barnes GT, Yang Q et al (2003) Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. J Clin Invest 112:1821–1830
4. Langhans W (2000) Anorexia of infection: current prospects. Nutrition 16:996–1005
5. Nolan JP, Hare DK, McDevitt JJ, Ali MV (1977) In vitro studies of intestinal endotoxin absorption. I. Kinetics of absorption in the isolated everted gut sac. Gastroenterology 72:434–439
6. Wiedermann CJ, Kiechl S, Dunzendorfer S et al (1999) Association of endotoxemia with carotid atherosclerosis and cardiovascular disease: prospective results from the bruneck study. J Am Coll Cardiol 34:1975–1981
7. Stoll LL, Denning GM, Weintraub NL (2004) Potential role of endotoxin as a proinflammatory mediator of atherosclerosis. Arterioscler Thromb Vasc Biol 24:2227–2236
8. Van der Poll T, Braxton CC, Coyle SM et al (1995) Effect of hypertriglyceridemia on endotoxin responsiveness in humans. Infect Immun 63:3396–3400
9. McClain C, Hill D, Schmidt J, Diehl AM (1993) Cytokines and alcoholic liver disease. Semin Liver Dis 13:170–182
10. Vyberg M, Ravn V, Andersen B (1987) Pattern of progression in liver injury following jejunoileal bypass for morbid obesity. Liver 7:271–276
11. Roden M (2006) Mechanisms of disease: hepatic steatosis in type 2 diabetes-pathogenesis and clinical relevance. Nat Clin Pract Endocrinol Metab 2:335–348
12. Yang SQ, Lin HZ, Lane MD, Clemens M, Diehl AM (1997) Obesity increases sensitivity to endotoxin liver injury: implications for the pathogenesis of steatohepatitis. Proc Natl Acad Sci USA 94:2557–2562
13. Pappo I, Becovier H, Berry EM, Freund HR (1991) Polymyxin B reduces cecal flora, TNF production and hepatic steatosis during total parenteral nutrition in the rat. J Surg Res 51:106–112
14. Li Z, Soloski MJ, Diehl AM (2005) Dietary factors alter hepatic innate immune system in mice with nonalcoholic fatty liver disease. Hepatology 42:880–885
15. Kolek MJ, Carlquist JF, Muhlestein JB et al (2004) Toll-like receptor 4 gene Asp299Gly polymorphism is associated with reductions in vascular inflammation, angiographic coronary artery disease, and clinical diabetes. Am Heart J 148:1034–1040
16. Illig T, Bongardt F, Schopfer A et al (2003) The endotoxin receptor TLR4 polymorphism is not associated with diabetes or components of the metabolic syndrome. Diabetes 52:2861–2864
17. Takeda K (2005) Evolution and integration of innate immune recognition systems: the toll-like receptors. J Endotoxin Res 11:51–55
18. Lin Y, Lee H, Berg AH et al (2000) The lipopolysaccharide-activated toll-like receptor (TLR)-4 induces synthesis of the closely related receptor TLR-2 in adipocytes. J Biol Chem 275:24255–24263
19. Bes-Houtmann S, Roche R, Hoareau L et al (2007) Presence of functional TLR2 and TLR4 on human adipocytes. Histochem Cell Biol 127:131–137
20. Poltorak A, He X, Smirnova I et al (1998) Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science 282:2085–2088
21. Declerck PJ, Verstreken M, Collen D (1995) Immunoassay of murine t-PA, u-PA and PAI-1 using monoclonal antibodies raised in gene-inactivated mice. Thromb Haemost 74:1305–1309
22. Folch J, Lees M, Sloane Stanley GH (1957) A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 226:497–509
23. Burcelin R, Dolci W, Thorens B (2000) Portal glucose infusion in the mouse induces hypoglycemia: evidence that the hepatoportal glucose sensor stimulates glucose utilization. Diabetes 49:1635–1642
24. Burcelin R, Crivelli V, Dacosta A, Roy-Tirelli A, Thorens B (2002) Heterogeneous metabolic adaptation of C57BL/6J mice to high-fat diet. Am J Physiol Endocrinol Metab 282:E834–E842
25. Negrel R, Dani C (2001) Cultures of adipose precursor cells and cells of clonal lines from animal white adipose tissue. Methods Mol Biol 155:225–237

26. Jager J, Gremeaux T, Cormont M, Le Marchand-Brustel Y, Tanti JF (2007) Interleukin-1β-induced insulin resistance in adipocytes through down-regulation of insulin receptor substrate-1 expression. Endocrinology 148:241–251
27. Johnson GB, Riggs BL, Platt JL (2004) A genetic basis for the "Adonis" phenotype of low adiposity and strong bones. FASEB J 18:1282–1284
28. Bruun JM, Helge JW, Richelsen B, Stallknecht B (2006) Diet and exercise reduce low-grade inflammation and macrophage infiltration in adipose tissue but not in skeletal muscle in severely obese subjects. Am J Physiol Endocrinol Metab 290:E961–E967
29. Norris AW, Chen L, Fisher SJ et al (2003) Muscle-specific PPARgamma-deficient mice develop increased adiposity and insulin resistance but respond to thiazolidinediones. J Clin Invest 112:608–618
30. Uysal KT, Wiesbrock SM, Marino MW, Hotamisligil GS (1997) Protection from obesity-induced insulin resistance in mice lacking TNF-alpha function. Nature 389:610–614
31. Moraes RC, Blondet A, Birkenkamp-Demtroeder K et al (2003) Study of the alteration of gene expression in adipose tissue of diet-induced obese mice by microarray and reverse transcription-polymerase chain reaction analyses. Endocrinology 144:4773–4782
32. Somm E, Henrichot E, Pernin A et al (2005) Decreased fat mass in interleukin-1 receptor antagonist-deficient mice: impact on adipogenesis, food intake, and energy expenditure. Diabetes 54:3503–3509
33. McCullough AJ (2004) The clinical features, diagnosis and natural history of nonalcoholic fatty liver disease. Clin Liver Dis 8:521–533 (viii)
34. Shi H, Kokoeva MV, Inouye K et al (2006) TLR4 links innate immunity and fatty acid-induced insulin resistance. J Clin Invest 116:3015–3025
35. Song MJ, Kim KH, Yoon JM, Kim JB (2006) Activation of toll-like receptor 4 is associated with insulin resistance in adipocytes. Biochem Biophys Res Commun 346:739–745
36. Suganami T, Tanimoto-Koyama K, Nishida J et al (2007) Role of the toll-like receptor 4/NF-kappaB pathway in saturated fatty acid-induced inflammatory changes in the interaction between adipocytes and macrophages. Arterioscler Thromb Vasc Biol 27:84–91
37. Xu H, Sethi JK, Hotamisligil GS (1999) Transmembrane tumor necrosis factor (TNF)-alpha inhibits adipocyte differentiation by selectively activating TNF receptor 1. J Biol Chem 274: 26287–26295
38. Petruschke T, Hauner H (1993) Tumor necrosis factor-alpha prevents the differentiation of human adipocyte precursor cells and causes delipidation of newly developed fat cells. J Clin Endocrinol Metab 76:742–747
39. Ruan H, Hacohen N, Golub TR, Van Parijs L, Lodish HF (2002) Tumor necrosis factor-alpha suppresses adipocyte-specific genes and activates expression of preadipocyte genes in 3T3-L1 adipocytes: nuclear factor-kappaB activation by TNF-alpha is obligatory. Diabetes 51:1319–1336
40. Gual P, Le Marchand-Brustel Y, Tanti JF (2005) Positive and negative regulation of insulin signaling through IRS-1 phosphorylation. Biochimie 87:99–109
41. Hotamisligil GS, Peraldi P, Budavari A et al (1996) IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF-alpha- and obesity-induced insulin resistance. Science 271:665–668
42. Allan SM, Rothwell NJ (2001) Cytokines and acute neurodegeneration. Nat Rev Neurosci 2:734–744
43. Meier CA, Bobbioni E, Gabay C et al (2002) IL-1 receptor antagonist serum levels are increased in human obesity: a possible link to the resistance to leptin? J Clin Endocrinol Metab 87:1184–1188
44. Juge-Aubry CE, Somm E, Giusti V et al (2003) Adipose tissue is a major source of interleukin-1 receptor antagonist: upregulation in obesity and inflammation. Diabetes 52:1104–1110
45. Matsuki T, Horai R, Sudo K, Iwakura Y (2003) IL-1 plays an important role in lipid metabolism by regulating insulin levels under physiological conditions. J Exp Med 198:877–888
46. Di Gregorio GB, Yao-Borengasser A, Rasouli N et al (2005) Expression of CD68 and macrophage chemoattractant protein-1 genes in human adipose and muscle tissues: association with cytokine expression, insulin resistance, and reduction by pioglitazone. Diabetes 54:2305–2313
47. Chawla A, Schwarz EJ, Dimaculangan DD, Lazar MA (1994) Peroxisome proliferator-activated receptor (PPAR) gamma: adipose-predominant expression and induction early in adipocyte differentiation. Endocrinology 135:798–800
48. Somm E, Cettour-Rose P, Asensio C et al (2006) Interleukin-1 receptor antagonist is upregulated during diet-induced obesity and regulates insulin sensitivity in rodents. Diabetologia 387–393
49. Keshavarzian A, Choudhary S, Holmes EW et al (2001) Preventing gut leakiness by oats supplementation ameliorates alcohol-induced liver damage in rats. J Pharmacol Exp Ther 299:442–448
50. Starkel P, Sempoux C, Leclercq I et al (2003) Oxidative stress, KLF6 and transforming growth factor-beta up-regulation differentiate non-alcoholic steatohepatitis progressing to fibrosis from uncomplicated steatosis in rats. J Hepatol 39:538–546
51. Munford RS (2005) Detoxifying endotoxin: time, place and person. J Endotoxin Res 11:69–84