Epidermal growth factor receptor inhibitor PKI-166 governs cardiovascular protection without beneficial effects on the kidney in hypertensive 5/6 nephrectomised rats


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a. Chronic EGFR inhibition prevents hypertension

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d. Non-standard Abbreviations:
ACE: Angiotensin converting enzyme
ACh: Acetylcholine
ADAM: A disintegrin and metalloprotease
AG1478: 4-(3’-Chloroanilino)-6,7-dimethoxyquinazoline
Ang II: Angiotensin II
AT₁R: Angiotensin II type 1 receptor
CKD: Chronic kidney disease
CV: Cardiovascular
DBP: Diastolic blood pressure
DMSO: Dimethyl sulfoxide
EDPVR: End diastolic pressure volume relationship
EGFR: Epidermal growth factor receptor
EGTA: Ethyleneglycol-bis-(b-aminoethylether) tetra-acetic acid
FGS: Focal glomerulosclerosis
GPCR: G-protein-coupled receptors
HB-EGF: Heparin binding EGF-like growth factor
HRP: Horseradish peroxidase
L-NAME: N (G)-nitro-L- arginine methyl ester
LV: Left ventricle
LVEDP: Left ventricle end-diastolic pressure
LVSP: Left ventricle systolic pressure
PBS: Phosphate buffered saline
PE: Phenylephrine
pEGFR: phosphorylated epidermal growth factor receptor
PKI-166: 4-[4-([(1R)-1-phenylethyl]amino]-7H-pyrrolo[2,3-d]pyrimidin-6-yl]phenol
RAS: Renin–angiotensin system
SBP: Systolic blood pressure
Tau: Isovolumetric relaxation time
U46619: (5Z)-7-[(1R,4S,5S,6R)-6-[(1E,3S)-3-Hydroxy-1-octenyl]-2-oxabicyclo[2.2.1]hept-5-yl]-5-heptenoic acid

α-SMA: α-smooth muscle actin

α₁-AR: α₁-adrenoceptor

5/6Nx: 5/6 nephrectomy

+\text{dP}/\text{dt}_{\text{max}}: \text{the maximal rate of increase in left ventricle pressure}

-\text{dP}/\text{dt}_{\text{max}}: \text{the maximal rate of decrease in left ventricle pressure}

e. \text{Recommended section: Cardiovascular}
Abstract

Transactivation of epidermal growth factor receptor (EGFR) signaling by G-protein-coupled receptors has been implicated in several cardiovascular (CV) conditions, including hypertension, heart failure, cardiac and vascular hypertrophy. However, the therapeutic potential of EGFR inhibition in these conditions is currently unknown. Main objective of the present study was to investigate cardiac, vascular and renal effects of EGFR inhibition by PKI-166 in the hypertensive chronic kidney disease (CKD) model. Rats underwent 5/6 nephrectomy (5/6Nx) and were treated with PKI-166 or lisinopril or vehicle from week 6 after disease induction until week 12. Sham animals received either PKI-166 or vehicle. Treatment with PKI-166 did not affect the development of the characteristic renal features in 5/6Nx including proteinuria, diminished creatinine clearance and increased glomerulosclerosis, whereas these were attenuated by lisinopril. Despite absence of effects on progressive renal damage, PKI-166 attenuated the progression of hypertension and maintained cardiac function (LVEDP) to a similar extent as lisinopril. Also, PKI-166 attenuated the increase in phosphorylated EGFR in heart as induced by 5/6Nx. Moreover, PKI-166 and lisinopril restored the impaired contraction of isolated thoracic aortic rings to phenylephrine and angiotensin II and impaired myogenic constriction of small mesenteric arteries in 5/6Nx rats. Blockade of the EGFR displays a CV benefit independently of limiting the progression of renal injury. Our findings extend the evidence on EGFR signaling as a target in CV disorders.
Introduction

A growing body of evidence demonstrates G-protein-coupled receptors (GPCRs) to be implicated in hypertension-associated oxidative stress, cardiac hypertrophy, vascular remodeling and endothelial dysfunction (Griendling et al., 2000; Asakura et al., 2002; Fortuno et al., 2005; Griol-Charhbili et al., 2011). Recently, it has been recognized that the transactivation of epidermal growth factor receptor (EGFR) by many different Gq/11 protein-coupled receptors may constitute an important part of their signaling (Fernandez-Patron, 2007). Therefore, instead of targeting various GPCRs, for example angiotensin II (Ang II) type 1 receptor (AT1R) or α1-adrenoceptor (α1-AR), it is conceivable that blockade of EGFR transactivation may have a significant potential in cardiovascular (CV) disorders (Supplemental Figure 1). EGFR transactivation has been implied in several CV conditions including hypertension (Fernandez-Patron, 2007), heart failure (Xu et al., 2009), cardiac (Kagiyama et al., 2002; Jaffre et al., 2009) and vascular hypertrophy (Ohtsu et al., 2006). Furthermore, it may also play a role in renal disease (Francois et al., 2004) and/or associated CV complications, the major cause of death in chronic kidney disease (CKD) (Parfrey and Foley, 1999). Hypertension represents the most frequent CV complication in CKD; it not only predicts mortality but also is a major determinant of progression of renal injury (Bakris et al., 2000). The mechanism of development of hypertension in CKD is complex and includes activation of the renin–angiotensin (RAS) (Gretz, 1995) and sympathetic nervous systems (Rump et al., 2000). Regardless of the origin of hypertension, the increased blood pressure leads to a progression of renal injury, thereby initiating a vicious circle (Johnson et al., 2002).
In recent years, a strong connection between hypertension and EGFR signaling has been demonstrated. First, increased expression level of EGFR relates to blood pressure in genetic (Swaminathan et al., 1996) and experimental (Northcott et al., 2001) models of hypertension. Further, EGF acts as a potent vasoconstrictor of arteries (Florian and Watts, 1999) and increased EGFR expression was found in hypertrophied left ventricle (LV) of SHR rats (Fujino et al., 1998). Also, receptor tyrosine kinase inhibitors such as EGFR tyrosine kinase inhibitor AG1478, and EGFR antisense oligonucleotides attenuate vasoconstriction and elevation of blood pressure in Ang II-induced hypertension (Kagiyama et al., 2002; Kagiyama et al., 2003).

EGFR transactivation also modulates vascular reactivity. For example, in heart failure, increased myogenic constriction of mesenteric artery is due to AT1R mediated transactivation of EGFR (Xu et al., 2009). Moreover, we recently showed that transactivation of the EGFR governs part of the α1-AR induced contraction of rat aorta (Ulu et al., 2010). A potential mechanism involved in EGFR transactivation is GPCR-induced shedding of heparin binding EGF-like growth factor (HB-EGF), which subsequently activates EGFR (Asakura et al., 2002). These findings collectively imply the possible therapeutic potential of EGFR inhibition in hypertension, as hypothesized previously (Fernandez-Patron, 2007; Beltowski and Lowicka, 2009).

To investigate whether EGFR inhibitors represent a novel class of drugs in renal disease associated CV complications, the effects of PKI-166 were investigated in 5/6 nephrectomised (5/6Nx) rats, by assessment of renal function and damage, blood pressure, cardiac parameters, vasoreactivity by measuring sensitivity to AT1R and α1-AR stimulation (aorta) and myogenic tone (mesenteric artery). The angiotensin converting...
enzyme (ACE) inhibitor (lisinopril; positive control) and vehicle groups were used as controls.
Methods

Animals

Experiments were performed on 12 weeks old male Wistar rats (n=60, 330 to 400 g, Harlan, Zeist, the Netherlands). Animals were housed under standard conditions of temperature (21-24°C), humidity (40-60%) and 12: 12 h light: dark cycle at the animal facilities of the University of Groningen. Animals had free access to food (standard rat chow; Hope Farms, Woerden, the Netherlands) and drinking water throughout the study. Animal experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Committee for Animal Experiments of the University Medical Centre Groningen.

Experimental Protocol

Before the induction of kidney damage in rats, blood samples and baseline measurements of systolic and diastolic blood pressures (SBP and DBP) were obtained by means of the tail-cuff method (PS-200A; Riken-Kaihatsu; Tokyo, Japan and IITC Life Sciences, Woodland Hills, CA, USA). Thereafter, rats underwent right nephrectomy and resection of two-thirds of the left kidney by ligation of 2-3 branches of the left renal artery (Vettoretti et al., 2006). Sham operated rats underwent the same procedure without the surgical reduction of kidney mass.

Six weeks after the operation, 5/6Nx rats were initiated on treatment with either vehicle (5/6Nx+Vehicle; n=12), EGFR kinase inhibitor (5/6Nx+PKI-166; n=10) or lisinopril (5/6Nx+Lisinopril; n=8) until week 12. Sham animals received either vehicle (Sham+Vehicle; n=10) or EGFR kinase inhibitor (Sham+PKI-166; n=12). Vehicle, PKI-
166 (50 mg/kg/day) and lisinopril (5 mg/kg/day) treatments were provided daily by oral 
gavages. PKI-166 was dissolved in 10% DMSO+0.5% Tween-80 diluted 1:20 (vol/vol) 
in water at a final concentration of 50 mg mL\(^{-1}\). Ten percent DMSO+0.5% Tween-80 
diluted 1:20 (vol/vol) was also added in the vehicle solution. During the treatment period, 
proteinuria and tail-cuff blood pressure was assessed every 3 weeks. As blood pressure 
levels stabilize around 6 weeks after 5/6Nx (Gschwend et al., 2002; Vettoretti et al., 
2006; Windt et al., 2008b), we initiated drug treatments at this time point, in order to 
alleviate effects of PKI-166 on development of the disease model.

At the end of the protocol, under brief anesthesia with 2.5% isoflurane, cardiac 
performance was measured by a pressure transducer catheter which was inserted through 
the right carotid artery (Micro-Tip 3-French; Millar Instruments Inc., Houston, Tex., 
USA). Heart rate, LV end-diastolic pressure (LVEDP), LV systolic pressure (LVSP), and 
the maximal rates of increase and decrease in LV pressure (+dP/dt\(_{\text{max}}\) and –dP/dt\(_{\text{max}}\) ) 
(Ulu et al., 2009) were recorded. Central systolic and diastolic blood pressures were 
measured after withdrawal of the catheter into the aortic root. Blood samples (2-3 mL) 
were collected from the abdominal aorta for biochemical analyses. Heart and kidneys 
were harvested for further analysis after confirmation of the absence of withdrawal reflex 
and tail pinch response for each animal prior to being euthanized. Thoracic aorta and 
third-order branches of superior mesenteric arteries were obtained and placed into ice-
cold Krebs solution.

**Proteinuria and blood pressure measurement**
Rats were placed in metabolic cages for 24 h and proteinuria was determined by trichloroacetic acid precipitation (Nephelometer Analyzer II; Dade Behring, Marburg, Germany).

Blood pressure was measured by means of the tail-cuff method (Vettoretti et al., 2006). In brief, animals were adapted to the procedure in a two weeks training period before the experimental protocol. For each animal, blood pressure values represent the mean of three to five recordings obtained in a single session.

**Biochemical analysis**

Plasma and urine creatinine were measured by means of a photometric assay with the Jaffé method without deproteinisation (DiaSys Diagnostic Systems, Holzheim, Germany) in the same week at the end of the protocol (i.e. 12 weeks after 5/6Nx) and creatinine clearance was calculated as (Urine Creatinine x Urine flow) / (Plasma Creatinine x Body Weight).

**Immunohistochemistry, morphometry and immunoblotting**

Immunostaining for α-smooth muscle actin (α-SMA) was performed on cryosections of the kidneys using anti-α-SMA antibody (Clone 1A4; Sigma, St. Louis, MO, USA) incubated for 1 h at 1:15000 dilution, followed by horseradish peroxidase (HRP)-conjugated rabbit-anti-mouse and subsequently HRP-conjugated goat-anti-rabbit antibodies. Positive cortical interstitial and glomerular α-SMA staining was measured by Aperio ImageScope software (version 9.1.772.1570, Aperio Technologies Inc, Vista, CA,
USA) at 200x magnification. Data from α-SMA immunostaining are presented as the intensity of the positive pixels.

Renal damage was assessed in paraffin embedded sections of kidneys stained with Periodic Acid Schiff reagent. The incidence of focal glomerulosclerosis (FGS) was microscopically evaluated semi-quantitatively by scoring of 50 glomeruli per slide on a scale of 0 to 4 by an examiner blinded for the groups as described previously (Van Dokkum et al., 1999). FGS score is presented as the sum of 50 glomeruli scores, thus ranging from 0 to 200.

Immunostaining for phosphorylated EGFR (pEGFR) was performed on frozen sections of the rat hearts using an anti-EGF receptor [pY1173] phosphospecific antibody (Invitrogen, Breda, the Netherlands). The antibody was diluted in PBS + 1% bovine serum albumin and applied for 1 h at room temperature. Peroxidase-labeled secondary antibodies with rat or human serum (1%) were used for sequential incubation. Peroxidase activity was developed using 3-amino-9-ethylcarbazole.

For immunoblotting, heart and kidneys samples were homogenized in 500 µL of ice-cold homogenization buffer (1% Igepal ca-630, 1% SDS, 5 mg mL\(^{-1}\) sodium deoxycholate, 1 mM sodium orthovanadate, 10 mM β-mercapto-ethanol, 40 µg mL\(^{-1}\) PMSF, 100 µg mL\(^{-1}\) benzamidine, 500 ng mL\(^{-1}\) pepstatin A, 500 ng mL\(^{-1}\) leupeptine and 500 ng mL\(^{-1}\) aprotinin in PBS) with Dispomix Homogenization (L&M Biotech, Cary, NC, USA). The homogenate was clarified by centrifugation at 2000 g for 10 min at 4 °C, the supernatant was collected and protein concentration was determined by using Qubit Protein Assay (Invitrogen, Carlsbad, CA, USA). Whole cardiac extracts were boiled in sample buffer for 3 min, separated by using 7% resolving gels and transferred electrically
to nitrocellulose membranes. Membranes were incubated overnight with the anti-EGFR or phosphospecific anti-EGFR antibodies (1:500 dilutions; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 4 ºC. The next day, membranes were washed with TBS/T-20 (0.05 M Tris, 150 mM NaCl and 0.04% Tween-20) and re-incubated with the secondary antibodies for 1 h at room temperature. Anti-GAPDH antibody (1:10000 dilutions; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was used to confirm equal loading conditions. Finally, blots were incubated with ECL Western Blotting reagent (Santa Cruz, CA, USA) for 1 min and exposed to X-ray film for 45-90 sec.

**Vascular reactivity of mesenteric arteries**

Third-order branches of superior mesenteric arteries were cleaned from perivascular tissue and transferred to an arteriograph system for pressurized arteries (Living System Instrumentation, Burlington, VT, USA) as described previously (Gschwend et al., 2002).

Intraluminal pressure was set at 80 mmHg and arteries were allowed to equilibrate for 40 minutes. Subsequently, smooth muscle and endothelium viability was checked by a single dose of phenylephrine (PE; 0.3 μM) and acetylcholine (ACh; 30 μM). Following wash out, intraluminal pressure was decreased to 20 mmHg and myogenic reactivity was studied by obtaining active pressure-diameter curves over a pressure range of 20-160 mmHg in steps of 20 mmHg. Each step was held for 5 minutes to reach stable contraction. Thereafter, Krebs solution was exchanged for calcium-free Krebs solution supplemented with ethyleneglycol-bis-(b-aminoethylether) tetra-acetic acid (EGTA, 2
mM) and passive pressure-diameter curves were obtained over the 20-160 mmHg pressure range.

**Endothelium-dependent relaxation of mesenteric arteries**

Following the determination of myogenic curves, intraluminal pressure was set to 80 mmHg, arteries were washed and stabilized for additional 20 minutes. Subsequently, arteries were pre-constricted with U46619 (30 nM) and endothelium-dependent relaxation was assessed by administering cumulative doses of ACh (1 nM –10 µM) to the recirculating bath.

**Contractility of thoracic aorta segments**

Aorta segments (approximately 2 mm) were cleaned from perivascular tissue and mounted in an organ bath with Krebs solution at 37 °C and continuously bubbled with 95% O₂ and 5% CO₂. Viability of smooth muscle cells was checked by pre-constriction with PE (1 µM). After wash out and 30 minutes of stabilization, contractility was measured in response to cumulative concentrations of Ang II (1 nM –1 µM) in endothelium-denuded rings. Finally, KCl (60 mM) was added to the organ baths. In additional endothelium-denuded rings, PE (1 nM –10 µM) mediated aorta contractility was also studied. Each experimental condition was studied in duplicate rings.

**Solutions and drugs**

The composition of Krebs solution was (in mM): NaCl (120.4), KCl (5.9), CaCl₂ (2.5), MgCl₂ (1.2), NaH₂PO₄ (1.2), glucose (11.5), NaHCO₃ (25.0) at pH 7.4. All
compounds for Krebs solution and all other drugs were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). PKI-166 was kindly provided by Dr. Giorgio Caravatti (Novartis Pharma AG, Basel, Switzerland). The dose of lisinopril was selected based on our previous study (Windt et al., 2008a) in which lisinopril at a dose 5 mg/kg/day was able to decrease blood pressure in exactly the same model of chronic kidney disease.

**Statistical analysis**

Data are expressed as mean ± SEM; n values represent the number of investigated rats. SPSS 17.0 for Windows (SPSS Inc., Chicago, Illinois, USA) was used for statistical analysis. Concentration-response and myogenic constriction curves were compared by ANOVA for repeated measures followed by Bonferroni *post hoc* test for multiple comparisons. Myogenic tone was expressed as the percentage decrease in active diameter from the maximally dilated (passive) diameter determined at the same pressure in calcium-free/EGTA solution, i.e., myogenic tone (\\%) = 100 \[1/(D_{Ca-free} - D_{Ca}) / D_{Ca-free}\], where D is the diameter in calcium-free (D_{Ca-free}) or calcium-containing (D_{Ca}) Krebs. Group comparison of animal parameters was performed by One-Way ANOVA followed by Dunnett’s or Bonferroni *post hoc* tests. Comparison of pEGFR expression was carried out with the Mann–Whitney *U*-test. Differences were considered significant at *P* < 0.05 (two-tailed).
Results

Animal characteristics and effects of 5/6Nx

Animal characteristics are presented in Table 1 and Table 2. Vehicle-treated 5/6Nx animals had increased kidney weight/body weight ratio, lower creatinine clearance (Table 1), and increased water intake and urinary output between week 5 and 11 (Table 2) compared to Sham. Body weights were similar in vehicle-treated Sham and 5/6Nx rats (Table 2). 5/6Nx induced a gradual increase in proteinuria in the first 5 weeks after the nephrectomy (Figure 1, *P*<0.05 vs. Sham groups), which continued to increase in vehicle-treated rats until week 12. At the end of the experimental protocol, 5/6Nx+Vehicle rats had higher FGS score (Figure 2C1 and F) and interstitial α-SMA staining (Figure 2C2 and G) compared to Sham (Figure 2A1 and A2). 5/6Nx induced a significant increase in systolic blood pressure (SBP) at 5 weeks after the operation. In vehicle-treated 5/6Nx rats, SBP continued to increase up to 8 weeks and remained stable afterwards (Figure 3A). Diastolic blood pressure (DBP) was slightly (but not significantly) higher in 5/6Nx animals at week 5, but increased significantly in vehicle-treated 5/6Nx rats afterwards (Figure 3B). Also, LV weight/body weight ratio, LVSP and LVEDP were increased in vehicle-treated 5/6Nx rats compared to Sham (Table 1). Together, these data demonstrate the successful induction of experimental CKD and related changes in CV parameters induced by 5/6Nx.

Above measurements were also obtained in Sham animals treated from week 6 to 12 with PKI-166. Importantly, no difference in any of the parameters was observed in Sham+PKI animals compared to vehicle treated Sham rats (Figures 1-3, Table 1 and 2).
The effects of PKI-166 on renal damage

Six weeks of treatment with PKI-166 between weeks 6 and 12 after 5/6Nx did not prevent higher water intake and increase in urine output (Table 2). Neither PKI-166 nor lisinopril was able to prevent renal hypertrophy (Table 1). Creatinine clearance was decreased in PKI-166-treated, similarly to vehicle-treated rats, and to a lesser extent in lisinopril-treated 5/6Nx rats (Table 1). Whereas PKI-166 did not affect the progression of proteinuria throughout the treatment period, lisinopril treatment prevented the increase in proteinuria (Figure 1). In line with proteinuria data, PKI-166 treatment did not influence both FGS score (Figure 2D1 and F) and interstitial α-SMA staining (Figure 2D2 and G). However, lisinopril treatment partially protected the kidneys from injury as evidenced by a lower FGS score (Figure 2E1 and F). Despite numerous attempts and adaptations in procedures, detection of the expression of pEGFR in kidney tissue with western blot was extremely hard. High loading with protein rendered only very low pEGFR signals, while detection of housekeeping genes was without problem. Moreover, pEGFR blots needed substantial amplification. In general, it seems that groups do not differ substantially in expression of pEGFR (Supplemental Figure 2). However, because of the difficulty to obtain adequate signals, we feel unqualified to draw finite conclusions on changes in pEGFR expression in kidney in the different groups. Collectively, these data demonstrate that PKI-166 treatment did not affect kidney injury after 5/6Nx, whereas lisinopril limited its progression.

The effects of PKI-166 on hypertension and cardiac function
Treatment with PKI-166 blunted the increase in SBP in 5/6Nx animals and completely restored DBP back to Sham levels, whereas lisinopril completely restored both (Figure 3A and B). Prior to being euthanized (i.e. 12 weeks after the induction of 5/6Nx), SBP and DBP was also measured under brief anesthesia by Millar catheter. In accord with the conscious tail-cuff blood pressure measurements, PKI-166 treatment significantly lowered SBP and DBP (Figure 3C and D). Lisinopril completely restored the increased SBP and DBP back to Sham levels at week 12 (Figure 3C and D).

Neither PKI-166 nor lisinopril significantly influenced the heart rate (Table 1). Whereas PKI-166 was ineffective in preventing the increase in LV weight, lisinopril treatment completely prevented it (Table 1). Nevertheless, increased LVSP in vehicle-treated 5/6Nx rats was attenuated by PKI-166 and lisinopril (Table 1). Remarkably, PKI-166 completely prevented the increase in LVEDP after 5/6Nx, as lisinopril did (Table 1).

To substantiate that PKI-166 inhibits phosphorylation of EGFR in 5/6Nx, immunoblottings for pEGFR and total EGFR (tEGFR) were performed in randomly selected heart samples from Sham, and vehicle, PKI-166 and lisinopril treated 5/6Nx rats (n=3-5 for each group). Twelve weeks after 5/6Nx, a marked increase in pEGFR protein was detected in vehicle treated 5/6Nx rats compared to Sham rats, which was inhibited by chronic PKI-166 and lisinopril treatment (Figure 4A and B). In contrast, tEGFR was similar among all groups (Figure 4A). To establish that the regulation of pEGFR as detected by western blot did not involve a difference in the localization of cardiac pEGFR, immunostaining for pEGFR was also performed in frozen heart sections. pEGFR protein was localized in the vascular structures of the heart, without staining of cardiomyocytes (Figure 4C, D, E, F and G).
Vascular effects of 5/6Nx and PKI-166

To further explore the alterations in systemic vascular reactivity after 5/6Nx and the effects of PKI-166, we investigated myogenic constriction in the mesenteric artery and sensitivity to GPCR agonists in the thoracic aorta.

Myogenic constriction: Passive diameters of mesenteric arteries did not differ among the experimental groups over the pressure range (Figure 5A), suggesting no apparent structural changes in the investigated arteries. Active diameters were increased only in the 5/6Nx+Vehicle group (Figure 5B), signifying a gross impairment of myogenic constriction in mesenteric artery at 12 weeks after 5/6Nx (Figure 5C), as reported previously (Vettoretti et al., 2006). Chronic treatment of 5/6Nx rats either with PKI-166 or lisinopril completely restored the impaired myogenic tone back to the Sham values (Figure 5C).

Ang II and PE mediated aorta contractility: To assess the involvement of EGFR in Ang II and PE mediated contraction in thoracic aorta rings, full concentration-response curves of Ang II and PE were obtained. Twelve weeks after 5/6Nx, contraction to Ang II was significantly diminished in thoracic aorta (Figure 6A). The contractile response to Ang II in 5/6Nx was partially restored by PKI-166 and completely by lisinopril (Figure 6A). Similar to our findings with Ang II, PE mediated aorta contractility was attenuated in 5/6Nx+Vehicle group (Figure 6C). Both lisinopril and PKI-166 completely restored the impairment of PE mediated contractions (Figure 6C).

To further investigate the role of hypertension in the attenuated Ang II and PE mediated aorta contractility, we analyzed the relationship between arterial SBP and
maximal contraction of aortic rings. SBP correlated negatively with the maximal contractions to Ang II (Figure 6B; $R=-0.515$, $P<0.01$) and in particular to PE (Figure 6D; $R=-0.848$, $P<0.0001$).

**Endothelial function in mesenteric artery:** Endothelial function was assessed by obtaining concentration-response curves to ACh in pressurized arteries (80 mmHg) precontracted with U46619 (30 nM). ACh dose-dependently relaxed mesenteric arteries isolated from all five different experimental groups (Figure 7). Our data showed that endothelial function was unchanged after 5/6Nx as shown previously (Jolma et al., Exp Nephrol 2002;10:348–354). Drug treatment did not affect ACh induced relaxations.
Discussion

This is the first study exploring the therapeutic action of long-term EGFR inhibition in the advanced stage of a reno-cardiovascular disease model. Our findings show that chronic inhibition of the EGFR by PKI-166 in 5/6Nx prevents the progression of hypertension, independently of limiting the progression of functional and structural changes in the kidney. The beneficial effect of PKI-166 treatment on the CV system is further substantiated by preservation of LVEDP and attenuation of cardiac EGFR phosphorylation, and by the normalization of the impaired myogenic tone in mesenteric artery and of the contractile response of thoracic aortic rings to Ang II and PE. Collectively, these data constitute the first evidence that CV protection in progressive hypertensive kidney disease can be achieved by EGFR inhibition independently of attenuation of the progression of renal injury.

Our data strongly suggest that the beneficial action of PKI-166 is mediated primarily through the extra renal inhibition of EGFR, as the compound attenuated the increase in blood pressure, but did not affect the progressive renal injury of 5/6Nx. In addition to its lowering of blood pressure, PKI-166 restored myogenic tone in small mesenteric arteries in 5/6Nx rats. Most likely, this action reflects its capability to maintain normal peripheral resistance, which would imply that decreased peripheral resistance in 5/6Nx is caused by hypertension, rather than by the uremic state (Vettoretti et al., 2006). Thirdly, PKI-166 attenuated the reduction in contractile response to Ang II and PE in the aorta of 5/6Nx rats. Decrease in aortic contractility following 5/6Nx is in accord with previous studies demonstrating a down regulation in vascular α1-AR (Meggs et al., 1986) in partial nephrectomy, possibly related to increased levels of circulating...
catecholamines (Meggs et al., 1986; Ghosh et al., 2009). Also, reduction in the sensitivity to Ang II was found in the spontaneous hypertensive rat (SHR) and in obese Zucker Diabetic rat (Harker et al., 1993). Thus, it is most likely that PKI-166 restores aortic sensitivity to PE and Ang II by normalizing blood pressure. Indeed, this is supported by the significant negative correlation between SBP and the maximal contraction response to Ang II or PE. As PKI-166 normalized 5/6Nx induced changes in mesenteric and aortic vasculature in the absence of renoprotection, the present study also suggests that normalization of blood pressure may suffice to preserve vascular function in 5/6Nx, perhaps also in case of ACE inhibition.

Renal mass reduction clearly induced cardiac alterations in vehicle-treated 5/6Nx rats, including LV hypertrophy and elevated LVSP and LVEDP, as previously described (Amann et al., 1998; Kennedy et al., 2003; Windt et al., 2008a). Although PKI-166 did not lower heart weight, indicating that it did not prevent LV hypertrophy, the drug completely normalized the 5/6Nx induced increase in LVEDP. An increase in LVEDP is an early sign for LV diastolic dysfunction (Zile et al., 2001) and has been shown to be an independent predictor of future clinical heart failure events (Liang et al., 2006) and mortality (Judge et al., 1991; Bella et al., 2002). These results indicate that PKI-166 displays therapeutic potential to lower LVEDP, possibly limiting the cardiac risk in CKD. It is well known that LV hypertrophy develops when the myocardium is subjected to sustained pressure overload (Grossman et al., 1975) as was the case in this study. LV hypertrophy increases wall stress during systole and leads to increased thickness of the ventricular wall. This subsequently causes decreased chamber compliance and as a result, LVSP and LVEDP are elevated. Although we did not measure more robust markers of
diastolic function such as the passive and active diastolic ventricular properties known as end diastolic pressure volume relationship (EDPVR) and isovolumetric relaxation time (Tau), the observed increase in LVSP after 5/6Nx was attenuated by chronic PKI-166 and lisinopril treatment, which likely implies an increased Tau in vehicle-treated 5/6Nx rats. Higher values of Tau associates with all forms of myocardial hypertrophy (Maurer et al., 2004) including the load-dependent LV hypertrophy together with leftward/upward shift of the EDPVR which is indicative of passive diastolic dysfunction (Klotz et al., 2006). Therefore, it is likely that leftward/upward shift of the EDPVR would have been present in vehicle-treated 5/6Nx rats which might be partially preserved by both treatments.

It is still elusive what brings about the blood pressure lowering action of PKI-166 in 5/6Nx. We show that PKI-166 treatment did not affect cardiac hypertrophy, but normalized LVEDP and the increased pEGFR expression in the vascular tissue of heart in 5/6Nx animals, while cardiomyocytes were negative for receptor expression. Crucial for the interpretation of these results is to realize that treatment only started after development of hypertension and renal symptoms. However, proper establishment of PKI-166 effects is hampered by the gradual decrease in the efficacy of its blood pressure lowering action towards the end of the treatment period. Thus, our data may be interpreted as a desensitization of the effect of PKI-166. Alternatively, as suggested by the reduction in cardiac pEGFR expression and its vascular localization, PKI-166 may mainly counteract changes in the coronary vasculature, possibly improving capillary flow, without affecting hypertrophy.

Previously, EGFR signaling has been reported to be affected by several antihypertensive therapies such as RAS inhibitors, endothelin 1 receptor antagonists and
antioxidants (Fujino et al., 1998; Dorrance et al., 2001; Nakano et al., 2005; Portik-Dobos et al., 2006), which matches the observed decrease in pEGFR expression in lisinopril treated 5/6Nx animals. Moreover, the ACE inhibitor, imidapril (Nakano et al., 2005), the AT₁R antagonist, losartan (Lautrette et al., 2005), and the aldosterone antagonist, spironolactone (Dorrance et al., 2001) also affect EGFR signaling. Thus, our observation that PKI-166 treatment successfully lowered the blood pressure in absence of renoprotection fuels the idea that these antihypertensive agents may decrease blood pressure, at least in part, by interfering with EGFR signaling.

Several experimental studies have suggested that EGFR inhibitors possess renoprotective effects (Gilbert et al., 2001; Lassila et al., 2005; Schellings et al., 2006; Advani et al., 2011). Of note is the contribution of Ang II in the development of renal fibrotic lesions (Chatziantoniou et al., 1998; Boffa et al., 1999; Francois et al., 2004). Previously, the renal fibrotic role of Ang II was linked to EGFR transactivation via AT₁R-induced shedding of membrane-bound EGFR ligands through activation of ADAM (a disintegrin and metalloprotease) (Uchiyama-Tanaka et al., 2001; Chen et al., 2006). In a study by Francois et al., EGFR inhibition by gefitinib (also an inhibitor of EGFR tyrosine kinase) in nitric oxide deficiency-induced hypertension (L-NAME) model limited renal fibrosis, but did not display an antihypertensive action (Francois et al., 2004). In contrast to above mentioned report, Benter et al. found a moderate reduction in the mean arterial blood pressure by a chronic, but shorter (3 weeks), treatment with another EGFR kinase inhibitor (AG1478) in deoxycorticosterone acetate (DOCA)–salt-induced hypertensive rats (115±4 mmHg vs 135±5 mmHg) (Benter et al., 2009). A more recent study, however, shows exactly the opposite of what we found.

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induced diabetes in female hypertensive rats, the latter due to heterozygous renin-overexpression (TGR(mRen-2)), chronic PKI-166 treatment (100 mg/kg/day for 16 weeks) attenuated renal enlargement, podocyte loss and albuminuria, while not affecting the increased blood pressure (Advani et al., 2011). There are, however, main differences with our current study, including the animal strain, experimental models and sex of the animals. The fact that PKI-166 lowers blood pressure in 5/6Nx but not in TGR(mRen-2), albeit both models in which hypertension is provoked by increased renin production, may signify that PKI-166 acts through mechanisms upstream of renin production. Further, both studies differ substantially in their drug treatment, which may have affected the therapeutic action of PKI-166. First, we instituted PKI-166 treatment only at an advanced stage of the renal disease when blood pressure levels are stably increased. Secondly, we employed half the dose of PKI-166 during a course which was 10 weeks shorter in duration. The main reason for using a lower dose was because we experienced some moderate adverse effects of PKI-166 in a previous study in rats also at 100 mg/kg/day, including a decrease in water and food intake and hence a reduction in body weight (Mulder et al., 2010). These adverse effects have been reported previously by others in rat studies at a dose of 100 mg/kg/day (Wassef et al., 2004; Advani et al., 2011). Moreover, in a human phase I study with continuous PKI-166 treatment at 50 and 100 mg/kg/day, adverse events such as transaminase elevations, diarrhea, cutaneous toxicity, nausea, and vomiting were reported (Hoekstra et al., 2005). As these adverse events were thought to originate from drug accumulation, we selected the dose of 50 mg/kg/d PKI-166 in order to limit the influence of potential adverse effects on the study outcome. Despite the lower dose of 50 mg/kg/day, our study clearly shows that this dosing regimen
of PKI-166 decreases pEGFR expression in the hearts of 5/6Nx rats compared to untreated 5/6Nx controls. As a similar expression of total EGFR and GAPDH was found in all groups, this analysis confirms the in vivo activity of the drug. Taken together, the 4 studies conducted so far in rat models of cardio-renal disease (including ours) show large differences in the action of EGFR kinase inhibitors with respect to CV and renal actions. While this may signify differences in the contribution of EGFR in several rat models to pathogenesis, alternatively, drug characteristics such as tissue penetration need also to be considered.

In conclusion, we demonstrate that EGFR inhibition, initiated at an advanced disease stage in reno-cardiovascular disease, attenuated the progression of hypertension and preserved cardiac and vascular function in the rat without beneficial effects on renal disease progression. To the best of our knowledge, this is the very first in vivo study demonstrating CV protective effects of an EGFR inhibitor in CKD to be independent of modulation of renal injury. Therefore, this study extends the evidence on EGFR signaling as a target in CV disorders.

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Contributed new reagents or analytic tools: Ulu, Mulder, Gurdal, van Dokkum, van Goor and Henning.

Performed data analysis: Ulu, Mulder, Vavrinec, Landheer, Duman-Dalkilic, Goris, Henning.

Wrote or contributed to the writing of the manuscript: Ulu, Mulder, Gurdal, van Goor, and Henning.
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Footnotes:

*Both authors contributed equally to this work

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Legends for Figures

Figure 1. The effect of treatment with PKI-166 or lisinopril on proteinuria levels. Treatments were initiated 6 weeks after induction of 5/6Nx. Data are expressed as mean±SEM. *P<0.05 versus Sham+Vehicle and #P<0.05 versus Sham+PKI-166.

Figure 2. Assessment of renal damage. Fifty glomeruli per slide (A1-E1) were microscopically evaluated and scored semi-quantitatively (F) for the incidence of focal glomerulosclerosis (FGS). For the assessment of prefibrotic changes, the intensity of the positive cortical interstitial and glomerular (A2-E2) pixels was measured by α-smooth muscle actin (α-SMA) staining (G). Representative photomicrographs of kidney sections from Sham+Vehicle (A1 and A2), Sham+PKI-166 (B1 and B2), 5/6Nx+Vehicle (C1 and C2), 5/6Nx+PKI-166 (D1 and D2) and 5/6Nx+Lisinopril (E1 and E2) groups. Data are expressed as mean±SEM. *P<0.05 versus Sham+Vehicle and #P<0.05 versus Sham+PKI-166.

Figure 3. The effect of treatment with PKI-166 or lisinopril on conscious arterial systolic (A) and diastolic blood pressure (B). Twelve weeks after 5/6Nx, arterial blood pressure was also measured under short anesthesia by Millar catheter (C and D). Data are expressed as mean±SEM. *P<0.05 versus Sham+Vehicle, #P<0.05 versus Sham+PKI-166, †P<0.05 versus 5/6Nx+PKI-166, and ‡P<0.05 versus 5/6Nx+Lisinopril.

Figure 4. The strongly increased phosphorylation of cardiac EGFR (pEGFR) in 5/6Nx is attenuated by chronic treatment with PKI-166 and lisinopril. Representative immunoblots
for pEGFR and total EGFR (tEGFR) in randomly selected rat heart samples from Sham, 5/6Nx+Vehicle, 5/6Nx+PKI-166 and 5/6Nx+lisinopril groups (A) twelve weeks after 5/6Nx are presented. pEGFR expression was normalized to GAPDH (B, n=3-5 for each group). Representative photomicrographs of immunostaining for pEGFR on frozen sections of the rat hearts from Sham+Vehicle (C), Sham+PKI-166 (D), 5/6Nx+Vehicle (E), and 5/6Nx+PKI-166 (F),and 5/6Nx+lisinopril (G) groups for the establishment of the localization of cardiac pEGFR. *P<0.05 versus Sham and †P<0.05 versus 5/6Nx+Vehicle (Mann–Whitney U-test).

**Figure 5.** Vascular reactivity of small mesenteric arteries. Diameters of small mesenteric arteries in response to stepwise increase of intraluminal pressure in the absence (A) or presence (B) of extracellular calcium and calculated myogenic tone (C). Data are expressed as mean±SEM. *P<0.05 versus all groups.

**Figure 6.** Full concentration-response curves of angiotensin II (A) and phenylephrine (C) mediated contraction and relationship between arterial SBP and maximal contraction response of aortic rings to angiotensin II (B) and phenylephrine (D) in rat thoracic aorta rings. Data are expressed as mean±SEM. *P<0.05 versus all groups.

**Figure 7.** Endothelial function in small mesenteric arteries. Endothelium-dependent relaxation after cumulative doses of acetylcholine (1 nM –10 µM) in the mesenteric arteries pre-constricted with U46619 (30 nM). Data are expressed as mean±SEM.
### Table 1. In vivo characteristics of untreated and treated sham and 5/6Nx rats 12 week after sham or 5/6Nx operation. Data are given as means±S.E.M. LVSP: Left ventricular systolic pressure, LVEDP: Left ventricular end-diastolic pressure. \( aP<0.05 \) versus Sham+Vehicle, \( bP<0.05 \) versus Sham+PKI-166, \( cP<0.05 \) versus 5/6Nx+PKI-166, \( dP<0.05 \) versus 5/6Nx+Lisinopril.

<table>
<thead>
<tr>
<th></th>
<th>Sham+Vehicle (n=10)</th>
<th>Sham+PKI-166 (n=12)</th>
<th>5/6Nx+Vehicle (n=12)</th>
<th>5/6Nx+PKI-166 (n=10)</th>
<th>5/6Nx+Lisinopril (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney weight (g)</td>
<td>1.59±0.03</td>
<td>1.39±0.05</td>
<td>2.32±0.11 ( ^{a,b} )</td>
<td>2.13±0.13 ( ^{a,b} )</td>
<td>2.08±0.09 ( ^{a,b} )</td>
</tr>
<tr>
<td>Kidney weight/Body weight</td>
<td>0.35±0.02</td>
<td>0.33±0.01</td>
<td>0.49±0.02 ( ^{a,b} )</td>
<td>0.48±0.04 ( ^{a,b} )</td>
<td>0.43±0.01 ( ^{b} )</td>
</tr>
<tr>
<td>Creatinine clearance (mL min(^{-1}) kg(^{-1}))</td>
<td>7.8±0.7</td>
<td>7.3±0.4</td>
<td>3.6±0.4 ( ^{a,b} )</td>
<td>2.9±0.5 ( ^{a,b} )</td>
<td>4.7±0.6 ( ^{a,b} )</td>
</tr>
<tr>
<td>Heart rate (beats min(^{-1}))</td>
<td>355±12</td>
<td>333±12</td>
<td>355±16</td>
<td>322±15</td>
<td>358±16</td>
</tr>
<tr>
<td>Left ventricular weight/Body weight</td>
<td>0.21±0.01</td>
<td>0.20±0.003</td>
<td>0.25±0.01 ( ^{a,b,d} )</td>
<td>0.26±0.01 ( ^{a,b,d} )</td>
<td>0.20±0.01</td>
</tr>
<tr>
<td>+dP/dt(_{\text{max}})</td>
<td>9569±505</td>
<td>8687±462</td>
<td>10326±404</td>
<td>8900±482</td>
<td>8393±685</td>
</tr>
<tr>
<td>-dP/dt(_{\text{max}})</td>
<td>-7252±354</td>
<td>-7322±314</td>
<td>-8762±398 ( ^{a,d} )</td>
<td>-7674±602</td>
<td>-6334±437</td>
</tr>
<tr>
<td>LVSP (mmHg)</td>
<td>119±4</td>
<td>116±4</td>
<td>151±5 ( ^{a,b,d} )</td>
<td>135±7 ( ^{d} )</td>
<td>108±5</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>4±1</td>
<td>3±1</td>
<td>13±4 ( ^{b,c} )</td>
<td>3±1</td>
<td>4±1</td>
</tr>
</tbody>
</table>
Table 2. Body weight, water intake and urine output of untreated and treated sham and 5/6 Nx rats between week 0 and week 11 after sham or 5/6 Nx operations. Data are given as means±S.E.M. \textsuperscript{a}P<0.05 versus Sham+Vehicle, \textsuperscript{b}P<0.05 versus Sham+PKI-166.

<table>
<thead>
<tr>
<th>Week 0</th>
<th>Body weight (g)</th>
<th>Sham+Vehicle (n=10)</th>
<th>Sham+PKI-166 (n=12)</th>
<th>5/6 Nx+Vehicle (n=12)</th>
<th>5/6 Nx+PKI-166 (n=10)</th>
<th>5/6+ Nx Lisinopril (n=8)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Water intake (mL/day)</td>
<td>353±6</td>
<td>357±3</td>
<td>353±3</td>
<td>359±6</td>
<td>356±3</td>
</tr>
<tr>
<td></td>
<td>Urine volume (mL/day)</td>
<td>26±2</td>
<td>27±2</td>
<td>30±2</td>
<td>24±1</td>
<td>24±2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13±2</td>
<td>14±2</td>
<td>18±1</td>
<td>13±1</td>
<td>15±3</td>
</tr>
</tbody>
</table>

| Week 5 | Body weight (g) | 433±10             | 449±4               | 428±8                | 431±11                | 429±9                  |
|        | Water intake (mL/day) | 23±2               | 24±2                | 35±3\textsuperscript{a,b} | 33±3\textsuperscript{a,b} | 31±3                   |
|        | Urine volume (mL/day) | 14±2               | 16±2                | 22±2\textsuperscript{a} | 19±2                  | 19±2                   |

| Week 8 | Body weight (g) | 459±11             | 446±6               | 456±10               | 442±16                | 461±10                 |
|        | Water intake (mL/day) | 19±1               | 26±1                | 35±4\textsuperscript{a} | 36±4\textsuperscript{a} | 26±3                   |
|        | Urine volume (mL/day) | 12±1               | 13±1                | 25±3\textsuperscript{a,b} | 21±2\textsuperscript{a,b} | 21±2\textsuperscript{a,b} |

| Week 11 | Body weight (g) | 464±12             | 428±11              | 468±13               | 453±18                | 474±9                  |
|         | Water intake (mL/day) | 22±2               | 29±4                | 34±4                 | 34±5                  | 23±3                   |
|         | Urine volume (mL/day) | 15±2               | 11±1                | 27±3\textsuperscript{a,b} | 21±1\textsuperscript{b} | 20±2\textsuperscript{b} |
Figure 2
Figure 3
Figure 4