IMPROVEMENT OF RENAL HEMODYNAMICS DURING HYPERTENSION-INDUCED CHRONIC RENAL DISEASE: ROLE OF EGF RECEPTOR ANTAGONISM

Frank Helle1, Charlotte Jouzel1,2, Christos Chadjichristos1,2, Sandrine Placier1, Martin Flamant1, Dominique Guerrot1,2, Hélène François1, Jean-Claude Dussaule1,2,3, and Christos Chatziantoniou1,2 *

Short title: EGF receptor, regression, and renal hemodynamics

1 : INSERM U702, Hôpital Tenon, Paris 75020, France
2 : Pierre et Marie Curie University,
3 : AP-HP, Laboratoire de Physiologie, Hôpital St-Antoine, Paris 75012, France.
Tel: (331) 56 01 66 53
Fax: (331) 56 01 66 59
e-mail: christos.chatziantoniou@chusa.jussieu.fr

Key words: Renal hemodynamics, Angiotensin, EGF receptor, Chronic Renal Disease

* To whom reprint requests should be addressed.
**ABSTRACT**

The present study investigated mechanisms of regression of renal disease after severe proteinuria by focusing on the interaction between Epidermal Growth Factor (EGF) receptors, renal hemodynamics and structural lesions.

The nitric oxide (NO) inhibitor N\(^\text{G}\)-nitro-L-arginine-methyl ester (L-NAME) was administered chronically in Sprague-Dawley rats. When proteinuria exceeded 2 g/mmolCreat, animals were divided in three groups for an experimental period of therapy of 2 weeks; in one group, L-NAME was removed to allow reactivation of endogenous NO synthesis; in the two other groups, L-NAME removal was combined with EGF or angiotensin receptor type 1 (AT1) antagonism.

L-NAME removal partially reduced mean arterial pressure and proteinuria, and increased renal blood flow (RBF), but not microvascular hypertrophy. Progression of structural damage was stopped, but not reversed. The administration of an EGF receptor antagonist did not have an additional effect on lowering blood pressure, neither on renal inflammation, but normalized RBF and afferent arteriole hypertrophy; the administration of an AT1 antagonist normalized all measured functional and structural parameters. Staining with a specific marker of endothelial integrity indicated loss of functional endothelial cells in the L-NAME removal group; in contrast, in the animals treated with an EGF or AT1 receptor antagonist functional endothelial cells re-appeared at levels equal to control animals. In addition, afferent arterioles freshly isolated from the L-NAME removal group showed an exaggerated constrictor response to endothelin; this response was blunted in the vessels isolated from the EGF or AT1 receptor antagonist groups.

EGF receptor is an important mediator of endothelial dysfunction and contributes to the decline of RBF in the chronic kidney disease induced by NO deficiency. The EGF receptor antagonist-induced improvement of RBF is important, but not sufficient for a complete reversal of renal disease, because it has little effect on renal inflammation. To achieve full recovery is necessary to apply AT1 receptor antagonism.
INTRODUCTION

Renal chronic disease is a complex degenerative process involving hemodynamic alterations, endothelial dysfunction, inflammation, protein leaking, alterations in extracellular matrix assembly and changes of renal structure that ultimately lead to the decline of renal function. Since the solutions existing to date are replacement strategies such as dialysis and transplantation, an important challenge for public health is to stop the decline of renal function and, if possible, to achieve regression of renal fibrosis and restoration of renal structure.

In previous studies investigating the mechanisms of progression of renal disease during nitric oxide (NO) deficiency (N(G)-nitro-L-arginine-méthyl ester or L-NAME model), we have observed that angiotensin receptor type 1 (AT1) antagonism reversed renal fibrosis and restored renal structure independently of systemic hemodynamics (5), and that NO inhibition increased, whereas arrest of L-NAME administration decreased, the activation of collagen I gene in transgenic mice (6, 24). Again, expression of collagen I was independent of systemic hemodynamics. Other investigators have also observed arrest or reversal of renal disease independently of blood pressure in experimental models (2, 22). In other studies, we found that inhibition of the Epidermal Growth Factor (EGF) receptor prevented the development of renal failure independently of variations of blood pressure (12). In additional studies we observed that transactivation of the EGF receptor mediated the hemodynamic and fibrogenic affects of endothelin (ET) (10, 12). However, the curative efficiency of EGF receptor antagonism, the role of renal hemodynamics and its interaction with the regression of renal inflammatory or structural lesions was not addressed in these studies. In addition, the above-mentioned regression studies did not consider the degree of proteinuria before starting therapy.

The present study was designed to address the above concerns. Therapy was introduced when animals reached a severe degree of proteinuria, and renal blood flow was measured in addition to structural and functional parameters. Our findings indicate that EGF receptor antagonism plays an important role in the improvement of renal hemodynamics, but is less efficient in alleviating renal inflammatory lesions.
METHODS

Animal Treatment

Male Sprague-Dawley rats weighing 250g were maintained on a normal salt diet and had free access to chow and tap water. NO synthesis was inhibited by L-NAME (orally 30 mg/kg/day, orally). We have previously found that this dose produced a gradual elevation of blood pressure accompanied by the progression of renal disease (5). When proteinuria exceeded 2 g/mmolCreat (between 4-6 wk), a group of animals was sacrificed to allow estimations of renal hemodynamics and morphological parameters just before the beginning of therapy (L-NAME group, n=14). The remaining animals were divided in three subgroups for an additional experimental period of 2 wk: in the first subgroup, L-NAME was removed from the drinking water to allow reactivation of endogenous NO synthesis (removal group, n=20); in the second and third subgroups L-NAME removal was accompanied by the administration of an AT1 receptor antagonist (losartan orally 30mg/kg/day, MSD, removal+losartan group, n=18) or an EGF receptor antagonist (gefitinib orally 50mg/kg/day, AstraZeneca, removal+gefitinib group, n=20). The doses of the drugs were based on pilot experiments and previously published studies (5). Control animals were sacrificed at 4-, 6- and 8- wk. Since control animals gave similar results to all measured parameters, the pooled data are presented in the figures of results (control group, n=16). Because of the extra preparation time and use of anaesthesia during RBF measurements, separate animals were used for preparation of ex vivo afferent arteriole contractility measurements and assessment of microvascular hypertrophy, totalling 5 in each group. All protocols and treatments were performed with the approval of the French government ethics committee.

Systolic Blood Pressure

Systolic blood pressure was measured by the tail-cuff method as previously described (5). To avoid variations in blood pressure due to day cycle, all measurements were carried out
between 9 and 11 am. Eight measurements from each rat were taken at 2-min intervals, and a mean value was determined.

Renal Hemodynamics

After anesthesia by pentobarbital sodium (50–60 mg/kg body weight ip, Nembutal, Abbott, Chicago, IL), animals were placed on a servo-controlled table kept at 37°C and the trachea was cannulated to facilitate respiration. The left femoral artery was catheterized for measurement of arterial pressure, and a femoral venous catheter was used for infusion of volume replacement. An ultrasound transit-time flow probe (1RB, Transonic, Ithaca, NY) was placed around the left renal artery. Bovine serum albumin (4.75 g/dl of saline solution) was infused initially at 50 µl/min to replace surgical losses, and then at 10 µl/min for maintenance.

Arterial pressure was measured via a pressure transducer (Statham P23 DB); renal blood flow (RBF) was measured by a flowmeter (T 420, Transonic; low-pass filter 40 Hz). RBF values were controlled for zero offset determined at the end of an experiment after cardiac arrest. Data were recorded, stored and analyzed using DataTranslation analog-to-digital converter and the IOX software (EMKA Technologies, Paris France).

Isolation and preparation of Afferent Arterioles.

Afferent arterioles were isolated for diameter measurements by use of the agarose infusion / enzyme treatment technique originally described by Loutzenhisser and Loutzenhisser (21) and later on adapted and modified in our laboratory (16). In short, the kidneys were infused with agarose (37°C) and incubated for 10 min at 4°C for solidification of the agarose. Two cortical sections 100 µm thick containing mostly superficial nephrones were sliced from each kidney using a Thomas slicer (Thomas scientific) and incubated at 37°C for 1 hour in a medium enzyme-solution containing 0.5 mg/ml protease (Sigma P3417, 0.5 U/ml), 0.3 mg/ml collagenase (Sigma C5138, 700 U/ml) and 0.05 mg/ml trypsin inhibitor. After wash-out of the enzymes, free-floating afferent arterioles free of connective tissue and with an agar-filled
lumen were harvested using a 150 μm tip-diameter pressure-controlled glass pipette. Since afferent arterioles from areas with strong vascular fibrosis are embedded in more matrix protein, it is possible that fibrotic vessels are underrepresented. The isolation procedure was performed in Ca\(^{2+}\)-free buffer which improved viability of the vessels. Vessels were kept up to 4 hours after isolation before recordings.

**Mean lumen diameter and afferent arteriole hypertrophy measurements.**

Isolated vessels were attached by self-adhesion to the clean glass surface of a No 0 cover slip mounted in a Petri dish (MatTek P35G-0-14-C) containing 3 ml medium without Ca\(^{2+}\). Before the experiments, Ca\(^{2+}\) concentration in the medium was gradually increased to 2 mmol/L in 3 steps (20 μmol/L, 200 μmol/L, and 2 mmol/L) with 3-5 min incubation in between. To stimulate the vessels, 300 μl medium was aspirated from the dish, and the same volume containing 10\(^{-8}\) mol/L endothelin-1 was gently superfused (~100 μl/s) over the vessels using a 1 ml pipette (Gilson), producing a final concentration of 10\(^{-9}\) mol/L endothelin-1 in the vessel bath. Brightfield images (Leica SP2 camera with 40x objective, 512x512 pixels, field of view 480x480 μm\(^2\), optical resolution 0.9 μm) were acquired at baseline and every 30 seconds for 6 minutes after addition of the agonist. The inner outline of the vessel-wall was traced using Olympus DP-Soft 5.0 software. Mean lumen diameter was calculated using the formula mean lumen diameter = lumen area / lumen length. Mean vessel wall thickness was calculated using the formula mean vessel wall = ((arteriole area – lumen area) / 2) / arteriole length. Media / lumen ratio was calculated as mean vessel wall thickness / mean lumen diameter. Up to two arterioles from each kidney were used for contractility experiments, 10 to 17 arterioles were used in each experimental group. For measurements of afferent arteriole vessel wall thickness and media/lumen ratio, 1-3 arterioles were harvested from each kidney, 17 to 28 arterioles were used in each experimental group.

**Urinary Protein Excretion and Plasma Creatinine**
Urine samples were collected for 4h period every second day. Urinary protein concentration was normalized to creatinine concentration and values were expressed as g protein/mmol creat. Blood samples were withdrawn on the last day of the study and plasma creatinine (µmol/L) was measured by automated Jaffe’s method.

Renal Histology

Kidneys from at least 10 animals/group were stained with Masson’s trichromic solution. Sections of kidneys were examined on a blinded basis by two investigators independently to estimate inflammation, glomerular ischemia, glomerular sclerosis, and vascular necrosis using a 0 to 4+ injury scale as described previously (3-5). Lesion indexes from individual sections were averaged to calculate a sclerotic index for each mouse. At least 100 glomeruli were scored to estimate the lesion index of an animal.

Immunohistochemistry for CD3 and CD68

Four-µm-thick sections of paraffin-embedded kidneys were dewaxed, heated in citric acid solution at 98° C for 30 min and incubated first with a polyclonal goat anti-rat CD3 antibody recognizing lymphocytes (Santa Cruz Biotechnology, Santa Cruz, CA) or a monoclonal mouse anti-rat CD68 antibody recognizing macrophages/monocytes (Serotec, Oxford, UK), respectively, and then, incubated at room temperature with a second antibody from Envision kit (Dako, Carpinteria, CA). Staining was revealed by applying AEC (Dako), counterstained with hematoxylin QS (Vector, Burlingame, CA) and finalized with Permanent Aqueous Mounting Media (Innovex). Quantification of CD3 and CD68 positive cells was performed using the Olympus analysis software.

Immunohistochemistry for RECA-1
RECA-1 expression was revealed using a monoclonal mouse anti-rat RECA-1 antibody (1:50, MCA970R, Serotec) on paraffin-embedded kidney tissue. After incubation with the primary antibody, sections were washed in PBS and incubated for 30 min with a streptavidin secondary antibody (Histofine Kit Solution), and then counterstained with hematoxylin followed by immersion in Scott’s tap water. Finally, slides were mounted with aqueous mounting medium (DAKO).

Statistical Methods

Statistical analyses for the in vivo studies were performed using ANOVA followed by Protected Least Significance Difference Fisher's test of the Statview software package. Diameter values, vessel wall thickness and media/lumen ratio were compared using One way ANOVA with Student-Newman-Keuls post hoc test using the SigmaStat 3.1 software. Results with $p < 0.05$ were considered statistically significant. All values are means ± SEM.

RESULTS

Partial improvement of renal hemodynamics and function following arrest of L-NAME administration

In agreement with previous results, NO inhibition was accompanied by a gradual increase of mean arterial pressure reaching 190-200 mmHg after 3 weeks. Mean arterial pressure remained at this level until the beginning of therapy (Fig 1). Abnormal proteinuria ($>0.5 \text{ g/mmole Creat}$) was detected as early as one week after L-NAME administration and progressed to $>2 \text{ g/mmole Creat}$ after 4-6 weeks. Pilot experiments showed that when proteinuria exceeded 3 g/mmole Creat, animals were vanishing rapidly showing symptoms of malignant hypertension and/or stroke. In contrast, when proteinuria was lower than 2 g/mmole Creat, the degree of renal lesions was not very pronounced. For these reasons, the endpoint for L-NAME treatment was set to values of proteinuria exceeding 2 g/mmole Creat. At the therapy
starting point, RBF was profoundly decreased and concentration of plasma creatinine was increased (Fig. 1).

To test whether renal disease is a reversible process when animals showed such high degree of proteinuria, L-NAME was removed from the drinking water. It has been previously shown that this manipulation reacti"
The addition of EGF receptor antagonist did not have an additive effect on the reduction of blood pressure observed after removal of L-NAME alone; blood pressure remained increased in this group of animals compared to control or to removal + losartan groups (p>0.3 vs removal; p<0.01 vs control or removal + losartan group, Fig 1). In contrast, renal blood flow was increased and reached values similar to control or to removal + losartan groups (Fig 1).

**Impairment and recovery of renal morphology**

The above-mentioned functional and hemodynamic changes were associated with important structural alterations (Fig 2). Confirming previous results, semi-quantitative estimation of renal morphological parameters showed that L-NAME treatment was accompanied by glomerular ischemia (1.16 ± 0.20), glomerulosclerosis (0.85 ± 0.21), vascular necrosis (1.28 ± 0.30) and inflammation (Fig 3) (p<0.01 for these four parameters). Mixed results were obtained with the evaluation of morphological parameters after the arrest of L-NAME: vascular necrosis almost disappeared (0.20 ± 0.10), whereas glomerular ischemia (0.95 ± 0.19), glomerulosclerosis (1.08 ± 0.30) and inflammation (Fig 3) remained similar to the L-NAME group. The addition of the EGF receptor antagonist gefitinib improved glomerular ischemia (0.16 ± 0.10, p<0.01 vs L-NAME), glomerulosclerosis (0.42 ± 0.14, p<0.05 vs L-NAME) and vascular necrosis (0.10 ± 0.05, p<0.01 vs L-NAME), but had little effect on cellular infiltration (Fig 3). To better characterize cell infiltrates, experiments were performed with antibodies specific to macrophages (CD68 positive cells) and lymphocytes (CD3 positive cells). As shown in Fig 3 (middle and left panels), the abnormal presence of macrophages and lymphocytes persisted during gefitinib treatment despite the improvement of RBF.

The inflammatory index and CD3 and CD68 staining were significantly decreased only in animals treated with AT1 receptor antagonist (no statistical difference vs. control group; p < 0.05 vs. removal of L-NAME, Fig 3), indicating that this therapeutic approach produced an additional protective effect that attained an almost complete reversal of renal disease despite the extensive proteinuria at the beginning of the treatment.
**Impairment and recovery of endothelial integrity**

The clear peritubular staining of RECA-1 in control animals (Fig 4A) became negligible in the L-NAME group (Fig 4B), and was not improved substantially after removal of L-NAME (Fig 4C). In contrast, it reappeared to normal levels after losartan or gefitinib treatment (Fig 4D&E).

**Ex vivo contractility of freshly isolated afferent arterioles**

To test whether the applied treatments altered differently the function and contractile properties of renal resistance vessels, contractility to endothelin-1 was measured in freshly isolated afferent arterioles (1-2 afferent arterioles from each kidney in 5 animals from all groups Fig 5). Ang II administration was not considered suitable for this purpose, because a group of animals was chronically treated with an AT1 antagonist and in preliminary experiments we found that vessels from this group were not responding to Ang II. In addition, we have previously observed that the major pathological mechanism leading to renal disease in the L-NAME model is an Ang II-induced activation of endothelin within renal resistance vessels (3, 4, 29). In preliminary dose-response experiments, we observed that afferent arterioles from control animals did not contract to $10^{-9}$ M endothelin-1, and thus we considered that contractility at this concentration would signify an abnormal sensitivity to endothelin-1. Indeed, vessels isolated from the L-NAME group gradually contracted at $10^{-9}$ M endothelin-1: 8%, 13%, 25% and 38% decrease of lumen diameter at 40 sec, and 2-, 4-, and 6 min, respectively (Fig 5). In vessels isolated from the removal group contractile response to endothelin-1 remained abnormal. This contrasted responses of vessels from losartan- or gefitinib-treated animals which were similar to control (Fig 5). Statistical differences were the same whether results are expressed as absolute changes or percentile change from baseline.
**Afferent arteriole vessel wall thickness**

As a measure of microvascular hypertrophy, vessel-wall thickness was analyzed in isolated afferent arterioles *ex vivo*. The afferent arteriole vessel wall and media / lumen ratio was significantly increased in arterioles from L-NAME and removal groups (Fig 6). With losartan or gefitinib, however, microvascular hypertrophy regressed completely (Fig 6).

**DISCUSSION**

In the present study, we investigated mechanisms of regression of renal disease by focusing on the relationship between renal hemodynamics and structural and functional alterations. A partial regression was achieved by correcting the cause of the pathology (inhibition of NO synthesis). A novel finding is that improvement of renal hemodynamics is associated with EGF receptor antagonism, and is under the control of local vasoactive systems operating independently of their systemic effects. Interestingly, this beneficial effect does not apply to the inflammation. To achieve a complete recovery of renal structure is necessary to associate angiotensin II receptor antagonism.

Alterations in endothelial function and in the activity of NO play a major role in vascular remodeling. NO is an important inhibitor of vascular smooth muscle cell growth and extracellular matrix synthesis in vitro and in vivo (15), whereas chronic inhibition of NO synthesis is accompanied by renal vascular fibrosis (1, 32). Conversely, removal of L-NAME from the drinking water normalized collagen I gene expression in transgenic mice harbouring the luciferase gene under the control of the promoter of collagen I-chain α2 gene (24). However, mice are resistant compare to rats in developing proteinuria or renal lesions. Even if L-NAME administration was prolonged up to 30 weeks, no proteinuria or important alterations in renal structure was observed (24). For this reason we used rats as experimental model in the present study, and we waited until the appearance of considerable proteinuria (>2 g/mmol Creat) before removing L-NAME. The major observation from this part of the study is that correction of the cause certainly improved, but was not sufficient to completely restore renal
hemodynamics, structure and function which remained below normal values. This partial improvement could be due to the systemic vasodilatory action of NO. For instance, vascular necrosis was decreased reflecting apparently the lowering of blood pressure after the removal of L-NAME. In contrast, glomerular ischemia or afferent arteriole hypertrophy remained abnormal, reflecting the incomplete improvement of renal blood flow. Similarly, removal of L-NAME was inefficient against renal inflammation, as the measured inflammatory indexes remained abnormally high. Furthermore, staining with the RECA-1 antibody, a marker of rat endothelial cells, demonstrated severe endothelial dysfunction during L-NAME treatment, which did not improve unless rats were treated with losartan or gefitinib. These observations imply that other vasoactive and/or pro-fibrotic systems are still operating after L-NAME removal.

One possible candidate is endothelin, which is known to be activated in conditions of NO inhibition. Indeed, in previous studies we found that inhibition of NO synthesis was accompanied by increased expression of endothelin which in turn activates collagen I gene (6, 29). In following studies however, we demonstrated that the activation of endothelin during NO inhibition was due to angiotensin II and that the progression of renal fibrosis in the L-NAME model was controlled by the interaction between NO and the renin-angiotensin system (3, 9). In agreement with this hypothesis, other investigators found that the intrarenal RAS is activated during NO inhibition (14). In addition, we have observed that endothelin transactivated the EGF receptor and the MAP kinase pathway to stimulate collagen I gene and to induce fibrosis (12). Based on these previous observations, we postulated that an additional EGF and/or AT1 receptor blockade could be an appropriate supplementary therapy. The fact that combination of L-NAME removal with EGF or AT1 antagonism fully restored renal hemodynamics, and reversed most of renal lesions, clearly indicates that both therapies can be used to achieve regression of renal disease, at least in this model. The AT1 receptor antagonism however, appeared to be a better treatment because of its efficiency on renal inflammatory lesions.
The mechanism(s) by which blockade of angiotensin receptor contributes to reversal of renal disease are probably multiple. We have previously shown that angiotensin II antagonism can regress renal fibrosis by inhibiting the de novo synthesis of extracellular matrix (due to the blockade of TGFβ action) associated to increased matrix degradation (due to metalloproteinase activity), both mechanisms acting independently of systemic hemodynamics (5). In the present study, we report additional mechanisms: Improvement of renal hemodynamics, suppression of inflammation and regression of microvascular hypertrophy. The information obtained from the histology and the afferent arterioles contractility experiments (an ex vivo setting in which circulating factors are not implied) supports the notion that the improvement of renal hemodynamics during AT1 antagonism was due to local mechanisms improving the structure of renal resistance vessels. It is interesting to note that changes in the endothelial phenotype may increase vascular resistance, since animals displaying low or absent RECA-1 staining (L-NAME and removal groups) showed also exaggerated vascular reactivity to endothelin-1. We have previously showed that Ang II activates endothelin during L-NAME treatment (5). The action of endothelin under normal conditions can be considered as complicated, mediating constriction (through ET_A) or relaxation (through ET_B) depending on the cell population. Both receptor subtypes are expressed on renal vessels (31), and depending on the conditions either ET_B antagonizes endothelin-1 action through NO and NO-independent mechanisms (19) or mediates a part of endothelin-1-induced decrease of renal blood flow (18, 28). Our previous findings clearly indicated that both receptor subtypes are involved in collagen I gene activation and renal fibrosis during chronic inhibition of NO synthesis (5, 6, 29).

An exaggerated local action of Ang II can also explain the effectiveness of AT1 antagonism against renal inflammation. Indeed, in previous studies we have observed that the renin-angiotensin system was activated in the renal cortex after 4-6 weeks of L-NAME, because of an upregulation of the angiotensin converting enzyme activity (30), and several investigators have described the pro-inflammatory action of angiotensin II in renal vessels and glomeruli (26, 27). In agreement with these observations, we have found important influx of
inflammatory cells in the renal cortical tissue after chronic infusion of angiotensin II or in transgenic mice expressing high levels of renin (10, 17).

Growth factors and their receptors have been proposed to mediate angiotensin II-induced alterations on renal structure and hemodynamics. For instance, the PDGF family has been recently involved in the pro-fibrotic action of angiotensin II in chronic renal disease (8, 11). Regarding renal vascular contractility and hemodynamics, it was demonstrated that the angiotensin induced-calcium influx in afferent arterioles was partly mediated through activation of the EGF receptor (7) and it has been proposed that EGF receptor mediates contraction in arteries from DOCA-salt, but not normotensive rats (23). Mice overexpressing a dominant negative isoform of EGF receptor were protected from renal lesions during chronic Ang II infusion, and a similar protection was also observed in mice lacking TGF-alpha, an endogenous ligand of EGF receptor (20). Moreover, we have observed that EGF receptor was activated within glomeruli concomitantly to the development of glomerulosclerosis in the NO deficiency model and that use of an EGF receptor antagonist in a preventive way, cancelled the activation of collagen I gene expression and prevented kidneys against the development of renal fibrosis (10, 12). In the present study, we show that the antagonism of EGF receptor can have curative effects with regards to renal hemodynamics and microvascular hypertrophy. However, blockade of the EGF receptor did not reverse the inflammatory action of angiotensin II, indicating that the angiotensin II effect on renal hemodynamics is distinct from that on inflammation. In this regard, we have demonstrated recently that the discoidin domain receptor 1 (DDR1), a tyrosine-kinase collagen receptor, is a major mediator of the angiotensin II-induced renal inflammation (10). In agreement with this hypothesis, DDR1 expression is induced in renal vessels after chronic inhibition of NO, whereas mice lacking DDR1 are protected against L-NAME-induced renal inflammation (data not shown).

In conclusion, the present study investigated reversal of renal disease by starting therapy after reaching a substantial degree of proteinuria. Correction of the cause partially improved renal hemodynamics and incompletely reversed renal structural lesions. Renal
hemodynamics and microvascular hypertrophy, but not renal inflammation, were restored when correction of the cause was combined with EGF receptor antagonism. Reversal of renal disease was obtained only when correction of the cause was combined with angiotensin blockade. These results provide novel insights in the mechanisms of progression and/or regression of chronic renal disease and suggest that improvement of renal hemodynamics and reversal of inflammation are important targets in order to achieve therapy.
ACKNOWLEDGEMENTS

This work was financially supported by the "Institut National de la Santé et de la Recherche Médicale" and the "University Pierre et Marie Curie". An important part was performed in the technical platform of “Explorations Fonctionnelles Rénales du petit animal” of IFR65-St Antoine. Parts of this work have been presented as an abstract in the ERA-EDTA 2007 annual meeting.

F. Helle and C. Chadjichristos were post-doctoral fellows of the "European Renal Association-European Dialysis and Transplantation Association". C. Jouzel and D. Guerrot were research fellows of the “Fondation pour la Recherche Médicale” and “Assistance Publique”, respectively.
REFERENCES


LEGENDS

FIGURE 1. Mean arterial pressure, renal blood flow, plasma creatinine concentration and urinary protein excretion measured in controls (open) and rats treated with L-NAME (closed bars); animals in which L-NAME was removed without or with addition of losartan or gefitinib for 2 additional wk are depicted in gray, diagonal, or dotted-line bars, respectively. Values are means ± SEM; n=16, 14, 20, 18 and 20 for control, L-NAME, removal, removal+losartan and removal+gefitinib groups, respectively. * P < 0.01 vs. control; # P < 0.01 vs L-NAME; § P < 0.05 vs removal.

FIGURE 2. Representative examples of extracellular matrix (Masson’s trichrome) in control animals (A), rats treated with L-NAME (B), and rats in which L-NAME was removed without (C) and with addition of losartan (D) or gefitinib (E). Note the extracellular matrix accumulation and the inflammatory cell infiltration during chronic inhibition of NO (B); inflammation persisted after removal of L-NAME (C), whereas all types of lesions clearly regressed when removal of L-NAME was combined with losartan administration (D); in contrast in the gefitinib group(E) the inflammatory lesions persisted.

FIGURE 3. Quantification of inflammatory index (left), and lymphocyte and macrophage staining (middle and right, respectively) in controls (open) and rats treated with L-NAME (closed bars); animals in which L-NAME was removed without or with addition of losartan or gefitinib for 2 additional wk are depicted in gray, diagonal, or dotted-line bars, respectively. Values are means ± SEM; n=16, 14, 20, 18 and 20 for control, L-NAME, removal, removal+losartan and removal+gefitinib groups, respectively. * P < 0.01 vs. control; # P < 0.05 vs L-NAME; § P < 0.05 vs removal.

FIGURE 4. Representative examples of the endothelial marker RECA-1 staining in control animals (A), rats treated with L-NAME (B), and rats in which L-NAME was removed without (C) and with addition of losartan (D) or gefitinib (E). Note that RECA-1 staining was substantially decreased in glomerular and peritubular capillaries during chronic inhibition of
NO (B) and remained low after removal of L-NAME (C). In contrast, RECA-1 staining was restored in glomeruli and peritubular capillaries (arrows) after losartan (D) or gefitinib (E) administration. (F): Quantification of functional endothelium in controls (open) and rats treated with L-NAME (closed bars); animals in which L-NAME was removed without or with addition of losartan or gefitinib for 2 additional wk are depicted in gray, diagonal, or dotted-line bars, respectively. Values are means ± SEM; n=16, 14, 20, 18 and 20 for control, L-NAME, removal, removal+losartan and removal+gefitinib groups, respectively. * P < 0.01 vs. control; # P < 0.01 vs L-NAME; § P < 0.01 vs removal.

FIGURE 5. Endothelin-1-induced vasocontraction expressed either as absolute (left) or as % of baseline (right panel) changes of the diameter of afferent arterioles freshly isolated from kidneys of control (open bars) and rats treated with L-NAME during 4-6 wk (closed bars); animals in which L-NAME was removed after 4-6 wk without or with losartan for 2 additional wk are depicted in gray and diagonal bars, respectively. Endothelin-1 concentration was 10⁻⁹ M. Values are means ± SEM; n = 11, 17, 14, 13 and 12 afferent arterioles from 5 animals in control, L-NAME, removal, removal+losartan and removal+gefitinib groups, respectively. * P < 0.05 vs. control.

FIGURE 6. Vessel-wall thickness and media/lumen ratio in the afferent arterioles were increased in animals from the L-NAME (closed bars) and removal groups (gray bars) compared to controls (open bars). In losartan (diagonal bars) and gefitinib (dotted-line bars) treated animals, however, microvascular hypertrophy was completely regressed; n = 20, 20, 25, 17 and 28 afferent arterioles from 5 animals in control, L-NAME, removal, removal+losartan and removal+gefitinib groups, respectively. * P < 0.05 and ** P < 0.01 vs. control, # P < 0.01 vs L-NAME; § P < 0.01 vs removal.
Mean Arterial Pressure (mm Hg)

Renal Blood Flow (ml/min)

Plasma Creatinine (µmol/l)

Proteinuria (g/mmol creat)

Figure 1
Figure 2
Figure 3
Figure 4
Figure 5

Afferent arteriole diameter (µm)

ET-1 10⁻⁹M

Afferent arteriole diameter (% of baseline)

Removal + AT1 ant
L-NAME
Removal
Removal + EGFR ant

Control

Figure 5
Figure 6