The balance between gluco- and mineralo-corticoid action critically determines inflammatory adipocyte responses

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Abstract

Obesity is associated with chronic inflammation. Pro-inflammatory adipokines may promote metabolic disorders and cardiovascular morbidity. However, the key mechanisms leading to obesity-related inflammation are poorly understood. The corticosteroid metabolism in adipose tissue plays a crucial role in the pathogenesis of the metabolic syndrome. Both the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR) mediate corticosteroid action in adipose tissue. The significance of the interplay of these receptors in mediating an inflammatory adipokine response is virtually unexplored. In the present study, we investigated the differential roles of the GR and MR in controlling the key adipose tissue functions including inflammatory adipokine expression and adipogenesis using selective stimulation with receptor agonists, acute receptor knockdown via RNA interference and newly generated knockout adipose cell lines. Selective GR stimulation of white adipocytes with dexamethasone inhibited the expression of interleukin 6 (IL6), monocyte chemoattractant protein-1 (MCP1 or CCL2 as listed in the MGI Database), tumour necrosis factor-α, chemerin and leptin. By contrast, selective MR stimulation with aldosterone promoted the expression of IL6, plasminogen activator inhibitor 1, chemerin and leptin. Furthermore, in the presence of an acute GR knockdown as well as in GR knockout adipocytes, corticosterone increased the gene expression of the pro-inflammatory adipokines IL6 and MCP1. Whereas GR knockout adipocytes displayed a mildly impaired adipogenesis during early differentiation, MR knockout cells completely failed to accumulate lipids. Taken together, our data demonstrate a critical role for the balance between gluco- and mineralocorticoid action in determining adipocyte responses implicated in obesity-associated inflammation and cardiovascular complications.
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Introduction

Obesity is associated with chronic subclinical inflammation, which is characterised by an increased production of pro-inflammatory cytokines and an altered endocrine and metabolic adipose tissue function (Wellen & Hotamisligil 2005). The inflammatory state seen in obesity is considered to be responsible for the development of obesity-related disorders such as insulin resistance, hyperlipidaemia and cardiovascular morbidity (Pickup & Crook 1998, Fernandez-Real & Ricart 2003, Yudkin 2003). Increased plasma levels of interleukin 6 (IL6) and plasminogen activator inhibitor 1 (PAI-1 or SERPINE1 as listed in the MGI Database) considerably impair insulin sensitivity and predict the development of type 2 diabetes and cardiovascular disease in humans (Kern *et al.* 2001, Pradhan *et al.* 2001, Mertens & Van Gaal 2002, Spranger *et al.* 2003). The pro-inflammatory monocyte chemoattractant protein-1 (MCP1 or CCL2 as listed in the MGI Database) which is elevated in obesity promotes monocyte infiltration in vessels and atherosclerotic plaques (Takahashi *et al.* 2003, Kanda *et al.* 2006). A vast array of data also implicates the pro-inflammatory cytokine tumour necrosis factor-α (TNF-α) in the pathophysiology of the metabolic syndrome (Hotamisligil *et al.* 1993, 1995). The crosstalk between adipocytes and macrophages which infiltrate adipose tissue in obesity is considered to be crucial for the development of the pro-inflammatory state (Bluher 2008). To date, the molecular mechanisms that lead to obesity-related inflammation have not been elucidated completely.

Similarities between the metabolic syndrome and the glucocorticoid excess seen in Cushing's syndrome have led to the assumption that a deranged corticosteroid metabolism in adipose tissue plays a pivotal role in the development of obesity and associated metabolic and cardiovascular disorders. Indeed, adipose tissue-specific amplification of glucocorticoids by selective overexpression of 11β-hydroxysteroid dehydrogenase type 1 (HSD11B1) was shown to induce all the characteristic features of the metabolic syndrome in mice, including central obesity, diabetes, dyslipidaemia and hypertension (Masuzaki *et al.* 2001, 2003). In contrast, the inactivation of glucocorticoid action in adipose tissue enhances insulin sensitivity in these mice and protects them

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against obesity and diet-induced metabolic disease (Morton *et al.* 2004, Kershaw *et al.* 2005). Although cortisol plasma concentrations were shown not to be consistently elevated in obese people (Seckl *et al.* 2004), there is accumulating evidence for increased endogenous adipose tissue-specific glucocorticoid reactivation in human obesity (Mariniello *et al.* 2006, Alberti *et al.* 2007, Paulsen *et al.* 2007).

Glucocorticoid action in adipose tissue has long been considered to be exclusively mediated by the cytosolic glucocorticoid receptor (GR listed as NR3C1 in the MGI Database). Recently, it has been found that the mineralocorticoid receptor (MR listed as NR3C2 in the MGI Database) is also expressed in white adipocytes as well as in brown adipocytes (Rondinone *et al.* 1993, Zennaro *et al.* 1998). The MR possesses the same affinity for aldosterone and glucocorticoid hormones (Arriza *et al.* 1987). Considering that the glucocorticoid-deactivating enzyme HSD11B2 is not significantly expressed in adipose tissue, glucocorticoids can also act via the MR in adipocytes (Bujalska *et al.* 1997, Yang *et al.* 1997, Engeli *et al.* 2004). We and others have recently identified metabolic and endocrine functions of brown adipose tissue as a novel target for mineralocorticoid action (Viengchareun *et al.* 2001, Kraus *et al.* 2005). A previous study suggests an involvement of the MR in corticosteroid-induced adipogenesis (Caprio *et al.* 2007). Moreover, in recent murine *in vivo* and *in vitro* studies, stimulation of the MR was implicated to promote inflammation (Guo *et al.* 2008). To date, however, the physiological significance of the MR in mature adipocytes remains virtually unexplored and only little is known about the interplay of GR- and MR-mediated regulation of endocrine and metabolic adipocyte functions. Given that MR/GR dimers can mediate and differentially modulate corticosteroid action, it is even more difficult to dissect the separate roles of each receptor in controlling adipocyte responses.

In the present study, we therefore investigated the different roles of the corticosteroid receptors GR and MR in mediating an inflammatory adipokine response in mature adipocytes using three different approaches: selective stimulation with receptor agonists, acute knockdown of the GR in differentiated adipocytes using RNA interference and the generation and characterisation of GR and MR knockout cell lines from newborn homozygous knockout mice which die shortly after birth (Cole *et al.* 1995, Berger *et al.* 1998).

Our study demonstrates opposing and complementary effects of the GR and MR in regulating an inflammatory adipokine response. While selective GR activation induces an anti-inflammatory effect, GR knockdown and knockout as well as selective MR stimulation promote a pro-inflammatory adipokine profile in mature adipocytes. Our data provide support for the notion that the balance between GR and MR activation in adipocytes critically determines inflammatory and metabolic adipocyte responses. Selective corticosteroid receptor modulation may have therapeutic implications for the prevention and treatment of obesity and insulin resistance.

Materials and Methods

Materials

Aldosterone, corticosterone and dexamethasone were purchased from Sigma–Aldrich. Antibodies against the MR and GR were ordered from Santa Cruz Biotechnology (Santa Cruz, CA, USA), while antibodies against Pref-1, C/EBPα and aP2 as well as secondary anti-rabbit and anti-mouse antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Primers for gene expression analyses were ordered from Biometra (Göttingen, Germany). All other materials were purchased from Sigma–Aldrich, unless stated otherwise.

Generation of adipose cell lines

Novel GR and MR knockout adipose cell lines from newborn homozygous knockout mice (ordered from the European Mouse Mutant Archive, Munich, Germany) as well as wild-type control cell lines were generated as originally described by Klein *et al.* (1999, 2002). In brief, 10 mg interscapular brown fat was removed from newborn mice, minced and subjected to collagenase solution (1 mg collagenase in 1 ml isolation buffer containing 0·123 mM NaCl, 5 mM KCl, 1·3 mM $CaCl_2$, 5 mM glucose, 100 mM Hepes, 100 U/ml penicillin/streptomycin and 4% BSA (w/v)). Digestion was performed in a shaking water bath at 37 °C for 40 min. The digested tissue was filtered through a 100-μm filter and centrifuged at 500 ***g*** for 5 min. The supernatant was removed and the pellet was washed carefully. Centrifugation was repeated at 500 ***g*** for 5 min. After resuspending the remaining pellet in culture medium, cells were transferred to plates and grown in a humidified atmosphere of 5% CO_2 (v/v) at 37 °C. The following day, cells were carefully washed with PBS. Subsequently, the culture medium was changed every day. After reaching 70% confluence, cells were infected with the puromycin resistance-conferring retroviral vector pBabe coding for the SV40 Large T antigen for 24 h. Cells were maintained in the culture medium for 48 h before a 3-week selection with puromycin (2 μg/ml) was begun. Preadipocytes were split whenever reaching 80% confluence.

Cell culture

Cells were cultured as described previously (Klein *et al.* 1999, 2002). Immortalised white epididymal adipose cell lines as well as newly generated brown GR knockout, MR knockout and control wild-type cell lines were differentiated on 10-cm plates in DMEM supplemented with 20% foetal bovine serum (v/v), 4·5 g/l glucose, 20 nM insulin and 1 nM triiodothyronine (differentiation medium) in a humidified atmosphere of 5% CO_2 (v/v) at 37 °C. When preadipocytes reached confluence, cell differentiation was induced by 250 μM indomethacine, 500 μM isobutylmethylxanthine and 2 μg/ml dexamethasone for 20 h (induction medium). Subsequently, cells were grown in differentiation medium

for another 6 days, and the culture medium was changed every day. Prior to the treatment with dexamethasone, aldosterone or corticosterone, adipocytes were serum starved in DMEM for 24 h. All experiments were carried out by using at least three independent cell lines from passages 10 to 30.

RNA interference

Preadipocytes were cultured in six-well plates and differentiated as described above. The induction was performed in dexamethasone-depleted induction medium. On day 3 after induction, the adherent cells were carefully washed twice with PBS, trypsinised and resuspended in differentiation medium. Adipocytes were centrifuged at 800 ***g*** for 5 min. The supernatant was carefully removed and the pellet was dissolved in PBS. Centrifugation was repeated at 800 ***g*** for 5 min. For RNA silencing, adipocytes were mixed with the manufacturer's nucleofection reagent (Cell line nucleofector kit V, Lonza, Cologne, Germany) and 200 pmol GR-specific siRNA or negative control siRNA (scr. RNA) (Qiagen) in a total volume of 100 µl. Electroporation was performed using a Nucleofector (Lonza) according to the manufacturer's protocol. Transfected cells were cultured in a six-well plate for another 2 days and were subsequently starved in serum-depleted medium overnight. On day 6, adipocytes were treated with 100 nM corticosterone for 2 h. The extent of the receptor knockdown was quantified by western blot and quantitative real-time PCR.

Western blot analysis

Proteins were isolated from adipocytes using cell lysis buffer containing 2 mM vanadate, 10 µg/ml aprotinin, 10 µg/ml leupeptin and 2 mM phenylmethylsulphonyl fluoride. The protein content was quantified using the Bradford protein assay according to the manufacturer's instructions (Bio-Rad). Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Membranes were blocked with rinsing buffer (10 mM Tris, 150 mM NaCl and 0·05% Tween (v/v), pH 7·2) including 3% BSA (w/v) at 4 °C overnight. For the detection of proteins, membranes were exposed to specific primary antibodies against the nuclear receptors GR and MR or the differentiation markers Pref-1, C/EBPα (CEBPA) and aP2. Gel loading was normalised using actin as a control protein. Protein bands were visualised using chemiluminescence (Perkin Elmer, Rodgau-Jügesheim, Germany). The subsequent quantification was performed using Quantity One software (Bio-Rad). Representative western blots are shown using actin as a loading control.

Genomic DNA isolation

Genomic DNA was extracted from cultured preadipocytes of newly generated adipose cell lines using the Genomic DNA Purification Kit from Gentra Systems (Minneapolis, MN, USA) in accordance with the manufacturer's instruction.

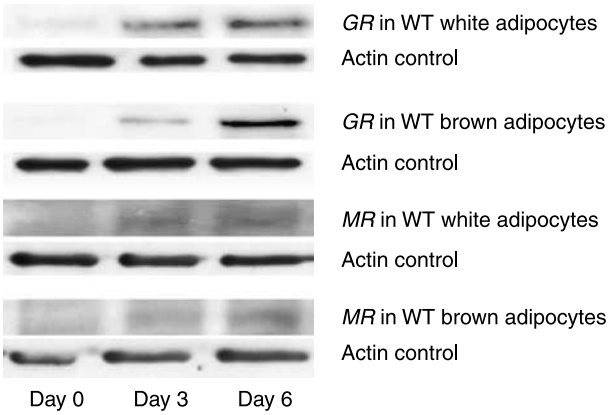

Figure 1 Expression of GR and MR in white and brown adipocytes during differentiation. Protein levels were determined by western blot analysis on days 0, 3 and 6 of adipocyte differentiation. Representative western blots of three independent experiments are illustrated.

RNA isolation and reverse transcription

Total RNA was isolated using QIAzol reagent (Qiagen). To optimise RNA quality, clean-up and genomic DNA digestion were performed using RNAeasy kit and RNase-Free DNase Set (Qiagen). Standardised RNA amounts of 2 µg were reverse-transcribed in a 20 µl reaction mixture containing Superscript II (Invitrogen), oligo p(dt)15 primers and RNase inhibitor (Roche Molecular Biochemicals).

Quantitative real-time PCR

Two microlitres of prediluted DNA were amplified in a total volume of 12 µl containing 1× SYBR Premix Ex Taq (TaKaRa, Otsu, Japan) and gene-specific primers using the Mastercycler ep realplex (Eppendorf, Hamburg, Germany). Quantitative real-time PCR was performed as follows: initial denaturation at 95 °C for 300 s, 40 cycles at 95 °C for 20 s, 60 °C for 30 s. Amplification of the genomic *GR*-DNA was carried out using: initial denaturation at 95 °C for 10 min, 40 cycles at 95 °C for 30 s, 69·1 °C for 1 min and a final elongation step at 68 °C for 1 min. For detecting the genomic *MR*-DNA, the following PCR programme was used: initial denaturation at 95 °C for 10 min, 40 cycles at 95 °C for 30 s, 67·1 °C for 1 min and 72 °C for 1 min. The identity of gene products was verified by melting curve analysis and agarose gel electrophoresis. For optimised relative quantification, 36B4 served as a housekeeping gene. Primer sequences are available upon request.

Determination of leptin concentrations

Leptin concentrations were determined using the RayBio Mouse Leptin ELISA Kit (RayBiotech, Norcross, GA, USA) according to the manufacturer's manual.

Oil Red O staining

Cells were cultivated in 10-cm plates and fat-specific staining was performed with Oil Red O after induction on days 0, 3 and 6. For this purpose, adipocytes were washed twice with PBS and were subsequently fixed with 10% formalin (v/v) for at least 15 min. Afterwards, cells were exposed to Oil Red O for 1 h at room temperature (stock solution: 0·5 g Oil Red O in 100 ml isopropanol; working

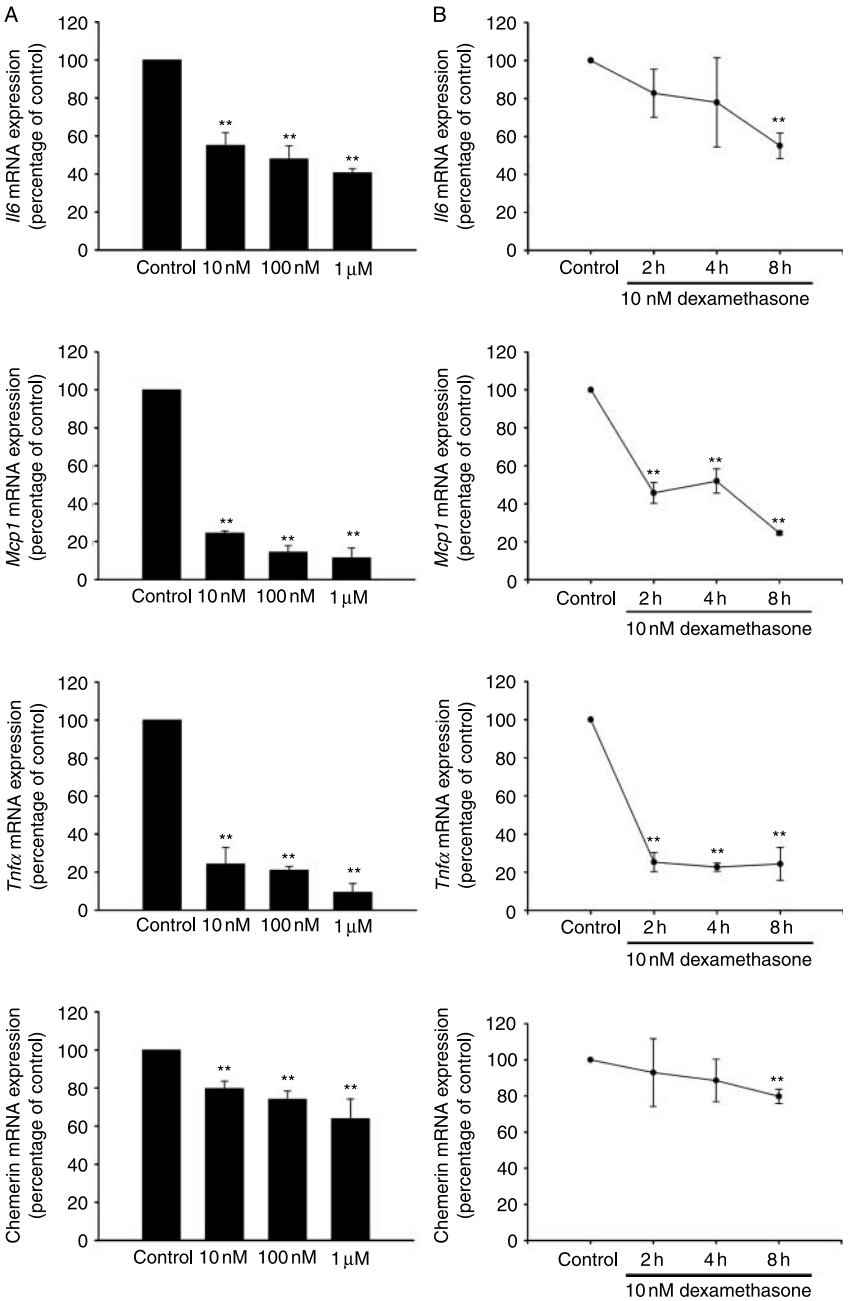

Figure 2 (A) Dose-dependent inhibition of *Il6*, *Mcp1*, *Tnfα* and chemerin in differentiated white adipocytes by dexamethasone. Adipocytes were treated with 10 nM, 100 nM and 1 μM for 8 h or left untreated. mRNA expression was quantified by RT-PCR. (B) Time-dependent decrease of *Il6*, *Mcp1*, *Tnfα* and chemerin in differentiated white adipocytes by dexamethasone. Cells were exposed to 10 nM dexamethasone for 2, 4 and 8 h. Gene expression was measured by RT-PCR. Results are shown as mean±S.E.M. of three independent experiments. $^{**}P \leq 0{\cdot}01$ comparing non-treated cells to dexamethasone-treated cells.

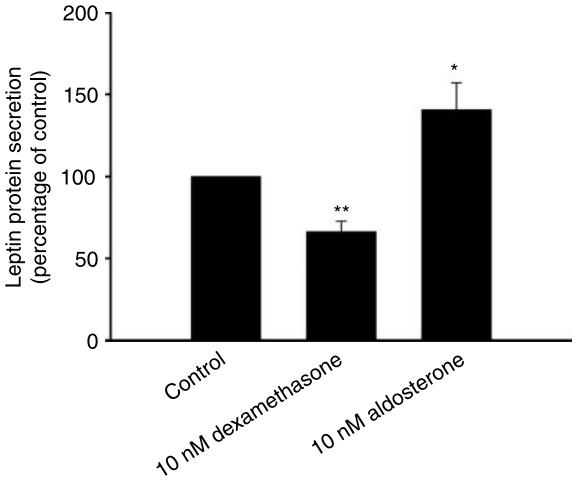

Figure 3 Reciprocal regulation of leptin secretion by dexamethasone and aldosterone. Adipocytes were treated with 10 nM dexamethasone or aldosterone for 24 h or left untreated. Protein concentrations were quantified by ELISA analyses. Results are shown as mean ± S.E.M. of at least three independent experiments. *$P \leq 0{\cdot}05$ and **$P \leq 0{\cdot}01$ comparing non-treated cells to treated cells.

solution: 60% stock solution (v/v) and 40% H_2O (v/v)). To remove the staining solution, cells were washed several times with distilled water. Representative macroscopic as well as microscopic pictures at a 40-fold magnification were taken using a digital camera (Olympus E330, Olympus Imaging Europa, Hamburg, Germany). For densitometric analysis, Oil Red O stain was removed by washing cells with isopropanol for 15 min. Optical density was measured at 500 nm.

Statistical analysis

Statistical analyses were performed with Sigma Plot (SPSS Science, Chicago, IL, USA). Results are presented as mean values ± S.E.M. Statistical significance was determined using the unpaired Student's *t*-test. *P* values $\leq 0{\cdot}05$ were considered significant, and those $\leq 0{\cdot}01$ were considered highly significant.

Results

GR and MR are expressed in white and brown adipocytes and are up-regulated during adipocyte differentiation

Both the GR and the MR were expressed in white adipocytes as well as in brown adipocytes. Protein analysis revealed an increase in the expression of both receptors during adipogenesis (Fig. 1).

Selective GR stimulation with dexamethasone inhibits the expression of pro-inflammatory adipokines

To define the role of the corticosteroid receptors MR and GR in mediating an inflammatory adipokine response in white adipose tissue, we first investigated the effect of selective GR stimulation on the adipokine profile. Acute dexamethasone treatment of fully differentiated white adipocytes dose- and time-dependently inhibited mRNA expression of the pro-inflammatory adipokines *Il6*, *Mcp1*, *Tnfα* and chemerin.

Il6 mRNA concentrations were dose-dependently decreased by up to 60% after stimulation with dexamethasone for 8 h (Fig. 2A). Furthermore, the inhibitory effect of

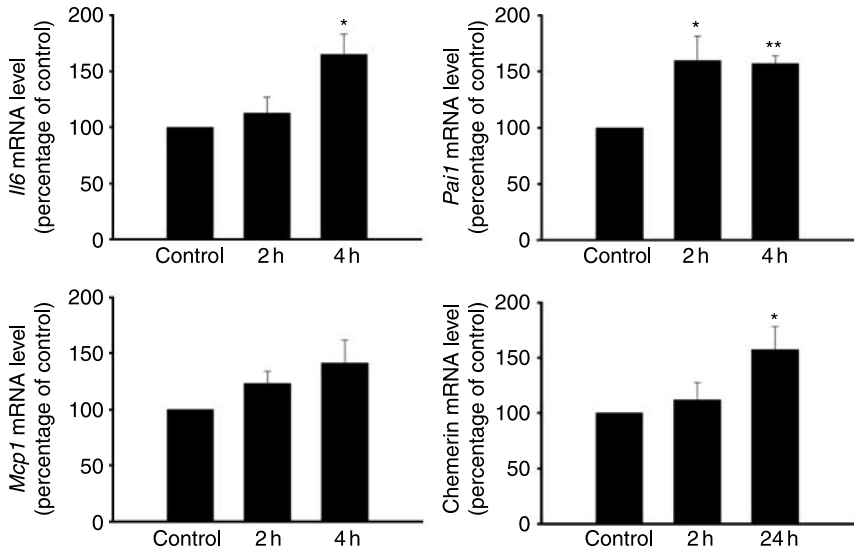

Figure 4 Time-dependent regulation of *Il6, Mcp1, Pai1* and chemerin gene expression in differentiated white adipocytes by aldosterone. Adipocytes were treated with 10 nM aldosterone for 2, 4 and 24 h or left untreated. mRNA expression of *Il6, Mcp1, Pai1* and chemerin was analysed by RT-PCR. Results are shown as mean ± S.E.M. of at least three independent experiments. *$P \leq 0{\cdot}05$ and **$P \leq 0{\cdot}01$ comparing non-treated cells to aldosterone-treated cells.

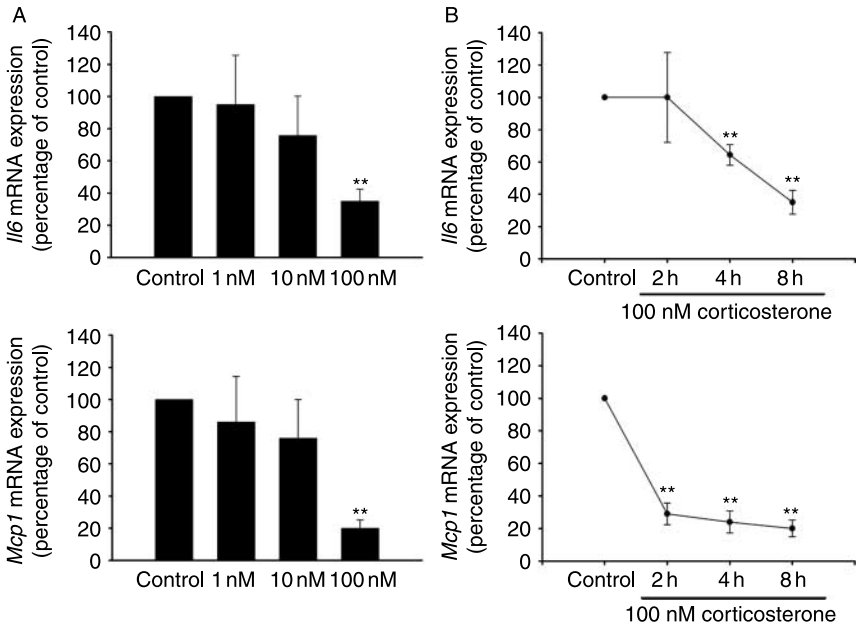

Figure 5 (A) Dose-dependent inhibition of *Il6* and *Mcp1* gene expression in differentiated white adipocytes by corticosterone. Adipocytes were treated with 1, 10 and 100 nM corticosterone for 8 h or left untreated. mRNA expression was quantified by RT-PCR. (B) Time-dependent decrease of *Il6* and *Mcp1* in differentiated white adipocytes by corticosterone. Cells were exposed to 100 nM corticosterone for 2, 4 and 8 h. Gene expression was measured by RT-PCR. Results are shown as mean ± S.E.M. of three independent experiments. $^{**}P \leq 0{\cdot}01$ comparing non-treated cells to corticosterone-treated cells.

dexamethasone was time-dependent. *Il6* gene expression was slightly reduced by 20% after 2 h in response to 10 nM dexamethasone treatment and reached a maximal reduction by 45% after 8 h (Fig. 2B).

Similarly, selective GR stimulation with dexamethasone for 8 h markedly suppressed the gene expression of the pro-inflammatory adipokine MCP1 in terminally differentiated adipocytes (Fig. 2A). A maximal decrease of MCP1 mRNA by 90% was observed when adipocytes were stimulated with 1 μM dexamethasone (Fig. 2A). Exposure of adipocytes to 10 nM dexamethasone for 2 h markedly suppressed MCP1 mRNA expression by 55% as compared to non-treated cells. The gene expression time-dependently declined and was maximally decreased by 75% after 8 h (Fig. 2B).

Treatment with dexamethasone also resulted in a significant reduction of the pro-inflammatory adipokine TNF-α. Gene expression of *Tnfα* was maximally decreased by 90% after exposure to 1 μM dexamethasone for 8 h (Fig. 2A). Even low concentrations of dexamethasone (10 nM) strongly suppressed the mRNA expression by ∼75% after 2, 4 and 8 h (Fig. 2A).

Moreover, GR activation by dexamethasone decreased mRNA levels of the novel pro-inflammatory adipokine chemerin. Chemerin gene expression was dose-dependently suppressed by up to 35% after treatment with 10 nM–1 μM dexamethasone for 8 h in terminally differentiated white adipocytes (Fig. 2A). The inhibitory effect was time-dependent (Fig. 2B).

Leptin was recently shown to be involved in immune responses as a pro-inflammatory factor. Exposure of differentiated adipocytes to 10 nM dexamethasone for 24 h significantly decreased the protein secretion of leptin by 35% when compared to untreated cells (Fig. 3).

Selective MR stimulation with aldosterone promotes a pro-inflammatory and diabetogenic adipokine expression profile

In contrast to the findings described above, selective MR stimulation of mature white adipocytes with low concentrations of aldosterone induced the gene expression of the pro-inflammatory adipokines *Il6* and *Pai1*, while *Mcp1* was unaffected. Gene expression of *Il6* tended to be increased by 15% after 2 h of 10 nM aldosterone treatment and was maximally elevated by 65% after 4 h as compared to non-treated cells (Fig. 4). Similar pro-inflammatory effects of MR stimulation were observed with regard to *Pai1* gene expression. The mRNA expression level of this adipokine was enhanced by about 60% after 2 and 4 h of treatment with 10 nM aldosterone in terminally differentiated white adipocytes (Fig. 4). There was a non-significant trend towards an up-regulation of *Mcp1* gene expression by 25 and 40% as compared to non-treated cells after 2 and 4 h of treatment with 10 nM aldosterone (Fig. 4). Gene expression levels of the pro-inflammatory adipokine chemerin were slightly elevated by 10% after MR activation by 10 nM aldosterone for 2 h and

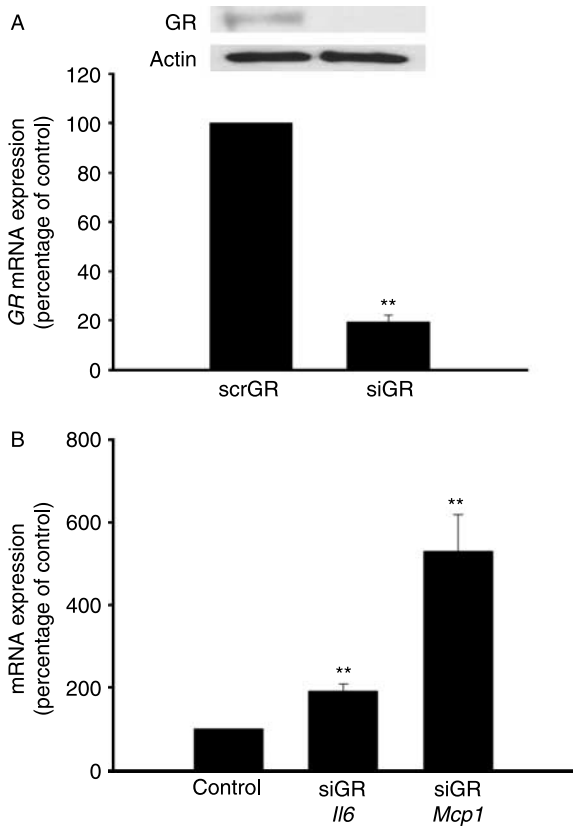

Figure 6 (A) Validation of *GR* knockdown in fully differentiated white adipocytes. Acute knockdown of the GR was performed on day 3 of adipocyte differentiation. On day 6, mRNA expression as well as protein expression of the GR was determined by RT-PCR and western blot. (B) Increase of *Il6* and *Mcp1* in GR knockdown adipocytes by corticosterone. *GR* knockdown was performed in white adipocytes on day 3 of adipocyte differentiation. Seventy-two hours after transfection with specific siGR or scrRNA, adipocytes were treated with 100 nM corticosterone for 2 h. mRNA expression of *Il6* and *Mcp1* was analysed by RT-PCR. Results are shown as mean ± S.E.M. of three independent experiments. $^{**}P \leq 0{\cdot}01$ comparing scrRNA-treated control cells to siGR-treated cells.

were maximally increased by 60% after 24 h (Fig. 4). Finally, protein secretion of the pro-inflammatory factor leptin revealed an up-regulation by 40% after stimulation with 10 nM aldosterone for 24 h (Fig. 3).

Unselective stimulation of corticosteroid receptors in adipocytes with corticosterone decreased the expression of pro-inflammatory adipokines

To investigate whether the anti-inflammatory GR-mediated effect or the mainly pro-inflammatory effect of the MR predominates the response to the unselective corticosteroid receptor agonist corticosterone in adipocytes, we investigated the effect of corticosterone on the adipokine profile. Whereas low concentrations of corticosterone did not have a significant effect on the gene expression of *Il6* and *Mcp1*, treatment with higher corticosterone concentrations resulted in a decrease of these pro-inflammatory adipokines (Fig. 5A). This suppression was time-dependent and reached a maximal reduction of *Il6* and *Mcp1* gene expression after 8 h by 65 and 80% respectively (Fig. 5B).

GR *knockdown and* MR *stimulation by corticosterone in mature white adipocytes lead to an increase in* Il6 *and* Mcp1 *gene expression*

We further examined the effect of an acute *GR* knockdown in fully differentiated white adipocytes on the adipokine profile. Western blot and mRNA expression analyses confirmed the *GR* down-regulation by ~80% in white adipocytes on day 6 of adipocyte differentiation using specific RNA interference (Fig. 6A). Treatment with 100 nM corticosterone for 2 h increased the gene expression of IL6 by 90% in siRNA-treated adipocytes when compared to control cells (Fig. 6B). Similarly, the mRNA concentrations of *Mcp1* in GR-deficient adipocytes were markedly elevated by 430% after 2 h (Fig. 6B).

GR *knockout adipose cell lines show mildly impaired adipocyte differentiation, while* MR *knockout preadipocytes completely fail to accumulate lipids*

We investigated the different roles of the GR and MR in adipocyte biology by generating novel brown adipose cell lines from newborn homozygous *GR* and *MR* knockout

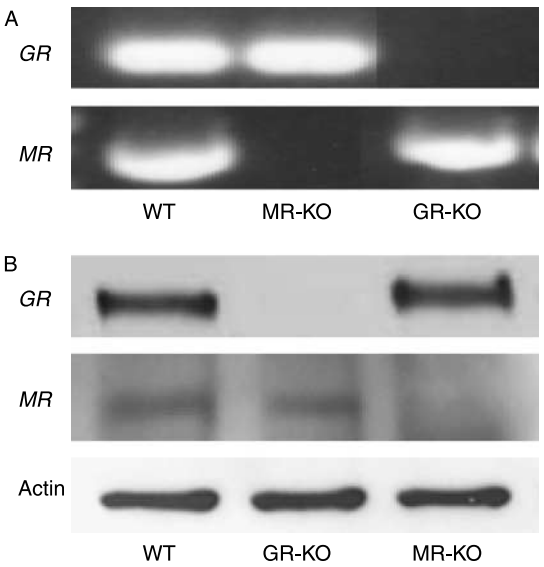

Figure 7 Expression of *GR* and *MR* in newly generated wild-type, *GR* and *MR* knockout cell lines. (A) Genotyping of wild-type, *GR* and *MR* knockout cell lines. Genomic DNA was extracted from cultured cells, amplified by PCR and separated by agarose gel electrophoresis. (B) Validation of *GR* and *MR* knockout in the novel adipose cell lines at protein level. Protein expression of GR and MR was detected in fully differentiated adipocytes by western blot analysis. Representative images of three independent experiments are shown.

mice which die shortly after birth. Genotyping of these cell lines confirmed the absence of the *GR* and *MR* gene (Fig. 7A) resulting in a complete lack of GR and MR protein expression respectively as shown by western blot analysis (Fig. 7B). The *GR* knockout in adipocytes had no effect on the expression of the MR protein. In *MR* knockout cells, the protein expression of the GR also remained unchanged when compared to wild-type cells (Fig. 7B).

To test whether the receptor deletion had any effect on adipocyte differentiation, the standard differentiation protocol was used, and fat-specific Oil Red O staining and microscopy were performed on days 0, 3 and 6 after induction. Preadipocytes from all newly generated cell lines did not show any differences on microscopic examination (data not shown). However, during the differentiation course, *GR* knockout adipocytes displayed an impaired accumulation of lipid droplets as compared to the wild-type control cell line (Fig. 8A and B: middle row). Newly generated preadipocytes from *MR* knockout mice completely failed to accumulate lipid droplets (Fig. 8A and B: bottom row).

GR knockout cell lines showed a considerable decrease of lipid accumulation by ~50% in early differentiation when compared to wild-type adipocytes as quantified by densitometry (Fig. 8C). This deficit was compensated in later differentiation stages. Densitometric analysis revealed a strong deficit in lipid accumulation by ~70% in *MR* knockout cell lines on days 3 and 6 of adipocyte differentiation (Fig. 8C). Pref-1, an early inhibitory differentiation marker, was highly up-regulated by 900% in *MR* knockout cells (Fig. 8D). Furthermore, immunoblot analysis on day 3 demonstrated a reduction of the differentiation marker C/EBPα in MR-deficient cells (Fig. 8D). The late differentiation marker

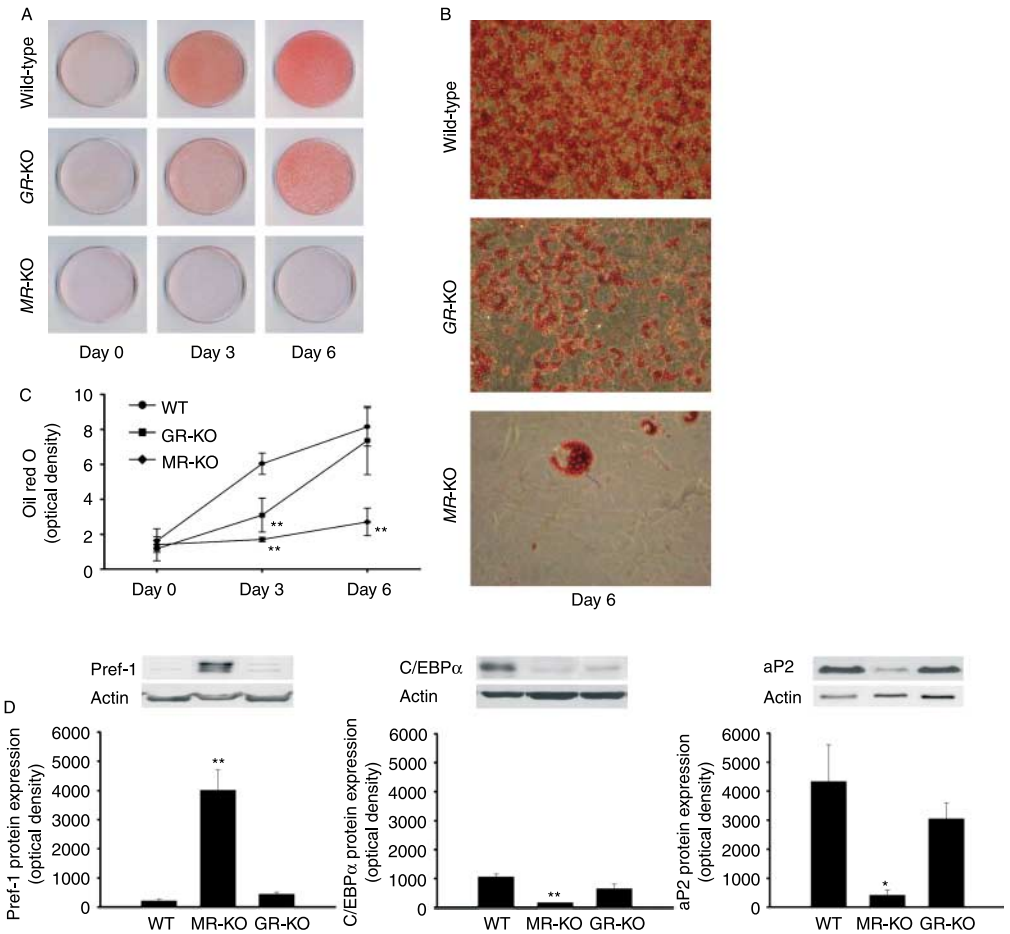

Figure 8 Adipocyte differentiation of newly established *GR* and *MR* knockout adipose cell lines. Lipid accumulation in wild-type, *GR* and *MR* knockout cell lines was visualised by fat-specific Oil Red O staining. (A) The extent of fat accumulation on days 0, 3 and 6 after cell induction. (B) Microscopy images in 40-fold magnification. (C) Densitometric analyses of Oil Red O staining. (D) Protein expression of the differentiation markers Pref-1, C/EBPα and aP2 was determined by immunoblotting on day 3 of adipocyte differentiation. Results are shown as mean ± S.E.M. of at least three independent experiments. $*P \leq 0{\cdot}05$ and $*P \leq 0{\cdot}01$ comparing wild-type cells to knockout cells.

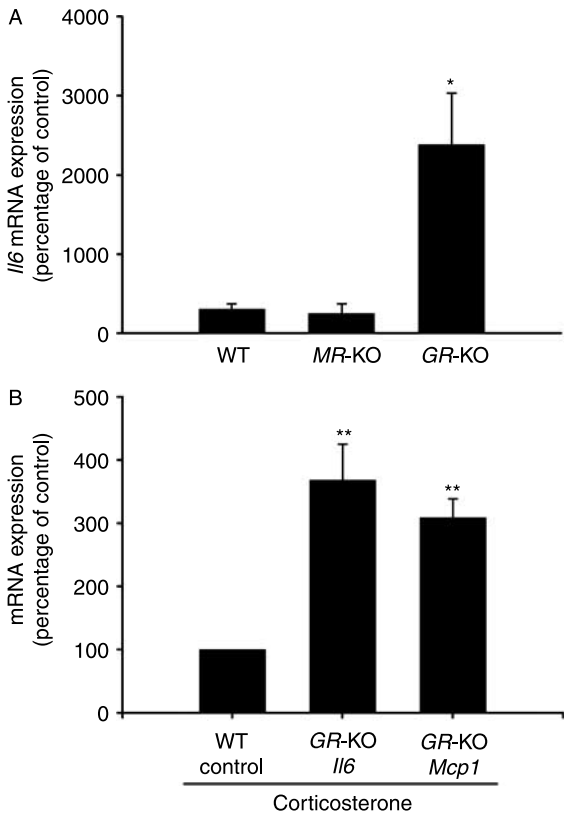

Figure 9 (A) Basal *Il6* gene expression in preadipocytes of novel wild-type, *MR* and *GR* knockout adipose cell lines. mRNA expression of *Il6* was analysed by RT-PCR. Results are shown as mean ± S.E.M. of three independent experiments. (B) Stimulation of pro-inflammatory adipokines by corticosterone in *GR* knockout cell lines. Wild-type and *GR* knockout cell lines were chronically treated with 100 nM corticosterone during the entire differentiation process. mRNA expression levels of *Il6* and *Mcp1* were quantified by RT-PCR. Results are shown as mean ± S.E.M. of seven independent experiments. $*P \leq 0{\cdot}05$ and $**P \leq 0{\cdot}01$ comparing wild-type cell lines to knockout cells.

aP2 was also markedly decreased by more than 90% in *MR* knockout cell lines when compared to the wild-type control (Fig. 8D). No significant differences between *GR* knockout and wild-type control cell lines were found with regard to the expression of differentiation markers during adipocyte differentiation.

GR knockout leads to a rise in pro-inflammatory adipokines

To examine the consequences of the *GR* or *MR* knockout and the physiological functions of the remaining corticosteroid receptors in these cell lines for the regulation of the adipokine profile, the expression levels of the pro-inflammatory adipokine *Il6* in wild-type, *MR* and *GR* knockout preadipocytes were analysed. Whereas *Il6* expression in *MR* knockout preadipocytes was unaltered when compared to wild-type cells, *GR* knockout in preadipocytes resulted in a strong increase of *Il6* by 2300% (Fig. 9A). Furthermore, wild-type and *GR* knockout adipocytes were chronically exposed to 100 nM corticosterone from day 0 until day 6 of adipocyte differentiation. Subsequent mRNA expression analysis revealed a robust increase of the pro-inflammatory adipokines *Il6* and *Mcp1* by ~270 and 210% respectively when compared to the wild-type control (Fig. 9B).

Discussion

Corticosteroid metabolism in adipose tissue plays a crucial role in the pathogenesis of the metabolic syndrome. Adipose tissue-specific amplification of glucocorticoids induces all the characteristic features of the metabolic syndrome (Masuzaki *et al.* 2001). Our study for the first time dissects the different metabolic and endocrine roles of the adipose GR and MR in native wild-type, knockdown and newly generated knockout cell lines. Selective GR stimulation of differentiated white adipocytes with dexamethasone considerably inhibited the expression of *Il6*, *Mcp1*, *Tnfα*, chemerin and leptin. By contrast, selective MR stimulation with aldosterone promoted the expression of pro-inflammatory adipokines. These cytokines were also elevated when the GR was acutely knocked down by siRNA or absent in knockout cell lines. Whereas adipocytes from *GR* knockout mice showed only mildly impaired adipogenesis, especially during early differentiation, *MR* knockout preadipocytes completely failed to accumulate lipids.

These data newly define the significance of the corticosteroid receptors GR and MR for adipocyte biology by demonstrating opposing effects of the GR and MR in mediating a pro-inflammatory and diabetogenic adipokine response. Furthermore, these data clearly demonstrate the crucial role of the MR for adipocyte differentiation.

To our knowledge, this is the first study to demonstrate that MR activation in fully differentiated white adipocytes promotes the gene expression of important pro-inflammatory adipokines. These data suggest that the MR indirectly impairs insulin sensitivity and promotes atherosclerosis via the stimulation of pro-inflammatory and pro-diabetogenic adipokines in adipose tissue.

These results are in accordance with a recent study illustrating that aldosterone treatment increases the mRNA levels of pro-inflammatory adipokines such as *Il6* and *Tnfα* in undifferentiated preadipocytes (Guo *et al.* 2008). Consistent with our findings, MR blockade with eplerenone was shown to reverse obesity-associated changes in the expression of pro-inflammatory adipokines in murine adipose tissue (Guo *et al.* 2008). Accordingly, spironolactone, an MR antagonist, reduced basal IL6 secretion in human adipose tissue (Corbould 2007). However, in contrast to previous studies in differentiated brown adipocytes and white preadipocytes (Kraus *et al.* 2005, Guo *et al.* 2008), in this study there was only a trend towards an up-regulation of *Mcp1*. This may suggest that mature white adipocytes are differentially

conducive to the pro-inflammatory effect of aldosterone. Interestingly, mineralocorticoid blockade with eplerenone suppressed macrophage infiltration and CD11c, a marker of M1 macrophages, in adipose tissue of obese mice (Hirata *et al.* 2009). This might be due to the inhibitory effect of eplerenone on the secretion of chemokines in adipocytes. In obese mice, mineralocorticoid blockade by eplerenone markedly reduced the number of hypertrophic adipocytes which are known to have an increased production of pro-inflammatory adipokines (Skurk *et al.* 2007, Hirata *et al.* 2009). Furthermore, treatment of obese mice with the MR antagonist eplerenone also improved obesity-associated metabolic disorders such as insulin resistance and hypertriglyceridaemia (Guo *et al.* 2008, Hirata *et al.* 2009). These data are in accordance with our findings indicating that the MR plays a crucial role in the regulation of inflammatory and metabolic adipocyte responses.

Clinical data point to the potential implication of the adipose tissue MR in obesity-related inflammation, insulin resistance and cardiovascular complications. An association between plasma aldosterone levels on the one hand and obesity and insulin resistance on the other hand has been demonstrated (Goodfriend *et al.* 1998, Corry & Tuck 2003, Colussi *et al.* 2007, Lastra-Gonzalez *et al.* 2007, Rossi *et al.* 2008). Recent studies even describe a higher prevalence of the metabolic syndrome and of cardiovascular events in patients with primary aldosteronism when compared to patients with essential hypertension (Milliez *et al.* 2005, Fallo *et al.* 2006, 2007). Treatment of patients with an MR antagonist improved insulin sensitivity and reduced markers of inflammation (Catena *et al.* 2006, Matsumoto *et al.* 2006).

In contrast to the MR-mediated pro-inflammatory, pro-diabetogenic effect, GR activation strongly inhibited the expression of the pro-inflammatory adipokines *Il6*, *Mcp1*, *Tnfα*, chemerin and leptin in this study. These findings are consistent with recent studies investigating the effect of dexamethasone on the adipokine profile (Fried *et al.* 1998, Zhang *et al.* 2001, Vicennati *et al.* 2002, Fasshauer *et al.* 2004). Our data provide evidence that the GR has an anti-inflammatory effect in adipose tissue. In accordance with these findings, it has been very recently shown by others that glucocorticoids prevent adipocyte-induced recruitment of macrophages into adipose tissue in obese mice (Patsouris *et al.* 2009). Consistent with our data on the anti-inflammatory action of the GR, this inhibitory effect on macrophage accumulation was mediated by the adipocyte GR (Patsouris *et al.* 2009). Furthermore, the study lends support to the notion that the crosstalk between adipocytes and macrophages via pro-inflammatory adipokines is crucial for the development of obesity-associated subclinical inflammation.

Taking into account the great anti-inflammatory effect of the adipocyte GR, corticosteroid action exclusively via the adipocyte GR does not explain the subclinical inflammatory state seen in obesity. Our data suggest that the adipocyte MR activated by mineralocorticoids as well as by glucocorticoids may be significant for the induction of inflammation in adipose tissue.

Whereas stimulation of wild-type adipocytes with low concentrations of the unselective corticosteroid corticosterone did not significantly regulate the expression of *Il6* and *Mcp1*, high concentrations resulted in a suppression of these pro-inflammatory adipokines. These novel data indicate that in a physiological state, in wild-type adipocytes the anti-inflammatory response mediated by the GR preponderates. However, given the fact that the MR possesses affinity to glucocorticoids as well as to mineralocorticoids and corticosterone as an unselective agonist also binds to the MR, it is likely that the pro-inflammatory MR is simultaneously activated and counteracts the anti-inflammatory GR action. This might potentially explain the rather small anti-inflammatory effect of corticosterone, particularly in low concentrations, when compared to the strong suppression of *Il6* and *Mcp1* by the same concentrations of dexamethasone.

Moreover, we found that *MR* knockout in adipocytes did not result in a significant regulation of pro-inflammatory adipokines when compared to wild-type cells. Interestingly, GR knockout resulted in a very strong increase of IL6 in preadipocytes. These data underline our hypothesis of a permissive pro-inflammatory effect of the MR whose action in a physiological state appears to be overlapped by the anti-inflammatory GR effect and becomes obvious when the balance between GR and MR expression in adipocytes is altered.

Interestingly, it has recently been shown by others that in obese mice, the *MR* expression is significantly up-regulated in adipose tissue (Hirata *et al.* 2009). Furthermore, elevated aldosterone levels were found in obese subjects. These obesity-associated changes may result in an increased activation of the adipocyte MR in obesity. A disturbance of the balance between GR and MR activation in adipose tissue may potentially decrease anti-inflammatory adipocyte responses and promote pro-inflammatory adipocyte responses in obesity.

However, our study investigating MR and GR action in a cell model cannot test the physiological significance of these findings for the pathophysiology of obesity in human beings. It might hence be of particular interest to investigate obesity-induced changes in GR and MR activation in obese subjects.

Finally, we have characterised newly generated knockout adipose cell lines from newborn homozygous GR and MR knockout mice (Cole *et al.* 1995, Berger *et al.* 1998). Since these mice die shortly after birth, immortalised cell lines represent a valuable tool to explore the adipose tissue of these GR- and MR-deficient mice (Klein *et al.* 2002). This is the first study to reveal the consequences of a complete deletion of the corticosteroid receptors MR and GR in adipocytes. Both dexamethasone and aldosterone have been found to be potent stimulators of adipocyte differentiation (Rondinone *et al.* 1993, Gregoire 2001). Surprisingly, *GR* knockout adipocytes mainly showed a deficiency in early differentiation, which was nearly compensated during the differentiation

course when compared to the wild-type control. In contrast, *MR* knockout preadipocytes completely failed to accumulate lipids. These findings demonstrate the great significance of the adipose MR for physiological adipocyte differentiation.

In line with our results, several studies have described that aldosterone promotes adipocyte differentiation (Rondinone *et al.* 1993, Penfornis *et al.* 2000, Caprio *et al.* 2007). Moreover, it has been recently reported that the MR antagonist spironolactone and specific MR down-regulation by siRNA inhibit adipocyte differentiation of 3T3-L1 cells (Caprio *et al.* 2007).

Furthermore, our findings suggest that the GR plays an important role during the early differentiation stage. These data are consistent with the findings from another group demonstrating that GR activation within the first days of adipocyte differentiation is a potent proadipogenic factor in 3T3-L1 cells, while late and long-term stimulation results in an inhibition of the terminal adipogenesis (Caprio *et al.* 2007).

There is accumulating evidence that corticosteroid action in adipose tissue is mediated by the GR as well as the MR. Our data lend support to a model in which the inflammatory response of adipocytes is highly and oppositely regulated by the activation of the GR and MR by corticosteroids in adipose tissue. The increased secretion of pro-inflammatory adipokines due to an enhanced MR activation in obesity may promote the migration of macrophages into adipose tissue. The release of further cytokines by macrophages might in turn further deteriorate the inflammatory process. Our results suggest that the corticosteroid action in adipose tissue is instrumental in controlling obesity-associated subclinical inflammation which may promote associated metabolic and cardiovascular complications.

In summary, the present study reveals differential and opposite roles of the MR and GR in controlling adipose metabolic and inflammatory responses. Corticosteroid action via the MR is essential for adipocyte differentiation and promotes a pro-inflammatory and pro-diabetogenic adipokine expression profile. This may play an important role in the pathogenesis of obesity and associated cardiovascular complications. Selective corticosteroid receptor modulation may offer new perspectives for the prevention and treatment of the metabolic syndrome.

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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