The renal antifibrotic effects of angiotensin-converting enzyme inhibition involve bradykinin B2 receptor activation in angiotensin II-dependent hypertension

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Objective **The renoprotective action of angiotensin I-converting enzyme inhibitors (ACE-Is) is well established, but the role played by bradykinin (BK) remains unclear. We therefore investigated whether an enhanced BK effect on B2 receptor subtype mediated the antifibrotic effect of ACE-Is and whether neutral endopeptidase (NEP) inhibition, which can blunt BK degradation more effectively than ACE inhibition, provided further renoprotection in a rat model of angiotensin (Ang) II-dependent renal damage.**

Methods **Five-week-old *Ren-2* transgenic rats (TGRen2) received, for 8 weeks, a placebo, ramipril (5 mg/kg body weight) or the dual ACE + NEP inhibitor MDL 100,240 (MDL) (40 mg/kg body weight). After 4 weeks, the B2 receptor antagonist icatibant (0.5 mg/kg body weight) was administered on top of active treatment for 4 weeks to 50% of the TGRen2 rats. Blood pressure was measured weekly by a tail-cuff method and, after sacrifice, kidney weight, glomerular volume, density of glomerular profiles were measured; tubulo-interstitial fibrosis, glomerular and perivascular fibrosis were quantified by histomorphometry.**

Results **The development of hypertension and tubulo-interstitial fibrosis was prevented by both ramipril and MDL ($P = 0.0001$ versus placebo); icatibant annulled the latter effect. Glomerular and perivascular fibrosis were unaffected by either ramipril or MDL alone; however, combined treatment with icatibant enhanced glomerular fibrosis ($P = 0.0001$ versus placebo).**

Conclusion **Enhanced BK effect on B2 subtype receptors is essential for the prevention of tubulo-interstitial fibrosis with ACE or dual ACE + NEP inhibition in TGRen2 rats. *J Hypertens* 24:1419–1427 © 2006 Lippincott Williams & Wilkins.**

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Introduction

Arterial hypertension involves progressive renal damage that can be prevented by antihypertensive treatment with ACE inhibitors (ACE-Is) or angiotensin type 1 receptor blockers [1–3]. Understanding of the renoprotective mechanisms of these agents, albeit still imperfect, is therefore important. Abundant evidence for a pivotal role of angiotensin (Ang) II in inducing renal fibrosis [4,5], a marker of renal damage [6], exists. Hence, ACE-Is can exert their favourable actions not only by lowering blood pressure (BP), but also by decreasing Ang II formation [7,8]. However, ACE-Is also blunt bradykinin (BK) degradation [9], and therefore their beneficial effects could involve enhanced BK effects [10]. Nonetheless, data on the antifibrotic effects of BK and the involvement of B2 receptor subtype are scarce and conflicting [11–16].

Neutral endopeptidase (NEP) 24.11 (EC 3.4.24.11) is the prototype of a family of zinc metallopeptidases participating in the post-secretory processing and metabolism of such vasoactive peptides as BK, atrial (ANP) and brain natriuretic peptide (BNP) and adrenomedullin [17,18]. NEP entails a substantial amount (about 4–5%) of the protein content in the renal epithelial cell brush border, where it may regulate the bioactivity of these peptides [18]. Therefore, NEP blockade might enhance the effects of peptides that inhibit cell proliferation and collagen deposition in the kidney. Hence, dual ACE- and NEP-inhibitors may provide a tool to gain insight on the renoprotective role of these peptides, including BK.

We therefore sought to investigate whether the antifibrotic effects of an ACE-I and of a dual ACE + NEP inhibitor involved enhanced BK effects on B2 receptor,

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and whether dual ACE + NEP inhibitor conferred additional renoprotection, as compared to the ACE-I alone, in the transgenic rat carrying the mouse *Ren2* gene (TGRen2), a model of severe hypertension and cardiovascular and renal damage induced by excess tissue Ang II [19–21].

Methods

Animals

The protocol of this experiment was described in detail previously [22] and therefore will be only briefly reported. Male heterozygous TGRen2 rats at age 5 weeks, body weight (bw)- and BP-matched, were allocated randomly to receiving by gavage one of the following treatments: a placebo ($n = 5$), the ACE-I ramipril (5 mg/kg bw) ($n = 10$) [23] or the dual inhibitor MDL 100,240 (MDL) (40 mg/kg bw) ($n = 11$), which has a high affinity and a balanced competitive activity against both NEP and ACE (reviewed by Rossi [24]).

As at this age the rats are still growing, drug dosages were adjusted weekly to each rat body weight, as described previously [22]. After 4 weeks of either active treatment, animals were assigned randomly to either continue for additional 4 weeks with ramipril ($n = 5$) or MDL alone ($n = 6$), or to receive also the BK B2 receptor antagonist icatibant ($n = 5$, each group). The latter was administered subcutaneously (0.5 mg/kg bw) via osmotic minipumps (Alzet 2ML4; Charles River, Calco, Italy).

These dosages of MDL and ramipril were chosen because they were shown to induce antihypertensive effects; the dosage of icatibant was selected because it provided BK B2 receptor blockade in chronic studies [22].

Body weight and BP levels (tail-cuff method) were measured at weekly intervals. At the end of the treatment period, the rats were weighed and sacrificed by cervical dislocation. The kidneys were quickly removed, weighed, and snap frozen in isopentane pre-cooled on dry ice. They were thereafter kept stored in liquid nitrogen until analysed.

Drugs

Ramipril and icatibant were the kind gift of Dr Juergen Puenter (Aventis Pharma, Frankfurt am Main, Germany); MDL was a gift of Dr Joel Covinsky (Aventis Inc., Parsippany, New Jersey, USA). The study protocol and animal handling followed our institutional guidelines for animal studies.

Biochemical variables

Blood samples were collected at sacrifice from the open chest cage for measurements of serum creatinine (standard biochemical technique), plasma ANP (Eiken Chemical Ltd, Tokyo, Japan; radioimmunoassay) and aldosterone (Ares Serono, Milan, Italy; radioimmunoassay) levels.

Glomerular volume and profile numerical density

Transverse serial sections (5 μm thick) of the kidney stained with Masson's trichrome were examined by a Leitz Laborlux D microscope (Leica Microsystems GmbH, Heidelberg, Germany) equipped with SC Casti Imaging software (Casti Imaging, Mira, Italy). Fifteen cortical areas were selected randomly in each section at magnification 4×; the numerical density, expressed as glomerular profile numerical density per area was estimated in each area [25]. Glomeruli were counted, considering only well-preserved structures and not glomerular areas crossing the outline of the examined field. Maximum and minimum diameters were traced on at least 25 profiles per rat and the mean glomerular volume was calculated by applying Schwartz's transformation for spheres [26]. The glomerular volume index was calculated as the glomerular volume/kidney weight ratio.

Quantitative analysis of kidney fibrosis

Kidney sections were stained with Sirius red (0.5% Sirius red F3BA in saturated picric acid) to visualize selectively fibrillar collagen. A quantitative analysis was performed by a single examiner blind to treatment, using a photomicroscope Leica DM equipped with QWin Standard LeicaTM Image Analysis Software (Leica Microsystems GmbH), and running specific routines that were created to estimate fibrosis in an operator-independent fashion. After acquiring full-colour (24-bit) images, colour thresholding was applied to identify the Sirius red-stained structures, with exclusion of the pale-yellow picric-acid-stained tissue. We used a combination of hue, saturation and brightness (HSB) values to feature each pixel colour [27] with the 'hue' component recognizing the site where staining is located, 'saturation' estimating the stain amount and 'brightness' indicating the overall density of the stained specimen. Sirius red-stained structures were identified by setting thresholds for the 'hue' component leading to pixel selection in the red range, and levels for 'saturation' lower than the mean saturation level measured in the background minus two standard deviations. The binary image resulting from the colour thresholding allowed us to measure the area corresponding to Sirius red-stained collagen in an accurate and highly reproducible fashion.

Since Sirius red staining increases the birefringence of the fibrillar collagen, thicker fibres (type I collagen) appear in red-orange shades, whereas thinner ones (type III collagen) appear as greenish-yellow shades under polarization microscopy [28]. Red-orange and greenish-yellow areas were identified by setting appropriate thresholds of the 'hue' component in the polarization-light image. The ratio of red-orange to greenish-yellow

areas (thick-to-thin fibrillar collagen ratio) was used as an estimate of collagen composition.

Glomerular fibrosis

Three sections were examined for each rat, at magnification ×10. Five views were captured randomly in each section in the cortical area in order to evaluate at least 100 glomeruli/rat. To measure collagen contents, which can be scarce in the glomeruli, identification of glomerular profiles was first performed under dark-field light microscopy, thus allowing an easy tracing of the glomerular profiles. The measurement of Sirius red-stained collagens was then performed only on glomerular profiles under bright field. Glomerular fibrosis was estimated as the percentage of total glomerular surface area pertaining to fibrillar collagen.

Tubulo-interstitial fibrosis

Four sections and 10 views corresponding to 294 mm^2 of the cortical region were examined for each rat, at magnification ×10. A pilot study showed that 10 views randomly captured from each section and roughly corresponding to 70–80 mm^2 accurately represented the entire section ($r = 0.91$). Interstitial collagen automatically detected as described above was quantified in each field as the percentage of total surface area pertaining to fibrillar collagen.

Perivascular fibrosis

Sirius red-stained sections were likewise examined, at magnification ×10, to evaluate small arteries, ranging between 50 and 250 μm in diameter, that roughly correspond to interlobar, arcuate and interlobular arteries. A variable number of views in 4–6 sections were examined to collect at least 50 arteries for each rat, because only cross-sectionally captured arteries were selected for the analysis. After identifying the Sirius red-stained collagen as described above, the stained area pertaining to the vessel profile (perivascular fibrosis) was selected and measured. To correct for differences in vessel size, the ratio perivascular fibrosis to the arterial section was then calculated for each artery. Strongly (red-orange) or weakly (greenish-yellow) birefringent fibres examined under polarization microscopy, roughly corresponding to thick (type I) and thin (type III) fibrillar collagen, respectively [21] provided an index of the collagen composition.

Statistical analysis

Results are expressed as mean ± SD or SEM. One-way analysis of variance (ANOVA) followed by least significant differences (LSD) post-hoc test for multiple comparisons and Student's *t*-test for unpaired data were used to evaluate differences across groups. Logarithmic or square-root transformations were adopted for variables that were not normally distributed; attainment of a Gaussian distribution was verified by the Kolmogorov–Smirnov test. For the thick-to-thin fibrillar collagen ratio, a normal distribution could not be achieved with the aforementioned transformations. Therefore, results are expressed as median and range for this variable, and the Kruskall–Wallis test was used for multiple comparisons across treatments groups. A P value lower than 0.05 was considered statistically significant. Analyses were carried out with the SPSS for Windows statistical package (version 13.0; SPSS Inc., Chicago, Illinois, USA).

Results

Blood pressure and kidney weights

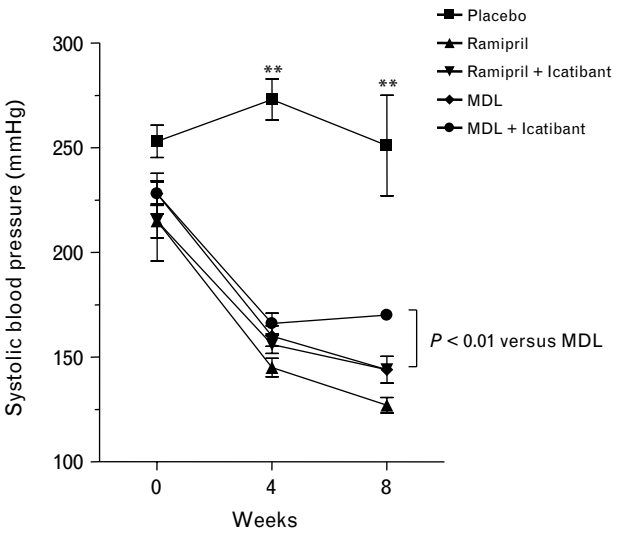
There were no significant differences in BP across groups at baseline. Both ramipril and MDL similarly decreased BP ($P < 0.01$), as compared to placebo. Icatibant blunted ($P < 0.01$) the BP-lowering effect of MDL (Fig. 1), but did not significantly affect that of ramipril. No significant differences in kidney weights were seen across groups (Table 1).

Biochemical variables

Both ramipril and MDL significantly lowered serum creatinine levels in treated rats, as compared to placebo ($P < 0.01$); icatibant blunted the decrease induced by either treatment.

ANP plasma levels were not significantly affected by ramipril or MDL; by contrast, plasma aldosterone levels

Fig. 1



Systolic blood pressure (BP, tail-cuff) measured in *Ren-2* transgenic rats (TGRen2 rats) receiving a placebo, ramipril [5 mg/kg body weight (bw)], the dual angiotensin-converting enzyme (ACE) + neutral endopeptidase (NEP) inhibitor MDL 100,240 (MDL) (40 mg/kg bw), or the active treatment combined with the bradykinin B2 receptor antagonist, icatibant (0.5 mg/kg bw), after 4 and 8 weeks of treatment. Both ramipril and MDL similarly decreased BP levels, as compared to placebo. Icatibant added from the beginning of the fifth week of the experiment blunted the BP-lowering effect of MDL, but not of ramipril. Values are means ± SEM. $^{**}P < 0.01$ versus all other groups.

Table 1 Atrial natriuretic peptide (ANP), aldosterone plasma levels and renal parameters after angiotensin-converting enzyme (ACE) inhibition or dual ACE and neutral endopeptidase (NEP) inhibition, and/or addition of the bradykinin B2 receptor antagonist icatibant in *Ren-2* transgenic(TGRen2) rats

	Placebo	Ramipril	Ramipril + icatibant	MDL 100,240	MDL 100,240 + icatibant
Kidney weight (mg)	1783 ± 25	1599 ± 30	1619 ± 25	1849 ± 50	1659 ± 45
Kidney weight/body weight (mg/g)	3.89 ± 0.13	3.72 ± 0.16	3.64 ± 0.03	3.81 ± 0.12	3.74 ± 0.07
Serum creatinine (μmol/l)	0.77 ± 0.05	0.50 ± 0.03**	0.61 ± 0.04	0.50 ± 0.02**	0.59 ± 0.02
ANP (pmol/l)	609 ± 175	555 ± 61	692 ± 142	722 ± 41	638 ± 132
Plasma aldosterone (pmol/l)	710 ± 153	339 ± 117	284 ± 32*	213 ± 43**	236 ± 30*
Profile numerical density (number/mm^3)	32.3 ± 4.3	34.7 ± 3.3	32.6 ± 2.1	28.7 ± 1.6	31.2 ± 1.7
Glomerular volume (μm^3 × 10^6)	4.64 ± 0.13	4.05 ± 0.10**	3.84 ± 0.91**	4.25 ± 0.13*	4.02 ± 0.11**
Glomerular volume index (μm^3 × 10^6/mg kidney weight)	49 ± 0.78	49 ± 0.69	47 ± 0.61	46 ± 0.65**	47 ± 0.67
Glomerular fibrosis (%)	19.0 ± 0.7	19.1 ± 0.6	24.8 ± 0.7***,§§	21.8 ± 0.8	24.6 ± 0.8***,°°
Perivascular fibrosis (%)	0.61 ± 0.03	0.72 ± 0.04	0.48 ± 0.02**,§§	0.65 ± 0.03	0.64 ± 0.04
Thick-to-thin fibrillar collagen ratio	0.21 (0–4.76)	0.23 (0–5.99)	0.17 (0–9.80)	0.21 (0–7.91)	0.24 (0–5.30)

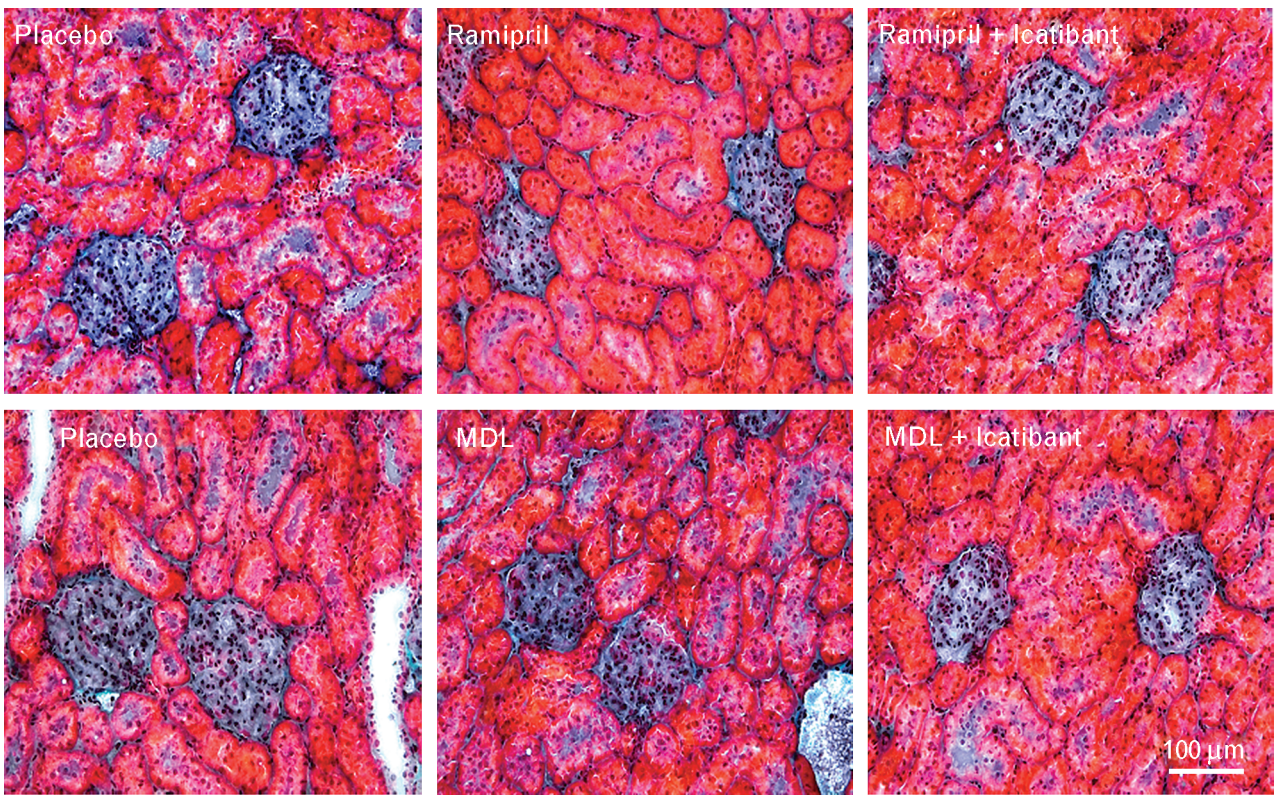
Values are means ± SEM, or median and range for thick-to-thin fibrillar collagen ratio. *$P < 0.05$, **$P < 0.01$, ***$P < 0.0001$ versus placebo; °°$P < 0.01$ versus MDL alone; §§$P < 0.01$ versus ramipril alone.

were lowered by MDL, while the decrease with ramipril did not achieve statistical significance (Table 1). Co-administration of icatibant did not induce any significant changes, as compared to the respective active treatment (Table 1).

Glomerular density and volume

The number of glomeruli identified in the serial cross-sectional sections of the kidney sections (Fig. 2) was similar in the ramipril and MDL treatment groups. The glomerular volume was significantly decreased by both ramipril and MDL, as compared to placebo (Fig. 2 and Table 1), but the decrease was unaffected by concomitant icatibant treatment. A trend toward a decrease in glomerular volume index was also noticed with all treatments; however, it reached statistical significance only in the MDL group ($P < 0.01$).

Fig. 2



Examples of 5-μm-thick slices stained with Masson's trichrome and chosen to entail equatorial section of glomeruli. These sections were used for the measurements of glomerular volume and profile numerical density in the different treatment groups. A decrease of glomerular volume was found after either ramipril or MDL alone, or combined treatments with icatibant (see Table 1).

Glomerular fibrosis and tubulo-interstitial fibrosis

Glomerular fibrosis was unaffected by either ramipril or MDL, as compared to placebo. However, icatibant, when administered concomitantly to either active treatment, increased glomerular fibrosis as compared to either active treatment (Table 1). By contrast, tubulo-interstitial fibrosis was markedly lowered by either ramipril or MDL; this effect was abolished by icatibant (Figs 3 and 4). Measurement of the thick-to-thin fibrillar collagen ratio was not feasible at either the glomerular or interstitial level, because of the faint signal detectable at these sites.

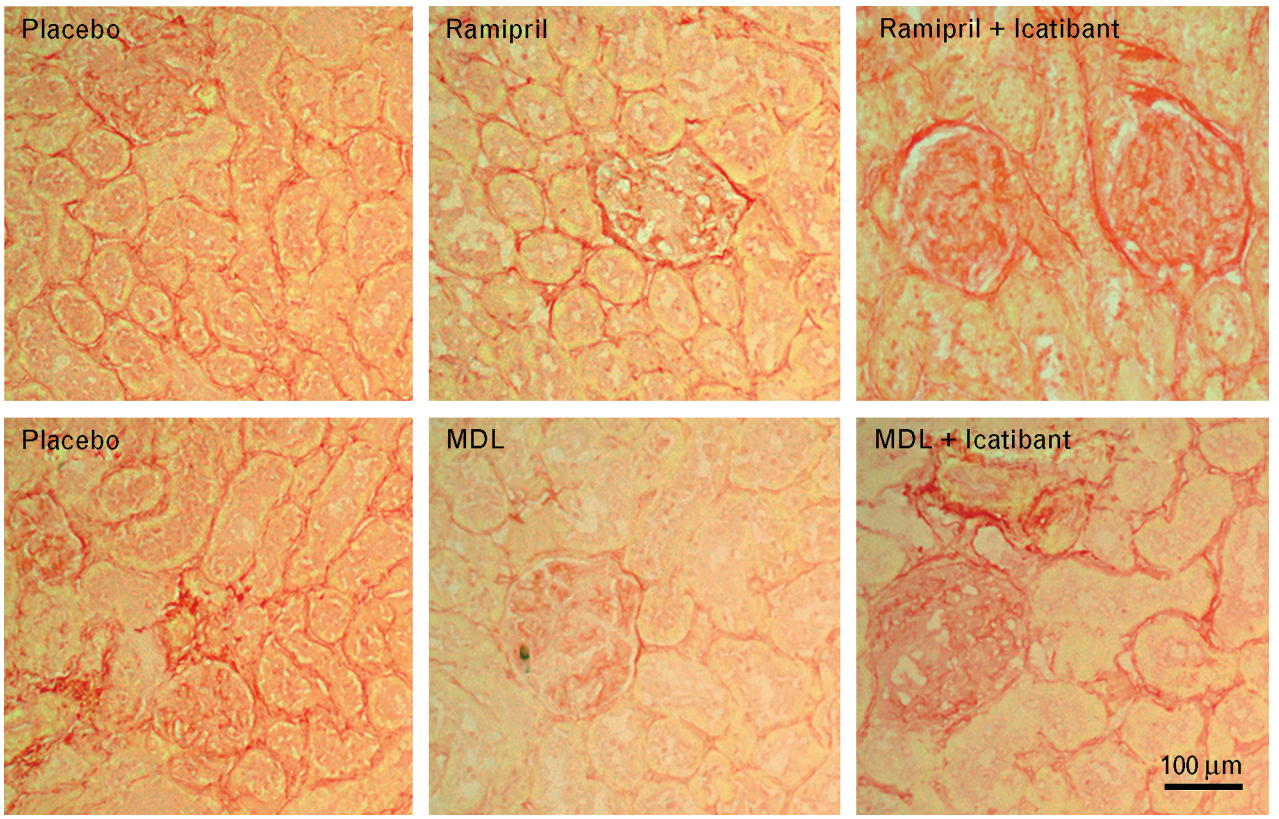
Perivascular fibrosis

Perivascular fibrosis was not affected by either ramipril or MDL, and by icatibant on top of MDL, whereas it was decreased by icatibant on top of ramipril. Although there was a trend for thick (red-orange) and thin (greenish-yellow) collagen fibrils to be, respectively, more and less abundant in ramipril- and MDL-treated rats, than in groups receiving placebo and icatibant (Fig. 5), there were no statistical differences of thick-to-thin fibrillar collagen ratio across treatment groups (Table 1).

Discussion

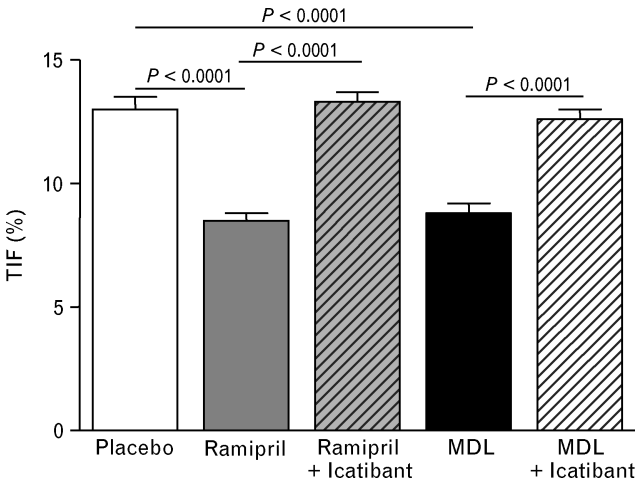
The prevention of renal fibrosis, which is a hallmark of kidney damage in several diseases involving the kidney, including arterial hypertension, and portends progression to end-stage kidney disease [5,29,30], is a major therapeutic challenge. In this context there is compelling evidence for a role of activation of the renin–angiotensin–aldosterone system, along with a high dietary salt [31]. Data also implicate this system in releasing cytokines, such as transforming growth factor-β1 (TGFβ1), plasminogen activator inhibitor-1 (PAI-1) and monocyte chemoattractant protein-1 (MCP-1), which can promote inflammatory responses and collagen accumulation in the kidney. By contrast, scant information exists on the role of BK in renal fibrosis [6,15,32,33]. It was recently shown that the dual ACE and NEP inhibitor omapatrilat prevented the development of glomerulosclerosis and collagen deposition in the renal cortex and medulla of TGRen2, although no information on tubulo-interstitial and glomerular fibrosis was provided [34]. An important renoprotective role of NEP inhibition, which can involve enhanced BK effects, was therefore suggested, albeit not

Fig. 3



Examples of 5-μm-thick slices of Sirius red-stained sections performed to selectively visualize and quantify fibrillar collagen of the kidneys in the different treatment groups. A decrease of tubulo-interstitial fibrosis is evident in the rats receiving either the angiotensin-converting enzyme (ACE) inhibitor or the dual ACE + neutral endopeptidase (NEP) antagonist. This decrease was abolished by the addition of icatibant. Quantitative analysis (see Fig. 4) confirmed these findings.

Fig. 4



Tubulo-interstitial fibrosis measured in 5 μm transverse serial sections of the kidneys of *Ren-2* transgenic (TGRen2) rats after angiotensin-converting enzyme (ACE) or dual ACE and neutral endopeptidase (NEP) inhibition, with ramipril and MDL, respectively, and/or addition of the bradykinin B2 receptor antagonist icatibant. Sections were stained with Sirius red (0.5% Sirius red F3BA in saturated picric acid) to visualize selectively fibrillar collagen, and examined blindly to treatment by a specific routine allowing operator-independent identification of the Sirius red-stained fibrils in the cortex. Four sections and 10 views corresponding to 294 mm^2 were examined for each rat, at magnification ×10. Tubulo-interstitial fibrosis was quantified in each field as the percentage of total surface area pertaining to fibrillar collagen. Values are mean ± SEM.

conclusively proven. The present results furnish some novel information on this issue by showing that a dual ACE + NEP inhibitor and an ACE-I, namely two drugs that can differentially enhance BK actions, similarly prevented renal fibrosis.

Tubulo-interstitial fibrosis

By combining a selective staining for collagen and a painstaking quantitative analysis of the distribution of this protein, we could detect the site at which ramipril and MDL exerted their antifibrotic effects. Importantly, we found that both ramipril and MDL decreased tubulo-interstitial fibrosis by 70% (Figs 3 and 4).

Previous studies suggested a major role of the B2 subtype receptor in mediating interstitial collagen deposition in rodent models of tubulo-interstitial fibrosis: activation of the B2 receptor subtype was claimed to result in extracellular matrix degradation and decreased renal tubular-interstitial fibrosis during ACE inhibition [11,15]. Our present findings that B2 BK receptor activation was necessary for prevention of tubulo-interstitial fibrosis in a transgenic rat model of severe hypertension caused by enhanced local synthesis of Ang II in the kidney are fully consistent with this hypothesis [20].

Of interest and consistent with results of clinical trials [1–3], the prevention of tubulo-interstitial fibrosis did not closely mirror the haemodynamic changes: icatibant blunted the prevention of tubulo-interstitial fibrosis with both the ACE-I and the dual ACE + NEP inhibitor, whereas it blunted the BP-lowering effects only of the latter. Thus, the enhanced BK effects on B2 subtype receptor mediate the antifibrotic effects of both agents, but is less important for the antihypertensive effect of ramipril than MDL.

Glomerular volume

The glomerular volume values obtained in our TGRen2 rats (Table 1) were similar to those reported with a similar methodology in other rat strains [25]. The detection of a higher glomerular volume in placebo-treated TGRen2 rats is novel because previous ultra-structural investigation of untreated heterozygous TGRen2 rats aged 4–6 months documented marked vascular and glomerular changes, but gave no information on glomerular volume [20]. This finding is in accordance with the increased glomerular volume found in other models of hypertension- and diabetes-driven renal damage [35,36]. Of note, glomerular volume was lowered by 9–14% with ramipril and MDL. This lowering, which can suggest a decrease of glomerular hypertrophy and/or of hyperfiltration, must be interpreted with caution because the perfusion fixed technique for collecting the kidney was not used in this study as in other, albeit not all, previous studies [35–37]. Nonetheless, it accords well with the hypothesis of systemic hypertension causing glomerular hypertension and hyperfiltration, ultimately leading to glomerulosclerosis [38].

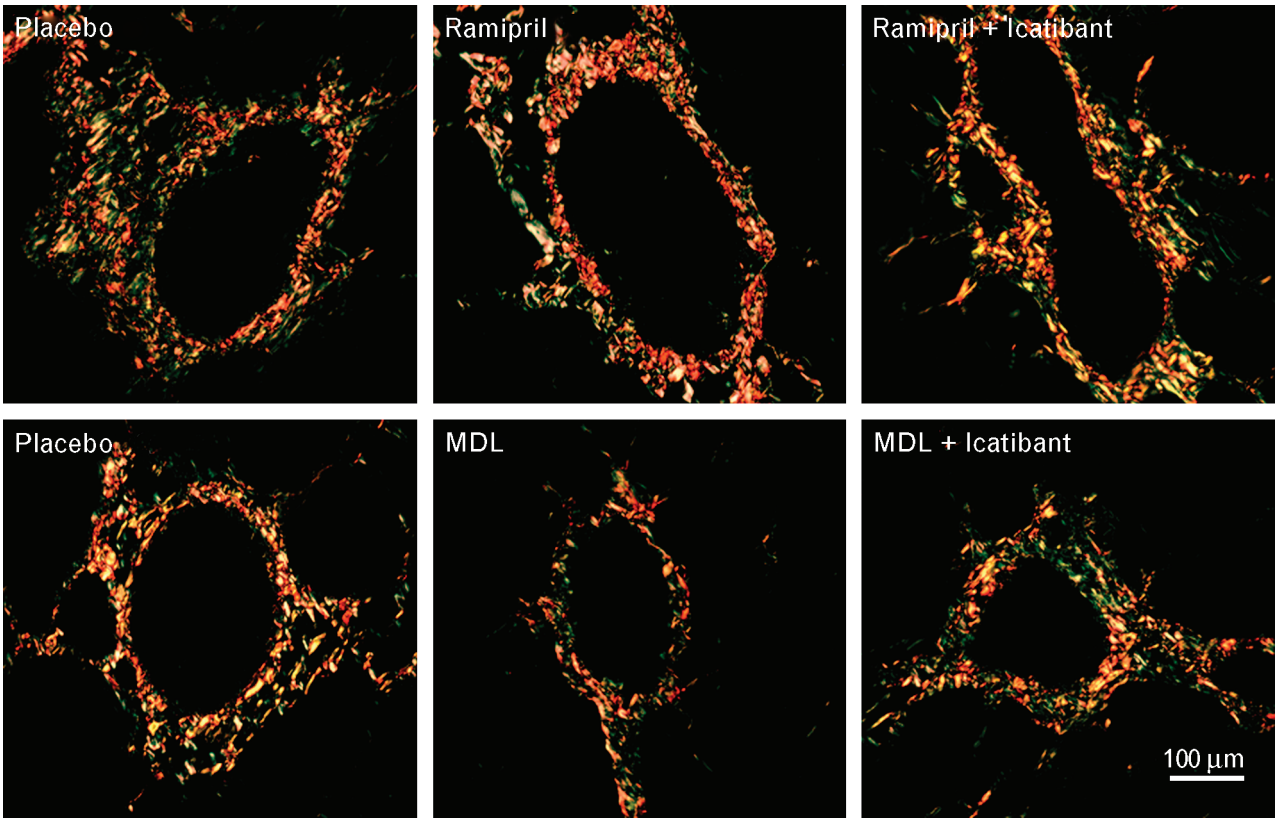
Of further interest, bradykinin B2 receptor blockade combined with ramipril or MDL did not affect glomerular volume. These data are also novel and fully consistent with findings concerning another bradykinin B2 receptor antagonist S16118 in spontaneously hypertensive rats (SHR) [37]. Overall, it indicates that glomerular volume is determined by multiple factors, rather than BK alone.

Glomerular and perivascular fibrosis

Glomerular fibrosis was not significantly prevented by either ACE-I or dual ACE + NEP inhibitor treatment alone, in keeping with data from deoxycorticosterone acetate (DOCA)-salt hypertensive rats [39]. However, it was enhanced by B2 subtype receptor blockade with icatibant on top of ramipril or MDL, thus further supporting the contention that the B2 receptor subtype activation in TGRen2 is implicated in the renoprotective effect of ACE-I and dual ACE + NEP inhibitors.

Prevention of tubulo-interstitial fibrosis with either inhibitor was not paralleled by a reduction of perivascular fibrosis or changes of the thick-to-thin fibrillar collagen

Fig. 5



Perivascular fibrosis in the kidney of *Ren-2* transgenic (TGRen2) rats after angiotensin-converting enzyme (ACE) or dual ACE and neutral endopeptidase (NEP) inhibition, with ramipril and MDL, respectively, and/or addition of the bradykinin B2 receptor antagonist icatibant, examined at polarization microscopy. Strongly (red-orange) and weakly (greenish-yellow) birefringent fibres roughly correspond to thick (type I) and thin (type III) fibrillar collagen, respectively. No significant difference was found in thick-to-thin fibrillar collagen ratio between treatments.

ratio. Hence, no blunting of these variables could be observed after B2 receptor subtype blockade. Unexpectedly, icatibant reduced perivascular fibrosis, as compared to ACE-I alone (Table 1). These intriguing observations may indicate that the renin–angiotensin–aldosterone and the BK systems, and possibly other factors, may contribute to fibrosis to different extents at different sites in the kidney in Ang II-dependent hypertension. We would like to recall in this context that in the heart of the TGRen2 rats perivascular fibrosis could be prevented by antagonizing the endothelin (ET)-1 system with bosentan, a mixed ET_A/ET_B receptor blocker [40,41]. The evidence for a role of ET-1 in tissue fibrosis, along with the demonstration that Ang II activates collagen synthesis in the renal vasculature via an ET-1-mediated mechanism, would therefore support an involvement of the ET-1 system [40,41]. Furthermore, B2 subtype blockade caused by icatibant could up-stimulate B1 subtype receptors [42] that, although undetectable in most tissues, may be rapidly induced by stress conditions, such as ischaemia or exposure to inflammatory cytokines, and can play a favourable role in the vessel wall remodelling during ACE inhibition [43]. Whether the B1 subtype receptor may protect the kidney against perivascular fibrosis remains to be investigated.

Role of hormonal changes

The decrease in plasma aldosterone levels under dual ACE + NEP inhibitor would suggest that blunted aldosterone production contributes to prevention of tubulo-interstitial fibrosis. However, the decrease of aldosterone levels with the dual ACE + NEP inhibitor alone and with the combined treatment with MDL and icatibant were similar, but only the latter treatment failed to prevent tubulo-interstitial fibrosis. This finding suggests that the favourable effect of lowering aldosterone was counterbalanced by a blunted extracellular matrix degradation resulting from B2 subtype receptor blockade [15].

In contrast with our expectations, but in accordance with previous findings with other dual ACE + NEP inhibitors [34,44,45], no significant changes of plasma ANP levels after treatment with MDL was observed. This result might be explained by several potential mechanisms, as discussed previously [22,34]. Furthermore, as TGRen2

is a model of activated natriuretic peptide system [46], no further increase of ANP levels can conceivably occur during chronic dual ACE + NEP inhibitor treatment.

In conclusion, this study shows that enhanced BK action on B2 receptors is crucial for the prevention of tubulo-interstitial fibrosis with ACE inhibition and dual ACE + NEP inhibition in a rat model of severe Ang II-dependent hypertension and renal damage. As both ACE and dual ACE + NEP inhibition effectively prevented tubulo-interstitial fibrosis, but not glomerular and perivascular fibrosis, we would like to suggest that the former is an earlier and more easily reversible marker of kidney disease than the latter. The alternative contention that the underlying mechanisms involve mediators other than BK deserves further specific research.

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