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REVIEW

INTERROGATION OF MICROARRAY DATASETS INDICATES THAT MACROPHAGE-SECRETED FACTORS STIMULATE THE EXPRESSION OF GENES ASSOCIATED WITH VITAMIN D METABOLISM (*VDR* AND *CYP27B1*) IN HUMAN ADIPOCYTES

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Abstract

Microarray datasets have been interrogated to determine whether the expression of vitamin D-related genes is modulated in adipocytes during inflammation. The datasets were from human adipocytes and preadipocytes incubated with macrophage-conditioned medium (U937 cells). In adipocytes, exposure to the conditioned medium for 24 h resulted in a major increase (82.2-fold) in mRNA level of *CYP27B1*, the gene encoding the enzyme that converts 25-hydroxycholecalciferol to the active form of vitamin D_3, 1,25-dihydroxycholecalciferol; exposure for 4 h also raised CYP27B1 mRNA level (10.9-fold). The level of the mRNA encoding the vitamin D receptor (VDR) was increased after 24 h (7.7-fold), but there was no change at 4 h. In contrast, incubation with conditioned medium for either 4 or 24 h had no effect on the expression of the *CYP24* and *CYP2R1* genes, which encode enzymes that catalyse a 24-hydroxylation and the conversion of vitamin D_3 to 25-hydroxycholecalciferol, respectively. In preadipocytes, the only effect of the macrophage-conditioned medium was to stimulate *CYP27B1* expression (5.7-fold) after 24 h. It is concluded that the capacity of adipocytes to produce active vitamin D_3 hormone and its nuclear receptor is strongly upregulated by secretory products from macrophages; this is consistent with a counter-regulatory effect of the vitamin D system to ameliorate inflammation.

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Introduction

Microarray studies have examined global gene expression in adipose tissue and in adipocytes in particular, and the changes that take place in response to specific stimuli (1-5). Our own studies have focused on the effects of macrophage secretions on gene expression in human adipocytes and preadipocytes in culture (6-7). Bioinformatic analysis of microarray data allows major networks and pathways that are modulated in particular conditions, or in response to specific stimuli, to be identified. These pathways, together with the individual genes whose expression is altered most extensively, are normally the main focus of interest; this is certainly so during the initial analysis. For example, in our studies on the effect of macrophage secretions on adipocyte gene expression, a group of matrix metalloproteinases (*MMP1*, *MMP3*, *MMP10*) where the mRNA level increased by >1000-fold were of key interest, as were the major pathways

activated - which included those associated with inflammation and macrophage infiltration (6).

Microarrays are a rich source of data beyond the major pathways and highly differentially expressed genes, and interrogation of the datasets can provide important insight in unexpected areas. One of the emerging interests in adipobiology is the extent to which adipocytes are involved in vitamin D metabolism, both in relation to the storage of the hormone and as a target tissue. In this short article we describe the outcome of examination of our microarray data for genes encoding the vitamin D receptor (*VDR*) and enzymes involved in the synthesis of the active hormone (*CYP27B1* and *CYP2R1*).

Vitamin D

The vitamin D endocrine system has been linked historically to calcium homeostasis and bone metabolism, with rickets being the classical deficiency disease (8-10). However, it is increasingly evident that this hormonal system is involved in a range of physiological functions. The main route by which vitamin D is obtained is by endogenous synthesis in the skin, though provision through the diet is important for those populations who live in regions where sunlight is limited, or where exposure to the sun is restricted for cultural reasons or because of factors such as age. The main dietary sources of vitamin D are oily fishes (especially the liver), eggs, and full-fat milk.

The term 'vitamin D' is used somewhat loosely and it is important to note that it is not strictly a vitamin in the sense of being an essential dietary nutrient, given the synthesis in the skin. Furthermore, vitamin D in practise refers essentially to D_3 (cholecalciferol), the only form of the vitamin that is found naturally in humans and other animals (8-9).

Vitamin D metabolism

Vitamin D_3 is synthesised in the skin non-enzymatically from 7-dehydrocholesterol through irradiation by UV light. The product of irradiation, precalciferol, isomerises to cholecalciferol. This is then transported to the liver bound to a plasma transport protein, Gc-globulin (also known as transcalciferin). Dietary-derived vitamin D_3 is absorbed and transferred to the liver in chylomicrons. Vitamin D_3, whether from endogenous synthesis in the skin or from the diet, is then hydroxylated in the liver to form 25-hydroxycholecalciferol (or calcidiol). The released $25(OH)D_3$, which is the main circulating form of vitamin D_3 hormone, is further hydroxylated to form 1,25-dihydroxycholecalciferol (or calcitriol), and this occurs primarily in the kidney (8-9). The enzyme that catalyses this second hydroxylation is $25(OH)D_3$-1α-hydroxylase, and is present in at least ten other tissues additional to the kidney (9). A 24-hydroxylation can also occur in the kidney (and other tissues) to form 24,25-dihydroxycholecalciferol. The main route for the degradation of $1,25(OH)_2D_3$ is also through a 24-hydroxylation.

There are two modes of action of $1,25(OH)_2D_3$ – genomic and non-genomic (11). The actions on gene transcription are mediated through a specific nuclear receptor, the vitamin D receptor (VDR). This receptor is widely expressed, expression being reported in a number of tissues (11). The transcription of as many as 500 genes may be regulated through VDR. There are also rapid responses to $1,25(OH)_2D_3$ which occur within minutes and which do not involve gene transcription, such as the stimulation of intestinal calcium transport. The current view is that VDR is present not only in the nucleus, but also in calveolae associated with the plasma membrane of target cells, and is the receptor for the non-genomic as well as genomic actions of $1,25(OH)_2D_3$ (11).

Functions of vitamin D

Vitamin D has long been recognised to play a central role in the stimulation of calcium absorption by the intestine and in the mineralisation and re-modelling of bone (8-10). Indeed, vitamin D deficiency is closely associated with the development of rickets in children and osteomalacia in adults. Other functions are now increasingly evident, particularly in relation to the immune system (10,12,13). Examples include an anti-inflammatory action in macrophages, with the down-regulation of the production of tumor necrosis factor-alpha (TNF-α) through a decrease in NFkB activity (14-15). Indeed, an anti-inflammatory action seems to be a characteristic of $1,25(OH)_2D_3$. Other major actions of the hormone include the stimulation of insulin secretion by pancreatic β cells, vitamin D deficiency leading to the inhibition of secretion (9, 16).

A growing number of diseases have been linked to vitamin D status, apart from rickets and osteomalacia, and these include several types of cancer (breast, colon and prostate) (9). In addition, inflammatory bowel disease, hypertension, peridontal disease, multiple sclerosis and muscle weakness (especially in the elderly) have each been associated with vitamin D insufficiency (9-10,13). Of particular relevance to obesity is the proposed link with cardiometabolic diseases (atherosclerosis, hypertension, type 2 diabetes and the metabolic syndrome). Indeed, a recent editorial in the *Journal of Clinical Endocrinology and Metabolism* was provocatively titled "25-OH vitamin D: is it the universal panacea for metabolic syndrome and type 2 diabetes?" (17).

Vitamin D status is considered to be best assessed by the serum level of $25(OH)D_3$ (9). The circulating level of $1,25(OH)_2D_3$ is of the order of 1000 times lower than that of $25(OH)D_3$. Several epidemiological studies have demonstrated an inverse corre-

lation in adults between body mass index (BMI) and circulating $25(OH)D_3$ level (18-20). In practise, it is the amount of body fat that is important. The explanation for the relationship with obesity is not clear, but it is possible that the hormone is sequestered within adipose tissue by virtue of the high lipid solubility. If this is the case, then low serum $25(OH)D_3$ levels are not indicative of an insufficiency of vitamin D *per se*. An alternative explanation for low serum levels in obesity is that adipocytes, or other cells within adipose tissue, have the capacity to degrade the steroid hormone.

Vitamin D and adipose tissue

White adipose tissue and adipocytes are among the extra-renal sites of expression of the 1α-hydroxylase gene (CYP27B1), and the conversion of $25(OH)D_3$ to $1,25(OH)_2D_3$ has been directly demonstrated, including in preadipocytes and in human mammary adipocytes (21-22). The vitamin D receptor is also expressed in mouse preadipocytes and adipocytes (22-26). Treatment of 3T3-L1 cells with $25(OH)D_3$ has been reported to induce expression of CYP24, a $1,25(OH)_2D_3$ responsive gene (21). There is also evidence that $1,25(OH)_2D_3$ may increase lipid synthesis in human adipocytes; however, contrastingly, the expression of Insig-2, a gene that encodes a protein that is involved in inhibiting fat synthesis, is stimulated by $1,25(OH)_2D_3$ (27). The augmentation of glucocorticoid production in adipocytes through a (small) upregulation of 11β-hydroxysteroid dehydrogenase type 1 expression by $1,25(OH)_2D_3$ has been observed (28). The favouring of inflammatory cytokine production (TNF-α and IL-6) in adipocytes and the suppression of anti-inflammatory cytokine production by $1,25(OH)_2D_3$ has also been reported in 3T3-L1 adipocytes and in human adipose cells (29). However, a very recent study showed that $1,25(OH)_2D_3$ reduced the release of monocyte chemotactic protein-1 (MCP-1), also known as chemokine (C-C motif) ligand 2 (CCL2), and adiponectin by human adipocytes (30). This suggests that vitamin D_3 may have both pro- and anti-inflammatory effects in human fat cells.

Several studies have investigated the effects of $1,25(OH)_2D_3$ on adipocyte differentiation and proliferation, and while both an augmentation and an inhibition have been reported the growing consensus is that the hormone has primarily an inhibitory action on fat cell recruitment (22,31-32).

Interrogation of microarrays

In a preliminary exploration of the extent to which adipose tissue may be a significant site of vitamin D_3 metabolism and a target tissue of the hormone, we examined two different microarray datasets for the expression of genes involved in the vitamin D_3 endocrine system. The first microarrays were from a study on the effect of macrophage-conditioned (MC) medium on global gene expression in human adipocytes (Simpson-Golabi-Beymel Syndrome - SGBS) at short and longer time points – 4 and 24 h (6). The other was on the effects of MC medium on gene expression in human preadipocytes (SGBS), following a 24 h incubation with the medium (7). In both sets of studies, the comparison was with adipocytes/preadipocytes incubated in unconditioned macrophage (UC) medium – the medium used to culture macrophages, but which had not been exposed to cells (150 μl of UC or MC medium added per ml of preadipocyte/adipocyte medium). The macrophages were derived from U937 monocytes, which had been induced to differentiate through the addition of phorbol myristic acid.

The microarray data was interrogated for whether the treatments affected the expression of four genes associated with the metabolism and action of the vitamin D_3 system. These genes were: (*i*) *CYP27B1* (encodes the 1α-hydroxylase), (*ii*) *CYP2R1* (encodes the 25-hydroxylase), (*iii*) *CYP24* (encodes the 24-hydroxylase that degrades $1,25(OH)_2D_3$), and (*iv*) *VDR* (encodes the vitamin D receptor). In human adipocytes exposed to MC medium, major changes in *CYP27B1* expression were observed; the CYP27B1 mRNA level was increased 10.9 fold at 4 h and by as much as 82-fold at 24 h (Fig 1). At 24 h this gene was ranked number 40 out of 1307 genes whose expression was up-regulated by the conditioned medium, indicating that it is one of the most highly responsive genes to macrophage-secreted factors in human adipocytes. In contrast, there was no change in *CYP24* expression, nor in *CYP2R1*. VDR mRNA level increased 7.7-fold at 24 h, though there was no change at 4 h, indicating that expression of the vitamin D receptor is increased by prolonged exposure to MC medium (Fig 1).

In human preadipocytes exposed to MC medium for 24 h, no changes in VDR, CYP2R1, or CYP24 mRNA levels were observed (Fig 2). However, there was an increase in CYP27B1 mRNA level (5.7-fold) and this gene ranked at number 34 in the list of 401 genes that were up-regulated in preadipocytes by MC medium (Fig 2). The up-regulation of *CYP27B1* gene expression in preadipocytes induced by macrophage secretions indicates that the capacity of adipocytes to hydroxylate $25(OH)D_3$ is not differentiation-dependent.

A further microarray dataset that was available to us on the effects of exposure to hypoxia (1% O_2) for 24 h on global gene expression in human adipocytes was also examined. No changes in expression were found for *CYP27B1*, *CYP24*, *CYP2R1,* or *VDR*, the mRNA levels for each of these genes being similar in adipocytes exposed to normoxia (21% O_2) or hypoxia. Thus vitamin D_3 system genes are not hypoxia-sensitive in adipocytes.

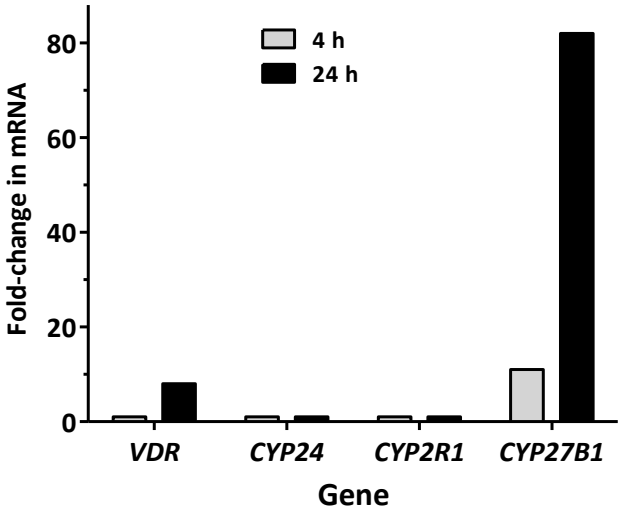

Figure 1. Effect of macrophage-conditioned medium on the expression of vitamin D_3-related genes in human adipocytes. Gene expression was assessed by microarrays following 4 and 24 h exposure to the conditioned medium. The fold-changes relate to the conditioned medium compared to unconditioned medium. The data may be reviewed through the following link:
http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=rtmtvcmessiqutm&acc=
The GEO accession number is GSE14312.

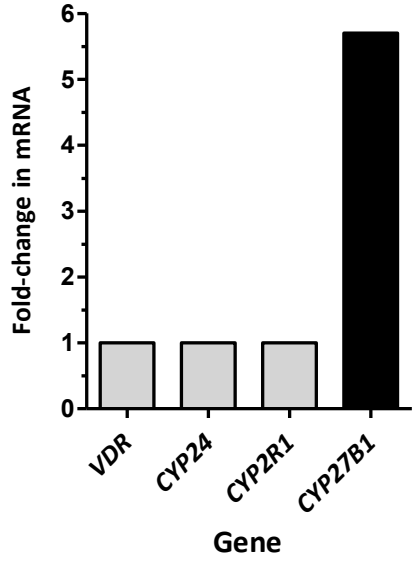

Figure 2. Effect of macrophage-conditioned medium on the expression of vitamin D_3-related genes in human preadipocytes. Gene expression was assessed by microarrays following 24 h exposure to the conditioned medium. The fold-changes relate to the conditioned medium compared to unconditioned medium. The data may be reviewed through the following link:
http://www. ncbi. nlm. nih.gov/geo/query/acc.cgi?token = rfqfjamewuwmors&acc=
The GEO accession number is GSE27503.

Conclusion

This analysis of microarray datasets confirms that human adipocytes express the genes encoding the 1α-hydroxylase that converts 25(OH)D_3 to 1,25$(OH)_2D_3$, and the VDR, and demonstrates that the expression of both genes is markedly stimulated by MC medium. Thus the capacity of adipocytes to produce the active vitamin D_3 hormone and its nuclear receptor would appear to be strongly upregulated by secretory products released by macrophages (assuming that the changes in mRNA are mirrored at the protein level). Given the apparent anti-inflammatory actions of 1,25$(OH)_2D_3$, the upregulation of *VDR* and *CYP27B1* expression in adipocytes on exposure to macrophage-secreted products would suggest a counter-regulatory response to ameliorate inflammation. The stimulation of VDR expression by inflammatory mediators is consistent with a local action within adipose tissue for increased 1,25$(OH)_2D_3$ produced during inflammation.

The strong upregulation of *CYP27B1* by MC medium raises the intriguing possibility that adipocytes are a significant site of the generation of 1,25$(OH)_2D_3$ under inflammatory conditions. Obesity is, of course, characterised by chronic mild inflammation, and adipose tissue exhibits a substantial inflammatory response in the obese (33-35). This involves macrophage recruitment in the tissue, as well as the production of inflammation-related factors by adipocytes including pro-inflammatory cytokines and chemokines (36,37). The nature of the factors secreted from adipocytes that stimulate transcription of the *VDR* and *CYP27B1* genes in adipocytes is unclear. However, cytokines such as TNF-α and IL-1β may well play a key role, similar to their action in stimulating matrix metalloproteinase expression and release (6).

In conclusion, the present report illustrates the information and insight that can be obtained through the mining of microarray datasets.

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