Imatinib Attenuates End-Organ Damage in Hypertensive Homozygous TGR(mRen2)27 Rats

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Abstract—Imatinib specifically inhibits receptor tyrosine kinase signaling and is clinically used to treat leukemia. Receptor tyrosine kinases not only mediate tumor growth but also initiate adverse signaling in heart failure. We investigated whether imatinib, by inhibiting the platelet-derived growth factor receptor-β (PDGFRβ), prevents cardiac and renal damage in TGR(mRen2)27 (Ren2) rats. Eight-week-old male homozygous Ren2 and Sprague Dawley rats were treated either with imatinib (30 mg/kg; STI-571) or placebo for 8 weeks (Ren2 n = 12 for each group; Sprague Dawley n = 6 for each group). Imatinib did not affect blood pressure or left ventricular (LV) hypertrophy in both groups. Imatinib attenuated the decline in fractional shortening (imatinib versus Ren2 placebo 45 ± 4.5% versus 32 ± 3%; n = 7–11; P < 0.05) and in diastolic function in Ren2 rats (baseline diastolic dP/dt corrected for systolic blood pressure Ren2 imatinib versus Ren2 placebo 38.6 ± 0.67 versus 35.3 ± 0.41 [1 · s⁻¹]; n = 7–11; P < 0.05). This was associated with decreased cardiac fibrosis and decreased activation of PDGFRβ and extracellular signal-regulated kinase 1/2. Renal microvascular hypertrophy and perivascular fibrosis in Ren2 rats were significantly decreased by imatinib. In vitro, imatinib blocked angiotensin II–mediated activation of the PDGFRβ and significantly decreased fibroblast proliferation and collagen production. In conclusion, imatinib did not affect LV hypertrophy but attenuated the decline in cardiac function and reduced renal microvascular damage associated with reduced activation of the PDGFRβ. The simultaneous improvement in both heart and kidneys suggests that inhibition of the PDGFRβ has broad protective effects that may provide novel avenues for a blood pressure–independent protection against end-organ damage.

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Key Words: platelet-derived growth factor • angiotensin II • hypertrophy • fibrosis

Angiotensin II (Ang II)–mediated hypertension causes end-organ damage not only via increased blood pressure but also via the direct effects of Ang II. Ang II is known to play a crucial role in the development of cardiomyocyte hypertrophy1-2 and interstitial cardiac fibrosis,3-4 and sustained increases of Ang II result in severe left ventricular (LV) dysfunction.5-7 We have shown that the homozygous hypertensive TGR(mRen2)27 (Ren2) rat, a model of Ang II–driven hypertension, develops accelerated heart failure accompanied by severe LV dysfunction and cardiac collagen accumulation.8,9 Ang II–mediated cardiac hypertrophy in Ren2 rats is associated with increased extracellular signal-regulated kinase (ERK) and Smad signaling.7 ERK1/2 is an important downstream target of receptor tyrosine kinases, like the epidermal growth factor receptor (EGFR) and the platelet-derived growth factor receptors (PDGFRs). Recently, Ang II–mediated transactivation of these receptor tyrosine kinases has gained much interest,10 and we demonstrated that inhibition of the EGFR attenuates ERK1/2 activation in the Ren2 rat, which was paralleled by decreased cardiac fibrosis.7

Imatinib mesylate (Gleevec), a specific tyrosine kinase inhibitor, is a novel, orally active drug used to treat chronic myeloid leukemia.11 Imatinib blocks the continuously increased tyrosine kinase activity of bcr/abl. Bcr/abl is a fusion protein resulting from a translocation between chromosome 9 and 22, which is the causative gene defect in chronic myeloid leukemia. However, imatinib blocks other tyrosine kinases as well, including the tyrosine kinase activity of PDGFR-α, PDGFR-β, and c-kit.11

We therefore hypothesized that imatinib-mediated inhibition of PDGFRβ tyrosine kinase activity may attenuate downstream ERK1/2 activation, thereby protecting against the direct adverse effects of Ang II such as cardiac and renal fibrosis or dysfunction.

The present study reveals that imatinib treatment in Ren2 rats protected against adverse cardiac and renal remodeling.
and dysfunction, associated with a decrease in PDGFRβ phosphorylation and ERK1/2 activation. In concordance, administration of imatinib in vitro decreased collagen production and proliferation of cardiac fibroblasts and attenuated Ang II–induced PDGFRβ phosphorylation.

In conclusion, imatinib treatment might provide a novel therapeutic tool to protect against cardiac dysfunction during Ang II–mediated hypertension.

Materials and Methods

Drug Treatment

Homozygous Ren2 and Sprague Dawley rats were purchased from the Moellegaard Breeding Company, Ry, Denmark. All described study protocols were approved by the animal care and use committee of the Universiteit Maastricht and were performed according to the official rules formulated in the Dutch law on care and use of experimental animals. At 8 weeks of age, 24 male Ren2 rats and 12 nontransgenic Sprague Dawley rats were randomly assigned to receive placebo treatment (distilled water) or imatinib dissolved in 0.1% FBS for 24 hours, and stained with Sirius Red to visualize collagen. LV interstitial collagen was quantified by computerized planimetry. Transversal renal sections of 2 µm were cut for investigation of glomerulosclerosis using periodic acid staining. Renal sections were cut at 4 µm and stained with α-smooth muscle actin and Sirius Red to investigate the renal microcirculation and to stain for collagen. The percentage of glomeruli that exhibited focal or global glomerulosclerosis was determined as described previously. Tubulointerstitial injury was defined as inflammatory cell infiltrates, tubular dilation or atrophy, or interstitial fibrosis. Injury was graded according to Shih et al on a scale of 0 to 4 (0 normal; 0.5 small focal areas of damage; 1 involvement of <10% of the cortex; 2 involvement of 10% to 25% of the cortex; 3 involvement of 25% to 75% of the cortex; and 4 extensive damage involving >75% of the cortex).

Protein Isolation, Western Blotting, and Immunoprecipitation

Protein was isolated after grinding frozen heart tissue with radioimmunoprecipitation assay (RIPA) buffer containing PBS, pH 7.4, Igepal (1%), deoxycholic acid (0.5%), sodium dodecyl sulfate (1%), β-mercapto-ethanol, and complete protease inhibitor tabs (Roche Diagnostics).

Western blotting was performed on cultured fibroblasts to identify pPDGFRβ, PDGFRβ, pEGFR, and EGFR (CST). Tyrosine phosphorylation state of PDGF (CST) and ERK1/2 (CST) in the heart homogenates was assessed by immunoprecipitation with an immobilized phosphotyrosine antibody (CST).

Protein samples were incubated with an immobilized phosphotyrosine antibody (1:10) at 4°C overnight. Subsequently, the samples were centrifuged at 2000 rpm, 4°C, and washed 3 times with RIPA buffer. Finally, the samples were prepared for Western blotting.

Cell Culture

DMEM and FBS were purchased from Gibco BRL. Culture plates were obtained from Costar. Cardiac fibroblasts were isolated from 2-day-old neonatal Lewis rats. All the experiments were performed on cells from the second passage. Cells were maintained in DMEM supplemented with 10% FBS along with 0.1% gentamyacin, incubated at 37°C in a humidified chamber, and grown to confluence, before synchronization in low-serum medium (0.1% FBS) 48 hours before the experiments. For DNA synthesis determination, cells were put on high (10% FBS) or left on low (0.1% FBS) serum for 24 hours after 30 minutes pretreatment with increased concentrations of imatinib (0 to 10 µmol/L). Synthesis of DNA was assessed by radiolabeled [H]thymidine incorporation assay. For collagen synthesis determination, cells were treated with imatinib (control 1 and 10 µmol/L) for 24 hours. Hereafter, [H]proline was added for 48 hours. The incorporated [H]proline was corrected for cell density by dividing the radioactivity in disintegrations per minute by the total amount of cellular proteins.

For transactivation experiments, cardiac fibroblasts were treated with imatinib or placebo 30 minutes before the addition of Ang II
(1 μmol/L), PDGF-BB (20 ng/mL), or EGF (10 ng/mL). One minute or 10 minutes after growth factor stimulation, cells were harvested and prepared for Western blotting.

**Statistical Analysis**

Data are presented as means±SEM. Statistical analysis was performed using paired and unpaired Student t test.

**Results**

**Imatinib Does Not Lower Blood Pressure But Improves Hemodynamics in the Ren2 Rat**

After 8 weeks of treatment, body weight was significantly lower in the Ren2 groups compared with the Sprague Dawley groups, but imatinib did not significantly alter body weight (Table). LV to body weight ratio was significantly increased in Ren2 rats compared with Sprague Dawley rats and was not altered by imatinib (Table). Ren2 rats had severely elevated blood pressures. Imatinib did not affect systolic blood pressure (Figure 1).

To assess cardiac function of the rats during the study, we performed echocardiography as described above. In placebo-treated rats, fractional shortening decreased significantly. Imatinib significantly attenuated this decrease (Table). After 8 weeks of treatment, baseline diastolic dP/dt was significantly higher in imatinib-treated Ren2 rats compared with placebo-treated Ren2 rats (Table). Imatinib treatment had no significant effects in Sprague Dawley rats (Table).

**Imatinib Decreases Cardiac Interstitial Fibrosis in Ren2 Rats**

Heart sections were stained with Sirius Red to visualize total collagen. Quantification of myocardial collagen with a computer-assisted densitometric analysis revealed a significant decrease in interstitial collagen content in Ren2 rats treated with imatinib compared with placebo-treated Ren2 rats (4.6±0.3% versus 5.5±0.3%, respectively; P<0.05).

**Imatinib Decreases Renal Microcirculatory Changes in Ren2 Rats**

Renal afferent arterioles of Ren2 rats showed significant thickening of the vascular wall and a nonsignificant tendency toward a larger lumen (Figure 2). Imatinib fully prevented
vascular thickening and normalized vascular thickness. Perivascular collagen was markedly increased in afferent arterioles of untreated rats, which was significantly decreased by imatinib treatment (Figures 2 and 3).

Imatinib Attenuates Glomerular and Tubular Damage in Ren2 Rats

Tubular atrophy was markedly attenuated by imatinib (1.7±0.4 R2pl versus 0.8±0.3 R2ima; n=7; P<0.05), whereas glomerulosclerosis index decreased nonsignificantly (0.6±0.2 R2pl versus 0.3±0.1 R2ima) after imatinib in Ren2 rats (Figure 3). Glomeruli of Ren2 rats showed a slightly increased mesenchymal damage as semiquantitatively investigated, which was downregulated toward control level in imatinib-treated rats (0.5±0.1 R2pl versus 0.3±0.1 R2ima; n=7; P<0.05; Figure 3).

Imatinib Decreases Cardiac Fibroblast Collagen Production and Proliferation in Vitro

Collagen production by cardiac fibroblasts, measured by [3H]proline incorporation, was significantly reduced by imatinib (Figure 4A).

Proliferation of neonatal rat cardiac fibroblasts on 10% FBS was dose-dependently inhibited by imatinib as determined by [3H]thymidine incorporation (Figure 4B, black bars). Proliferation of neonatal rat cardiac fibroblasts on 0.1% FBS was significantly inhibited by 0.1 to 10 μmol/L imatinib (Figure 4B, gray bars).

Imatinib Attenuates PDGFR Signaling

To assess the phosphorylation state of the PDGFRβ and its downstream signaling protein ERK1/2 in LV homogenates, we immunoprecipitated proteins phosphorylated on their tyrosine residues and then immunoblotted for the PDGFRβ and for ERK1/2. Phosphorylation of the PDGFRβ was increased

Figure 3. Decreased perivascular fibrosis and renal damage in Ren2 rats treated with imatinib. A, Representative Sirius Red staining of a imatinib-treated Ren2 rat. B, Representative Sirius Red staining of a placebo-treated Ren2 rat.

Figure 4. Effects of imatinib on DNA and collagen synthesis in cultured cardiac fibroblasts. A, Synchronized cardiac fibroblasts were treated with imatinib (1 to 10 μmol/L), and [3H]proline was added for 48 hours followed by scintillation counting. Untreated cells were used as controls. *P<0.05 vs control; n=3. B, Synchronized cardiac fibroblasts were treated with imatinib (0.001 to 1000 μmol/L) for 24 hours, and DNA synthesis was assessed with [3H]thymidine incorporation followed by scintillation counting (black bars 10% FBS; gray bars 0.1% FBS) *P<0.01; †P<0.001 vs control; n=6.
in Ren2 rats but was significantly blunted by imatinib treatment (Figure 5A). Activation of ERK1/2 is dependent on threonine and tyrosine phosphorylation. Imatinib reduced tyrosine phosphorylation of ERK1/2, a downstream signaling protein in the PDGF pathway (Figure 5B).

Imatinib Inhibits Transactivation of Receptor Tyrosine Kinases
We examined whether phosphorylation of the PDGFRβ or EGFR is increased in cardiac fibroblasts treated with Ang II, PDGF-BB, or EGF. Ang II treatment for 1 minute resulted in both PDGFRβ and EGFR phosphorylation, with imatinib only inhibiting PDGFRβ phosphorylation (Figure 6A and 6B). Ten minutes of PDGF-BB treatment resulted in a marked increase in both PDGFRβ and EGFR phosphorylation. In contrast, EGF treatment only activated the EGFR but not the PDGFRβ. Imatinib inhibited the PDGF-BB induced effects but not the EGF induced phosphorylation of the EGFR (Figure 6C through 6F).

Discussion
The present study reveals that imatinib (Gleevec), a tyrosine kinase receptor blocker used to treat leukemia, attenuates the loss of cardiac function in a model of Ang II–driven hypertensive LV hypertrophy. Furthermore, imatinib improved renal microvascular damage in these hypertensive homozygous Ren2 rats. This confirms that inhibition of growth factor signaling, such as specifically provided by imatinib, might represent a novel therapy to protect against cardiac and renal dysfunction in Ang II–mediated hypertension.

Imatinib attenuated the loss of cardiac function in Ren2 rats as measured by echocardiography and direct LV pressure measurements. Furthermore, imatinib attenuated the accumulation of interstitial collagen in the heart and the kidney, reversed renal microvascular hypertrophy, and markedly reduced perivascular fibrosis in the renal microcirculation. To a smaller degree, glomerular, and, in particular, mesenchymal and tubular, damage were attenuated by imatinib. The improvement in cardiac function and architecture in imatinib-treated Ren2 rats was associated with decreased cardiac PDGFRβ–ERK1/2 signals. Apart from a general decreased collagen content in the heart, we were further able to distinguish an effect on renal perivascular collagen content, which may imply vascular localized action of imatinib. These in vivo findings were corroborated by the

Figure 5. Effects of imatinib treatment on tyrosine phosphorylation levels of PDGFRβ and ERK1/2 in Ren2 rats. Tyrosine phosphorylation levels of PDGFRβ and ERK1/2 are increased in Ren2 rats compared with Sprague Dawley rats. Imatinib treatment decreased the tyrosine phosphorylation levels of PDGFRβ and ERK1/2 in Ren2 rats. A, Top, Representative blots showing tyrosine phosphorylation state of PDGFRβ in Sprague Dawley (lanes 1 and 2), untreated (lanes 3 through 8), and imatinib-treated (lanes 9 through 14) Ren2 rats. Bottom, Quantification of blots from 2 experiments, corrected for total PDGFRβ; *P<0.05; †P<0.01. B, Top, Representative blots showing tyrosine phosphorylation state of ERK1/2 in Sprague Dawley (lanes 1 and 2), untreated (lanes 3 through 8), and imatinib-treated (lanes 9 through 14) Ren2 rats. Bottom, Quantification of blots from 2 experiments, corrected for total ERK1/2; *P<0.05. WB indicates Western blot.
action of imatinib in vitro on fibroblasts, where it inhibited collagen production and fibroblast proliferation. These findings substantiate the idea that targeted inhibition of growth factor systems, in particular tyrosine kinase inhibition, may have beneficial effects in hypertensive cardiac and renal damage.16

Effects of Imatinib in an Ang II–Driven Model of Cardiac Hypertrophy and Failure

Homozygous Ren2 rats have sustained local activation of the renin-angiotensin system, resulting in severe hypertension.17 The elevated levels of Ang II may directly or indirectly activate growth factor systems that can be inhibited by

Figure 6. Effects of imatinib treatment on PDGFRβ and EGFR phosphorylation in Ang II–, PDGF-BB–, or EGF-treated neonatal rat cardiac fibroblasts. A, Representative blot showing that Ang II treatment (1 μmol/L; 1 minute) increased PDGFRβ phosphorylation in cardiac fibroblasts. Treatment with imatinib (10 μmol/L) 30 minutes before Ang II blocked induced PDGFRβ phosphorylation; *P<0.05; lanes 1 and 2, control (n=3); lanes 3 and 4, Ang II (n=3); lanes 5 and 6, Ang II+imatinib (ima; n=3). B, Representative blot showing that Ang II treatment (1 μmol/L; 1 minute) increased EGFR phosphorylation in cardiac fibroblasts. Treatment with imatinib (10 μmol/L) 30 minutes before Ang II did not block induced EGFR phosphorylation; *P<0.05. Left lane, control (n=3); middle lane, Ang II (n=3); right lane, Ang II+ima (n=3). C, Representative blot showing that PDGF-BB treatment (20 ng/mL; 10 minutes) increased EGFR phosphorylation in cardiac fibroblasts. Treatment with imatinib (10 μmol/L) 30 minutes before PDGF-BB blocked induced EGFR phosphorylation; *P<0.05. Lanes 1 and 2, control (n=2); lanes 3 and 4, PDGF-BB (n=2); lanes 5 and 6, PDGF-BB+ima (n=2). D, Representative blot showing that PDGF-BB treatment (20 ng/mL; 10 minutes) increased PDGFRβ phosphorylation in cardiac fibroblasts. Treatment with imatinib (10 μmol/L) 30 minutes before PDGF-BB blocked induced PDGFRβ phosphorylation; *P<0.05. Lanes 1 and 2, control (n=2); lanes 3 and 4, PDGF-BB (n=2); lanes 5 and 6, PDGF-BB+ima (n=2). E, Representative blot showing that EGF treatment (10 ng/mL; 10 minutes) increased EGF phosphorylation in cardiac fibroblasts. *P<0.05. Left lane, control (n=2); right lane, EGF (n=2). F, Representative blot showing that EGF treatment (10 ng/mL; 10 minutes) did not increase PDGFRβ phosphorylation in cardiac fibroblasts. *P<0.05. Left lane, control (n=2); right lane, EGF (n=2). WB indicates Western blot.
Imatinib. First, Ang II, by binding to its Ang II type I receptor, can transactivate receptor tyrosine kinases, including the PDGFRβ.2,18 The concept of transactivation by Ang II has been reported in vitro19,20 and in vivo.2,7,21,22 Here, we show that a 1-minute stimulation with Ang II results in PDGFRβ phosphorylation in neonatal cardiac fibroblasts, and that this transactivation can be inhibited by imatinib treatment. These findings suggest that imatinib efficiently blocks the activation of the PDGFRβ in vitro as well as may explain the in vivo decreased cardiac fibrosis and improved cardiac function in imatinib-treated Ren2 rats.

Recently, our group showed that inhibition of EGF signaling also has beneficial effects in this model.7 Therefore, it is interesting to compare the role of these 2 growth factor receptors, EGFR and PDGFRβ, that share tyrosine kinase activity. However, the effects of inhibition of either EGFR or the PDGFR differ quite substantially. Whereas EGF inhibition by tyrphostin decreased cardiac fibrosis, and also attenuated the development of LV hypertrophy (LVH), tyrphostin treatment did not significantly ameliorate cardiac function. In contrast, in our current study, although imatinib also decreased cardiac fibrosis, it failed to attenuate LVH and, importantly, did improve cardiac dysfunction. Therefore, the effects of EGFR inhibition differ importantly from the here-described effects of inhibition of the PDGFRβ. To analyze this discrepancy, we investigated how the PDGFRβ transactivates the EGFR. Here, we show that when the PDGFRβ is activated by its native ligand PDGF, EGFR is transactivated, and this can be inhibited by imatinib. However, when the PDGFRβ is activated by Ang II, there is also concomitant activation of the EGFR, but this cannot be blocked by imatinib. This strongly suggests that Ang II uses other pathways beside the PDGFRβ to activate the EGFR, so that imatinib cannot block the effects of Ang II on the EGFR. As a result, imatinib only blocks the PDGFRβ effects in our model but not the EGFR effects. So, although seemingly related, EGFR or PDGFRβ inhibition results in different protective effects in the Ren2 rat.

Given the efficacy of direct inhibition of the PDGFRβ by imatinib, we propose that this activation of the PDGFRβ is a crucial step in the development of cardiac and renal end-organ damage. This explains also that direct inhibition of the PDGFRβ by imatinib is efficacious even without lowering blood pressure. Together, these findings implicate that imatinib attenuates the transition from LVH toward LV dysfunction by interrupting the Ang II–related activation of the PDGFRβ.

Effects of Imatinib in an Ang II–Driven Model of Accelerated Nephropathy

As mentioned above, homozygous Ren2 rats experience severe hypertension and high levels of local tissue Ang II, which together result in accelerated nephropathy.17 We show that imatinib treatment attenuates renal damage in the Ren2 rat. This result is in concordance with previous studies showing the protective effect of PDGF inhibition on nephropathy.23,24 Previous work of de Borst et al24 also revealed that ERK1/2 inhibition, using a mitogen-activated protein kinase blockade, protected against Ang II–mediated renal damage. Here, we show combined beneficial effects of imatinib on renal parenchymal and renovascular level in imatinib-treated Ren2 rats.

Other studies also have demonstrated the important role for the PDGFR. Aberrant PDGF signaling has been implicated in various pathological conditions, such as oncogenesis, atherosclerosis, lung fibrosis, and kidney fibrosis.25,26 Recently, Ponten et al showed that overexpression of PDGF-C results in dilated cardiomyopathy, cardiac fibrosis, and cardiac hypertrophy,27 whereas PDGF-D overexpression activated the PDGFRβ and resulted in cardiac fibrosis.28 These findings suggest that activation of PDGF signaling pathways plays an important role in the development of heart failure, in concordance with our finding of increased PDGFRβ activation in our model.

In conclusion, the present study is the first to demonstrate that imatinib, an orally active tyrosine kinase inhibitor, attenuates the development of hypertensive end-organ damage in the hypertensive homozygous Ren2 rat, probably by inhibition of the Ang II–related activation of the PDGFRβ pathway.

Perspectives

The findings presented in this study may have important implications for future treatment of cardiovascular diseases. The use of specific kinase inhibitors, already widely appreciated in the treatment of cancer, may also be very successful in cardiovascular diseases. It is known that growth factor receptors, such as the transforming growth factor-β (TGF-β) receptor and the EGFR, play important roles in the development of cardiac fibrosis and hypertension, and targeted inhibition of TGF-β and EGF signaling has beneficial effects.7,30 Now we also show that inhibition of PDGFRβ signaling has beneficial effects in a model of accelerated LV dysfunction. Because imatinib is already clinically successfully used for cancer therapy, our findings suggest that strategies aimed at inhibiting such tyrosine kinases may help to prevent hypertensive cardiac and renal damage.

References


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