

Differential effects of $1\alpha,25$ -dihydroxycholecalciferol on MCP-1 and adiponectin production in human white adipocytes

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Abstract

Background/aim Obesity is characterized by a low-grade inflammation in white adipose tissue (WAT), which promotes insulin resistance. Low serum levels of $1\alpha,25$ -dihydroxycholecalciferol (DHCC) associate with insulin resistance and higher body mass index although it is unclear whether vitamin D supplementation improves insulin sensitivity. We investigated the effects of DHCC on adipokine gene expression and secretion in adipocytes focusing on two key factors with pro-inflammatory [monocyte chemoattractant protein-1 (MCP-1/*CCL2*)] and anti-inflammatory [adiponectin (*ADIPOQ*)] effects.

Methods Pre-adipocytes were isolated from human subcutaneous WAT and cultured until full differentiation. Differentiated adipocytes were either pre-treated with

DHCC (10^{-7} M) and subsequently incubated with tumor necrosis factor- α (TNF α , 100 ng/mL) or concomitantly incubated with TNF α /DHCC. MCP1 and adiponectin mRNA expression was measured by RT-PCR and protein release by ELISA.

Results DHCC was not toxic and did not affect adipocyte morphology or the mRNA levels of adipocyte-specific genes. TNF α induced a significant increase in *CCL2* mRNA and protein secretion, while DHCC alone reduced *CCL2* mRNA expression ($\sim 25\%$, $p < 0.05$). DHCC attenuated TNF α -induced *CCL2* mRNA expression in both pre-incubation ($\sim 15\%$, $p < 0.05$) and concomitant ($\sim 60\%$, $p < 0.01$) treatments. TNF α reduced *ADIPOQ* mRNA ($\sim 80\%$) and secretion ($\sim 35\%$). DHCC alone decreased adiponectin secretion to a similar degree ($\sim 35\%$, $p < 0.05$). Concomitant treatment with DHCC/TNF α for 48 h had an additive effect, resulting in a pronounced reduction in adiponectin secretion ($\sim 70\%$).

Conclusions DHCC attenuates MCP-1 and adiponectin production in human adipocytes, thereby reducing the expression of both pro- and anti-inflammatory factors. These effects may explain the difficulties so far in determining the role of DHCC in insulin sensitivity and obesity in humans.

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Introduction

Insulin resistance in obesity is associated with a chronic low-grade inflammation in white adipose tissue (WAT). This is characterized by an increased infiltration of macrophages, which results in a disturbed production of

cyto- and chemokines [1]. Among the pro-inflammatory regulators, TNF α is considered to be a key factor, which links increased fat mass with insulin resistance and inflammation [2]. In man, TNF α acts in an auto-/paracrine manner [3] and induces several effects including stimulation of lipolysis [4], apoptosis [5], and inhibition of adipocyte differentiation [6]. TNF α also regulates the production of both pro- and anti-inflammatory adipokines in human adipocytes [7].

The primary factors initiating macrophage recruitment into WAT have been elucidated in recent years. One of the most interesting candidates is the chemokine monocyte chemoattractant protein-1 (MCP-1) [8]. The expression of this factor is believed to be up-regulated in adipocytes early in the development of obesity, which results in an increased recruitment of macrophages [9, 10]. The infiltrating macrophages also produce MCP-1, thereby stimulating further macrophage migration. Among factors that could potentially attenuate this process is adiponectin, an insulin-sensitizing hormone produced by adipocytes [11], whose levels are dramatically decreased in obesity and insulin resistance [12, 13]. TNF α plays an integrative role in the regulation of the expression of these adipokines since it stimulates MCP-1 and inhibits adiponectin secretion/expression [14].

An interesting factor that could play a role in promoting an inflammatory response in human adipose tissue is hypoxia. It has been proposed that the growth in size and possibly number of adipocytes in obesity result in an increased distance to the microvasculature and a reduced oxygen tension in WAT. In cultures of human adipocytes, hypoxia stimulates the expression of several pro-inflammatory adipokines and inhibits the secretion of anti-inflammatory factors such as adiponectin (reviewed in [15]).

Vitamin D is either taken up by the diet or produced in the skin under UV radiation. The circulating form of vitamin D is 25-hydroxycholecalciferol, which is further converted to the biologically active form 1 α ,25-dihydroxycholecalciferol (DHCC). Emerging evidence in the last few years has suggested a link between vitamin D and insulin sensitivity, as low serum 25-hydroxycholecalciferol levels have been associated with insulin resistance [16] and higher body mass index [17]. It was recently proposed that DHCC plays an important role in the regulation of adipocyte metabolism and thereby in the control of energy homeostasis [18]. DHCC modulates human adipocyte metabolism via several mechanisms. These include post-transcriptional effects (i.e., stimulation of Ca²⁺ influx) that affect lipogenesis and lipolysis [19] but also direct transcriptional regulation. The latter is mediated by activation of the nuclear vitamin D receptor (VDR) [20]. Upon ligand binding, VDR forms heterodimers with the retinoid X

receptor (RXR) and binds to genomic sites termed vitamin D response elements (VDREs) in the proximity of its target genes [21].

A number of studies have suggested a potential role for DHCC in adipose tissue inflammation. Microarray data from human adipocytes suggest that DHCC up-regulates genes involved in inflammation but also genes controlling cell proliferation, angiogenesis, and oxidative stress [22]. In another study, conducted in human adipocytes, DHCC was reported to stimulate the expression of inflammatory cytokines while inhibiting the production of anti-inflammatory cytokines [23, 24]. However, the role of DHCC as a pro-inflammatory compound is controversial since some studies have also described anti-inflammatory effects [23–25]. To our knowledge, there are no publically available data regarding the crosstalk between DHCC and TNF α in human adipocytes. In this study, we aimed to define possible reasons for the divergent findings regarding DHCC's effects in adipose inflammation. We therefore investigated the effects of DHCC in the regulation of MCP-1 and adiponectin production both alone or in combination with TNF α in *in vitro* differentiated adipocytes obtained from human subcutaneous WAT.

Materials and methods

Patient samples

This study was conducted in accordance with the guidelines in the Declaration of Helsinki and approved by the ethics committee at Karolinska University Hospital. The study was explained in detail to each subject, and written informed consent was obtained. Subcutaneous WAT was obtained from healthy women undergoing elective surgical procedures of fat removal for esthetic purposes (age 39.5 \pm 10.3 years; BMI 31.9 \pm 8.5 kg/m²).

Cell culture

Cell culture experiments were performed on *in vitro* differentiated adipocytes isolated from subcutaneous WAT. Isolation, culture, and *in vitro* differentiation of human pre-adipocytes were performed as previously described [26]. Experiments were performed in 12-well plates after 10–12 days of differentiation. At the end of the differentiation, the adipocytes displayed similar functional and morphological characteristics as mature fat cells. Most importantly, we have previously shown that the expression of adipocyte-specific genes and proteins, lipolytic responses, and GPDH activity (a marker of adipocyte differentiation) at day 12 in culture was in the same range as in mature primary fat cells [27].

Treatments

After 10–12 days of differentiation, adipocytes were treated with or without DHCC (10^{-7} M; Sigma–Aldrich, St. Louis MO) and/or TNF α (100 ng/mL, Sigma–Aldrich) for 24 or 48 h. The concentration of DHCC was determined in separate titration experiments, and the lowest concentration giving significant effects without affecting morphology or expression of adipocyte-specific genes of the cells was used throughout the study (data not shown). In pre-incubation wells, adipocytes were initially pre-treated with DHCC for 24 h after which DHCC was removed by washing and adipocytes were further incubated with TNF α for an additional time period of 24 h. In concomitant wells, adipocytes were treated with DHCC and TNF α (both agents added at the same time) for a total time of 48 h including a medium change after 24 h. DHCC and TNF α were dissolved in ethanol and water, respectively, according to the instructions of the manufacturer. Control cells were treated with the same amount of corresponding vehicle.

Quantitative real-time PCR

Cells were treated as described earlier, and total RNA was extracted using the NucleoSpin RNA II (Macherey–Nagel, Düren, Germany). RNA concentration and purity were assessed spectrophotometrically with a NanoDrop ND-1000 (Thermo Scientific, Wilmington, DE). One microgram of total RNA was reverse-transcribed to cDNA using Omniscript reverse transcription kit (QIAGEN, Hilden, Germany) and random hexamer primers (Invitrogen, Carlsbad, CA). For SYBR Green assays (*ADIPOQ* coding for adiponectin; *LIPE* coding for hormone-sensitive lipase; *PNPLA2* coding for adipose triglyceride lipase; *PLIN1* coding for perilipin-1; *PPARG* coding for peroxisome proliferator-activated receptor gamma), 5 ng of cDNA was mixed with 2x iQ SYBR Green Supermix (Eurogentec SA, Ougre'e, Belgium) and primers (Invitrogen,) in a final volume of 25 μ L as previously described [28–30]. For TaqMan assays, cDNA (10 ng) was mixed with 2x TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and TaqMan primers (*CCL2* coding for MCP-1; *SLC2A1* coding for glucose transporter 1, GLUT-1; Applied Biosystems) in a final volume of 20 μ L [14, 30]. Quantitative real-time PCR was performed in an iCycler IQ (Bio-Rad Laboratories, Hercules, CA). mRNA levels were determined by a comparative threshold cycle (Ct) method. Ct values were normalized to the reference gene *LDL receptor-related protein 10 (LRP10)*, according to the formula $2^{\Delta\Delta Ct} = \text{Arbitrary Units}$

(AU). The PCR efficiency in all runs was close to 100%, and all samples were run in duplicate.

Adipokine measurements

Human MCP-1 and adiponectin protein levels were measured in medium aliquots using a Quantikine Immunoassay (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions.

Statistical analysis

Results are means \pm SEM. Statistical analyses were performed by paired or unpaired Student's *t* test (two tailed) unless otherwise stated. Similar statistically significant results were obtained when using non-parametric tests (data not shown). Differences were considered as statistically significant at $p < 0.05$. The statistical analyses were performed using Statview software (version 5.01; SAS Institute, Cary, NC).

Results

Effects of DHCC on morphology and expression of adipocyte-specific genes

In initial experiments, we determined the effects of different concentrations of DHCC on adipocyte morphology. Concentrations from 10^{-8} to 10^{-6} M did not affect adipocyte morphology in our cell culture model at any stage of differentiation (data not shown). These concentrations are considered to be within the physiological and pharmacological range of DHCC as reported elsewhere [31]. The effect of different concentrations of DHCC on the expression of genes characteristic of fat cells [hormone-sensitive lipase (*LIPE*), adipose triglyceride lipase (*PNPLA2*), perilipin (*PLIN1*), and peroxisome proliferator-activated receptor γ (*PPARG*)] was assessed in fully differentiated adipocytes treated for 24 or 48 h. Incubation with DHCC did not affect gene expression at either time point or concentration (data not shown). As expected, treatment with 100 ng/mL TNF α down-regulated the mRNA levels of *LIPE*, *PNPLA2*, *PLIN1*, and *PPARG* (by approximately 90%). However, the addition of DHCC either prior to or concomitant with TNF α treatment did not affect the mRNA levels of any of these genes at any concentration tested (for 10^{-7} M, Fig. 1a). This demonstrates that in differentiated human adipocytes, DHCC, in the range between 10^{-8} and 10^{-6} M, is not toxic and does not affect adipocyte morphology or the expression of genes typical for fat cells. For all subsequent experiments, results are presented for 10^{-7} M DHCC.

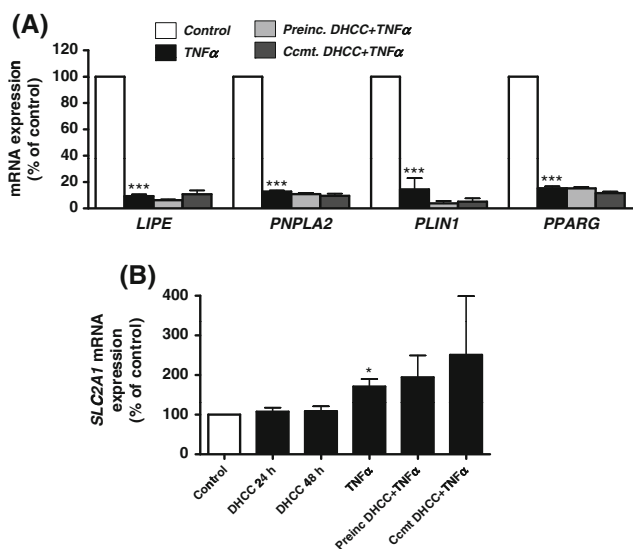


Fig. 1 Effects of TNF α and 1 α ,25-dihydroxycholecalciferol (DHCC) on the mRNA expression of adipocyte-specific genes and the hypoxia-responsive gene *SLC2A1*. Human adipocytes were treated with 100 ng/mL TNF α for 48 h with or without 10⁻⁷ M DHCC (Ccmt DHCC + TNF α and TNF α , respectively). In addition, cells were preincubated with DHCC for 24 h followed by a 24-h incubation with TNF α (Preinc DHCC + TNF α). Messenger RNA levels of *LIPE*, *PNPLA2*, *PLIN1*, *PPARG* (a), and *SLC2A1* (b) are displayed. * $p < 0.05$, *** $p < 0.001$ as compared to control adipocytes. Results are shown as a mean of up to six independent experiments. Data are expressed as mean \pm SEM

Effects of DHCC on the mRNA levels of the hypoxia-responsive gene *SLC2A1*

Previous studies have demonstrated that hypoxia affects the expression and secretion of inflammation-related adipokines in human adipocytes [32]. To assess whether DHCC could affect the expression of hypoxia-responsive genes, we determined the mRNA expression levels of *SLC2A1* (coding for the protein GLUT-1), a gene previously shown to be highly up-regulated under hypoxic conditions in human adipocytes [33]. The presence of DHCC alone, prior to or concomitantly with TNF α , did not alter *SLC2A1* levels (Fig. 1b). In addition, although TNF α up-regulated ($p < 0.05$) *SLC2A1* mRNA expression, this effect was not modified by DHCC. This suggests that the effects of DHCC on adipokine expression are not secondary to changes in hypoxia-related genes.

Effects of DHCC on MCP-1 production in human adipocytes

DHCC induced a $\sim 30\%$ decrease in *CCL2* mRNA expression after both 24 h ($p < 0.01$) and 48 h ($p < 0.05$) of treatment compared to control adipocytes (Fig. 2a). As expected, TNF α induced a pronounced (30–40-fold)

induction of *CCL2* mRNA expression (Fig. 2b). Pre-incubation with DHCC for 24 h attenuated the increase in *CCL2* mRNA induced by TNF α by $\sim 15\%$ ($p < 0.05$). This effect was even more marked following concomitant treatment with DHCC and TNF α for 48 h ($\sim 60\%$, $p < 0.01$). DHCC alone (Fig. 2c) or a 24-h incubation prior to TNF α treatment (Fig. 2d) did not modify MCP-1 protein secretion. However, there was a trend ($p = 0.056$, two-tailed; $p = 0.028$ one-tailed test) toward decreased TNF α -induced MCP-1 protein release in cells concomitantly incubated with DHCC and TNF α (Fig. 2d). In adipocytes incubated with 10⁻⁶ M DHCC, the attenuating effect on MCP-1 secretion was significant ($p < 0.01$, graph not shown). Thus, in our experimental settings, DHCC reduces *CCL2* mRNA expression although the effects at the protein level are less pronounced.

Effects of DHCC on adiponectin secretion in human adipocytes

Incubation with DHCC did not affect *ADIPOQ* mRNA expression (Fig. 3a). However, DHCC alone induced a $\sim 35\%$ decrease in adiponectin secretion after treatment for both 24 and 48 h ($p < 0.05$; Fig. 3c). TNF α markedly reduced *ADIPOQ* production at both the mRNA ($\sim 80\%$, $p < 0.001$ Fig. 3b) and protein level ($\sim 35\text{--}40\%$, $p = 0.01$, Fig. 3d). Concomitant treatment with DHCC and TNF α decreased adiponectin protein secretion in an additive manner ($\sim 75\%$, $p < 0.01$ Fig. 3d). In contrast, DHCC either prior to or concomitantly with TNF α did not significantly modify *ADIPOQ* mRNA levels (Fig. 3b). This suggests that DHCC mainly affects adiponectin secretion/release at the post-transcriptional level.

Discussion

Several studies have demonstrated the ability of DHCC to modulate energy metabolism by regulating lipogenesis and lipolysis [19] as well as adipokine expression in human adipocytes [34]. However, its interaction with inflammatory factors has not been elucidated before in human fat cells. In this work, we demonstrate that DHCC has differential effects on TNF α regulation of MCP-1 and adiponectin, two important adipokines with opposite effects on insulin sensitivity and inflammation. DHCC reduces the mRNA levels of *CCL2* and to a lesser extent also protein secretion. Therefore, DHCC might attenuate adipose tissue inflammation by reducing MCP-1. On the other hand, DHCC inhibited adiponectin secretion, an adipokine that improves insulin sensitivity and inflammation [35, 36]. These novel data suggest a dual role of vitamin D in the adipose inflammation.

Fig. 2 Effects of 1 α ,25-dihydroxycholecalciferol (DHCC) and TNF α on *CCL2* mRNA expression and MCP-1 secretion. Human adipocytes were incubated as described in Fig. 1 (b, d) or with DHCC alone for 24 h or 48 h (a, c). Concentrations of DHCC and TNF α were the same as in the experiments described in Fig. 1. The effects of DHCC on MCP-1 secretion were only statistically significant in cells treated concomitantly with TNF α and DHCC when using a one-sided *t* test (d). **p* < 0.05, ***p* < 0.01 as compared to control or TNF α -treated adipocytes. * \S *p* < 0.05 compared to TNF α -stimulated adipocytes using one-tailed *t* test. Results are shown as a mean of up to six independent experiments. Data are expressed as mean \pm SEM

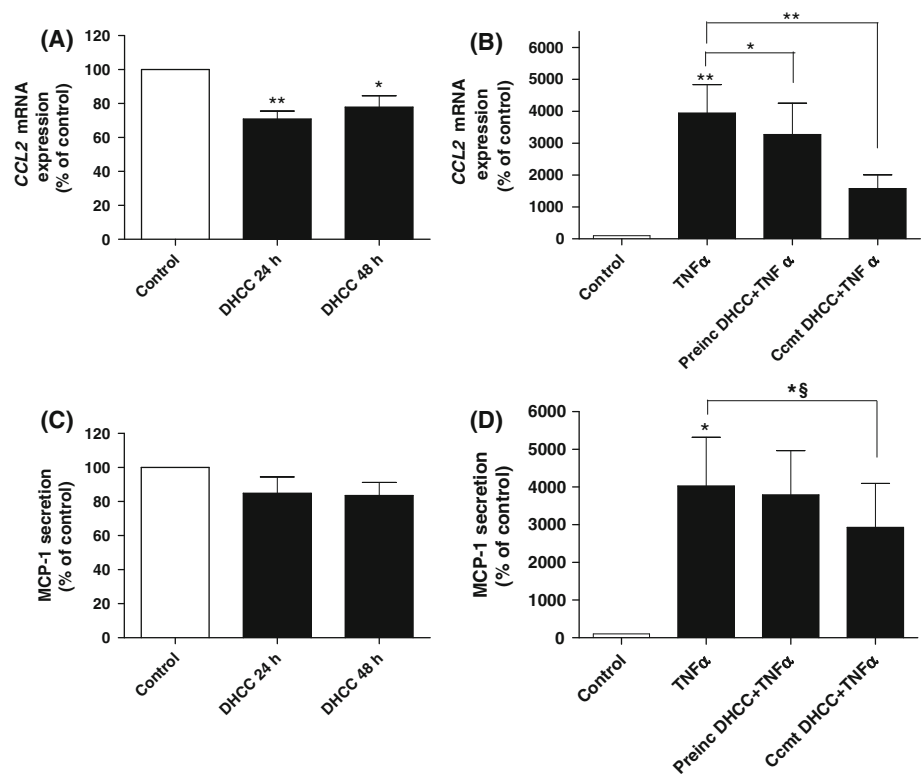
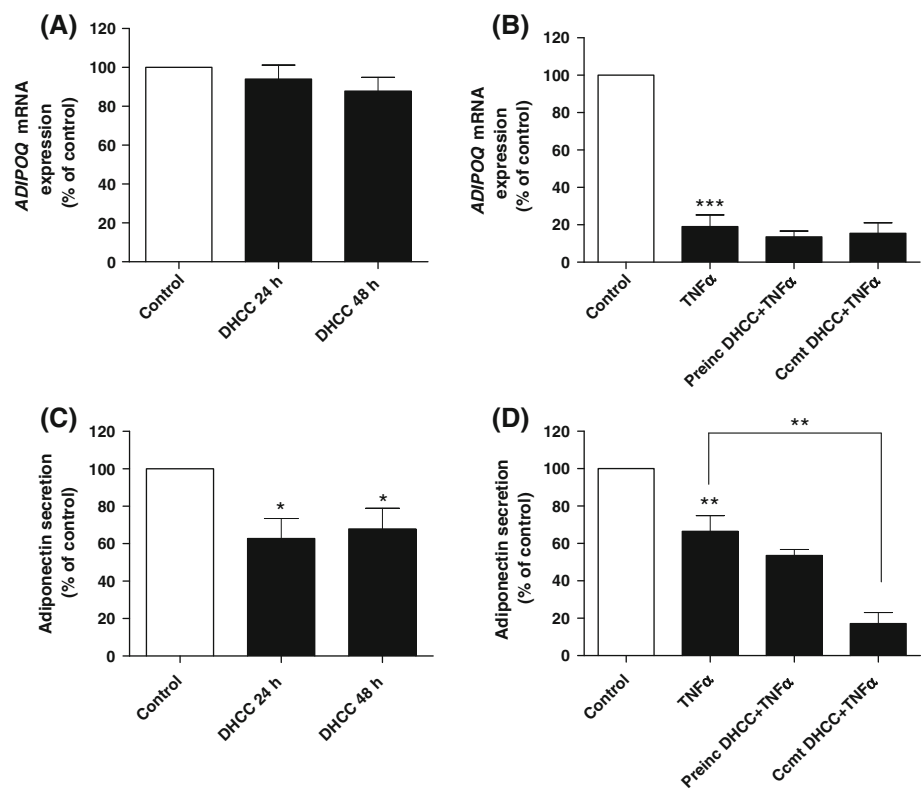


Fig. 3 Effects of 1 α ,25-dihydroxycholecalciferol (DHCC) and TNF α on *ADIPOQ* expression and adiponectin secretion. Human adipocytes were incubated as described in Fig. 2. Effects on *ADIPOQ* mRNA levels (a, b) and adiponectin secretion (c, d) are displayed. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 as compared to control or TNF α -treated adipocytes. Results are shown as a mean of up to six independent experiments. Data are expressed as mean \pm SEM



DHCC appears to regulate the expression of these two factors via different mechanisms. Thus, adiponectin secretion seems to be controlled at the post-transcriptional

level. It has previously been demonstrated that TNF α inhibits adiponectin production via down-regulation of pro-adipogenic factors, such as PPAR γ [37]. However, it is not

likely that DHCC attenuates adiponectin secretion via reduced adipocyte differentiation since DHCC did not affect the morphology or the expression of fat cell-specific genes. Therefore, the mechanism(s) through which DHCC affects adiponectin secretion remain to be defined.

In contrast to *ADIPOQ*, *CCL2* is most probably a direct VDR target gene. The latter is supported by an *in silico* promoter analysis we have performed. We identified several VDREs in the human *CCL2* gene promoter using the *MatInspector* software (www.genomatix.de/). Admittedly, there was only a trend regarding the effect of DHCC on MCP-1 secretion, but it is possible that longer incubation times would have resulted in more prominent changes. In any case, our results are in agreement with the previous studies, showing that DHCC down-regulates *CCL2* expression in primary kidney cells [38] and renal epithelial cells [39]. In the latter study, the authors reported that DHCC suppressed TNF α -induced *MCP-1* mRNA expression and suggested a possible role for DHCC as a modulator of renal inflammation, an effect proposed to be mediated via nuclear factor κ B. However, our findings are in contrast to the results published by Sun and Zemel [40] who reported that 10^{-8} M DHCC up-regulated *MCP-1* mRNA expression in differentiated 3T3-L1 adipocytes after 48 h of treatment. Differences in concentrations but more probably cell systems (murine 3T3-L1 adipocytes vs. human adipocytes) could explain these discrepancies.

We also show that DHCC did not affect the expression of the hypoxia-responsive gene *SLC2A1*, suggesting that the effects of DHCC on adipokine expression are not secondary to changes in hypoxia-responsive genes. This, however, does not preclude that DHCC could regulate hypoxia-sensitive genes in other cell types within adipose tissue, a question that will be addressed in future experiments.

The use of human adipocytes in this study constitutes an advantage when interpreting the results in a physiological context. We have recently shown that these cells express genes and phenotypical traits comparable to those of mature primary fat cells freshly isolated from adipose tissue [27]. In contrast, murine adipocytes and other available fat cell lines are qualitatively different in many important aspects [41].

The concentration of DHCC used in the present study might be a point for potential controversies, since Sun and Zemel reported a pro-apoptotic role for DHCC at high (pharmacological) concentrations of the hormone (\geq to 10^{-7} M) in human adipocytes *in vitro* [42]. However, under the conditions used in our experiments, we did not observe any toxic effects at concentrations up to 10^{-6} M and no effects on the mRNA expression of adipocyte-specific genes, such as *LIPE*, *PNPLA2*, *PLIN1* and *PPARG*. This is in agreement with a study in porcine adipocytes [43], where 10^{-6} M DHCC had no effects on cell viability.

In any case, it should be stressed that it is difficult to compare concentrations used *in vitro* with the levels of DHCC in plasma or adipose tissue *in vivo*.

The effect of vitamin D supplementation on insulin sensitivity in clinical trials is not clear (reviewed in [44]). While some authors have reported improvements in parameters of insulin secretion and/or resistance, others have not observed any changes. A caveat in most of these studies is that they were rather small and neither randomized nor placebo-controlled, which therefore warrants further research. This study sheds new light on the complex role of DHCC in human inflammation. It could be speculated that the dual effects of DHCC on adipokine secretion could to some extent explain the lack of clear effects on insulin sensitivity of vitamin D supplementation in humans. Clinical studies are underway in order to elucidate the role of DHCC in obesity and insulin resistance.

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Conflict of interest All the authors have read and approved submission of the manuscript and declare no conflict of interest.

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