Calcitriol and calcium regulate cytokine production and adipocyte–macrophage cross-talk

Xiaocun Sun, Michael B. Zemel*

Department of Nutrition, University of Tennessee, Knoxville, TN 37996-1900, USA

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Abstract

Objective: The objective of this study was to investigate the effects of calcitriol on adipocyte and macrophage cytokine expression as well as release and on adipocyte–macrophage cross-talk in local modulation of inflammation.

Research Procedures and Results: We investigated calcitriol modulation of the expression of macrophage inhibitory factor (MIF) and macrophage surface-specific protein CD14, two key factors in regulating macrophage function and survival, in differentiated human adipocytes. Calcitriol significantly increased MIF and CD14 expression by 59% and 33%, respectively, while calcium-channel antagonism with nifedipine completely reversed these effects, indicating that calcitriol stimulates MIF and CD14 expression via a calcium-dependent mechanism. Similar results were also found in cultured 3T3-L1 adipocytes; in addition, calcitriol also up-regulated macrophage colony-stimulating factor, macrophage inflammatory protein, interleukin-6 (IL-6) as well as monocyte chemoattractant protein-1 expression in 3T3-L1 adipocytes and stimulated tumor necrosis factor as well as IL-6 expression in RAW 264 macrophages. These effects were blocked by either a calcium-channel antagonist (nifedipine) or a mitochondrial uncoupler (dinitrophenol). Moreover, co-culture of 3T3-L1 adipocytes with RAW 264 macrophages significantly increased the expression and production of multiple inflammatory cytokines in response to calcitriol in both cell types.

Conclusions: These data demonstrate that calcitriol regulates local inflammation via modulating the interaction between adipocytes and macrophages as well as regulating inflammatory cytokine production in each cell type via calcium-dependent and mitochondrial uncoupling-dependent mechanisms. These data provide further mechanistic explanation for our recent observations that suppression of calcitriol by dietary calcium reduces inflammatory cytokine expression and oxidative stress in adipose tissue.

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1. Introduction

Obesity is characterized by increased oxidative and inflammatory stress [1–4]. Adipose tissue is a significant source of reactive oxygen species (ROS) and expresses and secretes a wide variety of inflammatory components, such as tumor necrosis factor (TNFα) and interleukin-6 (IL-6), in obese individuals [5]. Adipose tissue also includes a stromal–vascular fraction that contains blood cells, endothelial cells and macrophages [6–8]. Although adipocytes directly generate inflammatory mediators, adipose tissue-derived cytokines also originate substantially from these non-fat cells, among which the infiltrated macrophages appear to play a prominent role [9]. Infiltration and differentiation of adipose tissue-resident macrophages are under the local control of chemokines, many of which are produced by adipocytes. Accordingly, cross-talk between adipocytes and macrophages may be a key factor in mediating inflammatory and oxidative changes in obesity. This hypothesis is supported by the observation that co-culture of rodent adipocytes and macrophages results in marked elevation of a pro-inflammatory cytokine (TNFα) and down-regulation of an anti-inflammatory factor (adiponectin) in adipocytes [10]. However, the impact of adipocyte-derived factors on macrophage secretory activity has yet to be investigated.

Previous data from this laboratory demonstrate that dietary calcium exerts an antiobesity effect that is mediated,

* Corresponding author. Tel.: +1 865 974 6238; fax: +1 865 974 3491.
E-mail address: mzemel@utk.edu (M.B. Zemel).

in part, by inhibiting calcitriol [1,25(OH)$_2$-D$_3$] secretion [11–13]. We have shown that calcitriol induces an increase in intracellular calcium ([Ca^{2+}]i) in adipocytes via the 1,25 (OH)$_2$-D$_3$ membrane-associated rapid response steroid hormone (1,25D$_3$-MARRS) binding protein; this increase in [Ca^{2+}]i stimulates lipogenesis [14] as well as ROS production [15] and inhibits lipolysis [16]. We have also shown calcitriol to exert a dose-responsive inhibition of uncoupling protein-2 (UCP2) expression in adipocytes mediated by the classic nuclear vitamin D receptor [17,18]. Moreover, increasing dietary calcium suppressed adipocyte [Ca^{2+}]i, increased mitochondrial UCP2 expression [19] and promoted adipocyte apoptosis [20] in white adipose tissue of mice fed with an obesigenic diet, and it also reduced metabolic efficiency, adiposity and oxidative stress in an animal model of obesity, suggesting that dietary strategies designed to suppress circulating calcitriol levels may reduce adiposity and oxidative stress.

We have recently found dietary calcium to attenuate obesity-associated inflammatory stress as well as oxidative stress in a mouse model of obesity [15,21]. Because calcitriol regulates Ca^{2+} signaling and ROS production, and each of these modulates cytokine production and release, it is likely that calcitriol regulates cytokine production by adipocytes and macrophages and that dietary calcium modulation of inflammatory stress is mediated by suppression of calcitriol. However, the role of calcitriol in modulating adipocyte and macrophage cytokine production and that in adipocyte–macrophage cross-talk have not yet been investigated. We thus hypothesized that treatment of adipocytes with calcitriol would promote expression of pro-inflammatory cytokines and inflammatory markers and that this effect is mediated by increased [Ca^{2+}]i and reduced mitochondrial uncoupling. We further reasoned that macrophage–adipocyte interactions would modify these outcomes.

2. Materials and methods

2.1. Chemicals

Calcitriol, dinitrophenol (DNP), nifedipine, dexamethasone, penicillin/streptomycin, fetal bovine serum (FBS) and IBMX were obtained from Sigma (St. Louis, MO, USA). Calcitriol, DNP and nifedipine were diluted in 100% ethanol to make 10,000× stock before use.

2.2. Cell culture

The human adipocytes used in this study were cultured and supplied by Zen-Bio (Research Triangle, NC, USA). Cells were originally pooled from the subcutaneous fat of six healthy female subjects (body mass index range= 26.56–32.77 kg/m^2) and then cultured until 85–90% differentiated. Briefly, preadipocytes were inoculated in Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F-10 medium (DMEM/F-10) (1:1, vol/vol) containing 10% FBS, 15 mmol/L of HEPES and antibiotics at a density of 30,000 cells/cm^2. Confluent monolayers of preadipocytes were induced to differentiate with a standard differentiation medium consisting of DMEM/F-10 (1:1, vol/vol) supplemented with 15 mmol/L of HEPES, 3% FBS, 33 µmol/L of biotin, 17 µmol/L of pantothenate, 100 nmol/L of insulin, 0.25 µmol/L of methylisobutylxanthine, 1 µmol/L of dexamethasone, 1 µmol/L of BRL49653 and antibiotics. Preadipocytes were maintained in this differentiation medium for 3 days and subsequently cultured in an adipocyte medium in which BRL49653 and MIX were omitted. Cultures were refed every 2–3 days.

RAW 264 macrophages and 3T3-L1 preadipocytes (American Type Culture Collection) were incubated at a density of 8000 cells/cm^2 (10-cm^2 dish) and grown in high-glucose DMEM (Gibco) containing 10% FBS and 1% penicillin/streptomycin (adipocyte medium; Gibco) at 37°C in 5% CO$_2$ in air. Confluence in 3T3-L1 preadipocytes was reached in 2–3 days, after which cells were induced to differentiate in the absence of added insulin with a standard differentiation medium consisting of DMEM supplemented with 10% FBS, 1 µM of dexamethasone, IBMX (0.5 mM) and 1% penicillin/streptomycin. Preadipocytes were maintained in this differentiation medium for 3 days and subsequently cultured in the adipocyte medium. Cultures were refed every 2–3 days to allow 90% cells to reach full differentiation for 3T3-L1 adipocytes or grow to a confluence for RAW 264 before conducting chemical treatment. Cells were treated without (vehicle only) or with calcitriol (10 nmol/L), DNP (100 µmol/L) and/or nifedipine (10 µmol/L) for 48 h, as indicated in each figure.

Cells were washed with fresh adipocyte medium, refed with medium containing the indicated treatments and incubated at 37°C in 5% CO$_2$ for 48 h before analysis. Cell viability was measured via trypan blue exclusion.

2.3. Co-culture of adipocytes and macrophages

Cells were co-cultured by using transwell inserts with a 0.4-µm porous membrane (Corning) to separate adipocytes and macrophages. After incubation for 48 h, the cells in the lower well were harvested for further analysis. Experiments were repeated with each cell type (3T3-L1 and RAW 264) in the lower well.

2.4. Total RNA extraction

A total cellular RNA isolation kit (Ambion, Austin, TX, USA) was used to extract total RNA from cells according to the manufacturer's instruction. The concentration and purity of the isolated RNA were measured spectrophotometrically, and the integrity of the RNA sample was analyzed with the use of a BioAnalyzer (Agilent 2100, Agilent Technologies).

2.5. Quantitative real-time polymerase chain reaction

Adipocyte and muscle 18s, CD14, TNFα, macrophage inflammatory protein (MIP), macrophage colony-stimulating factor (M-CSF), IL-6 and monocyte chemoattractant

protein-1 (MCP-1) were quantitatively measured using a Smart Cycler Real-Time Polymerase Chain Reaction (PCR) System (Cepheid, Sunnyvale, CA, USA) with a TaqMan 1000 Core Reagent Kit (Applied Biosystems, Branchburg, NJ, USA). The primer and probe sets were obtained from the Applied Biosystems TaqMan Assays-on-Demand Gene Expression primer and probe set collection according to the manufacturer's instruction. Pooled adipocyte total RNA was serial diluted in the range of 1.5625–25 ng and used to establish a liner standard curve that was first constructed from an RNA of known concentration; total RNAs for samples were also diluted in this range and then calculated for quantitative information for messenger RNA (mRNA) targets of unknown concentrations according to the standard curve. This approach evaluates the C_t changes for each target gene (including 18s) to report the amount of each target in arbitrary units, which are then normalized as ratios to 18s arbitrary units. Reactions of quantitative real-time PCR for standards and unknown samples were also performed according to the instructions of the Smart Cycler System and TaqMan Real Time PCR Core Kit (Applied Biosystems).

2.6. Cytokine antibody array

A TransSignal mouse cytokine antibody array kit (Panomics, Fremont, CA, USA) was used to detect cytokine protein released in culture medium according to the manufacturer's instruction. Briefly, membranes immobilized with capture antibodies specific to particular cytokine proteins were incubated with 1× blocking buffer for 2 h, and then the blocking buffer was washed three times with washing buffer. Then, the membranes were incubated in samples for 2 h to allow cytokine protein in the culture medium to bind to the capture antibody on the membrane. At the end of the incubation, unbound protein was washed away with washing buffer. The membranes were then incubated with biotin-conjugated antibody mix, which binds to a second epitope on the protein. The membranes were then washed and incubated with streptavidin–horse radish peroxide to visualize the antibody–protein complexes on the array to determine which cytokines are present in the sample via a chemiluminescent signal that was detected using X-ray film.

2.7. Statistical analysis

Each treatment was performed using six independent biological replicates, and data were expressed as mean± S.E.M. Data were evaluated for statistical significance using analysis of variance (ANOVA), and significantly different group means were then separated by the least significant difference test using SPSS (SPSS, Chicago, IL, USA). The co-culture experiments were analyzed via two-way (Treatment×Culture condition) ANOVA.

3. Results

Calcitriol increased macrophage inhibitory factor (MIF) and CD14 expression by 59% (Fig. 1A) and 33% (Fig. 1B), respectively, in human adipocytes, while calcium-channel antagonism with nifedipine reversed these effects, indicating that calcitriol stimulates MIF and CD14 expression by regulating $[Ca^{2+}]i$ signaling. Similar results were found in cultured 3T3-L1 adipocytes, with calcitriol stimulating MIF expression by 50% (Fig. 1C) and CD14 expression by 45% (Fig. 1D) and nifedipine exerting the opposite effects. In addition, calcitriol up-regulated M-CSF (Fig. 2A), MIP (Fig. 2B), IL-6 (Fig. 2C) and MCP-1 (Fig. 2D) expression in differentiated 3T3-L1 adipocytes cultured alone by two- to threefold, and these effects were blocked by either nifedipine
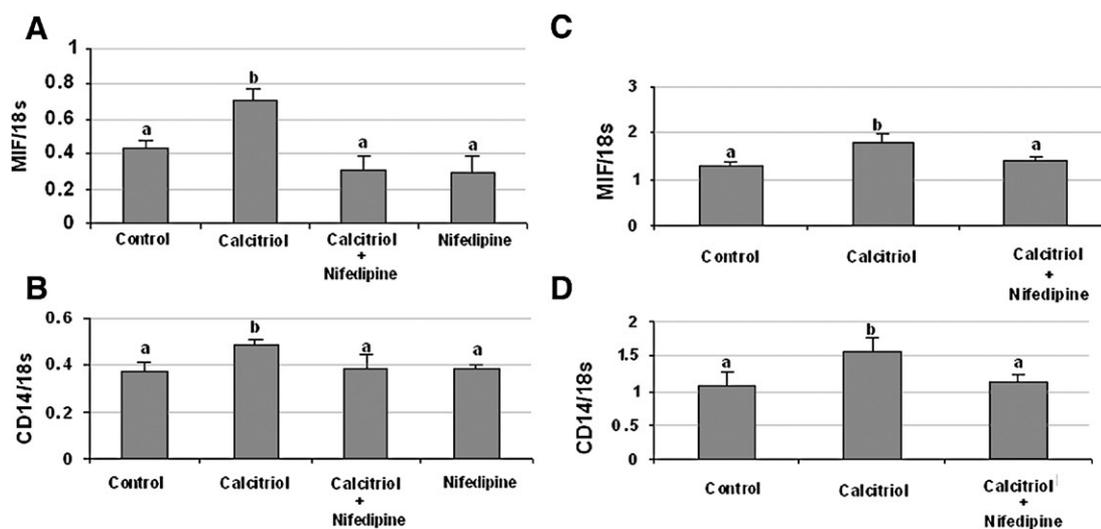

Fig. 1. Effects of calcitriol and calcium on the MIF and 18s expression ratio (A) and CD14 and 18s expression ratio (B) in differentiated Zen-Bio human adipocytes. The MIF and 18s expression ratio as well as CD14 and 18s expression ratio in differentiated 3T3-L1 adipocytes are shown in Panels (C) and (D), respectively. Means with different letters differ: $P=.001$ for Panel (A), $P=.008$ for Panel (B), $P<.01$ for Panel (C) and $P<.05$ for Panel (D).
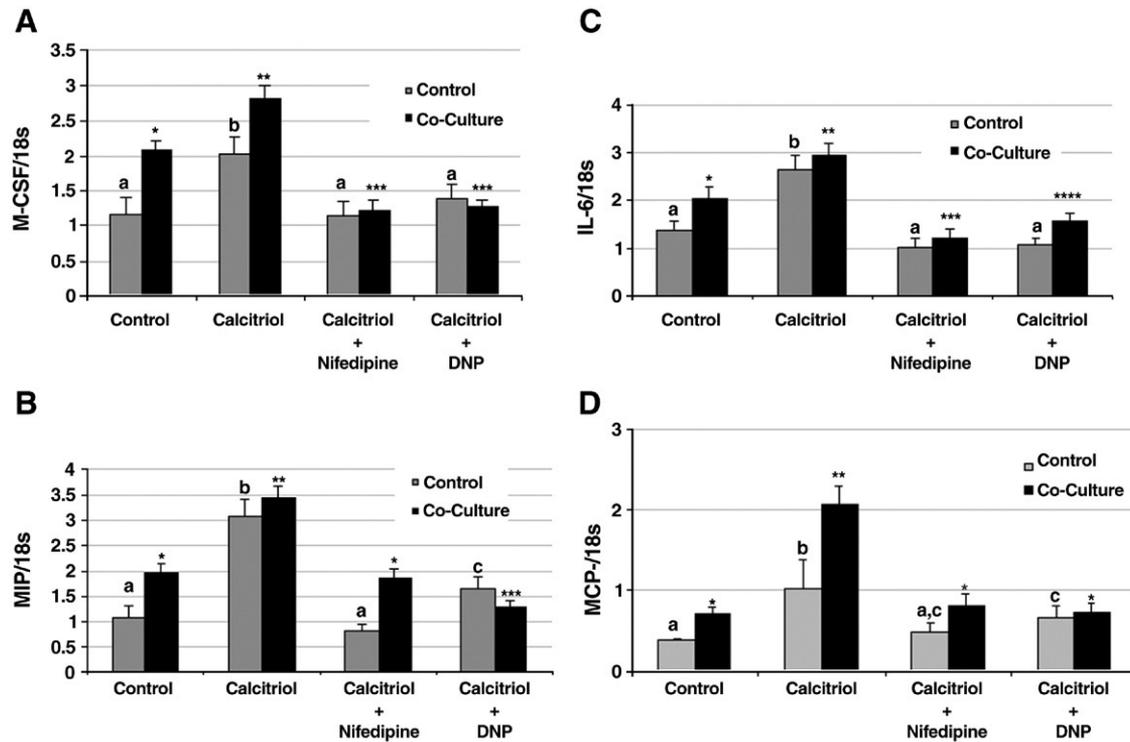
Fig. 2. Effects of calcitriol on the M-CSF and 18s expression ratio (A), MIP and 18s expression ratio (B), IL-6 and 18s expression ratio (C) as well as MCP-1 and 18s expression ratio (D) in differentiated 3T3-L1 adipocytes with or without co-culture with RAW 264 macrophages. Values are presented as mean±S.E.M., n=6. (A) Means with different letters differ for the control culture (P=.039), and means with different symbols differ for the co-culture (P<.001); main effects of chemical treatment and culture status were significant (P=.015). (B) Means with different letters differ for the control culture (P=.004), and means with different symbols differ for the co-culture (P<.001); main effects of chemical treatment and culture status were significant (P<.01). (C) Means with different letters differ for the control culture (P=.001), and means with different symbols differ for the co-culture (P<.001); main effects of chemical treatment and culture status were significant (P<.02). (D) Means with different letters differ for the control culture (P<.001), and means with different symbols differ for the co-culture (P<.001); main effects of chemical treatment and culture status were significant (P=.001).

or a mitochondrial uncoupler (DNP). Moreover, co-culture of 3T3-L1 adipocytes with RAW 264 macrophages significantly increased the expression of these inflammatory cytokines, suggesting that calcitriol may regulate macrophage activity by modulating adipocyte production of factors associated with macrophage function. Calcitriol also markedly stimulated TNFα expression by 91% (Fig. 3A) and IL-6 expression by 796% (Fig. 3B) in RAW 264 macrophages cultured alone, and these effects were blocked by adding nifedipine or DNP. Co-culture of macrophages with differentiated 3T3-L1 adipocytes markedly augmented TNFα (Fig. 3A) and IL-6 (Fig. 3B) expression in macrophages, and these effects were further enhanced by calcitriol.

We used a cytokine antibody array to further investigate the effects of calcitriol on the release of major inflammatory cytokines from adipocytes. These protein data support the gene expression observations as calcitriol up-regulated the production of multiple inflammatory cytokine proteins in differentiated 3T3-L1 adipocytes cultured alone (Figs. 4A and 5A); these include TNFα, IL-6, IL-2, interferon (IFN)-inducible protein-10, IL-4, IL-13, macrophage-induced gene, RANTES (regulated upon activation, normal T-cell expressed and secreted), IL-5, MIP-1α and vascular endothelial growth factor. Co-culture of 3T3-L1 adipocytes

with macrophages resulted in markedly greater production of cytokines such as IFN-γ, TNFα, granulocyte colony-stimulating factor and MIP-1α, as compared with 3T3-L1 cultured alone (Figs. 4B and 5B), although we cannot discern the source of the additional secretion (adipocyte or macrophage), and calcitriol further stimulated inflammatory cytokine production.

4. Discussion

Data from this study demonstrate that calcitriol stimulates production of adipokines associated with macrophage function and increases inflammatory cytokine expression in macrophages and adipocytes; these include CD14, MIF, M-CSF, MIP, TNFα, IL-6 and MCP-1 in adipocytes as well as TNFα and IL-6 in macrophages. Consistent with this, the cytokine protein array identified multiple additional inflammatory cytokines that were up-regulated by calcitriol in adipocytes. Moreover, calcitriol also regulated cross-talk between macrophages and adipocytes, as shown by the augmentation of expression and production of inflammatory cytokines from adipocytes and macrophages in co-culture versus individual culture. These effects were
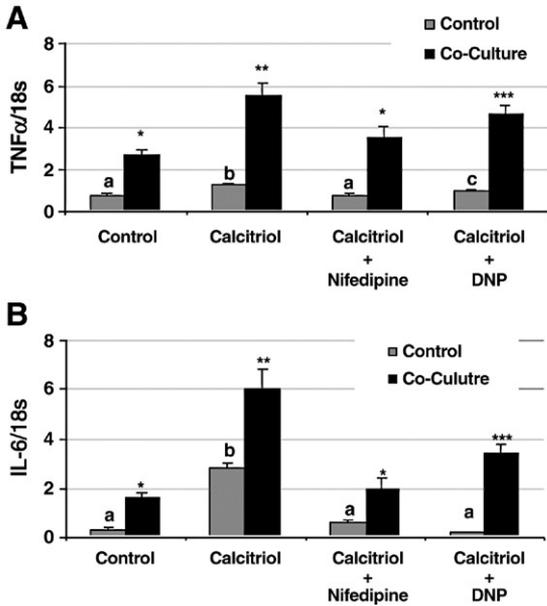
Fig. 3. Effects of calcitriol on the TNFα and 18s expression ratio (A) and IL-6 and 18s expression ratio (B) in RAW 264 macrophages with or without co-culture with differentiated 3T3-L1 adipocytes. Values are presented as mean±S.E.M., n=6. (A) Means with different letters differ for the control culture ($P<.001$), and means with different symbols differ for the co-culture (P=.01); main effects of chemical treatment and culture status were significant ($P<.001$). (B) Means with different letters differ for the control culture ($P<.001$), and means with different symbols differ for the co-culture ($P<.001$); main effects of chemical treatment and culture status were significant ($P<.001$).

attenuated by either calcium-channel antagonism or mitochondrial uncoupling, indicating that the pro-inflammatory effects of calcitriol are mediated by calcitriol-

induced stimulation of Ca^{2+} signaling and attenuation of mitochondrial uncoupling.

Mounting evidence indicate that obesity is associated with subclinical chronic inflammation [22,23]. Adipose tissue not merely is a simple reservoir of energy stored as triglyceride but also serves as an active secretory organ, releasing many peptides and cytokines into circulation [24–26]. In the presence of obesity, the balance between these numerous molecules is altered such that enlarged adipocytes produce more pro-inflammatory cytokines, such as TNFα and IL-6, and less anti-inflammatory factors, such as adiponectin. This dysregulation of adipokine production participates in the development of metabolic disorders associated with obesity [27]. However, adipose tissue not only is composed of adipocytes but also contains a stromal–vascular fraction that consists of endothelial cells, cells with characteristics of progenitor cells and leukocytes. Moreover, adipose tissue appears to be infiltrated by more macrophages as the degree of obesity increases [6]. Such macrophages may be the major source of cytokines that initiate the inflammatory state preceding the development of insulin resistance and atherosclerosis [9]. The local interaction between adipocytes and tissue-resident macrophages has thus received considerable attention in that adipocytes secrete a wide range of chemokines and growth factors that favor infiltration and differentiation of macrophages, while macrophages produce cytokines that regulate adipocyte growth, metabolism and secretory activity. For example, adipocyte-derived MCP-1 plays a crucial role in the recruitment of monocytes and T lymphocytes into adipose tissue [27] and obesity is associated with increased expression of MCP-1 in adipose tissue in rodent and humans
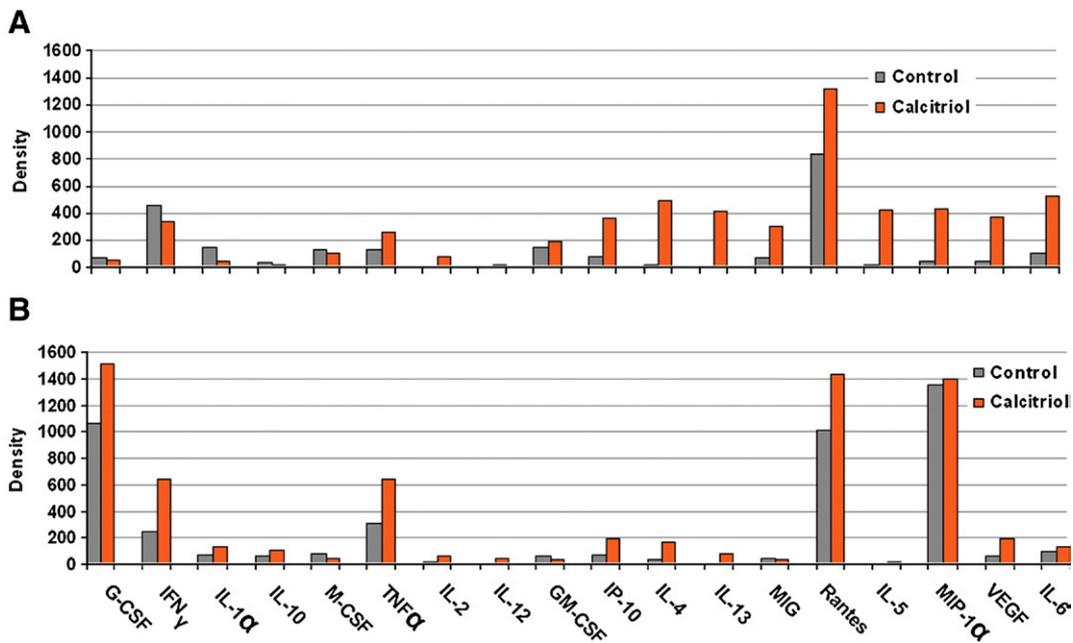

Fig. 4. Effects of calcitriol on inflammatory cytokine secretion: (A) signal intensities quantified by densitometry for differentiated 3T3-L1 adipocytes cultured alone and (B) signal intensities quantified by densitometry for differentiated 3T3-L1 adipocytes co-cultured with RAW 264 macrophages.
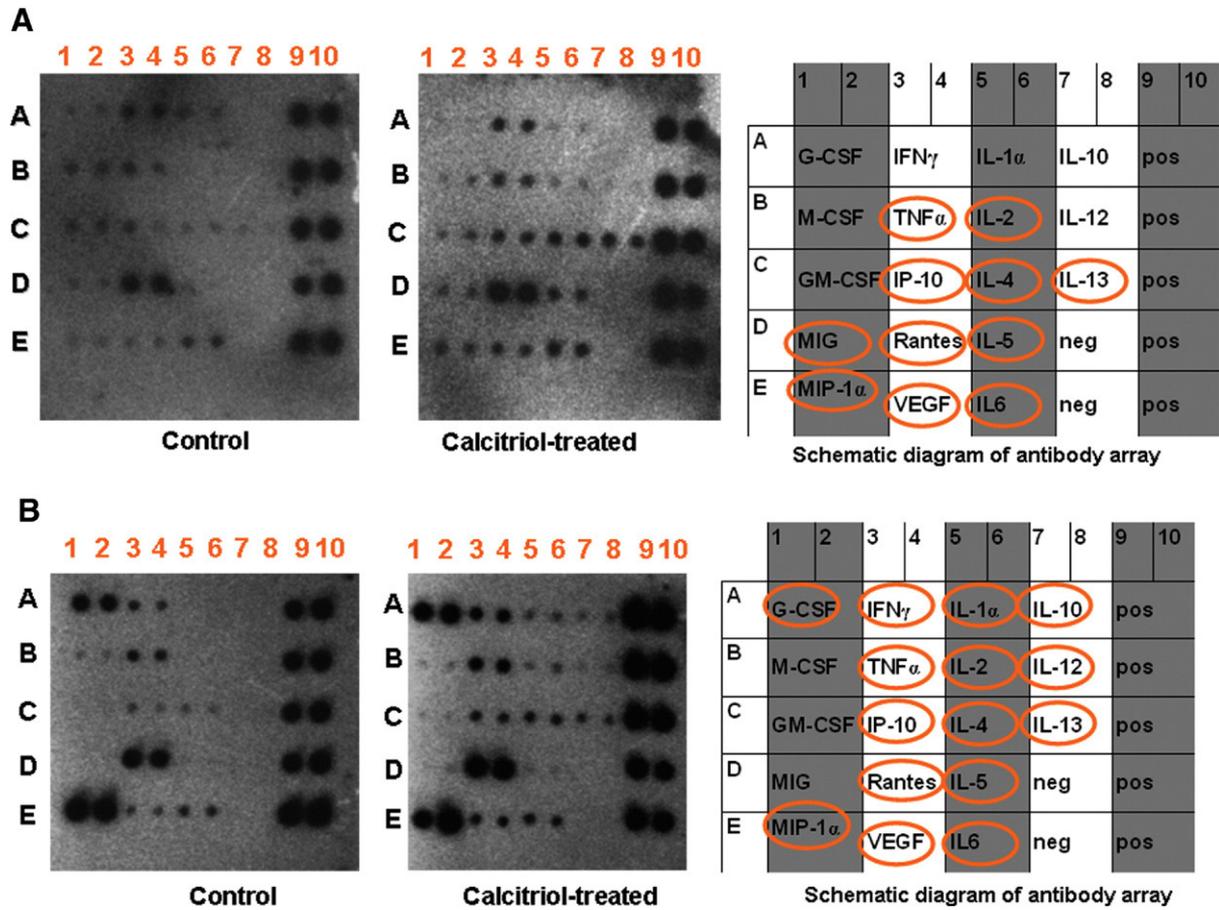
Fig. 5. Effects of calcitriol on inflammatory cytokine secretion: (A) membranes were incubated with control media and calcitriol-treated media collected from differentiated 3T3-L1 adipocytes cultured alone and (B) membranes were incubated with control media and calcitriol-treated media collected from differentiated 3T3-L1 adipocytes co-cultured with RAW 264 macrophages. Images were collected using an X-ray film.

[28–30]. These concepts are also supported by our present observations and data from other laboratories [31,32] that conditioned medium and co-culture significantly increase inflammatory cytokine production in adipocytes and macrophages, suggesting an interaction between these two cell types that contributes to increased inflammatory stress.

Oxidative stress also appears to play a role in regulating inflammatory status in adipose tissue as well as in modulating the adipocyte–macrophage interaction. Oxidative stress is augmented with increasing adiposity, and addition of oxidants has been found to suppress the expression of adiponectin and increase that of inflammatory factors [2]. These suggest that a local increase in adipose tissue oxidative stress results in dysregulated adipokine production. The role of adiposity in the up-regulation of oxidative stress and inflammation has been investigated intensively. Fat accumulation stimulates nicotinamide adenine dinucleotide phosphate (NADPH) oxidase expression in white adipose tissue [15,33,34], and NOX4, an isoform of NADPH oxidase, is expressed in adipocytes but not in macrophages [35,36]. Weisberg et al. [6] and Xu et al. [7] also reported that ROS stimulate macrophage infiltration of

adipose tissue via ROS-induced MCP-1 production as well as local NADPH expression and ROS production, indicating that adipocytes and macrophages contribute to elevated oxidative stress in obesity. In support of this concept, our current observations of calcitriol regulation of inflammatory cytokine expression and production in adipocytes and macrophages follow the same pattern as our previous observations of calcitriol regulation of oxidative stress [15] and demonstrate similar regulation by Ca^{2+} signaling and mitochondrial uncoupling. These suggest that calcitriol regulation of both ROS and inflammatory cytokine production may share a common pathway, although calcitriol-induced oxidative stress would be expected to further exacerbate inflammatory cytokine production as well.

We have previously shown that dietary calcium attenuates diet-induced obesity in humans and mice [11]. A key mechanism believed to underlie this antiobesity effect is suppression of calcitriol, which modulates $[Ca^{2+}]i$ signaling as well as mitochondrial uncoupling in adipocytes and consequently results in reduced adiposity and oxidative stress [15,37,38]. In contrast to our previous data, Boon et al. [39] recently reported that short-term (7 days) administration

of calcitriol to healthy subjects exerted no effect on adipose tissue mRNA levels of UCP2, fatty acid synthase, glycerol phosphate dehydrogenase, hormone-sensitive lipase and peroxisome proliferator-activated receptor-gamma. However, theirs was a short-term study performed in healthy nonobese males, and circulating calcitriol did not increase significantly until day 5 of their 7-day study. Accordingly, there may have been insufficient duration of exposure to elevated calcitriol to observe a significant change in gene expression [39].

Because obesity is highly associated with inflammation and increasing dietary calcium was found to suppress indices of inflammation in a mouse model of obesity [21], it is reasonable to propose that calcitriol, as well as $[Ca^{2+}]i$ signaling and mitochondrial uncoupling, may also play a role in regulating inflammatory factor production in adipocytes and/or macrophages. The present data support this hypothesis as calcitriol modulated the expression of inflammatory cytokines associated with macrophage function in human and murine adipocytes, indicating a potential role of calcitriol in the modulation of the interaction between adipocytes and macrophages. We also found a striking calcitriol stimulation of expression and production of inflammatory cytokines in differentiated 3T3-L1 adipocytes cultured alone or co-cultured with macrophages, and this effect was attenuated by either a calcium-channel antagonist or a mitochondrial uncoupler. However, these data must be interpreted with caution as these experiments only utilized two mouse cell lines (3T3-L1 adipocytes and RAW 264 macrophages) to investigate the local environment in mouse adipose tissue. Due to the complexity of adipose tissue, alternative approaches, such as establishing an in vitro system using the stromal–vascular fraction and adipocytes isolated from adipose tissue, are important complementary steps.

Although our previous observation of reduced inflammatory stress on a high calcium diet [21] might have resulted from reduced adiposity, the present in vitro evidence suggests an independent role of calcitriol in regulating inflammatory cytokine expression in adipocytes and macrophages, independent of weight and adipose loss. Accordingly, calcitriol may play a direct role in regulating inflammatory status in adipose tissue as well as in regulating the interaction between adipocytes and macrophages in the development of inflammatory and oxidative stress. In contrast to our data, those of Giulietti et al. [40] showed an anti-inflammatory effect of calcitriol in monocytes isolated from diabetic subjects. However, their data only demonstrated calcitriol attenuation of IFN-γ-stimulated inflammatory gene expression and did not assess an independent effect of calcitriol, thereby precluding direct comparison with our data. Moreover, previous reports demonstrated a pro-inflammatory effect of calcitriol, although this effect is dependent on cell type and dose [41,42].

In summary, these data demonstrate that calcitriol regulates both adipocyte and macrophage production of inflammatory factors via calcium-dependent and mitochondrial uncoupling-dependent mechanisms and that these effects are amplified with the co-culture of both cell types. These data further suggest that strategies for reducing circulating calcitriol levels, such as increasing dietary calcium, may regulate the adipocyte–macrophage interaction and thereby attenuate local inflammation in adipose tissue.

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