Platelet-Derived Growth Factor Receptor Transactivation Mediates the Trophic Effects of Angiotensin II In Vivo

Darren J. Kelly, Alison J. Cox, Renae M. Gow, Yuan Zhang, Bruce E. Kemp, Richard E. Gilbert

Abstract—In addition to the modulation of vascular tone, angiotensin II (Ang II) has growth factor–like effects in vascular tissue. The mechanisms whereby Ang II mediates these trophic actions are incompletely understood but are thought to include effects on systemic blood pressure, stimulation of transforming growth factor-β (TGF-β) expression, and transactivation of growth factor receptor kinases. To investigate the role of platelet-derived growth factor receptor (PDGFR) transactivation in mediating the growth factor–like effects of Ang II we administered Ang II (200 ng/kg per minute) or saline to male Sprague-Dawley rats by osmotic minipump for 12 days and treated with imatinib mesylate, an inhibitor of the PDGFR tyrosine kinase. In addition to systolic blood pressure elevation, Ang II infusion led to increased vascular weight, media:lumen ratio, matrix expansion, and overexpression of TGF-β mRNA in mesenteric arteries. Without affecting blood pressure or PDGF ligand expression, imatinib attenuated the changes in vessel morphology but further increased TGF-β mRNA. Western blot analysis of mesenteric arterial tissue demonstrated the presence of the β- but not the α-isoform of PDGFR. Phosphorylation of PDGFR-β was increased by Ang II in vascular smooth muscle cells, and this was inhibited by imatinib. The findings of attenuation of vascular hypertrophy and matrix deposition by imatinib indicate that transactivation of the PDGFR in vivo contributes to the growth factor–like effects of Ang II, independent of its hemodynamic effects or its ability to induce TGF-β gene expression. (Hypertension. 2004; 44:195-202.)

Key Words: angiotensin II ■ platelet-derived growth factor ■ transforming growth factors ■ vascular diseases

The renin-angiotensin system (RAS) is a well recognized contributor to the pathogenesis of cardiovascular disease.1 While initially viewed as a purely vasoactive hormone, evidence accumulated through more than a decade suggests that angiotensin II (Ang II), the dominant effector molecule of the RAS, may also act as a growth factor in responsive tissues, such as those of the cardiovascular system.2 These growth factor–like effects include vascular smooth muscle hypertrophy3 and the synthesis of extracellular matrix,4 together contributing to Ang II–induced hypertrophic vascular remodeling.5

A range of mechanisms may contribute to the growth factor–like effects of Ang II.6 For instance, Ang II may induce hypertrophic remodeling as a consequence of its direct effects on blood pressure. In addition, angiotensin II potently stimulates the expression of transforming growth factor-β (TGF-β), a powerful inducer of matrix synthesis and cell hypertrophy.7 Furthermore, and of particular relevance to the current study, Ang II, by way of its type-1 receptor (AT1R), may also transactivate growth factor receptor tyrosine kinases, including those of the platelet-derived growth factor receptor (PDGFR)8 and epidermal growth factor receptor (EGFR).9 However, to date, studies exploring the trophic mechanisms of Ang II have been confined to cell culture or ex vivo organ baths10 that do not permit differentiation between the primary growth factor-like effects of Ang II and the secondary hemodynamic changes induced by this hormone. Furthermore, given the redundancy of the many signaling pathways of Ang II, the confirmation that a specific pathway has a key role in mediating organ injury requires the demonstration that its specific interruption attenuates injury in the in vivo setting.11 However, this has not yet been shown for the Ang II–PDGFR pathway.

In the present study we took advantage of a recently developed inhibitor of the PDGFR kinase to determine the role of the Ang II–PDGFR pathway in the in vivo mediation of vascular hypertrophy. We report that despite continued hypertension, inhibition of PDGFR signaling ameliorated Ang II-induced vascular remodeling in the superior mesenteric arterial tree, a site of extensive study in Ang II-mediated vascular injury.12,13

Methods

Animals

Eighty-eight, 8-week-old, male Sprague Dawley rats (Animal Resources Centre, Canning Vale, Western Australia) were anesthetized...
with enflurane (Abbott Australasia), and an osmotic minipump (Alzet Model 2002, Alzet Corporation) was inserted in the interscapular region. Rats were randomized to receive minipumps filled with vehicle (0.15 mol/L NaCl, 1 mmol/L acetic acid) either with or without Ang II (200 ng/kg per minute), as previously described.14 Animals were then further randomized to receive either vehicle (methylcellulose) or the PDGFR tyrosine kinase inhibitor, imatinib (methylcellulose) or the PDGFR tyrosine kinase inhibitor, imatinib mesylate (gift of Dr Elisabeth Buchdunger, Novartis, Basel, Switzerland) at a dose of 60 mg/kg by daily gavage and were euthanized after 12 days of Ang II infusion.

Mesenteric Vessel Isolation and Analysis
In each group of 22 animals, 8 were used for the measurement of mesenteric weight and gene expression analyses. In this group, animals were anesthetized and the first four-order branches of the mesenteric arterial tree mesenteric vessels were dissected free of fat, connective tissue, and the attached veins, as previously described.15

Mesenteric Histomorphometry and Immunohistochemistry
Quantitative histomorphometry and immunohistochemistry, including the assessment of vascular dimensions, were performed in 8 animals per group, as previously described.16 Sections were stained with either Masson trichrome, mouse anti-smooth muscle α-actin antibody, anti-phospho-Smad2 antibody, and anti-phospho-akt. Quantification of collagenous matrix, phospho-Smad2, and phospho-akt was performed on trichrome and immunostained sections, respectively, using computer-assisted image analysis, as previously described17 and detailed in an online supplement available at http://www.hypertensionaha.org.

RNA Extraction and cDNA Synthesis
Frozen mesenteric vascular tissue, stored at −80°C was homogenized (Polytron, Kinematica Gmbh, Littau, Switzerland), and total RNA was isolated using TRizol reagent (Life Technologies). DNase treated RNA was then reverse transcribed, as previously reported18 and detailed online (http://www.hypertensionaha.org).

Quantitative Real-Time Reverse Transcription–Polymerase Chain Reaction
TGF-β1, PDGF-A, and PDGF-B gene expression were quantified by real-time reverse transcription–polymerase chain reaction (RT-PCR) using sequence-specific primers, as previously reported by our group18 (Table), and a commercial, predesigned 18S control kit (see http://www.hypertensionaha.org).

Immunoprecipitation and Immunoblot Analyses of Mesenteric Arterial Tissues
Immunoprecipitation and Western blot analysis were performed as previously described19 and detailed in the online supplement using polyclonal rabbit anti-rat antibodies for PDGFR-β, PDGFR-α, and c-abl antibody (Pharmingen, San Diego, Calif) and anti-phosphotyrosine antibody 4G10 (anti-pY, Upstate). Immunoblot analysis of c-kit was also performed by immunoblotting with anti-pY followed by anti-c-kit. Positive controls for PDGFR-α and c-kit were rat vascular smooth muscle cells (gift of Michael Hill, RMIT, Bundoora, Australia) and gastrointestinal stromal tumor lysates (gift of Dr. David Thomas, University of Melbourne, Australia) for PDGFR-α and c-kit, respectively.

Cell Culture
Rat aortic vascular smooth muscle cells (VSMCs) were isolated from the thoracic aorta of Sprague-Dawley rats and maintained. Ang II stimulation studies were then performed, as previously described.20 Samples of cell protein lysate were electrophoresed, transferred to membranes, and immunoblotted with a rabbit polyclonal phospho-specific PDGFR-β antibody (Tyr 751, Cell Signaling, Beverly, Mass), then anti-PDGFR-β, or with rabbit polyclonal phospho-specific c-abl antibody (Tyr 245, Cell Signaling) followed by anti-c-abl, stripping the membranes between each immunoblotting. Chronic myeloid leukemia BCR-ABL positive cell line, K562, was used as a positive control for c-abl phosphorylation.21 Band intensities were quantified using MCID M4 software (Image Research Inc.), as previously described.22 Results are the mean of at least 3 experiments.

Statistics
All data are shown as mean±SE unless otherwise specified. Data were analyzed by ANOVA using the StatView IV program (Brainpower) on a Macintosh G4. Comparisons between group means were performed by the Fisher least significant difference method. P<0.05 was considered statistically significant.

Results
Animal Data
Ang II infusion was associated with increased systolic blood pressure (SBP) compared with vehicle-treated animals (141±2 mm Hg versus 202±7 mm Hg, vehicle versus Ang II, P<0.001). Mesenteric vessel weight was also increased in Ang II–infused rats, compared with control animals receiving vehicle (Figure 1). Treatment with imatinib had no effect on SBP in either Ang II–infused rats (205±10 mm Hg) or in control animals (138±3 mm Hg). However, imatinib significantly reduced mesenteric weight in animals receiving Ang II (Figure 1). Similarly, medial thickness was also increased.

Histology and Immunohistochemistry
Histomorphometric analysis revealed a significant increase in the medial thickness, matrix expansion, and media:lumen ratio (Figure 1) in Ang II–infused rats. Treatment with imatinib significantly reduced each of these parameters to levels approaching those in control animals. (Figures 1 and 2).

Vessels from Ang II–infused rats showed increased immunostaining for phospho-Smad2 and phospho-akt with significant reductions in both phospho-proteins in animals treated with imatinib (Figures 3 and 4).

Gene Expression
Mesenteric vessel TGF-β1 mRNA was increased 2-fold in animals receiving Ang II infusions when compared with control animals receiving vehicle. In animals receiving imatinib treatment, a further increase in TGF-β expression was noted when compared with their untreated counterparts also subjected to Ang II infusion (Figure 5). In contrast, PDGF-A

<table>
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<tr>
<th>Primers and Probe Used for RT-PCR Analysis</th>
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<tr>
<td><strong>TGF-β</strong></td>
</tr>
<tr>
<td>Forward: 5'-AGA AGT CAT CAC CGG GTG GCT A-3'</td>
</tr>
<tr>
<td>Reverse: 5'-GTG GTG ATG TCT TTG GTG TCA-3'</td>
</tr>
<tr>
<td>Probe: FAM-TGG TGG ACC GCA ACA ACC CAA TAMRA</td>
</tr>
<tr>
<td><strong>PDGF-A</strong></td>
</tr>
<tr>
<td>Forward: 5'-TGAACAGCGACGGCAGTCAAGT-3'</td>
</tr>
<tr>
<td>Reverse: 5'-CAATTTGGCTCTTCCGTACATAAC-3'</td>
</tr>
<tr>
<td>Probe: FAM-CTCAAGG6GTCCACACCGCAGTGF TAMRA</td>
</tr>
<tr>
<td><strong>PDGF-B</strong></td>
</tr>
<tr>
<td>Forward: 5'-CGA CTG CAA GAC GGC TAC A-3'</td>
</tr>
<tr>
<td>Reverse: 5'-GCG ATT GTG GGC ATC GA-3'</td>
</tr>
<tr>
<td>Probe: FAM-AGG TGT TCC AGA TCT CCG GGA ACC TC-TAMRA</td>
</tr>
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These primers and probes were used for RT-PCR analysis.
and PDGF-B gene expression were not increased in Ang II–infused animals and was unaffected by treatment with imatinib (Figure 5).

**Immunoblots of Mesenteric Tissue**

Imatinib is a potent inhibitor of PDGFR (α and β) and also c-abl and c-kit. We therefore examined the expression of all of these tyrosine kinases in the mesenteric arterial tissue. PDGFR-β and c-abl were both readily detected in mesenteric lysates when immunoblotted (Figure 6). In contrast, PDGFR-α was not detected after immunoprecipitation nor could c-kit be detected in mesenteric lysates, though Coomassie-stained filters showed similar protein loading to gastrointestinal stromal tumor (GIST) cell lysate that served as a positive control (Figure 6). Expression of each of these kinases was unchanged by Ang II, with or without imatinib.

**In Vitro Phosphorylation Studies**

Having found that only PDGFR-β and c-abl were both present in vivo, activation (tyrosine phosphorylation) of these 2 kinases in response to angiotensin II and coadministration of imatinib was then examined in vitro. Phosphorylation of the 180 kDa PDGFR-β in VSMC lysates was demonstrated by immunoblotting filters with the phosphospecific PDGFR-β antibody (Tyr 751). Stimulation with 100 nM Ang II for 1 minute led to an approximate 2-fold increase in phosphorylated PDGFR-β that was prevented by preincubation with 1 μmol/L imatinib (Figure 7). Although c-abl was present in VSMC it was neither phosphorylated at baseline nor in response to Ang II (Figure 7). K562 cells (positive controls) showed evidence of phosphorylation of both c-abl and bcr-abl.

**Discussion**

Blockade of the RAS is an important therapeutic strategy in reducing morbidity and mortality in patients with cardiovascular disease. However, the mechanisms by which such therapy exerts its vasoprotective effects, including those associated with tissue remodeling remain the subject of ongoing debate. The present study demonstrates that despite continued hypertension, the hypertrophic remodeling induced by Ang II can be attenuated by inhibiting PDGF signal transduction, thereby providing a mechanistic explanation for at least some of the growth factor–like effects of Ang II in vivo setting.

Previous studies have explored the ability of Ang II to transactivate the PDGFR in vitro and ex vivo in an organ bath. Other studies have also shown that Ang II infusion leads to activation of the PDGFR in experimental animals. However, Ang II activates a range of intracellular signaling pathways that, in addition to growth-factor receptors, include pp60 c-src kinase (c-src), focal adhesion kinase, and Janus kinases (JAK2/TYK2). Thus, the confirmation of a pathophysiological role of any particular pathway requires the in vivo demonstration that specific blockade leads to attenuation of injury. To our knowledge, the present study is the first to report that blockade of Ang II–induced PDGFR signaling in vivo leads to a reduction in Ang II–induced hypertrophy and matrix accumulation.

Cross-talk with receptor tyrosine kinases has been shown to be responsible for the growth factor–like effects of a
number of G protein–coupled receptor activating ligands. In particular, several aspects of Ang II signal transduction also resemble those of growth factors with in vitro studies documenting Ang II–induced transactivation of the PDGFR and EGFR. In the present study we used a pharmacological approach to examine the PDGFR transactivation pathway in vivo. Inhibition of the PDGFR tyrosine kinase attenuated several aspects of Ang II–induced hypertrophy, including the increased vessel weight, media:lumen ratio, and matrix deposition. Moreover, these effects occurred independently of any change in blood pressure. Examination of tissue expression in our experimental model showed that whereas the β-isofrom of PDGFR was abundantly expressed, as in previous studies, the PDGFR-α isoform was not, indicating that transactivation of PDGFR-β is sufficient to induce growth factor–like effects of Ang II in the in vivo setting.

In mesenteric arterial tissue we found the presence of PDGFR-β and evidence of activation by Ang II, as indicated by the increased expression of phosphorylated-akt. Using cultured VSMCs we then went on to examine PDGFR-β phosphorylation in response to Ang II and in the presence of the PDGFR tyrosine kinase inhibitor, imatinib. In these studies we showed that not only does Ang II induce PDGFR-β phosphorylation, but also that this phosphorylation could be prevented by pretreatment with imatinib. However, imatinib did not result in complete abrogation of the effects of Ang II on mesenteric hypertrophy. These findings indicate that hemodynamically responsive mechanisms or other downstream pathways in addition to PDGFR-β may also contribute to the trophic effects of Ang II. In contrast to PDGFR-β, whereas c-abl was also present in mesenteric
arterial tissue and in VSMCs, it was neither phosphorylated at baseline nor in response to Ang II.

Despite continued hypertension, imatinib attenuated angiotensin-induced vascular remodeling. These findings raise the possibility that both the mechanical effects of hypertension and Ang II may signal by common intracellular transduction pathways involving the PDGFR. Indeed, recent organ bath studies have shown that pressure and Ang II lead to activation of extracellular signal-regulated kinase 1/2 (ERK 1/2), a central signaling molecule in vascular remodeling.10 Moreover, the finding that both pressure and Ang II-induced ERK 1/2 activation could be inhibited by both a PDGFR-neutralizing antibody and PDGFR kinase blocker10 suggest that both activate PDGFR-dependent pathways.

The ability of Ang II to induce TGF-β has been repeatedly suggested to be a key mechanism underlying the development of hypertrophy and matrix expansion in cardiac and renal disease.32,33 For instance, reduction in TGF-β by blockade of the RAS with either angiotensin-converting enzyme inhibition or AT₁R antagonism is thought to contribute to the cardio- and renoprotective effects of these therapies.34,35 TGF-β is synthesized as a 391 amino acid precursor molecule with little biological activity, requiring cleavage of its N-terminal latency associated peptide to give rise to its active form.36 In addition, its biological effects may also be modified by the presence of the proteoglycan decorin37 and the scavenging protein α2-macroglobulin.38 Thus, increased TGF-β1 mRNA or protein may not necessarily reflect parallel changes in TGF-β1 activity. In the present study, in addition to examining its mRNA, we therefore also assessed the biological effects of TGF-β by examining one of its
specific intracellular actions, the phosphorylation of the TGF-β receptor–activated protein, Smad2. In contrast to control rats, prominent nuclear staining of phosphorylated Smad2 was noted in vessels from Ang II–infused animals and was substantially diminished by treatment with imatinib, consistent with the previously described interactions between the TGF-β and PDGFR signaling pathways. However, in contrast to the reduction in TGF-β signaling with imatinib, an increase in gene expression was noted. The possible reasons...
for the apparent discordance between TGF-β mRNA and biological activity are many and underscore the importance of assessing the biological activity of this growth factor.

Like TGF-β, the actions of PDGF also include matrix synthesis and cell growth. We therefore considered the possibility that Ang II induction of PDGF ligand might account for the observed changes in our study. However, in contrast its effects on TGF-β, PDGF expression was not increased by Ang II and remained unaffected by imatinib. These findings indicate that the effects of Ang II and imatinib, as demonstrated in the present study, do not reflect a paracrine or autocrine effect of Ang II–induced PDGF ligand expression.

Whereas imatinib is a potent inhibitor of the PDGFR tyrosine kinase, it has no significant effect on a range of other receptor tyrosine kinases such as those of the EGF, insulin, and insulin-like growth factor-1 receptors. In particular, the 100-fold difference in the IC50 of imatinib for PDGFR compared with EGFR is of particular relevance to the present study as transactivation of the EGFR has also been implicated in the mediation of the growth factor–like effects of Ang II. However, although the inhibitor activity of imatinib is narrow, it does include the c-abl and c-kit tyrosine kinases. In the present study, c-kit was not detected in vivo; whereas c-abl was present in mesenteric arterial tissue, it was not phosphorylated at baseline or in response to Ang II. These finding indicate that neither c-abl nor c-kit contribute to the trophic effects of Ang II or to their attenuation by imatinib, as observed in the present report. Indeed, although the signal transduction pathway of Ang II includes the activation of a number of tyrosine kinases (reviewed in Reference 6) these do not include phosphorylation of either c-abl or c-kit. Alternative inhibitors of PDGF tyrosine kinases include staurosporine and the tyrphostins. However, staurosporine is a relatively nonspecific tyrosine kinase inhibitor and tyrphostins such as AG1296, used as a specific PDGFR blocker, also potently inhibits c-kit. Moreover, none of these compounds are suitable for long-term in vivo administration, contrasting the 2-phenylaminopyrimidine compound used in the present study, which is both suitable for in vivo use and has had the specificity of its inhibitory activity more extensively defined.

Perspectives

The results of the present study demonstrate attenuation of Ang II–induced vascular hypertrophy by imatinib despite persistent hypertension. These findings indicate that the PDGFR signal transduction pathway is a significant contributor to the trophic, as distinct from the hemodynamic, effects of Ang II in the mesenteric arterial tree of the rat. Further investigation will be needed to determine whether PDGFR transduction inhibition may provide a new strategy for adverse remodeling in cardiovascular tissues.

Acknowledgments

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