Calcitriol ameliorates capillary deficit and fibrosis of the heart in subtotally nephrectomized rats

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Abstract

Background. Remodelling of the heart, characterized by hypertrophy, fibrosis and capillary/myocyte mismatch, is observed in patients with chronic renal failure. Low vitamin D levels have been associated with increased cardiovascular risk. In the present experimental study, we studied the effects of non-hypercalcaemic doses of calcitriol on microvascular disease and interstitial fibrosis of the heart.

Methods. Three-month-old male Sprague-Dawley rats were randomized to subtotal nephrectomy (SNX) or sham operation and received calcitriol (6 ng/kg) or vehicle starting immediately thereafter. Blood pressure was measured by tail plethysmography. Albuminuria was measured by ratspecific ELISA. Capillary length density, volume density of interstitial tissue, immunohistochemistry and western blots (vitamin D receptor, collagen I, III, TGF-β1, MAP kinases and nitrotyrosine) were assessed after 12 weeks of treatment.

Results. After SNX blood pressure, albuminuria and heart weight were elevated, capillary length density reduced and interstitial fibrosis increased. Treatment with calcitriol reduced albuminuria and prevented reduction of capillary density and expansion of interstitium without affecting significant blood pressure and heart weight after perfusion fixation. Calcitriol left high VEGF unchanged, but upregulated VEGF receptor 2 (presumably reversing VEGF resistance). Calcitriol reduced expression of profibrotic TGF-β1 and the accumulation of collagens I and III.

Conclusions. Non-hypercalcaemic doses of calcitriol ameliorated, directly or indirectly, cardiac remodelling in subtotally nephrectomized rats.

Keywords: calcitriol; cardiac remodelling; chronic renal failure; secondary hyperparathyroidism; vitamin D

Introduction

Chronic kidney disease is a major risk factor for cardiovascular (CV) disease [1]. In uraemic patients, remodelling of the myocardium is found with interstitial fibrosis and capillary/myocyte mismatch [2] and the same is observed in subtotally nephrectomized rats [3]. Low vitamin D concentrations are associated with increased CV risk [4]. Vitamin D is widely used for the management of secondary hyperparathyroidism (sHPT). Observational data suggest increased survival when patients on dialysis are supplemented with active vitamin D compounds, e.g. calcitriol or paricalcitol [5,6].

Since most dialysis patients die of cardiac causes and the use of vitamin D in this population have a survival benefit, the effect of active vitamin D on the heart is of obvious interest. Vitamin D receptor (VDR) knockout mice have cardiomyocyte hypertrophy [7]. In cardiomyocyte cultures, calcitriol reduces cardiomyocyte volume [8] and proliferation [9] and paricalcitol prevents cardiac hypertrophy in the Dahl salt-sensitive rats [10]. Moreover, calcitriol is known to inhibit renin secretion from the juxtaglomerular apparatus [11].

It was the purpose of the present study to investigate the effects of active vitamin D (calcitriol) on capillary density, interstitial fibrosis and expression of molecules relevant to fibrosis and angiogenesis in subtotally nephrectomized rats.

Materials and methods

Animals and subtotal nephrectomy

All animal procedures were approved by the local ethic committee for animal experiments (Regierungspraesidium Karlsruhe). Twelve-week-old male Sprague-Dawley rats (Charles River Co., Sulzfeld, Germany), with mean body weight 331 ± 85 g, were housed at constant room temperature (21 ± 1°C) and humidity (75 ± 5%) and exposed to a 12-h light on, 12-h light off cycle. The animals had free access to water and were fed a standard rodent diet ad libitum (19.0% protein, 4.0% fat, 0.90% calcium, 0.70%...
phosphorus; Saniff, Soest, Germany). After a 7-day adaptation period, the rats were randomly allotted to sham operation or 5/6 surgical nephrectomy (SNX) as previously described [12]. After the second operation, SNX and sham-op animals were randomized to the following treatments:

1. Sham-op, treated with vehicle (sham+vehicle, n = 16),
2. Sham-op, treated with calcitriol (sham+calcitriol, n = 20),
3. SNX, treated with vehicle (SNX+vehicle, n = 17),
4. SNX, treated with calcitriol (SNX+calcitriol, n = 17).

Calcitriol (Calbiochem, Darmstadt, Germany) was administered daily (6 ng/kg body weight) by subcutaneous injection (0.1 ml) for 12 weeks. Control animals received vehicle (saline). The dose of calcitriol was deliberately chosen not to increase serum calcium and phosphorus levels based on a pilot experiment. Body weight was measured at weekly intervals. At Weeks 4 and 12, the animals were kept in metabolic cages for 24-h urine collection.

Systolic blood pressure measurement

Systolic blood pressure (SBP) was measured at Week 12, using a semi-automatic tail-cuff system (TSE Systems, Bad Homburg, Germany). The animals were accustomed to the instrument for 5 consecutive days before the actual recorded measurements to minimize procedure-induced stress. The first 5 of 15 blood pressure values recorded at each session were disregarded, and the remaining 10 values were averaged and used for analysis.

Tissue preparation

The abdominal aorta was catheterized under ketamine/xylazine anaesthesia (100 mg/kg and 3 mg/kg, respectively), blood samples were taken and the experiment was terminated by retrograde aortic perfusion with 3% glutaraldehyde for morphometric and stereological investigations (n = 36) or with ice-cold 0.9% NaCl for molecular investigations (n = 34). Perfusion pressure was kept constant at 120 mmHg to avoid pressure-related artefacts. From each animal, eight random samples of randomly orientated left ventricular sections were embedded in epon araldite. Semithin sections (1 µm) were cut, stained with methylene blue/basic fuchsin and investigated using the orientator method [2].

After perfusion with ice-cold 0.9% NaCl, horizontal slices of the heart were snapfrozen in liquid nitrogen for western blotting or fixed in 4% formaldehyde for immunohistochemistry.

Urinary albumin measurements and blood analysis

Urinary albumin excretion was measured using a rat-specific sandwich ELISA [12]. Blood was obtained at sacrifice by aortic puncture for measurements of creatinine, calcium, phosphorus, cholesterol and triglycerides by standard laboratory methods and serum parathormone (PTH) by the two-antibody method using a rat iPTH ELISA kit (Immutopics, Inc., San Clemente, CA, USA). The serum 1,25-dihydroxy vitamin D$_3$ (1,25(OH)$_2$D$_3$) concentration was measured by a RIA method. Serum angiotensin II was measured using an ELISA kit (Peninsula Laboratories LLC., San Carlos, CA, USA).

Quantitative stereology of the heart

All investigations were performed in a blinded manner, i.e. the observer was unaware of the animal group. The length density (Lv) of capillaries, i.e. the length of capillaries per unit tissue volume, and the volume density (Vv) of cardiac capillaries and interstitial tissue, i.e. the volume of a component per unit volume of myocardial tissue, were measured in eight systematically subsampled areas per section as previously described [13]. Intercapillary distance was calculated according to a formula by Henquell and Honig [14].

Myocardial total collagen content was measured in sections stained with 0.1% Sirius red F3BA saturated in picric acid (a percent of Sirius-red stained collagen area to total myocardial area) using a semiautomatic image analysis software (Optimas 6.0, Optimas Corp., Seattle, WA, USA).

Wall thickness and lumen diameter of intramyocardial arteries (diameter: 20–100 µm) were determined planimetrically using a semiautomatic image analysis software (Optimas 6.0) as described in detail elsewhere [15].

Immunohistochemistry and in situ hybridization

Immunohistochemical analysis was performed on paraffin sections using antibodies against VDR, vascular endothelial growth factor (VEGF), transforming growth factor beta-1 (TGF-β1), TGF-β receptor type 1 and type 2 (Santa Cruz Biotechnologies, Heidelberg, Germany), VEGF receptor type 1 and type 2 (Abcam, Cambridge, UK), collagen type I, and type III, (Biotrend, Cologne, Germany), nitrotyrosine (Upstate, New York, NY, USA) and prolyl-4-hydroxylysine (Acris Antibodies, Hiddenhausen, Germany) by the streptavidin-biotin technique using alkaline phosphatase as the labelling enzyme. All antibodies had been tested for specificity in rats, and the optimal concentration for staining was evaluated by testing different dilutions in a pilot study. Negative controls were performed by omitting the primary antibody.

The nonradioactive in situ hybridization for VDR (sequence NM 017058) was performed as previously described [12] using following primers: sense, 3'-TGAGGGCTGCAAAGGTTTCT-5'; antisense, 3'-TAGCTTGGGCCTCAGACTGT-5'.

The staining was analysed by an investigator blinded with respect to the animal group using the following semi-quantitative scoring system (0–4): 0, no expression; 1, weak expression; 2, moderate expression; 3, strong expression, 4, extremely strong expression. Intraobserver error was <5%.

Western blotting

Samples of myocardium from eight to nine animals per group were prepared by homogenization, and the protein concentration was assessed according to Bradford (Protein Assay Kit, Bio-Rad Laboratories, Munich, Germany).
Table 1. Animal data at Week 12

| Group                        | Body weight (g) | Body weight ratio (%) | Left ventricular weight (g) | Heart/body weight ratio | Albumin excretion (mg/24h) | Serum creatinine (mg/dl) | Serum total cholesterol (mg/dl) | Serum HDL cholesterol (mg/dl) | Systolic blood pressure (mmHg) | Serum triglycerides (mg/dl) | Serum total triglycerides (mg/dl) |
|------------------------------|-----------------|-----------------------|----------------------------|-------------------------|---------------------------|--------------------------|-----------------------------|--------------------------------|-----------------------------|-----------------------------|-----------------------------|
| sham + vehicle               | 506 ± 37        | 0.34 ± 0.12           | 0.83 ± 0.27                | 125 ± 23                | 0.55 ± 0.09               | 69 ± 25                   | 43 ± 15                     | 128 ± 36             | 125 ± 23                    | 0.8 ± 0.31                   | 39 ± 2.7                     |
| sham + calcitriol            | 501 ± 41        | 0.32 ± 0.08           | 0.82 ± 0.10                | 125 ± 12                | 0.61 ± 0.08               | 94 ± 96                   | 52 ± 12                     | 128 ± 36             | 124 ± 24                    | 7.7 ± 2.4                    | 61 ± 24                     |
| SNX + vehicle                | 462 ± 41        | 0.58 ± 0.09           | 1.35 ± 0.31                | 168 ± 20                | 1.05 ± 0.36              | 109 ± 39                  | 61 ± 24                     | 124 ± 36             | 124 ± 24                    | 116 ± 44                     | 62 ± 11                     |
| SNX + calcitriol             | 486 ± 40        | 0.65 ± 0.11           | 1.51 ± 0.26                | 158 ± 21                | 1.09 ± 0.30              | 123 ± 56                  | 62 ± 11                     | 124 ± 36             | 124 ± 24                    | 166 ± 107                    | 62 ± 11                     |
| ANOVA                        |                |                       |                            |                         |                          |                           |                             |                                |                             |                             |                             |

Equal amounts of protein (100 µg) were electrophoresed in SDS-PAGE gel and subsequently transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA, USA). The membranes were blocked with 5% non-fat dried milk and then incubated with primary antibody against endothelial nitric oxide synthase (eNOS, Affinity Bioreagents, Golden, CA, USA), phosphorylated ERK-1/2 (p-ERK-1/2, Novus Biologicals, Littleton, CO, USA), VDR, phosphorylated JNK (p-JNK), phosphorylated p38 (p-p38), collagen type I, VEGF, TGF-β1 (Santa Cruz Biotechnologies), inducible NO Synthase (iNOS, BD Biosciences, Heidelberg, Germany), matrix metalloproteinase-1 (MMP-1), MMP-2, tissue inhibitor of matrix metalloproteinase 1 (TIMP-1), tissue inhibitor of matrix metalloproteinase 1 (TIMP-2) (Calbiochem, Darmstadt, Germany) and collagen III (BioTrend, Cologne, Germany). Horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnologies) were used and followed by the ECL kit (GE Healthcare, Buckinghamshire, UK) according to manufacturer’s instruction. Specific bands were quantified by densitometric analyses (EasyWin32, Herolab, Wiesloch, Germany).

Statistics

Data are given as mean ± SD. For western blots, the vehicle-treated sham-op group served as reference and the mean value of individual measurements was set as 100%. The value for each animal was expressed as manifold of reference. After testing for normal distribution, ANOVA or the Kruskal–Wallis test, respectively, was used for analysis of variance. The differences between groups were assessed using Duncan’s multiple range test. The results were considered significant when the P-value was <0.05.

Results

Animal data (Table 1)

The heart and left ventricular weight were significantly higher in SNX compared with sham-op animals and were not influenced by calcitriol treatment.

At the end of the study, pre-dosis SBP measured by plethysmography was higher in both SNX groups as compared with sham-op animals. The treatment had no significant effect on SBP in sham-op or SNX animals.

Serum creatinine was significantly increased in both SNX groups compared with sham-op, but no differences were found between the treatment groups. Serum total and HDL cholesterol were significantly higher in vehicle-treated SNX compared with vehicle-treated sham-op. In sham-op animals treated with calcitriol, total and HDL cholesterol were significantly higher compared with vehicle-treated animals. In SNX+calcitriol, total cholesterol was significantly higher compared with SNX+vehicle.

The serum concentration of triglycerides was significantly higher in animals (sham-op and SNX) treated with calcitriol compared with vehicle-treated animals.

The serum angiotensin II concentration was significantly (P < 0.001) higher in SNX+vehicle (30.1 ± 13.1 ng/ml).
Table 2. Serum calcium, phosphate and PTH concentrations

| Group                  | Serum calcium (mmol/l) | Serum phosphate (mmol/l) | Ca × P product (mmol²/l²) | PTH (pg/ml) | 1,25(OH)₂D₃ (ng/l) |
|------------------------|------------------------|--------------------------|---------------------------|-------------|-------------------|
| sham + vehicle (n = 16)| 2.64 ± 0.18            | 2.28 ± 0.31              | 5.85 ± 0.72               | 393 ± 218   | 36.0 ± 12.9       |
| sham + calcitriol (n = 20) | 2.78 ± 0.15             | 2.21 ± 0.28              | 5.98 ± 1.32               | 366 ± 266   | 61.2 ± 12.6       |
| SNX + vehicle (n = 17) | 2.66 ± 0.25             | 2.78 ± 0.56              | 7.26 ± 2.00              | 1739 ± 1023 | 30.8 ± 4.9        |
| SNX + calcitriol (n = 17) | 2.73 ± 0.13             | 2.66 ± 0.37              | 7.13 ± 1.15              | 332 ± 87    | 54.6 ± 14.9       |

ANOVA NS P = 0.010 P = 0.012 P < 0.001 P = 0.018

NS—not significant.
Significant differences versus sham-op + vehicle; sham-op + calcitriol and SNX + vehicle.

Fig. 1. Quantitative morphology of the myocardium: capillary length density (A), mean intercapillary distance (B), volume density of interstitial matrix (C) and volume density of fibrocytes (D).

and SNX + calcitriol (29.3 ± 12.9) compared with sham-op + vehicle (5.9 ± 4.6) and sham-op + calcitriol (6.6 ± 6.6).

At 12 weeks, post-op albuminuria was increased in both SNX groups compared with sham-op animals. Albuminuria was highest in the untreated SNX group, and significantly lower in the SNX + calcitriol group.

Calcium and phosphorus metabolism (Table 2)

No significant difference in pre-dose serum calcium concentrations was found between the groups. The phosphorus concentration was significantly higher in untreated SNX and SNX + calcitriol compared with sham-op animals. The calcium–phosphate product was increased in parallel. The serum PTH concentration was significantly increased in untreated SNX compared with sham-op and was lower in SNX + calcitriol. The serum calcitriol concentration was not significantly different between untreated SNX and sham-op. In animals receiving calcitriol (sham-op and SNX), its serum concentration was significantly higher 12 h postdosing compared with untreated animals.

Quantitative morphology of the myocardium

Capillaries. In untreated SNX animals, the capillary Lv was significantly lower (Figure 1A) and the mean...
intercapillary distance significantly higher (Figure 1B) than in sham-op animals. In SNX animals treated with calcitriol capillary Lv was significantly higher and the mean intercapillary distance significantly lower compared with vehicle-treated SNX.

**Interstitium.** The volume densities of interstitial matrix (Figure 1C) and of fibrocytes (Figure 1D) were significantly higher in vehicle-treated SNX than those in sham-op or calcitriol-treated SNX.

Total collagen area (given as Sirius-red positive area) was significantly higher in vehicle-treated SNX than those in sham-op or calcitriol-treated SNX.

Markers of fibrosis

Collagen I abundance in the myocardium, assessed by immunohistochemistry (Table 3) and western blot (Figure 4A), was significantly higher in untreated SNX than that in sham-op and SNX+calcitriol.

Immunohistochemical staining for collagen III was more marked in untreated SNX than that in sham-op (Table 3). The myocardial expression of collagen III, assessed by western blotting (Figure 4B), was significantly higher in vehicle-treated SNX compared with all sham-op and significantly lower in SNX treated with calcitriol compared with SNX+vehicle.

The immunohistochemical staining for prolyl-4-hydroxylase, a marker for collagen synthesizing cells, was not significantly different between study groups (Table 3).

The expression of matrix metalloproteinase 1 (MMP-1) by western blot was significantly lower in vehicle- or calcitriol-treated SNX compared with sham-op (Figure 4C).

The expression of TIMP-1 by western blot was significantly higher in vehicle-treated SNX compared with sham-op and significantly lower in SNX+calcitriol compared with SNX+vehicle (Figure 4D).

No difference in the expression of TIMP-2 by western blot was found between the groups (Figure 4E).
Table 3. Markers for myocardial fibrosis assessed by immunohistochemistry

| Group                          | Collagen I | Collagen III | Prolyl-4-hydroxylase | TGF-β₁ | TGF-β receptor 1 | TGF-β receptor 2 |
|-------------------------------|------------|--------------|----------------------|--------|-----------------|------------------|
| sham-op+vehicle (n = 8)       | 1.41 ± 0.33| 0.99 ± 0.29  | 0.94 ± 0.29          | 0.84 ± 0.68 | 2.57 ± 0.71 | 1.96 ± 0.49     |
| sham-op+calcitriol (n = 10)   | 1.36 ± 0.27| 0.77 ± 0.43  | 0.83 ± 0.28          | 1.24 ± 0.42 | 2.45 ± 0.39 | 1.92 ± 0.94     |
| SNX+vehicle (n = 8)           | 2.24 ± 0.69| 1.49 ± 0.34  | 1.33 ± 0.27          | 1.97 ± 0.68 | 2.01 ± 0.25 | 2.15 ± 0.73     |
| SNX+calcitriol (n = 8)        | 1.30 ± 0.36| 1.09 ± 0.18  | 0.95 ± 0.72          | 1.34 ± 0.28 | 1.16 ± 0.51 | 1.91 ± 0.65     |

ANOVA  

\[ P < 0.001 \quad P = 0.015 \quad NS \quad P = 0.020 \quad P = 0.002 \quad NS \]

NS—not significant.  
Significant differences versus a sham-op+vehicle; b sham-op+calcitriol and c SNX+vehicle.

The protein expression of TGF-β₁ by immunohistochemistry was significantly increased in untreated SNX compared with sham-op animals on vehicle or on calcitriol (Table 3). In SNX rats, treatment with calcitriol diminished staining for TGF-β₁. All results were confirmed by western blot analysis (Figure 4F).

No difference in immunohistochemical staining for TGF-β receptor 1 and TGF-β receptor 2 was found between untreated SNX and sham-op animals (Table 3). Treatment with calcitriol caused significant reduction of staining for TGF-β receptor 1 in SNX.

**MAP kinases**

The expression of both ERK-2 and the phosphorylated form p-ERK-1/2 by western blot was not significantly different between vehicle-treated sham-op and SNX rats. Calcitriol increased expression of total ERK-2 and phosphorylated ERK-1/2 in SNX rats (Figure 5A, B).

The expression of p-JNK and p-p38 was not significantly different between the groups (Figure 5C, D).

**VEGF and VEGF receptors**

Immunohistochemical staining for VEGF was significantly more marked in untreated SNX compared with sham-op animals (Table 4). It was significantly lower in SNX+calcitriol compared with untreated SNX, but still higher than that in sham-op animals. The expression of VEGF assessed by western blotting was significantly higher in both SNX groups compared with sham-op (Table 4).

The expression of the VEGF receptor type 1 (flt-1) showed no significant differences between the study groups (Table 4).

Significantly weaker staining for VEGF receptor type 2 (flk-1) was observed in untreated SNX compared with sham-op (Table 4). Treatment with calcitriol resulted in
Table 4. Expression of VEGF by western blotting and myocardial staining for VEGF, VEGF receptor 1 (flt-1) and 2 (flk-1), and nitrotyrosine

| Group                      | VEGF (WB) (%) | VEGF (IHC) (score) | VEGF receptor 1 (flt−1) (score) | VEGF receptor 2 (flk−1) (score) | Nitrotyrosine (score) |
|----------------------------|---------------|--------------------|---------------------------------|---------------------------------|-----------------------|
| sham-op+vehicle (n=8)      | 100 ± 15      | 1.47 ± 0.33        | 1.83 ± 0.65                     | 1.73 ± 0.39                     | 1.07 ± 0.16           |
| sham-op+calcitriol (n=10)  | 89 ± 33       | 1.45 ± 0.46        | 1.63 ± 0.32                     | 1.31 ± 0.50                     | 0.63 ± 0.12           |
| SNX+vehicle (n=8)          | 126 ± 19b     | 2.63 ± 0.19a,b     | 1.66 ± 0.31                     | 1.06 ± 0.57a                    | 2.01 ± 0.55b          |
| SNX+calcitriol (n=8)       | 124 ± 17b     | 2.10 ± 0.35a,b,c   | 1.29 ± 0.54                     | 1.91 ± 0.68b,c                 | 1.70 ± 0.39b          |

ANOVA P = 0.029 P < 0.001 NS P = 0.019 P < 0.001

NS—not significant.

Significant differences versus *sham-op+vehicle; ^sham-op+calcitriol and ^SNX+vehicle.

Fig. 4. Representative western blots and quantitation of western blots of the myocardium for collagen I (A), collagen III (B), MMP-1 (C), TIMP-1 (D), TIMP-2 (E) and TGF-β1 (F).

stronger staining for flk-1 in SNX animals compared with untreated SNX.

Markers of oxidative stress

The staining for nitrotyrosine, a marker of oxidative stress, was significantly increased in untreated SNX and SNX+calcitriol compared with sham-op animals (Table 4).

The expression of iNOS was significantly (P = 0.003) lower in vehicle (50 ± 19%) or calcitriol (66 ± 20%)-treated SNX and sham-op treated with calcitriol (63 ± 16%) compared with vehicle-treated sham-op animals (100 ± 34%).

The expression of eNOS and HIF-1α and heat shock protein 70 (Hsp-70) was not significantly different between the groups (data not shown).
Discussion

The present experimental study provides clear evidence that calcitriol treatment after subtotal nephrectomy (SNX) selectively prevents the development of a capillary deficit (microvessel disease), and the expansion of the interstitial space (interstitial fibrosis) in the heart without affecting cardiac volume. These experimental data are of interest in view of recent observational clinical data of a beneficial effect of active vitamin D on survival in dialyzed patients in whom the major causes of death are cardiac events.

In patients with end-stage renal disease treated with active vitamin D, partial reversal of left ventricular hypertrophy had been observed [16]. In SNX rats treated with calcitriol, lower heart weight (more specifically heart weight after perfusion fixation, which may introduce artifacts) was not seen in the present experiment. Blood pressure is not likely to be a major confounder, because calcitriol at the given dose did not change significantly serum angiotensin II levels in SNX rats. Therefore, modulation of the systemic renin–angiotensin system by calcitriol was not the major pathway for the beneficial effect of calcitriol on cardiac microvasculature and interstitial fibrosis.

Of concern were previous studies showing reduced VDR expression in uraemia [18,19] that might have interfered with the efficacy of calcitriol treatment. In this study, however, we showed unchanged VDR expression in the heart.

In clinical studies, reduction in albuminuria has been linked to improvement in CV outcomes [20]. It is, therefore, of note that in our study albumin excretion was reduced in SNX rats treated with active vitamin D resembling the observations in humans [21].

The mechanism(s) underlying the mismatch between capillary growth and cardiomyocyte enlargement in previous findings [10,17]. Calcitriol is known to inhibit renin secretion from the juxtaglomerular apparatus [11]. We have shown, however, that calcitriol at the given dose did not change significantly serum angiotensin II levels in SNX rats. Therefore, modulation of the systemic renin–angiotensin system by calcitriol was not the major pathway for the beneficial effect of calcitriol on cardiac microvasculature and interstitial fibrosis.

Of concern were previous studies showing reduced VDR expression in uraemia [18,19] that might have interfered with the efficacy of calcitriol treatment. In this study, however, we showed unchanged VDR expression in the heart.

In clinical studies, reduction in albuminuria has been linked to improvement in CV outcomes [20]. It is, therefore, of note that in our study albumin excretion was reduced in SNX rats treated with active vitamin D resembling the observations in humans [21].

The mechanism(s) underlying the mismatch between capillary growth and cardiomyocyte enlargement in
uraemic cardiomyopathy have not been completely resolved. The expression of VEGF in the heart of SNX animals was significantly increased, but that of VEGF receptor 2 (flk) was reduced suggesting problems with VEGF signal transduction (VEGF resistance) as the potential cause of inadequate capillary growth. Such constellation of increased VEGF and decreased VEGF receptor expression resembles the findings in the heart of diabetic patients [22]; the authors of this study suggested VEGF resistance as a potential cause of microvessel disease. Microvessel disease has recently been recognized as an important cause of impaired cardiac ischaemia tolerance [23] in numerous cardiac conditions.

The increase in collagen fibre deposition in the cardiac interstitium [24] has important functional consequences, particularly diastolic dysfunction [25] and electric instability [26]. In the present model, increased amounts of both collagen type I and collagen type III were deposited in untreated SNX rats, but interstitial fibrosis was prevented by calcitriol. The constellation of lower expression of MMP-1 and higher abundance of TIMP-1 in vehicle-treated SNX rats suggest that the breakdown of collagen is slower, as has been observed by experimental models and clinical studies in hypertension and chronic heart failure [27]. As to the beneficial effect of calcitriol on cardiac fibrosis, the arguably most important aspect may be the normalization of the protease inhibitor TIMP-1 that was normalized by calcitriol treatment. In contrast, the expression of prolyl-4-hydroxylase, a marker of collagen synthesis, was unchanged consistent with the proposal that increased collagen deposition in myocardium is the result of diminished degradation rather than of enhanced synthesis.

In the heart, several types of injury trigger sustained TGF-β overproduction that has been recognized as an important causal factor for progressive deposition of extracellular matrix and tissue fibrosis [28]. In several tissues blockade of TGF-β signalling prevented deposition of extracellular matrix [28]. In the context of less fibrosis in the heart of calcitriol-treated SNX rats, it is of note that TGF-β1 expression was increased in untreated SNX rats and substantially lowered by calcitriol. The expression of both type 1 and 2 TGF-β receptors was unchanged. Although a link between oxidative stress and TGF-β1 has been described [29], we could demonstrate lower TGF-β1 in animals treated with calcitriol despite similar oxidative stress (at least as reflected by nitrotyrosine). TGF-β1 not only activates proteins of the Smad family and regulates gene transcription but also increases ROS production [30]. This effect of TGF-β1 was probably not responsible for the antifibrogenic effects in calcitriol-treated animals. The lack of change in redox-state-sensitive MAP kinases further supports this explanation.

Activators of the PPAR alpha nuclear receptor had been shown to ameliorate cardiac fibrosis [31,32]. As activation of VDR suppresses PPAR alpha [33], this seems not to be the mechanism of the antifibrotic effects of calcitriol in this study.

Cardiac hypertrophy is a compensatory response to external stressors [34]. When the stressors persist, the initially compensatory growth evolves into maladaptive remodelling as described by Selvetella [35]. MAP kinases play a central role in cardiac hypertrophy [36]. Activation of the ERK-1/2 causes adaptive hypertrophy as an effort to compensate for increased load thus normalizing increased wall stress and preventing the occurrence of fibrosis and microvessel pathology [34,37]. It is, therefore, of note that in SNX rats, calcitriol increased both total and phospho-rylated ERK. In SNX rats, heart weight (at least heart weight after perfusion/fixedation), in contrast to microves-sel disease and interstitial fibrosis, was unchanged. Therefore, one might argue that calcitriol treatment, although not preventing cardiac hypertrophy, shifted signalling towards adaptive growth that is characterized by the absence of interstitial fibrosis and microvessel disease.

Several confounders have to be considered in the interpretation of the above results. As anticipated, calcitriol lowered the PTH concentration in the treated SNX. The data are compatible with both a direct effect of calcitriol and an indirect effect via PTH that is known to play a role in the genesis of cardiomyopathy in renal failure [15]. A previous study documented a role of PTH in the genesis of interstitial fibrosis [38]. PTH lowering by parathyreoidectomy or by a calcimimetic reduced cardiac abnormalities in SNX rats [39]. In the study of Repo et al. [40], however, lowering of PTH was not sufficient to prevent cardiac fibrosis. A role of PTH in the genesis of capillary rarefaction—with potentially even greater functional significance—has never been recognized to date.

In a recent experimental study, paricalcitol aggravated cardiac fibrosis [40]. In our study, calcitriol had an opposite effect. This may indicate differences between the compound in respect to VDR activation or be explained by the different time course.

In our study, serum cholesterol levels were higher in rats treated with calcitriol, but rat is a high HDL animal and the relevance of the rat findings for humans is uncertain.

In summary, treatment with non-hypercalcaemic doses of calcitriol directly or indirectly abrogated interstitial fibrosis and microvessel disease of the heart in subtotal nephrectomized rats.

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Conflict of interest Statement. None declared.

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