Role of Rho-Kinase and p27 in Angiotensin II–Induced Vascular Injury

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Abstract—Angiotensin II enhances the development of atherosclerotic lesion in which cellular proliferation and/or migration are critical steps. Although cyclin-dependent kinase inhibitor, p27, and Rho/Rho-kinase pathway have recently been implicated as factors regulating these events cooperatively, their role in vivo has not been fully elucidated. We evaluated the contribution of p27 and Rho-kinase to angiotensin II–induced vascular injury using p27-deficient mice. Two-week angiotensin II (1500 ng/kg per minute SC) infusion elicited similar degrees of elevation in systolic blood pressure in wild-type mice (159 ± 1100 mm Hg) and p27-deficient mice (157 ± 5 mm Hg; P > 0.05). Angiotensin II infusion to wild-type mice resulted in increases in the medial thickness of aorta, proliferating cell number, and monocyte/macrophage infiltration within the vasculature. In p27-deficient mice, however, these changes were more prominent than those in wild-type mice. Treatment of wild-type mice with fasudil, a selective Rho-kinase inhibitor, did not alter blood pressure but significantly upregulated p27 expression, decreased medial thickness of aorta, reduced proliferating cell number, and prevented monocyte/macrophage infiltration. These protective effects of fasudil were attenuated in p27-deficient mice. In conclusion, p27 constitutes an important modulator of angiotensin II–induced vascular injury through p27-dependent and p27-independent mechanisms. (Hypertension. 2005;45[part 2]:724-729.)

Key Words: angiotensin II • remodeling • muscle, smooth, vascular • macrophages • hypertension, experimental

Sustained hypertension causes vascular injury and atherosclerosis that are mediated by multiple mechanisms. Although numerous factors participate in these disease processes, angiotensin II (Ang II) has emerged as an important growth factor that regulates cell proliferation.1 Ang II is responsible for the inflammatory response and cell migration by producing various cytokines and adhesion molecules2 and by direct effects on monocyte/macrophages.3 Of interest, recent studies have witnessed that Ang II also activates Rho-kinase,4,5 an effector of the small G protein Rho, which not only plays an important role in calcium sensitization of vascular smooth muscle cells (VSMCs) but also shares the facilitatory action on cell adhesion,4 proliferation, and migration7 with Ang II. Because excessive cellular proliferation and migration within the arterial wall are established as a central mechanism for the development of atherosclerosis,8,9 Ang II would contribute, whether directly or indirectly, to the development of vascular injury as a local detrimental factor, as well as a systemic hemodynamic hormone.

A growing body of evidence has accrued that p27, which plays a crucial role in cell proliferation as an inhibitor of cyclin/cyclin-dependent kinase complexes,10 participates in the regulation of vascular injury. The p27 expression inversely correlates with proliferation of macrophages and VSMCs within human atherosclerotic tissue.11 Mice doubly deficient for p27 and apolipoprotein E display accelerated atherogenesis with augmented proliferation of macrophages and VSMCs.12 In addition, p27 is reported to be a negative regulator of migration.13 The p27 expression is downregulated in infiltrating leukocytes after balloon injury.14 Targeted disruption of bone marrow p27 enhanced arterial leukocytes infiltration and inflammatory response, resulting in accelerated vascular injury.14,15 Of note, p27 expression is downregulated by Rho-kinase activation, leading to acceleration of the cell cycle progression.16–18 Rho/Rho-kinase pathway is reported to mediate cell migration, partly through p27-dependent pathways.13 Nevertheless, the effect of Ang II on p27 expression remains a matter of controversy,19–21 and definite evidence linking Rho/Rho-kinase pathway and p27 in mediating the Ang II–induced cellular proliferation and migration has not been accumulated.
The current study was designed to elucidate directly the role of p27 in Ang II–induced vascular injury. To clarify this issue, we used homozygous p27-deficient (p27KO) mice. We also examined whether Rho-kinase mediated the Ang II–induced changes in p27 expression and vascular injury with a selective Rho-kinase inhibitor, fasudil.

Methods

Animal Study

This study was performed in accordance with the animal experimentation guideline of Keio University School of Medicine. The role of p27 in Ang II–induced vascular injury and the interaction between p27 and Rho-kinase were evaluated in p27KO mice. Mice heterozygous for the p27 gene with C57BL/6 genetic background were kindly supplied by Nippon Roche Research Center (Kanakura, Japan). Male mice homozygous for the p27 gene and wild-type (WT) mice were selected from the offspring of heterozygous mating. The genotype was determined by polymerase chain reaction analysis on DNA isolated from tail biopsy specimens. Alzet osmotic minipumps (model 1002; Durect Corporation, Cupertino, Calif) were implanted into WT and p27KO mice at 2 months of age under anesthesia with inhaled isoflurane. Pumps were filled either with saline vehicle or with solutions of Ang II (Sigma-Aldrich, St. Louis, Mo) that delivered subcutaneously 1500 ng/kg per minute of Ang II for 14 days. Fasudil (30 mg/kg per day; Asahi Kasei, Tokyo, Japan) was given in drinking water for 14 days. Fasudil was used for Rho-kinase inhibition because it is the only Rho-kinase inhibitor practically available for long-term in vivo use. This agent acquires unique characteristics when administered in vivo; its metabolite, hydroxy fasudil, still retains its activity and possesses a more selective action than its parent drug on Rho-kinase. Thus, its specificity for Rho-kinase is 100-times higher for PKC and 1000-times higher for myosin light chain kinase. It is unequivocal that selective inhibition for Rho-kinase is reasonably practiced by the treatment with fasudil.

The systolic blood pressure was measured by the tail-cuff method using an automatic sphygmomanometer (Softron BP-98A; Softron, Tokyo, Japan). Before each measurement, mice were placed in a Plexiglas cage and subsequently heated at 37°C for 5 minutes.

Microscopic Analysis

For morphometric analysis of vascular hyperplasia, sections were stained with hematoxylin and eosin. Three serial sections from the abdominal aorta of each animal were photographed and stored in a digital format. Arterial wall thickness was measured in a blinded fashion with Scion Image software.

Immunohistochemistry

Immunohistochemical analysis of aortic cross-sections were performed using antibodies against Mac-3 (1:50; sc-19991; Santa Cruz Biotechnology, Santa Cruz, Calif), proliferating cell nuclear antigen (PCNA) (1:50, sc-7907; Santa Cruz Biotechnology), and p27 (1:800; sc-528; Santa Cruz Biotechnology). Quantification of nuclear staining for Mac3, PCNA, and p27 was performed from 4 to 6 independent aortic sections in each group. Mac 3-positive, PCNA-positive, and p27-positive cells were counted by computer-aided planimetry using the Scion Image software.

Statistical Analysis

Data are expressed as mean±SEM. Data were analyzed by 1-way or 2-way ANOVA as appropriate, followed by Bonferroni multiple comparison post hoc test. P<0.05 was considered statistically significant.

Results

Ang II–Induced Changes in Blood Pressure and the Effects of Fasudil Treatment

Baseline systolic blood pressure was comparable between p27KO mice (113±2 mm Hg, n=6) and WT mice (110±2 mm Hg, n=6). Two-week Ang II infusion elicited similar magnitude of elevations in systolic blood pressure in WT mice (159±5 mm Hg; P<0.01) and p27KO mice (157±5 mm Hg; P<0.01). The treatment with fasudil (30 mg/kg per day) had no effect on the changes in blood pressure in either WT (156±3 mm Hg) or p27KO mice (154±3 mm Hg).

p27 Expression in the Vascular Tissues of Ang II–Infused Mice

Two-week Ang II infusion reduced the p27-positive cells (B) as compared with saline (A). Treatment with fasudil significantly increased the number of p27-positive cells (C). The aorta from p27-deficient mice showed negative staining for p27 (D). Scale bar=50 μm. E, Summary of quantitative analysis of p27 expression in the vehicle-infused WT mice, Ang II–infused WT mice, and Ang II–infused WT mice treated with fasudil. Each bar represents the mean±SEM. *P<0.05 versus vehicle infusion; †P<0.01 versus vehicle infusion; ‡P<0.05 versus Ang II infusion.

Ang II–Induced Medial Thickness

To elucidate the role of p27 in Ang II–induced hypertension, we also infused Ang II to the p27KO mice. With the vehicle infusion, the medial thickness of the aorta in the p27KO mice...
Ang II–Induced Vascular Proliferation

We evaluated the number of PCNA-positive cells within the vascular tissues as a marker for cell proliferation and found no difference between the number of positive staining cells in WT and in p27KO mice treated with vehicle (Figure 3A, a and d). In WT mice, Ang II infusion resulted in increased PCNA staining in the vascular wall in comparison with vehicle infusion (Figure 3A, b; 12.3 ± 1.0 cells/section, n = 5). Ang II infusion to p27KO mice was also associated with a significant increase in PCNA-positive cells, and this increase was more prominent than that in Ang II–infused WT mice (Figure 3A, e; 20.5 ± 1.2 cells/section, n = 6, P < 0.01 versus Ang II–infused WT). In parallel with the results on the medial thickness, fasudil inhibited the Ang II–induced increase in the proliferating cell number in WT and p27KO mice. Of importance, the inhibitory effects were diminished in Ang II–induced p27KO mice (55 ± 5% inhibition of the Ang II–induced increase in cell number, n = 6) compared with those in WT mice (72 ± 4% inhibition of the Ang II–induced increase in cell number, n = 5; P < 0.05) (Figures 3A c, f, and 3B).

Ang II–Induced Accumulation of Macrophages

We next examined the accumulation of macrophages within the vascular tissues as assessed by Mac-3 staining. In mice treated with vehicle alone, no difference in the number of positive-staining cells was noted between WT and p27KO mice (Figure 3A c, d). In WT mice, Ang II infusion resulted in increased PCNA staining in the vascular wall in comparison with vehicle infusion (Figure 3A, b; 12.3 ± 1.0 cells/section, n = 5). Ang II infusion to p27KO mice was also associated with a significant increase in PCNA-positive cells, and this increase was more prominent than that in Ang II–infused WT mice (Figure 3A, e; 20.5 ± 1.2 cells/section, n = 6, P < 0.01 versus Ang II–infused WT). In parallel with the results on the medial thickness, fasudil inhibited the Ang II–induced increase in the proliferating cell number in WT and p27KO mice. Of importance, the inhibitory effects were diminished in Ang II–induced p27KO mice (55 ± 5% inhibition of the Ang II–induced increase in cell number, n = 6) compared with those in WT mice (72 ± 4% inhibition of the Ang II–induced increase in cell number, n = 5; P < 0.05) (Figures 3A c, f, and 3B).
mice (Figure 4A, a and d). Ang II caused a significant increase in the accumulation of Mac-3–positive cells (Figure 4A, b; 4.5 ± 0.4 cells/section). The disruption of p27 resulted in the enhancement of Ang II–induced macrophage accumulation. The increase in the number of MAC-3–positive cells was significantly augmented in Ang II–infused p27KO mice (12.4 ± 1.5 cells/section, n = 5), compared with that in Ang II–infused WT mice (P < 0.01; Figure 4A, e). Simultaneous treatment with fasudil markedly prevented the increase in Ang II–induced macrophage accumulation in WT mice (Figures 4A, c and 4B; 73 ± 5% inhibition of Ang II–induced accumulation, P < 0.01, n = 5), whereas this inhibitory action was less in p27KO mice (Figures 4A, f and 4B; 46 ± 5% inhibition of Ang II–induced accumulation, P < 0.05, n = 6).

**Discussion**

In the present study, we have demonstrated that p27KO mice manifest higher susceptibility to the vascular remodeling action of Ang II. This effect is accompanied by the enhanced accumulation of PCNA-positive cells and macrophage infiltration. Furthermore, in WT mice, chronic inhibition of Rho-kinase by fasudil upregulates the p27 expression level and ameliorates the Ang II–induced cell proliferation and macrophage infiltration within the vascular wall, independent of systemic blood pressure, but these effects are diminished in p27-deficient mice. These results indicate that p27 constitutes an important modulator of the progression of vascular remodeling and is under the regulation of Rho-kinase activity that mediates a part of the vascular action of Ang II.4–5 The strategy to increase the expression of p27 by the treatment with Rho-kinase inhibitor is expected to offer a promising approach to the treatment of cardiovascular diseases.

Although originally discovered as a potent vasoconstrictor substance, Ang II induces cell growth and migration that would precipitate vascular injury. One of the mechanisms for the growth-promoting and promigratory effects of Ang II is downregulation of p27.1 The results presented here show that Ang II induces the proliferation of vascular cells and decreases p27 expression in WT mice (Figures 1 and 3), a finding in good agreement with previous in vitro and in vivo reports by other laboratories.19,21 In the present study, medial thickness, cell proliferation, and macrophage infiltration are similar in saline-infused WT and p27 KO mice, which coincides with the demonstration by Diez-Juan et al.12 In contrast, Ang II–induced cell proliferation and macrophages infiltration in the vascular wall is augmented in p27KO mice (Figures 3 and 4). Because the p27-positive cells (Figure 1) are supposed to be VSMCs or infiltrated macrophages,11 it is likely that decreased p27 expression in the vascular tissues plays an important role only in the pathological state.12,14 Enhanced cell proliferation in p27KO mice indicates the contribution of additional factors to the Ang II–induced vascular proliferation; Ang II downregulates another cyclin-dependent kinase inhibitor, p21,21 and upregulates cyclin D1, a cell cycle-positive regulator,19 which might enhance proliferation in Ang II–infused p27KO mice. In concert, the inverse relationship between the p27 expression level and cell proliferation/macrophage migration lends support to the formulation that p27 contributes, at least in part, to the regulation of cell proliferation and migration as an inhibitory factor in Ang II–induced vascular remodeling and inflammation. Of interest, we found that macrophage infiltration was augmented in p27KO mice, particularly in adventitial regions (Figure 4), which could contribute to the increased medial thickness.26

A therapeutic strategy for targeting p27 expression has not been fully elucidated. It has been demonstrated that multiple factors affect p27 expression and the subsequent progression of vascular injury.13,16–27 Among these, Rho-kinase pathway has attracted much attention because it promotes cell cycle progression, possibly through destabilizing p27.17 Moreover, Rho/Rho-kinase pathway mediates migration through p27-dependent and independent pathways.13 Thus, several studies, including that of our laboratory, have reported that direct inhibition of Rho-kinase pathway increases the p27 expression and inhibits the proliferation of various cells and the infiltration of macrophages.16–18 In the present study, we have found that fasudil, a Rho-kinase inhibitor, restores the decreased p27 level induced by chronic Ang II infusion (Figure 1) and reduces PCNA (Figure 3) and Mac-3–positive cells in the vascular wall (Figure 4). In addition, these vascular protective effects of fasudil were attenuated in p27KO mice.
compared with WT mice. Although fasudil at the dose used in the present study (ie, 30 mg/kg per day) did not alter systemic blood pressure, this agent possesses hypotensive action and is demonstrated to reduce blood pressure.\textsuperscript{28} The inhibition of Rho-kinase therefore would prevent vascular injury in Ang II–induced hypertension through p27-dependent pathway, as well as hypotensive action.

Of importance, both antiproliferative and antimigratory effects by fasudil were observed in p27KO mice. These observations indicate that the inhibition of Rho-kinase can decrease vascular proliferation and macrophage infiltration through p27-independent mechanism. In this regard, some of the Ang II–related proliferative effects are mediated by redox-sensitive pathway,\textsuperscript{21} and Rho-kinase pathway is involved in the upregulation of NAD(P)H oxidase expression.\textsuperscript{4} Additionally, inhibition of Rho-kinase prevents the expression of MCP-1,\textsuperscript{1} which is implicated as promigratory factor in Ang II–induced monocyte/macrophages recruitments.\textsuperscript{26,29} These mechanisms could be responsible for the antiproliferative or antimigratory effect of fasudil in Ang II–infused p27KO mice. Alternatively, Ang II–induced downregulation of p27 may not completely depend on the activation of Rho/Rho-kinase signal transduction.\textsuperscript{30} Although precise mechanisms remain undetermined, it is reasonable to conclude that the inhibition of Rho-kinase by fasudil suppresses the proliferation of vascular cells and the macrophage infiltration through multiple mechanisms, including p27-dependent pathway.

Finally, the mechanism whereby Ang II accelerates atherosclerosis merits comment. As detailed, Ang II has direct effects on cellular components of the arterial wall.\textsuperscript{1,2} However, Ang II would facilitate vascular injury through the mechanical stress that occurs as a result of elevated systemic blood pressure.\textsuperscript{31} Although Ang II elevates systemic blood pressure to the same level in p27KO and WT mice, the platelet-derived growth factor,\textsuperscript{33} because platelet-derived growth factor causes VSMC proliferation through Rho/Rho-kinase signal transduction.\textsuperscript{30} Although precise mechanisms remain undetermined, it is reasonable to conclude that the inhibition of Rho-kinase by fasudil suppresses the proliferation of vascular cells and the macrophage infiltration through multiple mechanisms, including p27-dependent pathway.

Perspectives

Targeting vascular cell proliferation and migration constitutes a potential therapeutic approach to the prevention of vascular remodeling and inflammation. Our present study supports the notion that decreased p27 levels act as a pro-inflammatory and pro-atherosclerotic factor in Ang II–induced hypertension. Our findings also indicate that the chronic inhibition of Rho-kinase offers cardiovascular protection, partially through the restoration of p27 expression and partially through the p27-independent mechanism. It will be of potential importance in future works to address whether targeting p27 and/or Rho/Rho-kinase pathway in cardiovascular disease will yield therapeutic benefits in clinical trials.

References

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Hypertension. 2005;45:724-729; originally published online February 7, 2005;
doi: 10.1161/01.HYP.0000153316.59262.79
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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