The effect of vitamin D derivatives on vascular calcification associated with inflammation

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Abstract

Background. Vitamin D sterols may modulate vascular response to inflammation and vascular calcification (VC).

Methods. Rat aortic rings (RARs) and human vascular smooth muscle cells (HVSMCs) were treated in vitro with phosphate (P), tumour necrosis factor alpha (TNF-α), calcitriol (CTR) and paricalcitol (PCT). Rats having undergone subtotal nephrectomy (Nx) (n = 66) on a high-phosphorus diet were treated with Escherichia coli lipopolysacharide (LPS) (40–400 μg/kg/day) or LPS plus CTR (80 ng/kg/48 h) or LPS plus PCT (240 ng/kg/48 h) for 14 days.

Results. In vitro, the addition of TNF-α to the medium increased the mineral content of RAR and HVSMC. Treatment with both vitamin D analogues decreased bone morphogenetic protein 2 but did not modify Runx-2. Calcification was prevented only by PCT. In vivo, treatment with LPS increased plasma levels of TNF-α, monocyte chemotactic protein-1 and interleukin-1alpha and induced calcification. The concomitant administration of LPS with either CTR or PCT led to a significant decrease in cytokine plasma levels and the decrease was more accentuated after treatment with PCT than with CTR. Rats treated with CTR showed an elevation in aortic Ca and marked Von Kossa staining; however, rats treated with PCT did not increase aortic Ca and did not show Von Kossa staining.

Conclusion. Treatment with PCT resulted in more marked anti-inflammatory effect than treatment with CTR and, as opposed to CTR, PCT prevented VC.

Keywords: inflammation; LPS; TNF-α; vascular calcification; vitamin D

Introduction

Accelerated arteriosclerosis and atherosclerosis are common features in patients with chronic kidney disease (CKD). Vascular calcification (VC) represents an important contributor to the high rate of cardiovascular mortality associated to CKD [1, 2]. In uraemic patients, VC is caused in part by deranged mineral metabolism and is closely related to the control of secondary hyperparathyroidism (HPT). Patients with CKD also show a chronic inflammatory state, which is associated to VC, cardiovascular morbidity and mortality. In addition to promoting concurrent changes in mineral metabolism, inflammation favours vascular damage that ultimately might lead to the development of VC [3, 4].

Tumour necrosis factor-α (TNF-α) is an important cytokine in the inflammatory cascade that has been associated with the osteogenic transdifferentiation characteristic of arteriosclerosis [5–7]. The actions of TNF-α on vascular smooth muscle cells seem to be signalled through the Nuclear Factor kappa-light-chain enhancer of activated B cells (NF-κB) pathway and involve an up-regulation of bone morphogenetic protein 2 (BMP2) [8].

Vitamin D sterols are used in CKD for the treatment of secondary HPT. Vitamin D replacement therapy has been reported to increase survival in haemodialysis patients [9, 10]. However, administration of vitamin D often results in hypercalcaemia and hyperphosphataemia, which may lead to VC. Some vitamin D derivatives [e.g. paricalcitol (PCT)] have been reported to be less hypercalcaemic and to have less calcifying potential than calcitriol (CTR) [11–13].

In addition to its effects on mineral metabolism, vitamin D is known to modulate immune response and cell differentiation [14, 15]. As previously reported in other cell types (keratinocytes, chondrocytes, synoviocytes and dentritic cells), vitamin D receptor (VDR) activation may modulate vascular smooth muscle cell response to inflammation [16]. Moreover, VDR-mediated effects may not be identical when different VDR activators are used and these differential effects may have an impact on VC and on survival. Recent reports suggest that patients treated with PCT have a lower mortality risk than those treated with CTR [9].

We hypothesize that treatment with vitamin D derivatives will modify the vascular response to inflammatory mediators, a mechanism by which vitamin D may modulate VC. To test this hypothesis, the effects of CTR and PCT on
inflammation and VC were evaluated in cultures of aortic explants and vascular smooth muscle cells exposed to TNF-α and in uremic rats receiving *Escherichia coli* lipopolysaccharide (LPS).

**Materials and methods**

*In vitro studies*

**Rat aortic ring culture.** Rat aortic ring (RAR) culture was performed following the method used by Lomashvili et al. [17], with minor modifications. Thoracic aortas (from the aortic arch to the diaphragm) were removed in a sterile manner from Wistar rats (200–250 g). Aortas were cleaned with physiological solution at 4°C. Subsequently, the vessels were cut into 2- to 3-mm rings and cultured in the maintenance medium [Dulbecco’s modified Eagle’s medium (DMEM); Sigma, St Louis, MO] supplemented with 1 mmol/L Na pyruvate, 4.5 g/L glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin and 20 mM HEPES at 37°C in a humidified atmosphere of 5% CO₂. The aortic rings were randomly distributed in five groups: one group was cultured in maintenance medium (control) and in the other four groups, the maintenance medium was supplemented with 3.75 U/mL alkaline phosphatase (Promega, Fitchburg, WI) and the phosphate salts (Na₂HPO₄ and NaH₂PO₄ in 1:2 proportion; Sigma–Aldrich) necessary to reach the desired final phosphate concentration of 3.3 mmol/L; in three of these groups, TNF-α, 10 ng/mL, was added to the medium (P + TNF). Two groups were also supplemented with either CTR, 10⁸ M (P + TNF + CTR), or PCT, 3 × 10⁻⁸ M (P + TNF + PCT). The medium was replaced every 2 days. The rings were cultured for 9 days.

**Human vascular smooth muscle cell culture.** Human vascular smooth muscle cells (HVSMSCs) were obtained from Clonetics (Lonza Walkersville, Inc.,). Cells were cultured in DMEM supplemented with fetal bovine serum (20%) (Bio Whitaker, Verviers, Belgium), Na pyruvate (1 mmol/L), glutamine (4.5 g/L), penicillin (100 U/mL) and HEPES (20 mM/L) at 37°C in a humidified atmosphere with 5% CO₂. Cells were used after the fifth passage with 80% confluence. Cultures were randomly distributed in five groups: one group was cultured in maintenance medium (control), and in the other four groups, the maintenance medium was supplemented with phosphate (Na₂HPO₄ and NaH₂PO₄ in 1:2 proportion to reach a final concentration of 3.3 mM P) to allow mineralization; in three of these groups, TNF-α, 10 ng/mL, was added to the medium (P + TNF). Two groups were also supplemented with either CTR, 10⁸ M (P + TNF + CTR), or PCT, 3 × 10⁻⁸ M (P + TNF + PCT). The medium was replaced every 2 days. The cells were cultured for 9 days.

*In vivo studies*

**Animal model.** Female Wistar rats weighing 250 g were housed with a 12 h/12 h light/dark cycle and given *ad libitum* 12 h/12 h light/dark cycle and given access to normal diet and water. Animal model. Female Wistar rats weighing 250 g were housed with a 12 h/12 h light/dark cycle and given *ad libitum* dietary supplement. Uraemia was induced by 5/6 nephrectomy (5/6 Nx), a two-step procedure. Thoracic aortas (from the aortic arch to the diaphragm) were removed in a sterile manner from Wistar rats (200–250 g). Aortas were cleaned with physiological solution at 4°C. Subsequently, the vessels were cut into 2- to 3-mm rings and cultured in the maintenance medium [Dulbecco’s modified Eagle’s medium (DMEM); Sigma, St Louis, MO] supplemented with 1 mmol/L Na pyruvate, 4.5 g/L glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin and 20 mM HEPES at 37°C in a humidified atmosphere of 5% CO₂. The aortic rings were randomly distributed in five groups: one group was cultured in maintenance medium (control) and in the other four groups, the maintenance medium was supplemented with 3.75 U/mL alkaline phosphatase (Promega, Fitchburg, WI) and the phosphate salts (Na₂HPO₄ and NaH₂PO₄ in 1:2 proportion; Sigma–Aldrich) necessary to reach the desired final phosphate concentration of 3.3 mmol/L; in three of these groups, TNF-α, 10 ng/mL, was added to the medium (P + TNF). Two groups were also supplemented with either CTR, 10⁸ M (P + TNF + CTR), or PCT, 3 × 10⁻⁸ M (P + TNF + PCT). The medium was replaced every 2 days. The rings were cultured for 9 days.

Real-time polymerase chain reaction (RT-PCR)

For RNA isolation, 500 µL of phenol–guanidine isothiocyanate solution (Tri-Reagent; Sigma Chemical Company) was added to HVSMSCs. Thereafter, total RNA was extracted following a modification of the Chomczynski and Sacchi protocol [20] and dissolved in nuclease-free water. The primers used for polymerase chain reaction (PCR) amplification were [1] human β-actin forward 5’-GCA CTC TTC CAGCT TCC TT-3’ and reverse 5’-ATC CAC ATC TGC TGG TAG GT-3’, [2] human Runx-2 forward 5’-GCA GTT CCC AGG CAT TTC AT-3’ and reverse 5’-CGG ACA TAC CGA GGA ACA T-3’ and [3] human BMP-2 forward 5’-AGG GAC CAA AGA AAA GGA GCC GAC-3’ and reverse 5’-GGA AGC AGC AAC GCT AGA AGA CGA-3’. Real-time PCR was performed in duplicate with QuantiTect SYBR Green PCR (Qiagen) according to the manufacturer’s protocol. All PCR amplifications were carried out using Lightcyler 480 (Roche Molecular Biochemicals, Indianapolis, IN). The expression of target genes was
Results

In vitro studies

RARs that were incubated in medium with high phosphate (P 3.3 mM) showed a significant (P < 0.01) increase in calcium content (3.1 ± 0.3 mg/g of tissue) when compared with rings incubated without high phosphate (0.4 ± 0.1 mg/g of tissue). The addition of TNF-α to the medium induced a further increase (5.9 ± 0.8 mg/g of tissue, P = 0.03 versus P 3.3 mM) in the mineral content of the aortic tissue. Supplementation of the culture medium with CTR resulted in a slight and non-significant decrease in aortic calcium content (4.6 ± 0.4 mg/g of tissue); however, addition of PCT significantly decreased aortic calcium content to 2.7 ± 0.2 mg/g of tissue, P < 0.001 versus the group without PCT (Figure 1A). The results obtained in HVSMC cultures mirrored the results in RAR and also demonstrated a decrease in VC in cells treated with PCT (5.1 ± 0.5 versus 7.3 ± 0.9 μg/mg protein in the group not receiving PCT, P = 0.007) (Figure 1B). Mineral deposits in Von Kossa-stained sections of RAR and HVSMC correlated with the calcium content (Figure 2).

BMP-2 messenger RNA (mRNA) levels (ratio versus β-Actin) were increased by treatment with phosphate + TNF-α (3.5 ± 0.8 versus 1.1 ± 0.2 in the control group, P < 0.001). Treatment with both vitamin D analogues significantly decreased BMP-2 but the decrease was more marked with PCT (0.9 ± 0.3) than with CTR (1.7 ± 0.4) (Figure 3A). Runx-2 mRNA levels were significantly increased by incubation in a high phosphate medium (1.33 ± 0.09 versus 0.87 ± 0.17, P < 0.05) and did not further increase by the addition of TNF-α to the medium. HVSMCs treated with PCT showed lower expression of Runx-2 than HVSMCs treated with CTR (1.34 ± 0.11 versus 2.08 ± 0.19, P < 0.05).

In vivo studies. The experimental procedures, which included 5/6 nephrectomy, a moderate increase in dietary content of phosphate and the i.p. administration of LPS and vitamin D analogues, did not induce significant mortality. Survival was close to 100% with only sporadic losses scattered among groups.

Plasma biochemical values are shown in Table 1. As expected, all 5/6 Nx rats on a high-P diet had higher creatinine levels than the sham-operated animals (P < 0.05). Plasma creatinine concentrations were significantly higher in rats treated with LPS plus CTR (1.21 ± 0.14 mg/dL) as compared with other 5/6 Nx groups (P < 0.05). Treatment with LPS plus PCT did not cause a further increase in plasma creatinine in uremic rats. Plasma ionized calcium concentration was significantly (P < 0.001) reduced in 5/6 Nx groups on a high-P diet (range 1.00–1.03 mmol/L) as compared with sham-operated rats (1.20 ± 0.05 mmol/L). However, nephrectomized rats receiving LPS plus CTR had similar calcium values (1.16 ± 0.03 mmol/L) as the sham-operated animals. Rats treated with LPS plus PCT had intermediate calcium levels (1.08 ± 0.03 mmol/L). Plasma phosphorus was uniformly elevated in 5/6 Nx rats on a high-P diet (range 311–352 pg/mL) (P < 0.01). The highest plasma phosphorus levels were observed in 5/6 Nx rats treated with LPS plus CTR (11.71 ± 1.13 mg/dL); these values were significantly higher than in the LPS plus PCT-treated group (9.3 ± 0.70 mg/dL) (P = 0.02). Thus, the plasma Ca × P was lower in rats not receiving vitamin D, intermediate in animals treated with LPS plus PCT and higher in rats treated with LPS plus CTR. Plasma PTH concentration was significantly increased in 5/6 Nx rats on a high-P diet (range 311–352 pg/mL) (P < 0.001) as compared to sham-operated animals (range 31–51 pg/mL). Both sham-operated and 5/6 Nx rats experienced a modest, non-significant, increase in PTH levels after treatment with LPS. Treatment with vitamin D derivatives significantly reduced plasma PTH concentrations in 5/6 Nx rats.

Plasma TNF-α levels were slightly increased in 5/6 Nx rats on a high-P diet (68.7 ± 19.8 versus 59.5 ± 10.6 pg/mL).
Treatment with LPS also increased MCP-1 in 5/6 Nx rats (from 411.5 ± 18.3 to 482.5 ± 26.4 pg/mL, P = 0.04). IL-1α was as well increased, although the differences were not significant (from 18.8 ± 5.8 to 24.2 ± 7.1 pg/mL, P = 0.451). Both vitamin D derivatives decreased IL-1α and MCP-1 but significant differences were only found in MCP-1, which decreased from 482.5 ± 26.4 to 387.3 ± 34.5 pg/mL (P = 0.006) in rats receiving LPS plus PCT.

Uraemic rats that received LPS showed a significant increase (P < 0.05) in aortic calcium content (1.65 ± 0.13 mg/g of tissue) when compared with sham-operated and 5/6 Nx + vehicle rats (0.86 ± 0.12 mg/g). Treatment with LPS plus CTR induced a further elevation (3.27 ± 0.89 mg/g of tissue, P < 0.05) in aortic calcium content. However, 5/6 Nx rats treated with LPS plus CTR did not increase aortic calcium content (1.59 ± 0.60 mg/g of tissue) (Figure 4B).

Von Kossa-stained sections of the aortas from the in vivo experiments are shown in Figure 2. Mineral deposits correlated with the calcium content as shown in Figure 4B, although in the groups with lower calcium content it was difficult to distinguish the brown pigment. Treatment with
CTR resulted in extensive calcification, while treatment with PCT did not increase calcification.

**Discussion**

This study was designed to test the hypothesis that treatment with vitamin D derivatives would modulate VC by modifying the response to inflammatory mediators. Our results show that treatment with CTR and PCT reduced circulating cytokine levels in uraemic rats that received LPS. PCT was more effective than CTR in decreasing plasma levels of TNF-α and MCP-1. Although the inflammatory response was attenuated after receiving vitamin D, rats treated with CTR increased VC, while rats treated with PCT did not. *In vitro*, PCT but not CTR reduced VC in both RAR and HVSMC exposed to TNF-α.

Arteriosclerosis is common in uraemic patients, which are also known to suffer a chronic inflammatory status. Several studies point towards a relationship between the increase in inflammatory mediators and cardiovascular risk in uraemic patients [21, 22]. Moreover, *in vitro* experiments show that pro-inflammatory cytokines may induce calcification [23, 24]. Our results, that demonstrate an increase in the aortic mineral content of 5/6 Nx rats treated with LPS and in RAR and HVSMC exposed to high phosphate and TNF-α, provide further support to the hypothesis that chronic inflammation promotes VC in uraemia. Nonetheless, direct extrapolation of these data (which were obtained in rats and *in vitro*) to the human condition needs further evaluation.

In this study, we have used two *in vitro* models (RAR and HVSMC) to obtain more certainty and because each model has its advantages: RARs are superior for histology and HVSMCs provide better results in molecular biology. Calcification was comparable in both models (slightly higher in HVSMC but very similar), however, the differential effect of CTR and PCT on calcification seemed more accentuated in RARs. We believe that this finding may be related to preservation of vascular architecture in RARs, which would allow a more complex response to the inflammatory stimulus.

In our experimental model, nephrectomized rats showed an increase in TNF-α, MCP-1 and IL-1β that was potentiated by treatment with LPS. The LPS-induced increase in cytokines was more robust in nephrectomized rats than in sham-operated animals. Thus, uraemia seems to aggravate the inflammatory effects of LPS. This is similar to the clinical situation in dialysis patients in which uraemia plays a major role in the development of chronic inflammatory response [7]. Both vitamin D derivatives had a protective action against LPS-induced cytokine production, but PCT demonstrated a higher anti-inflammatory effect.

The causal relationship between inflammation (as evidenced by increased cytokine levels) and VC has been previously described [3, 4, 25]. Diabetic hyperlipidaemic mice have been reported to increase the expression of pro-

**Table 1.** Serum biochemical parameters in the study groups of *in vivo* experiments (sham = sham-operated animals, 5/6 Nx = rats having undergone subtotal nephrectomy) 

<table>
<thead>
<tr>
<th>Groups</th>
<th>Creatinine (mg/dL)</th>
<th>Ionized calcium (mmol/L)</th>
<th>Phosphorus (mg/dL)</th>
<th>PTH (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham + vehicle</td>
<td>0.46 ± 0.03</td>
<td>1.20 ± 0.05</td>
<td>6.20 ± 0.30</td>
<td>30.5 ± 6.3</td>
</tr>
<tr>
<td>Sham + LPS</td>
<td>0.49 ± 0.03</td>
<td>1.20 ± 0.04</td>
<td>7.13 ± 0.30</td>
<td>50.7 ± 7.2</td>
</tr>
<tr>
<td>5/6 Nx + vehicle</td>
<td>0.98 ± 0.06</td>
<td>1.03 ± 0.04</td>
<td>8.95 ± 0.44</td>
<td>311.4 ± 39.4</td>
</tr>
<tr>
<td>5/6 Nx + LPS</td>
<td>1.04 ± 0.03</td>
<td>1.00 ± 0.03</td>
<td>9.48 ± 0.44</td>
<td>352.1 ± 44.2</td>
</tr>
<tr>
<td>5/6 Nx + LPS + CTR</td>
<td>1.21 ± 0.14</td>
<td>1.16 ± 0.05</td>
<td>11.71 ± 1.13</td>
<td>91.6 ± 20.0</td>
</tr>
<tr>
<td>5/6 Nx + LPS + PCT</td>
<td>0.93 ± 0.07</td>
<td>1.08 ± 0.03</td>
<td>9.31 ± 0.70</td>
<td>141.2 ± 62.3</td>
</tr>
</tbody>
</table>

*a* All rats received high-P (0.9%) diet. LPS was injected i.p. every 24 h increasing the dose every 2 days (40, 80, 120, 160, 240, 300 and 400 μg/kg/day).

*b* P < 0.05 versus sham + vehicle.

*c* P < 0.05 versusNx + LPS + CTR.

*d* P < 0.05 versusNx + vehicle.

*e* P < 0.05 versusNx + LPS.
calcifying proteins (e.g. BMP-2) and to decrease the expression of proteins that inhibit calcification (e.g. fetuin) in response to increased TNF-α levels [26]. In our study, BMP-2, which is a key protein in the osteogenic transdifferentiation pathways associated to inflammation, was increased after *in vitro* exposure to TNF-α and was significantly decreased by treatment with PCT. However, Runx2 did not increase after exposure to TNF-α. These results are in agreement with previous reports [27–30]. Several studies have demonstrated that TNF-α induces an increase in BMP-2 gene expression mediated by the NF-κB pathway [29, 30]. Furthermore, it has been shown that the increase in BMP-2 subsequent to TNF-α exposure does not signal calcification through an elevation in Runx2 but rather through an increase in Dlx5 [8]. It is interesting to note that although Runx2-2 does not seem to play a major role in inflammation-induced VC, Runx2-2 levels are higher in HVSVMCs treated with CTR than in the same cells treated with PCT. This finding may be related to a direct effect of CTR on Runx2-2 [31].

Vitamin D replacement therapy plays an important role in the treatment of secondary HPT and has been reported to increase survival in haemodialysis patients [9, 10]. Low serum CTR levels and vitamin D deficiency are associated with cardiovascular mortality [32] and increased renal inflammation [33]. However, administration of pharmacological doses of vitamin D has also been associated with the development of VC [11–13]. Thus, both too low and too high vitamin D levels are undesirable. The doses of vitamin D derivatives were selected by their ability to control secondary HPT in rats as previously reported by our laboratory [12]. Not all vitamin D analogues have the same pro-calcifying properties and PCT has been reported to significantly reduce the risk of VC associated to vitamin D administration [34]. Our results support this contention since uremic rats exposed to LPS and treated with PCT achieved control of secondary HPT without increasing the aortic mineral content (same as untreated 5/6 Nx + LPS), while rats receiving CTR demonstrated significant VC. The beneficial effects of PCT preventing VC have been traditionally attributed to the fact that this drug is less hypercalcemic than other vitamin D derivatives (e.g. CTR) [34]. Our results confirm lower plasma Ca and P levels in PCT-treated animals than in rats receiving CTR; however, they also demonstrate that, in addition to its effects on mineral metabolism, PCT treatment reduces cytokine levels more efficiently than CTR. Thus, the more marked anti-inflammatory actions of PCT may also influence its less calcifying properties. This contention is further supported by the *in vitro* studies in which Ca and P were controlled and PCT but not CTR was able to reduce TNF-α-induced VC.

In conclusion, a systemic increase in cytokines has been found after administration of LPS to uremic rats and cytokines (TNF-α) generated by LPS administration had a pro-calcifying effect. Both vitamin D derivatives reduced cytokine levels although PCT was more efficient. *In vitro*, CTR increased LPS-induced VC but PCT did not. Moreover, *in vitro*, where any variables affecting calcium metabolism (e.g. changes in calcium) are controlled, PCT but not CTR showed a clear and significant protective effect on TNF-α-induced VC.

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Conflict of interest statement. The results presented in this paper have not been published previously in whole or part except in abstract form. Two abstracts containing part of the information reported here were presented at the Spanish Society of Nephrology Congress held in San Sebastian (Spain) in 2008 and at the ASN Congress held in San Diego (USA) in 2009.

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