Investigations of the Anti-inflammatory Effects of Vitamin D in Adipose Tissue: Results from an In Vitro Study and a Randomized Controlled Trial

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Key words
- obesity
- calcitriol
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- adiponectin

Abstract

Inflammation is a key feature of obesity and type 2 diabetes. The active vitamin D metabolite, 1,25-dihydroxyvitamin D [1,25$(OH)_2$D], modulates the inflammation *in vitro*. We studied whether inflammation in adipose tissue (AT) cultures could be reduced by incubation with 1,25$(OH)_2$D *in vitro*, or by oral treatment with vitamin D *in vivo* in obese subjects with low plasma levels of 25-hydroxyvitamin D. Samples of subcutaneous AT were stimulated with IL-1β to induce inflammation. In the *in vitro* study, samples were concomitantly incubated with or without 1,25$(OH)_2$D, and analyzed for mRNA and protein levels of inflammatory markers IL-6, IL-8, and MCP-1. In the *in vivo* study, samples of subcutaneous AT from obese subjects obtained before and after treatment with 7000 IU of vitamin D daily or placebo in a randomized controlled trial were stimulated with IL-1β. The samples were analyzed for AT gene expression and compared with plasma markers of inflammation. In the *in vitro* study, concomitant incubation with 1,25$(OH)_2$D reduced mRNA levels of MCP-1 by 45% ($p=0.01$), of IL-6 by 32% ($p=0.002$), and of IL-8 by 34% ($p=0.03$), and reduced secretion of IL-8 protein by 18% ($p=0.005$). In vivo treatment with vitamin D did not reduce AT expression or circulating levels of MCP-1, IL-6, or IL-8. 1,25$(OH)_2$D has significant anti-inflammatory effects in AT *in vitro*. However, a similar reduction in AT and systemic inflammation cannot be obtained by oral treatment with vitamin D in obese subjects.

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Introduction

Low plasma levels of 25-hydroxyvitamin D (25OHD) are common in obesity. Plasma levels of 25OHD have been inversely associated with obesity-related comorbidities such as insulin resistance [1,2], metabolic syndrome [3,4], type 2 diabetes [5], and cardiovascular disease [6]. Furthermore, vitamin D seems to play a role in both innate and adaptive immunity [7].

Adipose tissue (AT) consists of adipocytes and surrounding cells such as immune cells, vascular cells, and fibroblasts. In obesity, the AT is infiltrated with immune cells, especially macrophages [8], and chronic AT inflammation is a key feature of obesity, which promotes insulin resistance [9] and cardiovascular disease [10]. Adipose tissue macrophages produce proinflammatory cytokines, such as interleukin 1β (IL-1β), which may also contribute to obesity-related insulin resistance [11]. IL-1β stimulates the expression and secretion of markers of adipose tissue inflammation such as monocyte chemoattractant protein-1 (MCP-1) and the interleukines 6 and 8 (IL-6/IL-8) in adipose tissue cultures [12,13]. MCP-1 is a chemokine secreted from both adipocytes and macrophages, which is an important factor for the recruitment of macrophages to the adipose tissue [14,15]. AT expression and release of MCP-1 is increased in obesity along with other inflammation-related factors, such as IL-6 and IL-8 [16–18]. Likewise, a decreased adipocyte production of the anti-inflammatory and insulin-sensitizing hormone adiponectin is seen in obesity [19–21], and stimulation with IL-1β has been shown to reduce the expression of adiponectin in adipose tissue cultures [22].

Vitamin D is a prohormone produced in the skin after UVB exposure. Vitamin D is hydroxylated first into 25OHD, and subsequently to 1α,25-dihydroxyvitamin D [1,25$(OH)_2$D], which is the active form of the hormone. 1,25$(OH)_2$D acts through the vitamin D receptor (VDR), a nuclear receptor that heterodimerizes with retinoic X receptor and binds to VDR response elements to

initiate gene transcription [23]. VDR is abundant in almost all cell types including adipocytes and macrophages [24]. VDR stimulation modulates immune function [7] and has anti-inflammatory effects in several cells types, including mesangial cells [25], peripheral blood mononuclear cells (PBMCs) from healthy donors [26,27] and from type 2 diabetic patients [28], human peritoneal macrophages [29], and prostate cells [30,31]. However, data on the effects of VDR stimulation by $1,25(OH)_2D$ in adipose tissue are limited. In one study conducted in human isolated adipocytes, $1,25(OH)_2D$ reduced the expression and tended to decrease the secretion of MCP-1 [32] indicating immunomodulatory effects also in adipose tissue.

In the present study, we investigated the effects of $1,25(OH)_2D$ on AT and systemic inflammation. We aimed to determine whether $1,25(OH)_2D$ reduces the IL-1β-induced expression and secretion of inflammatory markers IL-6, IL-8, and MCP-1 in cultures of human AT fragments. Secondly, we aimed to determine whether IL-1β-induced expression of inflammatory markers in adipose tissue cultures as well as circulating levels of inflammatory markers could be reduced by vitamin D treatment for 6 months in obese subjects with low plasma levels of 25OHD.

Materials and Methods

Sample collection

The in vitro study: Samples of subcutaneous AT were obtained from 8 healthy, nonobese women (age: 31.0 years; BMI: 24.9 kg/m^2). Samples were collected after an overnight fast during liposuction for cosmetic purposes performed in general anesthesia at a clinic of plastic surgery. The AT was suspended in isotonic saline and transported to the laboratory in a sterile container.

The clinical intervention; in vivo study: Samples were collected during a randomized controlled trial investigating the effects of high-dose vitamin D treatment in obese adults with plasma levels of 25OHD below 50 nmol/l. Treatment was given as oral tablets of 175 µg of vitamin D (cholecalciferol) or placebo for 26 weeks as earlier described [33]. Fifty-five subjects were included in the trial. Those subjects who provided sufficient amounts of adipose tissue for cell culture experiments at both time points were included in the present study. Thus, we included samples from 40 subjects (age: 40.8 ± 7.4 years; BMI: 35.7 ± 3.5 kg/m^2). Twenty-two subjects were randomized to the vitamin D (VD) group and 18 subjects were randomized to the placebo (PL) group.

Blood samples for determination of inflammatory markers and AT samples for incubation studies were collected after an overnight fast at baseline and after treatment for 26 weeks. Subcutaneous abdominal AT samples were collected by liposuction, performed at our research clinic, as previously described [12]. Briefly, the skin was anesthetized with lidocain before a small incision was made. Then lidocain was injected into the subcutaneous adipose tissue, and approximately 10 g of tissue were removed by a blunt needle, to which a partial vacuum was applied. The adipose tissue was suspended in isotonic saline and transported to the laboratory in a sterile container.

All subjects were healthy except for their obesity and received no medication known to affect adipose tissue metabolism. All subjects provided written informed consent. The study was conducted in accordance with the Declaration of Helsinki II and the guidance on Good Clinical Practice (GCP). The GCP-unit of Aarhus University Hospital, Denmark, monitored the clinical trial. Approval was obtained from the Danish Medical Agency, the Ethics committee of central Denmark and the Danish Data Protection Agency. The trial was registered at ClinicalTrials.gov (NCT01037140).

Cell cultures

Cell culture experiments were performed on whole-adipose tissue fragments in both studies. The AT was treated essentially as previously described [13]. In short, the AT samples were cut into smaller pieces of approximately 10 mg each. The homogenized adipose tissue was washed free of blood clots and free lipid with saline, and incubated in organ cultures in medium 199. A total of 500 mg of adipose tissue fragments per 5 ml of incubation medium was used per tube. Medium 199 was supplemented with 25 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethenesulfonic acid] buffer; 1 % bovine albumin; 1 nM insulin, antipain and leupeptin 20 ml/100 ml medium, and penicillin and streptomycin (10 000 IU) 1 ml/100 ml medium. The adipose tissue was preincubated for 24 h. The medium was then replaced with medium 199 containing either medium 199 only, medium 199 containing IL-1β 2 ng/ml (Sigma-Aldrich, St. Louis, MO, USA) or medium 199 containing IL-1β 2 ng/ml and 100 nM 1,25-dihydroxycholecalciferol ($1,25(OH)_2D$) (Sigma-Aldrich) and then placed in a humidified incubator and maintained at 37 °C and at an atmosphere of 5 % CO_2. After incubating for 24 h, the adipose tissue and medium were harvested, snap frozen in liquid nitrogen and kept at −80 °C and at −20 °C, respectively. The medium was used for later measurement of adipokine secretion and the tissue was stored for RNA isolation.

In the *in vivo* study, the same procedures were applied for the cultures of AT collected before and after the treatment, except that no $1,25(OH)_2D$ were added to the AT incubation media. All incubations were performed in duplicate, and each data point represents the mean of duplicate incubations.

Isolation of RNA

Total RNA was isolated from the adipose tissue fragments using TriZol reagent (Gibco BRL, Life Technologies, Roskilde, Denmark); RNA was quantified by measuring absorbance at 260 and 280 nm, and the inclusion criteria was a ratio ≥ 1.8. Finally, the integrity of the RNA was checked by visual inspection of the 2 ribosomal RNAs, 18 S and 28 S, on an agarose gel.

Real-time RT-PCR for mRNA analysis

For real-time reverse transcriptase PCR, complementary DNA was constructed using random hexamer primers as described by the manufacturer (Verso cDNA Kit, VWR, Herlev, Denmark). PCR-mastermix, containing the specific primers, were added and real-time quantification of genes was performed by SYBR-green real-time reverse transcription PCR assay (KAPA SYBR Fast Universal kit, Ken-En-Tec, Taastrup, Denmark) using a LightCycler from Roche (Roche Diagnostics GmbH, Mannheim, Germany). The following primer pairs were used: IL8: sense primer 5′-TTGGCAGCCTTCCTGATTTC-3′ and antisense primer 5′-AACTTCTCCACAACCCTCTG-3′. IL6: sense primer 5′-AAATGCCAGCCTGCTGACGAAG-3′ and antisense primer 5′-AACAACAATCTGAGGTGCCCATGCTAC-3′. MCP1: sense primer 5′-ACTCTCGCCTCCAGCAAAGTC-3′ and antisense primer 5′-TGCAA AGACCCTCAAAACATCCCA-3′. Adiponectin: sense primer 5′-CATGACCAGGAAACCACGACT-3′ and antisense primer 5′-TGAATGCTGAGCGGTAT-3′. The housekeeping gene, GADPH, was amplified using sense primer 5′-AAGGTGAAGGTCGGAGTCAA-3′ and anti-

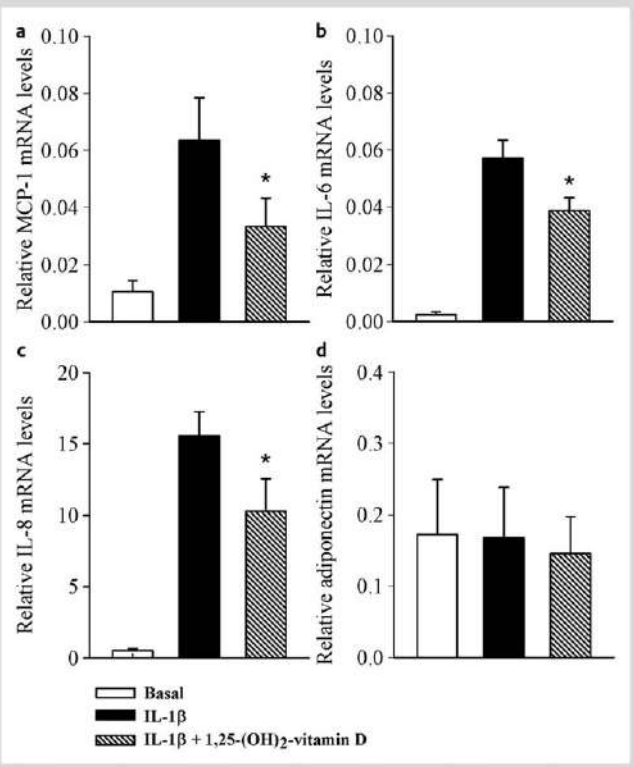

Fig. 1 Relative mRNA levels of inflammatory markers in human adipose tissue (n = 7/8). Whole adipose tissue fragments were incubated for 24 h with IL-1β (black bars), IL-1β and 1,25$(OH)_2$D (hatched bars) or as controls (blank bars). *Denotes p < 0.05 vs. IL-1β alone.

sense primer 5′-AATGAAGGGGTCATTGATGG-3′. All primers were from DNA Technology (Risskov, Denmark). cDNA with specific primers was amplified in separate tubes, and the increase in fluorescence was measured in real time. The threshold cycle was calculated, and the relative gene expression was calculated essentially as described in the User Bulletin No-2, 1997 from PerkinElmer (PerkinElmer Cetus, Norwalk, CT, USA) using the formula: $k^*2^{-(CTtarget-CTreference)}$, were k is a constant, set to 1. All samples were amplified in duplicate. A similar set-up was used for negative controls, except that the reverse transcriptase was omitted and no PCR products were detected under these conditions.

Assays

Specific high-sensitive human ELISAs were used to measure protein concentrations in culture media (Human IL-6 Duoset, Human IL-8 Duoset and Human MCP-1 Duoset) and in plasma (Quantikine HS ELISA Human IL-6 kit, QuantiGlo ELISA Human IL-8 and Quantikine ELISA Human MCP-1 kit). All assays were from R & D Systems Europe Ltd. Abingdon, UK.

Statistics

Data are presented as mean with standard deviations (SDs) or medians with 25th and 75th percentiles (25%; 75%) according to the distribution of data. The distribution of data was determined by visual inspection of QQ-plots and histograms, and data were log-transformed to achieve normality, if appropriate. Data were analyzed using a paired *t*-test, paired by experiment. Mann-Whitney test or Student's unpaired *t*-test was used to compare differences between intervention groups in the clinical trial. Wilcoxon Signed Rank test was applied to compare changes within groups when normality was not achieved. Differences were considered significant at p < 0.05. Statistical analyses were conducted using Stata version 11.2 (StataCorp LP, College Station, TX, USA).

Results

Pilot studies using various doses of 1,25$(OH)_2$D identified the concentration of 100 nM 1,25$(OH)_2$D as the concentration most efficient in reducing IL1β-induced inflammation in the adipose tissue cultures (data not shown). Thus, all further experiments were carried out using this dose of 1,25$(OH)_2$D.

The in vitro study

The effects of 1,25$(OH)_2$D on mRNA levels of inflammatory markers in AT were investigated in AT fragments incubated with IL-1β [± 1,25$(OH)_2$D] for 24 h. Incubation with IL-1β induced a 6-fold, a 24-fold, and a 30-fold increase in the expression of MCP-1 (p = 0.009), IL-6 (p < 0.001), and IL-8 (p = 0.002), respectively, compared to control samples (**Fig. 1**).

Concomitant incubation with 100 nM 1,25$(OH)_2$D significantly reduced the expression of MCP-1 by 45% (p = 0.01; **Fig. 1a**), the expression of IL-6 by 32% (p = 0.002; **Fig. 1b**), and the expression of IL-8 by 34% (p = 0.03; **Fig. 1c**), compared with incubation with IL-1β alone. The expression of adiponectin was not affected by addition of either IL-1β alone (p = 0.91) or in combination with 1,25$(OH)_2$D (p = 0.55; **Fig. 1d**).

Protein concentrations in the culture medium were measured after 24 h. The concentrations of MCP-1, IL-6, and IL-8 in control samples were 10.34 ± 5.6 ng/ml, 5.89 ± 3.9 ng/ml, and 19.15 ± 18.0 ng/ml, respectively. Addition of IL-1β caused a 4.6-fold increase in secretion of MCP-1 (p = 0.006), a 10-fold increase in secretion of IL-6 (p = 0.006), and a 15-fold increase in secretion of IL-8 (p = 0.001; **Fig. 2**).

Concomitant incubation with 1,25$(OH)_2$D reduced the IL1β-induced secretion of IL-8 protein by 18% (p = 0.005; **Fig. 2c**). The secretion of IL6 and MCP-1 protein was also reduced after addition of 1,25$(OH)_2$D but not to significant levels in this study (p = 0.22 and p = 0.27, respectively; **Fig. 2a, b**).

The clinical intervention; the in vivo study

After demonstration of a pronounced anti-inflammatory effect of vitamin D in human adipose tissue *in vitro*, we investigated whether oral treatment with vitamin D in obese subjects with low circulating levels of 25OHD would affect the inflammatory state in these subjects. We used samples from 40 healthy obese subjects with a mean age of 41 ± 7 years and a mean BMI of 36 ± 4 kg/m^2 treated with placebo (PL group) or vitamin D (VD group) for 26 weeks. Treatment increased plasma 25OHD from 33 ± 11 nmol/l to 110 ± 21 nmol/l in the VD group and from 33 ± 9 nmol/l to 47 ± 18 in the PL group, and decreased plasma PTH (median (25%; 75%) 5.3 pmol/l (3.6; 5.9) pmol/l to 4.5 pmol/l (3.7; 5.2) pmol/l in the VD group, but not in the PL group, as previously described [33].

Circulating levels of MCP-1, IL-6, IL-8, and adiponectin measured at baseline and after treatment, and the statistical tests for the comparison of relative changes from baseline between treatment groups are shown in **Table 1**. Overall, treatment with vitamin D did not affect circulating levels of inflammatory markers.

Circulating levels of adiponectin decreased from 6.1 ± 3.0 µg/ml at baseline to 5.9 ± 2.6 µg/ml after the intervention in the PL group (p = 0.82), and increased from 5.8 ± 2.3 µg/ml to 5.9 ± 1.9 µg/

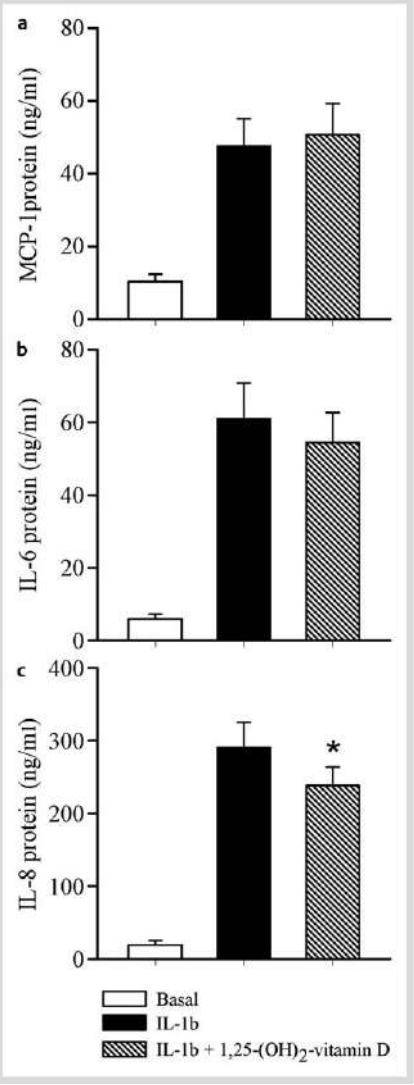

Fig. 2 Protein concentrations of inflammatory markers in human adipose tissue in incubation medias (n = 8). Whole adipose tissue fragments were stimulated for 24 h with IL-1β (black bars), IL-1β and $1,25(OH)_2D$ (hatched bars) or as controls (blank bars). *Denotes $p < 0.05$ vs. IL-1β alone.

ml in the VD group ($p = 0.26$) with no significant differences between groups ($p = 0.54$). Plasma IL-8 were below the detection limit of 1.6 pg/ml in 77 % of the samples at baseline, and was not affected by treatment (PL vs. VD, $p = 0.46$).

In vivo treatment with vitamin D on in vitro AT inflammation

We also investigated effects of oral treatment with vitamin D on mRNA levels of the inflammatory markers in AT. Subcutaneous abdominal AT samples from the 40 subjects were collected before and after the intervention and incubated for 24 h with or without IL-1β. The expression levels of MCP-1, IL-6, and IL-8 before and after treatment with placebo (PL) or vitamin D (VD) for 26 weeks are shown in **Fig. 3**. There were no significant differences between groups at baseline.

Stimulation of the AT fragments with IL-β induced the expression IL-6 by 19.1-fold at baseline ($p < 0.00001$ vs. control samples). After treatment, the IL-1β-stimulated expression of IL-6 was decreased by 27.7 % in the PL group ($p = 0.01$) and by 29.3 % in the VD group ($p = 0.007$) compared to baseline. However, the differences between groups were not significant (PL vs. VD, $p = 0.82$). Median basal expression of IL-8 decreased by 43 % in the VD group ($p = 0.02$ vs. baseline), but was not significantly altered in the PL group ($p = 0.50$ vs. baseline). However, there were no significant differences between relative changes between groups (PL vs. VD, $p = 0.89$).

Stimulation with IL-1β increased baseline expression of IL-8 by 26.8-fold ($p < 0.00001$ vs. control samples). After treatment, the IL-1β-stimulated expression of IL-8 was decreased by 24.5 % in the PL group ($p = 0.04$ vs. baseline) and by 30.1 % in the VD group ($p = 0.002$ vs. baseline). However, the differences between groups were not significant (PL vs. VD, $p = 0.66$). The median basal secretion of IL-8 protein at baseline in the intervention study did not significantly differ from the secretion of IL-8 protein in the control samples in the in vitro study (data not shown). The changes in the expression of IL-6 and IL-8 did not correlate with changes in plasma 25OHD.

Discussion

Experimental, epidemiological, and clinical evidence have linked inflammation to the pathogenesis of metabolic disorders [34, 35]. Dysfunctional adipose tissue with increased production of proinflammatory cytokines may be an important contributor in the development of insulin resistance in obesity [36, 37]. This is especially true for visceral adipose tissue, which produces more inflammatory mediators than subcutaneous adipose tissue [16, 38]. Thus, treatment with anti-inflammatory agents such as TNF-α antagonists has supported the role of inflammatory cytokines in human insulin resistance [39]. The active metabolite of vitamin D, $1,25(OH)_2D$ is another agent, which has been shown to have anti-inflammatory effects under experimental conditions. In mesangial cells, $1,25(OH)_2D$ reduced high-glucose-induced MCP-1 expression, and this was shown to be by a reduction in the activation of NFκB, which is a critical regulator of proinflammatory cytokine production [25]. Furthermore, in monocytes and macrophages, $1,25(OH)_2D$ inhibited lipopolysaccharide-induced p38 activation and thereby decreased the production of IL-6 and TNF-α [26]. Anti-inflammatory effects have been demonstrated with other VDR agonist and in other cells types: $1,25(OH)_2D$ reduced TNF-α-induced IL-8 mRNA and secreted protein in prostate cells lines [30, 31], and paricalcitrol reduced IL-8 protein in cultures of human peripheral blood mononuclear cells at both basal and LPS-induced states [27] and both tacalcitol and $1,25(OH)_2D$ decreased secretion of IL-6 and IL-8 in fibroblast cultures [40].

Few studies have examined the effects of $1,25(OH)_2D$ in adipose tissue. However, in isolated adipocytes, a reduction of MCP-1 expression was seen along with a tendency towards a decrease in MCP-1 secretion [32]. In 3T3-adipocytes, pretreatment with $1,25(OH)_2D$ was able to reduce the secretion of LPS-induced IL-6 protein, and in mice on high-fat diet, dietary treatment with $1,25(OH)_2D$ reduced IL-6 protein content in epididymal adipose tissue [41]. However, the exact mechanism of how $1,25(OH)_2D$ might decrease adipose tissue inflammation remains elusive.

In the present study, we showed that when directly administered to AT cultures *in vitro*, $1,25(OH)_2D$ regulated the expression of several of the inflammatory genes. We used IL-1β to stimulate an inflammatory response in the AT, and in parallel we studied the effects of added $1,25(OH)_2D$. Stimulation with IL-1β increased the expression of MCP-1, IL-6, and IL-8, and concomitant incubation with $1,25(OH)_2D$ reduced the expression of these proinflammatory cytokines, which is in agreement with findings from other studies, as mentioned above. However, the fact that the

Table 1 Plasma levels of inflammatory markers at baseline and at the end of study (EOS) in obese patients randomized to vitamin D 7 000 IU daily or placebo for 26 weeks.

	Placebo group		Vitamin D group		Difference between groups
	Baseline	EOS	Baseline	EOS	p-Value
MCP-1 (pg/ml)	104 (90; 123)	105 (91; 134)	113 (91; 135)	127 (107; 146)	0.54
IL-6 (pg/ml)	2.5 (1.6; 3.2)	2.1 (1.9; 2.8)	2.3 (2.1; 2.5)	2.6 (2.0; 3.3)	0.96
IL-8 (pg/ml)	2.0 ± 1.2	1.6 ± 0.2	1.7 ± 0.4	1.7 ± 0.3	0.46
Adiponectin (µg/ml)	6.1 ± 3.0	5.9 ± 2.6	5.8 ± 2.3	5.9 ± 1.9	0.54

Mean ± SD or median (25 %; 75 %)

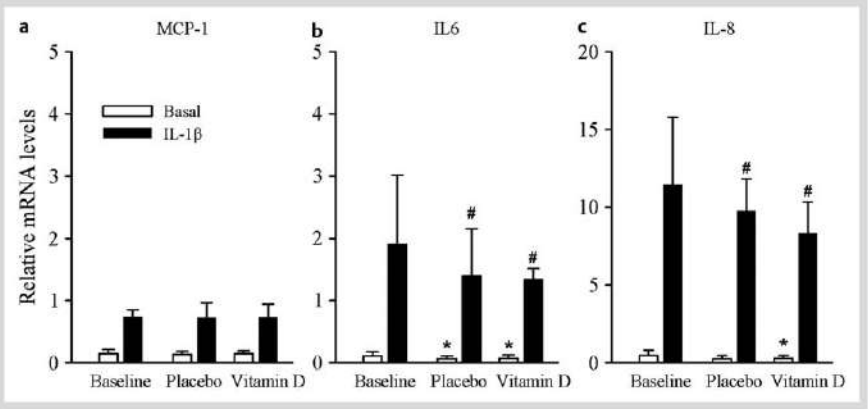

Fig. 3 IL-1β-stimulated mRNA levels of inflammatory markers in human adipose tissue after treatment with vitamin D or placebo for 26 weeks (n = 40). Whole adipose tissue fragments were incubated for 24 h with IL-1β (black columns) or as controls (blank columns) at baseline and after treatment. *p < 0.05 vs. baseline nonstimulated samples. #p < 0.05 vs. baseline IL-1β-stimulated samples.

expression of adiponectin was not decreased by IL-1β was in contrast to our expectations and to previous work [13].

When we examined the effects on protein secretion to the media, we found that IL-1β increased the secretion of MCP-1, IL-6, and IL-8. The addition of $1,25(OH)_2D$ decreased IL-8 secretion, but we were unable to demonstrate a statistically significant reduction of IL-6 or MCP-1 protein secretion. This might be explained by the fact that protein concentrations were measured in the media after 24 h, whereas a decrease in MCP-1 secretion was seen when protein concentrations were measured after 48 h [32]. The present data could also indicate that $1,25(OH)_2D$ only reduces mRNA expression, without affecting protein levels of IL-6 or of MCP-1. However, we cannot exclude that a reduction of IL-6 and MCP-1 protein concentrations would have been apparent if intracellular protein concentrations had been measured instead of protein concentrations in the media.

The reduced expression of IL-1β-induced inflammatory genes in whole adipose tissue cultures by concomitant incubation with 100 nM of $1,25(OH)_2D$ could be attributed to direct effects on the adipocytes as well as effects on macrophages within the stroma-vascular fraction (SVF). We recently showed that the VDR was expressed equally in isolated adipocytes and in the SVF [42] allowing for VDR-mediated effects in both cell fractions. Anti-inflammatory effects of VDR stimulation was shown in human peritoneal macrophages, as both $1,25(OH)_2D$ and other VDR agonists decreased expression of TNF-α mRNA and protein secretion [29]. However, in THP-1 macrophages, a short-term downregulation of IL-10 expression was followed by a later increase [43], and in another study on human macrophages, $1,25(OH)_2D$ increased the expression of IL-1β [44], stressing the complicated role of $1,25(OH)_2D$ in regulating immune cell function. As anti-inflammatory effects of $1,25(OH)_2D$ have been shown in both macrophages and in isolated adipocytes [32], it is plausible, that the effects on whole adipose tissue fragments observed in our study are a combination of effects on both the adipocytes and on the resident macrophages. However, separate measurements of the secretion of inflammatory proteins from isolated adipocytes and from the stroma-vascular cells fraction in response to $1,25(OH)_2D$ are needed to confirm this in future studies.

We found anti-inflammatory effects of direct VDR activation by $1,25(OH)_2D$ *in vitro* in adipose tissue fragments incubated with $1,25(OH)_2D$. However, after in vivo treatment with vitamin D, the precursor of $1,25(OH)_2D$, we were unable to demonstrate neither a treatment effect on adipose tissue nor on systemic inflammation. For all incubations samples of subcutaneous adipose tissue were used, which under basal conditions have a relatively low grade of inflammation. In contrast, visceral adipose tissue has more resident macrophages and produces more inflammatory cytokines [16, 38], and is more closely related to metabolic complications [45]. Thus, IL-1β was used to induce inflammation in the adipose tissue cultures. We speculate that a more pronounced anti-inflammatory effect may have been apparent if the visceral fat was used instead of subcutaneous fat. The lack of effect of *in vivo* vitamin D treatment on circulating levels of plasma markers of inflammation is in agreement with findings from other randomized clinical trials. In healthy adults, treatment for 3 years with 700 IU vitamin D plus calcium showed no effect on circulating levels of IL-6 or CRP [46]. Similarly, in healthy obese subjects, treatment for 1 year with a weekly dose of 40 000 IU vitamin D plus daily calcium showed no effect on circulating MCP-1, hs-CRP, or on any of several other inflammatory markers measured [47]. On the other hand, during a weight loss trial, treatment with 3 332 IU of vitamin D resulted in a greater decrease in TNF-α, but not CRP, compared to placebo [48]. Our findings add to the majority of these studies and do not support that oral vitamin D treatment could be used as anti-inflammatory agent to inhibit the proinflammatory response from the dysfunctional adipose tissue in obesity.

In our clinical trial, subjects were treated with vitamin D supplements containing vitamin D_3, which is recommended for the treatment and prevention of vitamin D deficiency [49]. We used a dose of 7 000 IU per day for the treatment study, which

increased mean plasma levels of 25OHD to 110 nmol/l in the intervention group. A minor increase of plasma 25OHD was seen in the placebo group, which might have been due to seasonal variation [33]. We did not measure plasma concentrations of $1,25(OH)_2D$ in this study. However, plasma concentrations of $1,25(OH)_2D$ are tightly regulated by PTH and plasma calcium. In a previous study including overweight and obese subjects the mean plasma concentration of $1,25(OH)_2D$ was 106.1 pmol/l (range: 10–256 pmol/l) [50]. This is in contrast to the concentration of 100 nmol/l used in our in incubations.

The steps of hydroxylation of VD into 25OHD and $1,25(OH)_2D$ occurs mainly in the liver and kidney, respectively. However, recently extra-renal 1α-hydroxylase activity is reported in many other tissues [51]. In fact, bioactivation of VD was shown in human mammary adipocytes [52] and in murine 3T3-L1-adipocytes [53]. But even though 1α-hydroxylation of 25OHD to $1,25(OH)_2D$ can also be exerted locally for paracrine effects, it is unknown how the plasma concentrations of 25OHD correspond to the tissue concentrations of $1,25(OH)_2D$ within the adipose tissue *in vivo*. Furthermore, we have recently shown that adipose tissue expression of the 25-hydroxylase CYP2J2 and the 1α-hydroxylase CYP27B1 are decreased in the subcutaneous adipose tissue in obesity [42]. Whether this results in an impaired bioactivation of VD for paracrine effects within adipose tissue in obesity, and whether this contribute to a lower amount of available $1,25(OH)_2D$ within the AT and thereby less VDR activation, demand further investigation. Nevertheless, we suspect that the concentrations of $1,25(OH)_2D$ achieved in plasma as well as within AT by vitamin D treatment, are too low to induce effects similar to those observed with concentrations of 100 nmol/l obtained by adding $1,25(OH)_2D$ directly to the culture medium *in vitro*, as we did for the incubations. Future studies should address whether anti-inflammatory effects of $1,25(OH)_2D$ could be obtained in adipose tissue *in vitro* by using concentrations similar to those obtained in plasma by vitamin D treatment of obese subjects.

Another explanation why vitamin D treatment was unable to decrease AT inflammation in our study could be that a sufficient vitamin D status might only contribute to prevent the obesity-related inflammatory changes, but when AT inflammation has already developed, a correction of vitamin D status might not be sufficient to reverse these changes.

A limitation of our study is that in order to demonstrate a detectable anti-inflammatory effect of $1,25(OH)_2D$, samples were stimulated with the proinflammatory IL-1β. This is a commonly used method to create an artificial state of massive inflammation in AT cultures. However, we cannot exclude that this method would conceal more discrete anti-inflammatory effects of vitamin D treatment. Another limitation is the fact that the levels of $1,25(OH)_2D$ in the circulation and in AT was not measured.

The strengths of our study include the comparison of results obtained *in vitro* under highly controlled conditions with results from a clinical experiment in the setting of the randomized controlled design, which allows for causative conclusions. Furthermore, the careful selection of patients both being obese and having low levels of 25OHD at baseline, distinguish the trial from previous studies.

In conclusion, we have found anti-inflammatory effects of $1,25(OH)_2D$ in adipose tissue *in vitro*, but no significant anti-inflammatory effects of vitamin D treatment *in vivo* was found neither in the circulation nor in AT. Based on our findings, a significant reduction in AT and systemic inflammation cannot be obtained by oral administration of vitamin D in obese subjects.

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Conflict of Interest

The authors declare that they have no conflicts of interest in the authorship or publication of this contribution.

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