Role of Jagged1-Hey1 Signal in Angiotensin II-induced Impairment of Myocardial Angiogenesis

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Abstract

Background: Angiotensin II (Ang II) is a major contributor to the development of heart failure. However, the molecular and cellular mechanisms that underlie this process remain elusive. Inadequate angiogenesis in the myocardium leads to a transition from cardiac hypertrophy to dysfunction, and our previous study showed that Ang II significantly impaired the angiogenesis response. The current study was designed to examine the role of Jagged1-Notch signaling in the effect of Ang II during impaired angiogenesis and cardiac hypertrophy.

Methods: Ang II was subcutaneously infused into 8-week-old male C57BL/6 mice at a dose of 200 ng·kg$^{-1}$·min$^{-1}$ for 2 weeks using Alzet micro-osmotic pumps. N-[N-(3, 5-difluorophenacetyl)-L-alanyl]‑S‑phenylglycine tert‑butyl ester (DAPT), a γ-secretase inhibitor, was injected subcutaneously during Ang II infusion at a dose of 10.0 mg·kg$^{-1}$·d$^{-1}$. Forty mice were divided into four groups (n = 10 per group): control group; Ang II group, treated with Ang II; DAPT group, treated with DAPT; and Ang II + DAPT group, treated with both Ang II and DAPT. At the end of experiments, myocardial (left ventricle [LV]) tissue from each experimental group was evaluated using immunohistochemistry, Western blotting, and real-time polymerase chain reaction. Data were analyzed using one-way analysis of variance test followed by the least significant difference method or independent samples t-test.

Results: Ang II treatment significantly induced cardiac hypertrophy and impaired the angiogenesis response compared to controls, as shown by hematoxylin and eosin (HE) staining and immunohistochemistry for CD31, a vascular marker. Jagged1 protein was significantly increased, but gene expression for both Jag1 and Hey1 was decreased in the LV following Ang II treatment, compared to those in controls (relative ratio for Jag1 gene: 0.45 ± 0.13 vs. 0.84 ± 0.15; relative ratio for Hey1 gene: 0.51 ± 0.08 vs. 0.91 ± 0.09; P < 0.05). All these cellular and molecular effects induced by Ang II in the hearts of mice were reduced by DAPT treatment. Interestingly, Ang II stimulated Hey1, a known Notch target, but did not affect the expression of Hey2, another Notch target gene.

Conclusions: A Jagged1-Hey1 signal might mediate the impairment of angiogenesis induced by Ang II during cardiac hypertrophy.

Key words: γ-secretase; Angiotensin II; Cardiac Angiogenesis; Hey; Jagged1

Introduction

Cardiac hypertrophy is classified as physiological when it is associated with normal cardiac function or pathological when associated with cardiac dysfunction. Pathological hypertrophy is induced by factors such as prolonged and abnormal hemodynamic stress due to hypertension, myocardial infarction, etc.[1] Adaptive angiogenesis has been reported to be one of the mechanisms by which cardiac hypertrophy maintains a compensatory state during pressure overload.[2] Enhanced angiogenesis in the hypertrophied heart is due to an increase in the production and secretion of angiogenic growth factors such as vascular endothelial growth factor (VEGF).[3] Another angiogenic growth factor, angiopoietin-II (Ang II), plays a crucial role in hypoxia-induced angiogenesis.[4] Inadequate angiogenesis, due to disorders in angiogenic factors during the development of cardiac hypertrophy, is the main reason that adaptive cardiac hypertrophy results in heart failure.[2] Overexpression of hypoxia-inducible factors (HIF)-1α...
results in normalization of diabetes-reduced VEGF concentrations, along with sustained myocardial capillary density and inhibition of cardiomyocyte hypertrophy.\cite{5} A sustained pressure overload induces accumulation of p53, which inhibits HIF-1 activity, thereby impairing cardiac angiogenesis during the development of cardiac hypertrophy.\cite{2} Activation of renin-angiotensin system (RAS) also participates in the development of cardiac hypertrophy after pressure overload.\cite{6} RAS appears to be involved in both rarefaction (structural degeneration) and expansion of the vascular network. Ang II, the main component of RAS, exerts effects not only on cardiomyocyte hypertrophy and cardiac fibrosis but also on dysregulation of myocardial angiogenesis.\cite{7} Ang II infusion has been shown to reduce the density of cardiac vessels and impair the angiogenic capacity of the aorta and coronary artery rings in rats.\cite{8} Ang II type 1 receptor blockers lower blood pressure (BP), which in turn induces vascular remodeling, resulting in more blood vessels. However, the dynamic vascular processes of degeneration and regeneration during angiogenesis have been more tightly correlated with the levels of circulating Ang II than with BP.\cite{9} Our recent study showed that a specific concentration of Ang II exerted an inhibitory effect on the formation of vasculature in cultured cardiac microvascular endothelial cells (CMVECs).\cite{10} In pressure overload-induced impairment of angiogenesis, accumulation and activation of p53 induced HIF-1 inhibition, by which Ang II impaired the ability of vasculature formation in CMVECs.\cite{11} Recent study has shown that Notch signaling is involved in the regulation of angiogenesis.\cite{12} In a previous study, we showed that Jagged1, but not DLL-4, is related to the Ang II-stimulated accumulation and phosphorylation of p53. Furthermore, both Jagged1 and p53 together result in the impairment of cardiac angiogenesis.\cite{13} However, the role of the Notch pathway in Ang II-induced impairment of cardiac angiogenesis is not clear. In the current study, we further investigate whether the downstream signaling molecules of Notch participate in the impairment of angiogenesis.

**Methods**

**Animals**

Forty 8-week-old male C57BL/6 mice were purchased from Shanghai Animal Administration Center (Shanghai, China). Ang II (200 ng kg\(^{-1}\)·min\(^{-1}\), Sigma-Aldrich, St. Louis, MO, USA) was continuously administered to mice for 2 weeks through Alzet micro-osmotic pumps (DURECT Corporation, Cupertino, CA USA) that were implanted subcutaneously.\cite{13,14} N-[N-(3, 5-difluorophenacetyl)-L-alanyl]-S-phenylglycine tert-butyl ester (DAPT; Sigma-Aldrich, St. Louis, MO, USA), a γ-secretase inhibitor, was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) and injected into the mice subcutaneously at a dose of 10.0 mg·kg\(^{-1}\)·d\(^{-1}\).\cite{15,16} Animals were divided into four groups (n = 10 per group): control group; Ang II group, mice treated with Ang II; DAPT group, mice treated with DAPT; and Ang II + DAPT group, mice treated with both Ang II and DAPT. At the end of the experiments, excised hearts were perfused with phosphate-buffered saline (PBS) and cut into two parts: one part containing the bottom was fixed in 10% formalin for immunohistochemistry and the other was frozen in liquid nitrogen for Western blotting and real-time polymerase chain reaction (PCR). All protocols were approved by the Animal Care and Use Committee of Qingdao Municipal Hospital and were in compliance with the Guidelines for the Care and Use of Laboratory Animals published by the National Academy Press (NIH Publication No. 85-23, revised 1996).

**Morphology and histological analysis**

The excised hearts were weighed, perfused with PBS followed by 4% paraformaldehyde for global morphometry, and fixed in 10% formalin for histological analysis. Paraffin-embedded hearts were sectioned at 4 µm thickness and stained with hematoxylin and eosin. High magnification images of the sections were used to identify cardiomyocytes, and the cross-sectional area (CSA) of cardiomyocytes was measured using a video camera (Leica Qwin 3) attached to a micrometer. Twenty randomly chosen regions from each cross-section of the left ventricular (LV) free wall were used for counting.

**Immunohistochemistry**

LV tissues were fixed in 10% formalin, embedded in paraffin, sectioned at 4 µm thickness, and stained with an anti-CD31 antibody (1:100, Santa Cruz Biotechnology, Dallas, TX, USA). For measurement, five random high-magnification fields were chosen from each section and quantified in a blinded study. CD31-positive vasculature was measured in 5 sections from each LV, and the mean value was calculated.

**Real-time polymerase chain reaction**

Total RNA was isolated from LV tissues using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Synthesis of complementary DNA and real-time reverse transcription polymerase chain reaction (RT-PCR) were performed using a PrimeScript® RT reagent kit and SYBR® Premix Ex Taq™II (Takara, Dalian, China), respectively, according to the manufacturer’s protocols. All PCR reactions were performed using standard PCR conditions according to the reagent kit. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. Expression of each gene was quantified by measuring cycle threshold (Ct) values and normalized using the 2^(-ΔΔCt) method, relative to one mouse from the control group. The three genes analyzed were Jag1, Hey1, and Hey2. Primers were synthesized by the Beijing Genomics Institute, China. Primer sequences were as follows: mouse Jag1: Forward, 5’-CAGTTATCCGGTTCAGGACG-3’; mouse Jag1: Reverse, 5’-ACACTACGCCATTGACGAC-3’; mouse Hey1: Forward, 5’-AAGGGTTATTTTGAGC-GCGC-3’;
mouse *Hey1*: Reverse, 5'-GGCATCGAGTCCTTCAATG-3'; mouse *Hey2*: Forward, 5'-ACAAGGATCTGCCAAGTTAG-3'; mouse *Hey2*: Reverse, 5'-CAATGCTCATGAGTTAGTGTG-3'; mouse *GAPDH*: Forward, 5'-GGCAGGCACTCAGATGC-3'; mouse *GAPDH*: Reverse, 5'-ACGACATACTCAGCAAGGC-3'.

**Western blotting analysis**

Total protein was isolated from the LV tissues. Isolated proteins (20 µg) were size fractionated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. Membranes were incubated

Figure 1: Effects of DAPT on Ang II-induced cardiac hypertrophy (*n* = 10). Ang II (200 ng·kg⁻¹·min⁻¹) was subcutaneously infused into mice for 2 weeks using Alzet micro-osmotic pumps. DAPT (10.0 mg·kg⁻¹·d⁻¹) was injected subcutaneously during the infusion. (a) hematoxylin and eosin staining of a left ventricle section of different groups (scale bar: 20 µm); (b) Quantification of cross-sectional area of cardiomyocytes; (c) Heart weight to body weight ratio of different groups. *P* < 0.05 versus control group; †*P* < 0.05 versus Ang II group. Ang II: Angiotensin II; DAPT: N-[N-(3, 5-difluorophenacetyl)-L-alanyl]-S-phenylglycine tert-butyl ester.

Figure 2: Immunostaining for myocardial CD31 (*n* = 10). Ang II (200 ng·kg⁻¹·min⁻¹) was subcutaneously infused into mice for 2 weeks using Alzet micro-osmotic pumps. DAPT (10.0 mg·kg⁻¹·d⁻¹) was injected subcutaneously during the infusion. Representative photographs from the left ventricle are shown (scale bar: 20 µm). Brown indicates CD31-positive capillaries. CD31-positive vasculature in the left ventricle wall was expressed as number per mm² area. Data are shown as mean ± SE. *P* < 0.05 versus control group; †*P* < 0.05 versus Ang II group. Ang II: Angiotensin II; DAPT: N-[N-(3, 5-difluorophenacetyl)-L-alanyl]-S-phenylglycine tert-butyl ester.
with primary antibodies followed by incubation with a HRP-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Primary antibodies used were polyclonal anti-rabbit antibody against Jagged1 (1:1,000; Abcam, England) and β-actin (1:5,000; Kang Chen, China).

Statistical analysis
All data are presented as mean ± standard error (SE) and were analyzed using one-way analysis of variance (ANOVA) test followed by the least significant difference method for multiple means comparison or independent samples t-test for two means comparison. A two-tailed value of $P < 0.05$ was considered statistically significant. All statistical analyses were performed using SPSS software version 16.0 (IBM company, USA) for Windows.

Results
Jagged1 functions in the angiotensin II-induced impairment of cardiac angiogenesis in mice
We confirmed the role of Jagged1 in Ang II-induced dysfunction of myocardial angiogenesis in mice. Ang II (200 ng·kg$^{-1}$·min$^{-1}$) was continuously administered to mice through an osmotic minipump for 2 weeks, as in our previous study, and blood pressure was not affected by Ang II administration at this dosage.[11,13] Histological analysis showed that Ang II infusion induced significant cardiac hypertrophy [Figure 1]. Immunohistochemical analysis of CD31-positive cells in the myocardium revealed that Ang II administration significantly reduced the density of CD31-positive vasculature, and that this reduction in vasculature was abrogated by treatment with DAPT (10.0 mg·kg$^{-1}$·d$^{-1}$) [Figure 2]. In addition, DAPT treatment ameliorated Ang II-induced cardiac hypertrophy [Figure 1]. Ang II infusion also significantly increased Jagged1 expression, as detected by Western blotting, and this was reduced by DAPT treatment [Figure 3].

Hey1 may participate in the angiotensin II-induced impairment of cardiac angiogenesis in mice
Real-time RT-PCR results showed that expression of Hey1 and Jag1 was significantly decreased in cardiac hypertrophy during Ang II infusion, and that this was reversed by DAPT treatment [Figure 4a and 4b]. Expression of Hey2 was not obviously affected by Ang II infusion [Figure 5]. Taken together, these results suggest that Jagged1-Hey1 may participate in the impairment of angiogenesis induced by Ang II [Figure 6].

![Figure 3: Jagged1 protein expression in LV tissue (n = 10). Total protein from LV tissues was subjected to Western blotting. β-actin was used as a loading control. Representative immunoblots are shown. Jagged1 expression was quantified as fold of β-actin. Data are expressed as mean ± SE. *$P < 0.05$ versus control group; †$P < 0.05$ versus Ang II group. LV: Left ventricle; Ang II: Angiotensin II. SE: Standard error.](image3)

![Figure 4: Jag1 and Hey1 gene expression in LV tissue (n = 10). RNA from LV tissue was subjected to real-time PCR. GAPDH was used as a loading control. Hey1 and Jag1 expression was quantified as fold of GAPDH (a and b), respectively. Data are expressed as mean ± SE. *$P < 0.05$ versus control group; †$P < 0.05$ versus Ang II group. LV: Left ventricle; PCR: Polymerase chain reaction; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; Ang II: Angiotensin II. SE: Standard error.](image4)
Ang II is a major pathogenic factor involved in cardiac remodeling and heart failure. Inhibition of Ang II and its type 1 receptor not only induces regression of cardiac hypertrophy but also improves cardiac dysfunction. Furthermore, the Ang type 1 receptor antagonist valsartan can also reduce the occurrence of cardio-cerebral thrombotic events in elderly patients with hypertension.

Hypertrophy of cardiomyocytes and hyperplasia of fibroblasts are known to be the two major responses triggered by Ang II during cardiac remodeling. It has been shown that Ang II-induced impairment of angiogenesis is secondary to progression of the cardiomyocyte hypertrophic response. We recently demonstrated the impairment of myocardial angiogenesis by Ang II, which may also contribute to cardiac remodeling, and that this impairment mechanism may be through p53-dependent downregulation of HIF-1, which is regulated by Jagged1/Notch1 signaling. In the present study, we provide evidence that a Jagged1-Notch signal acts directly on myocardial angiogenesis.

The transcriptional repressors Hey1 and Hey2 are primary target genes of Notch signaling in the cardiovascular system, and induction of Hey gene expression is often used to indicate activated Notch signaling. Here, we observed that Hey1 expression was reduced when angiogenesis was impaired by Ang II, but there was no significant change in Hey2 expression. In a study using a Matrigel plug assay, we found that downregulation of Hey1 caused a dramatic reduction in the formation of new blood vessels in mice. Interestingly, Jag1 gene expression was also downregulated in this model. Studies in tumors have shown that tangeretin treatment attenuated radiation-induced epithelial-mesenchymal transition, invasion, and migration in gastric cancer (GC) cells, which was accompanied by a decrease in Notch1.

Figure 5: Hey2 gene expression in LV tissue (n = 10). RNA from LV tissue was subjected to real-time PCR. GAPDH was used as a loading control. Hey2 expression was quantified as fold of GAPDH. Data are expressed as mean ± SE. P > 0.05. LV: Left ventricle; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; Ang II: Angiotensin II. SE: Standard error.

In conclusion, the present study demonstrated that Jagged1-Hey1 might participate in the impairment of angiogenesis induced by Ang II. In light of the current findings, it appears that Hey1 plays an important and direct role in the regulation of angiogenesis under pathological conditions, making DAPT a possible candidate for the treatment of cardiac hypertrophy. These data also suggest a new mechanism for the impairment of cardiac angiogenesis.

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