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Cover Letter

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W. Baumeister, Max Planck Institut (MPI) für Biochemie, Martinsried, Germany

Re: Vitamin D receptor ligands attenuate the inflammatory profile of IL-1β-stimulated human white preadipocytes via modulating the NF-κB and unfolded protein response pathways

Sent on behalf of all authors:
Dr Jingjing Zhu PhD
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Professor John Wilding DM FRCP Professor of Medicine

Correspondence address as above.

Dear Professor Baumeister,

Thank you for considering the above-titled article for publication in Biomedical and Biophysical Research Communications.

The potential metabolic benefit of vitamin D receptor ligands for health are increasingly recognized and the work described in this paper extends previous work from our group by demonstrating anti-inflammatory properties of vitamin D receptor ligands in human white preadipocytes, using IL-1β stimulation as a model for adipose tissue metaflammation. Furthermore, we have demonstrated that the inflammatory profile was attenuated via post-translationally modifying inflammatory associated signaling molecules including relA of the NF-κB pathway and eIF-2α of the unfolded protein pathway.

The authors believe that this manuscript addresses a subject that would be of interest to your readership. I can confirm that this manuscript has been approved by all the above named authors and has not been previously published and is not currently under consideration for publication elsewhere. I hope that you will consider this submission suitable for inclusion in Biomedical and Biophysical Research Communications, and look forward to hearing from you.

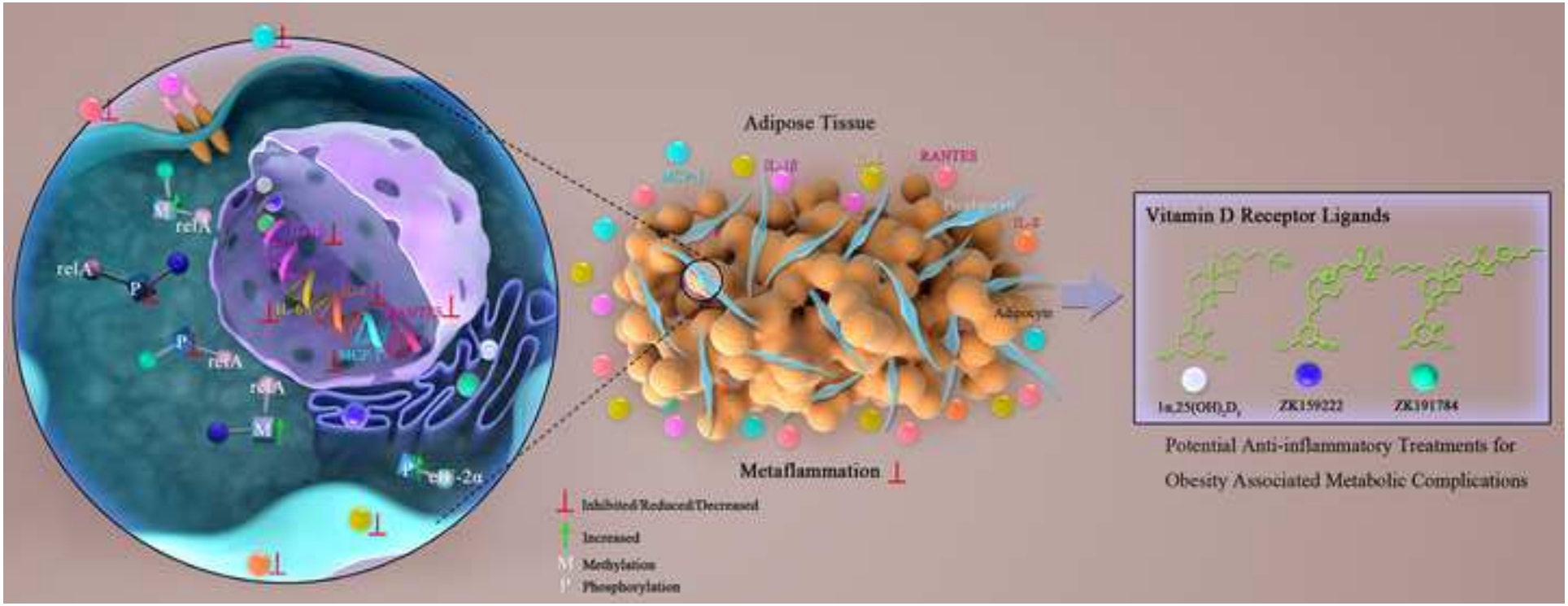
Yours sincerely

John Wilding DM FRCP
Professor of Medicine & Honorary Consultant Physician

Highlights (for review)

- IL-1β enhances the inflammatory profile of preadipocytes by post-translationally modifying relA of the NF-κB pathway.
- ZK159222 and ZK191784 inhibit the gene expression of IL-1β, IL-6, IL-8, MCP-1 and RANTES in IL-1β-stimulated preadipocytes by decreasing the phosphorylation or increasing the methylation of relA.
- $1\alpha,25(OH)_2D_3$, ZK159222 and ZK191784 reduce the secretion of IL-6, IL-8, MCP-1 and RANTES from IL-1β-stimulated preadipocytes by increasing the phosphorylation of eIF-2α of the UPR pathway.

Graphical Abstract



Vitamin D receptor ligands attenuate the inflammatory profile of IL-1β-stimulated human white preadipocytes via modulating the NF-κB and unfolded protein response pathways

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Abstract

Metaflammation in adipose tissue, which is characterized by increased local gene expression and secretion of pro-inflammatory factors, may contribute to the increased risk of metabolic complications in obesity. It has been suggested that IL-1β might induce metaflammation in adipose tissue via modulating the NF-κB signaling pathway. In our study, the mRNA and secretion levels of major pro-inflammatory factors including IL (interleukin)-1β, IL-6, IL-8, MCP (monocyte chemoattractant protein)-1 and RANTES (regulated on activation, normal T cell expressed and secreted) were measured as indicators of the inflammatory profile. We herein showed that IL-1β could induce adipose tissue metaflammation by enhancing the inflammatory profile of preadipocytes. Moreover, IL-1β could enhance pro-inflammatory gene expression by increasing the phosphorylation or decreasing the methylation of relA (NF-κB p65) of the NF-κB signaling pathway. VDR (vitamin D receptor) ligands have been shown to have anti-inflammatory properties in adipocytes. Likewise, our study demonstrated that the inflammatory profile of IL-1β-stimulated preadipocytes is significantly attenuated by VDR ligands 1α,25$(OH)_2D_3$, ZK159222 and ZK191785. Importantly, we showed that ZK159222 and ZK191785 could inhibit pro-inflammatory gene expression by decreasing the phosphorylation or increasing the methylation of relA. Furthermore, pro-inflammatory secretion could be reduced by 1α,25$(OH)_2D_3$, ZK159222 and ZK191785 via increasing the phosphorylation of eIF (eukaryotic translation initiation factor)-2α of the UPR (unfolded protein response) pathway. These observations suggest that the VDR ligands may be considered potential anti-inflammatory treatments for obesity

associated metabolic complications.

Key words

Preadipocyte; IL-1β; inflammatory profile; VDR ligands; relA; eIF-2α

1. Introduction

Obesity has become a leading global public health problem as it is considered as a risk factor for a variety of metabolic diseases including type 2 diabetes, hepatic steatosis, biliary disease, neurodegeneration, cardiovascular diseases, musculoskeletal abnormalities and even some cancers [1]. The risk of developing these obesity-related complications is determined in part by the degree of metaflammation in adipose tissue [2], characterized by moderate local expression of a plethora of pro-inflammatory factors [3]. Besides being triggered by ER (endoplasmic reticulum) stress during adipose tissue expansion in obesity [4], accumulated research evidence also indicates that metaflammation could be induced by IL-1β via activating IKKβ and the down-stream transcription factor NF-κB in the classical receptor-mediated way [5,6,7]. It is worth noting that mice lacking the IL-1β-receptor show attenuated adipose tissue metaflammation in response to a high fat diet, which coincides with protection against insulin resistance [8]. Hence, the metabolic outcomes of obese individuals might be improved by attenuating IL-1β-enhanced inflammatory profile in adipose tissue.

Abbreviations: EGF, epidermal growth factor; eIF, eukaryotic translation initiation factor; ER, endoplasmic reticulum; G-CSF, granulocyte colony-stimulating factor; IL, interleukin; IP, interferon gamma-induced protein; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; RANTES, regulated on activation, normal T cell expressed and secreted; UPR, unfolded protein response; VDR, vitamin D receptor.

Adipose tissue is the principal storage site of the fat soluble secosteroid vitamin D and its metabolites [9]. $1\alpha,25(OH)_2D_3$, acting as a VDR ligand, not only participates in bone and mineral metabolism, but also modulates cell proliferation, differentiation and immune-regulation [10,11,12]. Most intriguingly, recent studies reported that $1\alpha,25(OH)_2D_3$ could attenuate the inflammatory profile of human adipocytes [13,14]. However, the immuno-modulatory properties of $1\alpha,25(OH)_2D_3$ are mostly achieved at very high doses, that provoke hypercalcemia, which prevents its use as a practical clinical anti-inflammatory agent [15]. During recent decades, attempts have been made to synthesize other VDR ligands with anti-inflammatory efficacy but lower capacity to raise blood calcium levels [15].

In this study, we aimed to test whether $1\alpha,25(OH)_2D_3$, and two synthetic VDR ligands (ZK191784 and ZK15922) could attenuate the inflammatory profile of IL-1β-stimulated human white preadipocytes by inhibiting the gene expression and secretion of major pro-inflammatory factors. This was determined by examining the modifying effects of the VDR ligands on signaling molecules relA of the NF-κB pathway and eIF-2α of the UPR pathway activated during ER stress.

2. Materials and Methods

2.1. Culture media and conditions of human white preadipocytes

Human white preadipocytes derived from subcutaneous adipose tissue of a female Caucasian subject (BMI 21; age 44 years) were obtained from PromoCell (Germany). The preadipocytes

were cultured in T25 flasks then sub-cultured into 12-well plates (seeding density: 5000 cells per cm^2) in preadipocyte growth medium (PromoCell, Germany) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B, and incubated at 37°C in 95% air and 5% CO_2.

2.2. IL-1β stimulation and VDR ligand pre-treatment and treatment

VDR ligands ZK159222, ZK191784 (kindly provided by Bayer AG, Germany) and 1α,25$(OH)_2D_3$ (ENZO Life Sciences, USA) (reconstituted in DMSO to a concentration of 1 μg/μl) were diluted to final concentrations (indicated as below) in preadipocyte growth medium. The purpose of VDR ligand pre-treatments was to boost the efficacy as established previously [16]. When confluence was reached, preadipocytes (n=6 wells per group) were pre-treated with ZK 159222 (10 nM and 1 μM) or ZK191784 (10 nM and 1μM) or 1α,25$(OH)_2D_3$ (0.01-10 nM and 1 μM), or with preadipocyte growth medium alone as a control. After 48 h the pre-treatment media were discarded. Then, IL-1β (reconstituted in distilled water to a concentration of 0.1 μg/μl, R&D Systems, UK) was added 2 ml/well to induce metaflammation together with ZK159222, ZK191784 or 1α,25$(OH)_2D_3$ or control media at the same concentrations as used for the pre-treatment period and cultured for a further 24 h before cell and supernatant collection.

2.3. Inflammatory profile measurement

The mRNA levels of major pro-inflammatory factors including IL-1β, IL-6, IL-8, MCP-1 and

RANTES of preadipocytes were measured in duplicate using TaqMan gene expression assays (Applied Biosystems, UK), qPCR core kit (Eurogentec, Belgium) and Stratagene Mx3005P instrument system. The results were normalized to the values of reference gene PPIA [17] and presented as fold changes of Ct value relative to controls using the $2^{-\Delta\Delta ct}$ formula [18]. The concentrations of IL-6, IL-8, MCP-1 and RANTES of the cell supernatants were measured in duplicate using human ELISA kits following the manufacturer's instructions (R&D Systems, UK) and SPECTROstar Nano Microplate Reader (BMG LABTECH, Germany). The results were corrected for total cell protein content (measured by Thermo Scientific Pierce BCA Protein Assay Kit, UK) and presented μg(cytokine)/mg(cell protein). Cytokine secretion profile of the pooled cell supernatants (n=6) were determined by Proteome Profiler Human XL Cytokine Array following the manufacturer's instructions (R&D Systems, UK) and Molecular Imager ChemiDoc XRS+ System (Bio-Rad, UK). The results were presented as pixel density relative to the references of the arrays. The intracellular levels (densities) of signaling molecules including relA, phosphorylated relA, methylated relA, eIF-2α phosphorylated eIF-2α were measured using western blotting and Molecular Imager ChemiDoc XRS+ System (Bio-Rad, UK). All the antibodies used were purchased from Abcam (UK) and diluted according to the manufacturer's instructions. The results were calculated as ratios of phosphorylated relA to relA, methylated relA to relA and phosphorylated eIF-2α to eIF-2α, normalized to loading control vinculin, and presented as fold changes of density relative to controls. Methods of qPCR and western blotting were as previously described [16].

2.4. Statistical analysis

Results are presented as means ± SEM. For statistical analysis, one-way ANOVA test for independent samples was used, followed by Tukey's test for individual group comparisons. A value of $P<0.05$ was regarded as statistically significant. The analysis and presentation were performed using Prism 5 (GraphPad, USA).

3. Results

3.1. VDR ligands affect the gene expression of major pro-inflammatory factors in IL-1β-stimulated human white preadipocytes

IL-1β is critical in initiating and maintaining metaflammation in adipose tissue [8,19]. To investigate whether metaflammation induced by IL-1β might originate in preadipocytes – a critical component of adipose tissue [20], we treated human white preadipocytes with a moderate dose (0.5 ng/ml) of IL-1β. The qPCR results show that (Fig. 1) the mRNA levels of major pro-inflammatory factors including IL-1β, IL-6, IL-8, MCP-1 and RANTES were markedly increased, suggesting that IL-1β could target preadipocytes to propagate adipose tissue metaflammation.

We speculated that $1\alpha,25(OH)_2D_3$ might inhibit pro-inflammatory gene expression in preadipocytes, as has previously been shown in human adipocytes [13,14]. In preliminary work, a range of doses from 0.01 to 10 nM of $1\alpha,25(OH)_2D_3$ were tested, and the reducing effects on the

mRNA levels of the major pro-inflammatory factors were consistent within this dose range (Fig. S1). (Fig. 1) The mRNA levels of IL-1β, IL-6, IL-8, MCP-1 and RANTES were moderately decreased by 10 nM of 1α,25$(OH)_2D_3$, which were ~0.7 to 1.9-fold lower compared with IL-1β-stimulated preadipocytes. Synthetic VDR ligands, ZK159222 and ZK191784 (10 nM) were also tested in the same protocol and (Fig. 1) the pro-inflammatory mRNA levels were reduced by ~0.7 to 2.3 and ~1.5 to 1.8-fold, respectively. In parallel, considering the very high doses of VDR ligands applied clinically [15], a higher dose of 1 μM of 1α,25$(OH)_2D_3$ was also tested. This dose resulted in apoptosis of preadipocytes in 4 hours so was not tested further. In contrast, (Fig. 1) 1 μM of ZK159222 decreased the mRNA levels of IL-6, IL-8, MCP-1 and RANTES by ~0.5-1.6-fold but not IL-1β. Finally, (Fig. 1) 1 μM of ZK191784 reduced the major pro-inflammatory mRNA levels by ~1.4 to 2.9-fold. Taken together, these data show that all the VDR ligands tested attenuate the inflammatory profile of preadipocytes enhanced by IL-1β, by inhibiting pro-inflammatory gene expression. However, ZK159222 and ZK191784 might be pharmacologically less toxic than 1α,25$(OH)_2D_3$, for 1 μM of the latter induced apoptosis of preadipocytes.

3.2. VDR ligands affect the secretion of major pro-inflammatory factors from IL-1β-stimulated human white preadipocytes

Using ELISAs to measure concentrations of IL-6, IL-8, MCP-1 and RANTES in supernatants from cultured preadipocytes, (Fig. 2) the secretion levels of IL-6, IL-8, MCP-1 and RANTES were dramatically increased from IL-1β-stimulated preadipocytes, whereas the VDR ligands inhibited

the pro-inflammatory secretion. As observed in preliminary work, within the dose range the decreasing effects of $1\alpha,25(OH)_2D_3$ were consistent (Fig. S2). (Fig. 2) Compared with IL-1β-stimulated preadipocytes, 10 nM of $1\alpha,25(OH)_2D_3$, ZK159222 and ZK191784 decreased the secretion of the major pro-inflammatory factors by ~0.4 to 0.6-fold. In parallel, 1 μM of ZK159222 decreased the levels of IL-6, IL-8 and RANTES by ~0.4 to 0.5-fold, although concentrations of MCP-1 were numerically lower, this did not reach statistical significance. 1 μM of ZK191784 moderately reduced the levels of IL-6, IL-8 and MCP-1 by ~0.4 to 0.5-fold, but did not significantly affect RANTES. Therefore, the VDR ligands could also attenuate the inflammatory profile of preadipocytes by reducing secretion of pro-inflammatory factors.

Next, (Fig. 3A) we performed proteome array to explore whether 1 μM of ZK159222 and ZK191784 could affect the secretion of other cytokines from IL-1β-stimulated preadipocytes. The result revealed that (Fig. 3B) besides IL-6, IL-8, MCP-1 and RANTES, ZK159222 and ZK191784 reduced the levels of a variety of pro-inflammatory factors including EGF (epidermal growth factor), G-CSF (granulocyte colony-stimulating factor), GRO-a, IP (interferon gamma-induced protein)-10, MCP-3, MIP (macrophage inflammatory protein)-3a, pentraxin-3 and thrombospondin.

3.3. VDR ligands modify inflammatory associated signaling molecules in IL-1β-stimulated human white preadipocytes

The NF-κB signaling pathway could be activated by IL-1β via degrading I-κB to release NF-κB

p65/p50 heterodimer into the nucleus [7]. As a key transcription factor, post-translationally modified NF-κB regulates the expression of pro-inflammatory genes including IL-1β, IL-6 and MCP-1 [21]. As shown in Fig. 4A, the levels of relA were similar in control and IL-1β-stimulated preadipocytes, but (Fig. 4B) IL-1β significantly increased the phosphorylation level of relA by ~85%. By contrast, (Fig. 4C) the methylation level was reduced by ~33%. IκBα was also measured, but no effect of IL-1β on its intracellular concentration was observed (data not shown). The overall results suggest that IL-1β could enhance pro-inflammatory gene expression by increasing the phosphorylation or decreasing the methylation of relA of the NF-κB signaling pathway in preadipocytes.

In preliminary work, IκBα, relA, phosphorylated and methylated relA were probed to determine the modulating effects of $1\alpha,25(OH)_2D_3$ (0.01-10 nM) on the NF-κB pathway, but all the levels were comparable to IL-1β-stimulated preadipocytes (data not shown), which indicates that $1\alpha,25(OH)_2D_3$ does not modulate the NF-κB pathway in IL-1β-stimulated preadipocytes. Likewise, 10 nM and 1 μM of ZK159222 and ZK191784 had no effect on the levels of IκBα (data not shown) and (Fig. 4A) relA. However, (Fig. 4B) 1 μM of ZK159222 and ZK191784 decreased the phosphorylation levels of relA by ~34%. (Fig. 4C) In parallel, 10 nM and 1 μM of ZK159222 and 10 nM of ZK191784 were associated with moderately (~44%) higher methylation levels of relA in IL-1β-stimulated preadipocytes. However, (Fig. 4B and C) 10 nM of ZK159222 and ZK191784 and 1 μM of ZK191784 had no effect on the levels of phosphorylation and methylation of relA, respectively. Taken together, ZK159222 and ZK191784 could inhibit pro-inflammatory gene expression by decreasing the phosphorylation or increasing the methylation of relA in

IL-1β-stimulated preadipocytes.

The UPR can relieve ER stress [22,23], which is one of the potential causes of metaflammation in adipose tissue [4]. As one of the vital signaling molecules of the UPR pathways, phosphorylated eIF-2α could halt the general translation by ubiquitylation [24]. Therefore, we speculated that the VDR ligands might reduce the secretion of the major pro-inflammatory factors by increasing the phosphorylation of eIF-2α. (Fig. 4A) The levels of eIF-2α and phosphorylated eIF-2α were similar in control and IL-1β-stimulated preadipocytes. 0.1 to 10 nM of 1α,25$(OH)_2D_3$ also had no effect on the levels of eIF-2α, but consistently increased the phosphorylation levels of eIF-2α by ~43% to ~73%, compared to IL-1β-stimulated preadipocytes (Fig. S3 and Fig. 4A and D). Likewise, (Fig. 4A) 10 nM and 1 μM of ZK159222 and ZK191784 had no effect on the levels of eIF-2α, (Fig. 4D) but were associated with markedly (~47%) higher phosphorylation levels of eIF-2α. The overall results suggest by increasing the phosphorylation of eIF-2α of the UPR pathways, 1α,25$(OH)_2D_3$, ZK159222 and ZK191784 could reduce pro-inflammatory secretion from IL-1β-stimulated preadipocytes.

4. Discussion

IL-1β could induce adipose tissue metaflammation by modulating the NF-κB pathway [8,19]. Our study began with investigating whether metaflammation induced by IL-1β might originate in preadipocytes, by measuring the mRNA and secretion levels of major pro-inflammatory factors including IL-1β, IL-6, IL-8, MCP-1 and RANTES as indicators of the inflammatory profile in

keeping with published studies [25,26,27,28,29,30] . Our results indicate that the inflammatory profile of preadipocytes is significantly enhanced by IL-1β. Moreover, IL-1β could enhance pro-inflammatory gene expression via increasing the phosphorylation or decreasing the methylation of relA of the NF-κB signaling pathway, as previously suggested [31,32,33,34].

The risk for obese individuals to develop metabolic complications might be reduced by attenuating IL-1β-enhanced inflammatory profiles in preadipocytes, because the homeostasis of obese adipose tissue is dependent on preadipocytes exerting beneficial effects, protecting against metabolic complications [20,35], but IL-1β could enhance the major pro-inflammatory gene expression of preadipocytes as demonstrated in our study, which might sabotage the metabolic signaling of adipose tissue e.g. by causing insulin resistance [36].

VDR ligands have been shown to exert anti-inflammatory properties in adipose tissue [16,37,38]. Likewise, 1α,25$(OH)_2D_3$, ZK159222 and ZK191784 attenuate pro-inflammatory gene expression and secretion enhanced by IL-1β in preadipocytes. Moreover, ZK159222 and ZK191784 exhibited a more extensive inhibitory effect on secretion of pro-inflammatory factors including EGF, G-CSF, GRO-a, IP-10, MCP-3, MIP-3a, pentraxin-3 and thrombospondin. Besides VDR ligands directly regulate the gene expression of cytokines [39], by decreasing the phosphorylation or increasing the methylation of relA, ZK15922 and ZK191784 could inhibit pro-inflammatory gene expression, as formerly established [37,38]. Importantly, 1α,25$(OH)_2D_3$, ZK159222 and ZK191784 could also reduce pro-inflammatory secretion via increasing the phosphorylation of eIF-2α of the UPR pathway, thereby abating the general translation to relieve metaflammation triggered by ER stress

[4,22,23,24]. The potential mechanism might be the VDR ligands triggering Ca^{2+} release from ER stores [40].

In summary, we have demonstrated that IL-1β enhances the inflammatory profile of human preadipocytes by post-translationally modifying relA of the NF-κB pathway. Furthermore, we have shown that the VDR ligands inhibit the inflammatory profile, and that the anti-inflammatory mechanisms could be due to a decrease in the phosphorylation or an increase in the methylation of relA by ZK159222 and ZK191785, and finally that $1\alpha,25(OH)_2D_3$, ZK159222 and ZK191785 also increase the phosphorylation of eIF-2α of the UPR pathway. Therefore, the VDR ligands tested may be considered potential anti-inflammatory treatments for obesity associated metabolic complications.

Acknowledgements

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Fig. 1. Effects of VDR ligands on pro-inflammatory gene expression in IL-1β-stimulated human white preadipocytes.

Preadipocytes were either cultured alone (control), or with IL-1β (0.5 ng/ml) for 24 h. Further groups of cells were pre-treated with 1α,25$(OH)_2D_3$ (10 nM) or ZK159222 (10 nM and 1 μM) or ZK191784 (10 nM and 1 μM) for 48 h, followed by treatments with IL-1β (0.5 ng/ml) and 1α,25$(OH)_2D_3$ (10 nM) or ZK159222 (10 nM and 1 μM) or ZK191784 (10 nM and 1 μM) for a further 24 h before cell collection. The mRNA levels of pro-inflammatory factors (A) IL-1β, (B) IL-6, (C) IL-8, (D) MCP-1 and (E) RANTES were measured by qPCR. Data are means ± SEM for groups of 6. A significant difference to control was indicated by ***($p<0.001$); to IL-1β by #($p<0.05$), ##($p<0.01$) and ###($p<0.001$). The results were determined using one-way ANOVA

with Tukey's post hoc test and confirmed by three independent experiment

Fig. 2. Effects of VDR ligands on pro-inflammatory secretion from IL-1β-stimulated human white preadipocytes.

Preadipocytes were either cultured alone (control), or with IL-1β (0.5 ng/ml) for 24 h. Further groups of cells were pre-treated with 1α,25$(OH)_2D_3$ (10 nM) or ZK159222 (10 nM and 1 μM) or ZK191784 (10 nM and 1 μM) for 48 h, followed by treatments with IL-1β (0.5 ng/ml) and 1α,25$(OH)_2D_3$ (10 nM) or ZK159222 (10 nM and 1 μM) or ZK191784 (10 nM and 1 μM) for a further 24 h before supernatant collection. The release levels of pro-inflammatory factors (A) IL-6, (B) IL-8, (C) MCP-1 and (D) RANTES were measured by ELISA and normalized by total cell protein content. Data are means ± SEM for groups of 6. A significant difference to control was indicated by ***($p<0.001$); to IL-1β by #($p<0.05$), ##($p<0.01$) and ###($p<0.001$). The results were

determined using one-way ANOVA with Tukey's post hoc test and confirmed by three independent experiments.

Fig. 3. Effects of ZK159222 and ZK191784 on cytokine release from IL-1β-stimulated human white preadipocytes.

Preadipocytes were either cultured alone (control), or with IL-1β (0.5 ng/ml) for 24 h. Further groups of cells were pre-treated with ZK159222 (1 μM) or ZK191784 (1 μM) for 48 h, followed by treatments with IL-1β (0.5 ng/ml) and ZK159222 (1 μM) or ZK191784 (1 μM) for a further 24 h before supernatant collection. (A) The cytokine release levels were screened by cytokine array. (B) The results are presented as pixel density relative to the reference controls of the arrays.

Fig. 4. Modifying effects of VDR ligands on inflammatory associated signaling molecules in IL-1β-stimulated human white preadipocytes.

Preadipocytes were either cultured alone (control), or with IL-1β (0.5 ng/ml) for 24 h. Further groups of cells were pre-treated with ZK159222 (10 nM and 1 μM) or ZK191784 (10 nM and 1 μM) or 1α,25$(OH)_2D_3$ (10 nM) for 48 h, followed by treatments with IL-1β (0.5 ng/ml) and ZK159222 (10 nM and 1 μM) or ZK191784 (10 nM and 1 μM) or 1α,25$(OH)_2D_3$ (10 nM) for a further 24 h before cell collection. (A) The levels of relA, phosphorylated relA, methylated relA, eIF-2α and phosphorylated eIF-2α were measured by western blotting. The results were presented as fold changes of ratios of (B) phosphorylated relA to relA, (C) methylated relA to relA and (D) phosphorylated eIF-2α to eIF-2α to controls. Data are means ± SEM for groups of 6. A significant difference to control was indicated by ***($p<0.001$); to IL-1β by #($p<0.05$), ##($p<0.01$) and ###($p<0.001$). The results were determined using one-way ANOVA with Tukey's post hoc test and confirmed by three independent experiments.

Figure.1

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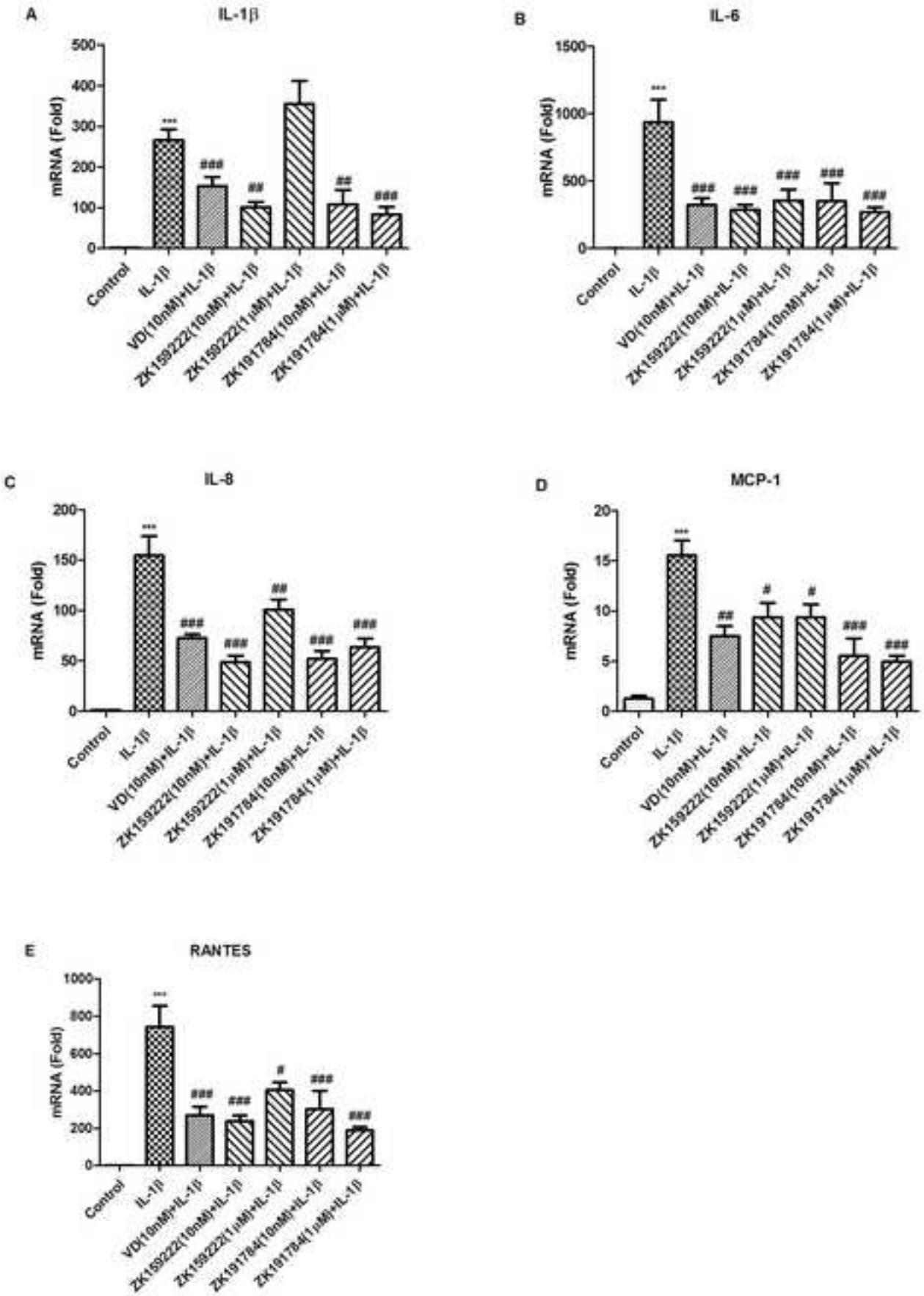

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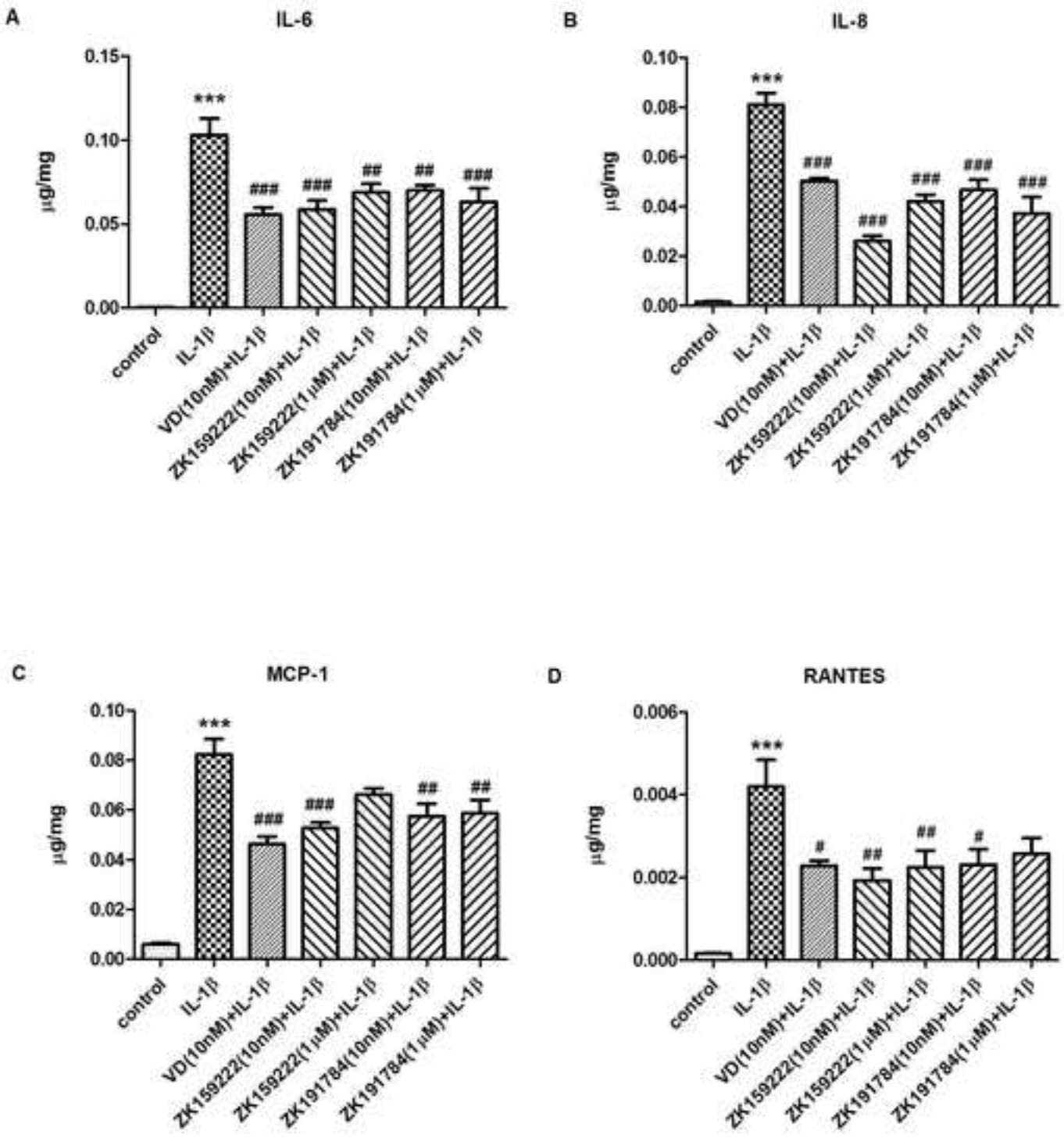

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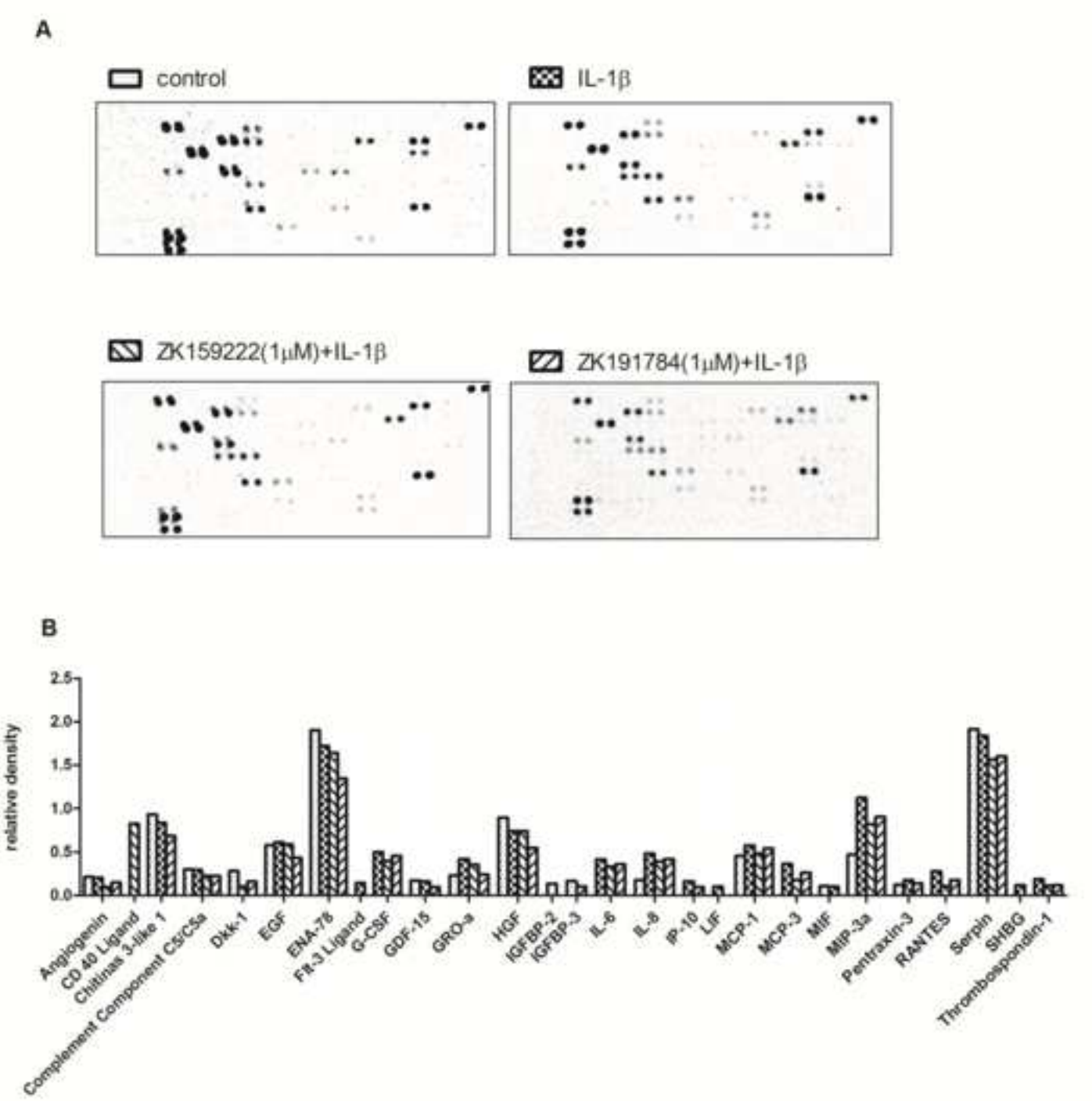

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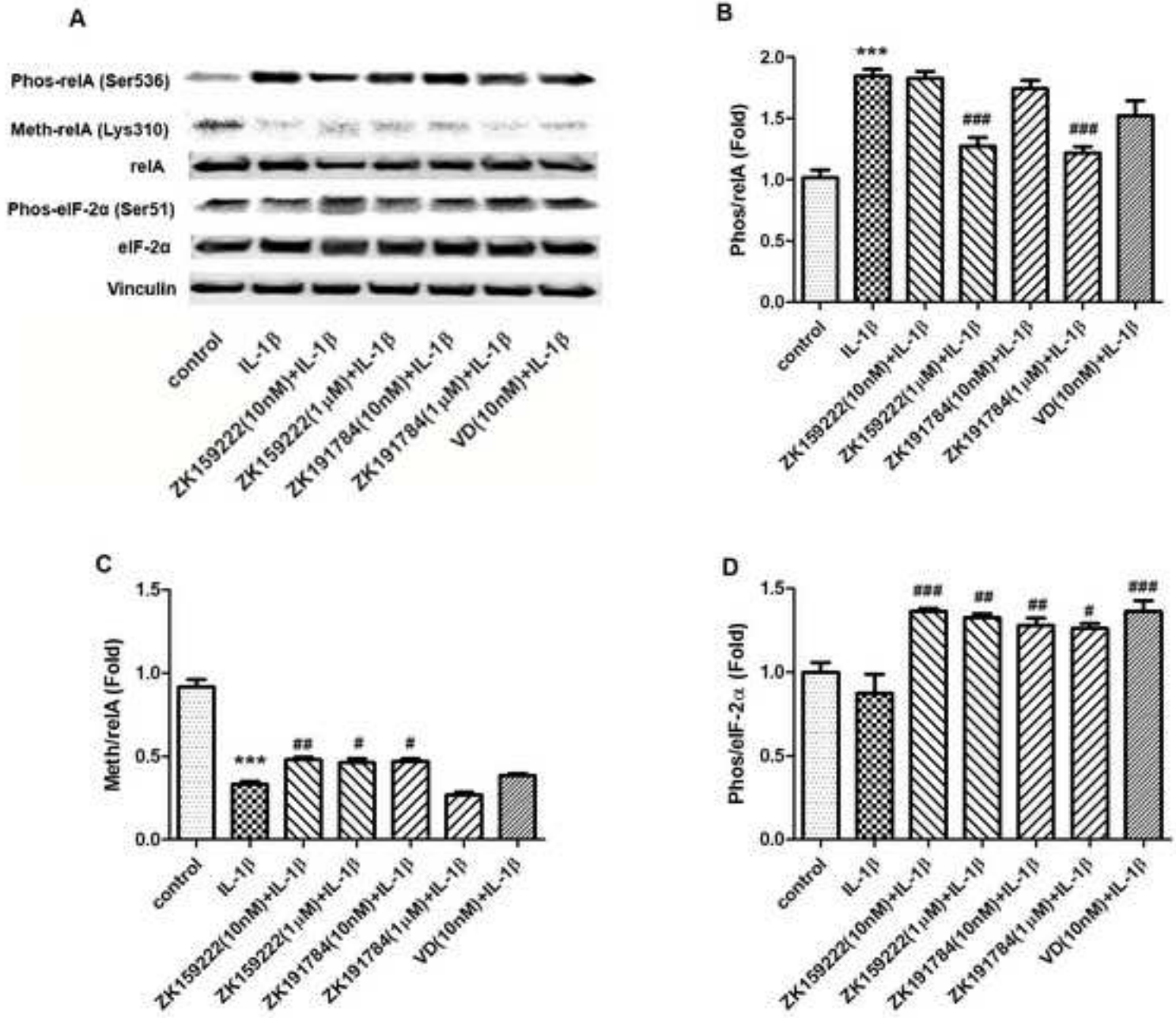

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***Conflict of Interest**