Research Article

AngiotensinII Preconditioning Promotes Angiogenesis *In Vitro* via ERKs Phosphorylation

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AngiotensinII (AngII) is involved in not only the formation of cardiac hypertrophy but also the development of cardiac remodeling both of which are associated with myocardial angiogenesis. This study was therefore performed to clarify the effects of AngII on the formation of vasculatures by cultured cardiac microvascular endothelial cells (CMVECs) after a long-period stimulation with or without the AngII preconditioning. Incubation with AngII for 18 hrs significantly impaired the formation of capillary-like tubes comparing to that without AngII. CMVECs with AngII pretreatment for 5 and 10 min formed more capillary-like tubes than those without AngII pretreatment, suggesting that preconditioning with AngII at a lower dose for a short period could prevent the further damage of CMVECs by a higher concentration of AngII. Moreover, AngII (10^{-7} M) stimulation for 5 and 10 min significantly induced the increase in extracellular signal-regulated protein kinases (ERKs) phosphorylation, and an ERKs inhibitor, PD98059, abrogated the increase in the formation of capillary-like tubes induced by the AngII-pretreatment. In conclusion, preconditioning with a lower concentration of AngII for a short period prevents the subsequent impairment of CMVECs by a higher dose of AngII, at least in part, through the increase in ERKs phosphorylation.

1. Introduction

Cardiac hypertrophy usually occurs as an adaptive response to increased workload to maintain cardiac function in early stages [1]. However, prolonged cardiac hypertrophy can lead to heart failure lately [2]. It has been postulated that a mismatch between the number of capillaries and the size of cardiomyocytes occurs during the development of cardiac hypertrophy and causes myocardial hypoxia [3, 4]. Various studies indicate that a potential relationship exists among cardiac angiogenesis, cardiac hypertrophy, and cardiac function [5–7]. Furthermore, rennin-angiotensin systems (RASs), especially angiotensinII(AngII), play a crucial role in the development of cardiac hypertrophy [8, 9]. AngII is proved to be related with myocardial cell apoptosis and remodeling [10–12]. Though both dysfunctions of angiogenesis in myocardium and AngII are critical to the progress of cardiac hypertrophy and heart failure [13], roles of AngII in myocardial angiogenesis at the different stages during the pathogenic process are unclear. The aim of this study was to identify effects of AngII on the formation of vasculatures by cultured cardiac microvascular endothelial cells (CMVECs) at an early or later time point after stimulation.

2. Materials and Methods

2.1. Cell Culture and AngiotensinII Treatment. Primary rat cardiac microvascular endothelial cells (CMVECs) were obtained from male Wistar rats (8-9 weeks old) by the method of planting myocardial tissue [14, 15] and were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) containing 20% fetal bovine serum (FBS, Hyclone), 100 u/mL penicillin, and 100 u/mL streptomycin (Sigma, USA). The 2nd and 3rd rat CMVECs were seeded on a 6-well plate at 5×10^5/well and cultured for

Control 0 min 5 min 10 min AngiotensinII 10^{-7} M
AngiotensinII 10^{-6} M
18 hours

15 min 30 min 60 min AngiotensinII 10^{-7} M
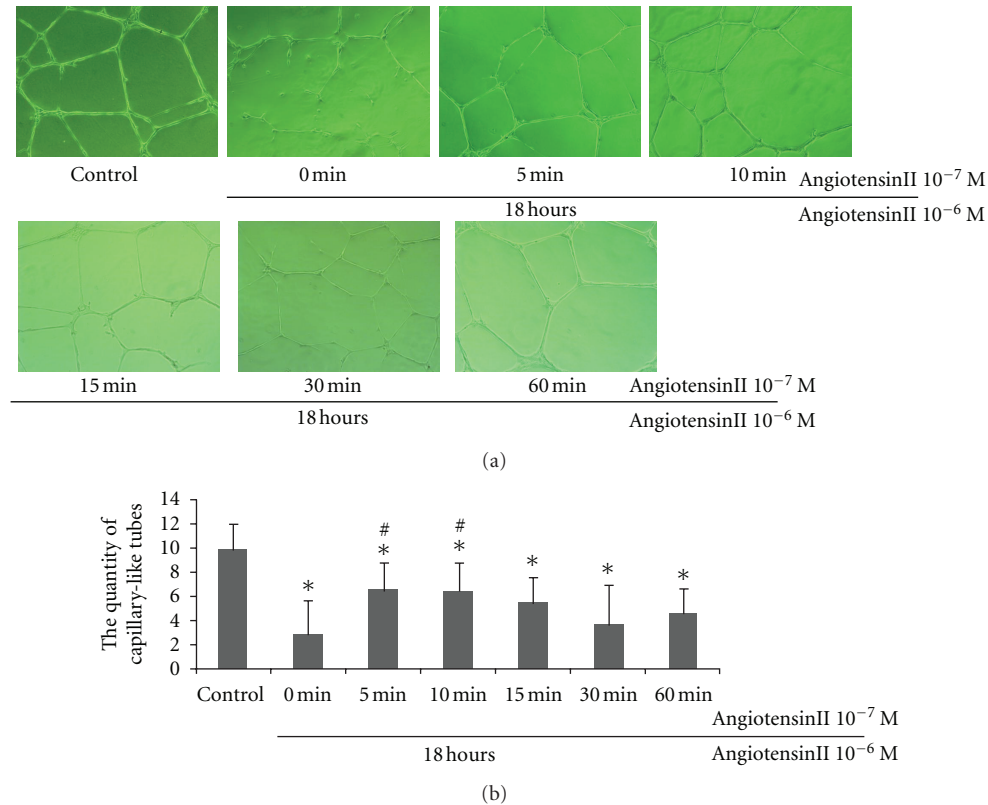AngiotensinII 10^{-6} M
18 hours

(a)

(b)

Figure 1: Time-dependent effects of AngII preconditioning on AngII-induced impairment of capillary-like tube formation in cultured CMVECs. CMVECs were pretreated with AngII (10^{-7} M) for the indicated times and then seeded onto the matrigel in plates containing AngII (10^{-6} M) or not (control). Eighteen hrs later, the formation of capillary-like tubes was observed under an optical microscope. (a) Representative photomicrographs of capillary-like tubes (100xmagnification). (b) Quantitative analysis for capillary-like tube formation. Capillary-like tubes were counted in randomly selected 5 fields for each plate. Data represent mean $\pm$ SD obtained from 15 independent experiments ($n = 15$); $^*P < 0.05$ versus control group; $^\#P < 0.05$ versus the group without AngII pretreatment (0 min).

24 hours. And 24 hours later, cells were cultured in serum-free DMEM for another 24 hours and then were incubated by AngII (10^{-7} M) for 5, 10, 15, 30, and 60 min, respectively. Finally, CMVECs were collected and lysed for the further western blotting analysis and capillary-like tube formation analysis.

2.2. PD98059 Treatment.
The 2nd and 3rd CMVECs were seeded on a 6-well plate at 5×10^5/well and cultured for 24 hours. Then, they were cultured in serum-free DMEM for another 24 hours, incubated by PD98059 (50 μM) for 30 min (DMSO as controls) and AngII (10^{-7} M) for a further 10 min. Finally, cells were collected and lysed for the further western blotting analysis and capillary-like tube formation analysis

2.3. Western Blotting Analysis.
Total protein was extracted from the cultured cells and then electrophoresed in 12% polyacrylamide gel and transferred to PVDF membrane. The expression of extracellular signal-regulated protein kinases (ERKs) phosphorylation was detected using a Phospho-p44/42 MAPK Rabbit mAb (1 : 1,000; Cell Signaling). The membrane was then incubated with peroxidase-conjugated rabbit anti-goat IgG (1 : 5,000; Jackson) and HRP-conjugated

monoclonal mouse antiglyceraldehyde-3-phosphate dehydrogenase (GAPDH, KangChen, China). Immunoreactive proteins were subjected to an ECL detection system (GE healthcare, RPN2106).

2.4. Capillary-Like Tube Formation.
200 μL growth-factor-reduced matrigel matrix (BD company) was homogenized and layered into a 24-well plate on a cooled planar surface and allowed to solidify at 37°C. CMVECs that AngII (10^{-7} M) pretreated or PD98059 pretreated were detached with trypsin EDTA, resuspended in high-glucose DMEM, and supplemented with 1% of FBS and AngII (10^{-6} M). And suspension was seeded into plates at 5×10^4 cells/well and cultivated at 37°C atmosphere with 5% CO_2/95% air for 18 hours. Images of tubes were taken at 100xmagnification with a digital output camera attached to an inverted phase-contrast microscope (Leica. Germany), and five random fields of view were counted to analyze the relative number of capillary-like tubes.

2.5. Statistical Analysis.
All the data were presented as mean $\pm$ SD and were analyzed using ANOVA tests (least significant
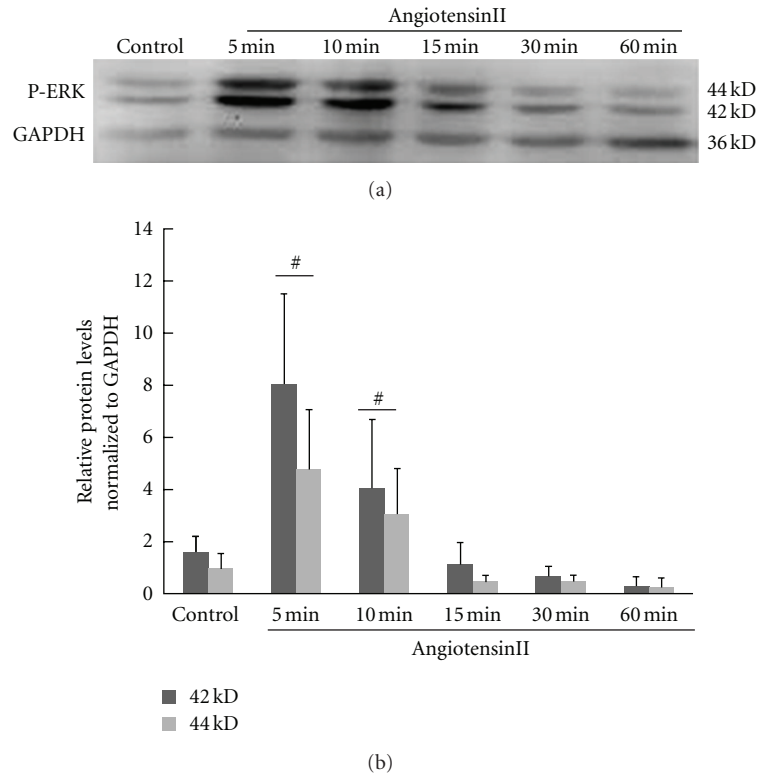
(a)

(b)

FIGURE 2: Time-dependent effects of AngII on ERKs phosphorylation in cultured CMVECs. CMVECs were treated with AngII (10^{-7} M) for the indicated times and then collected and lysed for western blot analysis. (a) Representative immunoblots showing the expression of phosphor ERKs. GAPDH was used as a loading control. (b) Quantitative analysis for the expression of phosphor ERKs. Data represent mean $\pm$ SD obtained from 6 independent experiments ($n = 6$); $^*P < 0.05$ versus control group (0 min).

difference t-tests) for multiple-means comparison. A two-tailed P value of <0.05 was considered statistically significant. All statistical analysis was performed by SPSS 16.0 for Windows (SPSS Inc., Chicago, IL).

3. Results

3.1. Impairment of CMVECs to Form Capillary-Like Tubes and the Protective Effect of AngII Pretreatment. In order to investigate the effect of AngII on CMVECs capillary-like tube formation, we select the function of its capillary-like tube formation to observe. The results showed that stimulation of cultured CMVECs with AngII (10^{-6} M) for 18 hours impaired its capillary-like tube formation, and cells that either AngII (10^{-7} M) pretreated for 5 or 10 min formed more capillary-like tubes than those without AngII pretreatment (Figures 1(a) and 1(b)), suggesting that precondition with AngII at a lower dose for a short period could prevent the subsequent impairment of CMVECs by a higher dose of AngII.

3.2. Phosphorylation of ERKs in Cultured CMVECs following AngII Stimulation. In order to identify the effect of AngII on ERKs phosphorylation in CMVECs, we observed levels of ERKs phosphorylation using western blotting analysis. The figures showed that ERKs phosphorylation in CMVECs under AngII (10^{-7} M) stimulation for 5 and 10 min was significantly increased more than those without AngII addition (Figures 2(a) and 2(b)).

3.3. ERKs Phosphorylation Participated in the Protective Effect by Preconditioning. The above results pointed out that pretreated CMVECs with AngII for 5 or 10 min could protect the subsequent impairment induced by a higher dose of AngII (Figure 1), and interestingly, levels of ERKs phosphorylation raised at that stimulation point as Figure 2 reveals. Therefore, we hypothesized that ERKs phosphorylation might take part in the protective effect by preconditioning. PD98059, an ERKs inhibitor, was selected to inhibit ERKs phosphorylation, and function of capillary-like tube formation was observed in CMVECs. Results indicated that 50 μM PD98059 could inhibit ERKs phosphorylation to the last penny (Figures 3(a) and 3(b)), and the number of capillary-like tubes of CMVECs significantly decreased when inhibited ERKs phosphorylation (Figures 4(a) and 4(b)). The figures suggested that preconditioning with a lower concentration of AngII for a short period prevents the subsequent impairment of CMVECs by a higher dose of AngII through the increase of ERKs phosphorylation.
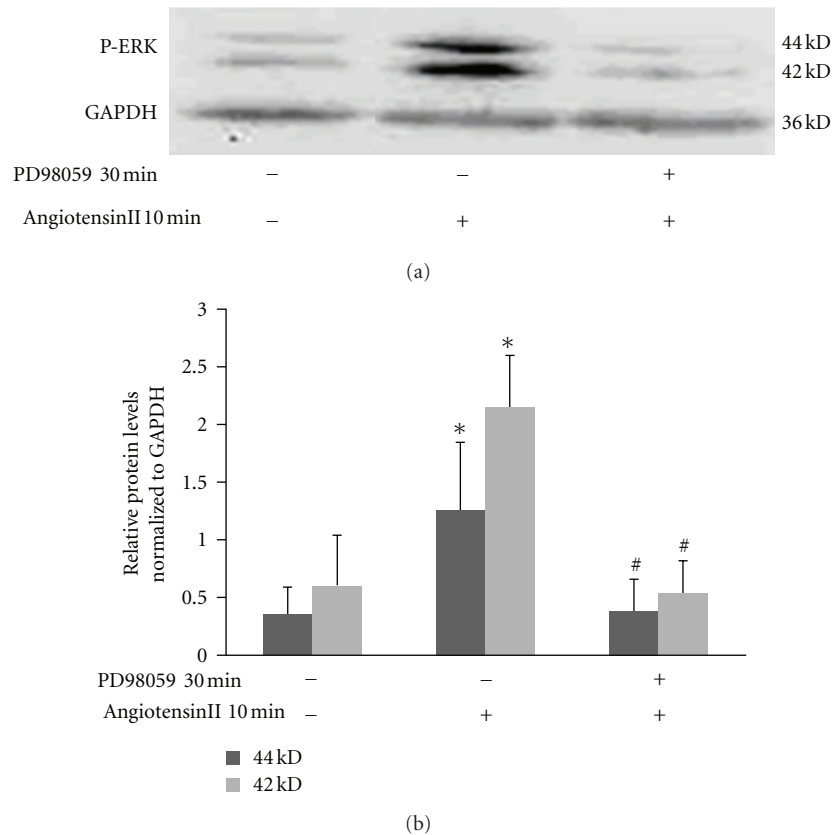
(a)

(b)

FIGURE 3: The effects of PD98059 on ERKs phosphorylation in cultured CMVECs. CMVECs were incubated with (+) or without (−) PD98059 (50 μM), an ERKs inhibitor, for 30 min and then treated with (+) or without (−) AngII (10^{-7} M) for 10 min. Thereafter, the cells were collected and lysed for western blotting. (a) Representative immunoblots showing the expression of phosphor ERKs. GAPDH was used as a loading control. (b) Quantitative analysis for the expression of phosphor ERKs. Data represent mean ± SD obtained from 4 independent experiments ($n = 4$). *$P < 0.05$ versus CMVECs with neither PD98059 nor AngII treatment; #$P < 0.05$ versus CMVECs without PD98059 but with AngII treatment.

4. Discussion

In normal mammalian myocardium, each cardiomyocyte is surrounded by an intricate network of capillaries and is next to cardiac microvascular endothelial cells (CMVECs) [16]. The unique structure between CMVECs and cardiomyocyte determines that CMVECs may participate in regulating cardiomyocyte function. In mature myocardium, CMVECs not only supply cardiomyocytes with oxygen and nutrient but also promote cardiomyocytes organization and survival with local protective signals. Cardiomyocytes cocultured with CMVECs may promote marked cardiomyocyte reorganization along the EC structures and inhibit cardiomyocytes apoptosis and necrosis [17, 18]. So, CMVECs are very important in the heart. A reduction of capillary length density has been found in several experimental models of cardiac hypertrophy [19]. In particular, major roles were shown for VEGF, angiopoietin, and Tie2 in cardiac vasculogenesis and angiogenesis after AngII infusion [20]. In the present study, our data provides that AngII incubated for 18 hours could damage functions of CMVECs capillary-like tube formation, AngII (10^{-7} M) pretreatment for 5~10 min

could diminish the subsequent impairment induced by a higher dose of AngII incubation. And the effect was related to ERKs phosphorylation which was considered to be one signal related to cell proliferation. Since the similar protective effect of AngII by preconditioning in ischemia-reperfusion models had been approved [21–29], we did not observe the preconditioning effect in perfusion heart here. Though the detailed mechanisms underlying ERKs phosphorylation in function of capillary-tube formations and other various pathological angiogenesis remain to be fully investigated, and the active preconditioning effect might be difficult to be realized in organism, our data had unraveled that this important molecular ERKs phosphorylation maintaining proliferation participated in the process of protection for CMVECs to form capillary-like tubes. Since AngII -induced angiogenesis is a critical physiological or pathological event in diverse cardiac diseases, these findings might be broadly applicable and significant in the therapeutics of cardiac diseases such as hypertension, acute myocardial infarction, and chronic systolic heart failure, particularly as how to explain that ACEI/ARB combination therapy was associated with more adverse effects without any increase in benefit. In

Control | 0 min | 10 min | 10 min AngiotensinII 10^{-7} M

18 hours AngiotensinII 10^{-6} M
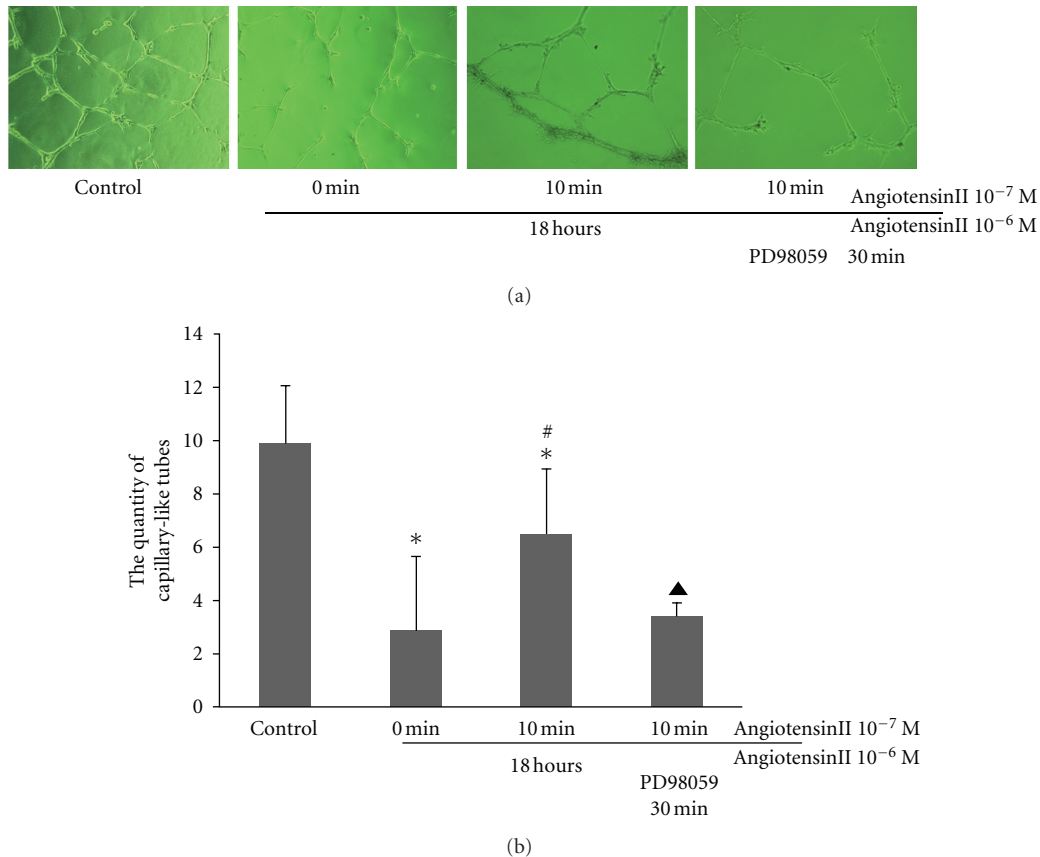
PD98059 30 min

(a)

(b)

FIGURE 4: Effects of ERKs inhibition on AngII preconditioning-induced improvement of vasculature formation under the condition of AngII stimulation. CMVECs preincubated with or without PD98059 (50 μM) for 30 min were treated with 10^{-7} M AngII for the indicated times, and then the cells were seeded onto the matrigel in plates containing AngII (10^{-6} M) or not (control). Eighteen hrs later, the formation of capillary-like tubes was observed under an optical microscope. (a) Representative photomicrographs of capillary-like tubes (100xmagnification). (b) Quantitative analysis for capillary-like tube formation. Capillary-like tubes were counted in randomly selected 5 fields for each plate. Data represent mean ± SD obtained from 12 independent experiments ($n = 12$). *$P < 0.05$ versus control group; #$P < 0.05$ versus the group without AngII pretreatment (0 min); ▲$P < 0.05$ versus the group pretreated with AngII (10 min) but without PD98059.

other words, that appropriate inhibition of AngII rather than complete inhibition of AngII is benefit of cardiac diseases.

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