Adipocyte glucocorticoid receptor has a minor contribution in adipose tissue growth

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Abstract

The glucocorticoids bind and activate both the glucocorticoid receptor (GR) as well as the mineralocorticoid receptor in adipocytes. Despite several studies to determine the function of these two receptors in mediating glucocorticoids effects, their relative contribution in adipose tissue expansion and obesity is unclear. To investigate the effect of GR in adipose tissue function, we generated an adipocyte-specific *Gr*-knockout mouse model (*Gr^{ad-ko}*). These mice were submitted either to a standard diet or a high-fat high sucrose diet. We found that adipocyte-specific deletion of *Gr* did not affect body weight gain or adipose tissue formation and distribution. However, the lack of *Gr* in adipocyte promotes a diet-induced inflammation determined by higher pro-inflammatory genes expression and macrophage infiltration in the fat pads. Surprisingly, the adipose tissue inflammation in *Gr^{ad-ko}* mice was not correlated with insulin resistance or dyslipidemia, but with disturbed glucose tolerance. Our data demonstrate that adipocyte-specific ablation of *Gr in vivo* may affect the adipose tissue function but not its expansion during a high calorie diet.

Key Words

- ▶ lipogenesis
- ▶ obesity
- ▶ high calorie diet
- ▶ corticosterone
- ▶ inflammation

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Introduction

Glucocorticoids, represented by cortisol in humans and corticosterone in rodents, play an essential role in several steps of adipocyte biology (Peckett *et al.* 2011). During adipogenesis, glucocorticoids are required to induce the adipogenic transcriptional program allowing the differentiation of preadipocytes to mature adipocytes (Hauner *et al.* 1989). In the final step of differentiation, glucocorticoids promote also the lipid uptake and lipid storage in mature adipocytes (Berdanier 1989, Fried *et al.* 1993). In mice, adipocyte-specific amplification of glucocorticoids through selective overexpression of 11 β HSD1, the enzyme converting the inactive cortisone

to active cortisol, induces central obesity and associated metabolic disorders (Masuzaki *et al.* 2001). Conversely, adipocyte-specific reduction of glucocorticoids through selective overexpression of 11 β HSD2, the enzyme converting the active cortisol to inactive cortisone, protects mice against obesity and diet-induced disorders (Morton *et al.* 2004, Kershaw *et al.* 2005). In humans, excess of glucocorticoids (Cushing's syndrome), caused by chronic steroid medications or by abnormal adrenal gland secretion, results in an increase in adipose tissues mass, particularly in the central region (Wajchenberg *et al.* 1995).

The absence of 11 β HSD2 expression in adipocytes allows the glucocorticoids to mediate their effects through binding to the glucocorticoid receptor (GR) as well as the mineralocorticoid receptor (MR). Once activated by the ligand, these receptors mediate two types of responses: an early and a late response. The early response also called the 'nongenomic response' is rapid and depends on the activation of secondary messengers triggering the cross-talk with additional signaling pathways (Dooley *et al.* 2012, Kadmiel & Cidlowski 2013, Faresse 2014). The molecular mechanisms behind the nongenomic responses induced by these steroid receptors are still unclear. The late response also called the 'classical response' is endorsed by the intrinsic transcriptional activity of the receptors. MR and GR are mainly cytoplasmic at the basal state. Once activated by the ligand, they translocate to the nucleus as homo- or heterodimers to regulate their target genes. To date, no specific MR response element has been identified in adipocytes and it is accepted that MR shares a common hormone-response-element with GR.

Involvement of MR and GR in the transduction of glucocorticoids effects in adipocytes is still under fervent debates (Armani *et al.* 2014, Lee & Fried 2014b). We previously found that pharmacological inhibition of both MR and GR abolished the adipogenesis and prevented adipose tissue expansion and adipocyte hypertrophy during a chronic high fat diet (Desarzens *et al.* 2014). Other studies evidenced that GR has a more prevalent role, compared with MR, in promoting the maturation of preadipocytes (Lee & Fried 2014a). However, divergent reports showed that MR inhibition blocked the adipogenesis *in vitro*, whereas downregulation of GR did not (Caprio *et al.* 2007, 2011). Besides their effects on adipocytes growth, glucocorticoids play a well-known anti-inflammatory role, limiting the obesity-induced inflammation in adipose tissue (Fried *et al.* 1998, Patsouris *et al.* 2009). However, the relative contribution of MR and GR in this process is not totally resolved. Pharmacological inhibition of MR by eplerenone reduces the expression of pro-inflammatory genes and macrophage infiltration into adipose tissue in obese mice (Guo *et al.* 2008, Hirata *et al.* 2009, Lee *et al.* 2014). Conversely, selective activation of GR by dexamethasone decreases pro-inflammatory cytokine expression and macrophage infiltration into adipose tissue (Fried *et al.* 1998, Patsouris *et al.* 2009, Singh *et al.* 2015). Given that the chronic low-grade inflammation and the activation of the immune system are central in the pathogenesis of obesity-related disorders (Esser *et al.* 2014, Chen *et al.* 2015), the clarification of the respective roles of MR and GR in adipocytes becomes crucial.

To better understand the physiological relevance of adipocyte GR on adipose tissue function, we have selectively disrupted GR expression in mouse adipose tissues using Cre-loxP-mediated recombination. We found that reduction of GR expression in adipose tissue was not essential for adipose tissue growth and did not lead to major metabolic disturbances on a standard or a high caloric diet. However, in accordance to the anti-inflammatory role of GR, we found that adipocyte *Gr* deletion promoted pro-inflammatory markers expression and macrophage infiltration in adipose tissue.

Materials and methods

Animal experiments

Animal studies were carried out in accordance to Swiss animal welfare regulations after successful evaluation by the veterinarian office of the Canton of Zurich, Switzerland. To establish the *Gr*^{ad-ko} mice, we first crossed homozygous *Gr* floxed mice (Tronche *et al.* 1999), with transgenic mice expressing the Adipoq-Cre (B6;FVB-Tg(Adipoq-cre)1Evdr/J, Jackson Laboratories). In this study, 8-week-old littermate male mice *Gr*^{fl/fl} and *Gr*^{ad-ko} in C57BL/6 background were fed either standard chow (4.2% fat, 10.8% sucrose by weight) or high-fat high sucrose diets (21.2% fat, 34.5% sucrose by weight, TD.88137 Harlan Laboratories, Venray, the Netherlands) and the body weight was monitored weekly. After 15 weeks, mice were singly housed in metabolic cages (Techniplast, Buguggiate, Italy) for 4 days after 3 days of gradual adaptation to measure manually water/food consumption and for collection of urine/feces. Mice were then anesthetized by isoflurane inhalation for blood collection and killed for tissue collection. For the ITT and GTT, insulin (0.75 U/kg body weight) or glucose (2.5 g/kg body weight) was injected intraperitoneally and blood was collected from the tail vein at indicated time points. Blood glucose was measured using the glucometer Accu-Check (Roche).

Adipocytes and stromal vascular fraction isolation

The epididymal, inguinal and peri-renal fat pads mice were dissected, pooled according to the genotype, washed with PBS and were subsequently finely minced. The extracellular matrix was digested by collagenase (1 mg/mL; 60 min at 37°C under constant agitation). Enzyme activity was neutralized with DMEM containing 10% FBS, and the digested tissue was filtered with a 100 μ m nylon filter

and centrifuged for 5 min at 1200g at room temperature. Floating adipocytes were collected and washed twice with PBS. The pelleted stromal vascular cells were washed twice with erythrocyte lysis buffer (155 mM NH₄Cl; 10 mM KHCO₃; 0.1 mM EDTA, pH=7.3). Cell lysates were extracted and submitted to immunoblotting as described previously (Faresse *et al.* 2012).

Blood metabolites

Blood was withdrawn from anesthetized animals between 09:00 h and 11:00 h. Quantification of corticosterone in plasma was performed using a commercial kit (ENZO, ADI-900-097). Insulin levels were determined using a commercial kit (Sigma, RAB017) as well as the FFAs (Sigma, MAK044) according to the manufacturer's protocol. The glucose, cholesterol, HDL cholesterol and triglycerides levels were measured by the Zurich Integrative Rodent Physiology (ZIRP) core facility of the University of Zurich using standard techniques. Liver triglycerides content was measured after lysis and extraction of the triglycerides using a commercial kit (Abcam).

Histology

Collected tissues were fixed with 4% paraformaldehyde in PBS for 48 h and embedded in paraffin blocs. Ten-micrometer sections were stained with hematoxylin and

eosin. Images were acquired under a light microscope and the size of adipocytes was determined using ImageJ software (NIH).

Real-time quantitative PCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen) and was reverse transcribed using RevertAid™ cDNA Synthesis Kit (Thermo Scientific). Sequences of primers are indicated in Table 1. Quantitative RT-PCR was performed in duplicate for each sample in a Light Cycler 480 Real-Time PCR System by using Master I SYBR Green qPCR Master Mix (Roche), according to the manufacturer's instructions. Relative amounts of mRNA were determined using the 2^{-ΔΔCt} method for the quantification and were normalized to *Gapdh* mRNA expression, for which Ct values remain unaltered between genotypes or diets.

Immunoblotting

Tissue extracts were prepared as previously described and 30 μg protein extracts were subjected to SDS-PAGE and immunoblotting (Faresse *et al.* 2010). The antibodies and dilutions used are indicated in Table 2. Detection of proteins was performed using a chemi-luminescence kit (GE HealthCare) and autoradiography films. Quantification of the immunoblots was performed using ImageJ software (NIH).

Table 1 Primer sequences used in qRT-PCR experiments.

Genes	Forward	Reverse
<i>Cebpa</i>	GACAAGAACAGCAACGAGTA	AGCTGGCGGAAGATGC
<i>Gr</i>	ATTATGGGGTGCTGACGTG	GACATTTTCGATAGCGGCAT
<i>Mr</i>	AGTTCCTTTCGGCTGTCAA	TGACACCCAGAAGCCTCATC
<i>11βHsd1</i>	CTTCCATGACGACATCCACT	AAAAGAACCCATCCAGAGCA
<i>Pparγ</i>	TTCAACAAGAGCTGACCCAAT	AAGCCTGATGCTTTATCCCC
<i>Cebpa</i>	GACAAGAACAGCAACGAGTA	AGCTGGCGGAAGATGC
<i>Lpl</i>	GGAGGTGGACATCGGAGAAC	ACACTGCTGAGTCCTTTCCC
<i>Glut4</i>	ACACTGGTCTAGCTGTATTCT	CCAGCCACGTTGCATTGTA
<i>Fas</i>	AAGCGGTCTGGAAGCTGAA	TCTGAACCACTCACACCCAC
<i>Srebp1</i>	AGCCACACTTCATCAAGGCA	GTGGATGGGCAGTTGTCTG
<i>Lipocalin 2</i>	GTCGCTACTGGATCAGAACA	CTTGGTTCTTCCATACAGGGT
<i>Il1β</i>	TGCCACCTTTTGACAGTGATG	TGATGTGCTGCTGCGAGATT
<i>Mcp1</i>	GTCACCAAGCTCAAGAGAGA	GTGGAAAAGGTAGTGGATGC
<i>Tnfα</i>	CCCCAAAGGGATGAGAAGTT	TCTTTGAGATCCATGCCGTT
<i>Il6</i>	TCTCTGGGAAATCGTGGAAG	CCAGGTAGCTATGGTACTCC
<i>Leptin</i>	CTGGCAGTCTATCAACAGGTC	TCCACCTCTGTGGAGTAGAG
<i>Vegf</i>	GTGGACATCTCCAGGAGTACC	TGCTGTAGGAAGCTCATTCTCT
<i>Adiponectin</i>	TGCCGAAGATGACGTTACTA	TCTACCCCTTAGGACCAAGA
<i>Hgf</i>	GGTGTTTCACAAGCAATCCA	GCCCTTGTCGGGATATCTTT
<i>Cd68</i>	AAAAGGCCGTTACTCTCCTG	TGTGGCATGAGAAATTGTGG
<i>F4/80</i>	GTGCCATCATTGCGGGATTC	TGGAAGCCCATAGCCAAAGG
<i>Gapdh</i>	CCA TCA CCA TCT TCC AGG AG	TCC ATG GTG GTG AAG ACA C

Table 2 Antibody origin and dilution.

Peptide/protein target	Name of antibody	Manufacturer catalog number	Species	Dilution
Glucocorticoid receptor	GR M-20	Santa Cruz No. sc1004	Rabbit polyclonal	1/1000
Mineralocorticoid receptor	MR	Provided by C Gomez-Sanchez	Mouse monoclonal	1/100
HSP90	HSP90	Cell Signaling No. 4877	Rabbit polyclonal	1/1000
p38 MAPK	p38	Cell Signaling No. 9212	Rabbit polyclonal	1/500
phospho-p38 MAPK	P-p38	Cell Signaling No. 4092	Rabbit polyclonal	1/1000
c-Jun N-terminal kinase	JNK	Santa Cruz No. sc-571	Rabbit polyclonal	1/500
phospho-JNK	P-JNK	Santa Cruz No. sc-6254	Rabbit polyclonal	1/1000
Extracellular signal-regulated kinases1/2	ERK1/2	Santa Cruz No. C-14	Rabbit polyclonal	1/1000
phospho-ERK1/2	P-ERK	Santa Cruz No. E-4	Rabbit polyclonal	1/1000
β -actin	anti-actin	Cell Signaling No. 3700	Mouse monoclonal	1/2000

Statistical analyses

All data are presented as mean $\pm$ S.E.M. and were analyzed by unpaired two-tailed Student's *t*-test for comparisons of two groups, and two-way ANOVA with Bonferroni *post hoc* analyses for comparisons of multiple groups. *P*-values of <0.05 were considered to be significant. The statistical analyses were performed using Prism 5 software (GraphPad).

Results

Generation of adipocyte-specific *Gr*-KO mice

To establish the adipocyte-specific deletion of *Gr*, we crossed homozygous *Gr* floxed mice in which the exon 3 was flanked with loxP sites (Tronche *et al.* 1999), with the transgenic Adipoq-Cre, constitutively expressing the Cre recombinase under the control of the adiponectin

Figure 1

Adipocyte-specific deletion of *Gr*. (A) The abundance of *Gr* mRNA expression relative to *Gapdh* was assessed by qRT-PCR in epididymal (eWAT), inguinal (ingWAT), peri-renal (prWAT) and interscapular brown (BAT) adipose tissue, and in brain, liver and kidney of *Gr^{fl/fl}* and *Gr^{ad-ko}* mice. (B) Epididymal and peri-renal fat lysates from *Gr^{fl/fl}* and *Gr^{ad-ko}* mice were subjected to immunoblotting using antibodies against GR and actin as a loading control. (C) Lysates from isolated SVF and adipocytes were subjected to immunoblotting using antibodies against GR and HSP90 as a loading control. (D) The abundance of *Mr* and *11 β Hsd1* mRNA relative to *Gapdh* in eWAT was measured by qRT-PCR. (E) Plasma concentration of corticosterone in anesthetized *Gr^{fl/fl}* and *Gr^{ad-ko}* mice. Values are mean $\pm$ S.E.M. ***P* < 0.01, ****P* < 0.001, *Gr^{fl/fl}* vs *Gr^{ad-ko}*.

promotor (Wang *et al.* 2010). The resulting Adipoq-Cre *Gr^{fl/fl}* mice (hereafter termed *Gr^{ad-ko}* mice) were viable and present no anatomical differences compared with the control group (*Gr^{fl/fl}* mice). To validate the GR deletion effectiveness at the transcriptional level, isolated mRNA from different tissues were analyzed by qRT-PCR. We found that GR expression was significantly reduced in the epididymal, inguinal and peri-renal white adipose tissue but not in other organs analyzed (Fig. 1A). The evaluation of GR protein level in epididymal and perirenal white adipose tissue showed also a major reduction in its expression of about 55% (Fig. 1B). To assess whether the remaining GR expression in *Gr^{ad-ko}* mice was due to the nonadipocyte cells or to an incomplete deletion, we evaluated the GR protein expression in isolated mature adipocytes and stromal vascular fraction by immunoblot. We found that the stromal fraction from *Gr^{ad-ko}* mice expressed a similar GR level than of *Gr^{fl/fl}* mice, but GR expression in isolated mature adipocyte was totally abrogated (Fig. 1C). Testing for a potential compensatory mechanism related to the GR deletion, we found that

the mRNA expression level of *Mr* was slightly but not significantly upregulated. Furthermore, the mRNA level of *11 β Hsd1* was also not increased as expected, but rather downregulated (Fig. 1D). Finally, evaluation of the plasmatic corticosterone levels under anesthesia, which may affect glucocorticoid levels (Teilmann *et al.* 2014), did not show differences between the genotypes (Fig. 1E). Taken together, these data suggested that *Gr^{ad-ko}* mice have an efficient deletion of *Gr* in the adipose tissue, with no detectable genetic or hormonal compensation.

Adipocyte-specific deletion of *Gr* has no effect on adipose tissue growth

To test the hypothesis that adipocyte GR plays a central role in adipose tissue formation, we placed *Gr^{fl/fl}* and *Gr^{ad-ko}* 8-week-old mice either on a standard chow diet (CD) or a high-fat high sucrose diet (HFSD) for 15 weeks and the body weight was assessed weekly. The HFSD significantly increased the body weight in *Gr^{fl/fl}* and *Gr^{ad-ko}* mice, but no differences were observed between the genotypes

Figure 2

Adipocyte *Gr* deletion did not affect adiposity. *Gr^{fl/fl}* and *Gr^{ad-ko}* male mice were fed a CD or HFSD for 15 weeks starting from 8 weeks of age ($n=8-11$ for each group). The absolute body weight (A), the ratio between food consumption and feces production (B), the ratio between water intake and urine production (C) and the fat depots mass (D) were determined for each mice. (E) Representative histological images of epididymal adipose tissue fixed and stained with hematoxylin and eosin. Scale bar, 50 μ m. (F) The distribution frequency of adipocytes according to cell surface was calculated using ImageJ software ($n=300$ adipocytes from three mice for each group). All values are given as mean $\pm$ s.e.m., $^{*}P<0.01$, $^{***}P<0.001$ CD vs HFSD.

Figure 3

Lipid handling is not disturbed in adipocyte *Gr^{ad-ko}* mice. Plasma concentration of free fatty acids (A), cholesterol (B), triglycerides (C), HDL cholesterol (D), liver mass (E) and liver triglycerides (F) were evaluated in *Gr^{fl/fl}* and *Gr^{ad-ko}* mice fed a CD or a HFSD for 15 weeks. All values are given as mean $\pm$ S.E.M. (n=6 for each group), *P<0.05, ###P<0.001 CD vs HFSD.

(Fig. 2A). *Gr^{fl/fl}* and *Gr^{ad-ko}* mice showed no differences in daily food and water intake, urine, feces production and observable activities either on CD or HFSD (Fig. 2B and C). We then evaluated the adiposity of the different groups of mice by isolation of white fat pads present

in the inguinal, epididymal, mesenteric and peri-renal regions. The HFSD significantly increased the fat mass in all regions, but no differences were detected between *Gr^{fl/fl}* and *Gr^{ad-ko}* mice. Moreover, histological analysis of the adipose tissue revealed no differences between genotypes

Figure 4

Normal insulin sensitivity and disturbed glucose handling in *Gr^{ad-ko}* mice. Plasma concentration of glucose (A) and insulin (B) in *Gr^{fl/fl}* and *Gr^{ad-ko}* mice fed a CD or a HFSD for 15 weeks. Glucose tolerance test (GTT) (C and D), insulin tolerance test (ITT) (F and G) and the area under the curve (AUC) (E and H) during the tests in *Gr^{fl/fl}* and *Gr^{ad-ko}* on a CD or HFSD. All values are given as mean $\pm$ S.E.M. (n=6 for each group), *P<0.05, ##P<0.01 CD vs HFSD.

concerning the adipocytes size (median on CD, $Gr^{fl/fl}$: 2406 μm^2 vs Gr^{ad-ko} : 2470 μm^2 , median on HFSD, $Gr^{fl/fl}$: 8118 μm^2 vs Gr^{ad-ko} : 8063 μm^2).

Gr^{ad-ko} mice did not present defects in lipid storage

Disorders in adipose tissue function may lead to defects in proper storage of energy in adipocytes. To test whether the lipid storage was affected in Gr^{ad-ko} mice, we measured the plasmatic levels of free fatty acids, triglycerides, cholesterol and HDL cholesterol in $Gr^{fl/fl}$ and Gr^{ad-ko} mice fed a CD or an HFSD. Except for a slight decrease in triglyceride levels on a CD, we did not observe abnormal concentrations of these metabolites in the plasma of Gr^{ad-ko} mice, suggesting a comparable lipids handling to $Gr^{fl/fl}$ mice (Fig. 3A, B, C and D). A dysfunction of adipose tissue may also lead to ectopic lipid deposition in the liver leading to an enlargement of the liver, inflammation, fibrosis and several associated disorders (Rasouli *et al.* 2007). Therefore, we evaluated the liver weight of $Gr^{fl/fl}$

and Gr^{ad-ko} mice on both diets. We found that the HFSD increased significantly the liver mass without differences between the genotypes (Fig. 3E). Assessment of the triglycerides content of the liver confirmed that the HFSD increased the lipid accumulation in the liver similarly in $Gr^{fl/fl}$ and Gr^{ad-ko} mice (Fig. 3F).

Normal insulin sensitivity and perturbed glucose tolerance in Gr^{ad-ko} mice

To further characterize the effects of *Gr* deletion in adipocytes, we examined the response of Gr^{ad-ko} mice to glucose and insulin. The glucose and insulin levels trended higher on HFSD compared with CD, but no differences were detected between $Gr^{fl/fl}$ and Gr^{ad-ko} mice (Fig. 4A and B). We then performed a glucose tolerance test by injecting 2.5 g/kg of body weight of glucose solution intra-peritoneally (Fig. 4C and D). If no difference was observed on CD, we found that control mice were more resistant to glucose loading compared with Gr^{ad-ko} mice

Figure 5

Adipocyte *Gr* deletion promotes diet-induced inflammation. (A) The abundance of key genes involved in lipogenesis, inflammation and adipokines expression was evaluated by qRT-PCR in epididymal fat from $Gr^{fl/fl}$ and Gr^{ad-ko} mice fed a HFSD ($n=5$ per group). (B) Representative histological images of epididymal adipose tissue fixed and stained with hematoxylin and eosin. The red arrows indicate the crown-like structures. Scale bar, 50 μm . (C) The abundance of macrophage markers *Cd68* and *F4/80* mRNA relative to *Gapdh* in adipose tissue was measured by qRT-PCR. (D) Plasma concentration of Lipocalin 2. (E) Epididymal lysates were submitted to immunoblotting using antibodies against MR, GR, phospho-JNK, JNK, phospho-ERK1/2, ERK1/2, phospho-P38, P38 and actin as a loading control. (F) Immunoblots were quantified by Image J and normalized to actin. * $P<0.05$, $Gr^{fl/fl}$ vs Gr^{ad-ko} . A full colour version of this figure is available at <http://dx.doi.org/10.1530/JOE-16-0121>.

on HFSD. The areas under the curves revealed a significant increase for *Gr^{ad-ko}* mice on HFSD vs CD, but not for *Gr^{fl/fl}* mice (Fig. 4E). Then, we performed an insulin tolerance test to evaluate the insulin sensitivity of each group of mice. Injection of 0.75 U/kg of body weight of insulin solution intra-peritoneally led to a similar lowering of glycemia between *Gr^{fl/fl}* and *Gr^{ad-ko}* mice in both CD and HFSD (Fig. 4F and G) and similar areas under the curves (Fig. 4H).

Gr deletion in adipocytes promotes obesity-mediated inflammation of the adipose tissue

Glucocorticoids via the glucocorticoid receptor have been proposed to regulate several aspects of adipocyte function including adipogenesis (Lee & Fried 2014a), inflammation (Lee *et al.* 2011, Singh *et al.* 2015) or endocrine functions (Lee & Fried 2014a, Lee *et al.* 2014). To test these previous findings *in vivo*, we analyzed the expression levels of a panel of key genes expressed by adipocytes involved in lipogenesis, inflammation and secreted adipokines in diet-induced obese *Gr^{fl/fl}* and *Gr^{ad-ko}* mice. As shown in Fig. 5A, *Gr* deletion has a slight or no effects on lipogenic genes as well as adipokines expression. However, pro-inflammatory markers tended to be higher in *Gr^{ad-ko}* mice, particularly TNF α and lipocalin-2, two important markers of obesity-mediated low-grade inflammation in adipose tissue (Fig. 5A). To test whether *Gr* deleted mice showed a higher adipose tissue inflammation, we assessed the macrophage infiltration in adipose tissue from *Gr^{fl/fl}* and *Gr^{ad-ko}* mice fed a HFSD. It has been shown that the large majority of macrophages infiltrating the adipose tissue of obese animals and humans is arranged around dead adipocytes, forming characteristic crown-like structures (Cinti *et al.* 2005). Histological analysis of adipose tissue from HFSD-fed *Gr^{fl/fl}* and *Gr^{ad-ko}* mice quantitatively showed more crown-like structures in *Gr^{ad-ko}* than *Gr^{fl/fl}* mice (Fig. 5B). This observation was confirmed by evaluating the expression levels of specific macrophage markers (CD68 and F4/80) that were higher in *Gr^{ad-ko}* compared with *Gr^{fl/fl}* (Fig. 5C). Clinical evidences demonstrated that serum concentrations of lipocalin-2 are closely associated with obesity and its related chronic inflammation (Wang *et al.* 2007). Quantification of this glycoprotein showed a significantly higher plasma concentration in *Gr^{ad-ko}* mice (Fig. 5D). Finally, pro-inflammatory changes in adipose tissue have been shown to activate stress kinase signaling pathways, including JNK, ERK1/2 and P38 (Hirosumi *et al.* 2002). In our model, *Gr* deletion significantly increased

the activation of JNK without detectable effects on ERK1/2 or P38 (Fig. 5E and F).

Discussion

Glucocorticoids excess is a well-known disorder mediating an increase in adiposity in humans and rodent models. At the cellular level, glucocorticoids are required to induce the differentiation of preadipocytes to mature adipocytes (Chapman *et al.* 1985, Lee *et al.* 2014), and act synergistically with insulin to upregulate lipogenesis (Minshull & Strong 1985, Gathercole *et al.* 2011). However, whether the glucocorticoids effects on adipose tissue are mediated by GR or MR remains unclear. Several studies attempted to elucidate the relative contribution of these steroid receptors in glucocorticoids effects (Caprio *et al.* 2007, Hirata *et al.* 2012, Armani *et al.* 2014, Lee & Fried 2014a). In addition to the conflicting results obtained, most of the deductions were based on *in vitro* observations and all the studies resulted in same conclusions about the crucial need of adipose-specific *Mr* or *Gr*-knockout mice. In this study, we found that adipocyte-specific *Gr* deletion did not affect adipose tissue expansion, fat distribution and did not induce major metabolic disturbances either on standard diet or during a high caloric diet. These findings would suggest that GR plays a minor role in the transduction of glucocorticoids-mediated lipogenesis in adipocytes *in vivo*. However, one limitation of this study is the tissue-specific adiponectin promotor that drives the expression of the Cre recombinase only in mature adipocytes and not in the early stages of differentiation, according to the adiponectin expression (Korner *et al.* 2005). Given the importance of glucocorticoids in the early steps of adipogenesis, our mouse model is suitable to study the function of GR in fully mature adipocytes but not in the formation of new adipocytes.

Our results differ from the recently published study from de Kloet *et al.*, showing a resistance to diet-induced obesity in a case of adipocyte GR deficiency (de Kloet *et al.* 2015). The authors observed that an acute exposure to a high fat diet (one week) was sufficient to detect a significant lower body weight in *Gr*-deficient mice. This difference was maintained until 5 weeks of diet and was principally due to a lower adiposity. In our study, despite a higher deletion efficiency of *Gr* (mRNA reduction of 74% vs 20% in eWAT), we did not observe any detectable difference between the genotypes even during the first weeks of diets. Such a discrepancy may have different explanations. In both studies, the same adiponectin-Cre mice

were crossed with different *Gr* floxed mice. In our case, the exon 3 encoding the DNA-binding domain of GR was floxed, whereas in de Kloet study, the exon 2 encoding the activation function-1 (*Af1*) domain was floxed. Notably, it was shown that disruption of exon 2 of *Gr* gene may yield to a ligand-responsive C-terminal fragment that maintains its ability to regulate gene expression (Mittelstadt & Ashwell 2003). Furthermore, in our study the food was supplemented with high sucrose and the fat distribution analysis was performed after 15 weeks of diet, whereas in de Kloet study, the mice were fed a high fat diet and the body composition was analyzed only after 5 weeks of diet.

In addition to adipose tissue growth, the potent anti-inflammatory role of glucocorticoids/GR signaling is well-studied and synthetic GR agonists have become widely used for therapy (De Bosscher & Haegeman 2009). This anti-inflammatory action is explained by the genomic and nongenomic properties of GR. The nongenomic pathway involves the direct interaction of GR with the MAPK protein JNK in the cytoplasm, suppressing the activation of this pro-inflammatory pathway (De Bosscher & Haegeman 2009). The genomic action is mediated through the nuclear translocation of the receptor and activation of key anti-inflammatory genes (*MKP1*, *IL10*, Annexin-1) and repression of pro-inflammatory genes (*TNF α* , *IL6*, *IL1 β*) (De Bosscher & Haegeman 2009, Nixon *et al.* 2012). Obesity is described as a low-grade inflammatory state with increased macrophage infiltration in adipose tissue contributing to insulin resistance and metabolic dysfunction (Gregor & Hotamisligil 2011). However, recently a provocative study showed that adipocyte inflammation is also essential for healthy adipose tissue expansion and remodeling (Wernstedt Asterholm *et al.* 2014). In our study, we found that adipocyte-specific *Gr* deletion increased pro-inflammatory cytokine expression, macrophage infiltration and activation of the stress kinase JNK in adipose tissue. These findings can be paralleled with *in vitro* and *in vivo* pharmacological studies showing that GR activators decrease whereas GR blockers increase pro-inflammatory adipokines expression and macrophage infiltration into adipose tissue (Patsouris *et al.* 2009, Hoppmann *et al.* 2010, Nikolic *et al.* 2013). Furthermore, the significant reduction of *11 β Hsd1* mRNA expression, that has been shown to be indirectly regulated by GR and dampen the inflammation process (Sai *et al.* 2008, Randall *et al.* 2014), may also contribute to the higher inflammation observed in *Gr*^{ad-ko} mice. Adipocytes size, another hallmark of obesity, is also highly correlated with systemic insulin resistance, dyslipidemia and development of type 2 diabetes (McLaughlin *et al.* 2010,

Yang *et al.* 2012). In our mouse model, we could not detect differences in adipocytes size, neither during chow nor high caloric diet and the higher inflammatory state was not paralleled with disturbances in insulin sensitivity or lipid handling. Nevertheless, the *Gr*^{ad-ko} appeared to be less tolerant to glucose loading when fed a high caloric diet that may be secondary to the increase of adipose tissue inflammation observed in these animals. Hence, the connection between inflammation, adipocytes size and metabolic disorders in our *Gr*^{ad-ko} mouse model would certainly benefit from deeper investigations.

Due to the higher accumulation of fat in the central region during glucocorticoids excess (Wajchenberg *et al.* 1995), it was thought that visceral fat is more sensitive to glucocorticoids than the subcutaneous fat. This theory was supported by the fact that GR expression is higher in the omentum than in the subcutaneous fat (Miller *et al.* 1987, Pedersen *et al.* 1994). However, in our *Gr*^{ad-ko} mouse model fed either a chow or a high caloric diet, the weight of the inguinal, epididymal, mesenteric or peri-renal fat pads was not affected, suggesting that the relative abundance of GR did not explain the depot-specific differences in glucocorticoids actions. Rather, the higher expression of MR and *11 β HSD1* in the visceral adipose tissue may have a more prominent role in these differences (Hirata *et al.* 2012). If our observations did not allow a conclusion about MR, the fact that glucocorticoids bind to MR with ten-fold higher affinity than GR (Arriza *et al.* 1987), the high concentration of circulating glucocorticoids (100–1000-fold higher than aldosterone) and the increase of adipocytes MR expression during adipogenesis and obesity (Caprio *et al.* 2007, Hirata *et al.* 2009), it may be probable that glucocorticoids mediate their effects mainly through MR.

In summary, our findings brought a first piece of *in vivo* evidences about the role of GR in adipose tissue. We found that adipocyte GR has a negligible role in the regulation of body weight and adiposity in a standard as well as a high caloric diet. Furthermore, the absence of GR in adipocytes did not cause major metabolic disorders but promoted the diet-induced inflammation in adipose tissue.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

N F conceived and designed the experiments; S D performed the experiments; S D and N F analyzed the data; and N F wrote the paper.

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