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Minor role of mature adipocyte mineralocorticoid receptor in high-fat diet-induced obesity

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Abstract

Obesity is a major risk factor that contributes to the development of cardiovascular disease and type 2 diabetes. Mineralocorticoid receptor (MR) expression is increased in the adipose tissue of obese patients and several studies provide evidence that MR pharmacological antagonism improves glucose metabolism in genetic and diet-induced mouse models of obesity. In order to investigate whether the lack of adipocyte MR is sufficient to explain these beneficial metabolic effects, we generated a mouse model with inducible adipocyte-specific deletion of *Nr3c2* gene encoding MR (adipo-MRKO). We observed a significant, yet not complete, reduction of *Nr3c2* transcript and MR protein expression in subcutaneous and visceral adipose depots of adipo-MRKO mice. Notably, only mature adipocyte fraction lacks MR, whereas the stromal vascular fraction maintains normal MR expression in our mouse model. Adipo-MRKO mice fed a 45% high-fat diet for 14 weeks did not show any significant difference in body weight and fat mass compared to control littermates. Glucose and insulin tolerance tests revealed that mature adipocyte MR deficiency did not improve insulin sensitivity in response to a metabolic homeostatic challenge. Accordingly, no significant changes were observed in gene expression profile of adipogenic and inflammatory markers in adipose tissue of adipo-MRKO mice. Moreover, pharmacological MR antagonism in mature primary murine adipocytes, which differentiated *ex vivo* from WT mice, did not display any effect on adipokine expression. Taken together, these data demonstrate that the depletion of MR in mature adipocytes displays a minor role in diet-induced obesity and metabolic dysfunctions.

Key Words

- ▶ adipose tissue
- ▶ corticosteroids
- ▶ metabolic syndrome
- ▶ insulin resistance

Journal of Endocrinology
(2018) **239**, 229–240

Introduction

Obesity represents a major risk factor for metabolic syndrome (MetS), which is characterized by the co-occurrence of clinical conditions strongly associated with the development of cardiovascular diseases (CDVs). Adipose tissue (AT) plays a pivotal role in the pathogenesis

of MetS (Cinti 2005, Vettor *et al.* 2005). In particular, the development of insulin resistance (IR) is related to chronic inflammation of AT in obesity (Hotamisligil 2006). The mineralocorticoid receptor (MR) is a member of the nuclear receptor superfamily and acts as a

ligand-dependent transcription factor. More specifically, it binds not only to its physiological ligand aldosterone (aldo), but also cortisol (Viengchareun *et al.* 2007). We previously demonstrated that MR activation is a pivotal player of adipogenesis *in vitro* and *in vivo*, mediating the effects of aldo and glucocorticoids (GCs) (Caprio *et al.* 2007). As a matter of fact, AT lacks 11 β -hydroxysteroid dehydrogenase type 2 (11 β -HSD2) activity, which converts cortisol to inactive cortisone, consequently exposing MR to GCs (Armani *et al.* 2015). Thus, MR activation in AT is mainly regulated by circulating GCs. Of note, increased AT-specific expression of 11 β -HSD1 leads to a local rise in GCs further promoting MR activity in obesity (Infante *et al.* 2017). Accordingly, it has been shown that MR expression is increased in AT of obese patients, as well as in mouse models of obesity (Urbanet *et al.* 2015). Moreover, mice with a conditional overexpression of MR in adipocytes developed insulin resistance, displaying features of MetS even in the absence of high-fat diet (HFD) (Urbanet *et al.* 2015). Several preclinical studies showed that MR blockade induces beneficial metabolic effects in *ob/ob*, *db/db* and diet-induced obese mice (Guo *et al.* 2008, Hirata *et al.* 2009, Wada *et al.* 2010, Armani *et al.* 2014). AT is made up of two cell types, white and brown adipocytes, with distinct phenotypes and functions (Armani *et al.* 2010, Cinti 2012). Notably, MR antagonism promotes browning of white adipose depots and prevents adipocyte dysfunction in HFD-fed mice (Armani *et al.* 2014). In order to gain more insight into the specific role of adipocyte MR, we evaluated weight gain, glucose metabolism and AT function in response to HFD challenge in a novel mouse model with inducible adipocyte-specific MR deletion.

Materials and methods

Adipocyte-specific MRKO mice

Animal studies were carried out by two distinct research groups (Laboratory of Cardiovascular Endocrinology IRCCS San Raffaele Pisana Rome and INSERM, UMR_S 1138, Teams 1, Centre de Recherche des Cordeliers, Paris). All study protocols were approved by the Italian National Institutes of Health Care and Use Committees (authorization number 493/2016-PR) and by Comité d'éthique en Expérimentation Animale Charles Darwin-CEEACD, Paris, France (number Ce5/2012/069). All experiments were conducted in accordance with the guidelines of the European Community for use of experimental animals (European Directive, 2010/63/

UE) and with the principles published in the Canadian Animals Research Act (R.S.O.1990-c22-s.17.1-3).

Adipocyte-specific MRKO mouse model was generated using the Cre-Lox approach: mice expressing tamoxifen-inducible Cre recombinase under the control of the adiponectin (Adipo-) promoter (kindly provided by S Offermans, Heidelberg, GE) (Sassmann *et al.* 2010) were crossed with mice containing *Nr3c2*-flox alleles (kindly provided by S Berger, Heidelberg, GE) (Berger *et al.* 2006) to generate mice (on the C57BL/6J background) in which *Nr3c2* can be conditionally deleted in adipocytes.

In vivo experiments were performed in 10- to 12-week-old male mice. Induction by tamoxifen was carried out in 6- to 8-week-old animals by injecting tamoxifen (1 mg/day in corn oil) on five consecutive days in order to transiently activate the CreERT2 recombinase as previously described (Sassmann *et al.* 2010). Mice were used for experiments 3 weeks after the last tamoxifen injection.

Nr3c2 excision was validated by PCR analysis of the genomic DNA from tail tips and qPCR and western blot analysis of subcutaneous and visceral adipose depots from MRKO and control mice (CRE negative, control-MR).

Studies in experimental animals

Animals were housed in a room kept at 22°C with a 12-h light/darkness cycle and provided with standard chow diet (ND) (SAFE number A04, 2791 kcal/kg, 10% lipids, 67% carbohydrates and 23% proteins; Augy, France) and water *ad libitum*. For the HFD protocol, mice were fed a HFD, (SAFE number U8955v7, 4365 kcal/kg, 46.4% lipids, 38.3% carbohydrates, and 15.3% proteins), for 14 weeks. Mice were killed by cervical dislocation after 4 (14–16 weeks old mice) and 14 (24–26 weeks old mice) weeks of HFD, and plasma and tissue biopsies were immediately collected and stored at –80°C. Intra-peritoneal glucose tolerance test (GTT) and insulin tolerance test (ITT) were performed in mice fed a HFD for 14 weeks, before killing.

For GTT, mice were fasted overnight and then injected intraperitoneally with a bolus of glucose (1 g/kg BW). Starting from the injection point (time 0), glycemia was monitored at 5, 10, 15, 20, 30, 40, 60, 90 and 120 min by analysis of tail blood (total blood volume <5 μ L); plasma samples were also collected from the tail (total blood volume 30–50 μ L) at 0, 15, 30, 60 and 120 min to monitor insulinemia during glucose stress. For ITT, mice were fasted for 5 h and then injected intraperitoneally with a bolus of insulin (1 U/kg body weight (BW)). Starting from the injection point, (time 0), glycemia was monitored at 5, 10, 15, 20, 30, 40, 60, 90 and 120 min. The procedures

were conducted in parallel in the control group and in the experimental group to avoid different stress-induced glucocorticoid release. Insulin resistance was evaluated by homeostasis assessment model (HOMA-IR) and calculated from fasting insulin and glucose concentration according to the formula: $\text{insulin } (\mu\text{IU/mL}) \times \text{glucose (mmol/L)} / 22.5$ (Matthews *et al.* 1985).

Food intake was measured in mice kept in individual cages for 1 week: 3 days for habituation and 4 days of actual measurements. Calories were deducted by multiplying the food intake by the energy density of each diet. Caloric efficiency (kcal/g body weight gain) was also calculated daily by multiplying food intake (g) by the caloric content of the diet and dividing this result by daily body weight gain (g) (Rising & Lifshitz 2006).

Biochemical assays

Glycemia was determined in blood tail samples using the Accu-check Aviva glucometer (Roche Diagnostics). Insulin concentration was quantified in plasma samples collected during GTT analysis using the Millipore Rat/Mouse Insulin ELISA kit (Merck KGaA).

Total triglycerides, total cholesterol and HDL cholesterol were quantified in plasma samples using Konelab 60 Prime Station with the appropriate kits (Thermo Scientific).

RNA isolation and qPCR analysis

Total RNA was extracted from tissues as well as cultured cells by using the Lipid RNeasy Mini Kit (Qiagen) and the TRIzol reagent (Life Technologies) respectively. After DNase treatment (Qiagen), reverse transcription and quantitative PCR (qPCR, sybr green PROMEGA) were performed as previously described (Armani *et al.* 2014). The mRNA levels were normalized using different housekeeping genes stable in adipose tissue, *18S*, *B2 microglobulin*, *TATAbox-binding protein* and they are expressed as fold increase of the control condition. Primer sequences are listed in Supplementary Table 1 (see section on [supplementary data](#) given at the end of this article).

Protein extraction and Western blot analysis

Specimens of epididymal AT were lysed in HNTG lysis buffer containing 1% Triton X-100, 50mM Hepes, 10% glycerol, 150mM NaCl, 1mM Na_3VO_4 and 75U of aprotinin and allowed to stand for 30min. After SDS-PAGE and blotting (Armani *et al.* 2014), membranes

were probed with mouse anti-MR (kindly provided by C Gomez-Sanchez) or goat polyclonal anti-actin (Santa Cruz Biotechnology sc-1615, Heidelberg, Germany). Bound antibodies were visualized with horseradish peroxidase-conjugated IgG (Sigma-Aldrich) and immunoreactivity was assessed by the chemiluminescence reaction, using the ECL Western detection system (Biorad). Densitometric scanning analysis was performed by Mac OS X (Apple Computer International, Milan, Italy) using NIH Image 1.62 software.

Histological analysis

Dissected AT depots were fixed in 4% paraformaldehyde and paraffin-embedded. Each paraffin-embedded depot was cut to obtain 6 μm sections stained with hematoxylin solution (#MHS16, Sigma-Aldrich). Two photographs ($\times 40$ final magnification) per section were taken and analyzed to determine the mean adipocyte size with ImageJ software. In every picture, adipocyte area was calculated by the software. Pictures were taken and analyzed by an investigator blinded to the identity of the samples. Only the lead researcher had access to sample identification.

Cell culture studies

The stromal vascular fraction (SVF) and mature adipocyte fraction (MAF) were isolated from epididymal AT of adiponectin receptor 1 knockout (MRKO) and control-MR mice as previously described (Soukas *et al.* 2001).

Primary cultures of murine preadipocytes derived from the SVF of inguinal fat depots of 10-week-old male C57BL/6J mice were prepared. The proliferation medium consisted of DMEM/Ham's F12 (Invitrogen) supplemented with 10% FCS, 100 U/mL penicillin and 100 $\mu\text{g/mL}$ streptomycin. To induce adipogenic differentiation, confluent cells were transferred in the same culture medium supplemented with 0.5 mM isobutylmethylxanthine, 125 nM indomethacin, 5 μM dexamethasone, 850 nM insulin and 1 nM triiodothyronine (T3). After day 2, cells were switched to maintenance medium containing 10% FCS, 850 nM insulin and 1 nM T3. The experimental design included both differentiating cells treated from confluence until day 6 with spironolactone (spiro, 10^{-5}M) and differentiated cells treated from day 6 with an additional 6 days with spiro. At the end of the experiments, adipocytes were harvested and total RNA was extracted as described earlier. Unless otherwise indicated, all chemicals were obtained from Sigma-Aldrich.

Statistical analysis

Data analysis and representation were accomplished by using GraphPad Prism 6 software (GraphPad). Results are reported as mean $\pm$ s.e.m. Statistical analyses were performed by Mann–Whitney *t* test, 1-way ANOVA test followed by Newman–Keuls multi-comparison correction test or 2-way ANOVA test followed by Fisher's least significant difference multi-comparison correction test.

Values of $P < 0.05$ were considered significant.

Results

Validation of inducible adipocyte-specific MR-null mice by CreLoxP system

Real-time qPCR and western blot analyses of AT from adipo-MRKO mice confirmed the tissue-specific nature of the knockout and showed reduced *Nr3c2* mRNA and MR protein levels in the AT, but not in the kidney where MR is normally expressed (Fig. 1A and B). The recombination efficiency in visceral depots of tamoxifen treated adipo-MRKO mice was approximately 50% by qPCR and Western blot, compared to control-MR mice (Fig. 1A and B). Interestingly, MAF isolated from visceral AT from adipo-MRKO mice displayed a significant reduction in *Nr3c2* mRNA levels, whereas the SVF also containing preadipocytes retains intact *Nr3c2* gene expression (Fig. 1C). Indeed, the adiponectin promoter controls Cre recombinase expression specifically in

mature adipocytes, thus promoting *Nr3c2* excision only in terminally differentiated adipocytes (and not in preadipocytes).

Adipo-MRKO mice do not significantly differ from control mice in terms of weight gain, glucose tolerance and lipid profile in response to HFD

Adipo-MRKO and control-MR mice were fed a ND or a HFD for 14 weeks to induce obesity and insulin resistance. Both adipo-MRKO and control littermates with intact *Nr3c2* gene fed a HFD showed a linear increase in body weight (Fig. 2A). Food intake was similar between adipo-MRKO and control-MR mice (data not shown). Caloric efficiency did not differ between the two groups fed a HFD (Fig. 2B). Interestingly, glucose and insulin tolerance were similarly impaired in both genotypes on HFD challenge compared to ND. Indeed, GTT was not significantly different between control-MR and adipo-MRKO mice, as confirmed by glucose area under the curve (AUC) (Fig. 2C). ITT analysis revealed no difference between the two groups (Fig. 2C). Adipo-MRKO mice showed similar HOMA-IR (Fig. 3A) compared to controls.

Moreover, we analyzed lipids profile in blood samples from adipo-MRKO as well as control-MR mice fed a HFD. No significant differences were observed in plasma levels of triglycerides, total cholesterol, HDL and non-HDL cholesterol and free fatty acids between the two groups (Fig. 3B).

Figure 1

Adipocyte-specific MRKO (adipo-MRKO) mice show reduced expression of *Nr3c2* (MR) in adipose tissue. (A) mRNA expression of *Nr3c2* in epididymal visceral adipose tissue (EVAT), subcutaneous adipose tissue (SAT) and kidney tissue of adipo-MRKO ($n=20$) and control-MR mice ($n=20$). mRNA levels were measured by qRT-PCR and expressed as fold increase compared with control-MR group. (B) Western blot analysis for MR performed in visceral fat depots of adipo-MRKO ($n=7$) and control-MR mice ($n=7$). Right panel: distribution of the densitometric analysis performed using goat polyclonal anti-actin as loading control. (C) mRNA levels of *Nr3c2* (MR) in stromal vascular (SVF) and mature adipose (MAF) fraction of adipo-MRKO ($n=7$) and control-MR mice ($n=7$). mRNA levels were measured by qRT-PCR and expressed as fold increase compared with control-MR SVF. Values are expressed as means $\pm$ s.e.m. $**P < 0.05$, $***P < 0.001$ vs control-MR group.

Figure 2

Adipo-MRKO mice are not protected from HFD-induced weight gain and glucose intolerance. (A) Body weight of adipo-MRKO ($n=20$) and control-MR mice ($n=20$) during 14 weeks of ND or HFD. (B) Caloric efficiency evaluated in adipo-MRKO and control mice fed a HFD. (C) Plasma glucose levels measured during glucose tolerance test (GTT; glucose 1 g/kg BW) and insulin tolerance test (ITT; insulin 1 U/kg BW), performed in four experimental groups ($n=10$) after 12 weeks of ND or HFD. AUC glucose was calculated using the trapezoidal rule. Values are expressed as means $\pm$ S.E.M. * $P<0.05$ HFD mice vs ND mice (for each genotype). ** $P<0.01$ HFD mice vs ND mice (for each genotype). *** $P<0.001$ HFD mice vs ND mice (for each genotype). **** $P<0.0001$ HFD mice vs ND mice (for each genotype).

Adipokine gene expression profile in adipose tissue of Adipo-MRKO mice

In order to determine if MR ablation in AT was able to influence the adipokine profile of visceral AT after HFD challenge, we analyzed total mRNA from epididymal adipose depots by qPCR. First, we evaluated the expression

of *serum- and glucocorticoid-regulated kinase 1 (Sgk1)*, a target of MR in several tissues including AT. No significant differences were observed between adipo-MRKO and control-MR mice.

According to the *in vivo* metabolic analysis, no differences between adipo-MRKO and control-MR mice were observed in AT expression of adipokines such

Figure 3

Adipo-MRKO and control-MR mice do not show significant differences in lipid profile, glucose and insulin plasma levels. (A) Fasting plasma levels of glucose and insulin were measured in adipo-MRKO ($n=10$) and control-MR mice ($n=10$) fed a HFD. HOMA index was calculated in both experimental groups. (B) Fasting plasma levels of triglycerides, free fatty acids, total cholesterol, HDL and non-HDL cholesterol in adipo-MRKO ($n=10$) and control-MR mice ($n=10$) fed a HFD. Values are expressed as means $\pm$ S.E.M.

as *Adiponectin*, *neutrophil gelatinase-associated lipocalin* (*Ngal*) and *Prostaglandin D2 Synthase* (*Ptgds*), which has been recently identified as a specific MR target in AT (Urbanet et al. 2015) (Fig. 4A). We also performed gene expression analysis in epididymal AT from adipo-MRKO as well as control-MR mice fed a HFD for only 4 weeks. This was done in order to get rid of potential confounding effects due to AT expansion in response to a long term HFD. Again, qPCR analysis did not reveal any difference in *Adiponectin*, *Tumor necrosis factor alpha* (*Tnfa*) and *Monocyte chemoattractant protein-1* (*Mcp1*) gene expression at an earlier stage of HFD (Fig. 4B).

Effect of adipocyte MR depletion on adipose tissue morphology

In order to investigate if MR depletion in adipocyte could influence AT morphology, histological analysis of epididymal adipose depots from adipo-MRKO and control-MR mice was carried out. Hematoxylin staining of epididymal AT did not show any significant difference in adipocyte size and morphology between adipo-MRKO and control-MR mice both in ND and HFD (Fig. 5A and B inset). Across the epididymal AT, frequency distribution of adipocyte (percentages of measured adipocyte) per size range showed no significant differences between adipo-MRKO and control-MR mice both in ND and HFD (Fig. 5A and B).

Moreover, no multilocular brown-like adipocytes were observed in any of the groups of mice, suggesting that removal of MR in AT does not affect conversion of white into brown AT (Fig. 5).

MR pharmacological antagonism on adipocyte differentiation and function *ex vivo*

In order to explore the impact of MR signaling in preadipocytes and in mature adipocytes on adipocyte function, we investigated the effects of the MR antagonist spiro in primary preadipocytes during differentiation as well as in terminally differentiated adipocytes (Fig. 6A). Preadipocytes isolated from the SVF of WT mice SAT and differentiated for 6 days in the presence of spiro showed increased *Ucp1* and decreased *Leptin* mRNA levels compared to untreated controls, consistent with previous data from our lab (Armani et al. 2014). *Tnfa* and *Mcp1* transcripts, which are physiologically expressed by terminally differentiated adipocytes *in vitro*, were also decreased upon spiro treatment during differentiation (Supplementary Fig. 1).

Notably, mature adipocytes differentiated from confluence (day 0) to day 6 in the absence of MR antagonist and then treated with spiro for additional 6 days, did not show any difference in terms of *Ucp1*, *Leptin* or inflammatory marker gene expression, as compared to untreated cells (Fig. 6B).

Figure 4

Adipo-MRKO mice fed a HFD do not show significant changes in adipokine and inflammatory marker expression. (A) mRNA expression of *Sgk1*, *Adiponectin*, *Ngal* and *Ptgds* in epididymal AT of adipo-MRKO ($n=15$) and control-MR mice ($n=15$) after 14 weeks of HFD. (B) mRNA levels of *Nr3c2*, *Adiponectin*, *Tnfa* and *Mcp1* in epididymal AT of both experimental groups ($n=10$) after 4 weeks of HFD. mRNA levels were measured by qRT-PCR and expressed as fold increase compared with control-MR group. Values are expressed as means $\pm$ s.e.m. ** $P<0.05$ vs control-MR group.

Figure 5

Adipo-MRKO mice do not show modified morphology of adipose tissue. Hematoxylin staining of epididymal AT in adipo-MRKO and control-MR mice after 14 weeks of ND (A) and HFD (B). Representative sections from either group fed ND or HFD. Scale bars=40 μm . Analysis of adipocyte size distribution in epididymal AT of adipo-MRKO ($n=6$) and control-MR mice ($n=6$) in both ND and HFD. Raw area data were transformed in a relative frequency distribution of adipocytes per size range (a range of 500 μm^2 was arbitrarily chosen to show frequency distribution). Statistical analysis was performed by two-way ANOVA test between the adipocyte distributions per range of adipocyte size in the two groups (Control-MR and Adipo-MRKO), both in ND and HFD.

Discussion

In this study, we hypothesized that the protective metabolic effects of pharmacological MR antagonism are specifically mediated by adipocyte MR. In order to gain more insight into the specific role of MR in adipocyte pathophysiology, MR was specifically depleted in the adipocyte, by using a tamoxifen-inducible CreLoxP-mediated recombination. In our model, depletion of MR in AT does not protect against diet-induced obesity and glucose dysfunctions.

In clinical and experimental obesity, increased levels of aldo contribute to the development and progression of metabolic and cardiovascular dysfunction (Ferrario & Schiffrin 2015). In obesity, not only can aldo production by adrenal glomerulosa cells be regulated by leptin secreted by AT (Huby *et al.* 2015), but also increased AT expression and activity of 11 β -hydroxysteroid dehydrogenase type 1 (11 β -HSD1) can enhance local conversion of cortisone

to active cortisol (Masuzaki *et al.* 2003, Morton & Seckl 2008, Hirata *et al.* 2009). Given the absence of the 11 β -HSD2 isoform in AT, adipocyte MR binds both aldo and GCs (Infante *et al.* 2017). However, obese subjects show increased expression of MR in AT, and this is also observed in obese mice (Urbanet *et al.* 2015). Preclinical studies have shown that MR activation influences adipocyte function inducing major changes in morphological and biochemical markers of AT differentiation (Guo *et al.* 2008, Hirata *et al.* 2009). Importantly, in these studies treatment of terminally differentiated 3T3-L1 adipocytes with aldo and MR antagonist has shown that MR can modulate expression of proinflammatory adipokines (*Tnfa*, *Il6*, *Mcp1*), *Adiponectin*, *Pparg*, *ROS-eliminating enzymes catalase* and *Cu,Zn-sod*, enzymes involved in ROS production, suggesting that MR has a key role in regulating mature adipocyte function. MR blockade reduces inflammation and ROS levels in mature adipocytes (Guo *et al.* 2008, Hirata *et al.* 2009). In these cells, MR activity also promotes

Figure 6

In terminally differentiated primary murine adipocytes, pharmacological MR antagonism does not affect expression of inflammatory and adipogenic markers. (A) Schematic representation of the procedure used to treat primary adipose cell cultures with spiro. (B) qRT-PCR analysis of *Ucp1*, *Leptin*, *Tnfa* and *Mcp1* transcripts was performed in murine terminally differentiated adipocytes, treated with spironolactone or vehicle after 6 days of differentiation. mRNA levels were expressed as fold increase compared with untreated cells (CTRL). Values are expressed as means $\pm$ S.E.M.

expression of the enzyme *11hsl*, resulting in local increase in GCs and further activation of MR (Guo *et al.* 2008, Hirata *et al.* 2009, Marzolla *et al.* 2012). Indeed, inflammation and oxidative stress are crucial factors in promoting local and systemic metabolic alterations (Manna & Jain 2015).

We have previously shown that increased MR expression in AT of mice contributes to MetS development with several metabolic dysfunctions, including visceral obesity and body weight gain, glucose intolerance, insulin resistance and dyslipidemia (Urbanet *et al.* 2015). In addition, both our group and other groups demonstrated that pharmacological MR blockade in mice induces beneficial effects on AT and glucose tolerance, consequently counteracting obesity (Hirata *et al.* 2009, Wada *et al.* 2010, Armani *et al.* 2014). Several studies have shown that metabolic changes occurring in AT, in response to MR antagonism (through modified expression of adipogenic and inflammatory markers) may mediate the observed improvement in glucose metabolism in obese mice (Hirata *et al.* 2009, Armani *et al.* 2014).

In our model, *Nr3c2* gene excision driven by the adiponectin promoter was limited to the mature adipocytes (Fig. 1C). As widely described in the literature (Lee *et al.* 2013, Jeffery *et al.* 2014, Liu *et al.* 2017), the transgenic Cre mouse line driven by the *adiponectin* gene promoter shows high recombination specificity in

mature adipocytes and does not display recombination in other tissues apart from AT. Notably, the adiponectin-Cre line displays more efficiency and specificity for adipocytes compared with adipocyte protein 2 (aP2)-Cre mouse line, which also exhibits recombination in the heart and skeletal muscle (Lee *et al.* 2013). AT is composed by different cell types expressing MR, including preadipocytes, mature adipocytes, endothelial cells and macrophages. Adiponectin represents a terminal differentiation marker of the adipocyte, and its expression is restricted to mature adipocytes. Indeed, the SVF isolated from adipo-MRKO mice showed similar *Nr3c2* mRNA levels compared to the SVF of control mice, thus indicating that preadipocytes from adipo-MRKO mice retain an intact *Nr3c2* gene. Adipo-MRKO and control-MR mice were challenged with HFD (45 kcal% fat) for 14 weeks. Notably, adipo-MRKO mice response to HFD did not differ from that of control littermates (Fig. 2). Indeed, MR depletion in mature adipocytes did not protect mice from HFD-induced glucose intolerance and insulin resistance, as shown by glucose and insulin tolerance tests (Fig. 2C) as well as by HOMA-IR index (Fig. 3A). No differences were observed in blood lipid profile in terms of free fatty acids, cholesterol and triglyceride levels (Fig. 3B).

SGK1 mediates aldo effects through MR activation in renal epithelia (McCormick *et al.* 2005). Of note, a role for SGK1 as a downstream target of GR activation has

also been suggested in the adipocyte, where its expression increases during differentiation in response to GCs (Di Pietro *et al.* 2010). Li *et al.* showed that *Sgk1* gene expression is increased in AT of obese subjects (Li *et al.* 2013). Furthermore, adipocyte MR overexpressing mice show increased mRNA levels of *Sgk1* in AT compared to control-MR mice (Nguyen Dinh *et al.* 2016). In our model, we did not observe any difference in AT *Sgk1* mRNA levels compared to littermates control animals. This suggest that *Sgk1* represents a potential downstream target of MR in preadipocytes. Previous studies provided evidence that treatment of genetically obese mice with the MR antagonist eplerenone leads to decreased expression of differentiation (*Adiponectin* and *Pparg*) and inflammatory markers (*Mcp1* and *Il6*) (Hirata *et al.* 2009) and attenuates activation of the inflammasome (Wada *et al.* 2017). Guo *et al.* also showed that 3T3-L1 adipocytes treated by aldo display increased mRNA levels of adipogenic and inflammatory markers, whereas eplerenone was able to blunt such an effect (Guo *et al.* 2008). In our model, *Adiponectin*, *Mcp1* and *Tnfa* mRNA levels in AT were similar to those of control littermates, indicating that adipo-MRKO mice do not show any major impairment in adipocyte differentiation nor showed decreased inflammation despite the absence of MR in mature adipocytes (Fig. 4A).

We recently performed a transcriptomic analysis of AT from adipocyte-specific MR overexpressing mice, which identified *Ptgds*, an enzyme regulating inflammatory cytokine expression (Peeraully *et al.* 2006), as a direct target of MR in AT (Urbanet *et al.* 2015). We studied *Ptgds* gene expression in AT of adipo-MRKO and control-MR mice and again we did not observe any difference in mRNA levels between the two groups (Fig. 4A). *Ngal* has been identified as a novel MR target in the cardiovascular system (Latouche *et al.* 2012, Tarjus *et al.* 2015). Moreover, NGAL is a novel adipokine whose expression is increased in AT of obese subjects (Catalan *et al.* 2009) and NGAL-knockout mice show improved insulin sensitivity and reduced inflammation in AT in obesity (Law *et al.* 2010). We did not observe any difference in *Ngal* gene expression between AT of adipo-MRKO and control-MR mice (Fig. 4A), further indicating that MR depletion in mature adipocyte does not affect the AT secretome. In order to get rid of any potential confounding effect due to AT expansion upon long-term HFD, we also performed gene expression analysis in epididymal AT from adipo-MRKO and control-MR mice fed a HFD for only 4 weeks. Similarly, we did not observe any difference in *Adiponectin*, *Tnfa*, and *Mcp1* mRNA levels at the early stage of HFD challenge (Fig. 4B).

In obesity, AT undergoes a complex process of tissue remodeling, leading to a marked increase in both number (hyperplasia) and size (hypertrophy) of adipocytes, with a major impact on the adipocyte secretome (Skurk *et al.* 2007, Mancuso 2016). Indeed, hypertrophic adipocytes show increased production of inflammatory molecules (Skurk *et al.* 2007). Hirata *et al.* showed that eplerenone increased the number of small adipocytes in obese mice (Hirata *et al.* 2009). Given the strict involvement of MR in regulating adipocyte size in murine and human adipose tissue, we hypothesized that the absence of MR in adipocyte could mimic the effect induced by MR pharmacological antagonists on adipocyte hypertrophy in obese mice. To this purpose, we evaluated the frequency distribution of adipocyte sizes across the epididymal depot and the number of total adipocytes per depot in both adipo-MRKO and control-MR mice. Adipocyte sizes and distribution were similar between the two genotypes, either fed ND or HFD (Fig. 5), indicating that in this model loss of MR in adipocyte has no impact on AT morphology.

There are a number of studies that show that browning of AT counters the development of obesity and alteration of glucose tolerance (Giordano *et al.* 2016). Previous studies by our group showed that pharmacological MR antagonism induces browning in AT of WT mice fed a HFD (Armani *et al.* 2014). In accordance with these studies, impaired browning may have a role in the development of obesity and glucose intolerance in adipo-MRKO mice. Importantly, adipo-MRKO mice did not show the presence of multilocular adipocytes, suggesting that browning, the conversion of white into brown adipocyte, does not occur in white AT of this model. Our data indicate that MR removal in mature adipocyte does not induce browning of white AT, raising the possibility that brown adipocytes detected in white AT of mice treated with MR antagonists (Armani *et al.* 2014) derive from MR blockade in adipocyte precursors present in the SVF of fat depots.

Collectively our data show that MR depletion in mature adipocytes is not sufficient to dampen expansion of AT and adipocyte dysfunction induced by HFD. Based on this finding, we further explored the impact of pharmacological MR blockade on primary preadipocytes, undergoing *in vitro* differentiation from the SVF of inguinal depot of WT mice. We previously demonstrated that there is a critical time frame for the inhibition of adipogenesis in 3T3-L1 by MR antagonists (Caprio *et al.* 2011). Indeed, MR blockade was effective in repression of adipogenesis only in differentiating preadipocytes, treated with the MR antagonist drospirenone from the early steps of differentiation (Caprio *et al.* 2011). Accordingly, in the

present study, pharmacological MR antagonism in mature primary adipocytes, differentiated *ex vivo* from WT mice, did not display any effect on adipogenic and inflammatory markers, whereas it reduced adipokines mRNA levels only when spiro was added from the early steps of adipose differentiation (Fig. 6 and Supplementary Fig. 1). Our data confirm that MR affects adipocyte function only in the early steps of differentiation, whereas terminally differentiated adipocytes function becomes relatively independent of MR activation.

A large body of evidence has shown that systemic pharmacological MR blockade induces beneficial metabolic effects in several mouse models of obesity. The present study shows that these metabolic benefits are not mediated by the blockade of mature adipocyte MR, suggesting that other tissue/cell types expressing MR are involved in the observed protective effect. Different cell types (i.e. macrophages) have been shown to play an important role in insulin resistance and type 2 diabetes in ob/ob mice (Zhang *et al.* 2017). However, a specific role for macrophage MR in AT dysfunction remains to be elucidated.

A limitation of the present study stems from the lack of a mouse that does not express the *Nr3c2* gene in the adipocyte precursor. Of note, our model displays a metabolic phenotype similar to adipose-specific glucocorticoid receptor (GR) KO mice, where deletion of the *Nr3c1* (encoding GR) in only mature adipocytes, (through recombination by an adiponectin-Cre), did not affect HFD-induced weight gain and glucose intolerance (Desarzens & Faresse 2016), whereas GR is known to play a major role in the early phase of adipogenesis (Steger *et al.* 2010). Unfortunately, a suitable transgenic model for specific deletion of MR or GR in preadipocytes is still not available.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/JOE-18-0314>.

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.

Funding

This work was supported by funding of the Italian Ministry of Health (Ricerca Corrente), by grants from the Italian Ministry of Health (Bando

2011–2012 Progetti Collaborazione Ricercatori Italiani all'Estero; Project Grant PE-2011-02347070 to M C and Bando Giovani Ricercatori 2013 Project Grant GR-2013-02357959) to AF, by a grant of MIUR (Progetti di Ricerca di interesse Nazionale 2015 project code 2015ZTT5KB to M C, work package leader) and by grants from the Institut National de la Santé et de la Recherche Médicale, Fondation de France (2014-00047968) and ANR Investissement Avenir CARMMA (ANR15-RHUS-0003). R Urbanet was supported by University of Padova (Italy) PhD fellowship and by Società Italiana dell'Iperensione Arteriosa (SIIA) postdoctoral fellowship. A Feraco was supported by FP7-funded COST ADMIRE network (BM1301). F Jaisser and M Caprio: Equal contribution to the paper.

Acknowledgements

The authors acknowledge networking support by the European Cooperation in Science and Technology (COST) Action Aldosterone and Mineralocorticoid Receptor (ADMIRE) BM1301. The authors wish to thank Dr Eirini Velliou from University of Surrey (UK) and Dr Amy Taheri from University of Zurich (CH) for language editing and for proofreading the manuscript. The authors thank Dr Aniko N Fejes-Toth from Dartmouth College (USA) for technical and scientific support.

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Received in final form 7 August 2018

Accepted 15 August 2018

Accepted Preprint published online 18 August 2018