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Original Article

Zoledronic acid induces a brown-like phenotype in white adipocytes

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Abstract: This study evaluated whether zoledronic acid (ZOL), which enhances bone mineral density, has a browning effect on white adipocytes. Oil Red O staining showed that ZOL ameliorated accumulation of lipid droplets in white adipocytes, and immunohistochemistry analysis further confirmed enhanced expression of the brown adipocytes-specific marker uncoupling protein 1 (UCP1). ZOL significantly increased expression of the *CIDEA* ($P < 0.05$) gene as well as the protein levels of peroxisome proliferator-activated receptor gamma coactivator 1α (PGC1α), PR domain containing 16 (PRDM16), and UCP1 (all $P < 0.05$), and slightly increased the gene expression level of PRDM16. Moreover, ZOL significantly inhibited aldosterone synthesis ($P < 0.05$), and treatment with eplerenone (an aldosterone inhibitor) markedly up-regulated gene expression levels of *CIDEA, PRDM16*, and *UCP1*. Overall, these results demonstrate that ZOL promotes a browning-like phenotype of white adipocytes, which is likely related to inhibition of aldosterone synthesis, highlighting a potential target for obesity treatment and prevention.

Keywords: Aldosterone, browning, white adipocytes, zoledronic acid

Introduction

Obesity, which involves excess accumulation of triglycerides in enlarged white adipocytes of white adipose tissue (WAT), is a prevalent risk factor for development of metabolic syndromes such as type 2 diabetes mellitus, hyperlipidaemia, hypertension, and atherosclerosis [1, 2]. Unlike white adipocytes, classical brown and beige adipocytes contribute to weight loss through their effects on energy expenditure and heat generation by increasing mitochondrial uncoupled respiration and ATP synthesis through the oxidation of free fatty acid (FFA), which is mediated by up-regulation of uncoupling protein 1 (UCP1) [3-5]. Classical brown adipocytes are only located in the brown adipose tissue (BAT) of infants, whereas beige adipocytes are sporadically distributed among the white adipocytes in adults [6].

Evidence from animal experiments demonstrates that under certain conditions, white adipocytes might differentiate into beige adipocytes, express a similar level of the brown adipocyte-specific protein marker UCP1, and stimulate UCP1-dependent thermogenic capacity [7]. Thus, determining the specific mechanisms and pathways involved in this differentiation could help to identify targets for treatment of obesity and related conditions. Such browning activation can be mediated through activation of the sympathetic nervous system. Norepinephrine, released from the sympathetic nervous system, binds to β3-adrenergic receptor, which activates cyclic adenosine monophosphate (c-AMP)-dependent protein kinase (PKA) [8, 9]. Peroxisome proliferator-activated receptor gamma coactivator 1α (PGC1α) and PR domain containing 16 (PRDM16) are important downstream targets of the β3-adrenergic/cAMP/PKA signalling pathway, and the functional consequences of this cascade result in significant up-regulation of UCP1 gene expression [10, 11]. Zoledronic acid (ZOL) is a nitrogen-containing bisphosphonate that is commonly used for treating osteoporosis since it blocks excessive bone resorption through down-regulating farnesyl diphosphate synthase (FPPS) by inhibiting the mevalonate pathway

[12]. By contrast, activation of the mevalonate pathway increases the synthesis of cholesterol [13], aldosterone precursor [14], and aldosterone-activated mineralocorticoid receptor (MR) to inhibit the expression of UCP1 through inhibiting the β3-adrenergic receptor pathway in white adipocytes [15-17]. Given this apparent link of the mevalonate pathway in osteoporosis and adipocyte differentiation, we hypothesized that ZOL might induce browning of white adipocytes.

To test this hypothesis, mouse mesenchymal cells (C3H10T1/2) and preadipocytes (3T3-L1) were stained with and without ZOL treatment by Oil Red O staining and immunohistochemistry to examine lipid deposition and the expression of brown adipocyte-specific markers, and screened for the relevant mechanisms and pathways involved in the observed effects by isobaric tags for relative and absolute quantitation (iTRAQ). Expression of browning-related molecules was validated through reverse transcription-quantitative polymerase chain reaction (qRT-PCR) and Western blotting.

Material and methods

Cell culture

C3H10T1/2 and 3T3-L1 cells were purchased from the Chinese Academy of Sciences (Shanghai, China). C3H10T1/2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin incubated in a 37°C, 5% CO_2 incubator. The cells were treated with bone morphogenetic protein-2 (BMP-2) to induce differentiation into the white adipocyte lineage. In brief, C3H10T1/2 cells were plated at a low density (20,000 cells/6-cm dish) and grown for 6 days until confluence in the presence of 50 ng/ml BMP-2. 3T3-L1 cells were propagated and maintained in DMEM containing 10% calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin incubated in a 37°C, 5% CO_2 incubator, and cell contact inhibition was applied for 2 days of culture. Adipocyte differentiation was then induced in both cell lines using DMEM containing 10% FBS, IBMX (0.5 mM), dexamethasone (250 nM), and insulin (1 μM) for 2 days, then fed with DMEM supplemented with 10% FBS and insulin (1 μM) for 2 days. When the cells showed obvious lipid accumulation, indicating successful differentiation into white adipocytes, they were treated with ZOL at serial concentrations (0, 1, 10, 25, 50, 100 nM) for 24 hours. The cells were then collected for western blotting analysis, qRT-PCR, and proteomics.

Oil Red O staining

The cells matured within 6-8 days, followed by washing with phosphate-buffered saline (PBS), and then fixed with 10% paraformaldehyde for 20 min at room temperature, and washed again with PBS. After washing the mixtures 2-3 times with deionized water, and the fat droplets in the cells were stained with 5% Oil Red O dye for 20 min and with 70% ethanol for colour separation, followed by hematoxylin redyeing and a phosphate rinse 2-3 times. The cells were sealed with gelatin and images were captured under Pannoramic Viewer.

Immunohistochemistry

Immunohistochemistry was performed to differentiate white and brown adipocytes using paraformaldehyde-fixed paraffin-embedded sections. The sections were stained with an anti-UCP1 (Proteintech 1:500) antibody and counterstained with haematoxylin.

Protein isolation, digestion, and labelling with iTRAQ reagents

Cells were lysed in RIPA lysis buffer, and broken by ultrasonic waves for 10 minutes on ice, and then cell debris were removed by centrifugation at 12,000 rpm for 30 minutes. The supernatant was collected, and the protein concentrations were determined with the BCA protein assay (Beyotime Biotechnology, Haimen, China).

For on-filter digestion, an aliquot of total protein (50 μg) was diluted to 100 μl with 0.5 M triethylammonium bicarbonate (TEAB), reduced with 5 mM tris (2-carboxyethyl) phosphine for 1 h at 55°C, and alkylated with 6.25 mM methyl methanethiosulfonate (MMTS) for 30 minutes at room temperature in the dark. The protein lysates were transferred to a 10k Vivacon filter following centrifugation at less than 12,000 rpm for 30 minutes to remove the solvent, and then washed three times with 0.5 M TEAB. Finally, trypsin was added onto the filter at a

Table 1. Sequences of primers for reference and adipogenic genes of C3H10T1/2 mice

Gene symbol	Upstream primer (5'-3')	Downstream primer (5'-3')
β-actin	TGTTACCAACTGGGAGGACA	GGGGTGTTGAAGGTCTCAAA
UCP1	AGGGTTCCAGTACCATTAGGT	CTGAGTGAGGCAAAGCTGATTT
PRDM16	CAGCACGGTGAAGCCATTC	GCGTGCATCCGCTTGTG
PGC1α	CCCTGCCATTGTTAAGACC	TGCTGCTGTTAATGTTTTC

proportion of 2% protein mass for the first digestion overnight and then at a proportion of 1% protein mass for a second digestion step at for 4 hours at 37°C.

The peptides were collected and labelled with iTRAQ Reagent-8 plex Multiplex Kit (AB Sciex UK Ltd.), according to the manufacturer's instructions. The peptides and labelled markers were as follows: three ZOL-treated samples were labelled with iTRAQ tag 116, iTRAQ tag 119, or iTRAQ tag 121; and three non ZOL-treated samples were labelled with iTRAQ tag 113, iTRAQ tag 114, or iTRAQ tag 115. All of the labelled samples were mixed at an equal amount. The labelled samples were fractionated using a high-performance liquid chromatography system (SHIMADZU) with a Durashell C18 column (5 μm, 100 Å, 4.6 × 250 mm). Ultimately, 12 fractions were collected for analysis.

Liquid chromatography-tandem mass spectrometry (LC MS/MS) analysis

Data were acquired with a Triple TOF 5600 System (AB SCIEX, Concord, ON, Canada). A 90-minute gradient from 2-30% [mobile phase A 0.1% (v/v) formic acid, mobile phase B 0.1% (v/v) formic acid, 5% (v/v) acetonitrile; 95% (v/v) acetonitrile] was used to chromatograph the samples after direct injection onto a 20-μm PicoFrit emitter (New Objective) packed to 12 cm with Magic C18 AQ 3 μm 120 Å stationary phase. The MS1 spectra were collected in the range of 350-1,500 m/z for 250 msec. The 20 most intense precursors with a charge state of 2-5 were selected for fragmentation, and MS2 spectra were collected in the range of 50-2,000 m/z for 100 msec. Precursor ions were excluded from reselection for 15 seconds.

The original MS/MS data were submitted to analysis by ProteinPilot Software (version 4.5, AB Sciex). MS/MS data were searched against the Mus musculus UniProt database (http://www.uniprot.org/proteomes/UP00000-0589) under the following search parameters: Triple-TOF 5600 instrument; iTRAQ quantification, cysteine modified with MMTS. T Biological modifications was selected as the ID focus, the quantitate was selected as trypsin digestion, and the Bias Correction and Background Correction option was selected for protein quantification and normalization. Automatic decoy database search strategy 77 was used to estimate the false discovery rate using the Proteomics System Performance Evaluation Pipeline Software algorithm. Only proteins with at least two unique peptides were considered for further analysis. After normalization, iTRAQ was used to measure the protein abundance ratios, and those with a p value < 0.05, and only fold changes > 1.5 or < 0.667 were considered significant.

Bioinformatics analysis

Expressed protein sequences identified to be differentially expressed were mapped with Gene Ontology terms (http://david.abcc.ncifcrf.gov). Kyoto Encyclopedia of Genes and Genomes (KEGG) was used to analyse the related pathways. Fisher's exact test determined the pathway enrichment statistics with a corrected p value < 0.05 considered to indicate the most significantly enriched pathways.

Western blotting

Cells were lysed in RIPA buffer with Protease Inhibitor Cocktail (Roche, Basel, Switzerland), and then proteins were extracted by centrifugation at 1200 rpm at 4°C for 20 minutes. Protein extracts were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore). The membrane was incubated with the following primary antibodies at 4°C overnight: rabbit polyclonal β-actin antibody (#4967, Cell Signaling Technology, Beverly, MA, USA), rabbit polyclonal UCP1 antibody (23673-1-AP, ProteinTech, Chicago, IL, USA), rabbit polyclonal PGC-1α antibody (ab54481, Abcam, Tokyo, Japan), and rabbit polyclonal

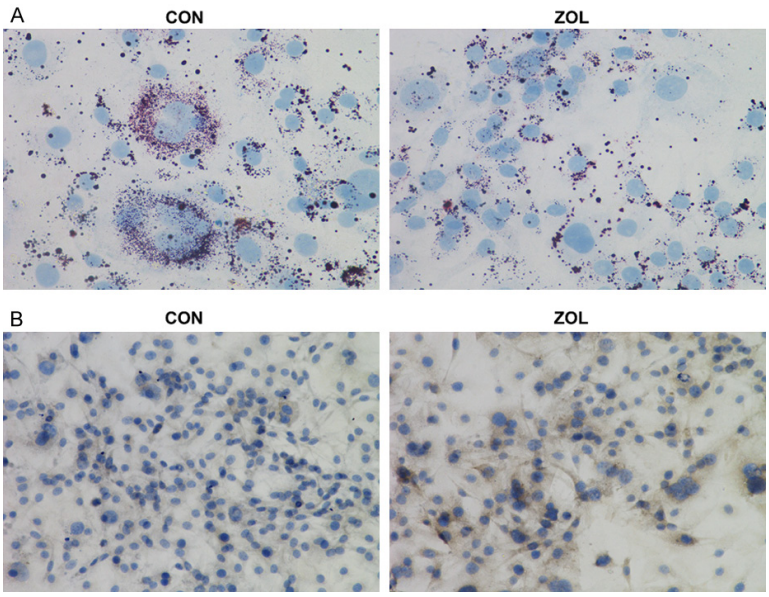

Figure 1. Effects of zoledronic acid (ZOL) on cellular lipid accumulation determined by Oil Red O staining (magnification 400×) (A) and expression of UCP1 protein by immunohistochemistry (B) in white adipocytes that were not treated (CON) or treated with ZOL (ZOL; magnification 200×). Representative images were selected for each group from three independent experiments.

PRDM16 antibody (ab106410, Abcam, Tokyo, Japan).

qRT-PCR

Total RNA from C3H10T1/2 and 3T3-L1 cells was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized from total RNA using a PrimeScript RT reagent Kit (Takara Bio, Otsu, Japan). Real-time PCR was performed on an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Grand Island, NY, USA) with SYBR Premix Ex Taq (Takara Bio). The primer sequences are listed in **Table 1**. The relative mRNA expression levels were calculated using the delta Cq method after normalizing to the level of β-actin, which was used as an internal control.

Measurement of aldosterone by enzyme-linked immunosorbent assay (ELISA)

Aldosterone was measured using an ELISA kit for mouse aldosterone (USCN Life Science Inc., Wuhan, China).

Statistical analysis

The data are expressed as the mean ± standard error of the mean. The differences among groups were analysed by one-way analysis of variance with the least significant difference or Dunnett T3 post-hoc comparison analysis, as appropriate. Values of $P < 0.05$ were considered statistically significant.

Results

ZOL elevated the expression of UCP1 protein in differentiated white adipocytes

Oil Red O staining demonstrated greater accumulation of lipid droplets in white adipocytes (**Figure 1A**). Importantly, immunohistochemistry analysis confirmed that ZOL elevated the expression of UCP1 protein, indicating a potential role in inducing the brown-like phenotype in white adipocytes (**Figure 1B**).

ZOL significantly upregulated the β-adrenergic receptor pathway

Of the total 3306 proteins quantified with iTRAQ-based proteomic analysis, 64 were up-regulated, including β-adrenergic receptor, PPARγ, and PGC1α (**Figure 2**).

ZOL increased the levels of genes and proteins associated with the browning of white cells

ZOL significantly enhanced the expression level of the brown fat-specific gene *CIDEA* (**Figure 3A**), and there was a slight increase in the expression level of *PRDM16* (**Figure 3B**). Brown fat-specific proteins (PRDM16, PGC1α, and UCP1) were also significantly up-regulated by ZOL in the white adipose tissue (**Figure 3C**).

ZOL inhibited aldosterone synthesis, and the MR antagonist up-regulated the β3-adrenergic receptor pathway

ZOL (1 nM) inhibited aldosterone synthesis, based on the reduced levels of intracellular aldosterone determined by ELISA (**Figure 4A**). ZOL treatment at 1 nM resulted in similarly up-regulated mRNA levels of *CIDEA, PRDM16*, and *UCP1* as detected with treatment of isoproterenol at 1 mM. Moreover, treatment with the MR

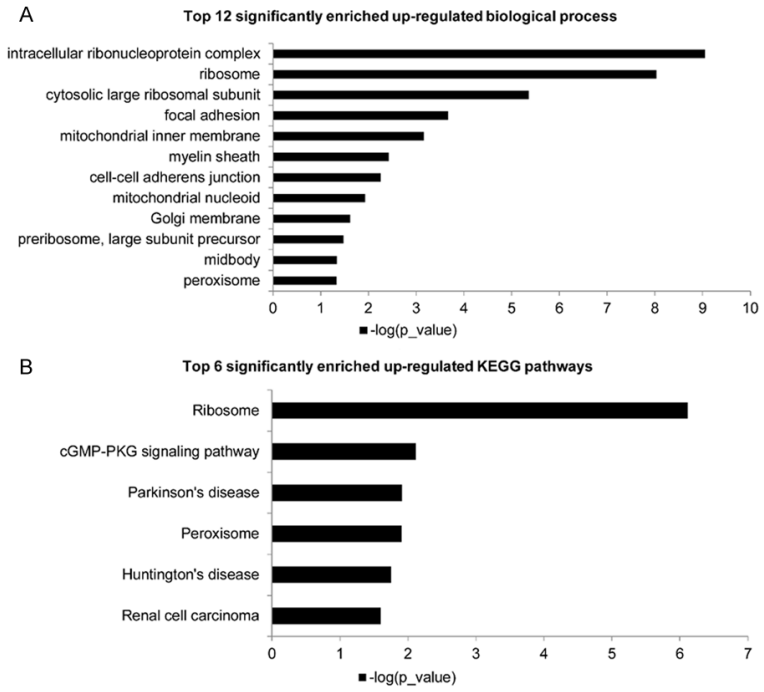

Figure 2. Gene Ontology and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment of 64 significantly dysregulated proteins between groups treated with and without zoledronic acid according to DAVID functional annotation. A. Top 12 significantly enriched biological processes associated with the up-regulated proteins quantified using the SWATH-MS approach. B. Top six significantly enriched KEGG pathways associated with the up-regulated proteins quantified using the SWATH-MS approach.

antagonist eplerenone increased the gene expression levels of the browning-specific markers *CIDEA, PRDM1*, and *UCP1* (**Figure 4B**).

Discussion

BAT activation promotes energy expenditure, reduces adiposity, and thus protects against obesity [18]. In this sense, inducing the white-to-brown switch is considered to be an effective therapeutic tool against obesity. Increasing bone mineral density is associated with an increase in BAT. Therefore, we speculated that anti-osteoporosis drugs might help to induce a brown-like phenotype in white adipocytes. In particular, we focused on ZOL, which is a widely used anti-osteoporosis drug, and confirmed for the first time that ZOL has potential to induce the browning of cultured white adipocytes by elevation of brown fat-specific genes and proteins. Pivotally, the reduced synthesis of aldosterone by ZOL might help to induce the browning effect. These observations help to elucidate the mechanisms underlying the occurrence and development of the browning of white adipocytes to contribute toward the discovery of new therapeutic candidates for obesity.

Chronic exposure to various factors, including environment, exercise, and endocrine factors, mediates the external cue-induced browning of white adipocytes by activation of the β3-adrenergic receptor pathway [19]. After release from the sympathetic nerve terminals, norepinephrine binds with the β3-adrenergic receptor to activate BAT thermogenic activity via PKA and p38-MAPK signalling, and then UCP1-mediated proton uncoupling stimulates lipolysis of FFAs [20, 21]. At the same time, these pathways powerfully provoke the expression of UCP1 through key transcriptional activators, including PRDM16, PGC1α, and PPARγ.

Animal experiments have confirmed that the decline of bone mineral density causes adipose accumulation, which is a prerequisite for obesity [22]. In addition to increasing bone mineral density by inhibiting FPPS of the mevalonate pathway, ZOL can simultaneously up-regulate the gene expression levels of the browning-specific markers *CIDEA* and *UCP1* in white adipocytes, and the protein levels of PRDM16, PGC1α, and UCP1 to induce a brown-like phenotype.

Aldosterone, a mineralocorticoid hormone in humans, is considered to be one of the major regulators of water, electrolyte balance, and blood pressure [23], and its effects are mediated through the MR of blood vessels. Recent data revealed that MR is also expressed in white adipose cells [17], and aldosterone could suppress the β3-adrenergic receptor pathway and UCP1 protein levels, thereby preventing browning of white cells [15]. ZOL also inhibits the mevalonate pathway, which is a synthesis pathway of the cholesterol precursor of aldo-

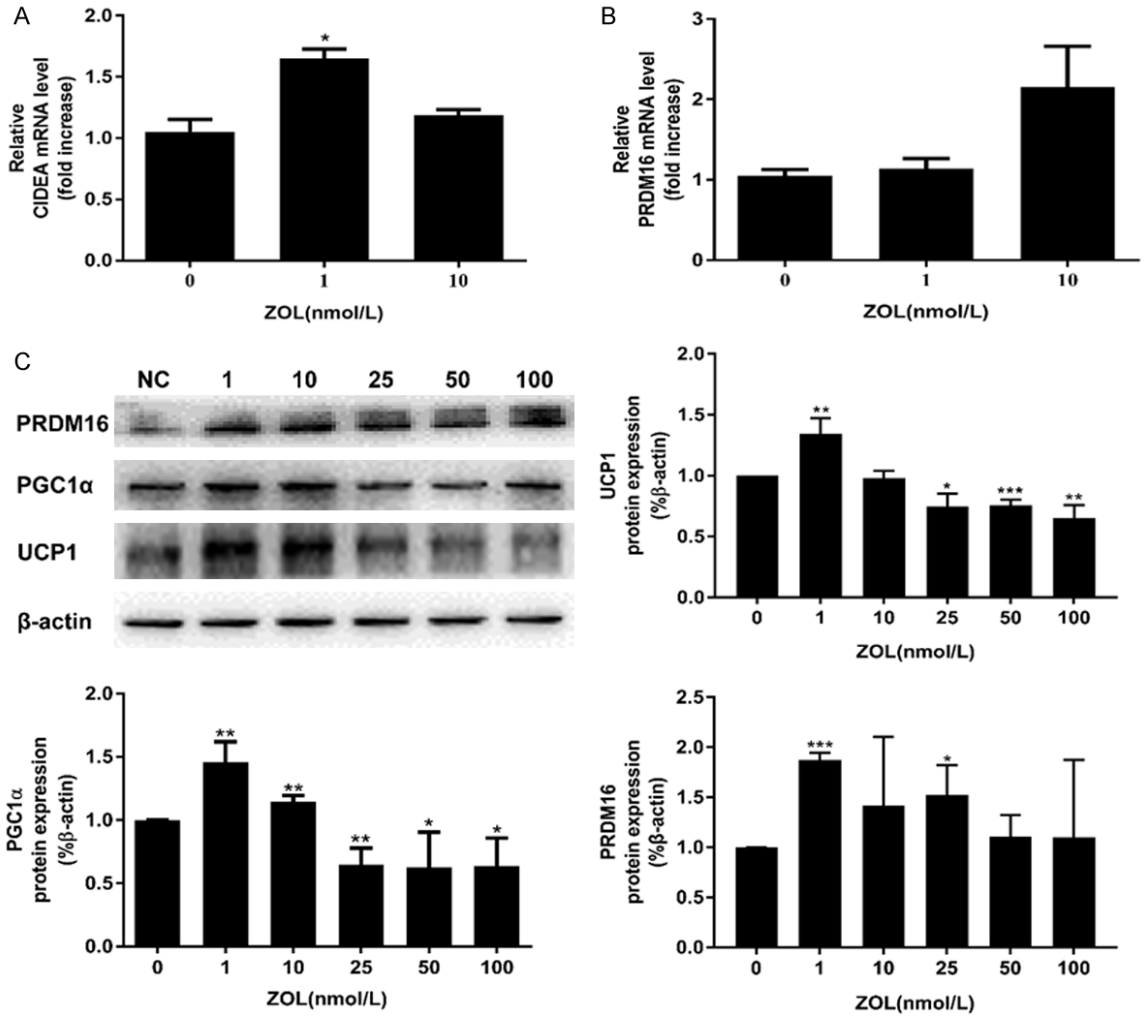

Figure 3. Expression of genes and proteins involved in browning up-regulated by zoledronic acid in C3H10T1/2 cells. A and B. Increased expression levels of CIDEA and PRDM16. C. Up-regulated protein expression of browning-specific markers (PRDM16, PGC1α, and UCP1). The data are presented as means ± standard error of the mean (n = 2-3); **P* < 0.05 and ***P* < 0.01 control vs. zoledronic acid-treated groups.

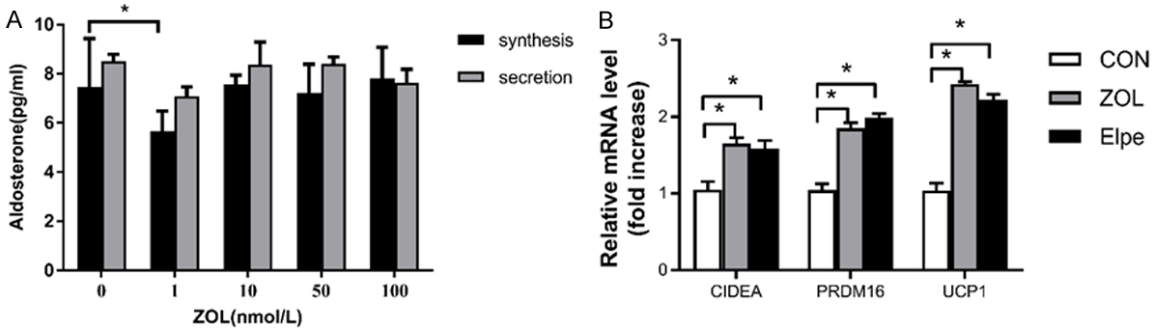

Figure 4. A. Intracellular and extracellular aldosterone levels (n = 3). B. Changes in the mRNA expression levels of *CIDEA, PRDM16*, and *UCP1* in C3H10T1/2 cells treated with zoledronic acid (ZOL, 1 nM) or eplerenone (Eple, 1 μM) determined by qPCR. Results are expressed as mean ± standard error of mean. *P < 0.05.

sterone, and eplerenone (an aldosterone antagonist) induced the gene expression levels of *CIDEA, PRDM16*, and *UCP1*, while ZOL inhibited the synthesis of aldosterone in white cells.

Thus, the suppressed aldosterone synthesis by ZOL might play a role in the browning of white adipocytes.

The main limitation of this study is that ZOL-induced browning of white adipocytes was only examined *in vitro*. Therefore, further studies are required using an animal obesity model and clinical experiments to confirm the effect of ZOL on the browning of white adipocytes and on aldosterone-treated white cells. Nevertheless, the ability of ZOL to up-regulate the β3-adrenergic receptor pathway, increase UCP1 expression, and induce browning of white adipocytes, largely through suppression of aldosterone synthesis, highlights important novel therapeutic targets for treating obesity and related metabolic syndromes.

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Disclosure of conflict of interest

None.

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