Role of angiotensin II, endothelin-1 and L-type calcium channel in the development of glomerular, tubulointerstitial and perivascular fibrosis

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Objective **Fibrosis is a hallmark of renal damage in several diseases, including arterial hypertension. We, therefore, investigated the role of angiotensin II, endothelin-1 and of L-type calcium channels in the development of the glomerular, vascular, and tubulointerstitial fibrosis in a model of severe angiotensin II-dependent hypertension.**

Methods **Five-week-old Ren-2 transgenic rats (TGRen2) received for 4 weeks a placebo, bosentan (100 mg/kg body weight), irbesartan (50 mg/kg body weight), the ET_A-selective endothelin receptor antagonist BMS-182874 (BMS; 52 mg/kg body weight), the combination of irbesartan (50 mg/kg body weight) plus BMS (52 mg/kg body weight), and nifedipine (30 mg/kg body weight).**

Results **Glomerular volume, tubulointerstitial fibrosis, glomerular, and perivascular fibrosis were accurately quantified by histomorphometry in four-to-six sections per kidney. Glomerular fibrosis was lowered by BMS ($P<0.001$), whereas tubulointerstitial fibrosis was blunted by bosentan ($P<0.001$) and irbesartan ($P<0.005$). Perivascular fibrosis was reduced by nifedipine and BMS. As only irbesartan and irbesartan plus BMS decreased blood pressure ($P<0.001$ vs. placebo), these effects on fibrosis were independent of blood pressure.**

Conclusion **Angiotensin II and L-type calcium channels modulate fibrosis selectively in the tubulointerstitial and in the perivascular compartments, respectively. The prevention of fibrosis with ET-1 receptor antagonism in all three compartments supports a major role of ET-1 in the development of renal fibrosis. *J Hypertens* 26:2022–2029 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Keywords: angiotensin-converting enzyme-inhibition, endothelin-1, kidney fibrosis, L-type calcium channels

Abbreviations: ACEI, angiotensin I converting enzyme inhibitors; Ang I, angiotensin I; Ang II, Angiotensin II; ARBs, angiotensin type I receptor antagonists; ASCEND, A Study of Cardiovascular Events in Diabetes; BMS, BMS-182874; Ca^{2+}, calcium; ET-1, Endothelin-1; IDNT, irbesartan diabetic nephropathy trial; PRA, plasma renin activity; RAS, renin angiotensin system; TGRen2, (mRen2)27 transgenic rats

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Introduction

The development of renal damage is common in arterial hypertension and can ultimately lead to renal failure and renal replacement treatment [1,2]. Fibrosis is a hallmark of renal damage associated with arterial hypertension and several diseases involving the kidneys [3]. The results of large trials employing angiotensin I (Ang I) converting enzyme inhibitors (ACEI) or angiotensin type I receptor antagonists (ARBs) [4–8], along with compelling experimental evidence (reviewed in [9]), implicate the renin–angiotensin system (RAS) in this pathogenic process. Angiotensin II (Ang II) affects Ca^{2+} fluxes, promotes collagen synthesis, and blunts collagenase activity, thus promoting collagen deposition in the kidney mostly acting via AT1. Endothelin-1 (ET-1) exerts similar effects (reviewed in [9]); it was held to induce renal damage mainly via ET_A, but it was also shown to act via ET_B receptors thus causing the release of aldosterone that, in the presence of excess sodium intake, is a trigger of collagen deposition [10]. The role of L-type Ca^{2+} channels in causing renal damage and fibrosis is also poorly known, and the few available studies have given conflicting results [11–15]. This is surprising inasmuch as Ca^{2+} participates in both Ang II and ET-1 intracellular signaling and thus might be involved in collagen deposition.

Moreover, the relative role of Ang II, ET-1 and of L-type Ca^{2+} channels in the development of fibrosis in the different compartments of the kidney, that is, glomeruli, the perivascular area and tubulointerstitium in the presence of arterial hypertension has never been investigated thus far. Therefore, by taking advantage of accurate histomorphometry techniques for quantifying the components of renal fibrosis and of pharmacologic tools, we investigated the role of Ang II, ET-1 and L-type Ca^{2+} channels in mediating renal fibrosis in a transgenic rat model of severe Ang II-dependent hypertension.

Methods

Animals

The protocol of this experiment was published and therefore will be only briefly described [16]. Male het-

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erozygous (mRen2)27 rat [TGRen2] at age 5 weeks, after measurement of body weight and systolic blood pressure (BP, tail-cuff method), were matched for both body weight and BP and then randomly allocated to receiving for 4 weeks one of the following treatments: placebo ($n=8$), mixed ET_A/ET_B endothelin receptor antagonist bosentan (100 mg/kg body weight) ($n=5$), AT-1 receptor selective antagonist irbesartan (50 mg/kg body weight) ($n=5$), ET_A-selective endothelin receptor antagonist BMS-182874 (BMS; 52 mg/kg body weight) ($n=4$), combination of irbesartan (50 mg/kg body weight) and BMS (52 mg/kg body weight) ($n=5$), and nifedipine (30 mg/kg body weight) ($n=5$). These dosages were previously shown to provide maximal antihypertensive effects when tested in animal models of hypertension [17]. Bosentan was a gift of Dr Martine Clozel (Actelion Ltd, Allschwil, Switzerland), BMS and irbesartan of Dr James Powell of Bristol Myers Squibb (Princeton, New Jersey, USA), and nifedipine of Dr Maurizio Verasani (Bayer SpA, Milan, Italy). The study protocol and animal handling followed available guidelines for animal studies [18].

We measured systolic BP levels (tail-cuff method) after rat training, in a noise-free environment, when the rats were quiet and relaxed. The mean value of at least three readings was considered. BP was measured at weekly intervals, until week 8, when rats were weighed and sacrificed. The kidneys were quickly removed, weighed, snap frozen in isopentane precooled on dry ice and were kept stored in liquid nitrogen until analyzed.

Biochemical variables

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

Glomerular volume

Transverse serial sections (5 μm thick) of the kidney were stained with Masson's trichrome and examined as described [18]. The analysis was performed blindly to treatment by a single examiner, using a photomicroscope Leica DM equipped with QWin Standard LeicaTM Image Analysis Software (Leica Microsystems GmbH, Wetzlar, Germany). Glomeruli were selected randomly in each section at 10× magnification; only glomerular areas crossing the outline of the field of view were examined. Maximum and minimum diameters were traced on at least 100 profiles per rat and the mean glomerular volume was calculated by applying Schwartz's transformation for spheres [18]. 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 fibrillar collagen. The quantitative analysis was performed with a Leica DM photomicroscope equipped with Image Analysis Software (Leica Microsystems GmbH), and running routines that were specifically created in our laboratory to estimate fibrosis in an operator-independent fashion. To minimize experimental variability, all sections were stained during the same experiment and were examined by a single operator blinded to treatments. Briefly, the area covered by Sirius red-stained structures, with the exclusion of the pale-yellow picric acid-stained tissue, was calculated as a measure of collagen fiber deposition. The intraassay and interassay coefficient of variation was less than 5 and 10%, respectively.

Glomerular fibrosis

Three sections were assessed for each rat at 20× magnification; the entire cortical area was examined to capture at least 100 glomeruli/rat. Glomerular fibrosis was estimated as the percentage of the glomerular surface area pertaining to fibrillar collagen.

Tubulointerstitial fibrosis

Four sections and 10 views corresponding to 294 μm^2 of the cortical region were examined for each rat, at magnification of 20×. This area was chosen based on results of a pilot study that showed that 10 randomly captured views from each section, corresponding to 70–80 μm^2, were necessary to obtain an accurate representation of the entire section. Automatically detected interstitial collagen was quantified in each field as the percentage of total surface area pertaining to fibrillar collagen.

Perivascular fibrosis

Sirius red-stained sections were examined at magnification 10×. To evaluate perivascular fibrosis involving the arteries ranging between 50 and 250 μm in diameter, which roughly correspond to interlobar, arcuate, and interlobular arteries, we selected at least 20 views in four to six sections to collect at least 50 arteries for each rat. Only arteries cross-sectionally captured in the sections were examined. After identifying the Sirius red-stained collagen as described earlier, the stained area pertaining to the vessel profile (perivascular fibrosis) was automatically selected and measured. As perivascular fibrosis and the ratio of thick-to-thin fibers showed a significant correlation with the size of renal resistance artery, we normalized perivascular fibrosis to the each arterial section. As Sirius red staining increases the birefringence of the fibrillar collagen, thicker fibres (type I collagen ranging between 1.6–2.4 μm diameter thick) can be visualized as red-orange shades, whereas thinner ones (type III collagen comprising fibers less than 0.8 μm diameter-thick) as greenish-yellow shades under polarization microscopy. By setting appropriate thresholds of the 'hue' component in the polarization light image, red-orange and greenish-yellow areas were identified and measured with Leica Image Analysis Software and a specific routine [19]. Red-orange and greenish-yellow

birefringent areas were then expressed as percentage of the total examined area [18,20].

Statistical analysis

Sample size was preliminarily calculated by nQuery Advisor software (version 6.0; nQuery Advisor Statistical Solutions, Saugus, Massachusetts, USA). When applied to glomerular volume we found that with the minimum sample size of five in each group, a one-way analysis of variance will have 84% power to detect at the 0.05 level a difference in means characterized by a variance of means, $V = \Sigma(\mu_i - \mu)^2/G$ of 1.064, assuming that the common standard deviation is 1.340. A higher power could be estimated for all the other histomorphometric variables that exhibited a lower spread of values.

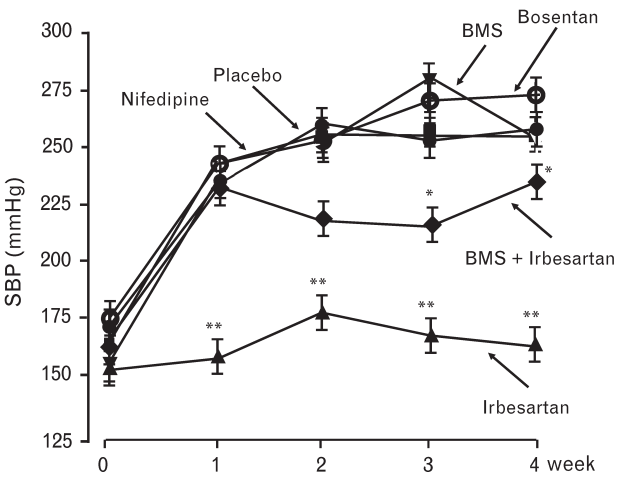
Results are expressed as mean ± SD, or SEM when repeated measures were obtained. Variables that showed a non-Gaussian distribution were log or square-root transformed until a normal distribution was attained. One-way analysis of variance (ANOVA) and Bonferroni post-hoc test for multiple comparisons were used to evaluate differences across groups. For the thick-to-thin fibrillar collagen ratio and the arterial section area, a normal distribution could not be achieved. Therefore, results are expressed as median and range, and comparisons across treatment groups was performed with the Kruskall–Wallis test. Linear regression (backward Wald) was employed to identify the predictors of perivascular fibrosis or the thick-to-thin fibrillar collagen ratio, and to calculate the unstandardized values of these variables, which were used for some analyses. *P* value less than 0.05 was considered statistically significant. Analyses were carried out with the SPSS for Windows statistical package (version 15.0; SPSS Inc., Chicago, Illinois, USA).

Results

Blood pressure and kidney weights

No significant differences in BP or in body weight were observed at baseline across treatment groups (Table 1, Fig. 1). Irbesartan and irbesartan plus BMS-treated rats showed a significantly ($P < 0.001$) lower BP, as compared with the other groups, starting from the end of week 1 and 3 of treatment, respectively (Fig. 1). No significant changes in kidney weight normalized to body weight were found across treatment groups at sacrifice.

Fig. 1



Changes in systolic blood pressure in the six experimental groups. A significant BP lowering was observed in irbesartan-treated groups ($P < 0.0001$) and irbesartan plus BMS ($P < 0.001$) treated groups, as compared with each other group. The decrease was significant starting from the end of the 1st and the 3rd week of treatment in irbesartan-treated rats and irbesartan and BMS-treated rats, respectively.

Biochemical variables

Serum creatinine levels showed no significant changes with any active treatment as compared with placebo (Table 1). In irbesartan-treated rats, but not in the other groups, plasma aldosterone levels were significantly lower ($P < 0.01$) than in placebo; no significant changes were observed in PRA (Table 1).

Glomerular volume and fibrosis

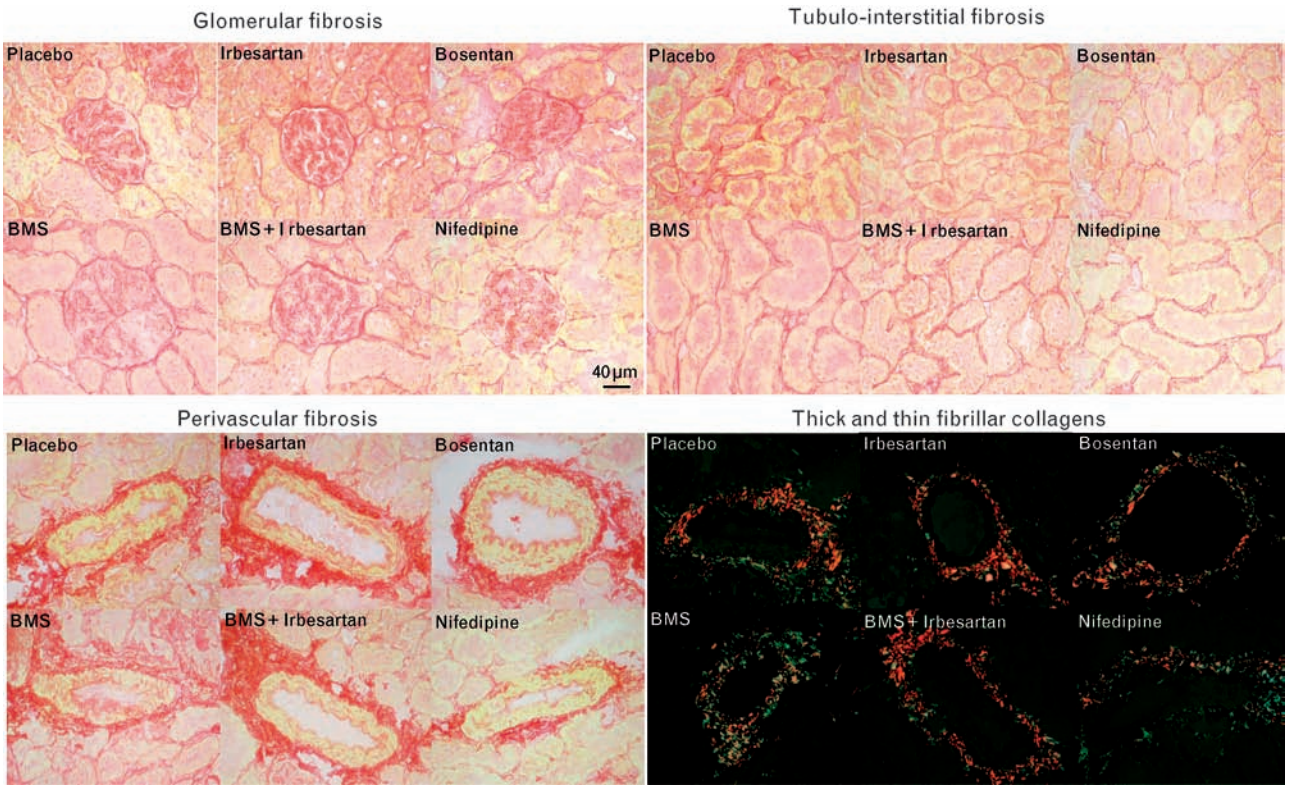
Glomerular volume was lower after irbesartan treatment ($P < 0.0001$) and higher after the combined treatment irbesartan and BMS ($P < 0.05$) than in the placebo group

Table 1 Renal and biochemical parameters in Ren2 transgenic rats after AT1 receptor selective blockade with irbesartan, non selective ET-1 receptor blockade with bosentan, ET_A subtype receptor selective blockade with BMS-182874 (BMS), combined blockade of AT1 and ET_A subtype receptors with BMS and irbesartan, or calcium channel blockade with nifedipine

	Placebo	Irbesartan	Bosentan	BMS	BMS and Irbesartan	Nifedipine
Kidney weight (mg)	1469 ± 74	1335 ± 45	1434 ± 41	1731 ± 93	1740 ± 128	1850 ± 73**
Kidney weight/body weight (mg/g)	4.6 ± 0.2	3.9 ± 0.1	4.4 ± 0.1	5.0 ± 0.1	4.3 ± 0.1	5.1 ± 0.3
Serum creatinine (μmol/l)	73.9 ± 12.3	60.1 ± 9.7	70.7 ± 8.8	114.0 ± 47.7	103.4 ± 33.5	96.3 ± 25.6
PRA (ng/ml per hour)	19.70 ± 2.43	22.82 ± 2.43	21.78 ± 1.31	20.57 ± 2.53	17.23 ± 0.99	22.72 ± 2.35
Plasma aldosterone (pmol/l)	60.1 ± 15.5	40.2 ± 6.6*	52.1 ± 5.2	50.6 ± 17.0	63.7 ± 16.7	70.2 ± 26.2
Glomerular volume ($\mu m^3 \times 10^6$)	10.19 ± 0.60	8.47 ± 0.51***	9.28 ± 0.47	11.13 ± 0.67	11.25 ± 0.69*	10.96 ± 0.61
Arterial section area (μm^2)	48.92 (15.91–281.36)	43.17 (13.80–210.36)*	46.20 (16.55–255.27)	65.44 (22.11–281.38)*	49.04 (18.38–254.56)	55.11 (11.50–322.94)*
Thick-to-thin fibrillar collagen ratio/arterial surface area × 10^3	0.07 (0–0.6)	0.12 (0–0.8)	0.07 (0–0.5)	0.05 (0–0.5)**	0.11 (0–0.6)	0.04 (0–0.8)**

Values are means ± SEM, or median and range for arterial section area and thick-to-thin fibrillar collagen ratio. * $P < 0.05$ vs. placebo. ** $P < 0.01$ vs. placebo. *** $P < 0.001$ vs. placebo.

Fig. 2



Representative 5 μm-thick kidney sections stained with Sirius red that selectively visualize fibrillar collagens in the glomeruli (left top panel), in the tubulointerstitium (right top panel) and in the perivascular compartment (left bottom panel) of the different treatment groups. A decrease in the glomerular fibrosis is evident in the rats receiving the ET_A-selective endothelin receptor antagonist BMS-182874, whereas a decrease in the tubulointerstitial fibrosis can be observed in both bosentan-treated rats and irbesartan-treated rats. Perivascular fibrosis is clearly lowered by nifedipine and the ET_A-selective endothelin receptor antagonist BMS-182874, which both also decreased thick fibrillar collagens, visualized as strongly birefringent red-orange fibers under polarized microscopy (right bottom panel) and corresponding to type I collagen. Quantitative analysis confirmed these findings (see Fig. 3 and Table 1).

(Table 1). Glomerular fibrosis was significantly ($P < 0.001$) lower in the BMS group than in the placebo group (Figs 2 and 3), but was unaffected by the other treatments, including the combined treatment BMS and irbesartan.

Tubulointerstitial fibrosis

Compared with placebo group tubulointerstitial fibrosis was markedly lowered by both bosentan ($P < 0.001$) and irbesartan ($P < 0.005$); it was unaffected by nifedipine and BMS alone, or by the concomitant treatment with BMS and irbesartan (Figs 2 and 3).

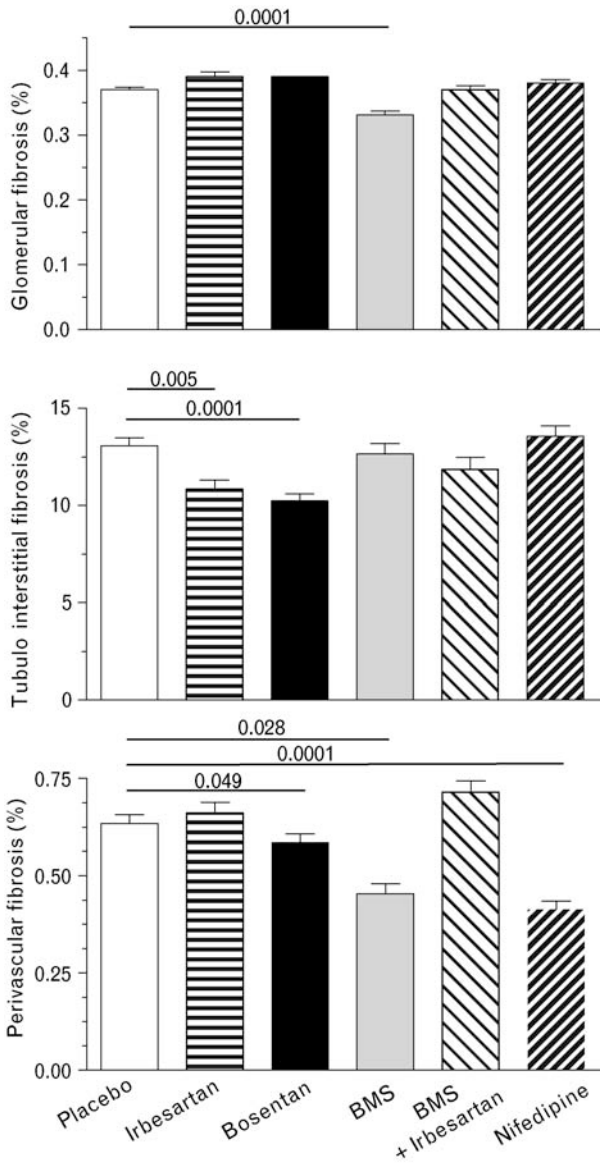
Perivascular fibrosis

Perivascular fibrosis was significantly lowered by nifedipine (by 34%; $P < 0.001$) and BMS (by 28%; $P < 0.028$) and, less markedly, by bosentan (by 7%; $P = 0.049$), as compared with placebo group (Figs 2–3). A reduction in thick (red-orange) to thin (greenish-yellow) collagen fibril ratio ($P < 0.001$) was also found after nifedipine (by 42%) and BMS (by 28%) treatments (Table 1; Fig. 2). The linear regression model showed that perivascular fibrosis was predicted by the artery cross-sectional area ($P < 0.001$, $r = 0.607$), and that thick-to-thin collagen fibril ratio was predicted by the artery cross-sectional area ($P < 0.001$, $r = 0.165$) and the BP at sacrifice ($P < 0.001$, $r = -0.186$). Moreover, there were differences of artery cross-sectional area across treatment groups: the area was significantly lower in the irbesartan, and higher in the nifedipine, and BMS groups. Hence, in addition to the raw perivascular fibrosis data we also compared the covariate-adjusted values of perivascular fibrosis and thick-to-thin collagen fibril ratio across groups. This showed that although the adjusted values of perivascular fibrosis did not differ across treatments, those of thick-to-thin collagen fibril ratio were increased in the irbesartan ($P < 0.001$) and the combined irbesartan and BMS ($P < 0.001$) groups, as compared with placebo.

Discussion

The histomorphometry technique developed for this study allowed us to gather novel information on the effect of the different pharmacologic interventions on the

Fig. 3



Glomerular, tubulointerstitial, and perivascular fibrosis measured in 5 μm transverse serial sections of the kidneys of Ren-2 transgenic (TGRen2) rats after a placebo, the AT-1 receptor selective antagonist irbesartan, the mixed ET_A/ ET_B endothelin receptor antagonist bosentan, the ET_A-selective endothelin receptor antagonist BMS-182874, the combination of irbesartan plus BMS-182874, and nifedipine. Sections were stained with Sirius red to selectively visualize fibrillar collagen, and examined blindly to treatment by a specific routine allowing operator-independent identification of the Sirius red-stained fibrils in the cortex. Values are mean ± SEM.

various components of renal fibrosis. Knowledge of this effect might be of utmost relevance for the early prevention of fibrosis in kidney diseases that predominantly affect the glomeruli, the tubule-interstitial, or the vascular compartment.

We could measure collagen using a very accurate and reproducible methodology, and examining several tissue sections and a large number of fields. This provided a large number of measurements with a tiny spread of the data, thus conferring to our study a high statistical power for addressing the questions that were posed, notwithstanding the apparent small number of rats examined. The relative role of Ang II and ET-1, and of L-type calcium channel blockade in mediating fibrosis in the glomerular, perivascular, and tubulointerstitial compartments could, therefore, be investigated in a rat model of Ang II-dependent hypertension that features a prominent cardiovascular organ damage involving also the kidney.

This study shows that blockade of Ang II AT1 receptors, ET-1 receptors, and L-type calcium channels has different effects on the development of renal fibrosis, apparently in a BP-independent fashion. ET-1 plays a predominant role in this process, as indicated by the prevention of all components of renal fibrosis, that is, glomerular fibrosis, tubulointerstitial, and perivascular fibrosis, with either an ET_A or a mixed ET_A/ET_B receptor antagonist or both. At variance, Ang II and L-type calcium channels seem to be involved only in the tubulointerstitial and perivascular fibrosis, respectively, inasmuch as blockade of the AT1 receptor with irbesartan and of the L-type Ca^{2+} channels with nifedipine resulted into a reduction of collagen deposition only in these compartments.

Glomerular fibrosis

The ET_A receptor antagonist BMS was the only treatment that exerted a significant antifibrogenic effect in this compartment, thus suggesting that glomerular fibrosis is predominantly mediated by ET_A subtype receptors. This contention agrees not only with available evidence of a predominant intrarenal distribution of ET_A subtype receptors on the mesangial and vascular smooth muscle cells [21], but also with previous studies showing a protective effects of ET_A receptor antagonists on the development of glomerulosclerosis [22–25]. However, caution should be exercised when comparing the data on glomerulosclerosis with those on glomerular fibrosis, because the two processes reflect different pathogenic mechanisms. The former, being characterized by either scarring or hardening of capillaries and hyaline deposits occurring in association with arteriolosclerosis, entails a degenerative process of all glomerular cell phenotypes, whereas the latter specifically refers to mesangial collagen deposition.

ET_A-selective blockade can promote ET_B receptor-mediated effects; furthermore, ET_B receptor activation elicits nitric oxide release and thereby can inhibit collagen synthesis [26,27]. The prevention of glomerular fibrosis with BMS can originate from both mechanisms and the lack of any significant effect with the mixed ET_A and ET_B blockade with bosentan is also consistent with this contention. However, we could not determine the

relative importance of the aforementioned mechanisms, as this study was not designed to this aim.

Unexpectedly, with irbesartan even despite, the significant lowering of BP, we found no protection from glomerular fibrosis; by contrast irbesartan exerted a clear-cut protective effect from tubulointerstitial where (see later). We can speculate that a dose of irbesartan higher than that providing antihypertensive effects might be required to fully contrast AT1-mediated effects in the glomeruli of TGR because of the markedly enhanced local production of Ang II [28]. However, the possibility that the development of glomerular fibrosis is not strictly an Ang II-dependent process cannot be excluded.

Tubulointerstitial fibrosis

In renal tubular epithelial cells and fibroblasts, Ang II was shown to stimulate TGFβ production; furthermore, RAS inhibition reduced tubulointerstitial in various models of kidney disease [29,30], and a decreased tubulointerstitial collagen type I deposition was found in TGRen2 after losartan even at sub-antihypertensive doses [31]. In the tubular cells, intracellular RAS and active AT1-mediated internalization of Ang II would induce the local production of proinflammatory cytokines and growth factors, thereby promoting cell proliferation and fibrosis [32]. Thus, the finding that irbesartan markedly protected from tubulointerstitial, even despite its lack of effect on glomerular fibrosis, could be anticipated.

The mixed ET_A and ET_B receptor blockade with bosentan also markedly prevented tubulointerstitial, even despite no effect on glomerular fibrosis, whereas, in contrast, the ET_A-selective blockade with BMS exerted no significant effects. The findings that at variance with irbesartan alone the combined treatment BMS and irbesartan failed to protect the kidney against fibrosis; bosentan but not BMS prevented tubulointerstitial collectively suggest that ET-1 fibrotic effect is mediated by ET_B. It may also imply that selective ET_A blockade, presumably by enhancing ET_B activation, may revert the antifibrotic effect of irbesartan. This contention is consistent with the more prominent immune reactivity of epithelial tubular cells for ET_B, rather than for ET_A, [21].

Moreover, they suggest a different role of ET-1 subtype receptors in the development of fibrosis in the glomerular or tubulointerstitial compartments.

Noteworthy, no protective effects of nifedipine on either tubulointerstitial or glomerular fibrosis were found, thus suggesting that L-type calcium channels do not play a crucial role for renal fibrosis at these compartments.

Thus, overall, these findings suggest an involvement of AT-1 and ET_B subtype receptors in the development of tubulointerstitial in contrast with the prevailing view that the ET_A receptor would be the main mediator of the fibrogenic effects of ET-1 [33]. Noteworthy, the relevant role of both ET_A and ET_B subtype receptors in renal fibrosis herein identified might account for the negative findings and the premature stop of ASCEND (A Study of Cardiovascular Events in Diabetes), a recent large trial designed to prove the hypothesis that selective ET_A receptor blockade delays the progression of renal damage in diabetic nephropathy (http://www.speedel.com).

Perivascular fibrosis

Both ET-1 antagonists prevented perivascular fibrosis, a finding which, in is our view, even more remarkable considering that they did not lower BP at all; BMS induced a significant reduction of thick-to-thin fibrillar collagen ratio (Figs 1 and 2). Hence, the protective effects of BMS and bosentan from perivascular fibrosis support an involvement of ET-1 in renal fibrogenesis in all compartments.

Although irbesartan exerted no appreciable effect on perivascular fibrosis, we also found a protective effect of nifedipine, which accords well with the central role of calcium in cell growth and proliferation [32], and also with the prevention of vascular damage, but not of glomerular fibrosis and tubulointerstitial alterations, documented with nifedipine in subtotally nephrectomized rats [13–15,34]. Moreover, because L-type Ca^{2+}channels channels play a major role in the constrictor response to either ET-1 or Ang II or both in the renal microcirculation, but provide a minor or minimal contribute to Ang II or ET-1-induced mesangial growth, we would like to propose an inter-relationship between ET-1 and L-type Ca^{2+}channels. Unfortunately, nifedipine cannot discriminate between Ang II- or ET-1- associated L-type Ca^{2+}channels and other L-type Ca^{2+}channels and therefore further research is necessary to better define the type(s) of Ca^{2+}channels that are implicated in this effect [35–38]. The reduction of thick-to-thin fibrillar collagen ratio induced by nifedipine further supports a beneficial effects of L-type calcium channel blockade on the vascular remodeling, given the different mechanical properties of collagen types I and III: the former is mainly endowed of tensile strength, the latter with distensibility [36].

Blood pressure independent fibrogenic effects

Available evidences support the concept that renal damage and fibrosis are partly independent of systemic hemodynamics [39]. In the irbesartan diabetic nephropathy trial (IDNT) different renoprotection was conferred by irbesartan and amlodipine despite a similar lowering of BP [5]. Moreover, transgenic mice overexpressing human ET-1 gene were reported to develop glomerulosclerosis and tubulointerstitial despite no overt change in BP [39]. Furthermore, chronically L-NAME-treated rats receiving an ET_A subtype selective receptor antagonist showed a blunted fibrosis development despite no normalization of BP [40], a finding consistent

with the observation that bosentan markedly protected the kidneys from L-NAME-induced fibrosis despite no correction of hypertension [41].

As we used drug dosages that previously provided antihypertensive effects in other models of hypertension, we did not expect a lack of significant BP lowering with some of the drugs that we used. However, the quite different BP-lowering effect that was recorded allowed us to discriminate BP-dependent from BP-independent effects. Of interest, we found a significant BP lowering only with irbesartan and, to a lesser extent, with irbesartan and BMS. However, only the former treatment was associated with a decrease of tubulointerstitial. It is interesting to note that notwithstanding their ineffectiveness in lowering BP bosentan, BMS, and nifedipine also reduced fibrosis, even though to different extents and in different compartments.

Thus, even though a high BP can be a prerequisite for the development of renal damage and fibrosis, lowering of BP does not warrant *per se* an antifibrogenic effects of pharmacologic treatments, a conclusion that is in accordance with the results of large clinical trials [5,6,8].

Limitations of the study

It is known that several factors, including BP and the density of packed collagen fibers, may affect birefringence properties of the perivascular collagen [19,42]. A first limitation of this study that may pertain to perivascular fibrosis is the fact that we could not pressure-perfusion fix the kidneys; therefore, the data on perivascular fibrosis and on the thick-to-thin fibrillar collagen ratio might be influenced by the different intraluminal pressure at the time of sacrifice. To address this potential limitation we used a regression analysis to identify the predictors of perivascular fibrosis and of the thick-to-thin fibrillar collagen ratio and to calculate predictor-adjusted values of perivascular fibrosis and the thick-to-thin fibrillar collagen ratio. This showed that the covariate-adjusted values of perivascular fibrosis did not differ across treatments, whereas those of thick-to-thin collagen fibril ratio were increased in the irbesartan and the combined irbesartan and BMS groups. Thus, the results and overall conclusions can change markedly depending on the fact that relevant predictors are taken into consideration or not. Hence, further studies using pressure-controlled perfusion fixation and algorithms that allow estimating the relationship between birefringence and density of packed fibers [42], which might be affected by BP and thereby artery cross-sectional area are required to clarify these issues.

A second limitation of this study is the lack of data on urine albumin excretion and proteinuria. We measured only serum creatinine levels which is a marker of advanced renal impairment. Therefore, we could not determine whether the changes in the kidney structure were accompanied by an improvement in glomerular permeability.

Finally, even though we could pinpoint the differential effects of pharmacologic manipulation of RAS, ET_A, ET_B receptors, and calcium channels, notwithstanding the relatively short duration of this study, because of the prominent renal damage occurring in TGRen-2 rats it remains unknown whether the same or more marked protective effects could be seen with a longer treatment.

Conclusion

Using a thorough methodology to estimate collagen in the tubulointerstitial, glomerular, and perivascular compartments, we could show that AT-1 receptors and L-type calcium channels selectively modulate tubulointerstitial and perivascular fibrosis, respectively. The protection conferred by ET-1 receptor antagonism on all three compartments supports a predominant role of ET-1 in the development of renal fibrosis. Moreover, the fact that ET-1 exerts its fibrogenetic effects through both ET_A and ET_B subtype receptor subtypes, albeit with differences across the glomerular, perivascular, and the tubulointerstitium compartments, support the need of testing mixed ET_A and ET_B receptor antagonists in adequately powered clinical trials. Whether blockade of L-type calcium channels and mixed ET_A and ET_B receptor antagonists can have an additive protective effect also remain to be investigated. Identification of the mechanisms underlying kidney fibrosis in the glomerular, tubulointerstitial and perivascular compartments could represent the basis for selective antifibrotic treatment.

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There are no conflicts of interests.

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