HNF4alpha Dysfunction as a Molecular Rational for Cyclosporine Induced Hypertension

Monika Niehof1, Jürgen Borlak1,2*

1 Center of Molecular Medicine and Medical Biotechnology, Fraunhofer Institute for Toxicology and Experimental Medicine, Hannover, Germany, 2 Center of Pharmacology and Toxicology, Medical School of Hannover, Hannover, Germany

Abstract

Induction of tolerance against grafted organs is achieved by the immunosuppressive agent cyclosporine, a prominent member of the calcineurin inhibitors. Unfortunately, its lifetime use is associated with hypertension and nephrotoxicity. Several mechanism for cyclosporine induced hypertension have been proposed, i.e. activation of the sympathetic nervous system, endothelin-mediated systemic vasoconstriction, impaired vasodilatation secondary to reduction in prostaglandin and nitric oxide, altered cytosolic calcium translocation, and activation of the renin-angiotensin system (RAS). In this regard the molecular basis for undue RAS activation and an increased signaling of the vasoactive oligopeptide angiotensin II (AngII) remain elusive. Notably, angiotensinogen (AGT) is the precursor of AngII and transcriptional regulation of AGT is controlled by the hepatic nuclear factor HNF4alpha. To better understand the molecular events associated with cyclosporine induced hypertension, we investigated the effect of cyclosporine on HNF4alpha expression and activity and searched for novel HNF4alpha target genes among members of the RAS cascade. Using bioinformatic algorithm and EMSA bandshift assays we identified angiotensin II receptor type 1 (AGTR1), angiotensin I converting enzyme (ACE), and angiotensin I converting enzyme 2 (ACE2) as genes targeted by HNF4alpha. Notably, cyclosporine represses HNF4alpha gene and protein expression and its DNA-binding activity at consensus sequences to AGT, AGTR1, ACE, and ACE2. Consequently, the gene expression of AGT, AGTR1, and ACE2 was significantly reduced as evidenced by quantitative real-time RT-PCR. While RAS is composed of a sophisticated interplay between multiple factors we propose a decrease of ACE2 to enforce AngII signaling via AGTR1 to ultimately result in vasoconstriction and hypertension. Taken collectively we demonstrate cyclosporine to repress HNF4alpha activity through calcineurin inhibitor mediated inhibition of nuclear factor of activation of T-cells (NFAT) which in turn represses HNF4alpha that leads to a disturbed balance of RAS.

Introduction

Cyclosporine is a potent immunosuppressive agent and widely used in transplantation medicine and in the treatment of several autoimmune diseases. However, it is known for a long time that its clinical application is confounded by unwanted secondary effects, notably new-onset diabetes, renal dysfunction, renal vascular damage and arterial hypertension [1–4]. A systematic review of cyclosporine’s effects on blood pressure was recently reported [5]. There is definitive proof for cyclosporine to increase blood pressure in a dose-related fashion and was associated with an increased risk of stroke, myocardial infarction and heart failure. Likewise, cyclosporine-induced hypertension was observed in various animal models in vivo, e.g. in mouse [6], rats [7–12], dogs [13,14], sheep [15], and primates [8,16]. Several mechanism, including activation of the sympathetic nervous system, endothelin-mediated systemic vasoconstriction, impaired vasodilatation secondary to reduction in prostaglandin and nitric oxide, altered cytosolic calcium translocation, and activation of the renin-angiotensin system (RAS) have been proposed to underlie cyclosporine-induced hypertension [17–19]. Notably, the RAS system is a coordinated hormonal cascade playing a role in the regulation of blood pressure with the peptide angiotensin II (AngII) as principle effector. Cyclosporine was reported to elevate RAS components in transplant patients, e.g. plasma renin activity [20–22], AngII levels [20–22], angiotensin converting enzyme (ACE) activity [23,24], or angiotensin receptors (AGTR1) [25–28], even though the effects of cyclosporine on RAS in man are to some extent contradictory, since normal or even lower plasma renin activity had been reported as well [18,29–31]. However, the lack of increase in plasma renin activity in some clinical studies does not exclude activation of tissue RAS, which plays additional important functions but is not necessarily seen as a change in plasma renin activity [31–33]. Furthermore, cyclosporine also exerts structural nephrotoxicity which may further increase plasma renin activity [18,25,34]. Thus, RAS activation may be both a cause and a consequence of cyclosporine-induced renal damage [18]. Nevertheless, cyclosporine induced blood pressure changes occur prior to renal damage [18]. Diverse antihypertensive drugs are available to treat high blood pressure and clinical trials evidenced the benefit of inhibitors of RAS, i.e. ACE inhibitors and AGTR blockers for the prevention of cardiovascular diseases in the general population [35] as well as in transplant recipients [36]. While several mechanisms including RAS activation had been
discussed as possible cause for cyclosporine induced hypertension, a detailed molecular rational has not been proposed as yet.

Recently, we proposed cyclosporine to repress HNF4α/HNF1α and subsequent regulation of genes coding for glucose metabolism and of pancreatic beta-cell function as a molecular rational for posttransplantation diabetes mellitus, which is an other acknowledged complication in calcineurin inhibitor immunosuppressive therapies [37]. HNF4α is a master regulatory protein in liver biology and an important transcription factor in angiotensinogen (AGT) gene regulation [38]. Specifically, AGT is synthesized in the liver and secreted into the circulation [39]. It is the substrate of renin, the rate-limiting enzyme of the RAS enzymatic cascade that generates the decapeptide angiotensin I (AngI), which then becomes further processed to the functional vasoactive octapeptide AngII by activity of the angiotensin I converting enzyme (ACE). It is known that variation in AGT transcription influences control of blood pressure [38]. Here, we studied the effect of cyclosporine on HNF4α and various RAS components to better understand cyclosporine induced hypertension and proposed HNF4α dysfunction and subsequent regulation of AGTR1, ACE, and ACE2 as a molecular rational.

Materials and Methods

Cell culture experiments

The human hepatoma cell line HepG2 was obtained from LGC-ATCC Standards (Wesel, Germany). In a dose-range finding study concentrations of up to 30 µg/ml were tested for cell viability with the MTS assay (CellTiter 96® Aqueous One Solution Cell Proliferation Assay, Promega, Mannheim, Germany). Thus, HepG2 cells were treated daily with 10 µg/ml cyclosporine (Sandimmun, Novartis, Nuernberg, Germany) for 72 h that represents 8 to 10 times of the Cmax seen in patients [40]. Treatment started at 40-50% confluence. Additionally, HepG2 cells were treated with a combination of cyclosporine and captopril at 10 µg/ml for 72 h, or alternatively for 4 h with captopril alone after treatment with cyclosporine for 72 h.

Additionally, experiments were carried out with primary human hepatocytes. Notably, liver specimens were obtained from patients undergoing hepatic resections at the Medical School of Hannover, Germany. Written approval for the use of human liver specimens was considered significant when the p value was less than 0.05. Statistical analysis was performed using the non-parametric two-tailed Mann-Whitney-U-Test. Therefore, the antigen-antibody complexes were visualized using the enhanced chemiluminescence (ECL) detection system (PerkinElmer Life Sciences, Rodgau-Jugdesh, Germany). Light signal detection was done with the CCD camera Imager system Kodak IS 440 CF (Kodak, Biostep GmbH, Jahnusdorf, Germany)

Quantitative real-time RT-PCR

Quantitative real-time RT-PCR measurement was done with the Lightcycler (Roche Diagnostics, Mannheim, Germany) with the following conditions: denaturation at 95°C, annealing at different temperatures for 8 sec, elongation at 72°C for different times and detection of SYBR-Green I-fluorescence at different temperatures. Detailed primer specific conditions and oligonucleotide sequence information are given in Table 2. Relative quantification was performed using the "Fit Points Method" of the LightCycler3 Data Analysis Software version 3.5.28 (Roche Diagnostics, Mannheim, Germany) by comparing the sample values to a standard curve within the linear range of amplification. The standardized sample values for each gene of interest were divided by the standardized values of the housekeeping gene (mitATPase), which was found to be stably expressed.

Statistical analysis

Experiments are performed at least in triplicate. All values are expressed as mean ± standard deviation. To determine significance between two groups, comparison was made using the non-parametric two-tailed Mann-Whitney-U-Test. Therefore, Statistics software, version 9 (StatSoft) was used. The results are considered significant when the p value was less than 0.05.

Results

Gene expression of members of the renin-angiotensin system after cyclosporine treatment

Angiotensinogen, a member of the renin-angiotensin system (RAS), is mainly synthesized in the liver [30]. We therefore used the HepG2 human hepatoma cell line as model system. Initially, we studied expression of HNF4α and of members of the RAS

Table 1. Shift-probe sequences.

| Gene     | Oligo name | Accession number | Sequence          |
|----------|------------|------------------|-------------------|
| HNF4α    | HNF1       | NM_000545        | AGGGCTGAAAGTCCAAAGTCTCCTC |
| AGT      | AGT        | NM_000299        | TGCAGAGGGCAGAGGGCAGGGGA |
| AGTR1    | AGTR1      | NM_000685        | TAAGGAGGCGAAGTCTAGTGA |
| ACE      | ACE1       | NM_000789        | TGGCGCTACCAAGTCTAGAGA |
| ACE2     | ACE2(a)    | NM_021804        | AGCTATGATCTTGTCTGCTAGCA |
| ACE2     | ACE2(b)    | NM_021804        | CCGGAGGATGATCTTGTCTAGCTTTC |

doi:10.1371/journal.pone.0016319.t001
cascade in cyclosporine treated HepG2 cells. By quantitative real-time RT-PCR we found HNF4α, angiotensinogen (AGT), angiotensin receptor 1 (AGTR1), and angiotensin converting enzyme 2 (ACE2) to be significantly repressed after treatment with 10 μg/ml cyclosporine for 72 h (Fig. 1A). Expression of renin and angiotensin converting enzyme (ACE) was not detected in HepG2 cells.

Furthermore, at least five splice variants have been identified for the AGTR1 protein, but only exon 5 encodes the open reading frame [43,44]. Therefore, we analyzed AGTR1 transcript splice variants and evidenced expression of AGTR1 for exon 2 [ACE2] to be significantly repressed after treatment with 10 μg/ml cyclosporine and after treatment with cyclosporine for 72 h (Fig. 1A). Expression of renin and angiotensin converting enzyme (ACE) was not detected in HepG2 cells.

Addition of a specific HNF4α antibody shifted the bands, therefore specific for HNF4α binding-sites in proximal promoter regions of further members of the renin-angiotensin system. Essentially, the transcription start site (TSS, +1) of the NCBI mRNA reference sequence (RefSeq) was aligned using the UCSC Genome Browser (http://genome.ucsc.edu/) for promoter annotation of the respective genes. Proximal promoters (up to −1000 bp) of human ACE and ACE2, and intronic regions of further members of the renin-angiotensin system. Locating binding-sites of HNF4α was confirmed by EMSA bandshift assays using an antibody specific for HNF4α. We observed strong binding of nuclear extracts of untreated cell cultures to all predicted sites (Fig. 2A). Addition of a specific HNF4α antibody shifted the bands, therefore providing clear evidence for the specificity and selectivity of the assay. Strikingly, cyclosporin significantly reduced binding of HNF4α to all EMSA probes employed to approximately 60% when compared to untreated cell cultures (Fig. 2B).

Cyclosporin inhibits protein expression of HNF4α
HNF4α protein expression was significantly repressed after treatment of HepG2 cells with 10 μg/ml cyclosporine for 72 h (Fig. 3A). For comparison Western blotting of actin was used as housekeeping protein and to control loading onto the gel (Fig. 3A, B). Protein expression of AGT and AGTR1 remained unchanged after treatment with cyclosporine (Fig. 3C).

Discussion
Cyclosporine had been introduced for more then 20 years and represents a major breakthrough in transplantation medicine. Unfortunately, treatment of patients with calcineurin inhibitors results in unwanted side effects, notably hypertension and nephrotoxicity [1–3,5]. Although these adverse effects are already

Table 2. Real-Time PCR primer sequences and amplification settings.

| Gene       | Accession number | Primer sequence | Fragment length | Annealing | Elongation | Fluorescence |
|------------|------------------|-----------------|-----------------|-----------|------------|-------------|
| HNF4α      | NM_000457        | fwd: CTGCTGGAGCCACAAAGAGATCCATG  
               |                  | rev: ATCACCCTGAGCGTGGTCGCA     | 371bp     | 60°C       | 15sec       | 88°C        |
| AGT        | NM_000029        | fwd: GGATGAGAGAGAGAGCCACACG   
               |                  | rev: CTGCTGGAGCCACAAAGAGATCCATG | 351bp     | 68°C       | 15sec       | 90°C        |
| ACE        | NM_152830        | fwd: ATGTAATGACGAGCGAACTCG   
               |                  | rev: CCAGGGAGGTGAAGAAATCA      | 342bp     | 64°C       | 14sec       | 88°C        |
| ACE2       | NM_021804        | fwd: GATCCCCAGTCTACAGAGGA   
               |                  | rev: GCCAGGAAGGACTTGACATC      | 228bp     | 60°C       | 20sec       | 82°C        |
| AGTR1      | NM_000685        | Exon2/Exon5: CTGATGCCATCCCAGAAAGT 
               |                  | rev: CTGATGCCATCCCAGAAAGT       | 144bp     | 60°C       | 20sec       | 78°C        |
|            |                  | Exon5/Exon5: CAAGACAAGACAAAAGCACA  
               |                  | rev: CAAGACAAGACAAAAGCACA       | 136bp     | 60°C       | 20sec       | 78°C        |
| mitATPase  | NC_012920        | fwd: CTGAAGAGACAGACCTGA     
               |                  | rev: TGGCCTGCAGTAATG          | 315bp     | 55°C       | 13sec       | 83°C        |

doi:10.1371/journal.pone.0016319.t002
Figure 1. Regulation of gene expression in HepG2 cells after cyclosporine treatment. A. Gene expression was measured by quantitative real-time RT-PCR in HepG2 cells 72 h after treatment with 10 μg/ml cyclosporine (n = 3, respectively) and was determined relative to expression of mitATPase6, which served as a housekeeping gene. Gene expression in untreated HepG2 cells was set to 100 and values for cyclosporine treatment represent transcript abundance relative to control. Non-parametric Mann-Whitney-U-Test was used to compare cyclosporine treated and control.
known for a long time and several mechanism had been explored, the molecular basis for cyclosporine induced hypertension remains elusive.

Specifically, cyclosporine binds to the cyclophilin receptor. This drug–receptor complex inhibits activation of the calcineurin phosphatase, a secondary messenger in the dephosphorylation and activation of nuclear factor of activation of T-cells (NFAT). NFAT is a DNA-binding protein that promotes transcription of interleukin-2 (IL-2) but inhibition of IL-2 production stops proliferation and activation of T-cells to mediate the immunosuppressive effect of cyclosporine [46]. Indeed, activation of the RAS system is one of the important mechanism involved in cyclosporine induced hypertension and represents a coordinated hormonal cascade of major importance in the control of cardiovascular and renal tonus [18]. Cyclosporine causes RAS activation by an increase in plasma renin and ACE activity with subsequent generation of elevated AngII levels [18,20–24,34]. Furthermore, long-term treatment with cyclosporine is associated with up-regulated AGTR1 receptors in vascular and renal tissues [25–28]. The exact molecular causes leading to RAS activation and AngII increase are unknown; its activation is probably a multifactorial process [18]. In addition to calcineurin inhibition, cyclosporine stimulates PDGF [47] and TGFβ [48,49] signaling that seems to be involved in mediating renin secretory effects of cyclosporine and it is well established that binding of AngII to AGTR1 leads to vasoconstriction and hypertension. Thus, an increase in blood pressure may contribute to renal damage and a subsequent increase in plasma renin activity [18]. This process may cause a vicious circle in RAS activation, which further perpetuates cyclosporine toxicity. However, cyclosporine also induces structural nephrotoxicity, which is independent of blood pressure changes [18,25,34]. Taken collectively, cyclosporine stimulates both, the systemic (circulating) RAS and the local (tissue) RAS components, which were identified in many organs [20]. In regards to the tissue RAS, AngII exerts further direct effects on cardiac myocytes contributing to hypertension and cardiac failure [50]. In this complex interplay HNF4α is one of the direct NFAT target genes [37,51]. Recently, we demonstrated recently its gene and protein expression to be significantly repressed in response to cyclosporine treatment [37]. AGT is known to be regulated by HNF4α [38] and we identified AGTR1 and ACE as further HNF4α target genes among RAS members. We applied position weight matrices to predict HNF4α consensus sites and confirmed the predictions experimentally by EMSA assays to determine its DNA binding activity. Thus, we demonstrate cyclosporine treatment of human hepatoma cells to significantly reduce DNA-binding of HNF4α to AGT and AGTR1 at gene specific promoters to cause impaired gene expression. As alternative splicing might be an important mechanism in the regulation of AGTR1 expression [44] we analyzed AGTR1 isoforms before and after cyclosporine treatment. Here, expression of splice variants was unchanged in HepG2 cells. Furthermore, we analyzed the consequences of inhibition of ACE in cyclosporine treated HepG2 cells. Captopril

### Table 3. Gene expression in HepG2 cells after treatment with captopril.

| Gene  | Treatment          | Transcript abundance (relative units, mean ± SD) | % of control |
|-------|--------------------|-----------------------------------------------|--------------|
|       |                    |                                               |              |
| HNF4α | Control            | 1.846 ± 0.127                                   | 100          |
|       | Cyclosporine       | 0.993 ± 0.024                                   | 53.8         |
|       | Cyclosporine + Captopril, 72h | 1.204 ± 0.138                               | 65.2         |
|       | Cyclosporine + Captopril, 4h  | 1.106 ± 0.053                                | 59.9         |
| AGT   | Control            | 0.186 ± 0.026                                   | 100          |
|       | Cyclosporine       | 0.071 ± 0.005                                   | 38.2         |
|       | Cyclosporine + Captopril, 72h | 0.078 ± 0.007                               | 41.9         |
|       | Cyclosporine + Captopril, 4h  | 0.073 ± 0.005                                | 39.2         |
| AGTR1 | Control            | 0.335 ± 0.185                                   | 100          |
|       | Cyclosporine       | 0.075 ± 0.010                                   | 22.4         |
|       | Cyclosporine + Captopril, 72h | 0.087 ± 0.022                               | 26.0         |
|       | Cyclosporine + Captopril, 4h  | 0.095 ± 0.006                                | 28.4         |

Gene expression was measured by quantitative real-time RT-PCR in HepG2 cells after treatment with 10 μg/ml cyclosporine (n = 3, respectively) for 72 h. Furthermore, cells were treated in parallel with 10 μg/ml ACE inhibitor captopril (cyclosporine + captopril, 72h), or with cyclosporine for 72 h and subsequently with 10 μg/ml captopril for 4 h (cyclosporine + captopril, 4h). Transcript abundance of HNF4α, AGT, and AGTR1 are determined relative to expression of mitATPase6, which served as a housekeeping gene. Data are given as relative units (mean ± SD). Furthermore, transcript abundance of HNF4α, AGT, and AGTR1 in untreated HepG2 cells was set to 100 and values for cyclosporine or cyclosporine/captopril treatment represent transcript abundance relative to the control.
did not influence gene expression of various RAS members in cyclosporine treated HepG2 cells. Hence, captopril selectively inhibits ACE enzymatic activity, but did not affect gene expression via feedback loops. However, RAS does not represent a linear cascade, but is composed of a sophisticated interplay between multiple mediators, receptors, and enzymes. The principal RAS

Figure 2. Cyclosporine inhibits binding of HNF4α to target genes. A. Electrophoretic mobility shift assays with 5 μg HepG2 cell nuclear extract (control or cyclosporine treatment, 10 μg/ml for 72 h, n = 3, respectively) and 32P labeled oligonucleotides to probe for DNA binding to HNF4α binding-sites within HNF1α (HNF1), angiotensinogen (AGT), angiotensin II receptor, type 1 (AGTR1), angiotensin I converting enzyme (ACE1), and angiotensin I converting enzyme 2 [ACE2(a), ACE2(b)] genes. In EMSA supershift assays an antibody directed against HNF4α (+) was added. Shifted (HNF4α s) and supershifted bands (HNF4α ss) were marked. B. Dried EMSA gels were analyzed with a Molecular Imager (BioRad, Muenchen, Germany) using the Quantity One software (BioRad, Muenchen, Germany). HNF4α binding of control extracts to the respective binding sites was set to 100% and inhibition of binding to the respective binding sites after treatment with cyclosporine (10 μg/ml for 72 h) was quantified. Non-parametric Mann-Whitney-U-Test was used to compare cyclosporine treated and control groups. Results are considered significant at p<0.05 (marked with an asterisk). doi:10.1371/journal.pone.0016319.g002
effector molecule AngII modulates positive and negative feedback loops, e.g. induction of AGT [52] and a decrease in AGTR1 [44]. Furthermore, AngII acts at an additional membrane receptor, AGTR2, which antagonizes AGTR1 induced signaling thereby mediating vasodilatation, antiproliferative and apoptotic effects [53]. Importantly, RAS can be envisioned as a dual function system, in which the vasoconstrictor/vasodilator actions are primarily driven by the ACE/ACE2 balance. Indeed, ACE2, which was termed the alternative axis of RAS, efficiently hydrolyses the octapeptide AngII to Ang(1-7), a peptide that exerts actions opposite to those of AngII [54]. Ang(1-7) has protective function; it binds to the G protein coupled receptor Mas, acts as a vasodilator and antagonizes AngII-induced vasoconstriction [54]. The impact of cyclosporine on ACE2 activity or expression was not analyzed so far. Bioinformatic prediction of HNF4α binding sites and subsequent EMSA assays identified ACE2 as a further HNF4α target gene, which was significantly repressed after cyclosporine treatment of HepG2 cells. As reported elsewhere a decrease in ACE2 leads unambiguously to enhanced AngII action on AGTR1 and caused vasoconstriction and hypertension [55]. Interestingly, ACE2 is markedly reduced in rat models of hypertension [56]. It has been proposed that some of the beneficial effects of currently used protective drugs as ACE inhibitors and AGTR1 blockers are beyond their direct effects on suppression of the ACE-AngII-AGTR1 axis as they also increased Ang(1-7) production through feedback mechanisms [54]. Consequently, ACE2 may represent a novel therapeutic target within the RAS system by enhancing AngII degradation. Indeed, a potential

Table 4. Predicted binding sites for HNF4α in different genes of the renin-angiotensin system.

| Gene name | Oligo name | Accession number | bp relative to TSS | Sequence | Matrix | Score core/matrix |
|-----------|------------|------------------|-------------------|----------|--------|------------------|
| AGTR1     | AGTR1      | NM_000685        | +39426 to +39439  | GAGGCCAAGTTCA | V$HNF4_Q6_01 | 1.000/1.000 |
| ACE       | ACE1       | NM_000789        | -4690 to -4668    | CTACCAAGGTCA  | V$HNF4_Q6_01 | 1.000/0.909 |
| ACE2      | ACE2(a)    | NM_021804        | -4380 to -4358    | ATGACTTTGCTCT | V$HNF4_Q6_01 | 1.000/0.969 |
| ACE2      | ACE2(b)    | NM_021804        | -6283 to -6261    | GTGACTTTGAATCT | V$HNF4_Q6_01 | 1.000/0.896 |

*TSS = transcription start site, NCBI GenBank Version Build 36.1 (hg18).

doi:10.1371/journal.pone.0016319.t004

Figure 3. Cyclosporine inhibits protein expression of HNF4α. A. HNF4α western blotting of 20 μg HepG2 cell nuclear extracts (upper panel) and actin western blotting (lower panel) of 10 μg HepG2 cell nuclear extracts [control or cyclosporine treatment, 10 μg/ml for 72 h, n = 3, respectively]. B. The graph panel represent the quantification of protein amounts for HNF4α relative to the actin expression. Non-parametric Mann-Whitney-U-Test was used to compare cyclosporine treated and control groups. Results are considered significant at p < 0.05 (marked with an asterisk). C. AGT, AGTR1, and actin western blotting of 30 μg, 20 μg and 15 μg total cellular extract, respectively, isolated from control or cyclosporine treated HepG2 cells (10 μg/ml for 72 h, n = 3, respectively).

doi:10.1371/journal.pone.0016319.g003

Figure 3. Cyclosporine inhibits protein expression of HNF4α. A. HNF4α western blotting of 20 μg HepG2 cell nuclear extracts (upper panel) and actin western blotting (lower panel) of 10 μg HepG2 cell nuclear extracts [control or cyclosporine treatment, 10 μg/ml for 72 h, n = 3, respectively]. B. The graph panel represent the quantification of protein amounts for HNF4α relative to the actin expression. Non-parametric Mann-Whitney-U-Test was used to compare cyclosporine treated and control groups. Results are considered significant at p < 0.05 (marked with an asterisk). C. AGT, AGTR1, and actin western blotting of 30 μg, 20 μg and 15 μg total cellular extract, respectively, isolated from control or cyclosporine treated HepG2 cells (10 μg/ml for 72 h, n = 3, respectively).
therapeutic benefit of direct increasing ACE2 activity in the treatment of hypertension was recently suggested [53]. In conclusion, we propose cyclosporine to cause dysfunction of HNF4α which in turn disturbs the balance of the renin-angiotensin-system. Most recently, Bai et al reviewed the genetic basis for adverse drug effects of calcineurin inhibitors and proposed that the development of type 2 diabetes, hypertension, and renal failure may be associated with specific DNA genotypes [57]. They conclude that disease-associated genes may provide genomic biomarkers for exploring adverse drug effects. As there are large individual differences in HNF4α expression amongst patients as recently reported by us [37], patients with low HNF4α activity might be at higher risk for developing cyclosporine induced hypertension.

Acknowledgments
We thank A. Holzmann, S. Marschke and A. Schulmeyer for valuable technical assistance.

Author Contributions
Conceived and designed the experiments: MN JB. Performed the experiments: MN JB. Analyzed the data: MN JB. Contributed reagents/materials/analysis tools: MN JB. Wrote the paper: MN JB.
45. Kel A, Reymann S, Matys V, Nettesheim P, Wingender E, et al. (2004) A novel computational approach for the prediction of networked transcription factors of aryl hydrocarbon-receptor-regulated genes. Mol Pharmacol 66: 1557–1572.
46. Li C, Lam SW, Sun BK, Yang CW (2004) Chronic cyclosporine nephotoxicity: new insights and preventive strategies. Yonsei Med J 45: 1004–1016.
47. Shehata M, el Nahas M, Barkworth E, Cope GH, Raftery AT (1995) Localization of PDGF-BB in the juxtaglomerular cells of cyclosporin-treated rats. Exp Nephrol 3: 173–179.
48. Pichler RH, Franceschini N, Young RA, Hugo G, Andoh TF, et al. (1995) Pathogenesis of cyclosporine nephropathy: roles of angiotensin II and osteopontin. J Am Soc Nephrol 6: 1186–1196.
49. Wolf G, Thaiss F, Stahl RA (1995) Cyclosporine stimulates expression of transforming growth factor-beta in renal cells. Possible mechanism of cyclosporines antiproliferative effects. Transplantation 60: 237–241.
50. Varagic J, Frohlich ED (2002) Local cardiac renin-angiotensin system: hypertension and cardiac failure. J Mol Cell Cardiol 34: 1435–1442.
51. Heit JJ, Apcyquist AA, Gu X, Winlow MM, Nelson JR, et al. (2006) Calcineurin/NFAT signalling regulates pancreatic beta-cell growth and function. Nature 443: 345–349.
52. Jamaluddin M, Meng T, Sun J, Boldogh I, Han Y, et al. (2000) Angiotensin II induces nuclear factor (NF)-kappaB1 isoforms to bind the angiotensinogen gene acute-phase response element: a stimulus-specific pathway for NF-kappaB activation. Mol Endocrinol 14: 99–113.
53. Porrello ER, Delbridge LM, Thomas WG (2009) The angiotensin II type 2 (AT2) receptor: an enigmatic seven transmembrane receptor. Front Biosci 14: 950–972.
54. Keidar S, Kaplan M, Gamliel-Lazarovich A (2007) ACE2 of the heart: From angiotensin I to angiotensin (1-7). Cardiovasc Res 73: 463–469.
55. Batlle D, Soler MJ, Wysocki J (2008) New aspects of the renin-angiotensin system: angiotensin-converting enzyme 2 - a potential target for treatment of hypertension and diabetic nephropathy. Curr Opin Nephrol Hypertens 17: 250–257.
56. Oudit GY, Crackower MA, Racks PH, Penninger JM (2003) The role of ACE2 in cardiovascular physiology. Trends Cardiovasc Med 13: 93–101.
57. Bai JP, Lesko LJ, Burckart GJ (2010) Understanding the genetic basis for adverse drug effects: the calcineurin inhibitors. Pharmacotherapy 30: 195–209.