Low-Dose Calcitriol Decreases Aortic Renin, Blood Pressure, and Atherosclerosis in ApoE-Null Mice

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Aims: To determine whether low-dose calcitriol attenuates atherosclerosis in apoE-null mice and, if so, through which predominant mechanism.

Methods: Starting at the age of 6 weeks, mice received intraperitoneal injections of either 0.25 ng/g body weight of calcitriol or the vehicle, every other day for 8 weeks.

Results: Calcitriol treatment resulted in 35% reduction of atherosclerosis at the aortic sinus, and in a significant decrease in blood pressure. These effects were possibly mediated by downregulation of the renin-angiotensin system (RAS), as there was a 64% decrease in the aortic level of renin mRNA. None of the other components of the RAS or the prorenin receptor were affected by treatment. Low-dose calcitriol treatment did not modify the plasma level of monocyte chemoattractant protein-1, interferon γ, interleukin-4 and interleukin-10, which were similar in control and treated mice. Likewise, there was no difference in the percentage of splenic Foxp³⁺ regulatory T cells. Calcitriol treatment resulted in an unfavorable metabolic profile (glucose and lipids), as determined after a limited fast, a difference that disappeared after food was withheld for a longer time.

Conclusions: At a relatively low dosage, calcitriol attenuates the development of atherosclerosis in apoE-null mice, most probably by down regulation of RAS, and not through immunomodulation; however, even at this low dose, calcitriol appears to elevate calcium and to have potentially adverse metabolic effects. Exploring the potential antiatherogenic effects of non-calcemic and safer analogues is therefore warranted.


Key words: Atherosclerosis, Blood pressure, Renin, Vitamin D, Renin-angiotensin system, ApoE-null mice, T-regs lymphocytes, Immunomodulation
reduce cardiovascular mortality in hemodialysis patients\(^6\), \(^7\). The applicability of this finding to the general population is not clear since only a few studies have been conducted that addressed this issue. Several randomized trials, and a recent meta-analysis\(^8\)-\(^{10}\), failed to show a statistically significant reduction in cardiovascular morbidity and mortality with vitamin D supplementation, alone or in combination with calcium. However, there was wide methodological heterogeneity among these studies, and none had cardiovascular morbidity or mortality as a pre-defined endpoint. In contrast, a large meta-analysis found a decrease in total mortality with vitamin D supplementation; however, there was no relation to the cause of death\(^{11}\).

In vitro studies and some animal models have provided several lines of evidence for a potential antiatherogenic effect of vitamin D. Among those, vitamin D was shown to have favorable effects on inflammation, lipid metabolism, vascular function, and on potential mediators of atherogenesis\(^{12}-^{16}\). Calcitriol, the active form of vitamin D, decreased the expression of multiple proatherogenic cytokines\(^{19}\). Calcitriol limited in vitro cholesterol accumulation and foam cell formation in macrophages from diabetic subjects\(^{15}\). Vitamin D receptor-null mutant mice displayed hypercoagulability due to upregulation of tissue factor and downregulation of antithrombin and thrombomodulin\(^{17}\), while vitamin D reduced the expression of thrombogenic genes, but increased that of fibrinolytic and vasodilatory genes in coronary artery vascular smooth muscle cells\(^{16}\).

A prominent candidate for the potential antiatherogenic role of vitamin D is the renin-angiotensin system (RAS). It is now well established that calcitriol, via the vitamin D receptor (VDR), is a negative regulator of renin\(^{18}\), the rate-limiting step of the RAS, thereby limiting the production of angiotensin II (AII), in itself a potent inflammatory proatherogenic cytokine shown to be involved in the atherosclerosis that develops in apoE null mice\(^{19}-^{21}\).

Surprisingly, interventional studies of the effect of calcitriol treatment on atherosclerosis in animals are scarce. In the present study we sought to address the possibility that vitamin D treatment might reduce atherosclerosis in apoE-null mice by down-regulating tissue RAS.

**Methods**

**Animals and Treatment Protocol**

ApoE-null mice maintained at the Tel Aviv Sourasky Medical Center animal facility were used for the study. All protocols were approved by the institutional ethics committee for experiments in laboratory animals, and conformed to the NIH Guide for the Care and Use of Laboratory Animals. Mice were housed under a 12 h light/dark cycle with light between 06:00 and 18:00. At the age of 6 weeks, apoE-null mice were switched to an atherogenic diet (Harlan-Teklad 88137; Harlan, Madison, WI), and calcitriol treatment was initiated. Altogether 62 mice were used for the various parts of the study. Treated mice received intraperitoneal injections of an aqueous solution of calcitriol (Sigma-Aldrich, St. Louis, MO) at a dose of 0.25 ng/g body weight (BW) every other day \((n=36)\). Control mice received the vehicle only \((n=26)\) for 8 or 12 weeks. The rationale for choosing this dose was our previous experience with Tsukuba Hypertensive mice, a model of hypertension due to transgenic expression of the human renin-angiotensin system (RAS). When given for 3 weeks, it significantly lowered blood pressure and decreased plasma human renin levels without generating significant hypercalcemia\(^{22}\). At this dose, assayed in wild-type C57/B16 mice, plasma calcitriol concentrations reached a peak of approximately 1000 pmol/L (a supraphysiological concentration) 3 hours after injection, and were back to below the baseline, as a result of PTH suppression, within less than 24 h (see supplemental methods and Fig. 1).

**Blood Pressure Measurements**

In the final week of the experiment, after acclimatization, blood pressure was measured noninvasively in awake mice using the 3-channel computerized tail-cuff IITC system model 3M229 BP, attached to the 31BP software package (IITC Life Science Inc., Woodland Hills, CA), as described\(^{23}\).

**Serum Biochemistry, Analytical Procedures, and Lipoprotein Fraction Analysis**

After 6 weeks of treatment, a subset of mice was subjected to an intraperitoneal glucose tolerance test (details in supplemental material).

At the end of the study, following a 3-hour fast, under light isoflurane anesthesia, blood was sampled from the retro-orbital plexus. Serum biochemistry was assessed for glucose, BUN, triglycerides and cholesterol on a Bayer Advia 1650 autoanalyzer; however, because the serum lipids interfered with the automated measurements, calcium concentrations were determined after acid extraction by flame atomic absorptiometry.

Cholesterol fractions were quantified by fast performance liquid chromatography (FPLC) followed by
In both cases, the amount of mRNA was adjusted for beta-actin (further details in the supplemental methods). Renin and other components of RAS, angiotensinogen, AT1-R, and the angiotensin converting enzyme (ACE 1) were then assessed by real-time PCR. The following TaqMan Gene Expression Assays on demand were used: renin MM02342887_MH, angiotensinogen (AGT) MM00599662_M1, ACE1 MM00802048_M1, AT1-R AGTR1a MM00616371_M1, with HPRT as the endogenous gene MM00446968_M1. The reactions were carried out on a StepOne Real-Time System (Applied Biosystems, Life Technology, Carlsbad, CA).

**Evaluation of Aortic NADPH Oxidase Activity**

NADPH oxidase activity in the aorta, a reflection of the tissue oxidative stress caused by AII, was assessed by an in-house lucigenin-enhanced chemiluminescence assay. Aortas frozen in liquid nitrogen were thawed in ice-cold KHB and kept on ice. Under binocular magnification, aortas were meticulously cleaned of all adjacent tissues and cut into 3-5 mm rings. They were subsequently incubated at 37 °C for 45 min in pre-warmed KHB. Each ring was then placed in an optical plate well in 175 μL KHB containing freshly made NADPH (Sigma-Aldrich Cat. #M8010) to yield a final reaction concentration of 204 bp PCR product. In both cases, the amount of mRNA was adjusted for beta-actin (further details in the supplemental methods). Renin and other components of RAS, angiotensinogen, AT1-R, and the angiotensin converting enzyme (ACE 1) were then assessed by real-time PCR. The following TaqMan Gene Expression Assays on demand were used: renin MM02342887_MH, angiotensinogen (AGT) MM00599662_M1, ACE1 MM00802048_M1, AT1-R AGTR1a MM00616371_M1, with HPRT as the endogenous gene MM00446968_M1. The reactions were carried out on a StepOne Real-Time System (Applied Biosystems, Life Technology, Carlsbad, CA).

**Quantification of Atherosclerosis at the Aortic Sinus**

Mice were sacrificed by a lethal dose of isoflurane. After perfusion, and careful exclusion of all apparent fat and connective tissue, the heart was removed en bloc with the aorta down to the bifurcation. The heart was dissected out, its apex removed, and the upper 1/3, which included the aortic sinus, was embedded in OCT (Tissue-Tek; Sakura Finetek, The Netherlands) and rapidly stored at −80 °C. In mice that developed plaques throughout the aorta, in this study, the extent of atherosclerosis was derived from the quantification of Oil Red O sections at the aortic sinus as detailed (supplemental methods), using the NIS element Br 3.0 imaging system (Nikon Instruments Europe B.V., The Netherlands).

**Evaluation of the Aortic Expression of RAS**

RNA was extracted from the entire vessel, including the arch and the descending aorta, thus encompassing all the atherosclerotic plaques. The expression of renin was first assessed by semi-quantitative RT-PCR using the following primers: forward 5’-ATGAA-GGGGCTGTCTGAGGGTC-3’, reverse 5’-ATG-TGGGGAGGGTGCCACCTG-3’. The prorenin receptor (PRR) was also assessed semi-quantitatively with the following primers: forward 5’-TTT GGA TGA ACT TGG GAA GC-3’ and reverse 5’-AAG CCG ATC ATA ATC CAC AGT AC-3’, yielding a 204 bp PCR product. In both cases, the amount of mRNA was adjusted for beta-actin (further details in the supplemental methods). Renin and other components of RAS, angiotensinogen, AT1-R, and the angiotensin converting enzyme (ACE 1) were then assessed by real-time PCR. The following TaqMan Gene Expression Assays on demand were used: renin MM02342887_MH, angiotensinogen (AGT) MM00599662_M1, ACE1 MM00802048_M1, AT1-R AGTR1a MM00616371_M1, with HPRT as the endogenous gene MM00446968_M1. The reactions were carried out on a StepOne Real-Time System (Applied Biosystems, Life Technology, Carlsbad, CA).

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**Fig. 1.** A. Typical photomicrographs of the Oil Red O-stained aortic sinus area of apoE null mice at the end of 8 weeks of treatment, demonstrating a clear reduction in atherosclerotic plaque formation in calcitriol-treated animals (right panel) compared to the control (left). Scale bar is 100 μm. B. The extent of the effect was quantified in 6 control and 10 calcitriol-treated mice, and indicates a significant 35% reduction in plaque area at the aortic sinus with treatment.
Calcitriol Decreases Atherosclerosis

Luminex 200 (Luminex Corp., Austin, TX), a bead-based multiplex dual laser flow analyzer (details of both methods given in supplemental material).

Statistical Analysis

All data are presented as the mean ± SE. Statistical analysis was performed using SPSS package 15 (SPSS Inc., Chicago, IL). Continuous variables were compared by the t-test if normally distributed, and otherwise by the Mann-Whitney U test. Categorical variables were compared by the chi-squared or Fisher’s exact test. Statistical significance was defined at \( p < 0.05 \).

Results

Calcitriol Treatment Reduces Atherosclerosis at the Aortic Sinus

Calcitriol, administered for 8 weeks, significantly

Fig. 2. Calcitriol treatment suppressed renin mRNA expression by 64% in the aorta, as assessed by real-time PCR of a representative sample of treated (\( n=6 \)) and control (\( n=6 \)) animals, using HPRT as the endogenous gene (A). A similar significant effect was shown by semiquantitative PCR with beta-actin serving as the housekeeping gene (B). Other components of RAS: AT1-R (C), AGT (D) and ACE (E) were not significantly affected by treatment.
recently been suggested that the antiatherogenic effect of calcitriol might be the result of increased regulatory T cells and decreased maturation of dendritric cells. At the low dose used in this study, calcitriol had no effect on the prevalence of T regs that comprised 12.5 ± 0.7% of CD4 splenocytes in control animals (n=5), and 12.0 ± 0.4% in calcitriol-treated mice (n=6; NS) (FACS graphic shown in Fig. 5). Likewise, an array of cytokines assayed on a large number of control and treated animals (20 and 22 respectively, mice were added to the experiment to account for variance), failed to indicate any typical anti-inflammatory or antiatherogenic profile with treatment. As expected from the lack of effect on T regs, neither reduced the extent of atherosclerosis at the aortic sinus (Fig. 1A, B). This could be seen in the representative Oil Red O-stained section of the aortic sinus as seen at the end of the study (age 14 weeks) in a calcitriol-treated mouse, and in a control animal (Fig. 1A). Quantification of the plaque area (Fig. 1B) showed that it was 35.4% smaller in calcitriol-treated animals than in control animals that received the vehicle (p=0.003).

Effect of Calcitriol on the Expression of Aortic RAS

Based on our working hypothesis, we anticipated that the antiatherogenic effect of vitamin D in apoE-null mice might be mediated by a reduced expression of RAS in the aorta, the site of atherosclerotic plaque. This possibility was assessed by determining the level of mRNA in the various RAS components. As expected, renin mRNA decreased by 64% (real-time and semi-quantitative PCR) (p=0.026) in the aortas of calcitriol-treated mice (Fig. 2A, B). None of the other components of RAS (angiotensinogen, AT1-R, or ACE1) were significantly affected when assessed by either real-time PCR (Fig. 2C, D, E) or semi-quantitative PCR (not shown). Likewise, calcitriol treatment had no effect on the expression of the prorenin receptor in the aorta (Fig. 3).

Effect of Calcitriol Treatment on Aortic NADPH Oxidase Activity

The generation of reactive oxygen species by angiotensin II signaling is believed to be a major culprit in the proinflammatory and proatherogenic role of angiotensin II. A naturally abundant enzyme, NADPH oxidase is expressed not only in macrophages, but also in endothelial and vascular smooth muscle cells, and in fibroblasts. Given the evidence that, in the aorta, calcitriol treatment was associated with the downregulation of renin, the rate-limiting step in the generation of angiotensin II, along with a very significant reduction in atherosclerosis, we expected to see a reduction in the activity of NADPH oxidase at the tissue level; however, despite the suggestion of such an effect, the extent of the reduction in aortic NADPH oxidase activity under calcitriol treatment failed to achieve statistical significance (Fig. 4).

Injected Low-Dose Calcitriol has No Effect on Splenic Regulatory T Cells and Circulating Proinflammatory Cytokines

Calcitriol is a potent immunomodulatory hormone. The inflammatory process leading to atherosclerosis is mediated in part by several cellular and humoral players from the immune system. It has
Calcitriol Decreases Atherosclerosis

427

ently contradictory results. Indeed, plasma glucose levels determined on plasma samples obtained in the morning after a 3 h fast were significantly higher in calcitriol-treated mice (Table 2), however, there was no difference in intraperitoneal glucose tolerance (IPGTT) assessed in a subset of mice studied in the early afternoon hours (Fig. 7A). Likewise, plasma total cholesterol levels measured after withholding food for only 3 hr in the morning were higher in calcitriol-
treated mice (Table 2). In contrast, after an overnight fast of 12 hours, cholesterol fractions by FPLC were similar among the groups (Fig. 7B). Thus, the higher total cholesterol levels with calcitriol might possibly reflect a greater chylomicron content, which would be cleared with a longer fast (as well as being filtered out before FPLC).

These differences nonetheless suggest dissimilar postprandial processing of nutrients among the treatments, while more prolonged fasting periods seemed to cancel out those disparities.

Finally, when determined using a limited number of mice, calcium levels were insignificantly higher in calcitriol-treated mice (Table 2).

**Discussion**

The two main findings of the present study, are that low-dose calcitriol treatment profoundly lowers

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**Table 2.** Blood pressure and biochemical parameters after 8 weeks of treatment

<table>
<thead>
<tr>
<th></th>
<th>Control (n=20)</th>
<th>Calcitriol (n=16)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>109.5 ± 2.4</td>
<td>91.5 ± 3.4</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>64.8 ± 2.1</td>
<td>53.4 ± 2.8</td>
<td>0.005</td>
</tr>
<tr>
<td>Glucose (mM)*</td>
<td>8.75 ± 0.7</td>
<td>14.75 ± 1.7</td>
<td>0.002</td>
</tr>
<tr>
<td>Cholesterol (mM)</td>
<td>21.6 ± 1.5</td>
<td>28.9 ± 2.8</td>
<td>0.028</td>
</tr>
<tr>
<td>Triglycerides (mM)</td>
<td>1.52 ± 0.13</td>
<td>1.57 ± 0.12</td>
<td>NS</td>
</tr>
<tr>
<td>Calcium (mM) †</td>
<td>2.15 ± 0.07</td>
<td>2.57 ± 0.34</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± SE

*To convert to conventional units for glucose, multiply by 18; for cholesterol, divide by 0.0259; for triglycerides, divide by 0.0113; and for calcium, divide by 0.25

†Serum calcium was determined by atomic absorptiometry of 3 control and 4 calcitriol-treated animals

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**Table 3.** Time-course of blood pressure effect of calcitriol. Data are shown as the mean ± SE. Number of mice are shown in parentheses

<table>
<thead>
<tr>
<th>Time</th>
<th>Systolic blood pressure (mmHg)</th>
<th>Diastolic blood pressure (mmHg)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline (62)</td>
<td>103.6 ± 2.2</td>
<td>61.2 ± 1.6</td>
<td>N/A</td>
</tr>
<tr>
<td>8 weeks</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (20)</td>
<td>109.5 ± 2.4</td>
<td>64.8 ± 2.1</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>Calcitriol (16)</td>
<td>91.5 ± 3.4*</td>
<td>53.4 ± 2.8†</td>
<td>0.005</td>
</tr>
<tr>
<td>12 weeks</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (16)</td>
<td>111.9 ± 3.0</td>
<td>67.6 ± 1.8</td>
<td>0.02</td>
</tr>
<tr>
<td>Calcitriol (10)</td>
<td>100.6 ± 2.9</td>
<td>59.7 ± 1.9</td>
<td>0.01</td>
</tr>
</tbody>
</table>

p: column refers to comparisons between treatment groups. There were no differences at baseline; therefore, the values for the entire cohort are given.

* p=0.01 compared with baseline.

† p=0.03 compared with baseline.

Despite a trend toward rising blood pressure with time in control animals as they aged and gained weight, there was no statistically significant difference (p=0.09 by ANOVA).
Calcitriol Decreases Atherosclerosis

However, due to the relatively limited contribution of the endothelium, or that of plaque macrophages to total aortic RNA, a 64% global reduction in renin mRNA must reflect the effect of calcitriol on vascular smooth muscle cells. Although not directly assessed in this study because of technical limitations, but in keeping with the marked suppression of kidney renin mRNA observed, we assume that circulating renin (mostly kidney-derived) was also affected. As renin is the rate-limiting step of RAS, we presume that a reduction in circulating and tissue level of AII similarly occurred in response to calcitriol treatment. This could certainly underlie the significant and sustained reduction in blood pressure observed in treated animals.

These findings also concur with those of previous studies which showed that components of RAS play an important role in the development of atherosclerosis in mice. Although apoE-null mice are generally seen as a classic paradigm of hypercholesterolemia-induced atherosclerosis, it has been demonstrated that manipulations aimed at lowering RAS expression, such as pharmacological ACE inhibition \(^{30}\), blocking AII action either by knockout of the angiotensin II type 1 receptor \(^{21}\) or by pharmacological AT-1R antagonism \(^{19}\), significantly attenuate atherosclerosis in this animal. Moreover, the protection from atherosclerosis conferred by ACE inhibition has been shown to be dissociated from its blood pressure-lowering effect. Indeed, ramipril given at a dose that did not affect blood pressure was as efficient at decreasing atherosclerosis as a hypotensive dose \(^{30}\).

More relevantly, inhibition of renin by aliskiren significantly reduced atherosclerotic lesions in LDL receptor-null mice. Notably, a decrease in the extent of atherosclerotic plaques was also seen in LDL receptor-null mice that harbored aortic macrophages derived from bone marrow transplanted from renin-null donors. In these chimeric animals, blood pressure and systemic levels of renin were unaltered, highlighting the importance of renin modulation in situ, in cells directly involved in plaque formation \(^{31}\). Nonetheless, further research will be required to distinguish the relative contribution of aortic RAS downregulation in response to local renin suppression from that of classical RAS in the protection from atherosclerosis conferred on these mice by systemic calcitriol treatment.

In our study, as in most others, calcitriol treatment had no significant effect on the expression of other components of RAS. The apparent increase in the level of AT1-R in calcitriol-treated animals, although not significant, could represent upregulation of the receptor secondary to the decrease in circulating AII \(^{32}\). Finally, although its role in cardiovascular

Role of Calcitriol in Renin Suppression

First observed in the kidney, it is now well established that calcitriol is a negative regulator of the renin gene \(^{18}\). The mechanism by which this occurs (interference with CREB binding to CRE in the renin gene promoter) has also been elucidated \(^{20}\). In this study, we demonstrated marked downregulation of renin mRNA locally in the aorta. This observation could be shared by any of the cellular components of the aorta, as complete tissue RAS, including renin expression by vascular smooth muscle cells, endothelial cells, and macrophages has been well documented \(^{27-29}\). However,
pathogenesis is still a matter of debate\(^{33}\), as it has been shown to be expressed in aortic smooth muscle cells\(^{34}\), we examined the possibility that calcitriol affected the degree of expression of the prorenin receptor; however, contrary to its effect on renin, calcitriol was devoid of any effect on the level of the aortic prorenin receptor transcript.

Many of the proinflammatory and proatherogenic effects of AII are felt to be mediated by NADPH oxidase\(^{25}\); thus, the lack of a significant reduction in the activity of aortic NADPH oxidase in calcitriol-treated mice was somewhat unexpected. This could be due to the increase in blood glucose levels seen in calcitriol-treated mice, as hyperglycemia and high glucose conditions have been shown to activate NADPH oxidase both \textit{in vivo} and in vascular cells in culture\(^{35}\). Alternative potential mechanisms for the antiatherogenic benefit conferred by the suppression of renin with low-dose calcitriol may involve other effectors of RAS, such as a decrease in the expression of oxidized LDL receptor, suppression of chemotaxis factors and of endothelial adhesions molecules. Finally, upregulation of the ACE2-angiotensin 1-7-Mas axis, shown to improve atherosclerosis in mice\(^{36}\), could also be at play.

**Present Findings in Light of Previous Studies**

The findings are consistent with the outcome of the recent study by Takeda \textit{et al}. also performed in mice\(^{24}\); however, several divergent points between the studies should be emphasized. In their investigation, the authors administered calcitriol by gavage twice weekly at two different doses. The lower daily dose of 20 ng had no effect. Only at the highest dose of 200 ng twice a week, not dissimilar to common absolute human doses, was a reduction in atherosclerosis noted. Surprisingly, this dose, which extrapolated to humans would be undoubtedly toxic, did not result in hypercalcemia. In contrast, we administered calcitriol by injection at a total weekly dose of approximately 20 ng, or roughly 5% of the dose found to be effective by Takeda \textit{et al}. This dosage was chosen on the basis of our preliminary studies in mice where a dose twice as high (0.5 ng/g BW every other day) as the dose used in the current study caused significant hypercalcemia\(^{22}\). We believe that even the lower dose we administered was still mildly supraphysiologic as there was a trend for higher calcium levels in calcitriol-treated animals; therefore, the lack of an effect of orally administered high-dose calcitriol on either blood pressure or renin in the study by Takada \textit{et al}. remains to be explained.

**Potential Deleterious Metabolic Effects of Calcitriol**

In keeping with Takeda’s study however, calcitriol treatment resulted in a significant elevation of cholesterol concentration. This finding is not only counter-intuitive but also hard to reconcile with the amelioration in the extent of atherosclerosis; however, it is consistent with the report that vitamin D receptor-null mice fed a high fat diet have lower cholesterol and triglyceride levels than wild-type animals\(^{37}\). This has been attributed to increased fatty acid beta-oxidation and uncoupling protein expression in the absence of vitamin D action. The identical lipoprotein fraction profile generated after a 12 h overnight fast by FPLC suggests that the higher cholesterol concentration seen in treated animals just a few hours after night time feeding may indeed represent protracted lipoprotein metabolism. Nevertheless, the fact that this was not seen in control animals feeding on the same high fat diet is certainly a cause for concern.

The higher early morning fasted serum glucose concentrations we observed in vitamin D-treated mice were likewise unexpected, and not previously reported in laboratory animals. Rather, there have been several reports linking vitamin D administration with the prevention of autoimmune diabetes in NOD mice, and with improved glycemia in streptozotocin-diabetes rats\(^{38,39}\). These beneficial effects of vitamin D were attributed to the immunomodulatory effect of vitamin D in pancreatic beta cells, and to improved insulin secretion, possibly mediated by intracellular calcium; however, in all these studies these effects were achieved with doses far in excess of those employed here.

**Questionable Immunomodulatory Effect of Low-DoseCalcitriol**

An immunomodulatory effect was also the mechanism proposed for the antiatherogenic properties of calcitriol in Takeda’s paper. Indeed, under orally administered high doses of calcitriol, the authors saw a shift toward a greater fraction of regulatory T cells (Treg) and immature dendritic cells in the intestine, mesenteric lymph nodes and spleen, along with a decrease in the amount of mature dendritic cells. This shift was mirrored by the relative content of these cells in atherosclerotic plaque at the aortic sinus. In contrast, under a far more physiological dose of calcitriol we failed to observe such an effect. We saw no increase in the splenic Treg content, and no alteration in the circulating cytokine profile with active vitamin D treatment.

Although the role of calcitriol as a modulator of the immune system is not in doubt, it does not appear to be the mechanism for the antiatherogenic effect of
Calcitriol Decreases Atherosclerosis

Conflicts of Interest
None to declare.

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Limitations of the Study
The lipemic serum of mice on a high fat diet is a well-known obstacle to many biochemical and hormonal determinations. Indeed, this prevented us from directly assessing plasma renin activity and AI concentration, which we anticipated to decrease as a result of the downregulation of renin with calcitriol. This limitation in fully determining the activity of systemic RAS in these animals is another hurdle in separating the local from the systemic effect of treatment.

The lack of an immunomodulatory effect at the dose of calcitriol we used was inferred from the absence of an effect on Tregs and from the plasma levels of a limited number of cytokines. There is a possibility that other proinflammatory cytokines such as TNFα could have been affected. Likewise, the lymphocyte composition of the plaque, specifically its Tregs content, was not analyzed, although it is unlikely that it would have been at odds with the systemic/splenic Treg population.

Conclusion
In summary, this study demonstrated the antiatherogenic effect of a low dose of calcitriol in apo-E null mice. While complete elucidation of this effect awaits further research, it appears that downregulation of RAS through the suppression of renin is at least part of the process.

Before introducing these findings into the clinical setting, the potential adverse metabolic effects seen with treatment should not be overlooked; thus, safer and less calcemic vitamin D analogs should undergo extensive preclinical assessment before VDR activators are studied for the prevention of atherosclerosis in non-uremic subjects.

Acknowledgments
The authors wish to thank Dr. Ayelet Gonen for sharing her skills and experience in the art of heart sectioning, and Ms. Hana Levkovitz for helping with the FPLC determination of lipoprotein fractions.

Grant Support
This work was supported in part by a young investigator’s grant from the Israeli Society for Research, Prevention and Treatment of Atherosclerosis (to MIS)
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Calcitriol Decreases Atherosclerosis

Supplemental Method

Methods

Determination of Calcitriol Concentrations in C57/Bl6 Mice

Extreme hyperlipidemia in apoE-null mice precluded the determination of plasma steroid hormone concentrations (vitamin D metabolites as well as aldosterone). To elucidate the concentrations of calcitriol achieved under the injection regimen, we established the time course of plasma concentrations of this active metabolite of vitamin D in 10-12-week-old C57/Bl6 wild-type mice subjected to the same scheduling of injections at the dose used in the study. Plasma samples were obtained at baseline, 1, 2, 3, 4, 6, 12, 24 hours after injection, and just prior to the next injection after 48 hours. Calcitriol concentrations were determined by RIA (Dia Sorin, Stillwater, MN, USA), as shown in Supplemental Fig. 1.

Intraperitoneal Glucose Tolerance and Lipoprotein Fraction Analysis

Intraperitoneal glucose tolerance. After withdrawing food for 3 h, in the early afternoon hours, a 10% glucose solution was administered to deliver a dose of 1 g/kg BW. Glucose was determined at given intervals on a tail clip blood sample on an Accu-Check Aviva (Roche Diagnostics, Indianapolis, IN).

FPLC analysis of lipoprotein fractions: Lipoproteins were separated by size exclusion chromatography using a Superose-6 column (1 × 30 cm) on an FPLC system (Pharmacia). A 200 μL aliquot of mouse serum was injected onto the column and separated with buffer containing 150 mM NaCl, 10 mM Na2HPO4, and 100 mM EDTA, pH 7.5, at a flow rate of 0.5 mL/min. Fifty fractions of 0.5 mL each were collected, with the lipoproteins distributed between tubes 12 and 30, as shown in Fig. 7B.

Quantification of Atherosclerosis at the Aortic Sinus

The aortic sinus area was processed by cutting serial 10 μm sections of the heart on a cryostat, and collecting about 10 sections that encompassed the area of interest. Slides were then stained with Oil Red O. The extent of the atherosclerosis lesion was assessed by quantifying the surface of the Oil Red O-stained plaques on a Nikon Eclipse TE2000E microscope with the aid of the NIS element Br 3.0 imaging system (Nikon Instruments Europe B.V., The Netherlands). The quantification of atherosclerosis for each mouse was based on the average of 3 sections analyzed in a blinded fashion.

Evaluation of the Aortic Expression of the RAS

After being severed from the heart, the aorta and kidney were snap-frozen and stored in liquid nitrogen. Total RNA was extracted using TRIzol Reagent (Invitrogen Life Technology, Carlsbad, CA). One microgram of total RNA was reverse-transcribed (EZ-First Strand cDNA Synthesis Kit; Biological Industries, Kibbutz Beit Haemek, Israel).

For semi-quantitative evaluation of aortic renin and prorenin receptor (PRR) after amplification, the PCR product was electrophoresed on ethidium bromide-containing 2% agarose gels, captured with the 202D Bio-imaging system (Dinco, Rhenium, Jerusalem, Israel), and analyzed with TINA software (Raytest, Staubenhardt, Germany). The relative intensity of the bands was expressed after adjusting for beta-actin (forward 5'-GACTACCTCATGAAGATCCTGACC-3', reverse 5'-TGATCTTCATGGTGCTAGGAGCC-3').

FACS Analysis of Splenic Regulatory T Cells (Tregs), and Circulating Levels of Cytokines

Splenocytes were obtained by passing a spleen through a 70 μm nylon mesh, and then washing with PBS. Red blood cells were lysed using 155 mM ammonium chloride solution for 5 min at room temperature. One million cells in 100 μL PBS were stained with 10 μL anti-mouse CD4 FITC, and 10 μL anti-mouse CD25 PE (Miltenyi, Germany) for 30 min at 4°C, washed and then resuspended in 0.5 mL fixation buffer for 1 hour at 4°C, and finally washed twice with permeabilization buffer (E-Bioscience, San Diego,
Cells were then blocked with 2 μL normal rat serum and stained with Foxp3 APC antibody (0.5 μg/sample) for 30 min at 4°C. Cells were washed and resuspended in 300 μL PBS. Fifty thousand events were acquired on a FACSCanto II flow cytometer (BD Biosciences, Rockville, MD). The number of Tregs is expressed as the percentage of CD25⁺Foxp3⁺/CD4⁺.

For cytokine determination, the number of mice was increased to 22 control and 21 calcitriol-treated mice. Serum levels of the following cytokines: MCP-1 (CCL2), IL-4, IL-10 and INF-γ were measured by Luminex 200, a bead based multiplex dual laser flow analyzer (Luminex Corp., Austin, TX). Twenty-five microliters of serum was added to wells containing the appropriate bead mixture coated with mouse monoclonal antibodies specific for the above cytokines. In addition, serial dilutions of these cytokines were added to the plate to generate a standard curve. The plate was incubated overnight at 4°C to enable capture of the cytokines by the antibody-conjugated fluorescent beads. After three washes with the assay buffer, 25 μL biotinylated secondary cytokine antibody mixture was applied for 1.5 h, in the dark, at room temperature. The reactions were detected with streptavidin-phycoerythrin using a Luminex 200 xPONENT system.