Epidermal Growth Factor Receptor Inhibitor PKI-166 Governs Cardiovascular Protection without Beneficial Effects on the Kidney in Hypertensive 5/6 Nephrectomized Rats 

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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, and cardiac and vascular hypertrophy. However, the therapeutic potential of EGFR inhibition in these conditions is currently unknown. The main objective of the present study was to investigate cardiac, vascular, and renal effects of EGFR inhibition by 4-[[((1R)-1-phenylethyl)amino]-7H-pyrrolo[2,3-d]pyrimidin-6-yl]phenol (PKI-166) in the hypertensive chronic kidney disease model. Rats underwent 5/6 nephrectomy (5/6Nx) and were treated with PKI-166, 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 (left ventricle end-diastolic pressure) to a similar extent as lisinopril. Also, PKI-166 attenuated the increase in phosphorylated EGFR in the 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 independent 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 implicates G protein–coupled receptors (GPCRs) 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).

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N.U. and G.M.M. contributed equally to this work.


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ABBREVIATIONS: ACE, angiotensin-converting enzyme; ACh, acetylcholine; AG1478, 4-(3’-chloroanilino)-7,6-dimethoxyquinazoline; Ang II, angiotensin II; α1-AR, α1-adrenoceptor; AT1R, angiotensin II type 1 receptor; CKD, chronic kidney disease; CV, cardiovascular; DBP, diastolic blood pressure; EDPVR, end diastolic pressure volume relationship; EGFR, epidermal growth factor receptor; FGS, focal glomerulosclerosis; GPCR, G protein–coupled receptor; LV, left ventricle; LVEDP, left ventricle end-diastolic pressure; LVSVP, left ventricle systolic pressure; 5/6Nx, 5/6 nephrectomy; PE, phenylephrine; pEGFR, phosphorylated epidermal growth factor receptor; PKI-166, 4-[[((1R)-1-phenylethyl)amino]-7H-pyrrolo[2,3-d]pyrimidin-6-yl]phenol; SBP, systolic blood pressure; α-SMA, α-smooth muscle actin; Tau, isovolumetric relaxation time; U46619, (5Z)-7-[[1R,4S,5S,6S,6F]-6-[[1E,3S]-3-hydroxy-1-octenyl]-2-oxabicyclo[2.2.1]hept-5-yl]-5-heptenoic acid.

Recently, it has been recognized that the transactivation of epidermal growth factor receptor (EGFR) by many different Gαq/11 protein–coupled receptors may constitute an important part of their signaling (Fernandez-Patron, 2007). Therefore, instead of targeting various GPCRs, such as angiotensin II (Ang II) type 1 receptor (AT1R) or α1-adrenoceptor (α1-AR), it is conceivable that blockade of EGFR transactivation may have significant potential in cardiovascular (CV) disorders (Supplemental Fig. 1). EGFR transactivation has been implied in several CV conditions, including hypertension (Fernandez-Patron, 2007), heart failure (Xu et al., 2009), and cardiac (Kagiyma 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 (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, an increased expression level of EGFR relates to blood pressure in genetic (Swamnathan 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 the hypertrophied left ventricle (LV) of spontaneous hypertensive rats (Fujino et al., 1998). Also, receptor tyrosine kinase inhibitors, such as EGFR tyrosine kinase inhibitor 4-(3′-chloroanilino)-6,7-dimethoxyquinazoline (AG1478), and EGFR antisense oligonucleotides attenuate vasoconstriction and elevation of blood pressure in Ang II–induced hypertension (Kagiyama et al., 2002, 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, 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 Lowica, 2009).

To investigate whether EGFR inhibitors represent a novel class of drugs in renal disease–associated CV complications, the effects of 4-(4-[1R]-1-pheneyethyl)aminol-7H- pyrrolo[2,3-d]pyrimidin-6-yl)phenol (PKI-166) were investigated in 5/6 nephrectomized (5/6Nx) rats by assessment of renal function and damage, blood pressure, cardiac parameters, and vaso-reactivity 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.

Materials and Methods

Animals. Experiments were performed on 12-week-old male Wistar rats (n = 60, 330–400 g; Harlan, Zeist, The Netherlands). Animals were housed under standard conditions of temperature (21–24°C), humidity (40–60%), and a 12:12-hour 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 National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were 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). 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 started on treatment with 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% dimethylsulfoxide + 0.5% Tween-80 diluted 1:20 (v/v) in water at a final concentration of 50 mg ml⁻¹. Ten percent dimethylsulfoxide + 0.5% Tween-80 diluted 1:20 (v/v) was also added in the vehicle solution. During the treatment period, proteinuria and tail-cuff blood pressure were assessed every 3 weeks. As blood pressure levels stabilize around 6 weeks after 5/6Nx (Gischwend et al., 2002; Vettoretti et al., 2006; Windt et al., 2008b), we initiated drug treatments at this time point 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, TX). Heart rate, LV end-diastolic pressure (LVEDP), LV systolic pressure (LVSP), and the maximal rates of increase and decrease in LV pressure (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. The 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 hours, 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 2-week 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 Jaffe method without deproteinization (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 × urine flow) / (plasma creatinine × 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-Aldrich, St. Louis, MO) incubated for 1 hour at 1:9.1.772.1570; Aperio Technologies Inc., Vista, CA) at 200× 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 50 glomeruli per slide on a scale of 0–4 by an
examiner blinded to the groups, as described previously (Van Dokkum et al., 1999). The 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-EGFR (pY1173) phosphospecific antibody (Invitrogen, Breda, The Netherlands). The antibody was diluted in phosphate-buffered saline + 1% bovine serum albumin and applied for 1 hour at room temperature. Peroxidase-labeled secondary antibodies with rat or human serum (1%) were used for sequential incubation. Peroxidase activity was developed using 3-amin-9-ethylcarbazole.

For immunoblotting, heart and kidney samples were homogenized in 500 μl of ice-cold homogenization buffer (1% Igepal ca-630, 1% SDS, 5 mg/ml sodium deoxycholate, 1 mM sodium orthovanadate, 10 mM β-mercapto-ethanol, 40 μg/ml phenylmethylsulfonyl fluoride, 100 μg/ml benzamidine, 500 ng/ml peptatin A, 500 ng/ml leupeptin, and 500 ng/ml aprotinin in phosphate-buffered saline) with Dispomix Homogenization (L&M Biotech, Cary, NC). The homogenate was clarified by centrifugation at 2000g for 10 minutes at 4°C, the supernatant was collected, and protein concentration was determined using Qubit Protein Assay (Invitrogen, Carlsbad, CA). Whole cardiac extracts were boiled in sample buffer for 3 minutes, separated using 7% resolving gels, and transferred electronically to nitrocellulose membranes. Membranes were incubated overnight with the anti-EGFR or phosphospecific anti-EGFR antibodies (1:500 dilutions; Santa Cruz Biotechnology) was used to confirm equal loading conditions. Finally, blots were incubated with enhanced chemiluminescence Western Blotting reagent (Santa Cruz Biotechnology) for 1 minute and exposed to X-ray film for 45–90 seconds.

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) as described previously (Gschwend et al., 2002).

Intraluminal pressure was set at 80 mm Hg, and arteries were washed with Tris-buffered saline/Tween 20 (0.05 M Tris, 150 mM NaCl, and 0.04% Tween-20) and re-incubated with the secondary antibodies for 1 hour at room temperature. Anti-glyceraldehyde-3-phosphate dehydrogenase antibody (1:10,000 dilution; Santa Cruz Biotechnology) was used to confirm equal loading conditions. Finally, blots were incubated with enhanced chemiluminescence Western Blotting reagent (Santa Cruz Biotechnology) for 1 minute and exposed to X-ray film for 45–90 seconds.

TABLE 1
In vivo characteristics of untreated and treated sham and 5/6Nx rats 12 weeks after sham or 5/6Nx operation

<table>
<thead>
<tr>
<th></th>
<th>Sham + Vehicle (n = 10)</th>
<th>Sham + PKI-166 (n = 12)</th>
<th>5/6Nx + Vehicle (n = 10)</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</td>
<td>2.13 ± 0.13</td>
<td>2.08 ± 0.09</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</td>
<td>0.48 ± 0.04</td>
<td>0.43 ± 0.01</td>
</tr>
<tr>
<td>Creactineme clearance (ml/min.kg)</td>
<td>7.8 ± 0.7</td>
<td>7.3 ± 0.4</td>
<td>3.6 ± 0.4</td>
<td>2.9 ± 0.5</td>
<td>4.7 ± 0.6</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>355 ± 12</td>
<td>332 ± 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</td>
<td>0.26 ± 0.01</td>
<td>0.20 ± 0.01</td>
</tr>
<tr>
<td>+dP/dtmax</td>
<td>9669 ± 505</td>
<td>8887 ± 462</td>
<td>10326 ± 404</td>
<td>8900 ± 462</td>
<td>8393 ± 685</td>
</tr>
<tr>
<td>+dP/dtmax</td>
<td>-7251 ± 354</td>
<td>7322 ± 314</td>
<td>-801 ± 398</td>
<td>-792 ± 430</td>
<td>-620 ± 437</td>
</tr>
<tr>
<td>LVSP (mm Hg)</td>
<td>119 ± 4</td>
<td>116 ± 4</td>
<td>151 ± 5</td>
<td>135 ± 7</td>
<td>108 ± 5</td>
</tr>
<tr>
<td>LVEDP (mm Hg)</td>
<td>4 ± 1</td>
<td>3 ± 1</td>
<td>13 ± 4</td>
<td>3 ± 1</td>
<td>4 ± 1</td>
</tr>
</tbody>
</table>

*dP/dtmax maximal rate of increase in left ventricle pressure; –dP/dtmax maximal rate of decrease in left ventricle pressure.

P < 0.05 versus sham + vehicle.

P < 0.05 versus sham + PKI-166.

P < 0.05 versus 5/6Nx + vehicle.

P < 0.05 versus 5/6Nx + PKI-166.

P < 0.05 versus 5/6Nx + vehicle.

P < 0.05 versus 5/6Nx + PKI-166.

P < 0.05 versus 5/6Nx + PKI-166.

P < 0.05 versus 5/6Nx + PKI-166.
are expressed as the mean levels. Treatments were initiated 6 weeks after induction of 5/6Nx. Data The effect of treatment with PKI-166 or lisinopril on proteinuria Fig. 1. Six weeks of treatment with PKI-166 between weeks 6 and 12 reduced systolic blood pressure (SBP) compared with vehicle-treated 5/6Nx rats until week 12. At the end of the experimental protocol, 5/6Nx induced a significant increase in SBP at 5 weeks after the operation. In vehicle-treated 5/6Nx rats, SBP continued to increase up to 8 weeks, and remained stable afterward (Fig. 3A). DBP was slightly (but not significantly) higher in 5/6Nx animals at week 5, but increased significantly in vehicle-treated 5/6Nx rats afterward (Fig. 3B). Also, LV weight/body weight ratio, LVSP, and LVEDP were increased in vehicle-treated 5/6Nx rats compared with sham rats (Table 1). Together, these data demonstrate the successful induction of experimental CKD and related changes in CV parameters induced by 5/6Nx.

The previous measurements were also obtained in sham animals treated from weeks 6 to 12 with PKI-166. Importantly, no difference in any of the parameters was observed in sham + PKI-166 animals compared with vehicle-treated sham rats (Figs. 1–3; Tables 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 rats, similar 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 (Fig. 1). In line with proteinuria data, PKI-166 treatment did not influence FGS score (Fig. 2, D1 and F) or interstitial α-SMA staining (Fig. 2, D2 and G). However, lisinopril treatment partially protected the kidneys from injury, as evidenced by a lower FGS score (Fig. 2, E1 and F). Moreover, pEGFR blot need substantial amplification. In general, it seems that groups do not differ substantially in pEGFR expression in kidneys in the different groups.

Animal Characteristics and Effects of 5/6Nx

Animal characteristics are presented in Tables 1 and 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 weeks 5 and 11 (Table 2) compared with sham animals. 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 (Fig. 1; P < 0.05 versus 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 scores (Fig. 2, C1 and F) and interstitial α-SMA staining (Fig. 2, C2 and G) compared with sham rats (Fig. 2, A1 and A2). 5/6Nx induced a significant increase in SBP at 5 weeks after the operation. In vehicle-treated 5/6Nx rats, SBP continued to increase up to 8 weeks,

TABLE 2

Body weight, water intake, and urine output of untreated and treated sham and 5/6 Nx rats between weeks 0 and 11 after sham or 5/6 Nx operations

<table>
<thead>
<tr>
<th></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>
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<tr>
<td><strong>Week 0</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>353 ± 6</td>
<td>357 ± 3</td>
<td>353 ± 3</td>
<td>359 ± 6</td>
<td>356 ± 3</td>
</tr>
<tr>
<td>Water intake (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>Urine volume (ml/day)</td>
<td>13 ± 2</td>
<td>14 ± 2</td>
<td>18 ± 1</td>
<td>15 ± 1</td>
<td>15 ± 3</td>
</tr>
<tr>
<td><strong>Week 5</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>433 ± 10</td>
<td>449 ± 4</td>
<td>428 ± 8</td>
<td>431 ± 11</td>
<td>429 ± 9</td>
</tr>
<tr>
<td>Water intake (ml/day)</td>
<td>23 ± 2</td>
<td>24 ± 2</td>
<td>35 ± 3</td>
<td>33 ± 3</td>
<td>31 ± 3</td>
</tr>
<tr>
<td>Urine volume (ml/day)</td>
<td>14 ± 2</td>
<td>16 ± 2</td>
<td>22 ± 2</td>
<td>19 ± 2</td>
<td>19 ± 2</td>
</tr>
<tr>
<td><strong>Week 8</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>459 ± 11</td>
<td>466 ± 6</td>
<td>456 ± 10</td>
<td>442 ± 16</td>
<td>461 ± 10</td>
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<tr>
<td>Water intake (ml/day)</td>
<td>19 ± 1</td>
<td>26 ± 1</td>
<td>35 ± 4</td>
<td>36 ± 4</td>
<td>26 ± 3</td>
</tr>
<tr>
<td>Urine volume (ml/day)</td>
<td>12 ± 1</td>
<td>13 ± 1</td>
<td>25 ± 3</td>
<td>21 ± 2</td>
<td>21 ± 2</td>
</tr>
<tr>
<td><strong>Week 11</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>464 ± 12</td>
<td>428 ± 11</td>
<td>468 ± 13</td>
<td>453 ± 18</td>
<td>474 ± 9</td>
</tr>
<tr>
<td>Water intake (ml/day)</td>
<td>22 ± 2</td>
<td>29 ± 4</td>
<td>34 ± 4</td>
<td>34 ± 5</td>
<td>23 ± 3</td>
</tr>
<tr>
<td>Urine volume (ml/day)</td>
<td>15 ± 2</td>
<td>11 ± 1</td>
<td>27 ± 3</td>
<td>21 ± 1</td>
<td>20 ± 2</td>
</tr>
</tbody>
</table>

*p < 0.05 versus sham + vehicle.

Table: 3

Sham + Vehicle
Sham + PKI-166
5/6 Nx + Vehicle
5/6 Nx + PKI-166
5/6 Nx + Lisinopril

Week 0
Week 5
Week 8
Week 11

Results

Fig. 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 the mean ± S.E.M. *P < 0.05 versus sham + vehicle; #P < 0.05 versus sham + PKI-166.

Fig. 2. A1 and A2. 5/6Nx induced a significant increase in SBP at 5 weeks after the operation. In vehicle-treated 5/6Nx rats, SBP continued to increase up to 8 weeks,
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 (Fig. 3, A and B). Prior to being euthanized (i.e., 12 weeks after the induction of 5/6Nx), SBP and DBP were also measured under brief anesthesia by a Millar catheter. In accord with the conscious tail-cuff blood pressure measurements, PKI-166 treatment significantly lowered SBP and DBP (Fig. 3, C and D). Lisinopril completely restored the increased SBP and DBP back to sham levels at week 12 (Fig. 3, C and D).

Neither PKI-166 nor lisinopril significantly influenced 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 did lisinopril (Table 1).

To substantiate that PKI-166 inhibits phosphorylation of EGFR in 5/6Nx, immunoblottings for pEGFR and total EGFR 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 with sham rats, which was inhibited by continuous PKI-166 and lisinopril treatment (Fig. 4, A and B). In contrast, total EGFR was similar among all groups (Fig.
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 (Fig. 4, C–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 (Fig. 5A), suggesting no apparent structural changes in the investigated arteries. Active diameters were increased only in the 5/6Nx vehicle group (Fig. 5B), signifying a gross impairment of myogenic constriction in the mesenteric artery at 12 weeks after 5/6Nx (Fig. 5C), as reported previously (Vettoretti et al., 2006). Continuous treatment of 5/6Nx rats either with PKI-166 or lisinopril completely restored the impaired myogenic tone back to the sham values (Fig. 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 in response to Ang II was significantly diminished in the thoracic aorta (Fig. 6A). The contractile response to Ang II in 5/6Nx was partially restored by PKI-166 and completely by lisinopril (Fig. 6A). Similar to our findings with Ang II, PE-mediated aorta contractility was attenuated in the 5/6Nx vehicle group (Fig. 6C). Both lisinopril and PKI-166 completely restored the impairment of PE-mediated contractions (Fig. 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

Fig. 3. The effect of treatment with PKI-166 or lisinopril on conscious arterial systolic (A) and diastolic (B) blood pressure. Twelve weeks after 5/6Nx, arterial blood pressure was also measured under short anesthesia by a Millar catheter (C and D). Data are expressed as the mean ± S.E.M. *P < 0.05 versus sham + vehicle; #P < 0.05 versus sham + PKI-166; †P < 0.05 versus 5/6Nx + PKI-166; ‡P < 0.05 versus 5/6Nx + lisinopril.
maximal contractions in response to Ang II (Fig. 6B; R = −0.515; P < 0.01) and in particular to PE (Fig. 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 mm Hg) precontracted with U46619 (30 nM). ACh dose dependently relaxed mesenteric arteries isolated from all five different experimental groups (Fig. 7). Our data showed that endothelial function was unchanged after 5/6Nx, as shown previously (Jolma et al., 2002). 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 continuous inhibition of the EGFR by PKI-166 in 5/6Nx prevents the progression of hypertension, independently of limiting the

Fig. 4. The strongly increased phosphorylation of cardiac EGFR (pEGFR) in 5/6Nx is attenuated by continuous 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) 12 weeks after 5/6Nx are presented. pEGFR expression was normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (n = 3–5 for each group) (B). 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; †P < 0.05 versus 5/6Nx + vehicle (Mann-Whitney U test).

Chronic EGFR Inhibition Prevents Hypertension
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 lowering 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). In addition, PKI-166 attenuated the reduction in contractile response to Ang II and PE in the aorta of 5/6Nx rats. A decrease in aortic contractility following 5/6Nx is in accord with previous studies demonstrating a downregulation 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, a reduction in the sensitivity to Ang II was found in the spontaneous hypertensive rat and in the 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 the 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 of 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 is associated 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, whereas cardiomyocytes were negative for receptor expression. To interpret these results, it is crucial 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 toward 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 renin-angiotensin system 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 blood pressure in
the 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 a disintegrin and metalloprotease (Uchiyama-Tanaka et al., 2001; Chen et al., 2006). In a study by Francois et al. (2004), EGFR inhibition by gefitinib (also an inhibitor of EGFR tyrosine kinase) in a nitric oxide deficiency-induced hypertension model limited renal fibrosis, but did not display an antihypertensive action. In contrast to the previously mentioned report, Benter et al. (2009) found a moderate reduction in the mean arterial blood pressure by continuous, but shorter (3 weeks), treatment with another EGFR kinase inhibitor (AG1478) in doxycorticosterone acetate salt-induced hypertensive rats (115 ± 4 mm Hg versus 135 ± 5 mm Hg). A more recent study, however, shows exactly the opposite of what we found. In streptozocin-induced diabetes in female heterozygous renal overexpressing transgenic TGR(mRen-2)27 rats, continuous 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 hypertension is provoked by increased renin production in both models, may signify that PKI-166 acts through mechanisms upstream of renin production. Furthermore, 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. Second, we used 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 receiving 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, diarrhoea, 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/day PKI-166 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 with untreated 5/6Nx controls. As a similar expression of total EGFR and glyceraldehyde-3-phosphate dehydrogenase was found in all groups, this analysis confirms the in vivo activity of the drug. Taken together, the four studies conducted so far in rat models of cardiovascular disease (including ours) show large differences in the action of EGFR kinase inhibitors with respect to CV and renal actions. Although this may signify differences in the contribution of EGFR to pathogenesis in several rat models, alternatively, drug characteristics such as tissue penetration also need 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 rats 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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Authorship Contributions

Participated in research design: Ulu, Mulder, Vavrincev, Goris, Duin, van Goor, Henning.

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Contributed new reagents or analytic tools: Ulu, Mulder, Vavrincev, Landheer, Duman-Dalkılıç, Goris, Henning.

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

References


Supplemental Figure 1

GPCR

MMPs
ADAMs

$G_{q/i}$

Tyr kinase
Ser/Thr kinase

intracellular signals
Src, PKC, ROS, PI3K, Ca$^{2+}$

EGFR

EGFR ligands

Grb-2
Sos
Shc

Plasma membrane
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Supplemental Figure 2