Blockade of mineralocorticoid receptor reverses adipocyte dysfunction and insulin resistance in obese mice

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Aims In obesity, chronic low-grade inflammation and overproduction of reactive oxygen species (ROS) in fat contribute to the development of metabolic syndrome. Suppression of inflammation and ROS production in fat may attenuate the metabolic syndrome. Activation of mineralocorticoid receptor (MR) promotes inflammation in heart, kidney, and vasculature via ROS generation. However, the significance of MR in fat remains elusive. Here we investigated whether MR blockade attenuates obesity-related insulin resistance and improves adipocyte dysfunction.

Methods and results Obese ob/ob and db/db mice were treated with eplerenone, a MR antagonist, for 3 weeks. 3T3-L1 adipocytes were treated with aldosterone or H2O2, with and without eplerenone or MR-siRNA. High levels of MR mRNA were detected in adipose tissue of obese ob/ob and db/db mice. Eplerenone treatment significantly reduced insulin resistance, suppressed macrophage infiltration and ROS production in adipose tissues, and corrected the mRNA levels of obesity-related genes in obese mice. In 3T3-L1 adipocytes, aldosterone and H2O2 increased intracellular ROS levels and MR blockade inhibited such increases. H2O2 and aldosterone resulted in dysregulation of mRNAs of various genes related to ROS and cytokines, whereas MR blockade corrected such changes.

Conclusion MR blockade attenuates obesity-related insulin resistance partly through reduction of fat ROS production, inflammatory process, and induction of cytokines.

1. Introduction

Metabolic syndrome develops on the basis of obesity, and its molecular mechanism has been investigated but is not fully understood. Increasing clinical evidence indicates that excess fat, especially visceral fat accumulation, is closely associated with obesity-related metabolic disorders. We and others have indicated that obesity-associated dysregulation of adipose-derived secretory proteins, such as adiponectin, tumour necrosis factor-α (TNF-α), and monocyte chemoattractant protein-1 (MCP-1), is associated with the development of the metabolic syndrome. Recent studies suggested that chronic low-grade inflammatory process in the fat tissue of obesity plays an important role in insulin resistance. Our group also demonstrated that overproduction of reactive oxygen species (ROS) in obese fat tissue is a key underlying mechanism responsible for dysregulation of ‘adipocytokines’ and accelerated development of the metabolic syndrome. Thus, suppression of inflammation and ROS in fat may attenuate metabolic syndrome and insulin resistance in obesity.

The renin–angiotensin–aldosterone system (RAAS) is often activated in human subjects and animal models with metabolic syndrome. Importantly, RAAS exists in not only the circulation where it is driven by renal renin, but it is activated locally also in many tissues and cells. The contribution of fat RAAS is also evident in the development of hypertension and insulin resistance. In fact, blockade of angiotensin II receptor signalling cascade improved adipocytokine dysregulation with the reduction of fat ROS. Classically, aldosterone is the final mediator of the RAAS and activates mineralocorticoid receptor (MR) to enhance unidirectional transepithelial sodium transport. A number of studies demonstrated that the activation of MR promotes inflammation, proliferation, and fibrosis mainly in the heart, kidney, and vasculature via ROS generation. In fact, blockade of MR reduced cardiovascular mortality in subjects with heart failure. However, the significance of MR in adipocytes has not been fully elucidated. On the basis of...
the above background, we herein tested the effects of MR blockade on systemic insulin sensitivity, adipose inflammation, and ROS both in vivo (obese model mice) and in vitro (3T3-L1 adipocytes).

2. Methods

2.1 Animal preparation and evaluation of insulin sensitivity

Male B6.16-Lept+/−/J (ob/ob) and BKS.Cg-m+/+Lepr−/−/J (db/db) obese mice were purchased from Charles River Laboratories (Charles River Japan Inc., Yokohama, Japan). Their respective lean control male C57BL/6J mice and db/m+ heterozygous litters were purchased from the same supplier. At 7 weeks of age, mice were fed regular chow containing either eplerenone, a MR antagonist, at 1 mg/g of chow (eplerenone group; Eple) or no compound (control group; Cont) for 3 weeks. The dosage of Eple was determined not to influence blood pressures by referring several reports.15–17 To estimate glucose tolerance, at 8 weeks of age, mice were fasted for 16 h and a basal blood sample was taken, followed by intraperitoneal injection of (−)−Glucose (2 mg/g of body weight). Blood samples were collected from tail vein at 15, 30, 60, and 120 min after injection. For insulin tolerance test, at 9 weeks of age, mice were fasted for 4 h and a basal blood sample was taken, followed by intraperitoneal injection of human regular insulin (Novolin R; Novo Nordisk, 2 U/kg of body weight). Blood samples were collected from the tail vein at 15, 30, 60, and 90 min after injection. Mice were kept in rooms set at 22 °C with a 12–12 h dark–light cycle (light cycle, 8 a.m. to 8 p.m.). Finally, mice were sacrificed at 10 weeks of age. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Osaka University School of Medicine. This study also conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2 Measurement of metabolic parameters and oxidative stress

Blood samples were collected under 4 h-fasting conditions when mice were dissected. Plasma glucose, insulin, and triglyceride (TG) were measured by the Glucose CII-Test (Wako Pure Chemical, Osaka, Japan), the Insulin Measurement ELISA kit (Morinaga, Yokohama), and TG E-Test (Wako), respectively. Plasma adiponectin was measured by using ELISA kit (Otsuka, Tokushima, Japan). The levels of oxidative stress in plasma, tissue homogenates, and cell lysates were measured as thiobarbituric acid reactive substance (TBARS) with TBARS assay kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer protocol. Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using the equation: [fasting plasma insulin (ng/mL) × fasting plasma glucose (mg/dL)]/405.

2.3 Histological analysis

Epididymal fat pad was excised from each mouse at 10 weeks of age. The isolated adipose tissue was formalin-fixed, paraffin-embedded, and subsequently cut into 6 μm sections and mounted on glass slides using standard procedures. The sections were stained with haematoxylin and eosin. The area of adipocytes was traced manually and measured in >200 cells per mouse by using Win ROOF 5.5 software (Mittani Co, Fukui, Japan). For F4/80 immunostaining, sections were stained with monoclonal anti-mouse F4/80 antibody (Serotec, Raleigh, NC, USA). Subsequently, the sections were incubated with the biotinylated secondary antibody by using the Elite mouse IgG kit (Vector Laboratories, Burlingame, CA, USA). The number of crown-like structures (CLS), in which F4/80-positive macrophages surround a single adipocyte, was determined under low-power magnification as described previously.18,19

2.4 3T3-L1 cell cultures

3T3-L1 cells were maintained and differentiated as described previously.20 Small interfering RNA (siRNA) was transfected into the cells as follows. On day 6 after 3T3-L1 differentiation, the media of 3T3-L1 cells in 12-well plates were changed to OPTI-MEM (Invitrogen, San Diego, CA, USA), and the cells were transfected with MR- and control-siRNA (Qiagen, Valencia, CA, USA) using LipofectA-MINE 2000 reagent (Invitrogen) according to the instructions provided by the manufacturer. The sequences of the MR-siRNA sense were as follows: 5′-GGUCUCUGUCACAAUAAAAdTdT-3′. On day 7, media of 3T3-L1 adipocytes were changed to Dulbecco’s modified Eagle medium (DMEM), and 3T3-L1 adipocytes were treated for 24 h with 10−5 M of Eple, 10−8 M of aldosterone, or 0.2 mM of H2O2. Eple and aldosterone were dissolved in dimethyl sulfoxide (DMSO) and acetone, respectively. The selected concentrations of these reagents were based on previous studies.10,21,22 Neither DMSO nor acetone affected mRNA levels, such as MR, adiponectin, and MCP-1, and intracellular TBARS levels in the equal amounts to 10−3 M of Eple or 10−8 M of aldosterone, respectively (data not shown).

2.5 Quantification of mRNA levels

Total RNA was isolated from mouse tissues by using RNA STAT-60 (Tel-Test Inc., Friendswood, TX, USA) according to the protocol supplied by the manufacturer. The quantity and quality of total RNA were determined by using ND-1000 Spectrophotometer (Nano Drop Technologies, Wilmington, DE, USA). First-strand cDNA was synthesized from 200 ng of total RNA using Thermoscript RT (Invitrogen) and oligo dT primer. Real-time quantitative PCR amplification was conducted with the LightCycler 1.5 (Roche Diagnostics, Mannheim, Germany) using LightCycler-FastStart DNA Master SYBR Green I (Roche Diagnostics) according to the protocol recommended by the manufacturer. Primers are listed in Supplementary material online, Table S1. Primers for MR were purchased by Qiagen. The final result for each sample was normalized to the respective 36B4 value.

2.6 Statistical analysis

Results were expressed as mean ± SEM of n separate experiments. Differences between groups were examined for statistical significance using Student’s t-test or ANOVA with Fisher’s protected least significant difference test. A P-value less than 0.05 denoted the presence of a statistically significant difference.

3. Results

3.1 Effects of MR blockade on insulin sensitivity

Significantly high MR mRNA levels were observed in white adipose tissue (WAT) of obese ob/ob and db/db mice compared with lean control mice (Figure 1A). Treatment of mice for 3 weeks with Eple did not result in significant changes in body weight, WAT mass, systolic blood pressure (Table 1), liver weight, or food intake (data not shown) compared with the control groups. However, Eple treatment reduced the high levels of plasma glucose (Table 1) and HOMA-IR (Figure 1B) and decreased plasma TG levels in both groups of obese mice (Figure 1C). There were no apparent changes in plasma adiponectin levels in db/db mice, but Eple treatment significantly increased plasma levels of adiponectin, an anti-diabetic secretory protein,23 in ob/ob mice (Table 1).
3.3 Effects of MR blockade on ROS and gene expression levels in obese mice

Since high ROS production in adipocytes could contribute to the development of metabolic abnormalities in obesity,7 ROS levels were determined in the present study by measuring TBARS levels in plasma and WAT. Plasma TBARS levels were significantly higher in both ob/ob and db/db mice than in lean control mice, but were reduced in Eple-treated obese mice (Figure 3B). Eple also reduced TBARS levels in WAT of these obese mice (Figure 3B).

Next, mRNA levels related to ROS were determined in WAT (Figure 4). The mRNA levels of transcription factor P.1, which is known to increase NADPH oxidase subunits at transcriptional level,25 were increased in obese mice, and Eple reduced such elevation of P.1. NADPH oxidase subunits p22 and p47, located upstream of ROS production, were significantly increased in both groups of obese mice compared with lean control mice. Eple suppressed the increased mRNA levels of NADPH oxidase subunits p22 and p47 in obese mice. Catalase and Cu, Zn-SOD, which are ROS-eliminating enzymes, were reduced in both ob/ob and db/db mice...
compared with lean control mice and administration of Eple significantly reversed these mRNA levels. These mRNA changes were consistent with TBARS levels in plasma and WAT (Figure 3).

The mRNA levels of genes associated with metabolic disorders were also determined in WAT (Figure 4). Eple partly reversed the suppression of mRNA levels of adiponectin, an insulin-sensitizing secretory protein,2 in ob/ob mice. The mRNA levels of proinflammatory cytokines such as TNF-α (data not shown), MCP-1, and IL-6 were higher in obese mice than in lean control mice, whereas Eple administration reduced mRNA levels of these cytokines in obese mice. Eple significantly reversed the reduction in mRNA expression level of peroxisome proliferator-activated receptor γ2 (PPARγ2) in both db/db and ob/ob mice. Furthermore, Eple suppressed the increase in 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) mRNA level and increased the low mRNA level of uncoupling protein 1 (UCP1) (data not shown) in obese mice. In db/db mice, Eple significantly decreased the high levels of leptin mRNA (data not shown).

### 3.4 Effect of MR blockade in 3T3-L1 adipocytes

In the next series of experiments, 3T3-L1 differentiated adipocytes were used to investigate the direct effects of MR blockade, aldoosterone (Aldo) as a MR ligand, and H2O2 as a ROS. 3T3-L1 cell differentiation was accompanied by a parallel increase in MR mRNA level (data not shown). The addition of Aldo and H2O2 significantly increased the mRNA level of MR in 3T3-L1 adipocytes (Figure 5A). Interestingly, Aldo and H2O2 also increased intracellular TBARS levels, whereas MR blockade with Eple reversed both Aldo- and H2O2-induced rises in TBARS (Figure 5B and C).

Next, the mRNA levels of genes related to ROS and cytokines were examined in 3T3-L1 adipocytes by using siRNAs for MR and Eple (Figure 6). Transfection of MR-siRNAs caused 70% reduction in MR mRNA level compared with control-siRNA (data not shown). The mRNA levels of PU.1 and NADPH oxidase subunits p22 and p47 increased significantly by Aldo and H2O2 (Figure 6, lane 1 vs. 5 and 9), whereas such increases were reduced by Eple and MR-siRNA (lane 5 vs. 6 and 7, lane 9 vs. 10 and 11, respectively). Aldo and H2O2 reduced catalase and Cu, Zn-SOD mRNA levels (lane 1 vs. 5 and 9), whereas Eple and MR-siRNA partly reversed these mRNA levels (lane 5 vs. 6 and 7, lane 9 vs. 10 and 11, respectively). Both Aldo and H2O2 decreased adiponectin mRNA level and increased PPAR γ mRNA level of peroxisome proliferator-activated receptor type 1 (PPAR γ2) in both db/db and ob/ob mice. Eple significantly reversed the reduction in MR mRNA level (data not shown). The mRNA levels of proinflammatory cytokines such as TNF-α, MCP-1, and IL-6 were higher in ob/ob mice. The mRNA levels of these cytokines were lower in mice treated with Eple and MR-siRNA (lane 5 vs. 6 and 7, lane 9 vs. 10 and 11, respectively). Aldo and H2O2 induced mRNA levels of these cytokines were decreased in mice treated with Eple and MR-siRNA (lane 5 vs. 6 and 7, lane 9 vs. 10 and 11, respectively). Eple reversed the reduction in mRNA expression level of peroxisome proliferator-activated receptor γ2 (PPARγ2) in both db/db and ob/ob mice. Furthermore, Eple suppressed the increase in 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) mRNA level and increased the low mRNA level of uncoupling protein 1 (UCP1) (data not shown) in obese mice. In db/db mice, Eple significantly decreased the high levels of leptin mRNA (data not shown).

### 4. Discussion

The main findings of the present study were: (i) Eple ameliorated insulin resistance associated with the reduction of ROS and improved adipose dysregulated gene expression levels in...
obese model mice. (ii) Orally administered Eple decreased the numbers of hypertrophic adipocytes and infiltrated macrophages into adipose tissue of obese mice. (iii) Upregulation of MR in the obese fat tissue and Aldo- and H2O2-treated 3T3-L1 adipocytes. (iv) In cultured 3T3-L1 adipocytes, MR blockade by MR-siRNA as well as Eple corrected Aldo- and H2O2-induced ROS and dysregulated expression of genes related to obesity.

MR is mainly expressed in colon, brain, kidney, heart, and lung.26 The functional role of MR has been mainly investigated in the kidney as a major target organ of aldosterone. The expression of MR was verified in the recent15 and the present study in adipocytes, suggesting that aldosterone affects adipocytes. However, the direct effect of aldosterone on differentiated adipocytes has not been reported.

As shown in Figure 6, aldosterone treatment resulted in the dysregulation of proinflammatory cytokines, adiponectin, ROS, and PPAR-γ2 mRNA levels in 3T3-L1 adipocytes, indicating that aldosterone directly affects adipocyte function. Aldosterone induces ROS, activates proinflammatory transcription factors, and promotes inflammation.27,28 We also showed for the first time that stimulation with aldosterone as well as H2O2 increased intracellular ROS level in adipocytes (Figure 5B). Furthermore, MR blockade also reversed the dysregulation of adipose gene expressions induced by H2O2-mediated ROS in 3T3-L1 adipocytes (Figure 6), suggesting that MR blockade ameliorates fat ROS independent of aldosterone.

Interestingly, the results showed that Eple increased the proportion of small adipocytes in obese mice (Figure 2A and B),
Figure 3  Effects of eplerenone treatment on plasma and adipose tissue ROS levels. (A) Plasma TBARS levels. (B) TBARS levels in white adipose tissue (WAT). Data are mean ± SEM; n = 5–6 mice per group. *P < 0.05; **P < 0.01 vs. control group in ob/ob or db/db mice, †P < 0.05 vs. control group of C57BL/6J mice.

Figure 4  Effects of eplerenone treatment on the expression levels of adipose tissue genes in obese mice. Total RNA was isolated from white adipose tissue and first-strand cDNA was synthesized as described in Methods section. Real-time quantitative PCR was performed and values were normalized to the level of 36B4 mRNA. C, control group; E, eplerenone group; BL, C57BL/6J mice; ob/ob, ob/ob mice; db/m+, db/m+ mice; db/db, db/db mice. Data are mean ± SEM; n = 5–6 mice per group. *P < 0.05 vs. control group in ob/ob or db/db mice, †P < 0.05; ††P < 0.01 vs. control group of C57BL/6J mice.
but had no effect on the weight of WAT (Table 1). The reason for the increased number of small adipocytes by Eple remains uncertain. Since PPARγ activation is known to increase the number of small adipocytes in obese model animals,29 and it is possible that the increase in adipose PPARγ2 mRNA levels in Eple-treated obese mice (Figure 4) suppresses adipocyte hypertrophy or increases the number of small-size adipocytes. In addition, MR-dependent antagonism may interrupt the adipose vicious metabolic circle, resulting in suppression of adipocyte hypertrophy. Further studies are needed to investigate the small-size adipocyte phenomenon.

Adipose tissue macrophages (ATMs) infiltrate obese adipose tissue and contribute to insulin resistance.5,6 As shown in Figure 2C–F, treatment of obese mice with Eple reduced macrophage infiltration and CD11c mRNA level, indicating that MR blockade does not only suppress the infiltration of ATMs, but also alters the phenotypic switch of ATMs. Recently, Guo et al.15 reported that long-term treatment of db/db mice with Eple for 18 weeks ameliorated obesity-related disorders, such as hyperglycemia, hypertriacylglyceridemia, and dysregulation of cytokines in plasma and adipose mRNAs. In comparison, we investigated the metabolic effects of 3 weeks Eple treatment. The results showed for the first time that short-term Eple treatment of ob/ob and db/db mice suppressed the infiltration of ATMs, decreased the size of adipocytes, reversed adipose dysregulation of ROS and cytokine expression levels, and improved insulin resistance, although there were several differences in the metabolic response by Eple treatment between ob/ob and db/db mice. Especially, as shown in Figure 1F and G, Eple obviously ameliorated insulin resistance in db/db mice compared with ob/ob mice. There were several phenotypic differences in db/db and ob/ob mice treated with or without Eple, but MR blockade seems to improve obese fat tissue dysfunction associated with amelioration of insulin resistance.

Accumulation of visceral fat is located upstream of metabolic syndrome and fat distribution is important in metabolic disorders.2 Preliminary studies from our laboratory have shown higher MR mRNA levels in visceral (mesenteric) fat tissue than in subcutaneous fat tissue in human subjects (data not shown), suggesting the importance of adipose MR in human subjects. The role of MR has been examined and demonstrated mainly in kidney, heart, and vasculature.11,12 Importantly, the present study demonstrated for the first time the upregulation of MR in adipose tissues of obese mice (Figure 1A). The functional role and mechanism of such upregulation in obese WAT is uncertain, but, as shown in Figure 5A, treatment with H2O2 significantly increased MR mRNA level in 3T3-L1 adipocytes, suggesting that the increased adipose ROS in obesity may explain, at least in part, MR induction. Previous7 and present (Figure 4) studies showed the increase of NADPH oxidase subunits mRNA levels and the decrease of catalase and Cu, Zn-SOD mRNA levels in obese WAT. Although molecular mechanism for the dysregulation of such mRNAs relating to ROS in obese WAT has not been clarified, it is likely that the ROS-induced upregulation of adipose MR further augments fat ROS and causes inflammation of adipose tissue and dysregulation of adipocytokines.

The enzyme 11β-HSD1 plays a crucial role in determining intracellular glucocorticoid levels by regenerating active glucocorticoid (cortisol in humans, corticosterone in rodents) from inactive cortisone and 11-dehydrocorticosterone, suggesting to serve as tissue-specific amplifier of glucocorticoid action.30 Importantly, the activity and expression of 11β-HSD1 is elevated in obese adipose tissue. In fact, the adipose 11β-HSD1 and glucocorticoid action is closely associated with the development of obesity and metabolic syndrome.31,32 The MR binds not only aldosterone but also glucocorticoid with equal affinity.27 Taken together, in obesity, the increased glucocorticoid may activate MR in adipocytes and such MR activation may promote the inflammation and ROS generation, suggesting that the adipose...
MR blockade decreases inflammation and ROS even under conditions when endogenous aldosterone levels are not elevated, i.e. MR blockade may ameliorate the local inflammation and ROS in obese adipose tissue independently of aldosterone concentrations. Furthermore, angiotensinogen mRNA level is increased in obese WAT and adipocytes secrete mineralocorticoid-releasing factor, suggesting plasma levels of angiotensin II and aldosterone may be elevated in obesity. The high levels of plasma aldosterone were observed in obese human subjects. In addition, aldosterone potentiates angiotensin II signalling in vascular smooth muscle cells and induces mRNA level of angiotensin converting enzyme in endothelial cells. Angiotensin II infusion elevates mRNA levels of angiotensinogen and angiotensin II type 1 receptor (AT1R) in WAT. These interactions between aldosterone and angiotensin II may cause vicious circle not only in vasculature but also in adipose tissue. Collectively, blockade of MR or/and AT1R may cut off the vicious metabolic circle in obese adipose tissue and may be a potentially useful strategy for treatment of the metabolic syndrome.

The present results indicated that MR blockade with Eple ameliorated insulin resistance in obese diabetic mice. MR blockade is reported to improve cardiovascular mortality in patients with heart failure, but the effect of MR blockade on diabetes and metabolic syndrome has not been evaluated in human subjects. The present study suggests that MR blockade may provide another benefit to subjects of the metabolic syndrome with insulin resistance and type 2 diabetes. Future clinical trials using MR blockade strategy as treatment for the metabolic syndrome and its components are warranted.

Supplementary material
Supplementary material is available at *Cardiovascular Research* online.

![Figure 6](https://example.com/figure6.png)

**Figure 6** Effects of MR blockade on mRNA levels in 3T3-L1 adipocytes. 3T3-L1 adipocytes were transfected with MR-siRNA (MR si) or control-siRNA on day 6, treated with aldosterone (Aldo), H2O2, or eplerenone (Eple) on day 7, and harvested on day 8. Data are mean ± SEM; n = 6 per group. *P < 0.05; **P < 0.01. *P < 0.05; **P < 0.01; ***P < 0.001 vs. lane 1. †P < 0.05; ††P < 0.01; †††P < 0.001 vs. lane 1.
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References