Eplerenone prevented obesity-induced inflammasome activation and glucose intolerance

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Abstract

Obesity-associated activation of the renin-angiotensin-aldosterone system is implicated in the pathogenesis of insulin resistance; however, influences of mineralocorticoid receptor (MR) inhibition remain unclear. Therefore, we aimed to clarify the anti-inflammatory mechanisms of MR inhibition using eplerenone, a selective MR antagonist, in C57BL/6 mice fed a high-fat diet (HFD) for 12 weeks. Eplerenone prevented excessive body weight gain and fat accumulation, ameliorated glucose intolerance and insulin resistance and enhanced energy metabolism. In the epididymal white adipose tissue (eWAT), eplerenone prevented obesity-induced accumulation of F4/80⁺CD11c⁺CD206[−] M1-adipose tissue macrophage (ATM) and reduction of F4/80⁺CD11c[−]CD206⁺ M2-ATM. Interestingly, M1-macrophage exhibited lower expression levels of MR, compared with M2-macrophage, in the ATM of eWAT and *in vitro*-polarized bone marrow-derived macrophages (BMDM). Importantly, eplerenone and MR knockdown attenuated the increase in the expression levels of *prol1b*, *Il6* and *Tnfa*, in the eWAT and liver of HFD-fed mice and LPS-stimulated BMDM. Moreover, eplerenone suppressed IL1b secretion from eWAT of HFD-fed mice. To reveal the anti-inflammatory mechanism, we investigated the involvement of NLRP3-inflammasome activation, a key process of IL1b overproduction. Eplerenone suppressed the expression of the inflammasome components, *Nlrp3* and *Caspase1*, in the eWAT and liver. Concerning the second triggering factors, ROS production and ATP- and nigericin-induced IL1b secretion were suppressed by eplerenone in the LPS-primed BMDM. These results indicate that eplerenone inhibited both the priming and triggering signals that promote NLRP3-inflammasome activation. Therefore, we consider MR to be a crucial target to prevent metabolic disorders by suppressing inflammasome-mediated chronic inflammation in the adipose tissue and liver under obese conditions.

Key Words

- renin-angiotensin system
- glucose metabolism
- mineralocorticoid receptor
- NLRP3 inflammasome, adipose tissue macrophage

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Introduction

The renin-angiotensin-aldosterone system is a coordinated hormone cascade, maintaining the salt-water balance and blood pressure (Giacchetti *et al.* 2005). However, this cascade is incidentally activated in obesity and accelerates insulin resistance mainly through the actions of angiotensin 2 (Oliveres-Reyes *et al.* 2009). In addition, we and others identified the implications of mineralocorticoid receptor (MR) activation in the progression of obesity-associated insulin resistance. We have previously reported that eplerenone, a potent MR antagonist, was able to prevent obesity-associated metabolic abnormalities, chronic inflammation and histological features of steatohepatitis in a mouse model of non-alcoholic steatohepatitis (NASH) (Wada *et al.* 2013). However, the precise mechanism by which eplerenone attenuates chronic inflammation in obesity remains to be clarified.

The innate immune system refers to a nonspecific defense mechanism and contributes to the maintenance of host homeostasis. Accumulating evidence has been untangling the complex link between insulin resistance in obesity and innate immunity, particularly via macrophages (Lackey & Olefsky 2016). The macrophage is known to express at least two types of polarity, namely inflammatory M1 and anti-inflammatory M2 (Fujisaka *et al.* 2009). Most resident macrophages in the lean visceral adipose tissue are classified as M2, whereas M1-macrophages increase when adipose tissue expands in obesity and exacerbate local chronic inflammation. Interestingly, macrophages from myeloid-specific MR knockout mice exhibit the transcriptional profile of M2 macrophage (Usher *et al.* 2010). Currently, however, the expression of MR in M1- and M2-adipose tissue macrophages (ATMs) as well as the impact of MR inhibition on the M1/M2 polarity remains unknown.

Recent studies have demonstrated the significant involvement of interleukin 1 β (IL1 β) in obesity-associated insulin resistance and have clarified a precise regulatory mechanism of IL1 β production by Nod-like receptor, pyrin domain-containing 3 (NLRP3) inflammasome (Maedler *et al.* 2002, Stienstra *et al.* 2010, 2011, Vandanmagsar *et al.* 2011, Latz *et al.* 2013, Coll *et al.* 2015, Lackey *et al.* 2016). The activation of the NLRP3 inflammasome is achieved by two sequential signaling stimuli. In the first priming stimulus termed Signal 1, innate immune cells, such as macrophages, perceive pathogen-associated molecular patterns (PAMPs) through pattern recognition receptors such as toll-like receptor 4. Then, they transmit the

signals to downstream nuclear factor- κ B (NF κ B), thereby activating the transcription of *proIL1 β* and inflammasome component molecules. In the second triggering stimulus termed Signal 2, immune cells recognize danger-associated molecular patterns (DAMPs) and PAMPs, which trigger the inflammasome complex formation by assembling the components of NLRP3, pro-caspase 1 and apoptosis-associated speck-like protein containing CARD (ASC). Consequently, *proIL1 β* is cleaved by the inflammasome-associated caspase 1, resulting in the production of mature IL1 β . Therefore, the NLRP3 inflammasome is recognized as a potential therapeutic target of obesity-associated metabolic abnormalities. Indeed, genetic deletion of their components, such as NLRP3, caspase 1, ASC and IL1 β , improved glucose metabolism in diet-induced obesity (Stienstra *et al.* 2010, 2011). However, the pathophysiological relationship between the inflammasome and the renin-angiotensin-aldosterone system remains to be clarified.

In the present study, to clarify the impacts of MR inhibition on chronic inflammation in obese conditions and their underlying mechanisms, we investigated the effects of eplerenone in the adipose tissue and liver of diet-induced obese mice. Here, we showed that eplerenone prevented diet-induced obesity by ameliorating glucose and energy metabolism. In this process, eplerenone suppressed both Signal 1- and 2-mediated activation of the NLRP3 inflammasome, thereby attenuating obesity-associated overproduction of IL1 β in the epididymal white adipose tissue (eWAT) and liver. Thus, the present study demonstrated that MR plays a causal role in chronic inflammation under obese conditions and provided insight into a novel MR-targeted therapeutic strategy to prevent obesity-associated metabolic abnormalities.

Materials and methods

Animals and experimental design

Five-week-old male C57BL/6J mice purchased from Japan SLC (Shizuoka, Japan) were divided into 4 experimental groups: (1) mice fed control chow diet (referred to as chow), (2) mice fed chow diet and treated with eplerenone (referred to as chow+Ep), (3) mice fed a high-fat diet (referred to as HFD), and (4) mice fed a high-fat diet and treated with eplerenone (referred to as HFD+Ep). In the chow and chow+Ep groups, mice were maintained on a regular diet (Rodent Diet 20 5053; LabDiet, St. Louis, MO, USA).

In the HFD and HFD+Ep groups, mice were maintained on a 60 kcal% fat diet (D12492; Research Diets, New Brunswick, NJ, USA). Eplerenone (1.67 g/kg diet, provided by Pfizer) was mixed with each diet and administered. Because of the different food consumption, eplerenone dosage was 0.2 and 0.1 g/kg in chow and HFD mice, respectively. Metabolic phenotypes of these mice were analyzed after a 12-week diet and eplerenone challenge. In experiments using genetically obese db/db mice, 8-week-old male db/db mice and their control misty mice purchased from the Institute for Animal Reproduction (Ibaraki Japan) were administered eplerenone (1.67 g/kg diet; 0.17 and 0.18 g/kg body weight of misty and db/db mice, respectively) for 4 weeks, and their metabolic phenotypes were analyzed. Mice were housed on a 12:12-h light-darkness cycle (lights on at 07:00h) in a temperature-controlled colony room and were provided with food and water *ad libitum*. All experimental procedures used in this study were approved by the Committee of Animal Experiments at the University of Toyama.

Measurements of serum parameters, hepatic triglycerides and glucose/insulin tolerance test

Mice were deprived of food overnight, and blood samples were collected from the abdominal aorta under anesthesia. After centrifugation at 1500g for 20 min, the supernatants of the blood samples were separated and subjected to measurements. Blood glucose levels were measured with an Accu-Chek (Roche). Serum levels of insulin were measured using an ELISA kit (Morinaga, Kanagawa, Japan). Serum levels of cholesterol and triglycerides were determined with a colorimetric kit (Wako Pure Chemical, Osaka, Japan) (Wada *et al.* 2013, Sameshima *et al.* 2015). Hepatic triglyceride content was determined using a triglyceride colorimetric kit (Wako Pure Chemical) after extraction of the lipid fraction from frozen liver specimens by the method of Bligh and Dyer with minor modifications (Wada *et al.* 2013). Analyses with EIA and ELISA kits were conducted in duplicate. The inter-assay coefficients of variation were less than 10% in each analysis. Glucose tolerance test (GTT) and insulin tolerance test (ITT) were conducted as described previously (Sameshima *et al.* 2015, Onogi *et al.* 2017).

Analysis of body fat composition by MRI imaging

Body fat composition was analyzed by magnetic resonance imaging (MRI) under anesthesia 12 weeks after

the initiation of HFD feeding, as described previously (Yonezawa *et al.* 2012). Series of T1-weighted axial slices were analyzed with the software ImageJ (NIH, Bethesda, MD, USA).

Histological analysis and immunohistochemistry

Isolated adipose tissues and livers were fixed in 10% formaldehyde for 24 h and embedded in paraffin. Six- μ m-thick sections were stained with hematoxylin-eosin (HE) and used for the analysis of adipocyte size. Approximately 300 cells per mouse were measured with the ImageJ software (NIH). In CD11c immunostaining, paraffin-embedded sections were incubated with a hamster anti-mouse CD11c antibody (dilution, 1:100, 10 μ g/mL) for 3 h followed by a goat anti-hamster IgG antibody (1:100, 8 μ g/mL) for 1 h. The numbers of crown-like structures formed were then analyzed.

Flow cytometry analysis

The stromal-vascular fraction (SVF) was isolated from epididymal white adipose tissues (eWAT) and analyzed by FACSCanto II or FACSARIA II for cell sorting (BD Bioscience), as described previously (Sameshima *et al.* 2015, Onogi *et al.* 2017). M1 and M2 macrophages were defined as CD45⁺F4/80⁺CD11c⁺CD206⁻ cells and CD45⁺F4/80⁺CD11c⁻CD206⁺ cells, respectively. For MR staining, cells were fixed, permeabilized and intracellularly stained with anti-mouse MR (H10E4C9F; Abcam) and anti-mouse IgG (H+L), F(ab')₂-Alexa Fluor 488 conjugated secondary antibody (Cell Signaling) according to the manufacturer's instruction. Data were analyzed by FACS Diva 6.1.2 (BD Bioscience) or FCS Express (De Novo Software).

Real-time quantitative PCR, Western blotting and IL1b secretion assay

RNA extraction, reverse transcription and real-time PCR using SYBR green were conducted, as described previously (Wada *et al.* 2010, Yonezawa *et al.* 2012). The relative expression of objective mRNAs was calculated as a ratio to that of the 18S ribosomal RNA (animal experiments) or hypoxanthine-guanine phosphoribosyltransferase (HRPT; *in vitro* experiments). Primer sequences are listed in Table 1. Western blotting was conducted as described previously (Wada *et al.* 2013). For sampling of culture media, proteins in the media were precipitated by mixing

Table 1 Primer list.

Genes	Forward primer	Reverse primer
IL1b	TCCAGGATGAGGACATGAGCAC	GAACGTCACACACCAGCAGGTTA
IL6	ATGGAAACCACACGGTGACCT	TGAAGGACTCTGGCTTTGTCT
TNFa	AGCCTGTAGCCACGTCGTA	GGCACCCTAGTTGGTTGTCTTTG
NLRP3	CCACAGTGTAACCTGCAGAAGC	GGTGTGTGAAGTTCTGGTTGG
Caspase1	GGCAGGAATTCTGGAGCTTCAA	GTCAGTCCTGGAAATGTGCC
UCP1	TACCAAGCTGTGCGATGT	AAGCCCAATGATGTTCACT
PGC1a	GCCCGGTACAGTGAGTGTTT	CTGGGCCGTTTAGTCTTCCT
CIDEA	TGCTCTTCTGTATCGCCAGT	GCCGTGTTAAGGAATCTGCTG
PRDM16	CTTCTCCGAGATCCGAACTTC	GATCTCAGGCCGTTTGTCCAT
18s rRNA	GTAACCCGTTGAACCCCAT	CCATCCAATCGGTAGTAGCG
HPRT	GTCGTGATTAGCGATGATGAACC	GTCTTTCAGTCCTGTCCATAATCAG

CIDEA, cell death-inducing DNA fragmentation factor-like effector a; HPRT, hypoxanthine–guanine phosphoribosyltransferase; NLRP3, Nod-like receptor, pyrin domain containing 3; PGC1a, peroxisome proliferator-activated receptor γ coactivator 1a; PRDM16, PR-domain-containing 16; UCP1, uncoupling protein 1; TNFa, tumor necrosis factor α .

with MeOH and CHCl_3 and yielded pellets were suspended in Laemmli buffer. Antibodies utilized for immunoblotting were described below. Anti-NFkB p65, anti-Caspase 1 and anti-IL1b antibodies were purchased from Santa Cruz Biotechnology. Anti-phospho-Ser536-specific NFkB p65 and anti-beta actin antibodies were purchased from Cell Signaling Technology. Anti-MR antibody was from Abcam Japan (Tokyo, Japan). A densitometric analysis of the blotted membrane was conducted using an LAS-4000 Immunoimage analyzer system (Fujifilm, Tokyo, Japan). For the analysis of mature IL1b secretion from adipose tissue, minced eWAT was incubated in serum-free DMEM for 24 h, and IL1b secretion in the culture media from 1 g of eWAT was determined by ELISA (R&D Systems), as described previously (Honda *et al.* 2014).

Energy consumption, locomotor activity, blood pressure and core body temperature

Oxygen consumption (VO_2), production of carbon dioxide (VCO_2) and locomotor activity (counted by an inflated ray sensor system) were measured in metabolic chambers (MK-5000RQ, Muromachi Kikai, Tokyo, Japan) with free access to food and water, as described previously (Yonezawa *et al.* 2012, Sameshima *et al.* 2015). Blood pressure and heart rate were measured using a blood pressure monitor for mice and rats (MK-2000ST; Muromachi Kikai) (Yonezawa *et al.* 2012). Rectal temperature was monitored using an electronic thermometer (PTC-301, Unique Medical, Tokyo, Japan) under random-fed conditions (Yonezawa *et al.* 2012, Ichihara *et al.* 2013).

Culture, polarization and siRNA-mediated MR knockdown in bone marrow-derived macrophages (BMDM)

Bone marrow cells (BMC) isolated from the femur and tibia of male C57BL/6J mice between 8 and 12 weeks old were differentiated into BMDM by incubation in RPMI supplemented with 10% fetal bovine serum (FBS) and 20% L929 condition medium for 3–4 days, and another 3–4 days in RPMI supplemented with 10% FBS (Onogi *et al.* 2017, Schiller *et al.* 2004). Charcoal-treated FBS (Biological Industry, Crownwell, CT, USA) was used after BMC were differentiated into BMDM to avoid contamination of steroid hormones in the serum. BMDM were polarized into M1- and M2-BMDMs by incubation with interferon gamma (IFN γ) (10 ng/mL) and LPS (100 ng/mL) or IL4 (10 ng/mL) for 36 h, respectively. Untreated BMDM was used as M0-BMDM. For MR knockdown, siRNA for MR and scrambled negative control (Thermo Fisher Scientific) were incorporated into BMDM using TransIT-TKO transfection reagent (Mirus Bio LLC, Madison, WI, USA), as described in the manufacturers' protocol.

Detection of reactive oxygen species (ROS)

Intracellular ROS levels were determined using ROS detection reagent (Thermo Fisher Scientific). In brief, serum starved BMDM seeded in 96-well plates were pretreated with eplerenone (5 μM) and stimulated with aldosterone (10^{-10}M) or LPS (100 ng/mL), followed by incubation with CM- H_2DCFDA for 1 h. Intracellular ROS levels were determined by fluorescence intensity.

Statistical analysis

Data are expressed as the mean $\pm$ S.E. *P* values were determined by one-way ANOVA with Bonferroni's test, and *P* < 0.05 was considered significant.

Results

Eplerenone attenuates HFD-induced obesity and improves glucose metabolism

We firstly examined the effects of eplerenone on body weight and fat composition in C57BL/6 mice fed chow or HFD. Figure 1A shows profiles of body weight changes in experimental periods. Body weights were significantly heavier in HFD and HFD + Ep mice than chow-fed mice after 2 weeks of HFD feeding. Eplerenone effectively attenuated HFD-induced body weight gain after more than 4 weeks of administration. Regarding the body composition, increased volumes of visceral and subcutaneous fat by HFD feeding were significantly attenuated by eplerenone, whereas eplerenone did not affect body weights and body fat volumes during 12 weeks of chow-diet feeding. Lean mass did not differ among the four groups of mice (Fig. 1B and C).

In order to evaluate the impact of eplerenone on glucose metabolism, we conducted GTT and ITT in each group of mice (Fig. 1D, E and F). Glucose levels in GTT and ITT were almost similar between chow and chow + Ep mice. In contrast, fasting glucose levels as well as glucose levels during GTT and ITT were significantly higher in HFD mice. These abnormalities were significantly ameliorated in HFD + Ep mice. Similar results were observed in the glucose area under the curve (AUC). Serum insulin levels during GTT were elevated by HFD feeding, but HFD + Ep mice exhibited remarkably lower insulin levels than HFD mice (Fig. 1E).

Other metabolic parameters in each group of mice are listed in the Table 2. Both systolic and diastolic blood pressures were significantly higher in HFD mice compared with chow mice, and these elevations were attenuated by eplerenone treatment. Tissue weights in the eWAT, inguinal WAT (iWAT) and liver, and plasma cholesterol levels were significantly higher in HFD mice compared with chow mice, and these increases were again ameliorated by the eplerenone treatment.

To directly clarify the anti-obese effects of eplerenone, we examined the therapeutic impact in obese db/db mice. Eplerenone was administrated

Figure 1

Effects of eplerenone on body weight gain, body fat composition and glucose metabolism in mice. (A, B and C) Four groups of mice (chow mice: open symbols, chow + Ep mice: gray symbols, HFD mice: black symbols, HFD + Ep mice: hatched symbols) were used. Transition of body weight (*n* = 17–19 per group) (A), representative T1-weighted axial slices of MRI at the level of the urinary bladder (B), and quantified volumes of visceral and subcutaneous fat and lean mass from the diaphragm to the anus (C) are shown (*n* = 6–9 per group). (D) GTT (2 g of glucose/kg body weight, i.p.) was conducted in 6 h-fasted mice after 12 weeks of HFD-feeding. The averaged glucose area under the curve (AUC) over the course of 120 min in each group was shown (*n* = 6–9 per group). (E) Insulin levels at 0, 15, 30 min in GTT are shown. (F) ITT (0.75 units of insulin/kg body weight, i.p.) was conducted in 4 h-fasted mice. The averaged AUC over the course of 120 min for each group are shown (*n* = 7–8 per group). Data are shown as the mean $\pm$ S.E.M. ***P* < 0.01, significantly different from the chow mice; ††*P* < 0.01, significantly different between HFD and HFD + Ep mice.

Table 2 Characteristics of mice at 12 weeks of the experimental period.

	chow	chow+Ep	HFD	HFD +Ep
Systolic blood pressure (0 weeks) (mmHg)	72.7±1.7	72.9±2.3	72.4±2.8	77.6±1.8
Systolic blood pressure (12 weeks) (mmHg)	85.7±3.6	85.4±2.2	112.0±2.2**	94.5±2.7††
Diastolic blood pressure (0 weeks) (mmHg)	29.1±3.7	31.9±2.7	30.2±1.5	33.9±4.4
Diastolic blood pressure (12 weeks) (mmHg)	43.1±5.1	36.7±4.9	64.0±4.7**	41.6±4.4††
Heart rate (0 weeks) (bpm)	708±19	662±20	677±17	679±23
Heart rate (12 weeks) (bpm)	597±34	542±39	694±12**	677±28**
eWAT weight (g)	0.48±0.0	0.35±0.1	2.74±0.1**	2.40±0.1**,††
iWAT weight (g)	0.25±0.0	0.21±0.0	2.47±0.1**	1.53±0.1**,††
Liver weight (g)	1.04±0.0	0.98±0.0	1.63±0.1**	1.40±0.0**,††
Plasma triglycerides (mg/dL)	90.5±6.2	82.9±12.6	55.4±4.7**	44.1±3.4**
Plasma cholesterol (mg/dL)	71.0±4.0	69.6±2.3	173.0±5.6**	118.0±2.5**,††

Data are shown as the mean±S.E.M. (tissue weights, $n=17-19$; others, $n=6-9$). C57BL/6J mice were fed chow or HFD with or without eplerenone (Ep) for 12 weeks. ** $P<0.01$, significantly different from chow. †† $P<0.01$, significantly different from HFD.

to 8-week-old db/db (db/db+Ep) and control misty (m^+/m^+ +Ep) mice for 4 weeks and analyzed the metabolic phenotypes (Supplementary Fig. 1A and B, see section on supplementary data given at the end of this article). Significant reduction of body weights was observed in db/db+Ep mice compared with db/db mice only after 1 week of administration. In a body composition analysis, both visceral and subcutaneous fat volumes were significantly lower in db/db+Ep mice than those in db/db mice. Furthermore, glucose metabolism assessed by GTT and ITT was also significantly ameliorated by eplerenone treatment in db/db mice (Supplementary Fig. 1C and D).

Eplerenone attenuates HFD-induced deterioration of energy metabolisms

To clarify the mechanisms by which eplerenone ameliorated diet-induced body fat gain (Fig. 1), we analyzed food consumption and fecal triglyceride content in each group (Fig. 2A and B). Calorie intake did not differ among the groups. Fecal triglyceride levels were increased by HFD feeding, but eplerenone did not affect them. On the other hand, core body temperature was significantly lowered in HFD mice, whereas it was maintained in HFD+Ep (Fig. 2C). Eplerenone increased oxygen consumption (VO_2) and carbon dioxide productions

Figure 2

Effects of eplerenone on the energy metabolism in mice. Four groups of mice (chow mice: open columns, chow+Ep mice: gray columns, HFD mice: black columns, HFD+Ep mice: hatched columns) were used. Daily food consumption (A), triglyceride (TG) content in the feces (B) and core body temperature (C) in each group are shown. Oxygen consumption (VO_2) (D), carbon dioxide production (VCO_2) (E) and spontaneous locomotor activity (F) in the light and dark phase analyzed by metabolic chamber are shown. (G) mRNA expression of thermogenesis-related genes in the brown adipose tissue are shown. Data are shown as the mean±S.E.M. ($n=4-10$). * $P<0.05$ and ** $P<0.01$, significantly different from chow mice; † $P<0.05$ and †† $P<0.01$, significantly different between HFD and HFD+Ep mice.

(VCO_2) in chow mice (Fig. 2D and E). The VO_2 and VCO_2 in the dark phase were remarkably decreased in HFD mice compared with chow mice, but these abnormalities were ameliorated in HFD+Ep mice. Similarly, eplerenone increased spontaneous locomotor activity in chow mice (Fig. 2F). In HFD mice, the locomotor activity was decreased compared with chow mice, and the increasing effects of eplerenone did not reach a significant level in HFD+Ep mice (Fig. 2F). mRNA expression levels of thermogenic factors, *Ucp1*, *Pgc1a*, *CideA* and *Prdm16*, in the brown adipose tissue (BAT) were higher in HFD+Ep mice than those in HFD mice (Fig. 2G).

Effects of eplerenone on obesity-induced histological changes in eWAT and liver

To reveal the histological alterations in association with the changes in the adipose tissue volume by HFD-feeding and the eplerenone treatment (Fig. 1), we conducted HE-staining of the eWAT and subcutaneous WAT. The sizes of adipocytes were markedly increased by HFD feeding, but these changes were suppressed by eplerenone (Fig. 3A, Supplementary Fig. 2). Consistently, immunohistochemical analyses demonstrated that CD11c⁺ macrophages were almost absent in the eWAT of chow and chow+Ep mice, whereas a cluster of CD11c⁺ macrophages around dead adipocytes, i.e., a crown-like structure (CLS), was abundant in HFD mice. Importantly, these infiltrations and CLS formations were markedly decreased in HFD+Ep mice (Fig. 3B). In the liver, lipid accumulation in the hepatic lobules in HFD mice was apparently reduced in HFD+Ep mice (Fig. 3C). A similar tendency was observed in hepatic triglyceride content (Fig. 3D).

Effects of eplerenone on the polarity and MR expression in the ATM

To characterize the impact of eplerenone on the ATM, we analyzed the number of macrophages and their polarity in eWAT by flow cytometry (Fig. 4A, B, C and D). F4/80-positive macrophage markedly increased in the eWAT of HFD mice. In particular, F4/80⁺CD11c⁺CD206⁻ M1-ATM significantly increased, whereas F4/80⁺CD11c⁺CD206⁺ M2-ATM decreased markedly under HFD-fed conditions, as previously reported (Fujisaka *et al.* 2009). HFD-induced increase in M1-ATM and decrease in M2-ATM were significantly attenuated by eplerenone treatment.

Macrophage from myeloid-specific MR-knockout mice has been reported to exhibit gene expression

Figure 3

Histological analysis in the eWAT and liver of mice. Four groups of mice (chow mice: open columns, chow+Ep mice: gray columns, HFD mice: black columns, HFD+Ep mice: hatched columns) were used. (A) Representative photomicrograph of hematoxylin and eosin (HE)-stained sections of the eWAT, and the averaged size of adipocytes. Scale bar, 200 μm. (B) Representative photomicrograph of the eWAT immunostained with anti-CD11c antibody, and the number of crown-like structure (CLS). Scale bar, 200 μm. (C) Representative photomicrograph of HE-stained sections of the liver. Scale bar, 100 μm. (D) Triglyceride content in the liver. Data are shown as the mean ± S.E.M. (n=8–12). ***P*<0.01, significantly different from chow mice; †*P*<0.05 and ††*P*<0.01, significantly different between HFD and HFD+Ep mice.

profiles of M2-macrophage (Usher *et al.* 2010). However, the actual expression levels of MR in M1- and M2-ATM of obese mice remain unknown. Therefore, we analyzed MR expression in both M1- and M2-ATM of the eWAT in each group (Fig. 4E, F and G). MR-positive M1-ATM was only about 20–30% in all four groups. In contrast, approximately 80% of M2-ATM were MR positive, and neither HFD nor eplerenone treatment affected the ratio of MR-positive cells (Fig. 4E and F). Analysis of the fluorescence intensity of MR further demonstrated that the MR expression was more abundant in the M2-ATM than the M1-ATM (Fig. 4G). To clarify the relationship between macrophage polarity and MR expression, we analyzed mRNA expression of MR in M0-, M1- and M2-BMDMs *in vitro*. The expression levels of MR were higher in the M0- and M2-BMDMs, compared with

Figure 4

Effects of eplerenone on the polarity of macrophages and their MR expression in the eWAT of mice. Flow cytometry analysis was conducted, using stromal vascular cells in the eWAT from four groups of mice (chow mice: open columns, chow+Ep mice: gray columns, HFD mice: black columns, HFD+Ep mice: hatched columns). (A, B, C and D) Representative dot plots of total macrophages (A), CD11c⁺CD206⁻ M1- and CD11c⁺CD206⁺ M2-ATM (C), and respective quantitative data (B and D) are shown. (E and F) Representative dot plots of MR-positive cells in the M1- and M2-ATM (E), and quantitative data (F) are shown. (G) Analysis of MR fluorescence intensity in the M1- and M2-ATM. Data are shown as the mean $\pm$ s.e.m. ($n=6-9$). ** $P<0.01$, significantly different from chow mice; †† $P<0.01$, significantly different between HFD and HFD+Ep mice. (H) mRNA expression of *Mr* in M0-, M1-, and M2-polarized BMDM. Data are shown as the mean $\pm$ s.e.m. ($n=4$). ** $P<0.01$, significantly different from Ep-untreated M0-BMDM.

M1-BMDM. Again, eplerenone treatment did not affect the expression of any polarity-type of BMDM (Fig. 4H).

Effects of eplerenone on gene expression of proinflammatory cytokines and inflammasome component molecules in the eWAT and liver

To examine the effects of eplerenone on the obesity-associated chronic inflammation, we analyzed the mRNA levels of proinflammatory cytokines. Expression of *prol1b*, *Il6* and *Tnfa* was significantly increased both in

the eWAT and liver of HFD mice, while these expressions were apparently reduced in HFD+Ep mice (Fig. 5A and B), although the effects on *prol1b* in eWAT did not reach a significant level. In addition, the mRNA levels of *Nlrp3* and *Caspase1*, inflammasome-component molecules, were increased in HFD mice, but these changes were attenuated by eplerenone. Expression of these molecules is known to be regulated by the transcription factor NFkB, which is phosphorylated/activated by PAMPs in the process of the Signal 1-mediated NLRP3-inflammasome activation (Stenstra *et al.* 2011, Vandanmagsar *et al.* 2011,

eWAT

Liver

eWAT

Liver

chow
chow+Ep
HFD
HFD+Ep

Figure 5

Eplerenone attenuates diet-induced chronic inflammation in the eWAT and liver of mice. Four groups of mice (chow mice: open columns, chow + Ep mice: gray columns, HFD mice: black columns, HFD + Ep mice: hatched columns) were used. (A and B) mRNA expression levels of proinflammatory and NLRP3-inflammasome-related genes in the eWAT (A) and liver (B) of each group are shown. (C) Phosphorylation levels of NFκB in the eWAT and liver of each group are shown. Data are shown as the mean $\pm$ S.E.M. ($n=6-9$). * $P<0.05$ and ** $P<0.01$, significantly different from chow mice; † $P<0.05$ and †† $P<0.01$, significantly different between HFD and HFD-Ep mice.

Latz *et al.* 2013, Lackey *et al.* 2016). Consistently, the phosphorylation levels of NFκB were significantly increased in the eWAT and liver of HFD mice, whereas they were maintained at normal levels in the HFD+Ep mice (Fig. 5C). In addition, the eplerenone treatment caused decrease (or tended to decrease) in the expression of the proinflammatory genes in both the eWAT and liver of db/db mice (Supplementary Fig. 1E and F). Taken together, these results indicate that eplerenone inhibited inflammasome activation induced by Signal 1.

Attenuation of ROS production is a key anti-inflammatory mechanism of MR inhibition

To further clarify the mechanism behind the eplerenone-induced anti-inflammation, we conducted MR-knockdown experiments using siRNA *in vitro*. The mRNA and protein

levels of MR were reduced to $52.0 \pm 0.1\%$ and $51.7 \pm 0.1\%$ in siRNA-transfected BMDM, respectively, compared with scramble-transfected BMDM (Fig. 6A). LPS-induced induction of *prol1b* and *Il6* was effectively attenuated by the knockdown of MR. Pretreatment with aldosterone alone did not affect the expression of proinflammatory cytokines. On the other hand, aldosterone augmented or tended to increase the LPS-induced expression of proinflammatory cytokines, but MR knockdown suppressed them (Fig. 6B). Thus, MR inhibition had a great impact on the proinflammatory cytokine expression in BMDM.

Furthermore, we investigated the ROS generation in the macrophage as a causal mechanism for the attenuation of proinflammatory cytokines as pretreatment with N-acetyl-cysteine (NAC), a potent antioxidant, similarly attenuated these inductions by LPS

Figure 6

Influences of MR inhibition on the proinflammatory cytokine expression and ROS production in BMDM. (A) Efficiency of siRNA-mediated knockdown of MR in BMDM. (B) Effects of MR-knockdown on proinflammatory cytokine expression in BMDM. Serum-starved BMDM transfected with MR-siRNA and scrambled control were pretreated with aldosterone (Aldo) for 18 h, and subsequently stimulated with LPS for 3 h. Then, mRNA expression of *Prol1b*, *Il6* and *Tnfa* was analyzed. (C) The time-course of Aldo-induced ROS productions in the BMDM. (D, E) Effects of eplerenone (Ep) pretreatment on the ROS production induced by Aldo (D) and LPS (E). BMDM was stimulated with aldosterone or LPS. Eplerenone was treated 30 min prior to the stimulation. Intracellular ROS level was analyzed by colorimetric assay. Data are shown as the mean $\pm$ S.E.M. ($n=4-12$). * $P<0.05$ and ** $P<0.01$, significantly different from untreated cells; † $P<0.05$ and †† $P<0.01$, significantly different among two groups, as indicated.

(Supplementary Fig. 3). Aldosterone transiently induced ROS generation in BMDM in a time-dependent manner (Fig. 6C), and eplerenone almost completely abolished the ROS production (Fig. 6D). Moreover, eplerenone was able to suppress ROS production induced by LPS alone (Fig. 6E). Pretreatment with H_2O_2 augmented LPS-induced expression of these proinflammatory cytokines, and these changes were significantly attenuated by pretreatment with NAC (Supplementary Fig. 3). Thus, antioxidant activity of eplerenone appears to contribute to the attenuation of proinflammatory cytokine expression in the macrophage.

Eplerenone inhibits not only Signal 1- but also Signal 2-mediated NLRP3 inflammasome activation

As ROS is a typical triggering stimulus (Signal 2) for the activation of NLRP3 inflammasome formation that promotes overproduction of IL1b, a major factor to develop insulin resistance in obesity (Dostert *et al.* 2008, Stenstra *et al.* 2010, 2011), we finally examined the effects of eplerenone on the Signal 2-mediated inflammasome activation. The amounts of IL1b and mature Caspase 1 proteins in the eWAT and liver were significantly increased in HFD mice, whereas the protein levels were maintained at normal levels in HFD+Ep mice (Fig. 7A, B, C and D). In addition, an *ex vivo* tissue culture experiment

showed that IL1b secretion from eWAT was significantly elevated in HFD mice, whereas it was maintained at a normal level in HFD+EP mice (Fig. 7E). Furthermore, we conducted the *in vitro* experiment to determine the influences of eplerenone on the Signal 2-mediated NLRP3 inflammasome activation (Fig. 7F). To this end, BMDMs were treated with LPS (i.e., Signal 1 stimulus), and then washed and subsequently incubated with ATP or nigericin (i.e., Signal 2 stimulus). Eplerenone was treated 30 min prior to the addition of ATP or nigericin to avoid influencing the Signal 1-mediated activation process. LPS, ATP and nigericin alone failed to induce IL1b production. On the other hand, marked secretion of mature IL1b in the culture media was observed in LPS-primed BMDM after stimulation with ATP or nigericin. Under these conditions, eplerenone significantly attenuated IL1b production (Fig. 7G). These results demonstrated that eplerenone was able to directly inhibit Signal 2-mediated activation of NLRP3 inflammasome, independently of the inhibitory effect on Signal 1 as shown above.

Discussion

It is well known that obesity-associated activation of the renin-angiotensin-aldosterone system contributes to the development of obese pathogenesis such as insulin

Figure 7

Effects of eplerenone on the Signal 2-mediated NLRP3 inflammasome activation in the eWAT and liver of mice. (A, B, C, D and E) Four groups of mice (chow mice: open columns, chow+Ep mice: gray columns, HFD mice: black columns, HFD+Ep mice: hatched columns) were used. (A, B, C and D) Protein amounts of IL1b and mature caspase1 in the eWAT and liver. (E) Secreted IL1b levels in the media of *ex vivo*-cultured eWAT. Data are shown as the mean $\pm$ S.E.M. ($n=3-7$). * $P<0.05$ and ** $P<0.01$, significantly different from chow mice; † $P<0.05$ and †† $P<0.01$, significantly different between HFD and HFD-Ep mice. (F) Effects of eplerenone on the Signal 2-mediated inflammasome activation *in vitro*. BMDM was stimulated with LPS for 3 h, and subsequently treated with ATP or Nigericin for 40 min. Eplerenone was treated 30 min prior to the addition of ATP or nigericin. Secreted IL1b levels in the culture media were analyzed. Data are shown as the mean $\pm$ S.E.M. ($n=5$). ** $P<0.01$, significantly different from untreated cells; † $P<0.05$ and †† $P<0.01$, significantly different between two groups, as indicated.

resistance (Favre *et al.* 2015); however, the underlying mechanism by which aldosterone-induced insulin resistance and dysregulation of glucose metabolism, independently of angiotensin 2, remains unclear. In the present study, we found several important features of the aldosterone-induced insulin resistance in mice, as follows: (1) Eplerenone attenuated HFD-induced obesity by enhancing energy metabolism; (2) eplerenone ameliorated HFD-induced accumulation of M1-ATM and reduction of M2-ATM in the eWAT; (3) eplerenone attenuated both Signal 1- and Signal 2-mediated NLRP3-inflammasome activation and improved HFD-induced chronic inflammation in the eWAT and liver of obese mice and (4) MR expression in the macrophage from obese adipose tissue was lower in the M1-ATM than the M2-ATM. These new findings help to understand the

fundamental mechanism for amelioration of insulin resistance by MR inhibition.

Adipocyte-specific MR-transgenic mice have been reported to exhibit phenotypes of metabolic syndrome (Urbanet *et al.* 2015). Therefore, adipocytes have been implicated as target cells for the deterioration of glucose metabolism by MR activation. In this context, we have demonstrated that aldosterone promoted ROS-mediated insulin resistance in 3T3-L1 adipocytes (Wada *et al.* 2009). MR antagonists are reported to improve obesity-related insulin resistance by reducing the intracellular ROS level and increasing adiponectin expression in the adipose tissue (Guo *et al.* 2008, Hirata *et al.* 2009). In addition, MR has the ability to induce differentiation of adipocytes by increasing expression of PPAR γ and CCAAT/enhancer-binding protein a (C/EBP α) (Marzolla *et al.* 2012). MR knockdown and MR antagonists attenuate adipocyte differentiation and lipid accumulation in 3T3L1 adipocytes (Caprio *et al.* 2007, 2011). As the body fat mass was decreased in HFD+Ep mice compared with HFD mice (Fig. 1), we consider that eplerenone may reduce lipid accumulation by modulating adipocyte differentiation.

We demonstrated that eplerenone ameliorated the lowering of core body temperature and energy metabolism (VO_2 and VCO_2) in HFD mice, especially in the dark phase (Fig. 2). Furthermore, the expression of thermogenic genes in the BAT was greatly increased by the eplerenone treatment. Although the role of MR in the regulation of thermogenesis in BAT remains uncertain, a recent study using Twik-related acid-sensitive K^+ channel 1 (TASK1) knockout mice implies the functional relevance of MR (Pisani *et al.* 2016). TASK1 is a pH-sensitive potassium channel that mediates beta 3 adrenergic receptor-stimulated UCP1 expression and oxygen consumption in the BAT. TASK1 deficiency reduced the UCP1 expression, but this reduction was prevented by eplerenone and not by glucocorticoid receptor (GR) antagonists. Therefore, we suggest that the thermogenic activity of BAT is inhibited by MR signaling, and this inhibition may be eliminated in the BAT of HFD+Ep mice. Similarly, a recent report indicated that beige adipocytes emerged in the visceral and inguinal fat of HFD-mice when treated with MR antagonists, in association with a reduced rate of autophagy (Armani *et al.* 2014), although no such change was observed in HFD+Ep in our experimental conditions (Supplementary Fig. 2). As the functions of brown and beige adipocytes significantly influence obese pathology, further studies are needed to clarify the underlying mechanism.

Steroid hormone receptors in the macrophage, such as GR and estrogen receptor alpha (ER α), have been reported to play crucial roles for the polarity and inflammatory profile (Ogawa *et al.* 2005, Ribs *et al.* 2011). Therefore, macrophages are considered as another important target for the MR antagonism-induced metabolic improvement in mice. Indeed, macrophages in the eWAT changed their polarity from M1-ATM to M2-ATM by eplerenone administration in HFD mice. On the other hand, neither treatment with eplerenone nor transient knockdown of MR affected the expression of genes relevant to the polarization in BMDM (data not shown). Therefore, the eplerenone-induced change in the polarity of ATM may be caused by secondary effects of body fat reduction in HFD mice, rather than the direct effects of MR inhibition in the macrophage.

As macrophages from myeloid-specific MR knockout mice exhibited a M2-like transcriptional profile (Usher *et al.* 2010), it had been speculated that the MR expression may increase in the M1-ATM. Unexpectedly, however, the results indicate that the MR expression was lower in the M1-ATM and M1-BMDM than in the M2-ATM and M2-BMDM (Fig. 4). The previous comprehensive expression analysis of nuclear receptors indicated that MR expression in the macrophage was negatively regulated by LPS stimulation (Barish *et al.* 2005). These suggest that the expression of MR in the macrophage is suppressed by inflammatory signals. However, we observed that eplerenone or MR blockade effectively attenuated proinflammatory cytokine expression in the macrophage both *in vivo* and *in vitro*. A relatively small number of MR expressed in M1-macrophage may sufficiently promote chronic inflammation in the adipose tissue and liver of obese mice.

Eplerenone attenuated production of IL1b by inhibiting both Signal 1- and Signal 2-mediated activation of NLRP3 inflammasome in macrophage (Fig. 7). In particular, the present finding that eplerenone suppressed ROS generation strongly highlighted the potent inhibitory efficacy of this drug on Signal 2-mediated inflammasome activation. Moreover, it has recently been reported that aldosterone itself activates the NLRP3 inflammasome. Eplerenone is reported to attenuate tubuleintestinal damage by inhibiting production of IL1b activation in the NLRP3 inflammasome in the kidney of high-dose aldosterone-infused mice (Kadoya *et al.* 2015). These suggest that eplerenone functions as a potent NLRP3 inflammasome inhibitor. As evidence indicates that the inflammasome-mediated overproduction of IL1b is a key

event to cause insulin resistance in obesity and diabetes (Maedler *et al.* 2002), eplerenone appears to prevent metabolic abnormalities in obese mice by suppressing this exacerbating mechanism. Therefore, further studies to examine the effects of MR inhibition in other species, including humans, are warranted.

In conclusion, we provided novel evidence regarding the beneficial effects of MR antagonists in obesity. In particular, we uncovered the inhibitory mechanism of eplerenone against metabolic stress-induced NLRP3 inflammasome activation. Thus, the present study shed new light on the development of a novel MR-targeted therapy to treat inflammasome-related metabolic diseases, including obesity and type 2 diabetes.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-17-0351>.

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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References

- Armani A, Cinti F, Marzolla V, Morgan J, Cranston GA, Antelmi A, Carpinelli G, Canese R, Pagotto U, Quarta C, *et al.* 2014 Mineralocorticoid receptor antagonism induces browning of white adipose tissue through impairment of autophagy and prevents adipocyte dysfunction in high-fat-diet-fed mice. *FASEB Journal* **28** 3745–3757. (doi:10.1096/fj.13-245415)
- Barish GD, Downes M, Alaynick WA, Yu RT, Ocampo CB, Bookout AL, Mangelsdorf DJ & Evans RM 2005 A nuclear receptor Atlas: macrophage activation. *Molecular Endocrinology* **19** 2466–2477. (doi:10.1210/me.2004-0529)
- Caprio M, Fève B, Claës A, Viengchareun S, Lombès M & Zennaro MC 2007 Pivotal role of the mineralocorticoid receptor in corticosteroid-induced adipogenesis. *FASEB Journal* **21** 2185–2194. (doi:10.1096/fj.06-7970com)
- Caprio M, Antelmi A, Chetrite G, Muscat A, Mammi C, Marzolla V, Fabbri A, Zennaro MC & Fève B 2011 Antiadipogenic effects of the mineralocorticoid receptor antagonist drospirenone: potential implications for the treatment of metabolic syndrome. *Endocrinology* **152** 113–125. (doi:10.1210/en.2010-0674)
- Coll RC, Robertson AA, Chae JJ, Higgins SC, Muñoz-Planillo R, Inerra MC, Vetter I, Dungan LS, Monks BG, Stutz A, *et al.* 2015 A small-molecule inhibitor of the NLRP3 inflammasome for the treatment of inflammatory diseases. *Nature Medicine* **21** 248–255. (doi:10.1038/nm.3806)

- Dostert C, Pétrilli V, Van Bruggen R, Steele C, Mossman BT & Tschopp J 2008 Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. *Science* **320** 674–677. (doi:10.1126/science.1156995)
- Favre GA, Esnault VL, & Van Obberghen E 2015 Modulation of glucose metabolism by the renin-angiotensin-aldosterone system. *American Journal of Physiology: Endocrinology and Metabolism* **308** E435–E449. (doi:10.1152/ajpendo.00391.2014)
- Fujisaka S, Usui I, Bukhari A, Ikutani M, Oya T, Kanatani Y, Tsuneyama K, Nagai Y, Takatsu K, Urakaze M, *et al.* 2009 Regulatory mechanisms for adipose tissue M1 and M2 macrophages in diet-induced obese mice. *Diabetes* **58** 2574–2582. (doi:10.2337/db08-1475)
- Giacchetti G, Sechi LA, Rilli S & Carey RM 2005 The renin-angiotensin-aldosterone system, glucose metabolism and diabetes. *Trends in Endocrinology and Metabolism* **16** 120–126. (doi:10.1016/j.tem.2005.02.003)
- Guo C, Ricchiuti V, Lian BQ, Yao TM, Coutinho P, Romero JR, Li J, Williams GH & Adler GK 2008 Mineralocorticoid receptor blockade reverses obesity-related changes in expression of adiponectin, peroxisome proliferator-activated receptor-gamma, and proinflammatory adipokines. *Circulation* **117** 2253–2261. (doi:10.1161/CIRCULATIONAHA.107.748640)
- Hirata A, Maeda N, Hiuge A, Hibuse T, Fujita K, Okada T, Kihara S, Funahashi T & Shimomura I 2009 Blockade of mineralocorticoid receptor reverses adipocyte dysfunction and insulin resistance in obese mice. *Cardiovascular Research* **84** 164–172. (doi:10.1093/cvr/cvp191)
- Honda H, Nagai Y, Matsunaga T, Okamoto N, Watanabe Y, Tsuneyama K, Hayashi H, Fujii I, Ikutani M, Hirai Y, *et al.* 2014 Isoliqurritigenin is a potent inhibitor of NLRP3 inflammasome activation and diet-induced adipose tissue inflammation. *Journal of Leukocyte Biology* **96** 1087–1100. (doi:10.1189/jlb.3A0114-005RR)
- Ichihara Y, Wada T, Soeda Y, Ishii Y, Sasahara M, Tsuneki H & Sasaoka T 2013 SH2-Containing Inositol 5'-phosphatase 2 selectively impairs hypothalamic insulin signalling and regulation of food intake in mice. *Journal of Neuroendocrinology* **25** 372–382. (doi:10.1111/jne.12014)
- Kadoya H, Satoh M, Sasaki T, Taniguchi S, Takahashi M & Kashiara N 2015 Excess aldosterone is a critical danger signal for inflammasome activation in the development of renal fibrosis in mice. *FASEB Journal* **29** 3899–3910. (doi:10.1096/fj.15-271734)
- Lackey DE & Olefsky JM 2016 Regulation of metabolism by the innate immune system. *Nature Reviews Endocrinology* **12** 15–28. (doi:10.1038/nrendo.2015.189)
- Latz E, Xiao TS & Stutz A 2013 Activation and regulation of the inflammasomes. *Nature Reviews Immunology* **13** 397–411. (doi:10.1038/nri3452)
- Maedler K, Sergeev P, Ris E, Oberholzer J, Joller-Jemelka HI, Spinas GA, Kaiser N, Halban PA & Donath MY 2002 Glucose-induced beta cell production of IL-1beta contributes to glucotoxicity in human pancreatic islets. *Journal of Clinical Investigation* **110** 851–860. (doi:10.1172/JCI200215318)
- Marzolla V, Armani A, Zennaro MC, Cinti F, Mammi C, Fabbri A, Rosano GM & Caprio M 2012 The role of the mineralocorticoid receptor in adipocyte biology and fat metabolism. *Molecular and Cellular Endocrinology* **350** 281–288. (doi:10.1016/j.mce.2011.09.011)
- Olivares-Reyes JA, Arellano-Plancarte A & Castillo-Hernandez JR 2009 Angiotensin 2 and the development of insulin resistance: implications for diabetes. *Molecular and Cellular Endocrinology* **302** 128–139. (doi:10.1016/j.mce.2008.12.011)
- Ogawa S, Lozach J, Benner C, Pascual G, Tangirala RK, Westin S, Hoffmann A, Subramaniam S, David M, Rosenfeld MG, *et al.* 2005 Molecular determinants of crosstalk between nuclear receptors and toll-like receptors. *Cell* **122** 707–721. (doi:10.1016/j.cell.2005.06.029)
- Onogi Y, Wada T, Kamiya C, Inata K, Matsuzawa T, Inaba Y, Kimura K, Inoue H, Yamamoto S, Ishii Y, *et al.* 2017 PDGFRβ regulates adipose tissue expansion and glucose metabolism via vascular remodeling in diet-induced obesity. *Diabetes* **66** 1008–1021. (doi:10.2337/db16-0881)
- Pisani DF, Beranger GE, Corinus A, Giroud M, Ghandour RA, Altirriba J, Chambard JC, Mazure NM, Bendahhou S, Duranton C, *et al.* 2016 The K⁺ channel TASK1 modulates beta-adrenergic response in brown adipose tissue through the mineralocorticoid receptor pathway. *FASEB Journal* **30** 909–922. (doi:10.1096/fj.15-277475)
- Ribas V, Drew BG, Le JA, Soleymani T, Daraei P, Sitz D, Mohammad L, Henstridge DC, Febbraio MA, Hewitt SC, *et al.* 2011 Myeloid-specific estrogen receptor alpha deficiency impairs metabolic homeostasis and accelerates atherosclerotic lesion development. *PNAS* **108** 16457–16462. (doi:10.1073/pnas.1104533108)
- Sameshima A, Wada T, Ito T, Kashimura A, Sawakawa K, Yonezawa R, Tsuneki H, Ishii Y, Sasahara M, Saito S, *et al.* 2015 Teneligliptin improves metabolic abnormalities in a mouse model of postmenopausal obesity. *Journal of Endocrinology* **227** 25–36. (doi:10.1530/JOE-15-0239)
- Stienstra R, Joosten LA, Koenen T, van Tits B, van Diepen JA, van den Berg SA, Rensen PC, Voshol PJ, Fantuzzi G, Hijmans A, *et al.* 2010 The inflammasome-mediated caspase-1 activation controls adipocyte differentiation and insulin sensitivity. *Cell Metabolism* **12** 593–605. (doi:10.1016/j.cmet.2010.11.011)
- Stienstra R, van Diepen JA, Tack CJ, Zaki MH, van de Veerdonk FL, Perera D, Neale GA, Hooiveld GJ, Hijmans A, Vroegrijk I, *et al.* 2011 Inflammasome is a central player in the induction of obesity and insulin resistance. *PNAS* **108** 15324–15329. (doi:10.1073/pnas.1100255108)
- Schiller NK, Black AS, Bradshaw GP, Bonnet DJ & Curtiss LK 2004 Participation of macrophages in atherosclerotic lesion morphology in LDLr^{-/-} mice. *Journal of Lipid Research* **45** 1398–1409. (doi:10.1194/jlr.M400036-JLR200)
- Urbanet R, Nguyen DCA, Feraco A, Venteclef N, El Moghrabi S, Sierra-Ramos C, Alvarez de la Rosa D, Adler GK, Quilliot D, Rossignol P, *et al.* 2015 Adipocyte mineralocorticoid receptor activation leads to metabolic syndrome and induction of prostaglandin D2 synthase. *Hypertension* **66** 149–157. (doi:10.1161/HYPERTENSIONAHA.114.04981)
- Usher MG, Duan SZ, Ivaschenko CY, Frieler RA, Berger S, Schütz G, Lumeng CN & Mortensen RM 2010 Myeloid mineralocorticoid receptor controls macrophage polarization and cardiovascular hypertrophy and remodeling in mice. *Journal of Clinical Investigation* **120** 3350–3364. (doi:10.1172/JCI41080)
- Vandanmagsar B, Youm YH, Ravussin A, Galgani JE, Stadler K, Mynatt RL, Ravussin E, Stephens JM & Dixit VD 2011 The NLRP3 inflammasome instigates obesity-induced inflammation and insulin resistance. *Nature Medicine* **17** 179–188. (doi:10.1038/nm.2279)
- Wada T, Ohshima S, Fujisawa E, Koya D, Tsuneki H & Sasaoka T 2009 Aldosterone inhibits insulin-induced glucose uptake by degradation of insulin receptor substrate (IRS) 1 and IRS2 via a reactive oxygen species-mediated pathway in 3T3-L1 adipocytes. *Endocrinology* **150** 1662–1669. (doi:10.1210/en.2008-1018)
- Wada T, Kenmochi H, Miyashita Y, Sasaki M, Ojima M, Sasahara M, Koya D, Tsuneki H & Sasaoka T 2010 Spironolactone improves glucose and lipid metabolism by ameliorating hepatic steatosis and inflammation and suppressing enhanced gluconeogenesis induced by high-fat and high-fructose diet. *Endocrinology* **151** 2040–2049. (doi:10.1210/en.2009-0869)
- Wada T, Miyashita Y, Sasaki M, Aruga Y, Nakamura Y, Ishii Y, Sasahara M, Kanasaki K, Kitada M, Koya D, *et al.* 2013 Eplerenone ameliorates the phenotypes of metabolic syndrome with NASH in liver-specific SREBP-1c Tg mice fed high-fat and high-fructose diet. *American Journal of Physiology: Endocrinology and Metabolism* **305** E1415–E1425. (doi:10.1152/ajpendo.00419.2013)
- Yonezawa R, Wada T, Matsumoto N, Morita M, Sawakawa K, Ishii Y, Sasahara M, Tsuneki H, Saito S & Sasaoka T 2012 Central versus peripheral impact of estradiol on the impaired glucose metabolism in ovariectomized mice on a high-fat diet. *American Journal of Physiology: Endocrinology and Metabolism* **303** E445–E456. (doi:10.1152/ajpendo.00638.2011)

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