Cardioprotective effect of calcineurin inhibition in an animal model of renal disease

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Aims

Chronic kidney disease is directly associated with cardiovascular complications. Heart remodelling, including fibrosis, hypertrophy, and decreased vascularization, is frequently present in renal diseases. Our objective was to investigate the impact of calcineurin inhibitors (CNI) on cardiac remodelling and function in a rat model of renal disease.

Methods and results

Male Sprague Dawley rats were divided into six groups: sham-operated rats, 5/6 nephrectomized rats (Nx) treated with vehicle, CNI (cyclosporine A 5.0 or 7.5, or tacrolimus 0.5 mg/kg/day) or hydralazine (20 mg/kg twice a day) for 14 days, starting on the day of surgery. Creatinine clearance was significantly lower and blood pressure significantly higher in Nx rats when compared with controls. Morphological and echocardiographic analyses revealed increased left ventricular hypertrophy and decreased number of capillaries in Nx rats. Treatment with CNI affected neither the renal function nor the blood pressure, but prevented the development of cardiac hypertrophy and improved vascularization. In addition, regional blood volume improved as confirmed by contrast agent-based echocardiography. Hydralazine treatment did not avoid heart remodelling in this model. Gene expression analysis verified a decrease in hypertrophic genes in the heart of CNI-treated rats, while pro-angiogenic and stem cell-related genes were upregulated. Moreover, mobilization of stem/progenitor cells was increased through manipulation of the CD26/SDF-1 system.

Conclusion

We conclude from our studies that CNI-treatment significantly prevented cardiac remodelling and improved heart function in Nx rats without affecting renal function and blood pressure. This sheds new light on possible therapeutic strategies for renal patients at high cardiovascular risk.

Keywords

Chronic kidney disease • Cardiovascular protection • Calcineurin inhibition • Cyclosporine • Tacrolimus • Vascularization

Introduction

Chronic kidney disease (CKD) patients have a substantially increased risk for cardiovascular disease. Cardiac mortality is the main cause of death in this population. Marked cardiac remodelling, including myocardial fibrosis, hypertrophy, and decreased vascularization is frequently observed in uremic patients.¹⁻³ Left ventricular (LV) hypertrophy, a well-known feature of renal failure, and LV mass have been correlated with survival in renal patients.³⁻⁴ Thus, renal disease obviously does not only affect cardiac morphology; it leads to LV systolic and diastolic dysfunction, all of which could be related to the increased incidence of sudden death.⁴

Growing evidence suggests an important role of the calcineurin pathway in mediating the development of cardiac hypertrophy.⁵⁻⁷ In addition, insufficient or impaired angiogenesis, especially in response to hypoxia, can lead to profound ventricular remodelling.

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heart dysfunction, and subsequent failure. However, whether calcineurin is activated in renal disease and whether the expression of growth factors or their signalling pathways are impaired causing failure is not still completely understood.

In this context, decreasing hypertrophy and increasing capillary density in the heart seems to be an attractive preventive and therapeutic approach for renal patients. Recently, it was shown that cyclosporine A (CsA), a calcineurin inhibitor (CNI) largely employed as immunosuppressant in the context of transplantation, results in beneficial effects on angiogenesis and vascular endothelial growth factor (VEGF) expression. In addition, CsA has been described to inhibit cardiac hypertrophy to promote cardioprotection in vivo, and, at low to moderate doses, to be tolerable and safe, without induction of nephrotoxicity.

By using 5/6 nephrectomy as a model of progressive kidney disease, we have shown that calcineurin signalling is activated even after a sub-acute kidney injury (14 days after surgery), and that low therapeutic doses of CNI (CsA and tacrolimus) protect the heart against remodelling and loss of function.

## Methods

Experiments were approved by a governmental committee on animal welfare and were performed in accordance with national animal protection guidelines. Detailed methods are given as Supplemental Data.

### Animal model: 5/6 nephrectomized rat

Renal disease was induced in Sprague Dawley rats by 5/6-resection of renal tissue. The rats were randomized into seven groups: (i) Sham + vehicle (Sham, n = 19); and 5/6-nephrectomized (Nx) rats treated with (ii) vehicle (Saline); (iii) CsA 5 and (iv) CsA 7.5 mg/kg/day; (v) tacrolimus 0.5 mg/kg/day; (vi) hydralazine 20 mg/kg twice a day; and (vii) CsA 5 mg/kg/day + soluble VEGF receptor 1 (sFlt-1) 300 ng/h. The treatment started on the day of surgery and lasted for 14 days.

### Analysis of capillary density and hypertrophy in the left ventricular myocardium

Heart hypertrophy and vascularization was determined by means of the heart/body weight ratio and by counting the number of blood vessels/cardiomyocyte, respectively.

### Assessment of myocardial fibrosis

The extent of fibrosis was analysed by Sirius-red staining.

### Echocardiographic analysis

M-mode, 2D, and 3D data sets as well as pw-Doppler and tissue-Doppler recordings were acquired and analysed to assess wall and chamber dimensions, LV mass, systolic and diastolic LV function. Myocardial regional blood volume (rBV) was assessed by contrast perfusion imaging.

### Calcineurin activity assay

Calcineurin activity was determined by using a commercial kit.

### Gene and protein expression analysis

Gene expression was analysed by real-time PCR using the SYBR Green PCR Master Mix. Primer sequences are given in Supplemental material online, Table.
Flow cytometry
Circulating number of progenitor and CD26+ cells was determined by FACS analysis.

Determination of cytokine levels
Circulating cytokines were determined by using commercial ELISA kits.

In vivo inhibition of vascular endothelial growth factor
Vascular endothelial growth factor was blocked by using recombinant sFlt-1, a specific inhibitor of the Flt-1 VEGF receptor.

Statistical analysis
Values are expressed as mean ± SEM. Comparison among groups was performed by one-way ANOVA along with post hoc Tukey's test. A level of $P < 0.05$ was accepted as statistically significant. Analyses were performed using GraphPad Prism version 4.0.

Results
Renal injury is accompanied by increased heart hypertrophy and capillary deficit
As shown in Table 1, 5/6 nephrectomy resulted in decreased renal function and increased blood pressure when compared with sham-operated rats. Moreover, on postoperative Day 14, in Nx rats, the heart-to-body weight ratio increased by 45% when compared with Sham animals (0.48 ± 0.015 vs. 0.33 ± 0.007, respectively), whereas the number of capillaries/cardiomyocyte decreased by 25% (3.1 ± 0.08 vs. 2.3 ± 0.08, respectively) (Figure 1).

Effects of calcineurin inhibitor therapy on renal function, blood pressure, and histological markers of heart remodelling
To evaluate the effects of calcineurin inhibition on renal disease, Nx rats were treated with low therapeutic doses of CNI (CsA 5.0 and 7.5 mg/kg, and Tac 0.5 mg/kg daily), vehicle, or an antihypertensive drug (hydralazine) for 14 days starting on the day of surgery. As shown in Table 1, treatment with CNI affected neither the renal function nor the blood pressure, but attenuated ventricular hypertrophy and improved capillary density as measured by means of histological analysis (Figure 1). In addition, upon CNI treatment, the Nx rats demonstrated reduced collagen deposition and fibrosis when compared with vehicle-treated animals. There was no evidence of fibrosis in sham-operated animals (Figure 2). Hydralazine-treatment maintained blood pressure at the control level (Table 1); however, heart remodelling was still present (Figure 1).

![Figure 1](https://academic.oup.com/eurheartj/article-abstract/32/15/1935/564492)

**Figure 1** Effect of kidney injury and calcineurin inhibitor therapy on heart hypertrophy and capillary density. (A) Representative micrographs showing increased ventricular and cardiomyocyte hypertrophy. (B) Graphical representation of heart hypertrophy given in percentage of heart/body weight. (C) Graphical representation of capillary density analysis. Magnification: ×100. Sham: sham animals; 5/6 Nx: nephrectomy; CNI: calcineurin inhibitors; CsA: cyclosporine A 5 or 7.5 mg/kg/day; Tac: tacrolimus 0.5 mg/kg/day; Hydra: hydralazine 20 mg/kg/twice a day. Results are mean ± SEM. *$P < 0.05$ compared with sham; **$P < 0.05$ compared with vehicle.
Evaluation of renal histology 14 and 30 days after surgery/treatment confirmed that low doses of CNI did not aggravate renal damage induced by 5/6 nephrectomy. Thus, one can exclude additional nephrotoxicity by application of low dose CNI in this model (Supplementary material online, Figures S1 and S2).

**Effects of calcineurin inhibitor therapy on left ventricular hypertrophy, blood volume, and cardiac function: in vivo assessment**

To determine the in vivo preservation of the cardiac/left ventricular function in Nx rats by CNI treatment, echocardiographic analysis was performed in all experimental animals. As expected, Nx rats presented with increased LV mass and interventricular septum thickness indicating LV hypertrophy, while these parameters were lower in all Nx animals undergoing CNI treatment, but not hydralazine treatment (Figure 3A and B). Contrast echocardiography demonstrated significant differences in rBV as assessed by contrast replenishment (Figure 3C and D). Homogeneous filling of the LV microvasculature was demonstrated in sham animals, while Nx animals demonstrated only patchy perfusion signals even at the highest triggering interval (1:20 cardiac cycles), indicating decreased rBV at rest. Even after administering bolus injections of contrast agent with severe acoustic shadowing within the LV cavity, echo-contrast did not adequately fill in the antero-lateral myocardium (data not shown). Calcineurin inhibitor therapy significantly improved rBV in Nx rats, while hydralazine treatment had only a slight but not significant effect (Figure 3C and D).

When measuring heart function, we assessed a comparable systolic LV function among groups, whereas diastolic function was significantly altered (Figure 3E and F). In detail, E/E′ was very high in Nx rats treated with vehicle or hydralazine, while significant lower values were found in rats treated with CNI.

**Figure 2** Effect of kidney injury and calcineurin inhibitor therapy on myocardial fibrosis (picro-sirius red staining). (A) Representative images of myocardial fibrosis (dark red staining). Magnification: ×10. (B) Myocardial fibrosis area was quantified by ImageJ software. Sham: sham animals; 5/6 Nx: nephrectomy; CNI: calcineurin inhibitors; CsA: cyclosporine A 5 or 7.5 mg/kg/day; Tac: tacrolimus 0.5 mg/kg/day; Hydra: hydralazine 20 mg/kg/twice a day. Results are mean ± SEM. *P < 0.05 compared with sham; #P < 0.05 compared with vehicle.
Extending the treatment period to 30 days after 5/6 nephrectomy, we confirmed the results, e.g. improvement in heart remodeling and function, obtained on Day 14 (Supplementary material online, Figure S3).

Activation of calcineurin after renal injury

In the next step, we aimed to identify possible mechanisms responsible for decreased heart remodelling in these animals. Activation of calcineurin pathway was evidenced by an eight-fold increase in...
the mRNA expression and a 50% increase in the phosphatase activity of calcineurin in the hearts of Nx rats when compared with sham-operated animals. Calcineurin activity was not affected by treatment with hydralazine, but was reduced by 80% by treatment with CsA 5 mg/kg/day (Figure 4).

Effects of CNI therapy on heart gene expression

The activation of calcineurin has been reported to upregulate the expression of hypertrophic genes, while cyclosporine use is associated to ischaemia-induced progenitor cell mobilization and angiogenesis. Therefore, expression analysis of genes involved in pathological hypertrophy (foetal, extracellular matrix- and inflammation-related genes) as well as in the preservation of vascularization (angiogenic and stem cell-related genes) was conducted.

After 5/6 nephrectomy, the expression of foetal genes, such as atrial and brain natriuretic peptides and alpha actin, as well as extracellular matrix/fibrosis related genes (fibronectin and inhibitor of metalloproteinase type 1) was upregulated. Treatment with CNI avoided an increase in the expression levels of these genes (Figure 5A and B). Expression pattern of inflammatory genes was also altered in the hearts of Nx rats. However, this profile was not substantially influenced by CNI treatment (Supplementary material online, Figure S4).

Calcineurin inhibitor treatment modulates blood cytokine levels and increases the number of stem/progenitor cells

Since stem cell-related genes were upregulated under CNI treatment and a decreased number of circulating progenitor cells may also contribute to impaired angiogenesis in CKD, we were tempted to evaluate the effect of CNI treatment on circulating stem/progenitor cell number and associated cytokines.

Progenitor cells were defined by the surface expression of stem cell antigen-1 (Sca-1) and c-Kit antigens, while Sca-1+ cells were further analysed for the expression of CD31, a marker of endothelial differentiation of progenitor cells. Fourteen days after surgery, the number of peripheral white blood cells did not differ among groups (Figure 6A), while Nx rats displayed a significant decrease in the number of circulating Sca-1+/cKit+ cells and a slight decrease in the number of Sca-1+/CD31+ cells when compared with sham rats. The number of progenitor cells was re-established after CNI treatment (Figure 6B and C).

To investigate putative mechanisms of increased progenitor cell mobilization, we measured blood levels of stromal cell-derived factor 1 (SDF-1), SCF, and VEGF. Both SDF-1 and SCF increased after CNI treatment (Figure 6E and F), while no differences in VEGF levels were observed among the different groups (data not shown).

CD26 (dipeptidylpeptidase IV) is responsible for SDF-1 cleavage and inactivation. In this, the number of circulating CD26+ cells lowered in the group undergoing CNI treatment (Figure 6D), in compliance with the increase in SDF-1 levels and progenitor cell mobilization.

Effects of vascular endothelial growth factor blockade on heart remodelling and function in 5/6 nephrectomized rats treated with calcineurin inhibitor

Following the observation that CNI induces angiogenesis in Nx rats by activating the VEGF pathway, including the mobilization and homing of progenitor cells, we tested the hypothesis that VEGF blockade would impair CNI-related effects on heart vascularization. Vascular endothelial growth factor pathway was inhibited in vivo by sFlt-1, which suppresses VEGF activity by sequestering VEGF as well as by functioning as a dominant-negative inhibitor of VEGF receptors.
Vascular endothelial growth factor inhibition abolished the beneficial effect of CNI on heart vascularization (Figure 7A and B), but not on cardiac hypertrophy (Figure 7C). These data are corroborated by the fact that, in comparison to rats receiving CsA only, rats receiving CsA and sFlt-1 still present decreased expression of hypertrophic genes, but impaired upregulation of angiogenic genes (Supplementary material online, Figure S6). Moreover, sFlt-1-treated rats also presented decreased number of circulating Sca-1⁺/cKit⁺ and Sca-1⁺/CD31⁺ cells, even though the number of CD26⁺ cells remained unaltered (Supplementary material online, Figure S7).

The amelioration in the diastolic heart function of CsA-treated animals was only partially affected by VEGF blockade, evidencing the differential effect of CNI on heart remodelling (vascularization vs. hypertrophy) (Figure 7D).

These findings demonstrate that CNI enhances angiogenesis in Nx animal via a VEGF-dependent mechanism, while decreased hypertrophy is directly associated to calcineurin inhibition.

**Discussion**

Chronic kidney disease is associated with an increased risk of cardiovascular morbidity and mortality, and uremic patients regularly present marked remodelling of the heart, including hypertrophy, fibrosis, and capillary rarefaction. In turn, CsA and tacrolimus have been shown to avoid hypertrophy and fibrosis formation in different animal models of hypertrophy. Moreover, CsA has been described to increase VEGF expression and to induce angiogenesis. Thus, we hypothesized that inhibition of calcineurin-dependent pathways might also serve as a treatment option for the prevention of heart remodelling in renal disease.

After 5/6 nephrectomy, cardiac injury and cardiac calcineurin activation were observed, indicating that calcineurin pathway may play a role in renal injury-related heart remodelling. Interestingly, low doses of CNI prevent the development of LV hypertrophy and myocardial fibrosis in Nx rats without affecting renal function and blood pressure. In addition, treatment with CNI improved the number of capillaries/cardiomyocyte and rBV. Calcineurin inhibitor application not only prevents cardiac remodelling, but also ameliorates cardiac function.

Left ventricular hypertrophy is a well-known feature in renal failure and a potent predictor of death in uremic patients. Activation of calcineurin-dependent pathways is involved in the development of cardiac hypertrophy, suggesting that these pathways are putative pharmaco-therapeutic targets in the treatment of...
dysfunctional cardiac hypertrophy. After 5/6 nephrectomy, increased expression of genes associated with hypertrophy (such as foetal genes) was inhibited by CsA and tacrolimus, confirming the involvement of calcineurin in renal disease-associated heart hypertrophy (Figure 8). Blood pressure normalization through hydralazine application did not prevent heart remodelling and diastolic dysfunction, confirming that the effects observed are rather mediated by a kidney injury-specific mechanism independent of pressure-overload.

It should be noted that even though the cardiac expression of inflammatory genes was regulated to some extent after 5/6 nephrectomy, CNI treatment did not significantly interfere with this expression. Therefore, amelioration of local inflammation by CNI does not seem to play a major role in their cardioprotective capacity.

Structural cardiac changes are paralleled by functional changes in patients with CKD. Left ventricular dysfunction in these patients is principally noted as abnormal myocardial diastolic rather than systolic function. Correlates of abnormal function are, among others, hypertension and LV mass. In our current study, CNI treatment in rats with kidney injury did not develop significant cardiac hypertrophy, fibrosis, and capillary deficit. In line, they have improved diastolic function, confirming the protective effects of the therapy. This is in agreement with the findings of other groups showing a protective effect of CNI, especially CsA, on cardiac function.

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Figure 6 Effect of kidney injury and calcineurin inhibitor therapy on circulating stem/progenitor cells and serum cytokines. (A) Total number of peripheral white blood cells (WBC). Progenitor cells were defined by the surface expression of stem cell antigen-1 (Sca-1) and c-Kit antigens (B), while Sca-1+ cells were further analysed for the expression of CD31 (C), a marker of endothelial differentiation of progenitor cells. (D) The number of circulating CD26+ cells was also enumerated in peripheral blood 14 days after surgery/treatment. (E and F) Concentrations of stromal cell-derived factor-1 (SDF-1) and stem cell factor (SCF), respectively, were determined in the serum of all animals. Sham: sham animals; 5/6 Nx: nephrectomy; CNI: calcineurin inhibitor (cyclosporine A 5 mg/kg/day). Results are mean ± SEM.
Impaired angiogenesis, i.e. decreased pro-angiogenic response, was identified to participate critically in ventricular remodelling, heart dysfunction, and subsequent heart failure. Here we show that CNI therapy significantly increased the number of capillaries/cardiomyocyte, thus improving rBV in Nx rats. Angiogenesis is triggered by hypoxia inducing the expression of different growth factors such as VEGF and its receptors. During cardiomyocyte hypertrophy in uraemia, capillary growth does not keep pace with cardiomyocyte growth, exposing these cells to a hypoxic/ischaemic milieu. In this context, one might expect upregulation of angiogenic genes and homing of stem/progenitor cells. We and others have demonstrated normal and increased expression of growth factors, including VEGF, in the heart of Nx rats when compared with controls. However, the increase in VEGF expression was not associated with an appropriate angiogenic response, suggesting that the cardiac angiogenesis after kidney injury may be at least in part due to resistance at the receptor level or a signalling defect.

Cyclosporine is able to increase VEGF expression, to potentiate ischaemia-induced progenitor cell mobilization and homing, and to stimulate neoangiogenesis. The increase in cardiac pro-angiogenic factors in CNI-treated Nx rats might therefore restore the ischaemia-induced angiogenesis (Figure 8).

Recently, it has been shown that circulating progenitor cells (EPC) are reduced in CKD patients, and it has been suggested that altered EPC function contributes to the high cardiovascular risk of CKD patients. After 5/6 nephrectomy, we detected high expression levels of stem/progenitor cell markers in the sites of injury/new blood vessels. Consistent with these results, levels of circulating Sca-1+/cKit+ cells as well as blood levels of SDF-1 and SCF increase in CNI-treated Nx rats. Both factors are chemoattractant molecules for stem and progenitor cell populations. They are considered to be important components involved in migration, homing, and mobilization of these cells. The effect of CNI on progenitor cell mobilization was mainly attributed to changes in SDF-1 and decreased activity/availability of CD26, a membrane bound proteinase responsible for the cleavage and inactivation of SDF-1. Organ recipients receiving immunosuppressant drugs exhibit lower CD26 activity when compared with healthy individuals and that CsA treatment decreases the number of CD26+ cells in the peripheral blood of rats. Our results are in line with the findings of Wang et al., who showed that CsA induces neoangiogenesis and, thereby, improved perfusion in the hind-limb ischaemia model through mobilization and homing of progenitor cells (Figure 8).

Since the in vivo blockade of VEGF abrogated the CNI-related effects on heart vascularization, but not on cardiac hypertrophy, our data suggest that CNI enhances angiogenesis in Nx animals via a VEGF-dependent mechanism, while decreased hypertrophy is directly associated to calcineurin inhibition.
With regard to the safety of CNI treatment in renal patients, clinical experiences with low-to-moderate doses of CsA in patients with lupus nephritis, nephrotic syndrome, and even with renal transplantation suggest that the treatment is tolerable and safe. Experimentally, even in rats with reduced renal mass or spontaneously hypertension, long-term CsA treatment appears not to adversely affect blood pressure or to cause relevant nephrotoxicity. These findings support our data showing that both CsA and tacrolimus low-dose treatments were neither associated with renal impairment nor with increased blood pressure in Nx rats.

In conclusion, we show for the first time that calcineurin inhibition avoids kidney injury-related heart remodelling in an animal model. Moreover, this translates into improved cardiac function without affecting renal function and blood pressure. Our preclinical findings suggest that inhibition of calcineurin-dependent pathways might serve as a new therapeutic strategy for renal patients at high cardiovascular risk.

**Supplementary material**

Supplementary material is available at European Heart Journal online.

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**Conflict of interest:** none declared.

**References**

Cardioprotection through calcineurin inhibition in renal disease


