In Vivo Activation of Rat Aortic Platelet-Derived Growth Factor and Epidermal Growth Factor Receptors by Angiotensin II and Hypertension

Shokei Kim, Yumei Zhan, Yasukatsu Izumi, Hideo Yasumoto, Masahiko Yano, Hiroshi Iwao

Abstract—It is unclear whether the previous in vitro evidence of a link between angiotensin II (Ang II) and growth factor receptors can apply to the in vivo situation. In this study, we examined vascular platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) receptor activation in stroke-prone spontaneously hypertensive rats (SHRSP) and the role of Ang II. Tyrosyl phosphorylation of the growth factor receptors was determined by Western blot analysis coupled with immunoprecipitation. Tyrosyl phosphorylation of the aortic PDGF β-receptor, but not the EGF receptor, was chronically increased in SHRSP with hypertension, compared with normotensive rats, being accompanied by increased extracellular signal–regulated kinase (ERK) activity. Treatment of SHRSP with ACE inhibitors (perindopril or enalapril) significantly reduced aortic PDGF β-receptor tyrosyl phosphorylation and ERK activity, whereas treatment with hydralazine failed to reduce these activities. Therefore, these aortic changes in SHRSP were mediated by Ang II in response to vascular ACE. Ang II was infused into rats to examine the effects on aortic growth factor receptors. Chronic Ang II infusion, via the angiotensin type 1 receptor, significantly increased activation of the aortic PDGF β-receptor but not the EGF receptor. Thus, the aortic PDGF β-receptor, activated by ACE-mediated Ang II, seems to be responsible for vascular remodeling in hypertensive rats. (Arterioscler Thromb Vasc Biol. 2000;20:2539-2545.)

Key Words: angiotensin • vascular remodeling • platelet-derived growth factor • hypertension • tyrosine phosphorylation

A ccumulating evidence supports the notion that the renin-angiotensin system, via angiotensin II (Ang II), plays a major role not only in hypertension but also in various vascular diseases.1–5 Therefore, so far, the actions of Ang II in cultured vascular cells, particularly vascular smooth muscle cells (VSMCs), have been extensively studied, and multiple lines of in vitro studies have supported the notion that Ang II, independent of its blood pressure–elevating effect, can stimulate vascular remodeling via the regulation of multiple vascular remodeling–associated molecules, such as growth factors, cytokines, and extracellular matrix.1,3,5–7 However, most of the in vitro findings involving the action of Ang II on cultured vascular cells have not yet been demonstrated in conditions in vivo, and the in vivo molecular mechanism of Ang II–induced vascular remodeling is not fully understood.

Platelet-derived growth factor (PDGF), particularly PDGF-BB, potently stimulates either VSMC proliferation or migration8–11 and is the major growth factor involved in various vascular diseases, such as atherosclerosis and neointimal hyperplasia.11–15 It is well known that PDGF, like many other growth factors, initially activates the intrinsic receptor tyrosine kinase on binding to the receptors (PDGF α- and β-receptors), resulting in tyrosyl phosphorylation of the receptors themselves and cellular substrate proteins for receptor kinases.14 Thus, tyrosyl phosphorylation of the PDGF α- or β-receptor is the essential first step leading to the biological action of PDGF, thereby being regarded as a useful parameter to estimate the functional activity of PDGF. Interestingly, an in vitro report has shown that Ang II induced tyrosyl phosphorylation of the PDGF β-receptor in cultured rat VSMCs in a direct manner.16 On the other hand, a more recent report indicated that Ang II induced tyrosyl phosphorylation of the epidermal growth factor (EGF) receptor but not the PDGF receptor in cultured rat VSMCs.17,18 Thus, controversial evidence exists with respect to the activation of PDGF and EGF receptors by Ang II in vitro. However, the role of Ang II in these receptor activations in vivo is poorly understood.

In the present study, to examine the contribution of PDGF and EGF receptor activation in hypertensive vascular remodeling, we determined tyrosyl phosphorylation of these receptors in stroke-prone spontaneously hypertensive rats (SHRSP), the popular and useful model for investigation of the mechanism of not only hypertension but also vascular remodeling.19 We also examined the role of ACE and Ang II in these receptor activations. We obtained the evidence that...
the PDGF β-receptor is chronically activated in the vascular tissue of hypertensive rats and that this receptor activation is mainly due to ACE-mediated Ang II.

Methods

Animals

Male SHRSP and control Wistar-Kyoto rats (WKY), which were purchased from Japan SLC (Shizuoka, Japan), were fed standard laboratory chow (CE-2, Clea) and given tap water ad libitum.

Effects of ACE Inhibitors and Hydralazine on Aortic Growth Factor Receptors

All procedures were in accordance with institutional guidelines for the care and use of laboratory animals. Thirteen-week-old SHRSP were orally given perindopril (2 or 4 mg/kg), enalapril (10 mg/kg), hydralazine (≈30 mg/kg), or vehicle (0.5% carboxymethylcellulose solution) for 4 weeks (from 13 to 17 weeks of age). Except for hydralazine, all drugs were given to SHRSP by gastric gavage once a day. Hydralazine was dissolved in distilled water and given to rats as drinking water for the same period. Furthermore, in another group, to investigate the possible contribution of bradykinin to the effects of perindopril, SHRSP were not only given perindopril orally (4 mg/kg per day) but were also subcutaneously infused the bradykinin B2 receptor antagonist Hoe 140 at a dose of 300 µg/kg per day via osmotic minipump (Alza Corp). After 1 and 4 weeks of drug treatment, the systolic blood pressure of conscious rats was measured by the tail-cuff method at 3 or 24 hours after oral dosing. As shown in Figure 2, at 1 and 4 weeks after the start of drug treatment, blood pressure of SHRSP was measured at 3 and 24 hours after oral dosing of each drug (except for hydralazine). After either 1 or 4 weeks, at 3 hours after oral dosing, perindopril lowered the blood pressure of SHRSP in a dose-dependent fashion, and 4 mg/kg perindopril and 10 mg/kg enalapril had a comparable hypotensive effect. On the other hand, hydralazine lowered the blood pressure of SHRSP in a dose-dependent fashion, and 4 mg/kg perindopril–treated SHRSP was 1.6-fold (P<0.01) and 2.0-fold (P<0.01), respectively, greater than that in age-matched WKY. There was no significant difference in aortic PDGF β-receptor or EGFR receptor tyrosine phosphorylation between WKY and SHRSP at any age examined. Furthermore, no significant difference was found in aortic PDGF α- or β-receptor or EGFR receptor protein levels between WKY and SHRSP at any age examined.

We also compared aortic PDGF β-receptor tyrosine phosphorylation between 20-week-old WKY and SHRSP after endothelial removal. After endothelial denudation, aortic PDGF β-receptor tyrosine phosphorylation in SHRSP (n=5) was 2.3-fold larger (P<0.01) than that in WKY (n=5), whereas there was no difference in aortic PDGF β-receptor protein levels between these groups.

Results

Tyrosine Phosphorylation of Aortic PDGF α-Receptors, PDGF β-Receptors, and EGF Receptors in SHRSP

Figure 1 indicates tyrosine phosphorylation of each growth factor receptor in aortas from SHRSP at various ages compared with WKY. Blood pressure of 5-week-old SHRSP was similar to that of age-matched WKY (110±9 versus 101±2 mm Hg, respectively). However, SHRSP showed higher blood pressure than did WKY at 10 weeks of age (178±5 versus 124±3 mm Hg, respectively; P<0.01) and 20 weeks of age (211±4 versus 131±4 mm Hg, respectively; P<0.01). In normotensive 5-week-old SHRSP, tyrosine phosphorylation of aortic PDGF α- or β-receptors or EGFR receptors was similar to that in age-matched WKY. On the other hand, tyrosine phosphorylation of aortic PDGF β-receptors in 10- and 20-week-old SHRSP was 1.6-fold (P<0.01) and 2.0-fold (P<0.01), respectively, greater than that in age-matched WKY. There was no significant difference in aortic PDGF α-receptor or EGFR receptor tyrosine phosphorylation between WKY and SHRSP at any age examined. Furthermore, no significant difference was found in aortic PDGF α- or β-receptor or EGFR receptor protein levels between WKY and SHRSP at any age examined.

As shown in Figure 2, at 1 and 4 weeks after the start of drug treatment, blood pressure of SHRSP was measured at 3 and 24 hours after oral dosing of each drug (except for hydralazine). After either 1 or 4 weeks, at 3 hours after oral dosing, perindopril lowered the blood pressure of SHRSP in a dose-dependent fashion, and 4 mg/kg perindopril and 10 mg/kg enalapril had a comparable hypotensive effect. On the other hand, hydralazine lowered the blood pressure of SHRSP in a dose-dependent fashion, and 4 mg/kg perindopril–treated SHRSP was significantly lower than that of 10 mg/kg enalapril–treated SHRSP. The blood pressure of SHRSP treated with hydralazine as drinking water was similar or lower than that of 4 mg/kg perindopril–treated SHRSP at all time points examined.

Hoe 140 treatment did not apparently affect the hypotensive effects of perindopril (4 mg/kg per day) at 3 or 24 hours after oral dosing after 1 or 4 weeks of drug treatment.
Effects of ACE Inhibitors and Hydralazine on Aortic ACE Activity of SHRSP

Figure 3 indicates serum and aortic ACE activity of SHRSP after 4 weeks of drug treatment. Serum ACE activity of SHRSP treated with 2 mg/kg perindopril, 4 mg/kg perindopril, and enalapril was much lower than that of vehicle-treated SHRSP, and there was no significant difference in serum ACE activity among all ACE inhibitor–treated groups.

Aortic ACE activity of SHRSP treated with 2 mg/kg perindopril, 4 mg/kg perindopril, and enalapril (8.26±0.41, 4.23±0.26, and 8.85 nmol·min⁻¹·mg protein⁻¹, respectively) was lower than that of vehicle-treated SHRSP (32.1±1.4 nmol·min⁻¹·mg protein⁻¹). Perindopril at 4 mg/kg reduced aortic ACE activity to a greater extent than did perindopril at 2 mg/kg and enalapril (P<0.05).

Hydralazine did not significantly affect serum or aortic ACE activity of SHRSP. Hoe 140 treatment did not apparently affect serum and aortic ACE activity of perindopril-treated SHRSP.

Figure 2. Blood pressure (BP) of SHRSP at 1 week (A) and 4 weeks (B) after start of antihypertensive drug treatment. BP was measured at 3 and 24 hours after oral dosing of each drug. SHRSP were treated with vehicle (C), 2 mg/kg perindopril [P(2)], 4 mg/kg perindopril [P(4)], 4 mg/kg perindopril combined with Hoe 140 [300 μg·kg⁻¹·d⁻¹, P(4)+Hoe], 10 mg/kg enalapril [E(10)], or hydralazine (Hy). Values are mean±SEM (n=7 or 8). *P<0.05 vs control (C); †P<0.05 vs P(2).

Effects of ACE Inhibitors and Hydralazine on Aortic PDGF β-Receptors of SHRSP

As shown in Figure 4, either perindopril or enalapril reduced tyrosine phosphorylation of aortic PDGF β-receptors in SHRSP, and perindopril at 4 mg/kg decreased aortic PDGF β-receptor tyrosine phosphorylation to a larger extent than did enalapril (P<0.05). On the other hand, hydralazine treatment failed to reduce aortic PDGF β-receptor phosphorylation of SHRSP. Hoe 140 treatment did not apparently affect the reduction of aortic PDGF β-receptor tyrosine phosphorylation of SHRSP by perindopril.

Effects of ACE Inhibitors and Hydralazine on Aortic ERK of SHRSP

As shown in Figure 5, either perindopril or enalapril significantly reduced the phosphorylation of aortic extracellular signal–regulated kinase (ERK), p42ERK and p44ERK, of SHRSP, and perindopril at 4 mg/kg decreased aortic ERK phosphorylation to a larger extent than did enalapril (P<0.01). On the other hand, hydralazine treatment failed to
reduce aortic ERK phosphorylation of SHRSP. Hoe 140 treatment did not apparently affect the reduction of aortic ERK phosphorylation of SHRSP by perindopril.

**Effects of Acute Ang II Infusion on Aortic PDGF β-Receptors and EGF Receptors**

As shown in Figure 6, aortic EGF receptor tyrosine phosphorylation was increased by 3.1-fold ($P<0.01$; each time point, $n=5$) at 5 minutes after acute infusion of Ang II at 100 ng·kg$^{-1}$·min$^{-1}$ with a 31 mm Hg rise in mean blood pressure but had already returned to the control level at 15 minutes.

Unlike the EGF receptor, the aortic PDGF β-receptor tyrosine phosphorylation was not affected throughout 60 minutes of acute infusion of Ang II at 100 ng·kg$^{-1}$·min$^{-1}$ (each time point, $n=5$; Figure 6).

**Effects of Chronic Ang II Infusion on Aortic PDGF β-Receptors and EGF Receptors**

Rats subjected to Ang II infusion (400 ng·kg$^{-1}$·min$^{-1}$ SC) had significantly increased blood pressure at 3 days compared with control rats (140±6 versus 118±4 mm Hg, respectively; $P<0.01$), and this increase in blood pressure was completely blocked by treatment with CS-866 or hydralazine. As shown in Figure I (please see online at http://atvb.ahajournals.org), chronic Ang II infusion increased aortic PDGF β-receptor tyrosine phosphorylation by 1.8-, 2.7-, and 2.2-fold at 3 days, 1 week, and 2 weeks, respectively. Treatment with CS-866 completely blocked Ang II–induced PDGF β-receptor tyrosine phosphorylation, whereas normalization of blood pressure by hydralazine only partially suppressed this receptor phosphorylation. Unlike the case of the PDGF β-receptor, chronic Ang II infusion did not apparently increase aortic EGF receptor tyrosine phosphorylation throughout the infusion (data not shown).

**Discussion**

The mechanism underlying Ang II–mediated vascular diseases in vivo is not fully understood. In vitro evidence shows that Ang II itself induces hypertrophy, but not proliferation, of cultured VSMCs. However, in contrast to in vitro findings, in vivo studies have demonstrated that Ang II stimulates VSMC proliferation, indicating the difference between in vitro and in vivo actions of Ang II on VSMCs and suggesting that vascular proliferative action of Ang II in vivo may be mediated by the elevation of blood pressure or the activation of vascular remodeling–associated molecules. Interestingly, Ang II is reported to induce PDGF gene expression and PDGF β-receptor tyrosyl phosphorylation in cultured VSMCs. On the other hand, more recently,
other groups of investigators have reported that Ang II induces tyrosyl phosphorylation of EGF receptors in cultured rat VSMCs but does not induce tyrosyl phosphorylation of PDGF receptors and that this EGF receptor activation participates in Ang II–induced ERK activation and cell hypertrophy. Thus, there have been controversial findings regarding the activation of PDGF and EGF receptors by Ang II in cultured VSMCs. Moreover, in the in vivo regulation of these receptor activations by Ang II is poorly understood. Therefore, in the present study, we investigated these receptor activations in hypertensive rats and the role of Ang II.

In the present study, we found that the tyrosyl phosphorylation of the aortic PDGF β-receptor, but not of the PDGF α-receptor or EGF receptor, was enhanced in SHRSP with the development of hypertension, suggesting the involvement of the PDGF β-receptor in hypertensive vascular remodeling. This enhanced PDGF β-receptor phosphorylation in SHRSP seems to be at least in part due to smooth muscle cells, because PDGF β-receptor tyrosyl phosphorylation of aortas subjected to endothelial removal was greater in SHRSP than in WKY, and smooth muscle cells are the major component of vascular tissue. To elucidate the mechanism of this PDGF β-receptor activation in SHRSP, we examined the effect of ACE inhibitors and hydralazine on aortic receptors. Of note, although hydralazine lowered the blood pressure of SHRSP at least to a degree comparable to that produced by ACE inhibitors (perindopril and enalapril), hydralazine did not significantly reduce aortic PDGF β-receptor phosphorylation of SHRSP, providing no evidence for the major role of hypertension in this receptor activation. On the other hand, perindopril or enalapril significantly decreased this receptor activation of SHRSP. These observations provide evidence that ACE directly contributes to the increase in aortic PDGF β-receptor activation in SHRSP. Previously, we have reported that aortic ERK activity is increased in aortas of SHRSP with the development of hypertension. ERK in VSMCs is well known to be activated by Ang II, PDGF, or EGF and plays an important role in VSMC proliferation and migration. Therefore, in the present study, we also examined the effect of ACE inhibitors on aortic ERK activity and found that the increased aortic ERK activity in SHRSP is mediated by ACE, as shown by the reduction of ERK activity by ACE inhibitors but not by hydralazine. Thus, the enhanced aortic ERK activity in SHRSP may be partially mediated by PDGF β-receptor activation. However, further study is needed to demonstrate our proposal.

A growing body of evidence supports the notion that vascular effects of ACE inhibitors are mediated not only by the inhibition of Ang II generation but also by the accumulation of bradykinin. Therefore, to determine which mechanism is involved in the inhibitory effects of ACE inhibitors on aortic PDGF β-receptors and ERK, we examined the effect of Hoe 140, a specific bradykinin antagonist, and found no evidence of an important role of bradykinin in the present experimental conditions. Thus, the effects of ACE inhibitors in the present study seem to be mainly due to the suppression of Ang II generation, although the possible contribution of bradykinin cannot be completely ruled out.

In the present study, in spite of a comparable hypotensive effect between perindopril (4 mg/kg) and enalapril (10 mg/kg) at 3 hours after oral dosing, the hypotensive effect at 24 hours after oral dosing was greater in perindopril than enalapril, confirming previous findings on longer pharmacological action of perindopril than enalapril in vivo. Interestingly, despite no significant difference in the decrease in serum ACE activity between perindopril and enalapril, perindopril (4 mg/kg) reduced the aortic ACE activity of SHRSP more potently than did enalapril (10 mg/kg), and this greater reduction of aortic ACE activity by perindopril was associated with the larger decrease in PDGF β-receptor phosphorylation and ERK activity. These findings suggest that vascular ACE may be implicated in aortic PDGF β-receptor and ERK activation of SHRSP.

To our knowledge, so far, there has been no report on vascular immunoreactive PDGF or EGF receptor protein or the tyrosyl phosphorylation of either in a hypertensive model. Previous reports on other hypertensive rats have been limited to studies of mRNA levels. It has been reported that aortic PDGF β-receptor mRNA is increased in spontaneously hypertensive rats and deoxycorticosterone acetate-salt hypertensive rats, but mRNA levels for PDGF α-receptors or PDGF A or B chains are not increased in these hypertensive rats. On the other hand, being in disagreement with the above findings, another group of investigators reported an increased aortic PDGF A chain (but not B chain) mRNA in spontaneously hypertensive rats. However, it is well established that the protein level is regulated not only by the transcriptional rate but also by other mechanisms, such as the translational rate and the degradation rate, and so the mRNA level is not necessarily parallel with the protein level. Therefore, it is difficult to directly compare our present study on protein level and tyrosyl phosphorylation with previous findings on mRNA. Abe et al. who also examined immunoreactive PDGF and EGF receptor protein and their phosphorylation as in our present work, reported that tyrosyl phosphorylation of the PDGF receptor, but not the EGF receptor, is chronically enhanced in the balloon-injured rat artery, whereas PDGF or EGF receptor protein expression is not elevated in the balloon-injured artery. Thus, our present findings on hypertensive rats are similar to the case of the balloon-injury model.

To further elucidate the role of Ang II in vascular growth factor activation, we examined the effects of acute and chronic Ang II infusion. The observations on acute Ang II infusion showed that Ang II transiently induced aortic tyrosyl phosphorylation of the EGF receptor but not that of the PDGF β-receptor. Thus, our present in vivo work provided the first evidence for the in vivo acute activation of the EGF receptor by Ang II and did not support in vitro previous findings that Ang II acutely and transiently phosphorylates the PDGF β-receptor in cultured VSMCs. Therefore, there may be a significant difference between the phenotype of VSMCs in vitro and in vivo, and this difference may be in part explained by the fact that the VSMC in an in vivo situation is constantly and continuously exposed to high pressure, shear stress, or a variety of vascular remodeling–related molecules, such as vasoactive peptides, growth factors, or inflammatory cytokines, unlike VSMCs in an in vitro situation.

Unlike acute infusion, chronic Ang II infusion increased tyrosyl phosphorylation of the PDGF β-receptor but not the EGF receptor. This PDGF β-receptor activation by Ang II was completely suppressed by the angiotensin type 1 (AT1)
receptor antagonist but only partially prevented by normalization of blood pressure by hydralazine, a vasodilator. Hence, Ang II in vivo activates the aortic PDGF β-receptor not only by its hypertensive effect but also by AT1 receptor activation independent of blood pressure. Partial prevention of Ang II-induced PDGF β-receptor activation by hydralazine suggests that no suppression of this receptor in SHRSP by hydralazine might be due to the increase in circulating Ang II, because hydralazine significantly increased plasma renin activity in SHRSP (data not shown). Sambhi et al. reported that 4 weeks of Ang II infusion in rats increased aortic EGF receptor mRNA and aortic binding activity with 125I-labeled EGF. However, these investigators did not examine immunoreactive EGF receptor protein itself or its tyrosyl phosphorylation, which seems to account for the discrepancy between our present findings and the previous report. Furthermore, it is also possible that this discrepancy may be explained by the difference in the period of Ang II infusion (2 weeks in our present work versus 4 weeks in the previous work).

Ang II in vivo elicits AT1 receptor–mediated various biological effects, such as the activation of the sympathetic nervous system or the release of adrenal steroid hormone, which may have significant effects on vascular tissues. Therefore, it cannot be excluded that the activation of aortic PDGF β-receptor by Ang II infusion might be partially mediated by these indirect actions. Further study is needed to confirm that Ang II directly activates the vascular PDGF β-receptor in vivo.

In conclusion, tyrosyl phosphorylation of the aortic PDGF β-receptor, but not the EGF receptor, was chronically enhanced in SHRSP with the development of hypertension. This enhanced aortic PDGF β-receptor activation of SHRSP was mediated by ACE rather than high blood pressure. Chronic PDGF β-receptor activation seems to be implicated in Ang II–induced vascular remodeling in vivo. Thus, the present study provides new insight into the molecular mechanism of hypertensive vascular remodeling.

Acknowledgments

This work was in part supported by grants-in-aid for scientific research (09470527 and 11670098) from the Ministry of Education, Science, and Culture.

References

activities are continuously and differentially increased in aorta of hypertensive rats. Biochem Biophys Res Commun. 1997;236:199–204.


In Vivo Activation of Rat Aortic Platelet-Derived Growth Factor and Epidermal Growth Factor Receptors by Angiotensin II and Hypertension
Shokei Kim, Yumei Zhan, Yasukatsu Izumi, Hideo Yasumoto, Masahiko Yano and Hiroshi Iwao

Arterioscler Thromb Vasc Biol. 2000;20:2539-2545
doi: 10.1161/01.ATV.20.12.2539
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2000 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/20/12/2539

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2000/12/13/20.12.2539.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org/subscriptions/
Supplementary information of Methods section to be published online only

Effects of angiotensin II infusion on aortic growth factor receptors

To study the effects of acute Ang II infusion, 9-week-old male Sprague-Dawley rats (Clea Japan, Tokyo) were anesthetized with sodium pentobarbital (50 mg/kg i.p.), and polyethylene catheter was inserted into the left femoral vein for infusion of Ang II, and the catheter was tunneled through the subcutaneous tissue to exteriorize in the dorsal midcervical region of the neck. The rats were then allowed to recover from anesthesia and surgical procedure for about 20 hours until start of the experiments. Ang II (100 ng/kg/min) was i.v. infused to rats by syringe infusion pump (Harvard Apparatus, Ltd.) for 5, 15, 30 or 60 minutes.

To investigate the effects of chronic Ang II infusion, rats were separated into 4 groups: (1) saline-infused group (control group); (2) Ang II-infused group; (3) Ang II-infused and CS-866 (10 mg/kg/day)-treated group; (4) Ang II-infused and hydralazine (20 mg/kg/day)-treated group. Rats were anesthetized with ether, and subjected to a subcutaneous implantation of an Alzet osmotic minipump for infusion of Ang II (400 ng/kg/min), for 3 days or 1 or 2 weeks. CS-866 (10 mg/kg/day), a selective non-peptide angiotensin AT1 receptor antagonist, suspended with 0.5% carboxymethylcellulose solution, was orally given to rats by gastric gavage once a day from one day before implantation of osmotic minipump to the end of Ang II infusion. Hydralazine (20 mg/kg/day), dissolved in the drinking water, was orally given to rats from 24 hours before the start to the end of Ang II infusion. At the indicated times after acute or chronic Ang II infusion, rats were decapitated, and the thoracic aorta was immediately excised, carefully dissected from adherent fat and connective
tissues, frozen in liquid nitrogen and stored at -80 °C until use.

**Immunoprecipitation and western blot analysis**

The method of immunoprecipitation and Western blot analysis has been described in detail in our previous reports 20,21. In brief, aortic tissues were homogenized in lysis buffer, and the protein extracts (250 µg) were preabsorbed with protein A-sepharose or protein G-sepharose, were incubated with rabbit polyclonal anti-PDGF α receptor antibody (Santa Cruz Biotechnology, Inc.), rabbit polyclonal anti-PDGF β receptor antibody (Santa Cruz Biotechnology, Inc.), or sheep polyclonal anti-EGF receptor antibody (GibcoBRL), and the immunocomplexes were precipitated with protein A-sepharose for anti-PDGF α or β receptor antibody, or precipitated with protein G-sepharose for anti-EGF receptor antibody. The immunoprecipitates were boiled in Laemmli’s sample buffer, centrifuged, the resulting supernatants were electrophoresed on 8% SDS-polyacrylamide gel, transferred to Hybond-PVDF membranes (Amersham Life Sciences), and the membranes were immunoblotted with mouse monoclonal anti-phosphotyrosine IgG (4G10) (Upstate Biotechnology). Immunocomplexes were visualized by using the enhanced chemiluminescence (ECL) method (Amersham). The densities were measured using the public domain National Institutes of Health IMAGE program. After stripping off the previous antibody, the membranes were again immunoblotted with other antibodies, as described above. Western blot analysis of phospho-extracellular signal-regulated kinase (ERK) in aortic protein extracts with rabbit polyclonal phospho-specific ERK antibody (New England Biolabs, Inc., MA) was carried out, as previously described 20.
Measurement of ACE activity

Vascular and serum ACE activity was measured, as described previously in detail 22,23. In brief, aortic tissue extracts and serum were incubated with hippuryl-His-Leu (Peptide Institute, Inc., Osaka), a synthetic substrate specific for ACE, at 37 °C, and the cleaved hippuric acid was measured by reverse-phase high-performance liquid chromatography on C18 column (4 mm i.d. x 250 mm, IRICA Instrument, Kyoto, Japan).

Statistical analysis

Data are expressed as mean ± SEM. Statistical significance was determined with an unpaired Student’s t-test when two groups were compared, and with one-way analysis of variance (ANOVA) followed by Duncan’s multiple range test when more than two groups were compared. Differences were considered statistically significant at a value of P<0.05.
Supplementary information of Figure legend section to be published online only

Figure I. Effect of chronic angiotensin II infusion on aortic PDGF β receptor tyrosine phosphorylation

Upper panel shows representative immunoblot analysis of tyrosine phosphorylation and protein levels of aortic PDGF β receptor: (1) control; (2) 1 wk of angiotensin II-infused group, (3) 1 wk of angiotensin II-infused and CS-866-treated group; (4) control; (5) 2 wk of angiotensin II-infused group; (6) 2 wk of angiotensin II-infused and CS-866-treated group.

Abbreviations; control, saline-infused group; Ang II, angiotensin II-infused group; Ang II+CS-866, Ang II-infused and CS-866-treated group; Ang II+Hydralazine, Ang II-infused and hydralazine-treated group. Tyr Phosphorylation indicates tyrosine phosphorylation of PDGF β receptor corrected for the protein level. Values are mean±SEM (n=7-8). The mean value in control at each time point is represented as 1.

* P<0.01 vs. control