Renal mineralocorticoid receptor expression is reduced in lipoatrophy

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Obesity is a condition characterized by adipose tissue hypertrophy; it is estimated that the obesity epidemic accounted for 4 million deaths in 2015 and that 70% of these were due to cardiovascular disease (CVD). One of the mechanisms linking obesity to CVD is the ability of adipose tissue to secrete circulating factors. We hypothesized that adipose tissue and its secretory products may influence mineralocorticoid receptor (MR) expression. Here, we showed that expression of MR and its downstream targets (Cnksr3, Scnn1b, and Sgk1) were significantly reduced in the kidneys of peroxisome proliferator-activated receptor-γ null (Pparg¹/¹) and A-ZIP/F-1 (AZIPtg/+) lipoatrophic mice with respect to their controls. Intriguingly, MR expression was also found to be significantly reduced in the kidneys of genetically obese ob/ob mice. Our data suggest that adipose tissue contributes to the regulation of MR expression. Given that leptin deficiency seems to be the major feature shared by Pparg¹/¹, AZIPtg/+, and ob/ob mice, we speculate that adipose tissue modulates MR expression through the leptin system.

Abbreviations
CVD, cardiovascular disease; ENaC, epithelial Na channel; MR, mineralocorticoid receptor; Nr3c2, nuclear receptor subfamily 3 group C member 2; PPARγ, peroxisome proliferator-activated receptor-γ; RAAS, renin–angiotensin–aldosterone system; Scnn1b, sodium channel epithelial 1 beta subunit; Sgk1, serum/glucocorticoid regulated kinase 1.
We hypothesized that adipose tissue and its secretory products could influence MR regulation. To test this hypothesis, we evaluated the renal expression of MR in mouse models of lipoatrophy and compared it with that of mouse models of obesity.

**Materials and methods**

**Animal models**

Female PPARγ null (Pparg	extsuperscript{-/-}) mice were generated in our laboratory as previously described [5,6]. Littermates with a mixed C57BL/6J × 129 genetic background and two functional Pparg alleles were used as controls (CTL). Female A-ZIP/F-1 mice (AZIPtg/) and their wild-type controls on a FVB/N background (FVB/N), which were a kind gift from C. Vinson, were generated as previously reported [7]. Female B6.V-Lep	extsuperscript{+/}J (ob/ob) mice and their controls (C57BL/6J) were purchased from Charles River (Saint Germain Nuelles, France).

Mice were followed for different time-periods and sacrificed by CO₂ inhalation. Kidneys were homogenized for protein extraction or snap frozen for RNA analysis. Skin samples were snap frozen in 1 mL of TRI-reagent/sample (Thermo Fisher Scientific, Waltham, MA, USA). Leptin was measured by ELISA (R&D, Minneapolis, MN, USA; MOB00); creatinine, glucose, and plasma aldosterone were measured at the Nephrology Service (CHUV, Switzerland). Animal care and treatments were carried out in compliance with specific European laws (86/609/EEC). This study was approved by the Commission for Animal Experimentation of the Cantonal Veterinary Services (Canton of Vaud).

**Quantitative real-time RT-PCR**

Total RNA from kidney was isolated with TRI-Reagent and RNeasy Mini Kit (Qiagen, Hilden, Germany). Gene expression of Nr3c2 and downstream mediators (Cnksr3, Scnn1b, Sgk1) was analyzed by real-time quantitative PCR (FastStart Universal SYBR Green Master; Roche, Pleasanton, CA, USA) in a Stratagene MX3005P Detection System (Agilent Technologies, Santa Clara, CA, USA). Rps9 was used as the housekeeping gene. Primer sequences are available in Table 1.

**Western blot**

Fresh kidney samples were manually homogenized in ice-cold TEN buffer containing protease inhibitors. After centrifugation, cells were resuspended in buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EGTA pH8, 0.1 mM EDTA, 1 mMDTT) and subsequently lysed by the addition of 10% NP-40. The homogenate was centrifuged, and the supernatant containing the cytoplasmic fraction was collected and stored at −80 °C. The pellet was then resuspended in nuclear extraction buffer, put on ice, and mixed periodically for 20 min. After centrifugation, the supernatant containing the nuclear fraction was stored at −80 °C.

Protein quantification was performed by BCA protein assay kit (Thermo Scientific). Cytosolic and nuclear fractions were subjected to SDS/PAGE and blotted onto nitrocellulose filters. After blocking, the membranes were incubated with primary antibodies for MR (MRN 2B7, a kind gift from C. Gomez-Sanchez), followed by peroxidase-conjugated goat anti-mouse secondary antibodies.

**Table 1. List of primers.**

| Gene  | Primer pair                                                                 |
|-------|-----------------------------------------------------------------------------|
| Mouse |                                                                           |
| Rps9  | (F) 5′-GACCAGGAGCTAAGGTGATTAGGA-3′                                          |
|       | (R) 5′-TCTTGCCCGTTAAGCTTGAG-3′                                              |
| Nr3c2 | (F) 5′-GAGGATCCCCAGTACAGAAGCACTTCG-3′                                      |
|       | (R) 5′-TCTCCCGTCAAACCTTG-3′                                                 |
| Cnksr3| (F) 5′-AAGATCTCGTAGTTGACTAG-3′                                              |
|       | (R) 5′-TCTCCCGTCAAACCTTG-3′                                                 |
| Scnn1b| (F) 5′-GCTGTGATGACATTCTGAGC-3′                                             |
|       | (R) 5′-ATGCCCCAGTGGAGATGTA-3′                                               |
| Sgk1  | (F) 5′-CTTGAAGCTGGCTACCTGGCACTC-3′                                         |
|       | (R) 5′-GAACCTTTTCAAAAAGCTTGCACTCC-3′                                       |

**Table 2. General characteristics of the mice studied.**

| Parameters                          | CTL | Pparg	extsuperscript{+/-} | FVB/N | AZIP	extsuperscript{+/-} | C57BL/6J | ob/ob |
|-------------------------------------|-----|--------------------------|-------|--------------------------|---------|-------|
| Age [weeks]                         | 3   | 3                        | 3     | 3                        | 3       | 8     | 8     |
| Body weight (g)                     | 11.6±0.3 | 7.8±0.2	extsuperscript{I} | 12.6±0.5 | 8.7±0.7	extsuperscript{I} | 20.6±0.7 | 39.7±0.6	extsuperscript{I} |
| Index of renal hypertrophy (g/gl	imes100) | 1.26±0.02 | 1.62±0.04	extsuperscript{I} | 1.18±0.03 | 1.37±0.03	extsuperscript{I} | 0.75±0.01 | 1.23±0.02	extsuperscript{I} |
| Plasma creatinine (μmol/L)          | 10.3±0.2 | 10.9±0.6                 | 13.0±0.4 | 14.1±1.1                 | 15.9±1.8 | 21.2±7.1 |
| Glycemia (mmol/L)                   | 10.1±0.2 | 16.4±1.5	extsuperscript{I} | 12.2±0.3 | 16.3±0.9	extsuperscript{I} | 9.0±0.1  | 14.5±1.7	extsuperscript{I} |
| Leptin (pg/mL)                      | 1484.0±491.0 | 121.4±71.1	extsuperscript{I} | 1808.2±344.3 | 205.3±65.1	extsuperscript{I} | 1250.0±629.6 | Undetectable |
| Aldosterone (pg/mL)                 | 467.4±87.5 | 785.5±179.5             | 492.8±100.2 | 835.5±255.4             | 347.2±25.5 | 382.8±30.0 |

Data are presented as mean ± SEM (n = 4–11). P < 0.05, 

\*P < 0.01, \*P < 0.001, \*P < 0.0001. Pparg	extsuperscript{+/-} vs. CTL, AZIP	extsuperscript{+/-} vs. FVB/N, or ob/ob vs. C57BL/6J (Student’s t-test).
Renal MR in lipoatrophy

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A  

\textbf{Nr3c2 (MR gene)}

\begin{itemize}
  \item CT\L
  \item $\gamma^{\Delta/\Delta}$
\end{itemize}

Arbitrary value

\begin{itemize}
  \item 3 weeks
  \item 3 weeks
  \item 52 weeks
  \item 52 weeks
\end{itemize}

B  

\textbf{Nr3c2 (MR gene)}

\begin{itemize}
  \item FVB/N
  \item $\text{AZI}^{\text{tg}^+/+}$
\end{itemize}

Arbitrary value

\begin{itemize}
  \item 3 weeks
  \item 3 weeks
  \item 13 weeks
  \item 13 weeks
\end{itemize}

C  

\textbf{Cytosolic MR}

\begin{itemize}
  \item CT\L
  \item $\gamma^{\Delta/\Delta}$
\end{itemize}

MR

\begin{itemize}
  \item FVB/N
  \item $\text{AZI}^{\text{tg}^+/+}$
\end{itemize}

MR

\begin{itemize}
  \item U2AF
\end{itemize}

\begin{itemize}
  \item FVB/N
  \item $\text{AZI}^{\text{tg}^+/+}$
\end{itemize}

\begin{itemize}
  \item U2AF
\end{itemize}

\[ \text{MR/\beta-actin} \]

\[ \text{MR/GAPDH} \]

\[ \text{MR/U2AF} \]

D  

\textbf{Nuclear MR}

\begin{itemize}
  \item CT\L
  \item $\gamma^{\Delta/\Delta}$
\end{itemize}

\begin{itemize}
  \item FVB/N
  \item $\text{AZI}^{\text{tg}^+/+}$
\end{itemize}

\begin{itemize}
  \item U2AF
\end{itemize}

\[ \text{MR/U2AF} \]

E  

\textbf{Cnkr3}

\begin{itemize}
  \item CT\L
  \item $\gamma^{\Delta/\Delta}$
  \item FVB/N
  \item $\text{AZI}^{\text{tg}^+/+}$
\end{itemize}

Arbitrary value

\[ \text{Cnkr3} \]

F  

\textbf{Scnn1b}

\begin{itemize}
  \item CT\L
  \item $\gamma^{\Delta/\Delta}$
  \item FVB/N
  \item $\text{AZI}^{\text{tg}^+/+}$
\end{itemize}

Arbitrary value

\[ \text{Scnn1b} \]

G  

\textbf{Sgk1}

\begin{itemize}
  \item CT\L
  \item $\gamma^{\Delta/\Delta}$
  \item FVB/N
  \item $\text{AZI}^{\text{tg}^+/+}$
\end{itemize}

Arbitrary value

\[ \text{Sgk1} \]
Immunoreactivity was detected using the Supersignal West Pico chemiluminescent substrate (Thermo Scientific). β-Actin (Sigma-Aldrich, St. Louis, MO, USA), GAPDH (Cell Signaling Technology, Danvers, MA, USA), and U2AF (Sigma-Aldrich) were used as loading controls.

Statistical analysis

Values, expressed as mean ± SEM, were analyzed using PRISM 5.0 (GraphPad Software, San Diego, CA, USA). Student’s t-test was used to assess statistical significance. A P value < 0.05 was considered statistically significant.

Results

MR expression is significantly reduced in the kidneys of Pparg<sup>D/D</sup> and AZIPtg/+ mice

The general characteristics of the animal studied are reported in Table 2. Lipoatrophy was associated with
body weight and leptin reduction, as well as kidney hypertrophy [5,6]. Interestingly, 3-week-old Pparg<sup>Δ/Δ</sup> and AZIP<sup>tg/+</sup> mice exhibited a significant downregulation of MR gene (Nr3c2) expression in their kidneys, which was also observed in older animals (Fig. 1A,B). Similar results were found in the skin, suggesting that MR gene downregulation was tissue-independent (Fig. 2A). The reduction of MR gene expression was associated with a significant reduction of cytosolic and nuclear MR protein levels, as assessed by western blot analysis, in the kidneys of 3-week-old lipoatrophic mice with respect to their controls (Fig. 1C,D).

**Downstream targets of MR are significantly reduced in the kidneys of Pparg<sup>Δ/Δ</sup> and AZIP<sup>tg/+</sup> mice**

Classically, when aldosterone binds to MR, it increases renal sodium reabsorption by upregulating the epithelial Na channel (ENaC) and the sodium/potassium ATPase (Na-K-ATPase) in the collecting duct system [3]. Cnksr3, Scnn1b, and Sgk1 are involved in this aldosterone-mediated ENaC modulation through MR activation [8,9]. Consistent with the MR reduction, Cnksr3 and Scnn1b were downregulated in both Pparg<sup>Δ/Δ</sup> and AZIP<sup>tg/+</sup> mice, and Sgk1 was reduced in Pparg<sup>Δ/Δ</sup> mice (Fig. 1E–G). Conversely, both lipoatrophic models exhibited a progressive increase of aldosterone plasma levels, possibly due to the decrease of its specific receptor (Fig. 2B).

**MR expression is significantly decreased in the kidneys of ob/ob mice**

To evaluate whether renal MR reduction in lipoatrophic mice was due to the absence of fat, we measured MR expression in the kidneys of ob/ob mice, a model of extreme obesity, due to a mutation of the gene encoding for leptin [10,11]. The general characteristics of these mice are reported in Table 2. Interestingly, we found that ob/ob mice displayed a significant reduction of both cytosolic and nuclear MR expression (Fig. 3A,B). Contrary to lipoatrophic mice, this was not associated with significant changes in aldosterone levels and/or the gene expression of Cnksr3, Scnn1b, and Sgk1 (Fig. 3C–E).

**Discussion**

This study shows that renal MR is significantly reduced not only in lipoatrophic mice but also in a mouse model of extreme obesity. Notwithstanding the presence of minor interstrain differences, our data suggest that the adipose tissue contributes to the regulation of renal MR expression, which seems related to adipose tissue function, rather than adipose tissue mass per se. Typically, adipose tissue dysfunction includes not only visceral (ectopic) fat accumulation (as seen in lipoatrophy), but also changes in its composition as well as in mRNA and protein expression patterns (as seen in ob/ob mice) [12].

Fig. 3. MR is downregulated in kidneys of ob/ob mice. (A) Representative blots and densitometric analysis of MR protein in the renal cytosolic fraction of 8-week-old ob/ob and control (C57BL/6J) mice. Data are normalized to β-actin and expressed as mean ± SEM (n = 4). (B) Nuclear MR protein expression in kidneys of the same groups of mice. Data are normalized to U2AF and expressed as mean ± SEM (n = 4). **P < 0.01 ob/ob vs. control animals (Student’s t-test). (C) Renal protein expression of Cnksr3, (D) Scnn1b, and (E) Sgk1 in C57BL/6J and ob/ob mice at 8 weeks of age. Data show mean ± SEM (n = 4–5) (Student’s t-test).
In healthy conditions, adipocytes are metabolically active cells that secrete a wide variety of hormones and adipokines, such as leptin, which regulates several physiological functions [15] by binding to its specific receptors in different tissues, including kidney and skin [16,17]. Interestingly, leptin deficiency has been associated with insulin resistance, diabetes, and organ damage in humans and animals [5,13]. In addition, although transgenic overexpression of leptin and fat transplantation rescued the metabolic disorders in lipoatrophic AZIPtg/+ mice [16,17], the transplantation of adipose tissue from ob/ob mice was unable to reverse AZIPtg/+ diabetes phenotype, thus underlying the fundamental role of leptin and its signaling in maintaining body homeostasis [18].

Adipocytes produce [19] and regulate aldosterone release as well. In particular, Ehrhart-Bornstein observed that adipocytes isolated from healthy subjects secreted potent mineralocorticoid-releasing factors, with a major effect on aldosterone release [20]. It has been shown that leptin is one of these mineralocorticoid-releasing factors, as it was able to directly regulate aldosterone secretion from the adrenal cortex, independent of angiotensin, and the sympathetic nervous system [21]. Given that leptin deficiency seems the major feature that Pparγ<sup>−/−</sup>, AZIPtg/+<sup>−/−</sup>, and ob/ob mice have in common, we speculate that adipose tissue modulates renal MR expression through leptin or a leptin-activated factor. This is consistent with the observation that in high-fat diet-induced obesity, which is associated with an increase of circulating leptin levels [22], there is an increase in the renal nuclear fraction of MR [23].

The stimulatory effect that leptin might have on renal MR expression could be an additional way that leptin has to promote aldosterone actions. Further studies are needed to evaluate the effect of leptin replenishment on MR expression in these mouse models of leptin deficiency. Nevertheless, our data support the relationship between fat, aldosterone, and CVD; improve the understanding; and open new possibilities for the management of obesity-related disease burden.

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**Conflict of interest**

The authors declare no conflict of interest.

**Author contributions**

BT conceived the study, performed the experiments, analyzed data, and wrote the manuscript. SB analyzed and interpreted data and wrote the manuscript. CW performed the experiments. CC performed some experiments. FG conceived the study and participated to data interpretation. BD conceived and supervised the study and wrote the manuscript. All authors read and edited the manuscript.

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