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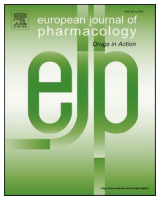

Esaxerenone, a selective mineralocorticoid receptor blocker, improves insulin sensitivity in mice consuming high-fat diet

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ABSTRACT

Background: Esaxerenone is a novel, non-steroidal selective mineralocorticoid receptor (MR) blocker. MR activation plays a crucial role in the development of cardiovascular and metabolic diseases. In this study, we investigated the effects of esaxerenone on various metabolic parameters in mice.

Materials and methods: Esaxerenone (3 mg/kg/day) was orally administered to high-fat diet (HFD)-fed male C57BL/6 mice. Mice fed a normal diet (ND) served as controls. Glucose and insulin tolerance, plasma lipid levels, and transaminase levels were assessed as metabolic parameters. Macrophage accumulation in the adipose tissue was evaluated using histological analysis. 3T3-L1 adipocytes, HepG2 cells, and C2C12 myotubes were used for in vitro experiments. Gene expression and insulin signaling were examined using quantitative RT-PCR and western blotting, respectively.

Results: HFD successfully induced insulin resistance compared with that in ND. Esaxerenone ameliorated insulin resistance ($P < 0.05$) without altering other metabolic parameters, such as the lipid profile. Esaxerenone administration tended to decrease plasma transaminase levels compared with those in the non-treated group. In the adipose tissue, esaxerenone decreased macrophage accumulation ($P < 0.05$) and increased the expression levels of adiponectin and PPARγ. Aldosterone significantly decreased the expression levels of PPARγ and adiponectin in 3T3-L1 adipocytes. Furthermore, aldosterone attenuated insulin-induced Akt phosphorylation in 3T3-L1 adipocytes, HepG2 cells, and C2C12 myotubes in a dose-dependent manner ($P < 0.01$). These effects were ameliorated by pretreatment with esaxerenone.

Conclusion: Esaxerenone ameliorated insulin resistance in HFD-fed mice. Reduction of inflammation and improvement in insulin signaling may underlie the beneficial effects of esaxerenone.

1. Introduction

Metabolic syndrome involves a cluster of risk factors, including hyperglycemia, hypertriglyceridemia, low high-density lipoprotein (HDL)-cholesterol level, high blood pressure, and obesity. The number of patients with metabolic syndrome is increasing rapidly worldwide. Obesity and insulin resistance are central features of metabolic syndrome; thus, organs/tissues targeted by insulin are considered to play key roles in the pathogenesis of metabolic syndrome (Ferguson et al., 2020). Recently, the relationship between aldosterone and metabolic syndrome has attracted considerable attention. In fact, plasma aldosterone levels are elevated in patients with obesity, hypertension, or dyslipidemia (Briones

Abbreviations: ANOVA, analysis of variance; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GTT, glucose tolerance test; HDL, high-density lipoprotein; HFD, high-fat diet; HRP, horseradish peroxidase; ITT, insulin tolerance test; Mac-3, macrophage antigen-3; MR, mineralocorticoid receptor; MRA, mineralocorticoid receptor antagonist; ND, normal diet; PPARγ, peroxisome proliferator-activated receptor γ; P/S, penicillin–streptomycin.

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et al., 2012; Ferguson et al., 2020; Flynn, 2014; Krug et al., 2008; La Sala et al., 2021).

Aldosterone is a mineralocorticoid hormone, mainly produced by the zona glomerulosa in the adrenal gland, that activates the mineralocorticoid receptor (MR) (Manosroi et al., 2020; Schütten et al., 2017). Aldosterone regulates water and electrolyte balance, as well as blood pressure. Patients with primary aldosteronism have impaired glucose tolerance, and MR antagonists (MRA) can improve glucose metabolism. Furthermore, genetic aldosterone deficiency improves insulin sensitivity (Hitomi et al., 2007; Luo et al., 2013). Aldosterone directly affects adipocytes through various pathways; for example, it reduces the expression levels of insulin receptor and insulin receptor substrate 1, as well as the insulin-induced phosphorylation of Akt (Wada et al., 2009). MRAs can prevent these effects. Additionally, MR expression levels are elevated in the adipose tissue of obesity models, leading to various metabolic dysfunctions, including body weight gain, visceral obesity, glucose intolerance, insulin resistance, and dyslipidemia (Urbanet et al., 2015). Several studies have shown the beneficial metabolic effects of currently available MRAs (Feraco et al., 2018; Marzolla et al., 2014). Spironolactone and eplerenone are traditionally available MRAs; however, because of their MR selectivity and steroidal structure, developing new MRAs remains a clinical challenge (Lainscak et al., 2015).

Esaxerenone is a recently introduced, orally bioavailable, selective, non-steroidal MR blocker with high MR-binding specificity (Arai et al., 2015; Takahashi et al., 2020). Esaxerenone has no agonistic effect on MR and does not show any antagonistic or agonistic effects on glucocorticoid, androgen, and progesterone receptors (Arai et al., 2015). Recent studies have reported the effects and pharmacological profile of esaxerenone in both clinical and preclinical settings (Duggan, 2019; Ito et al., 2020; Wan et al., 2021). Although the effect of esaxerenone on hypertension has been clarified, its metabolic effects remain unclear. In this study, we investigated the effect of esaxerenone on mice fed a high-fat diet (HFD).

2. Materials and methods

2.1. Animal and drug administration

All animal experiments were approved by the Ethics Review Committee for Animal Experimentation of the Tokushima University under the #T2020-127 protocol. Five-week-old C57BL/6J male mice were purchased from Japan SLC, Inc., and maintained under a standard 12 h light/dark cycle at 23 ± 1 °C. All mice were weighed and randomly housed in three groups: (1) mice fed a control normal diet (ND); (2) mice fed a high-fat diet and treated with vehicle (HFD); (3) mice fed a high-fat diet and treated with esaxerenone (HFD + Esax). Esaxerenone (3 mg/kg/day) or vehicle (0.5% solution of sodium carboxymethyl cellulose) was orally administered from the age of 5 weeks throughout the study period. Esaxerenone was provided by Daiichi Sankyo Co., Ltd. (Japan). The dose was determined based on previous studies (Arai et al., 2016, 2020). The mice were fed a HFD containing 60% fat (D12492, Research Diets, Inc.) from the age of 6 weeks. The control group was fed normal chow.

2.2. Measurement of blood pressure and lipid parameters

Blood pressure and heart rate were measured in conscious mice using a tail-cuff system (BP-98A, Softron) as described previously (Ganbaatar et al., 2020). The average value of three measurements per mouse was used for comparison. At the time of sacrifice, the mice were fasted overnight (16 h), and blood was collected from the heart into an EDTA-containing tube. The blood was centrifuged at 9000×*g* for 15 min at 4 °C to separate the plasma. Plasma lipid profiles and liver function were measured at the Sanritsu Zelcova Examination Center (Japan). The plasma concentration of insulin was determined using a mouse enzyme-linked immunosorbent assay kit (Fujifilm Wako, 634–01481) according to the manufacturer's protocol.

2.3. Metabolic studies

The metabolic studies were performed following previously reported protocols (Nishimoto et al., 2016). An intraperitoneal glucose tolerance test (GTT) was performed 12 weeks after HFD administration. Mice were fasted overnight, and then 1 g/kg glucose was injected intraperitoneally. One week after GTT, the insulin tolerance test (ITT) was performed. The mice received an intraperitoneal insulin injection (0.75 U/kg) after a 4-h fasting period. Blood samples were collected from the tail vein at 0, 30, 60, 90, and 120 min after injection, and glucose levels were measured using a Startstrip XP2 (NIPRO). Data are presented as the percentage of the pre-injection baseline (Nishimoto et al., 2016).

2.4. Histological and immunochemical analyses

Histological and immunohistochemical analyses were performed on paraffin-embedded samples as described previously (Nishimoto et al., 2016). The isolated tissues were fixed in 10% formalin and embedded in paraffin. The tissue was then sectioned at 5-μm intervals and mounted on glass slides. The sections were stained with macrophage antigen-3 (Mac-3) antibody (BD Pharmingen), followed by horseradish peroxidase (HRP)-conjugated anti-rat IgG (Abcam), and developed using an ImmPACT DAB Peroxidase Substrate Kit (Vector Laboratories, SK-4105). Each section was counterstained with Harris hematoxylin (Sigma-Aldrich). The number of Mac3-positive nuclei and total number of nuclei were counted, and the percentage of Mac3-positive cells was calculated. The average value evaluated in five random images was used for comparison.

2.5. Cell culture

3T3-L1 preadipocytes were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich D6429) containing 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (P/S). Differentiation into adipocytes was initiated with 1 μM dexamethasone, 500 μM 3-isobutyl-1-methylxanthine, and 10 μg/mL insulin 2 days after reaching confluence. Two days after initiation, the medium was changed to medium supplemented with insulin for another 2 days, and thereafter, the cells were maintained in medium without supplementation. Differentiated adipocytes, obtained 8–10 days after initiation, were cultured in DMEM with 10% charcoal/dextran-treated FBS (Cytiva) for 2 days and used for treatment. The human hepatoma cell line HepG2 was maintained in low-glucose DMEM (Sigma-Aldrich D6046) containing 10% FBS and 1% P/S. Mouse C2C12 myoblasts were maintained in DMEM supplemented with 10% FBS and 1% P/S. Twenty-four hours after reaching 80% confluence, the medium was changed to DMEM containing 2% horse serum and changed daily. Differentiated C2C12 myotubes were used for the experiments 8 days after initiation. All cells were cultured in a humidified atmosphere of 5% CO_2 at 37 °C. Insulin signaling in these cell types was examined after stimulation with 17 nM insulin (Sigma-Aldrich) for 15 min. The effects of aldosterone on insulin signaling were examined in cells treated with 100 or 1000 nM aldosterone (Sigma-Aldrich) for 4 h. Before stimulation with aldosterone, the cells were starved in DMEM containing 0.1% charcoal/dextran-treated FBS for 24 h. In some experiments, cells were pretreated with 10 or 100 nM esaxerenone for 8 h before aldosterone treatment.

2.6. Quantitative real-time PCR

Total RNA was extracted from the tissues and cells using a NucleoSpin® RNA kit (Takara Bio Inc., Japan). Reverse transcription was performed using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). Real-time quantitative PCR was performed on a StepOne™ Real-Time PCR System (Applied Biosystems) with Power

SYBR Green PCR Master Mix (Applied Biosystems) using gene-specific primers. The primer sequences used were as follows: F4/80, Fw 5′-TGCATCTAGCAATGGACAGC-3′ and Rev 5′-GCCTTCTGGATCCATTTGAA-3′; peroxisome proliferator-activated receptor-γ (PPARγ), Fw 5′-GATGGAAGACCACTCGCATT-3′ and Rev 5′-AACCATTGGGTCAGCTCTTG-3′; adiponectin, Fw 5′-ATGGCAGAGATGGCACTCCT-3′ and Rev 5′-CCTTCAGCTCCTGTCATTCCA-3′ and β-actin, Fw 5′-CCTGAGCGCAAGTACTCTGTGT-3′ and Rev 5′-GCTGATCCACATCTGCTGGAA-3′. The ΔΔCT method was used to determine mRNA levels, and the target gene expression level was normalized to that of β-actin.

2.7. Western blotting analyses

Protein lysates were extracted from frozen tissues or cultured cells using lysis buffer containing a protease and phosphatase inhibitor cocktail (Roche Life Science). Equal amounts of protein were separated on sodium dodecyl sulfate–polyacrylamide electrophoresis gels and transferred onto polyvinylidene difluoride membranes (Hybond-P; GE Healthcare). The membranes were blocked with 5% bovine serum albumin or 5% skim milk at room temperature for 1 h on a shaker. The membranes were incubated with primary antibodies against either phosphorylated-Akt Ser473, Akt (Cell Signaling Technology), or β-actin (Sigma) overnight at 4 °C. After washing with TBS-T buffer, the membranes were incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. The bands were visualized using an ECL Plus reagent kit (GE Healthcare) and a luminescent image analyzer (LAS-1000, Fuji Film). Band intensity was quantified using ImageJ software.

2.8. Statistical analyses

All numerical values are presented as the mean ± SEM. Comparisons of parameters between two groups were performed using Student's *t*-test, and differences among multiple groups were analyzed using analysis of variance (ANOVA) followed by Dunnett's test. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Esaxerenone attenuated insulin resistance in high-fat diet-fed mice

Mice fed HFD for 12 weeks significantly increased their body weight and metabolic parameters, such as fasting blood glucose levels. We examined the effects of esaxerenone on metabolic parameters and blood pressure in HFD-fed mice. In this study, no obvious acute toxic effects of esaxerenone were observed. Administration of esaxerenone did not affect body weight, food consumption, blood pressure, or tissue weight in the treated compared with the non-treated group. There was no significant difference in the plasma lipid profile and insulin level between the two groups, although esaxerenone slightly reduced plasma transaminase and lactate dehydrogenase levels in HFD-fed mice (Table 1 and Supplementary Fig. 1).

To investigate the effect of esaxerenone on glucose metabolism, we performed a GTT and ITT. HFD feeding for 12 weeks significantly impaired glucose metabolism, as determined by the GTT and ITT. There was no difference in the GTT results between the untreated and esaxerenone-treated groups. However, the results of ITT demonstrated that, overall, esaxerenone reduced plasma glucose levels although the effect was significant 120 min after insulin injection compared with that in the non-treated group ($P < 0.05$) (Fig. 1). These results indicate that administration of esaxerenone ameliorates insulin sensitivity in obese mice.

3.2. Esaxerenone altered characteristics of visceral adipose tissue

To evaluate the effect of esaxerenone on adipose tissue, we performed histological analysis of visceral fat obtained from mice. The number of Mac3-positive cells was markedly increased in the HFD-fed compared with that in the ND-fed group. Esaxerenone significantly reduced the number of Mac3-positive cells in the adipose tissue compared with that in the untreated group, indicating a reduction in macrophage accumulation (Fig. 2A). In accordance with this histological finding, the expression level of a macrophage marker, F4/80, in adipose tissue was elevated in the HFD-fed mice compared with that in the control ND-fed group. Administration of esaxerenone significantly decreased the expression level of F4/80 compared with vehicle-treated mice ($P < 0.05$). A reduction in the expression of genes associated with insulin-sensitizing factors, such as PPARγ and adiponectin, was observed in diet-induced obese mice. However, esaxerenone tended to increase the expression levels of these genes in adipose tissue compared with those in the non-treated group (Fig. 2B and Supplementary Fig. 2). In addition, aldosterone, a major MR agonist, reduced the expression levels of PPARγ and adiponectin in differentiated 3T3-L1 adipocytes (Fig. 2C and Supplementary Fig. 3), in a dose-dependent manner, suggesting direct effects of MR activation on adipocytes.

Table 1
Effects of esaxerenone on metabolic parameters.

Parameters	ND (n = 12)	HFD (n = 10)	HFD + Esax (n = 10)	P-value
Final body weight (g)	24.2 ± 0.2***	41.9 ± 1.3	39.9 ± 1.4	<0.0001
Fasting blood glucose (mg/dL)	66.1 ± 2.4**	96.5 ± 6.3	107.9 ± 8.4	<0.0001
Heart rate (bpm)	667.7 ± 11.9**	725.2 ± 10.3	716.3 ± 12.0	0.002
Systolic BP (mm Hg)	105.3 ± 3.8	101.1 ± 2.7	104.7 ± 3.5	0.66
Diastolic BP (mm Hg)	60.75 ± 3.6	63.8 ± 3.2	66.8 ± 4.3	0.51
Visceral fat (mg)	423.1 ± 26.6***	2589.7 ± 93.8	2662.6 ± 110.2	<0.0001
Subcutaneous fat (mg)	280.1 ± 12.6***	2134.4 ± 139.8	2248.3 ± 143.0	<0.0001
Liver (mg)	887.1 ± 48.26**	1259 ± 96.7	1109.7 ± 113.4	0.009
Total cholesterol (mg/dL)	98.2 ± 3.3***	209.5 ± 8.3	201.8 ± 10.3	<0.0001
Triglyceride (mg/dL)	119.6 ± 9.1**	85.5 ± 4.8	95.0 ± 8.3	0.01
HDL-cholesterol (mg/dL)	51.8 ± 2.1***	75.2 ± 2.0	75.2 ± 2.8	<0.0001
AST/GOT (IU/L)	98.7 ± 5.5**	153.8 ± 15.5	138.0 ± 17.1	0.01
ALT/GPT (IU/L)	25.4 ± 0.6**	103.8 ± 18.8	79.1 ± 24.3	0.01
LDH (IU/L)	420.9 ± 34.7	509.7 ± 46.7	358.9 ± 43.4	0.06
Insulin (ng/mL)	0.4 ± 0.1	1.61 ± 0.4	1.8 ± 0.5	0.03

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BP, blood pressure; HDL, high-density lipoprotein; HFD, mice fed high-fat diet; LDH, lactate dehydrogenase. Values are presented as the mean ± SEM. **; $P < 0.01$, ***; $P < 0.001$ vs. HFD group.

3.3. Aldosterone impaired insulin signaling

Considering that esaxerenone effectively ameliorated insulin resistance induced by high-fat diet feeding, we examined the direct effect of aldosterone on insulin signaling. In our in vitro experiments, aldosterone significantly abrogated insulin signaling, as determined by insulin-induced Akt phosphorylation in insulin target cells, 3T3-L1 adipocytes, HepG2 cells, and C2C12 myotubes, in a dose-dependent manner (Fig. 3). Importantly, pretreatment with esaxerenone significantly ameliorated insulin signaling in these cell types (Fig. 4).

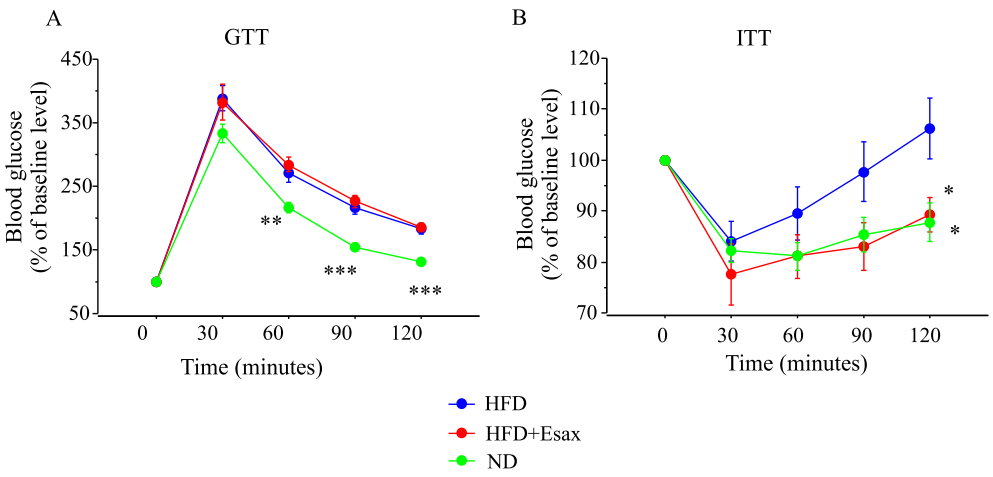

Fig. 1. Esaxerenone improved insulin sensitivity in HFD-fed mice.
To investigate the effect of esaxerenone on glucose metabolism, we performed GTT (A) and ITT (B) after 12 and 13 weeks of HFD feeding, respectively. High-fat feeding significantly impaired glucose metabolism compared with that of ND feeding. (A) In HFD-fed mice, the GTT results showed that glucose levels were not significantly different between the esaxerenone-treated and non-treated groups. (B) In HFD-fed mice, the ITT results demonstrate that esaxerenone improves the response to insulin. n = 10-12, per group. Data are presented as a percentage of the pre-injection baseline. Data are presented as the mean ± SEM. *; $P < 0.05$, **; $P < 0.01$, ***; $P < 0.001$ vs. HFD group.

4. Discussion

In this study, we examined the metabolic effects of a novel MR blocker, esaxerenone, in an HFD-induced obese insulin-resistant rodent model. We demonstrated that esaxerenone improved insulin sensitivity in HFD-fed mice without altering metabolic parameters, such as the lipid profile. Esaxerenone reduced macrophage infiltration and increased the expression of insulin sensitivity-associated genes in visceral fat. Moreover, esaxerenone reversed the reduction in insulin-induced Akt phosphorylation caused by aldosterone in insulin target cells, such as adipocytes, hepatocytes, and myocytes. These results suggest that esaxerenone may be a potential therapeutic option for metabolic disorders in obese individuals.

MRAs, such as spironolactone and eplerenone, ameliorate metabolic parameters, such as plasma triglyceride, insulin, and glucose levels, in obesity models (Guo et al., 2008; Hirata et al., 2009; Wada et al., 2013, 2017). These traditionally available MRAs are widely used; however, because of their relatively low MR selectivity and steroidal structure, several clinical issues remain (Lainscak et al., 2015). For example, spironolactone has low MR-binding specificity and a steroidal structure, inducing sex hormone-related side effects. Eplerenone has a higher MR-binding specificity than spironolactone and shows fewer sex hormone-related side effects, although hyperkalemia remains. Thus, the use of spironolactone and eplerenone remains limited (Jia et al., 2021; Rakugi, 2021). Because of its structure and differences in binding properties to those of aldosterone, esaxerenone is expected to demonstrate superior practical clinical utility (Duggan, 2019; Ito et al., 2020; Wan et al., 2021). However, the effect of esaxerenone on glucose metabolism has not yet been fully investigated. In this study, esaxerenone did not show any clear effects on metabolic parameters, such as body weight and lipid profiles, although it improved insulin sensitivity. Our data are consistent with the results of a previous study that examined the effects of another non-steroidal MR antagonist, finerenone, and reported prevention of glucose intolerance without affecting body weight gain in HFD-fed mice (Marzolla et al., 2020).

We determined that esaxerenone reduced macrophage infiltration and increased the expression levels of genes associated with insulin sensitivity. In vitro experiments using differentiated 3T3-L1 adipocytes have indicated the suppression of genes associated with insulin sensitivity by aldosterone. Inflammation in adipose tissue and reduction in adiponectin and/or PPARγ levels have been recognized as major factors in the development of insulin resistance (Ahmed et al., 2021; Caselli, 2014; Havel, 2004; Hotamisligil, 2008). Several clinical studies have reported elevated plasma aldosterone levels in obesity and/or metabolic syndrome patients (Engeli et al., 2003; Krug et al., 2008). Aldosterone secondarily impairs insulin sensitivity by affecting the production of inflammatory cytokines (Luther, 2014). Excessive aldosterone levels have also been associated with reduced circulating adiponectin (Williams et al., 2012). Furthermore, several studies have shown the overexpression of MR in genetically and diet-induced obese mice (Hirata et al., 2009). On the other hand, in genetically obese and diabetic mice, administration of MRA increased adipocyte PPARγ and adiponectin expression levels and reduced TNF-α and MCP1 expression levels, resulting in improved insulin sensitivity in vivo (Guo et al., 2008; Hirata et al., 2009). These findings indicate that MR activation is a key factor in regulating insulin sensitivity, at least in obesity. Our results are consistent with these findings. Thus, the reduction of inflammation and insulin resistance might be one of the underlying mechanisms for the improved insulin sensitivity in esaxerenone-treated mice.

Obesity is associated with insulin resistance, particularly in adipose tissue, liver, and skeletal muscle (Luther, 2014). Therefore, in this study, we examined the direct effects of aldosterone on insulin target cells. Our in vitro experiments revealed that aldosterone impaired insulin-stimulated Akt phosphorylation in adipocytes, hepatocytes, and myocytes. Furthermore, pretreatment with esaxerenone ameliorated the effect of aldosterone. These results are consistent with previous studies showing the role of aldosterone in insulin signaling (Flynn, 2014; Hitomi et al., 2007; Wada et al., 2009). Additionally, accumulating evidence suggests the contribution of indirect MR activation through Rac1 in several disease contexts (Kawarazaki et al., 2013; Nagase, 2010; Nagase et al., 2009). Augmented Rac1 expression and activity are associated with oxidative stress in obesity (Sun et al., 2012). Therefore, Rac1 signaling may contribute to MR activation in individuals with obesity. Further studies are needed to clarify the mechanism by which esaxerenone improves insulin resistance and develop therapeutic strategies targeting MR signaling.

We observed a reduction in the plasma transaminase levels in the esaxerenone-treated group. Combined with the improvement in insulin sensitivity, these results suggest that esaxerenone attenuates the development of fatty liver disease. Excess fat accumulation in the liver causes a fatty liver, leading to the development of insulin resistance (Asrih et al., 2015; Bosy-Westphal et al., 2019; Willis et al., 2021). Aldosterone increases hepatic glucose production. In addition, MRA or genetic deletion of MR improves dyslipidemia and prevents fatty liver disease (Noguchi et al., 2010; Wada et al., 2010, 2013). Aldosterone deficiency ameliorates HFD-induced fatty liver (Luo et al., 2013). Combined with these observations, our results suggest that these effects on the liver are one of the mechanisms underlying the effects of esaxerenone on insulin sensitivity.

In this study, esaxerenone did not lower blood pressure in HFD-fed mice. The blood pressure-lowering effect of MRAs depends on the mouse model (Arai, 2020; Li, 2019). Treatment with MRAs, including esaxerenone, lowered blood pressure in mice kept under high-salt conditions but not in mice kept under normal salt conditions. The results of

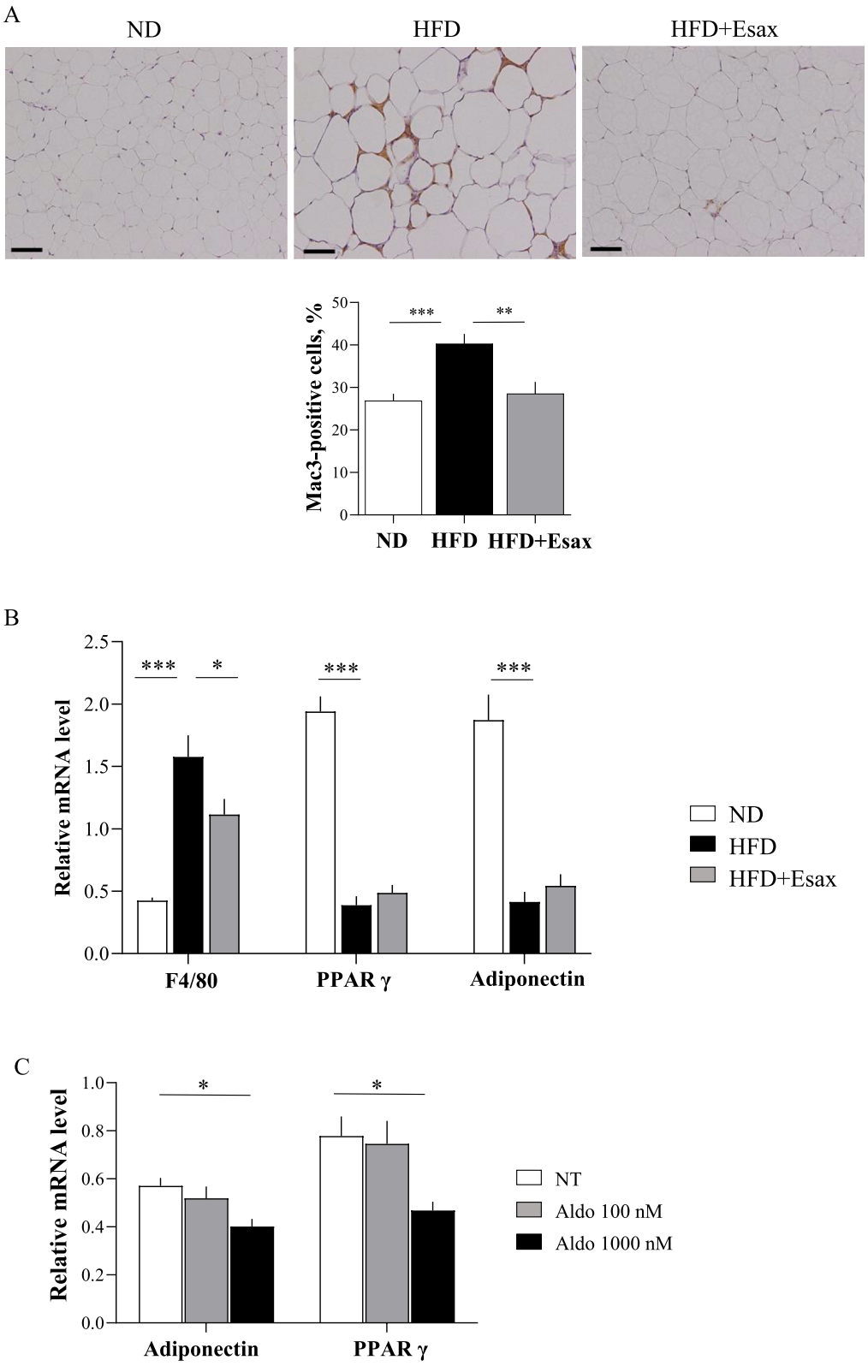

Fig. 2. Effects of esaxerenone on visceral fat and adipocytes.
(A) Representative photomicrograph of immunostaining against Mac3 of visceral fat. High-fat feeding markedly increased the number of Mac3-positive cells in adipose tissue, whereas esaxerenone reduced it. Scale bar 100 μm; n = 10–12. (B) Expression of macrophage marker F4/80 and genes related to insulin sensitivity (PPARγ and adiponectin) in visceral fat obtained from HFD-fed mice was examined using qPCR. Esaxerenone significantly reduced the expression level of the macrophage marker. The levels of genes related to insulin sensitivity increased with esaxerenone, but these increases were not statistically significant. n = 10–12. (C) The effects of aldosterone on mRNA expression in differentiated 3T3-L1 adipocytes were determined using qPCR. Aldosterone decreased the expression levels of genes related to insulin sensitivity in a dose-dependent manner. n = 6, per group. Values are presented as the mean ± SEM. *; $P < 0.05$, **; $P < 0.01$, ***; $P < 0.001$.

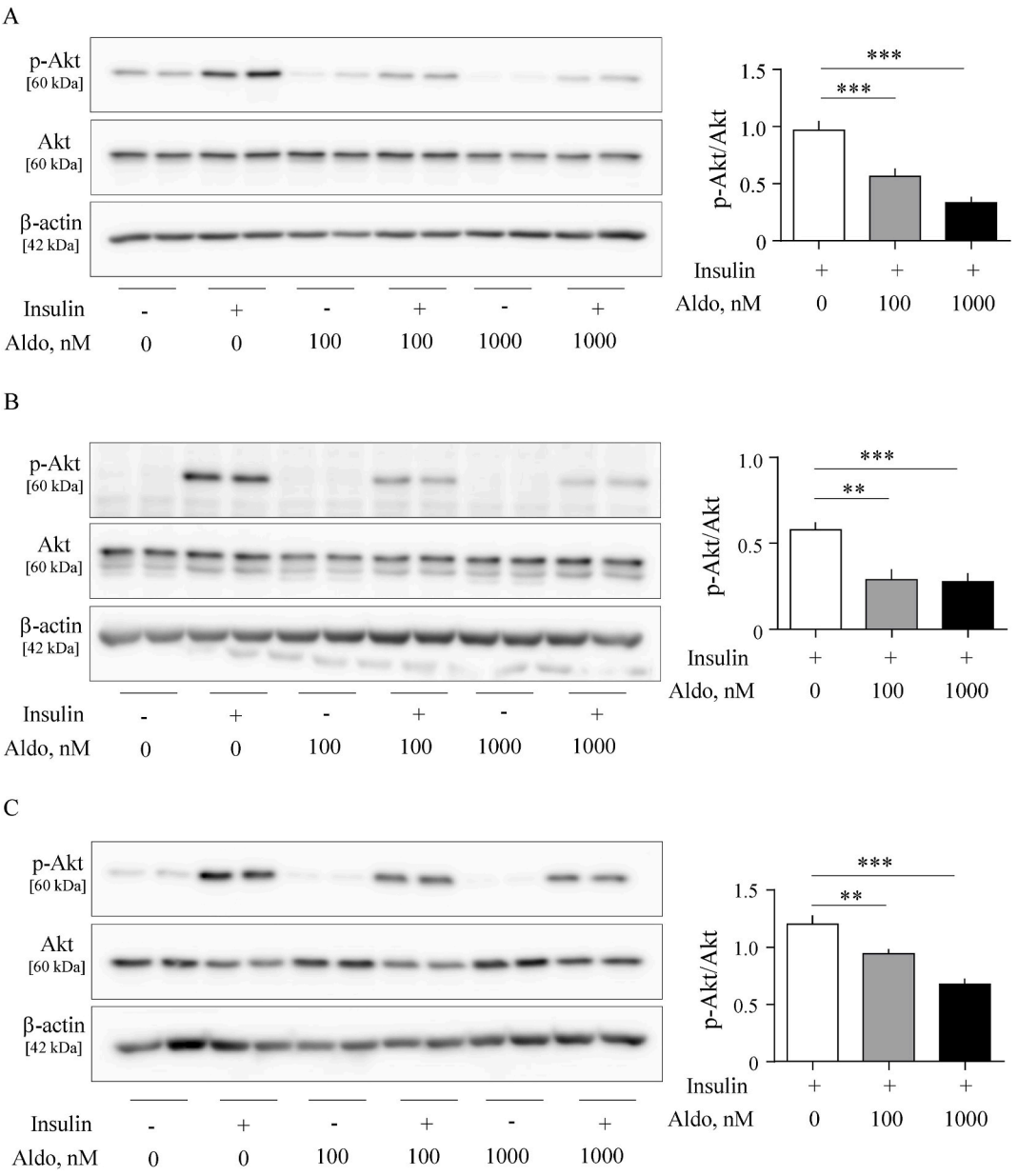

Fig. 3. Aldosterone inhibited Akt phosphorylation induced by insulin.
Insulin target cells, such as 3T3-L1 adipocytes (A), HepG2 cells (B), and C2C12 myotubes (C), were treated with aldosterone for 4 h and then stimulated with 17 nM insulin for 15 min. Western blot analysis demonstrated the abrogation of insulin-induced Akt phosphorylation by aldosterone in these cell types in a dose-dependent manner. n = 8, per group. Values are presented as the mean ± SEM. **; $P < 0.01$ and ***; $P < 0.001$.

our study were consistent with those of previous studies. Further studies using hypertension models are needed, as esaxerenone has been approved for the treatment of patients with hypertension in Japan (Duggan, 2019).

Increased aldosterone secretion and its impact on insulin resistance through various mechanisms, such as acceleration of inflammation and effects on insulin-signaling proteins, have been reported (Briet et al., 2011). Thus, MR activation may be a therapeutic target for insulin resistance. In conclusion, esaxerenone, a selective MR blocker, ameliorates insulin sensitivity in obese mice. Improvement of insulin sensitivity by promoting insulin signaling in adipocytes, liver, and muscle might be the underlying mechanism. In addition, the suppression of inflammatory changes in adipose tissue might have also contributed to our results. The results of our study suggest that esaxerenone may be a beneficial therapeutic option for patients with obesity. However, further studies are required to elucidate the underlying mechanisms.

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CRediT authorship contribution statement

Oyunbileg Bavuu: Methodology, Investigation, Visualization, Writing – original draft. **Daiju Fukuda:** Conceptualization, Methodology, Investigation, Validation, Writing – review & editing. **Byambasuren Ganbaatar:** Methodology, Investigation, Visualization, Writing – review & editing. **Tomomi Matsuura:** Visualization, Writing – review &

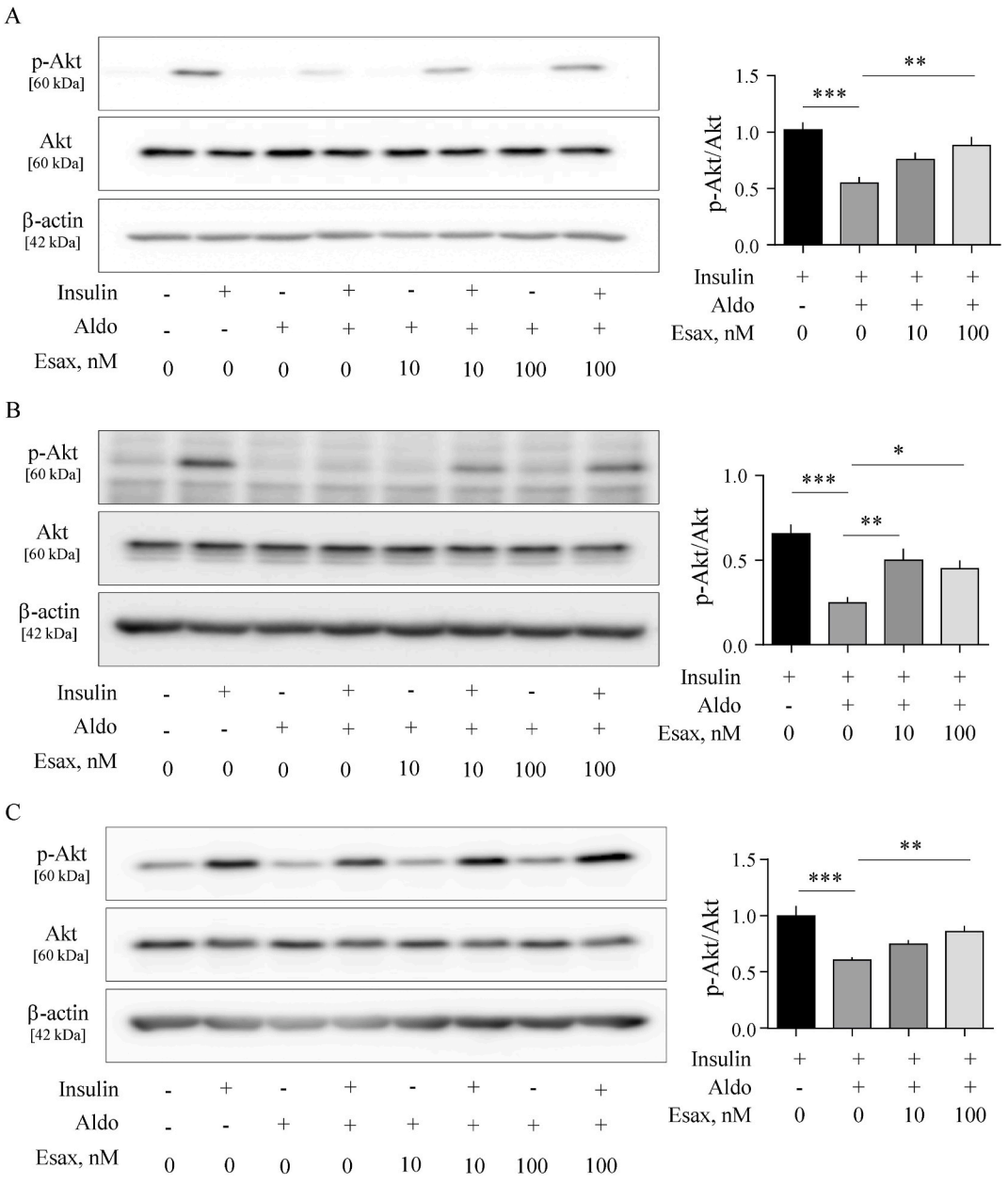

Fig. 4. Esaxerenone ameliorated aldosterone-induced impairment of insulin signaling.
3T3-L1 adipocytes (A), HepG2 cells (B), and C2C12 myotubes (C) were pretreated with esaxerenone (10 or 100 nM) for 8 h and then treated with aldosterone (1000 nM) for 4 h. Insulin signaling was determined by the phosphorylation of Akt after stimulation for 15 min with 17 nM insulin. Aldosterone inhibited insulin signaling in these cell types, whereas pretreatment with esaxerenone ameliorated the aldosterone-induced impairment of insulin signaling. n = 8–12, per group. Values are presented as the mean ± SEM. *; $P < 0.05$, **; $P < 0.01$ and ***; $P < 0.001$.

editing. **Takayuki Ise:** Visualization, Writing – review & editing. **Kenya Kusunose:** Visualization, Writing – review & editing. **Koji Yamaguchi:** Visualization, Writing – review & editing. **Shusuke Yagi:** Methodology, Visualization, Writing – review & editing. **Hirotsugu Yamada:** Visualization, Writing – review & editing. **Takeshi Soeki:** Visualization, Writing – review & editing. **Tetsuzo Wakatsuki:** Visualization, Writing – review & editing. **Masataka Sata:** Methodology, Validation, Visualization, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. Esaxerenone was provided by Daiichi Sankyo Co., Ltd.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.

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