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Original Article

Marine natural product des-*O*-methyllasiodiplodin effectively lowers the blood glucose level in *db/db* mice via ameliorating inflammation

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Aim: des-*O*-methyllasiodiplodin (DML) from *Cerbera manghas* has shown antagonistic activity against mineralocorticoid receptor (MR). Considering the involvement of MR in the insulin tolerance, we attempted to investigate the potential of DML in the treatment of type 2 diabetes mellitus (T2DM).

Methods: Surface plasmon resonance (SPR) technology and reporter gene-based assays were used to study protein-small molecule interactions. HepG2 and 3T3-L1 cells were treated with H_2O_2 (0.2 mmol/L) or aldosterone (10 nmol/L) for 24 h. The expression of MR in the cells was downregulated with siRNA. The anti-inflammatory effect of the compound was evaluated, respectively. *db/db* mice were administered DML (30 mg·kg^{-1}·d^{-1}) for 4 weeks. Serum biochemical parameters and insulin sensitivity were examined. The expression levels of pro-inflammatory cytokines (MCP-1, TNF-α, and IL-6) and ROS-related genes (NADPH p47 subunit and transcriptional factor PU.1) in adipose tissues and livers were analyzed using real-time RT-PCR.

Results: In HepG2 and 3T3-L1 cells, both H_2O_2 and aldosterone markedly stimulates the expression of MCP-1, TNFα, IL-6, p47, and PU.1 genes. Co-treatment with DML (10 μmol/L) significantly reduced the H_2O_2- or aldosterone-induced expression of these genes. SPR-based assay confirmed the antagonistic activity of DML against the interaction between SRC-1 and MR-LBD. Furthermore, DML decreased aldosterone-induced MR transcriptional activity in a dose-dependent manner. Downregulation of MR with siRNA in the cells prevented or significantly attenuated aldosterone-stimulated expression of these genes, whereas DML did no longer affect the expression of these genes except that of IL-6. Oral administration of DML effectively reduced the levels of blood glucose and glycosylated hemoglobin (HbA1c) in *db/db* mice. The treatment also rectified the expression of pro-inflammatory factor and ROS-related genes in *db/db* mice.

Conclusion: DML effectively lowers the blood glucose level in *db/db* mice possibly via ameliorating the expression of obesity-related pro-inflammatory cytokines, highlighting the potential of the marine natural product as a drug lead for the treatment of metabolic disorders.

Keywords: des-*O*-methyllasiodiplodin; diabetes; *db/db* mice; pro-inflammatory cytokine; ROS; mineralocorticoid receptor; aldosterone; blood glucose; glycosylated hemoglobin (HbA1c)

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Introduction

Chronic inflammation is closely linked to insulin resistance[1], a major risk factor for the development of type 2 diabetes mellitus (T2DM)[2, 3]. Many adipocyte-derived factors exhibit deviant increases in diabetic individuals, including free fatty acids, hormones and pro-inflammatory cytokines[4]. As such, malfunctions in the release of the principal pro-inflammatory cytokines monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) are strongly associated with the progression of insulin resistance. Therefore, reagents with anti-inflammatory activity are expected to show potential as treatments for metabolic disorders.

For many years, natural products have supplied major molecular structural resources for drug discovery based on their abundant chemical novelty and diversity[5]. Here, we screened and found a novel anti-inflammatory compound from the laboratory in-house natural product library accord-

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ing to the published approach[6].

Cerbera manghas (*C manghas*) is a type of mangrove plant[7], whose extracts exhibit various pharmacological activities, including cardiotonic, anti-proliferative, anti-estrogenic and anti-hypertensive activity[8–10], although little molecular target information regarding those beneficial effects has been disclosed to date. In the present study, we discovered that des-*O*-methyllasiodiplodin (DML, Figure 1A) from *C manghas* effectively attenuated pro-inflammatory cytokine expression in both 3T3-L1 and HepG2 cells. The related molecular mechanism of this marine product in the improvement of *db/db* diabetic status was also investigated.

In our previous work, we preliminarily reported that DML antagonized mineralocorticoid receptor (MR) activity (IC_{50}=8.9 μmol/L) in a yeast-two-hybrid system[11]. MR is a member of the steroid nuclear receptor (NR) family, and is a key modulator of electrolyte homeostasis and blood pressure[12]. Aldosterone (Aldo), as an agonist of MR, physiologically regulates the gene expression of the Na^+, K^+-ATPase[13] that is responsible for salt re-absorption in the kidney[14, 15]. Moreover, both basic and clinical research has revealed that Aldo not only mediates mineral balance in the kidney but also is directly involved in glucose tolerance[16–18]. Furthermore, the prevalence of metabolic syndrome in patients with primary aldosteronism (PAL) is higher than in patients with essential hypertension[19, 20]. Impaired insulin sensitivity was observed in patients with PAL and was ameliorated after surgery and medical treatment[21–23]. In addition, MR activation was linked to the renal inflammatory response in rodent models of diabetes mellitus[24].

Beneficial effects of MR blockade on coronary vascular protection and inflammation reduction have been reported in patients with diabetes[25–28]. Spironolactone (Spir) and eplerenone (Eple), known MR antagonists, have been shown to be useful in the treatment of hypertension and heart failure[29, 30], the reversal of obesity-related changes in pro-inflammatory adipokines and the amelioration of adipocyte dysfunction and insulin resistance[31–33]. In addition, the production of pro-inflammatory factors such as TNF-α, MCP-1, and IL-6 was attenuated by Eple both in liver and adipose tissue *via* the suppression of reactive oxygen species (ROS)[33], and Spir improved glucose and lipid metabolism by ameliorating inflammation in high-fat and high-fructose diet mice[31]. Many prior studies have addressed the potency of MR antagonists in the regulation of obesity-related pathological processes.

We report here that DML, as an antagonist of MR, effectively ameliorates the expression of obesity-related pro-inflammatory factors and lowers the blood glucose level. Our work demonstrates the potential of the marine natural product DML in the treatment of metabolic disorders.

Materials and methods

Materials

Cell culture plastic was purchased from Corning Inc (Corning, New York, USA). Dulbecco's modified Eagle's medium (DMEM), Eagle's minimum essential medium (MEM) and fetal bovine serum (FBS) were from Invitrogen. DML was synthesized as previously reported[11]. Aldo, corticosterone (Cort), Spir, dexamethasone (Dex) and 3-isobutyl-1-methylxanthine (IBXM) were obtained from Sigma-Aldrich. The RNAiso reagent kit, PrimeScript™ RT reagent kit and SYBR Premix Ex Taq Real-time PCR master mix were purchased from TaKaRa. Other solvents and reagents of analytical grade were purchased and used without further purification. HEK-293T (human embryonic kidney), 3T3-L1 (mouse embryo pre-adipose) and HepG2 (human hepatocellular carcinoma) cell lines were obtained from the American Type Culture Collection (ATCC numbers: CRL-11268, CL-173, and HB-8065). Male obese *db/db* mice were purchased from the Jackson Laboratory and bred to obtain eight-week-old male *db/db* mice.

The GFP-rat(r)MR vector was kindly donated by Dr Mayumi NISHI (Kyoto Prefectural University of Medicine, Japan). The expression plasmid pET28a-rMR-ligand binding domain $(LBD)_{aa.725\sim981}$ was constructed using GFP-rMR as the template. The fusion construct of pCMX-Gal4DBD-rMR-LBD was created using GFP-rMR as the template. The other reporter plasmids have been previously described[34, 35].

Protein preparation

For rMR-LBD expression, the recombinant plasmid pET28a-rMR-$LBD_{aa.725\sim981}$ was transformed into BL21(DE3) *E coli*, and the cells were grown in LB media at 37°C to an OD_{600} of approximately 1.0. Thereafter, the cells were pre-incubated with 50 μmol/L corticosterone for 30 min. The cells were then induced with 0.1 mmol/L isopropyl-β-*D*-thiogalactopyranoside (IPTG) for an additional 16 h at 16 °C. Then, 1 L of the induced culture was harvested and resuspended in 30 mL lysis buffer (25 mmol/L HEPES pH 8.6, 500 mmol/L NaCl, 10% glycerol, 3 mmol/L β-mercaptoethanol, 1 mmol/L PMSF, 0.5 mmol/L EDTA, 1 mg/mL lysozyme). The suspension was incubated at 4 °C with shaking for 60 min, followed by 5 min of sonication. The cell debris was pelleted by centrifugation at 13 000 r/min for 30 min and the supernatant was applied to a nickel column (Ni-NTA). The column was washed with 40 mL buffer A (25 mmol/L HEPES pH 7.5, 500 mmol/L NaCl, 40 mmol/L imidazole) and eluted with 30 mL buffer B (25 mmol/L HEPES pH 7.5, 500 mmol/L NaCl, 60 mmol/L imidazole). The eluted MR-ligand binding domain (MR-LBD) was concentrated to 2 mL and further purified through a Superdex-75 column.

Surface plasmon resonance (SPR)-based assays

The binding affinity of DML towards MR-LBD and the effect of DML on the MR-LBD/SRC-1 (steroid receptor coactivator-1) interaction were assayed using the SPR-based BIAcore 3000 instrument. Briefly, 0.1 mg/mL MR-LBD protein was immobilized on a chip, and different concentrations of DML were serially injected into the channel to evaluate binding affinity. To determine the effect of DML on the MR-LBD/SRC-1 interaction, $hSRC\text{-}1_{aa.613\text{-}773}$ protein was immobilized on a CM5 chip, and 0.2 mmol/L MR-LBD was pre-incubated with different concentrations of DML at 4 °C for 15 min. The protein-com-

pound mixture was then injected into the channel. Finally, the equilibrium dissociation constants (K_D) of the compound was obtained by fitting the data sets to a 1:1 Langmuir binding model using BIAevaluation software version 3.1 (BIAcore).

Cell culture

HEK-293T and 3T3-L1 cells were cultured in DMEM medium. HepG2 cells were cultured in MEM medium. All cells were cultured at 37 °C in media supplemented with 10% FBS and in a humidified atmosphere with 5% CO_2. Two days after confluence, differentiation of 3T3-L1 cells was induced by incubating the cells with a cocktail of 1.5 μg/mL insulin, 1 μmol/L Dex, and 0.5 mmol/L IBMX in DMEM supplemented with 10% FBS for 48 h. The culture medium was replaced every 48 h with DMEM supplemented with 10% FBS and 1.5 μg/mL insulin.

Transfection and luciferase assays

Transient transfections were carried out in 24-well plates. HEK-293T cells were transfected using a calcium phosphate cell transfection kit (Beyotime, Haimen, China) according to the manufacturer's protocols. The Renilla luciferase reporter pRL-SV40 was used to normalize the transfection efficiency. After a 6 h transfection, the medium was replaced with fresh phenol red-free DMEM supplemented with 5% charcoal/dextran-stripped FBS, and the cells were further stimulated with vehicle (DMSO) or compound(s) for another 18 h. Finally, the cells were lysed, and luciferase activities were measured using a Dual Luciferase Assay System kit (Promega, Madison, Wisconsin, USA).

Gene silencing with siRNA

Differentiated 3T3-L1 cells (or HepG2 cells) were transfected with MR ON-TAGERplus SMARTpool siRNA using Thermo Scientific DharmaFECT Transfection Reagent 3 (or Reagent 4 for HepG2 cells) according to the supplier's protocol (Thermo Fisher, San Jose, CA, USA). After 48 h, the cells were stimulated by 0.2 mmol/L H_2O_2 for another 24 h and co-incubated with compounds (10 μmol/L DML or 10 μmol/L Spir). The cells were subsequently collected and analyzed by real-time RT-PCR.

Real-time RT-PCR

Total RNA was extracted using the RNAiso reagent kit (TaKaRa). The reverse transcription reaction was performed using the PrimeScript™ RT reagent kit (TaKaRa, Otsu, Shiga, Japan). The real-time PCR product was detected using SYBR Green real-time PCR master mix (TaKaRa, Otsu, Shiga, Japan) with the DNA Engine Opticon 2 System (Bio-Rad Laboratories, Richmond, CA, USA). The data were taken from triplicate experiments. The relative mRNA levels were normalized to 18S RNA or GAPDH. PCR primer pairs are listed in Table S1 (supplementary data).

Animal procedures

Male obese *db/db* mice, 8 weeks of age, were acclimated to SPF microisolators for 2 d before any experimental intervention. The mice were treated with DML (30 mg/kg) or vehicle (sterilized 0.9% sodium chloride containing 5% Tween-80) daily *via* intragastric administration for 4 weeks (n=8 for each group). Each mouse was weighed twice a week. Blood glucose levels were detected weekly after a 6 h fast, and blood samples were collected from the tail vein. After euthanasia, the serum was collected and prepared for the biochemical assays. The white fat weight was recorded, and the tissues were frozen for further experiments. All animal experiments were approved by the Animal Ethics Committee of Shanghai Institute of Materia Medica.

Insulin tolerance test (ITT)

In the insulin tolerance test, the mice were fasted for 6 h and then were injected intraperitoneally with insulin (1.5 U/kg). Blood samples were collected from the tail vein at 0, 15, 30, 60, 90, and 120 min after injection. The area under the curve (AUC) was calculated using GraphPad Prism software to evaluate the insulin sensitivity.

Protein concentration detection and ELISA assay

Tissue homogenates were prepared using an ultrasonic method in RIPA lysis buffer (Thermo Fisher, San Jose, CA, USA). The total protein concentrations of the tissue lysates were determined by using a BCA kit (Thermo Fisher, San Jose, CA, USA). The lysates were used to measure the protein levels of TNFα and IL-6 according to the procedures of the mouse TNFα and IL-6 ELISA kits (Invitrogen, Carlsbad, CA, USA). The concentrations of TNFα and IL-6 were then normalized to the total protein concentration.

ROS activity measurement

The ROS levels of the tissue samples were evaluated using the DCFH-DA probe-based ROS kit (Beyotime, Haimen, China) according to the manufacturer's protocols. The activity of ROS was calculated and normalized to the total protein concentration.

Statistical analysis

The results are presented as the mean±SEM for at least three separate experiments for each group unless otherwise specified. Differences between groups were examined for statistical significance using Student's t-test, one-way or two-way ANOVA. P<0.05 was used to indicate a statistically significant difference.

Results

DML effectively ameliorated the H_2O_2-induced inflammatory actions

As reported, chronic inflammatory reactions play a key role in linking obesity to insulin resistance, and the attenuation of pro-inflammatory factors and/or ROS-related genes has been confirmed to ameliorate metabolic disorders and insulin resistance. Therefore, we evaluated the anti-inflammatory activities of the compounds using H_2O_2 stimulation.

In the assay, differentiated 3T3-L1 or HepG2 cells incubated

with compound(s) (10 μmol/L) or DMSO (0.1% *v/v*) were stimulated with 0.2 mmol/L H_2O_2 for 24 h. Total mRNA was then isolated and used for detecting the pro-inflammatory cytokine mRNA levels. We discovered that DML from *C manghas* effectively reduced the gene expression of H_2O_2-induced pro-inflammatory factors in both cell lines.

As shown in Figure 1, the mRNA levels of MCP-1, TNFα and IL-6 were significantly up-regulated by H_2O_2 stimulation in both 3T3-L1 and HepG2 cells. In 3T3-L1 cells, DML treatment efficiently reversed the elevations in MCP-1 and IL-6, but had no effect on TNFα (Figure 1B). However, in HepG2 cells, DML decreased the stimulation of both MCP-1 and TNFα without influencing IL-6 (Figure 1C).

Because H_2O_2 increases ROS levels and ROSs are produced by NADPH oxidase, we detected the mRNA levels of NADPH oxidase p47 subunit and its relevant transcription factor PU.1. The results demonstrated that the increases in the H_2O_2-induced p47 and PU.1 mRNA levels were abolished by DML treatment in these two cell lines (Figures 1B and 1C). Taken together, these results indicated that DML exhibited anti-inflammatory activity in HepG2 and 3T3-L1 cells.

DML exerted anti-inflammatory activity via MR modulation

We previously determined that DML antagonized the corticosterone-mediated interaction between the MR-LBD and SRC-1 in a yeast two-hybrid system. Considering that a MR antagonist could regulate obesity-related pro-inflammatory adipokine gene expression, we next investigated whether the effect of DML on the inflammatory response is mediated through MR.

To further identify the antagonism of DML against MR, SPR technology was used to determine its binding affinity. After MR-LBD was immobilized on the CM5 chip, serial concentrations of DML were injected automatically. The binding signals were continuously recorded in response units (RUs) and presented graphically as a function of time. As shown in Figure 2A, DML bound to MR-LBD in a concentration-dependent manner. The binding kinetic constants were fitted using the 1:1 (Langmuir) binding model contained in the BIAcore evaluation software, resulting in a K_D value of 2.29 μmol/L (Table 1). Then, the antagonistic activity of DML against the interaction between SRC-1 and MR-LBD was further verified by an SPR-based assay. In the assay, purified SRC-1 protein was immobilized on a CM5 chip, and MR-LBD at various concentrations (ranging from 0.03 to 0.48 μmol/L) was injected to interact with SRC-1. As shown in Figure 2B, the RU values evaluating MR-LBD binding to the immobilized SRC-1 revealed an obvious concentration-dependent manner, while such an interaction was strongly inhibited by the treatment of MR-LBD (0.2 μmol/L) pre-incubated with serial concentrations of DML (Figure 2C).

In addition, to determine the cellular effects of DML as a MR antagonist, we performed a mammalian one-hybrid assay.

Table 1. SPR-based kinetic analyses of DML binding to MR-LBD.

k_{on} [(mol/L)$^{-1}$·s^{-1}]	k_{off} (s^{-1})	K_D (mol/L)	χ^2
5.74	1.31×10^{-5}	2.29×10^{-6}	2.6

k_{on}, association rate constant; k_{off}, dissociation rate constant; K_D, equilibrium dissociation constant. $K_D=k_{off}/k_{on}$; χ^2, statistical value.

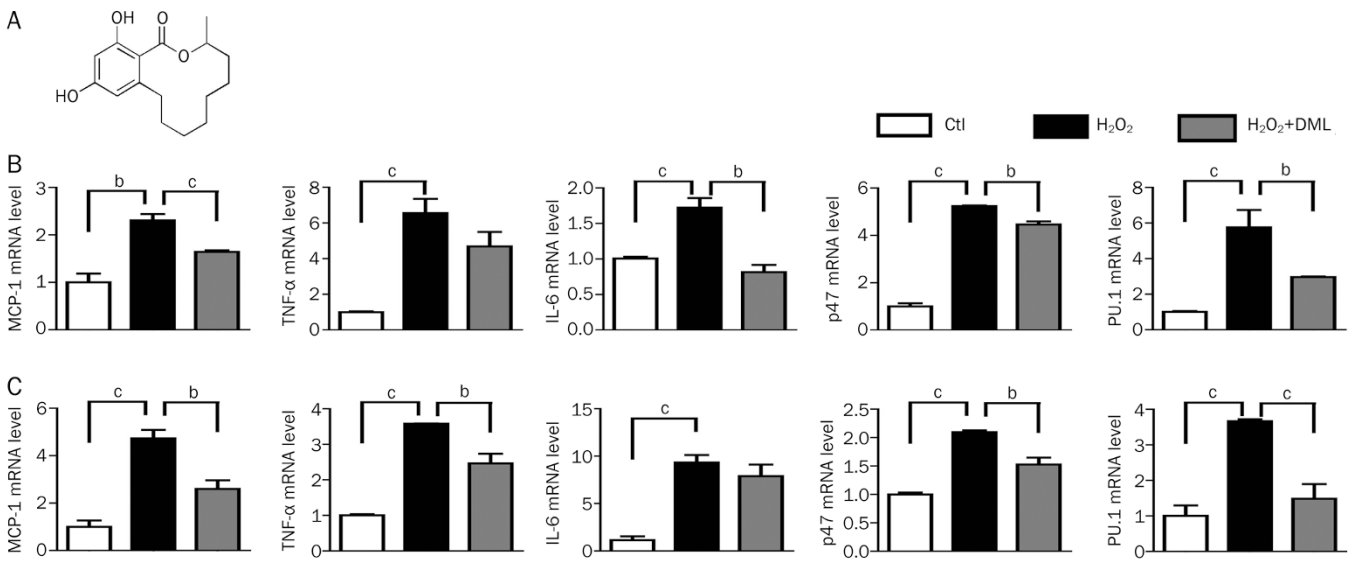

Figure 1. Regulation of the H_2O_2-stimulated pro-inflammatory factors and ROS-related genes by DML in 3T3-L1 adipocytes and HepG2 cells. (A) Chemical structure of DML (des-*O*-methyllasiodiplodin). (B) Differentiated 3T3-L1 cells or (C) HepG2 cells were treated with DMSO (labeled as Ctl), H_2O_2 (0.2 mmol/L) alone or simultaneously with DML (10 μmol/L) for 24 h. The treated cells were collected for RT-PCR analysis. The mRNA levels of MCP-1, TNF-α, IL-6, NADPH oxidase subunit p47 and PU.1 were detected. Their relative changes were normalized to GAPDH (for 3T3-L1 cells) or 18S RNA (for HepG2 cells) levels. The values are presented as the mean±SEM of three independent experiments. $^bP<0.05$, $^cP<0.01$, vehicle=1.

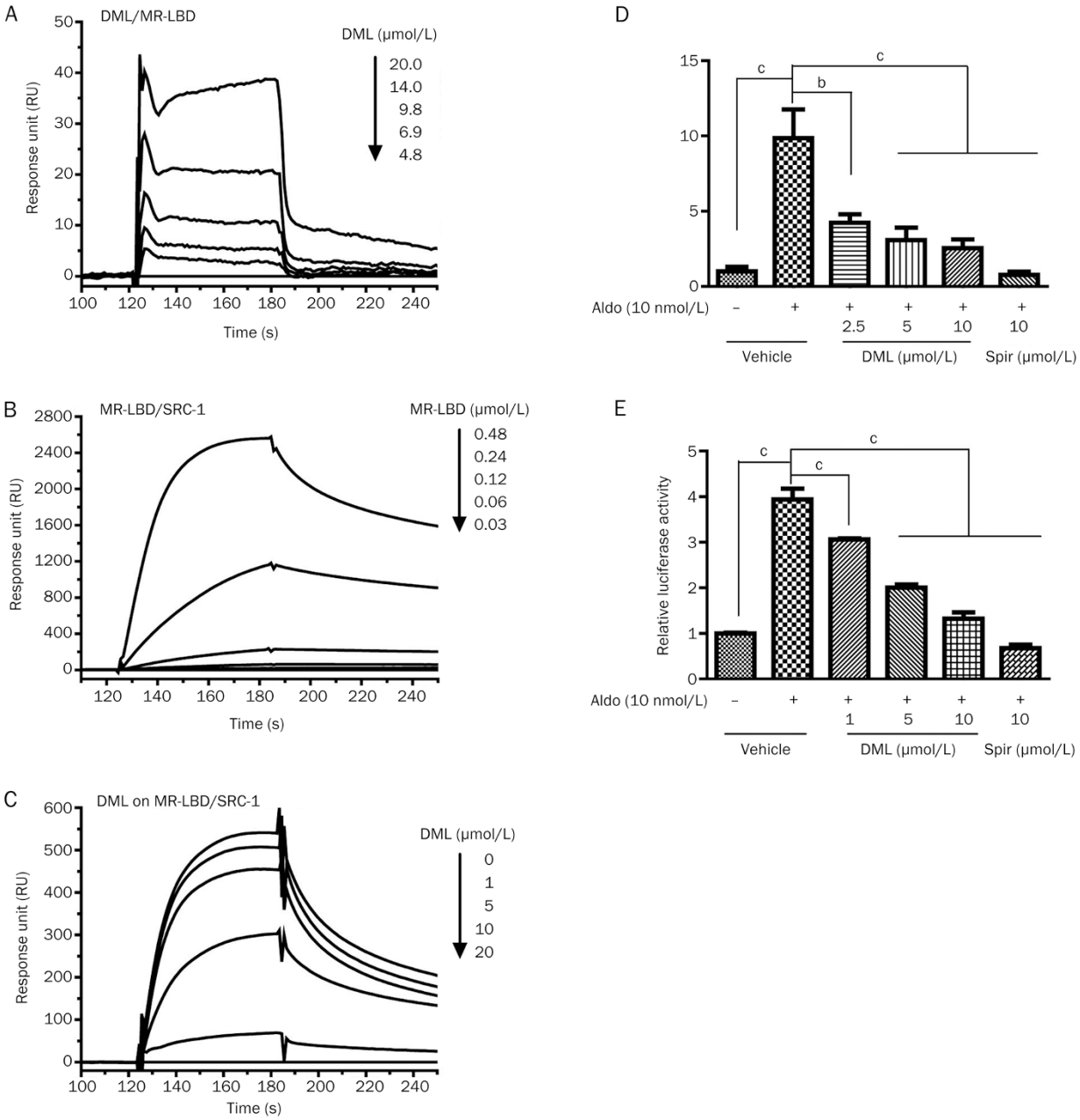

Figure 2. Effects of DML on MR. SPR-based assays were performed to show (A) the dose-dependently binding of DML to MR-LBD; (B) the interaction between MR-LBD and SRC-1; (C) the inhibition of DML to the interaction between MR-LBD and SRC-1. (D) MR antagonism of DML was evaluated by a mammalian one-hybrid assay. HEK-293T cells were transiently transfected with pCMX-Gal4-MRLBD, UAS-TK-Luc and pRL-SV40 plasmids, and treated with various concentrations of DML in the presence of Aldo (10 nmol/L) for 16 h, with Spir (10 μmol/L) as a control. (E) Cellular effects of DML as a MR antagonist were detected by a MR response element (MRE)-driven reporter gene assay. The MRE sequence was 5′-TGTACAGGATGTTCT-3′. HEK-293T cells were transiently transfected with GFP-rMR, pGL3-GRE/MRE, and pRL-SV40 plasmids, and treated accordingly. The data are presented as the mean±SEM of three independent experiments. $^{b}P<0.05$, $^{c}P<0.01$, vehicle=1.

HEK-293T cells were transiently co-transfected with pCMX-Gal4(DBD)-MR(LBD) fusion vector and UAS-TK-Luc reporter. The transfected cells were then incubated with vehicle, DML or Spir (MR positive antagonist) for 18 h in the presence of 100 nmol/L Aldo. As shown in Figure 2D, 10 nmol/L Aldo stimulated an approximate 8-fold increase in luciferase activity, which was completely abolished by treatment with 10 μmol/L Spir. Similarly, DML caused a significant decrease in the luciferase activity induced by Aldo in a dose-dependent manner. Subsequently, we identified the inhibitory activity of DML on MR transcription activation by a MR response element (MRE)-driven reporter gene assay. HEK-293T cells were transiently co-transfected with GFP-rMR and pGL3-MRE-Luc, and treated with DML, Spir or vehicle in the presence of 10

nmol/L Aldo. As indicated in Figure 2E, Aldo caused a 4-fold increase in MR transcriptional activity, while DML caused a similar decrease in a dose-dependent manner. We also performed the transcription activity assay in HepG2 cells, and similar results were found (data not shown).

In addition, we attempted to investigate whether the anti-inflammatory action of DML was dependent on its MR antagonistic activity in differentiated 3T3-L1 and HepG2 cells. In the assay, Aldo was used as a pro-inflammatory stimulus and Spir as a control. Meanwhile, RNA interference for the MR knockdown assay was also addressed. 3T3-L1 or HepG2 cells were transfected with control-siRNA or MR-siRNA for 48 h, followed by stimulating with 10 nmol/L Aldo for another 24 h, while co-incubating with 10 μmol/L DML, 10 μmol/L Spir or DMSO. Total mRNA was isolated and used for detecting the expression of pro-inflammatory cytokines and ROS-related genes.

In 3T3-L1 cells, as expected, Aldo significantly stimulated MCP-1, TNF-α, IL-6, p47, and PU.1 gene expression approximately 5-fold, 4-fold, 3.5-fold, 1.8-fold, and 3.4-fold, respectively (Figures 3B–3F). Both Spir and DML could efficiently reverse the Aldo-induced increases in gene expression (Figures 3B–3F). MR-siRNA decreased MR abundance by 70% (Figure 3A) and reversed the related Aldo-induced gene expression (Figures 3B–3F), which was in good accordance with published results. After MR knockdown, DML reduced none of the tested ROS related genes and pro-inflammatory factors except IL-6 (Figures 3B–3F), implying that other mech-

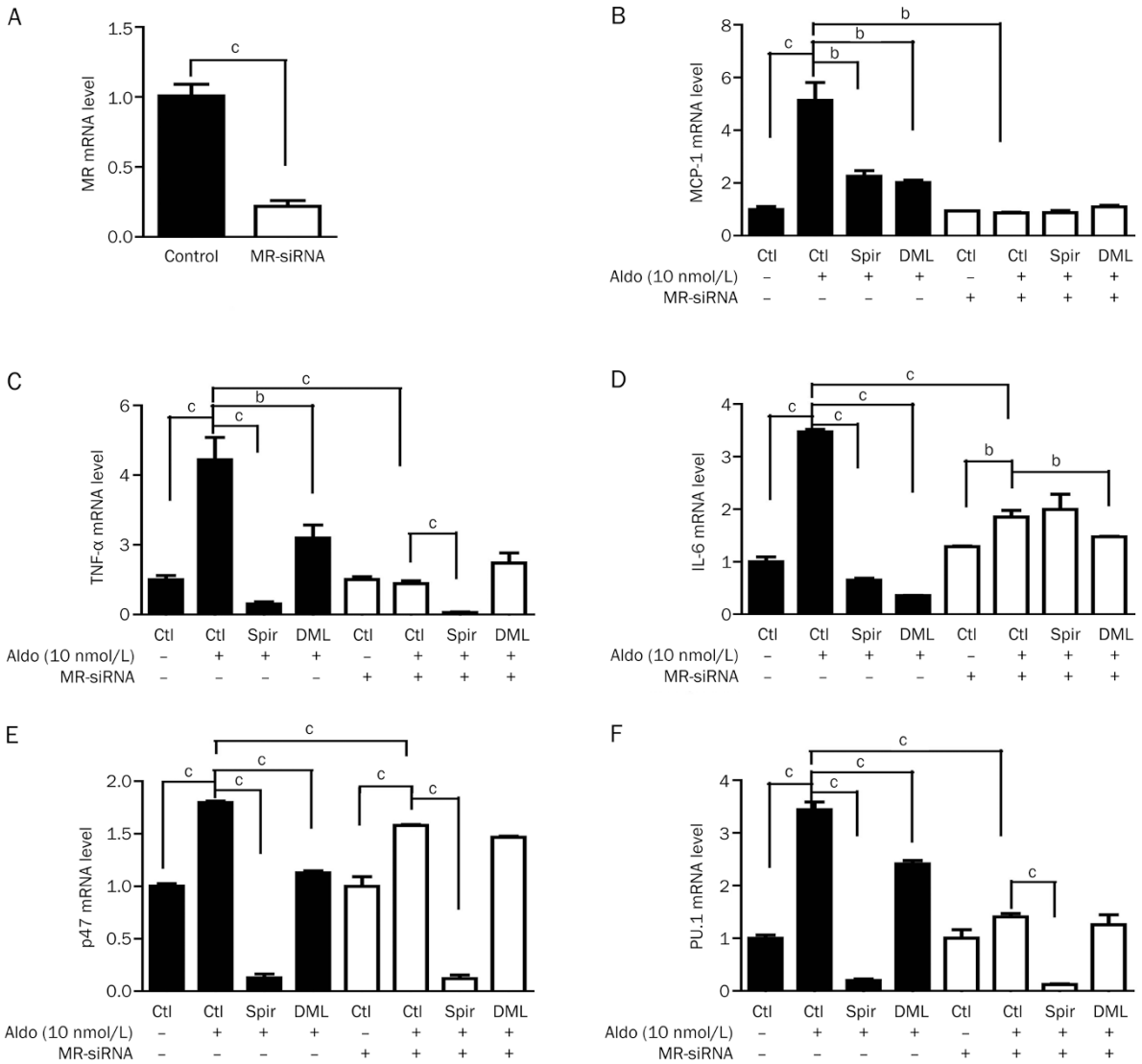

Figure 3. Regulation of Aldo-induced increases in pro-inflammatory factors and ROS-related genes by DML in 3T3-L1 adipocytes. Differentiated 3T3-L1 cells transfected with MR siRNA or control were cultured for 48 h and treated with DMSO (labeled as Ctl), Aldo (10 nmol/L) alone or simultaneously with DML (10 μmol/L) or Spir (10 μmol/L) for an additional 24 h. The treated cells were collected for RT-PCR analysis. The mRNA levels of (A) MR, (B) MCP-1, (C) TNFα, (D) IL-6, (E) NADPH oxidase subunit p47, and (F) PU.1 were detected. Their relative changes were normalized to GAPDH levels. The values are presented as the mean±SEM of three independent experiments. $^{b}P<0.05$, $^{c}P<0.01$. Ctl (control siRNA without Aldo treatment)=1.

anisms were involved. However, unlike DML, Spir exerted no influence on MCP-1 and IL-6, while it continued to strongly suppress TNF-α, p47 and PU.1 gene expression, implying a MR-independent mechanism in the regulation of these three genes.

In the same way, in HepG2 cells, Aldo also caused significant increases of these genes. MCP-1 and IL-6 mRNA levels increased more than 10-fold. Spir was able to reverse all the changes in gene expression that were stimulated by Aldo, and DML could also attenuate most of the Aldo-stimulated increases in gene expression, except for IL-6 (Figures 4B–4F). Likewise, this result identified the anti-inflammatory actions of DML. A similar MR-siRNA interference assay was also carried out in HepG2 cells. MR abundance was decreased by 70% with MR-targeted siRNA (Figure 4A). With MR knockdown, Aldo no longer promoted MCP-1 and IL-6 gene expression. Consequently, Spir and DML could no longer reverse the Aldo-stimulated effects (Figures 4B and 4D). These data supported that Aldo-induced MCP-1 and IL-6 gene expression was primarily MR-dependent. Similarly, Aldo-induced TNFα, p47 and PU.1 gene expression was also decreased by MR siRNA interference. In this case, neither Spir nor DML could change the mRNA levels of these three genes (Figures 4C, 4E, and 4F).

Taken together, the results showed that the Aldo-induced inflammatory response was primarily MR-dependent. Lower MR activity or MR abundance could reverse the Aldo-mediated inflammatory response. DML ameliorated Aldo-induced

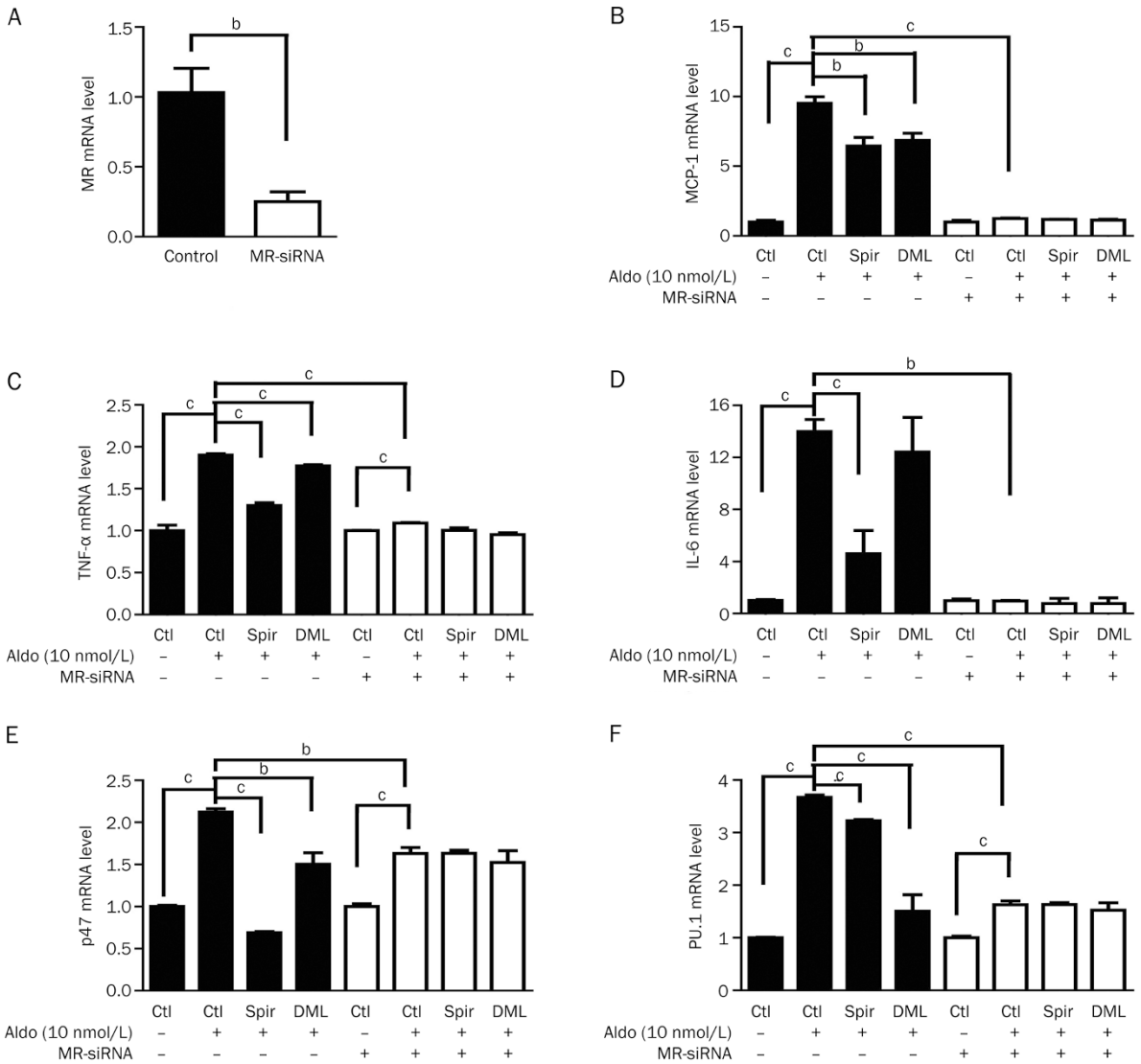

Figure 4. Regulation of the Aldo-stimulated pro-inflammatory factors and ROS-related genes by DML in HepG2 cells. HepG2 cells transfected with MR siRNA or control were cultured for 48 h and then treated with DMSO (labeled as Ctl), Aldo (10 nmol/L) alone or simultaneously with DML (10 μmol/L) or Spir (10 μmol/L) for another 24 h. The treated cells were collected for RT-PCR analysis. The mRNA levels of (A) MR, (B) MCP-1, (C) TNFα, (D) IL-6, (E) NADPH oxidase subunit p47, and (F) PU.1 were detected. The relative changes were normalized to 18S RNA levels. The values are presented as the mean±SEM of three independent experiments. $^{b}P<0.05$, $^{c}P<0.01$. Ctl (control siRNA without Aldo treatment)=1.

inflammation primarily via the MR-dependent pathway.

DML improved blood glucose and regulated glucose metabolism-related genes in *db/db* mice

As reported, MR antagonist Eple reduced the high levels of plasma glucose in diabetic mice. For this reason, we investigated the blood glucose levels in *db/db* mice. The results presented in Figure 5A suggested that DML treatment effectively reduced blood glucose within 4 weeks. In addition, the lowered glycosylated hemoglobin (HbA1c) level (Figure 5B) of the DML-treated group suggested its long-term action in blood glucose control. The ITT assay was also carried out to investigate the improvement of DML on insulin tolerance. As implied from Figure 5C and 5D, DML administration effectively ameliorated insulin resistance in *db/db* mice.

The body and fat weights, the serum insulin, lipid, AST (aspartate aminotransferase) and ALT (aminoleucine transferase) levels were also determined in *db/db* mice. Compared with the vehicle group, no changes in these indexes were observed in the DML group (Figure S1 A–I, supplementary data). Interestingly, it was noted that the triglyceride and cholesterol levels in the liver were reduced (Figure S1 J and K). In addition, histological examination of the liver and adipose tissues was carried out. In the liver, the vehicle group exerted some red-stained cytoplasm, while the DML-treated group was normal. No obvious morphological changes were detected in the adipose tissue (Figure S2).

Considering the potential role of a MR antagonist in controlling glucose metabolism, we also tested the mRNA expression of the genes involved in hepatic glucose output, including glucose-6-phosphatase (G6Pase), phosphoenolpyruvate carboxykinase (PEPCK) and the transcription factor PPARγ coactivator 1α (PGC1α) in mice livers. As expected, we found that DML decreased the expression of these three genes (Figure S3).

DML effectively regulated pro-inflammatory factors in *db/db* mice

After identifying the anti-inflammatory effect of DML in cultured cell lines, we studied its corresponding effects in *db/db* mice. After mice were sacrificed, mRNA isolated from epididymal fat tissues was used for detecting the gene transcription levels of pro-inflammatory cytokines. As expected, DML decreased the mRNA levels of MCP-1, TNFα and IL-6 by 59%, 47%, and 50%, respectively (Figures 6A–6C). Meanwhile, the expression of p47 subunit (Figure 6D) and PU.1 (Figure 6E) were decreased by 52% and 62% in the DML-treatment group.

Similarly, we evaluated the anti-inflammatory activity of DML in mice livers. As indicated in Figures 6F and 6G, compared with the vehicle group, the expression of MCP-1 and TNFα genes were markedly decreased, by 67% and 64%, respectively, in the DML-treated group. Unexpectedly, DML exhibited no effects on IL-6 gene expression (Figure 6H). However, the expression of p47 and PU.1 decreased approximately 13% and 45%, respectively, after treatment with DML (Figures 6I and 6J).

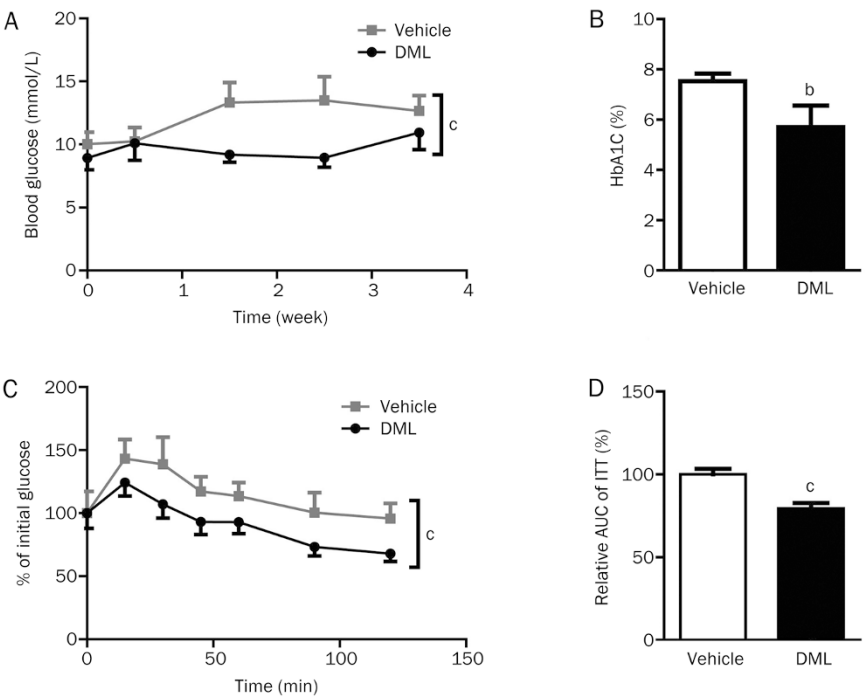

Figure 5. Amelioration of DML on blood glucose in *db/db* mice. (A) Blood glucose levels during the experiment. The values are the mean±SD. n=8 for each group. Two-way ANOVA was used. The P value=0.0042<0.01. (B) The serum HbA1c levels of each group. (C) The percent changes of initial blood glucose levels during the insulin tolerance test (ITT). Two-way ANOVA was used. The P value=0.0022<0.01. (D) The relative AUC for glucose level during ITT. The values are the mean±SEM; t-test was used unless indicated otherwise. ^bP<0.05, ^cP<0.01 *vs* vehicle.

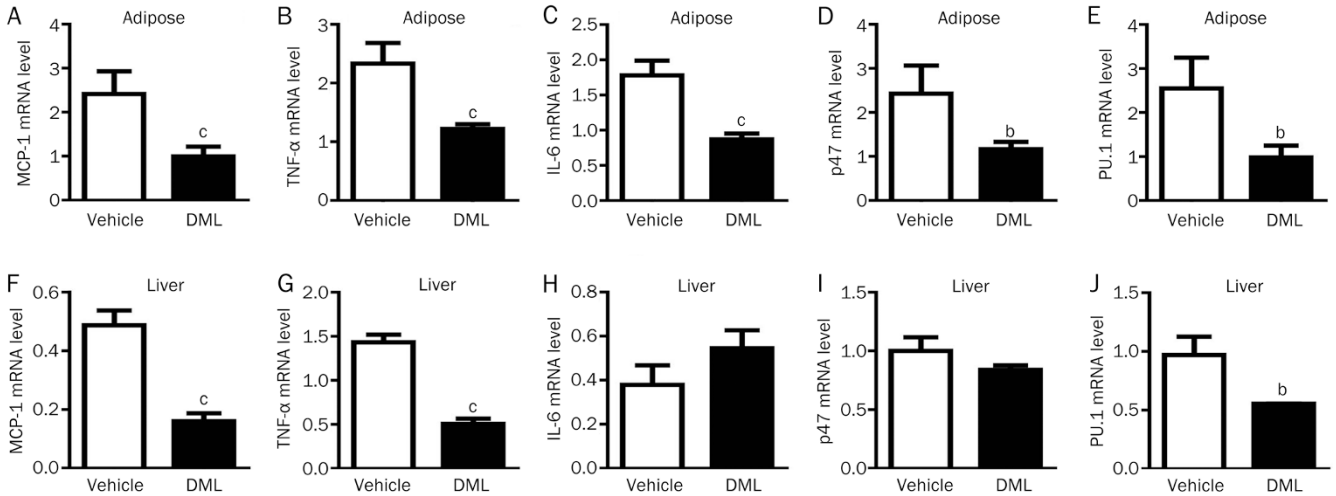

Figure 6. Decreases in pro-inflammatory factors and ROS-related gene expression by DML in *db/db* mice. The mRNA levels of MCP-1, TNFα, IL-6, NADPH oxidase subunit p47 and PU.1 were detected in *db/db* mice (A–E) adipose and (F–J) liver tissues by RT-PCR. The relative changes were normalized to GAPDH RNA levels. The values are presented as the mean±SEM of three independent experiments. $^{b}P<0.05$, $^{c}P<0.01$ *vs* vehicle.

Finally, we evaluated the protein levels of TNFα and IL-6 and the activity of ROS in the two tissues. In the liver, decreases in TNFα level and ROS activity were observed in the DML-treated group, but IL-6 was unaffected (Figure 7A–7C). In adipose tissue, both TNFα and IL-6 levels decreased with DML treatment, as well as the ROS activity (Figure 7D–7F). These results further demonstrated the anti-inflammatory action of DML *in vivo*.

Discussion

Diabetes and related obesity are associated with oxidative stress and inflammatory response, while pro-inflammatory factors such as TNFα and IL-6 contribute to insulin resistance by disturbing insulin signal transduction[36, 37]. In addition, NADPH oxidase-induced highly toxic ROS may cause tissue injury during inflammation[37]. In this respect, the transcription factor PU.1 is essential for NADPH oxidase subunit p47 promoter activity[38], and PU.1 and p47 subunit expression is closely involved in ROS generation[33]. Here, we discovered that the marine natural product DML showed effective anti-inflammatory activity, as indicated by its regulation of TNFα, MCP-1, IL-6, PU.1 and NADPH p47 subunit genes, both *in vitro* and *in vivo*. Moreover, we found that DML ameliorated inflammatory action through the MR-mediated pathway.

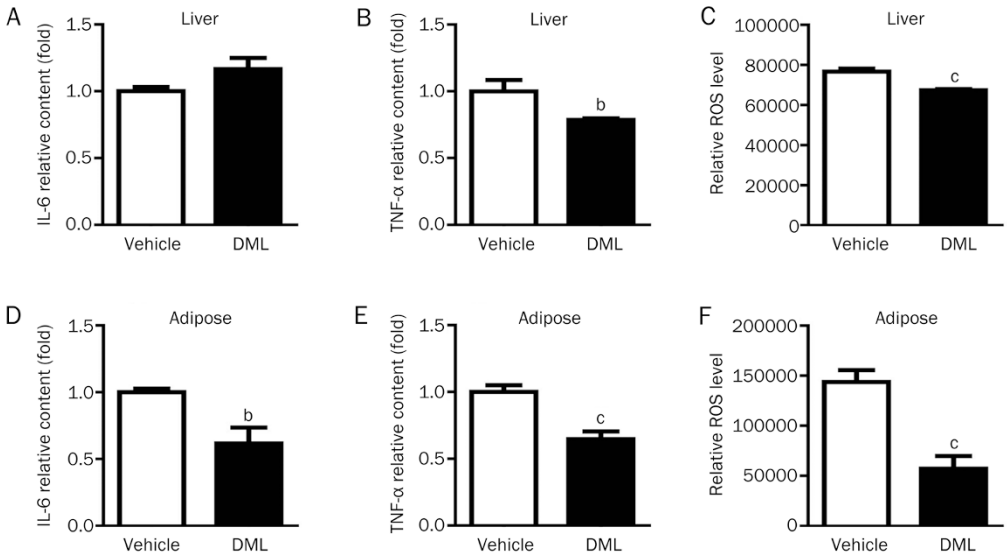

Figure 7. Regulation of TNF-α and IL-6 protein levels and ROS activity by DML in *db/db* mice. The protein levels of TNF-α and IL-6 were detected using an ELISA kit. ROS activity was measured by the DCFH-DA probe-based assay as described in the Materials and Methods section. The liver (A–C) and adipose (D–F) tissues were collected and analyzed. The values are presented as the mean±SEM of three independent experiments. $^{b}P<0.05$, $^{c}P<0.01$ *vs* vehicle.

MR is a metabolic nuclear receptor that plays a key role in the regulation of electrolyte homeostasis and blood pressure in the body[12]. MR activation mediates inflammation, proliferation and fibrosis[29, 39, 40], while MR blockade exhibits beneficial effects on cardiovascular morbidity and mortality in patients with heart failure[41, 42]. Recent studies have also shown that MR antagonists improve adipocyte dysfunction and insulin resistance in obese mice[31–33]. The two MR antagonists, Spir and Eple, have been used clinically as antihypertensive drugs[41, 42]. These antagonists regulate pro-inflammatory adipokines and ROS-related genes to ameliorate adipocyte dysfunction. However, Spir causes undesirable side effects because of its poor selectivity[41], while Eple is less potent than Spir[42]. Therefore, discovering novel non-steroidal specific small-molecule ligands of MR is of great interest in diabetes therapy. Fortunately, DML was identified as a MR antagonist exerting obvious anti-inflammatory action.

Here, we investigated the anti-inflammatory action of DML with two independent inflammatory stimuli[33]. Most of the elevations by H_2O_2 were obviously reversed by DML treatment, except for the regulation of TNF mRNA by DML (in 3T3-L1 cells, Figure 1B) and IL-6 mRNA (in HepG2 cells, Figure 1C). Interestingly, the change in TNF induced by H_2O_2 (approximately 6-fold) in 3T3-L1 cells was much stronger than the change in MCP-1 and IL-6 (2.4- and 1.7-fold), and a similar result for IL-6 (approximately 10-fold) was also obtained (MCP-1 and TNF were 3.5- and 5-fold) in HepG2 cells. DML failed to reverse the strong increases in corresponding cells.

In addition, we found that the changes in the H_2O_2-induced gene expression were mild after MR-siRNA interference in both 3T3-L1 and HepG2 cells. Once MR was deficient, the anti-inflammatory action of DML was abolished (Figure S4), implying that the reversal of DML on pro-inflammatory changes occurred through regulating MR.

Moreover, Aldo-induced elevations were mostly interrupted by DML or Spir treatment (Figures 3 and 4). However, both of these increases by Aldo and the reverses by DML were significantly attenuated under MR knockdown, except for IL-6. Therefore, we suggest that DML functions in a MR-dependent manner, and either down-regulation of MR activity or MR expression could ameliorate inflammatory action. Unexpectedly, Spir exhibited strong effects in decreasing TNFα, p47, and PU.1 gene expression with or without MR siRNA interference in 3T3-L1 cells but not in HepG2 cells. Previous results showed that the inflammatory action of Aldo was initiated after stimulation independently of MR transcription[43]. Considering that the non-MR action of Aldo might function in the pro-inflammatory reaction, we also performed a MR-siRNA interference experiment in the Aldo model to compare the anti-inflammatory effect of DML to the non-genomic action of Aldo.

In this experiment, we found that most of the Aldo-induced changes could not be reversed by DML with MR deficiency. As we know, Spir could regulate several nuclear receptors[41], and their actions against inflammatory reactions are different. Thus, its reductions in TNFα, p47 and PU.1 gene expression suggested its complicated mechanism. In addition, the different characteristic of gene expression[44] might be the reason for the different effects in the two cell lines.

As reported, mRNA levels related to ROS and pro-inflammatory factors in *db/db* mice were higher than in normal mice[33]. MR blockade could potently reverse these changes. Our work suggested that DML showed an anti-inflammatory effect in *db/db* mice due to its MR antagonistic activity, and further regulated glucose metabolism. DML potently reduced mRNA levels of PEPCK, G6Pase and PGC1α in *db/db* mice livers (Figure S3, supplementary data), consistent with the previous result that MR blockade decreased the hepatic glucose production genes G6Pase and PEPCK expression[31, 45]. The decreased level of gluconeogenesis in diabetic mice was helpful in lowering fasting blood glucose[46]. However, due to the complicated links between glucose and lipid metabolism[47], further studies are needed to elucidate the regulatory mechanism of MR against PEPCK and G6Pase. Tentatively, we hypothesize that the DML-exerted functions might be due to its antagonism of MR. In summary, our work has reinforced the conclusion that DML could be used as a promising drug lead compound against diabetes-related metabolic dysfunction.

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Author contribution

Prof Xu SHEN, Yue-wei GUO, and Li-li CHEN designed the research; Cheng-shi JIANG and Jing-xu GONG synthesized the compound DML; Rong ZHOU and Zhong-hui LIN studied the antagonism of DML against mineralocorticoid receptor; Rong ZHOU also performed related assays to reveal the anti-inflammatory action of DML; Yue-wei GUO, Li-li CHEN, and Xu SHEN supervised the project; Xu SHEN, Li-li CHEN, and Rong ZHOU contributed to the writing of the manuscript.

Supplementary information

Supplementary information is available at the Acta Pharmacologica Sinica website.

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