Impact of MR on mature adipocytes in high-fat/high-sucrose diet-induced obesity

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Abstract

Active glucocorticoid levels are elevated in the adipose tissue of obesity due to the enzyme 11 beta-hydroxysteroid dehydrogenase type 1. Glucocorticoids can bind and activate both glucocorticoid receptor (GR) and mineralocorticoid receptor (MR), and pharmacological blockades of MR prevent high-fat diet-induced obesity and glucose intolerance. To determine the significance of MR in adipocytes, we generated adipocyte-specific MR-knockout mice (AdipoMR-KO) and fed them high-fat/high-sucrose diet. We found that adipocyte-specific deletion of MR did not affect the body weight, fat weight, glucose tolerance or insulin sensitivity. While liver weight was slightly reduced in AdipoMR-KO, there were no significant differences in the mRNA expression levels of genes associated with lipogenesis, lipolysis, adipocytokines and oxidative stress in adipose tissues between the control and AdipoMR-KO mice. The results indicated that MR in mature adipocytes plays a minor role in the regulation of insulin resistance and inflammation in high-fat/high-sucrose diet-induced obese mice.

Key Words

- mineralocorticoid receptor
- adipocyte
- obesity
- insulin resistance

Introduction

The mineralocorticoid receptor (MR) is a member of the steroid receptor family of ligand activated transcription factors. MR is expressed in several tissues, including the kidney, heart, colon, brain and adipose tissue (Armani et al. 2015). In the kidney, its activation leads to the expression of ENaC or Na/K ATPase resulting in the reabsorption of sodium to maintain a normal salt concentration in the body. Consequently, systemic MR-deficient mice die from dehydration approximately 10 days after birth (Berger et al. 1998). MR binds both aldosterone and glucocorticoids (Reul & de Kloet 1985), and circulating levels of endogenous glucocorticoids are 100–1000 times higher than those of aldosterone. In epithelial tissues, 11 beta-hydroxysteroid dehydrogenase type 2 (11bHSD2) inactivates glucocorticoids, resulting in selective activation of MR by aldosterone (Funder 2009). On the contrary, in nonepithelial tissues lacking 11bHSD2, such as the adipose tissue, MR is activated by glucocorticoids due to their higher circulating concentrations than aldosterone (Funder 2000, Caprio et al. 2007).

In obesity, circulating glucocorticoid levels are normal; however, active glucocorticoid levels are elevated in the adipose tissue due to the enzyme 11 beta-hydroxysteroid dehydrogenase type 1 (11bHSD1) (Masuzaki et al. 2001; Engeli et al. 2004; Patsouris et al. 2009). Furthermore, MR is overexpressed in the adipose tissue of a mouse model of metabolic syndrome and also in obese individuals (Hirata et al. 2009, 2012; Urbanet et al. 2015).
Treatment with eplerenone, a specific MR blocker, partially reverses the obesity-associated dysfunction of adipocytes and insulin resistance in mice (Guo et al. 2008; Hirata et al. 2009). Moreover, transgenic mice with adipocyte-specific inducible overexpression of MR are overweight with large fat mass, insulin resistance and features of metabolic syndrome (Urbanet et al. 2015). Thus, MR seems a suitable target molecule for locally activated glucocorticoid in the adipose tissues of obesity. However, no information is available on adipocyte-specific MR-deficient mice. In this study, we describe the generation of adipocyte-specific MR-deficient (AdipoMR-KO) mice and report about the contribution of MR in obesity-related metabolic syndrome.

Materials and methods

Animal experiments

All animal studies were approved by the Ethics Review Committee for Animal Experimentation of Osaka University, Graduate School of Medicine and performed in accordance with the Osaka University Institutional Animal Care and Use Committee Guidelines. To establish the AdipoMR-KO mice, we crossed MR-floxed (McCurley et al. 2012) and Adiponectin-Cre (Eguchi et al. 2011) mice. The MR-floxed mice were kindly provided by Prof. Pierre Chambon (Institute for Genetics and Cellular and Molecular Biology, Strasbourg, France) and Prof. Iris Z Jaffe (Tufts University, Medford, MA, USA) and Adiponectin-Cre mice by from Drs E Rosen (Beth Israel Deaconess Medical Center, Boston, MA, USA) and J Eguchi (Okayama University, Okayama, Japan). Mice were fed a normal chow diet (protein, 25.7%; lipid, 12.8%; carbohydrate, 61.6% by calories; Oriental Yeast, Tokyo, Japan) or a high-fat/high-sucrose diet (HFHSD; protein, 17.2%; lipid, 54.5%; carbohydrate, 28.3% (sucrose, 16.6%) by calories; Oriental Yeast) from 5 weeks of age. All the mice were individually housed. There were eleven MR flox/flox (control) mice aged 5–13 weeks, ten aged 14–16 weeks, nine aged 17 weeks, seven aged 18–19 weeks, six aged 20–21 weeks and five aged >22 weeks. There were twelve AdipoMR-KO mice aged 5–13 weeks, eleven aged 14–16 weeks, ten aged 17 weeks, eight aged 18–19 weeks, seven aged 20–21 weeks and six aged >22 weeks. Their body weight (BW) was monitored every week and blood glucose levels were measured every 4 weeks after 4-h fasting ending at 13:00. After 14 weeks of HFHSD feeding, insulin (1.8 IU/kg BW) was intraperitoneally injected, and blood glucose levels were measured using samples obtained from the tail vein at 0, 15, 30, 60, 90 and 120 min after injection. Furthermore, after 16 weeks of HFHSD feeding, glucose (1 g/kg BW) was intraperitoneally injected and blood glucose levels were measured in samples obtained from the tail vein at 0, 15, 30, 60, 90 and 120 min after injection. Blood glucose levels were measured using Glutest Neo (Sanwa Kagaku Kenkyusho, Nagoya, Japan). After 18 weeks of HFHSD feeding, mice were fasted from 09:00 to 13:00, anesthetized using intraperitoneally injected pentobarbital (100 mg/kg BW), and finally killed. The harvested tissues were weighed and subsequently frozen in liquid nitrogen.

Isolation of adipocytes and stromal vascular fraction (SVF)

Male MR flox/flox and AdipoMR-KO mice (28-week-old; n=3 for each) that were individually housed and fed normal chow were fasted between 09:00 and 13:00, anesthetized, and subsequently killed. Epididymal fat (Epi fat) was washed with PBS and then finely minced in DMEM. The extracellular matrix was digested using collagenase (40,000 U/15 mL media) with DNase for over 40 min at 37°C in a water bath. The digested tissues were then filtered through a 70-μm nylon filter and mixed with DMEM to a final volume of 50 mL. The samples were then centrifuged at 500g for 5 min at room temperature. Floating adipocytes were collected and washed three times with DMEM. After removing DMEM, TRI reagent (Sigma) was poured over the adipocytes. The same reagent was also poured over pelleted stromal vascular cells. These lysates were frozen and processed for RNA extraction with an RNAeasy Mini kit (Qiagen).

Primary cell culture

Paired female MR flox/flox and AdipoMR-KO mice were individually housed and fed normal chow. The mice were killed at 14 weeks of age. Subcutaneous adipose tissues were obtained and immediately minced as previously described. The minced tissues were then strained by a 70-μm nylon membrane. The filtrated cells were collected and placed on the dish with DMEM. After confluence, the cells were differentiated with IBMX (0.5 mM), insulin (1 μM), dexamethasone (1 μM) and pioglitazone (10 μM). On day 7 from differentiation, the cells were harvested and used for mRNA extraction.
Quantitative real-time PCR (RT-PCR)

Frozen tissues or cells were treated with TRI reagent (Sigma), and total RNA was extracted with the RNeasy Mini kit (Qiagen). The RNA was reverse transcribed by Prime Script RT Master Mix (Takara). The sequences of primers are described in Supplementary Table 1 (see section on supplementary data given at the end of this article). Quantitative RT-PCR was performed in a Light Cycler System (Roche Diagnostics) with Thunderbird SYBR qPCR Mix (Toyobo, Osaka, Japan) according to the instructions provided by the manufacturer. The mRNA levels were expressed relative to those of cyclophilin A mRNA.

Measurement of blood metabolites and steroids

Blood samples were collected from anesthetized mice just before killing. Insulin levels were measured using a Morinaga Ultra-Sensitive Insulin ELISA kit (Morinaga, Kanagawa, Japan) according to the protocol supplied by the manufacturer. The levels of non-esterified fatty acid (NEFA), triglyceride (TG), total cholesterol (T-Chol) and high-density lipoprotein cholesterol (HDL-C) were determined by the NEFA C-test, TG E-test, Cholesterol E-test and HDL cholesterol E-test (Wako, Osaka, Japan), respectively. Plasma corticosterone levels were measured as previously reported (Bose et al. 2016). Plasma aldosterone levels were measured using Parameter Aldosterone Assay (R&D systems) according to the procedure provided by the manufacturer. Adipocytokines levels were measured using Adiponectin ELISA kit (Otsuka Pharma, Tokyo), Morinaga Leptin Mouse/Rat ELISA kit (Morinaga), Quantikine ELISA MCP-1 (R&D systems), and Quantikine ELISA Resistin (R&D systems) as described in the ELISA kits.

Measurement of tissue 8-isoprostane levels

Tissue 8-isoprostane in Epi fat was extracted and measured using the 8-Isoprostane EIA Kit (Cayman Chemical) according to the protocol recommended by the manufacturer. Briefly, Epi fat was lysed, incubated with potassium hydroxide, trapped with Sep-Pak (Waters, Milford, MA), washed with water and hexane, eluted with ethyl acetate, evaporated and reconstituted with EIA buffer. Tissue 8-isoprostane levels were expressed relative to the protein concentration estimated by a BCA Protein Assay Kit (Thermo Fisher Scientific).

Liver TG content

Frozen liver tissues of HFHSD-fed mice were treated with the detergent 5% nonidet-P40 (Nacalai tesque, Kyoto, Japan). The solution was sonicated and heated up to 90°C for 5 min twice. TG concentration in the supernatant was evaluated using the TG E-test, and the value was expressed relative to the protein concentration estimated by the BCA Protein Assay Kit (Thermo).

Data analysis

All the data are presented as the mean ± standard error (S.E.M.). Differences between groups were analyzed by the Student’s t-test. All P values of <0.05 were considered significant. The statistical analyses were performed using JMP software (SAS Institute).

Results

Generation of AdipoMR-KO mice

We generated the AdipoMR-KO mice by crossing homozygous MR-floxed (McCurley et al. 2012) and Adiponectin-Cre mice. They constitutively express the Cre recombinase under the control of adiponectin promoter (Eguchi et al. 2011). In contrast to the lethal phenotype of whole-body MR knockout (Berger et al. 1998), AdipoMR-KO were born at the expected Mendelian ratio and appeared grossly normal, with no apparent different features or signs. In these mice, Cre-mediated recombination was specifically noted in the adipose tissue but not in other tissues, such as the liver and kidney (Supplementary Fig. 1). To assess the extent of MR deletion at the transcriptional level, we isolated Mr mRNA from various tissues and analyzed its expression level by qRT-PCR. The expression levels of Mr were significantly reduced in the adipose tissue, including brown adipose tissue (BAT), subcutaneous adipose tissue (Sub fat) and Epi fat, but not in the liver and kidney (Fig. 1A). Next, we separated the epididymal adipose tissue into mature adipose fraction (MAF) and SVF. The fractionation efficiency was confirmed by the high levels of adiponectin and dipeptidyl peptidase-4 (Dpp4) in MAF (Maeda et al. 1996) and SVF (Shin et al. 2017), respectively (Supplementary Fig. 2). Fractionation analysis showed that Mr expression levels were approximately 70 times lower in the MAF of KO mice than those in the control but not in SVF (Fig. 1B).
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Effect of adipocyte MR deletion on body and organ weight

To validate the effect of adipocyte MR deletion on obesity-induced metabolic disorders, male mice were fed HFHSD from 5 weeks of age for 18 weeks. The change in BW during the study was similar in both the AdipoMR-KO and control mice (Fig. 2A). Furthermore, there were no differences in adipose tissue weights, including Sub fat, Epi fat and BAT between the control and AdipoMR-KO mice (Fig. 2B). There were no significant differences in adipocyte size between the two groups (Supplementary Fig. 3). However, the liver weight was lower in AdipoMR-KO than in the control mice (Fig. 2B).

When mice were fed normal chow diet, BW was similar in both the AdipoMR-KO and control mice (Supplementary Fig. 4A). Furthermore, there were no differences in organ weights, including liver, kidney, mesenteric fat (Mes fat), Epi fat, subcutaneous fat (Sub fat) and BAT between the control and AdipoMR-KO mice (Supplementary Fig. 4B).

Effect of adipocyte MR deletion on glucose or lipid metabolism and plasma adipocytokine levels

To evaluate the effect of MR deletion on glucose homeostasis, blood glucose levels were measured every 4 weeks during HFHSD feeding. There were no significant differences in fasting glucose levels between the two groups (Fig. 3A). Fasting insulin levels had no significant differences between the two groups (Fig. 3B). In addition, plasma NEFA, TG, T-Chol HDL-C, aldosterone and corticosterone levels were not significantly different between the two groups (Fig. 3C, D, E, F, G and H). There were no significant differences in plasma adipocytokine levels between the two groups, including adiponectin, leptin, resistin and MCP-1 levels (Fig. 3I, J, K and L). We performed an insulin tolerance test (ITT) at 14 weeks and an intraperitoneal glucose tolerance test (GTT) at 16 weeks after HFHSD feeding. The results showed no significant

Figure 1
Generation of adipocyte-specific MR KO mice. (A) Mr mRNA expression level relative to cyclophilin A was assessed by qRT-PCR in the liver, kidney, interscapular brown adipose tissue (BAT), subcutaneous white adipose tissue (Sub fat) and epididymal fat (Epi fat) of MR flox/flox and Adipocyte-specific MR-knockout mice (AdipoMR-KO). (n=3, for each). (B) Mr mRNA expression relative to cyclophilin A was also assessed in the mature adipocyte fraction (MAF) and stromal vascular fraction (SVF) of epididymal white adipose tissue (n=3, for each). Open bars, MR flox/flox mice; solid bars, AdipoMR-KO mice. Data are presented as mean ± s.e.m. *P<0.05, ***P<0.001.

Figure 2
Effect of adipocyte MR deletion on body and organ weight. (A) Male MR flox/flox mice and AdipoMR-KO mice were fed HFHSD for 18 weeks from 5 weeks of age (n=11 for mice aged 5–13 weeks, n=10 for mice aged 14–16 weeks, n=9 for mice aged 17 weeks, n=7 for mice aged 18–19 weeks, n=6 for mice aged 20–21 weeks and n=5 for mice aged >22 weeks in the MR flox/flox group; n=12 for mice aged 5–13 weeks, n=11 for mice aged 14–16 weeks, n=10 for mice aged 17 weeks, n=8 for mice aged 18–19 weeks, n=7 for mice aged 20–21 weeks and n=6 for mice aged >22 weeks in the AdipoMR-KO group). (B) Organ weights of MR flox/flox and AdipoMR-KO mice. Mice fed HFHSD from 5 weeks of age were killed at 23 weeks of age and the weights of different tissues were measured. The liver, kidney, mesenteric fat (Mes fat), epididymal fat (Epi fat), right soleus and gastrocnemius muscles (muscle), subcutaneous fat (Sub fat) and brown adipose tissue (BAT) were weighed (n=5 in the MR flox/flox group; n=6 in the AdipoMR-KO group). Data are presented as mean ± s.e.m. *P<0.05.
differences between the control and AdipoMR-KO mice (Fig. 4A and B). Collectively, deletion of adipose MR did not affect glucose or lipid metabolism and plasma adipocytokine levels. When mice were fed a normal chow diet, fasting plasma glucose and insulin levels were not significantly different between the two groups (Supplementary Fig. 4C and D).

**Effect of adipocyte MR deletion on fat mRNA and 8-isoprostane**

We analyzed the effects of MR deficiency on Epi fat mRNA (Fig. 5 and Supplementary Fig. 5). Regarding other steroid receptors (Fig. 5A), there were no significant differences in the expression levels of glucocorticoid receptor (Gr), estrogen receptor (Er) alpha and 11bHSD1 between the control and AdipoMR-KO mice. We also measured the mRNA levels of lipogenic and lipolytic enzymes and found no significant differences in Acaca, Fasn, Scd1, Acly, HSL, Atgl and Angptl4 between the two groups (Fig. 5B and C). We also validated the differentiation marker for adipocytes (Fig. 5D). No changes were observed in the mRNA levels of adiponectin, leptin and PPARγ. Regarding the inflammation-related genes, the mRNA levels of IL-6 and lipocalin 2 did not differ, whereas those of PAI1 tended to be lower in AdipoMR-KO mice (Fig. 5E).

Furthermore, there were no differences in the oxidative stress marker, 8-isoprostane (Fig. 5F), and the expression levels of related genes, such as Cyba, Sod1 and catalase (Fig. 5G). The mRNA levels of thermogenesis genes, such as Ucp1 and Pparg1a, were not different in Epi fat between the two groups (Fig. 5H). In BAT, the mRNA levels of Ucp1 were not different between the two groups (Fig. 5I).

Next, we measured mRNA levels of primary differentiated adipocytes from Sub fat of MR flox/flox and AdipoMR-KO mice. Both preadipocytes differentiated
into mature adipocytes with the accumulation of lipid droplets (Fig. 6A). We confirmed a lower expression of Mr in mature adipocytes from KO mice (Fig. 6B). In other genes, except lipocalin 2, there were no significant differences between the two groups (Fig. 6B, C, D, E and F). Expression levels of lipocalin 2 were significantly reduced in primary differentiated adipocytes of AdipoMR-KO mice compared with those of the MR flox/flox mice (Fig. 6F), which is consistent with a previous report (Urbanet et al. 2015).

Effect of adipocyte MR deletion on liver mRNA and TG content

Finally, we analyzed liver mRNA levels on lipogenesis (Fig. 7A). The levels of lipogenic genes were not significantly different between the two groups, whereas liver TG content tended to be lower in the AdipoMR-KO mice than the control mice (Fig. 7B). Furthermore, analysis of the mRNA levels of gluconeogenesis genes showed no effect of adipocyte MR deletion on G6Pase and PEPCK mRNA levels (Fig. 7C).
In obesity, active glucocorticoid levels are locally increased in adipose tissues (Masuzaki et al. 2001; Engeli et al. 2004; Patsouris et al. 2009). Adipocytes, immune cells, as well as other cells are considered to be possible target cells of glucocorticoid, which can bind and activate both MR and GR (Reul & de Kloet 1985). There is no consensus on the target cells and molecules for excess glucocorticoid in the adipose tissue of obesity. In the present study, we generated novel AdipoMR-KO mice and showed that adipose MR has a minor role in insulin resistance and inflammation. Recently, adipocyte-specific GR-deficient mice models were generated by three different groups (Desarzens & Faresse 2016; Mueller et al. 2017; Shen et al. 2017). Similar to AdipoMR-KO mice, these adipocyte-specific GR-deficient mice showed no significant differences in BW gain and adipose tissue formation compared with controls (Desarzens & Faresse 2016; Shen et al. 2017). Moreover, GR deficiency worsened diet-induced inflammation, as demonstrated by higher expression levels of proinflammatory genes and macrophage infiltration in the fat pads (Desarzens & Faresse 2016). In sharp contrast to adipocyte-specific MR-KO and GR-KO mice, obese myeloid-specific MR-KO mice exhibited improved glucose intolerance, insulin resistance and hepatic steatosis (Zhang et al. 2017). Additionally, Wada et al. (Wada et al. 2017) reported recently that aldosterone induces ROS generation in bone marrow-derived macrophages, and that eplerenone, a specific MR antagonist, inhibits the activation of NLRP3 inflammasome and ROS generation. Considering that eplerenone ameliorated inflammation and insulin resistance in ob/ob mice with suppression of macrophage infiltration and ROS production in the adipose tissues (Guo et al. 2008; Hirata et al. 2009), it is possible that MR in macrophage may play a role mainly in inflammation and insulin resistance induced by locally activated glucocorticoid in adipose tissues.

Our data showed significant reduction in liver weight of AdipoMR-KO mice in the HFHSD model. The liver TG content also tended to decrease, whereas the expression levels of lipogenic genes were not affected in AdipoMR-KO mice. The data were partially consistent with those of previous studies. In high-fat diet-induced obese animals, treatment with eplerenone induced significant reduction in liver mass and tended to reduce liver TG content (Wada et al. 2017). Another study reported that mice of ob/ob background with myeloid MR knockout had decreased liver mass and TG content without changes in Fasn and Acaca mRNA levels (Zhang et al. 2017). Ob/ob mice spontaneously develop more severe forms of obesity, insulin resistance and steatosis than those by the HFHSD model of C57BL/6 background. Therefore, it may be difficult to detect significant differences in liver TG content between the control and AdipoMR-KO in HFHSD models.

The influx of NEFA into the liver through lipolysis of adipose tissue leads to liver steatosis. In the present study, there were no significant changes in plasma NEFA levels or the expression levels of lipolytic genes in the adipose tissue. In addition to lipolytic gene expression, lipolysis is stimulated by catecholamines that act via the β-adrenergic cAMP signaling in adipose tissue of obesity, insulin resistance and steatosis than those by the HFHSD model of C57BL/6 background. Therefore, it may be difficult to detect significant differences in liver TG content between the control and AdipoMR-KO in HFHSD models.
Our results showed that adipocyte-specific MR deletion in the HFHSD model was not associated with overall significant changes. This finding may be related to the expression level of Mr. Mr expression in murine adipose tissue was approximately 2/3 of that in the kidney (Supplementary Fig. 6A). In addition, fractionation analysis indicated that the mRNA level of Mr in MAF was only 25% of that in SVE, including immune cells, endothelial cells and preadipocytes (Supplementary Fig. 6B). Furthermore, Mr expression further decreased by approximately 40% after differentiation of 3T3-L1 cells into mature adipocytes (Supplementary Fig. 6C). Taken together, it seems that Mr expression is relatively low in adipocytes. Interestingly, glucocorticoid and aldosterone can activate MR in 3T3-L1 cells; aldosterone was reported to downregulate the expressions of adiponectin and anti-oxidative enzymes but upregulate those of inflammatory cytokines and ROS-generating enzymes in a MR-dependent manner (Guo et al. 2008; Hirata et al. 2009). In 3T3-L1 cells, downregulation of MR markedly inhibits differentiation into mature adipocytes (Caprio et al. 2007). MR has a high affinity for glucocorticoids and occupied with basal endogenous glucocorticoid levels (Joels & de Kloet 2017), and corticosterone concentration in adipose tissue of obese mice is almost twice that of lean mice (Patsouris et al. 2009). Contrary to expectation, the results of the present study showed no significant differences in the expression levels of adipocytokines, ROS-related enzymes and tissue 8-isoprostane between the control and AdipoMR-KO mice. Considered together, minor effect of adipose MR deletion in the current study should be attributed to its low expression in adipocytes. Nevertheless, adipose MR may still play important roles in other conditions, such as excessive glucocorticoid- or aldosterone-induced metabolic syndrome models or caloric restriction models.

In the current study, we used adiponectin-Cre mice to delete Mr specifically in adipocytes. Adiponectin-Cre is active in fully mature adipocytes in adult animals (Eguchi et al. 2011); therefore, we could not detect the effect of Mr during differentiation of adipocytes. As described earlier, downregulation of Mr markedly inhibits the differentiation of preadipocytes into mature adipocytes (Caprio et al. 2007). Armani et al. reported that MR antagonists induce browning of white adipose tissue (Armani et al. 2014). It is controversial whether beige adipocytes are derived from the differentiation of precursor cells (Wu et al. 2012) or transdifferentiation of mature adipocytes (Cinti 2009). Taken together with our results, the effects of MR antagonist on the induction of browning in white adipose tissues may be attributed to its precursor cells but not mature adipocytes.

In summary, MR in mature adipocytes plays a minor role in controlling insulin resistance and inflammation in high-fat/high-sucrose diet-induced obese mice. Further studies are needed to understand the exact MR function(s) in adipocytes.

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**Supplementary data**

This is linked to the online version of the paper at [https://doi.org/10.1530/JOE-18-0026](https://doi.org/10.1530/JOE-18-0026).

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**Declaration of interest**

Dr Atsunori Fukuhara belongs to endowed department of the Takeda Pharmaceutical Company, Sanwa Kagaku Kenkyusho Co., Ltd., Rohto Pharmaceutical Co., Ltd., FUJI OIL HOLDINGS INC., and Roche DC Japan. Dr Jihoon Shin belongs to endowed department of the AstraZeneca K.K., Boehringer Ingelheim, Mitsubishi Tanabe Pharma Co., MSD, Novo Nordisk Pharma, Ono Pharmaceutical Co., and Taisho Toyama Pharmaceutical Co. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

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**Author contribution statement**

T H designed and performed the experiments and acquired data with the help of T Q, T M, Y Q, J S, A F, M O and I S designed the study, interpreted the data and wrote the manuscript. All authors reviewed the manuscript and provided final approval of the version to be published. A F is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and accuracy of the analysis.

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**References**


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