Original Paper

Repetitive Transient Ischemia-Induced Cardiac Angiogenesis is Mediated by Camkii Activation

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Key Words

Coronary angiogenesis • Ca²⁺/Calmodulin-dependent kinase II • Vascular endothelial growth factor • Ischemia

Abstract

Background/Aims: Coronary angiogenesis is an important protective mechanism in response to myocardial ischemia in coronary artery disease. However, the underlying mechanisms remain largely unclear. Here, we investigated the role of CaMKII activation in ischemia-induced cardiac angiogenesis. **Methods:** Repetitive transient ischemia model was established in C57/BL6 mice by daily multiple episodes (3 times/day) of short time (5 min) occlusion of the left anterior descending coronary artery for 7 days. Coronary angiogenesis was detected by immunofluorescent staining. RT-qPCR and Western blot analyses were used to detect the mRNA and protein levels of CaMKII, p-CaMKII and VEGF. Primary cardiac microvascular endothelial cells (CMECs) were isolated to investigate the effects of KN93 on cell proliferation and migration in hypoxic condition. **Results:** We found that angiogenesis was induced in the ischemic myocardium and suppressed by chronic intraperitoneal injection of CaMKII inhibitor KN93. RT-qPCR and Western blot analyses showed that myocardial ischemia induced an increased expression and autophosphorylation of CaMKII. VEGF expression was increased in the ischemia model but blunted by KN93. Moreover, KN93 suppressed the proliferation and migration of cardiac endothelial cells in hypoxic condition in which the protein expression of CaMKII, p-CaMKII and VEGF was increased. **Conclusion:** CaMKII is an important mediator for the ischemia-induced coronary angiogenesis, in which CaMKII-triggered VEGF expression plays a key role.

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Introduction

Ischemic heart disease (IHD) is a primary cause of death in cardiovascular diseases [1]. Although the prognosis has been significantly improved by percutaneous coronary intervention, there are still considerable patients who are not suitable for the intervention. Coronary angiogenesis is a compensatory coronary collateral vessel growth initiated by proliferation and migration of endothelial cells. Coronary angiogenesis significantly improves blood supply in the ischemic area, reduces the infarct size and mortality in patients with myocardial infarction [2-5]. Considerable studies have been conducted to investigate the mechanisms of coronary angiogenesis in order to develop better therapeutic strategies for IHD [6-8], but none of them showed promising effects in large clinical trials [9-11].

Ca²⁺/Calmodulin-dependent kinase II (CaMKII) is excessively activated in IHD, which mediates the inflammation and ischemia/reperfusion damage [12, 13]. Vascular endothelial growth factor (VEGF) is the vital stimulus for blood-vessel growth. Growing evidence showed that CaMKII activation may have potential connection with the increased expression of VEGF [14-16]. It has been demonstrated that CaMKII is capable of regulating the response of tumor to hypoxia via activating VEGF promoters [17]. However, whether CaMKII can stimulate VEGF expression and coronary angiogenesis in IHD has not yet been reported. Our aim is to decipher the role of CaMKII in mediating ischemia-induced angiogenesis in the heart.

Materials and Methods

Repetitive transient ischemia murine model

6 to 8-week-old male C57/BL6 mice at weight of 20-22g were used according to the protocol approved by the local Institutional Committee for Care and Use of Laboratory Animals (IACUC) at Zhongnan Hospital of Wuhan University, Wuhan. Mice were anesthetized with 1% sodium pentobarbital (80mg/kg i.p.), intubated, and mechanically ventilated. A mid line incision was made between the 2nd and 3rd ribs to expose the heart. To form a controllable occlusion, a 7-0 prolene suture was carefully passed through a 1 mm polyethylene tubing after threaded around the LAD area. Both ends of the suture were taken out of the thorax and fixed in a subcutaneous pocket. The skin was then closed with 5-0 prolene. Mice recovered from surgery for 3 days before ischemia experimentation. Coronary angiogenesis was then induced by episodes (3 times/day) of short time (5 min) occlusion in the left anterior descending artery for consecutive 7 days. The sham group underwent the same procedures except placing tension on the suture ends. The ischemia group underwent repetitive ischemia (transient LAD occlusion). CaMKII inhibitor KN93 and its inactive analogue KN92 were used to inhibit CaMKII and as a negative control of KN93, respectively. In the ischemia + KN93 or KN92 (Calbiochem) group, KN93 or KN92 (10μmol/kg, i.p. daily) was given prior to LAD occlusion to produce chronic CaMKII inhibition.

ECG recording

To determine successful ischemia induction, the limb leads ECG (Powerlab instrument PL3516B49, Labchart 7.0 software) was recorded in the conscious mice. ST-segment elevation in leads I and aVL was observed when placing tension in the suture and returning to the baseline when loosening the suture, indicating establishment of an onset of ischemia.

Echocardiography in conscious mice

Cardiac function was measured in conscious mice by Vevo 2100 system with a MX400 (18-38MHz) transducer. Left ventricular (LV) dimensions at diastole and systole (LVDD, LVDS) were measured digitally from the M-mode tracings. Based on these measurements, LV systolic volume (LVVS) was calculated as $[7.0 / (2.4 + LVDS)] \times LVDS^3$, LV diastolic volume (LVVD) was calculated as $[7.0 / (2.4 + LVDD)] \times LVDD^3$, ejection fraction (EF) % was calculated as $[(LVDD^3 - LVDS^3) / LVDD^3] \times 100$, Fractional shortening (FS) % was calculated as $(LVDD - LVDS) / LVDD \times 100$, stroke volume (SV) was calculated as LVVD - LVVS. Parameters were measured and calculated before ischemia intervention as a baseline, then every other day during the periodic ischemia for 7 days.

Immunohistochemistry analysis

Microvessel density (MVD) was assessed by CD31 immunofluorescent staining while endothelial cell proliferation was enumerated via double staining with the proliferation marker Ki67. Left ventricles from sham, ischemia and ischemia + KN93/KN92 mice were isolated at the end of experiments (day 7). Slides were treated with primary antibodies, including rat anti-CD31/PECAM 1:200 (BD) and rabbit anti-Ki67 1:200 (Santa Cruz Biotechnology). The secondary antibodies include rat anti-Cy3-conjugated antibody 1:300 (Goodbio technology) and rabbit anti-FITC-conjugated antibody 1:300 (Goodbio technology). The nuclei were labeled with 0.1 mg/ml DAPI (Goodbio technology). Images were acquired using fluorescence microscope (Nikon Eclipse Ti-SR). Angiogenesis index was determined as the ratio of the numbers of microvessels to the DAPI positive cells (CD31:DAPI). Cellular proliferation index was calculated as the ratio of Ki67 staining cells to the DAPI positive cells (Ki67:DAPI). Both of them were quantified at least in 4 independent fields from each heart.

Quantitative RT-PCR

RNA was isolated using Trizol (Invitrogen Life Technologies). cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo). Quantitative RT-PCR was performed by Applied Biosystems 7300 real Time PCR using SYBR green (Roche) based assay. Quantitative RT-PCR was performed with the specific primers shown in Table 1.

Western-blot

The isolated LV tissues or cultured cells were homogenized as described [18]. Primary antibodies used in this study include rabbit polyclonal anti-CaMKII antibody, rabbit polyclonal anti-phospho-CaMKII (Thr287) antibody, rabbit monoclonal anti-GAPDH antibody (Cell signaling technology), rabbit polyclonal anti-VEGF antibody (Santa Cruz Biotechnology) and rabbit polyclonal anti-CD31 antibody (Abcam). The second antibody used was goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology).

Isolation, culture and identification of primary Cardiac Microvascular Endothelial Cells (CMECs)

Isolation of CMECs was performed according to the published protocol [19]. Briefly, 6 neonatal Sprague-Dawley rat hearts were isolated and dissected into $\approx 1\text{mm}^3$ pieces and then digested by addition of 0.1% collagenase B (Roche) for 30 min and 0.25% Tyrosin-EDTA (hyclone) for 10 min at 37°C. After filtration and centrifugation, cells were resuspended with 20% Fetal Bovine Serum (gbico)-M199 medium (hyclone) and 50µg/ml heparin. 75µg/ml endothelial cell growth supplement (BD) was added to provide the best microenvironment for endothelial cells. CMECs were identified by microscope and CD31 immunofluorescent staining.

CMECs proliferation assay

The CMECs (5×10^3 cells/well) were cultured in 96-well plates overnight. The media was replaced with fresh M199 medium, M199+KN93 (2, 5, 10, 20µM) and M199+KN92 (2, 5, 10, 20µM), respectively. For hypoxia group, cells were incubated with 3% O₂, while the cells in normoxia group were incubated with 20% O₂ at 37°C for 24 hours. At the end of the indicated time-periods, cell proliferation was measured using a cell counting Kit-8 (CCK-8 kit; Dojindo Molecular Technologies) according to the manufacturer's instructions. The inhibition rate of cell proliferation was calculated by the following formula: Inhibition rate (IR%) = $(1 - \frac{\text{OD}_{\text{experimental group}}}{\text{OD}_{\text{control group}}}) \times 100$

CMECs migration assay

Cell mobility was assessed using the transwell assay (Corning Inc. Corning, NY, USA). CMEC cells (4×10^4 per well, treated with 2µM KN93, KN92 or blank control medium) were seeded in the upper wells with serum-free medium. Medium with 20% FBS was added into the bottom wells to act as a chemoattractant.

Table 1. Primer sequences for the target gene RT-qPCR

Gene		Primer Sequence
CaMKII	Forward	TTCGTGCTTTACGTCCTTCATC
	Reverse	ACGCTCTACTGTTGCCTCCAT
VEGF	Forward	AGGAGTACCCCGACGAGATAGA
	Reverse	CACATCTGCTGTGCTGTAGGAA
β-actin	Forward	GTGACGTTGACATCCGTAAAGA
	Reverse	GTAACAGTCCGCTAGAACAC

Cells in the hypoxia groups were incubated with 3% O₂, while cells in the normoxia groups were incubated with 20% O₂ at 37°C. After 12 hours of incubation, the cells were washed with PBS for 3 times and fixed with 4% paraformaldehyde for 30 min, then stained with 1% crystal violet for 15 min at 37°C. The cells on the upper chamber were removed by cotton swab, the migrated cells counting was then performed under microscope (Nikon Eclipse Ti-SR). Five random views were selected to count the migrated cells.

Statistical analysis

Statistical tests were performed using GraphPad Prism software (version 5.0). Statistical significance was evaluated using independent-sample *t*-test for comparison between 2 groups and one-way ANOVA with Dunnett's post test for comparison among 3 or more groups. Echocardiographic and morphometric data were reported as mean ± standard deviation, while other data were shown as mean ± SEM. *p* value less than 0.05 was considered statistically significant.

Results

Establishment of coronary angiogenesis model induced by repetitive ischemia

We performed a closed-chest ischemia/reperfusion (I/R) to induce repetitive transient cardiac ischemia in mice. Echocardiographic and morphometric data during myocardial ischemia are summarized in Table 2. The left ventricular function assessed by EF (%) remained constant after ischemic performance, indicating that the repetitive ischemia did not cause myocardial necrosis. ECG monitoring was conducted during each ischemia application to verify a successful induction of ischemia. ST segment was elevated during each onset of ischemia and recovered afterwards (Fig. 1), indicating that each episode resulted in an ischemic attack, i.e. the repetitive I/R model was well established.

Role of CaMKII activity in the I/R-induced cardiac angiogenesis

To determine whether CaMKII activation is associated with the I/R-induced cardiac angiogenesis, the immunofluorescent staining and Western blot against CD31 were performed and quantified in the ischemic myocardium 7 days after daily I/R. As shown in Fig. 2, the capillary densities in the LV myocardium in both ischemia and ischemia + KN92 groups were significantly increased compared to the sham-operated controls. However, the angiogenesis was eliminated in mice treated with daily intraperitoneal injection of CaMKII inhibitor KN93. In addition to the inhibition of capillary density, KN93 also blunted cellular proliferation (Ki67/DAPI ratio). These results suggested that CaMKII

Table 2. Echocardiographic and morphometric parameters monitored during periodic ischemia. Values are mean ± standard deviation. LVID: left ventricular internal dimension, EF: ejection fraction, FS: fractional shortening, SV: stroke volume, BW: body weight, HW: heart weight, s: systolic, d: diastolic, n: sample size in each group

Parameter	Day 0	Day 1	Day 3	Day 5	Day 7
(n=20)					
LVIDd(mm)	3.4±0.4	3.3±0.2	3.5±0.3	3.4±0.3	3.5±0.3
LVIDs(mm)	1.5±0.2	1.7±0.1	1.5±0.2	1.5±0.2	1.7±0.2
EF(%)	89.5±5.8	86.9±3.5	91.3±3.9	90.3±6.6	88.2±4.9
FS(%)	54.7±9.4	49.2±5.1	56.8±6.9	56.0±9.3	51.8±6.6
SV(μL)	43.9±14	38.6±6.9	46.9±8.5	44.7±12.3	44.2±10.8
BW(g)	21.2±0.4	20.8±0.9	20.9±0.9	21±0.6	21±0.7
HW/BW(mg/g)	5±0.4	5.3±0.6	5.3±0.6	5.6±0.3	5.4±0.2

Fig. 1. Changes of ECG at lead I before (a) during (b) and after (c) each brief ischemia episode. ST segment was elevated during ischemia and fell back after reperfusion.

activation plays a key role in the repetitive ischemia-induced angiogenesis.

CaMKII inhibition abrogated VEGF expression in the ischemic myocardium

To explore the underlying mechanism of coronary angiogenesis induced by ischemia, the mRNA levels of CaMKII and VEGF at the ischemic day 0, 1, 3, 5, 7 were detected by RT-qPCR. As shown in Fig. 3, the mRNA level of CaMKII increased significantly and reached a peak after 5 days ischemia in both ischemia (Fig. 3A), ischemia + KN92 (Fig. 3B) and ischemia + KN93 group (Fig. 3C). In contrast, VEGF mRNA level was increased in the ischemia and ischemia + KN92 groups but not in the ischemia + KN93 group. These results indicated that the repetitive ischemia induced CaMKII and VEGF expression while the VEGF expression was mediated by CaMKII activation. Coincide well with the RT-qPCR results, the phosphorylation level of CaMKII, expression of CaMKII and VEGF gradually increased with the ischemia episode. Injection of KN93 blocked phosphorylation of CaMKII and down-regulated the protein level of VEGF without changes in the expression of CaMKII (Fig. 4). These data demonstrated that the repetitive ischemia triggers coronary angiogenesis through the activation of CaMKII-VEGF pathway.

Fig. 2. Coronary angiogenesis detected using immunofluorescence. A: CD31/PECAM and Ki67 double-staining immunofluorescent images in sham, ischemia, ischemia + KN93, ischemia + KN92 groups. Scale bar = 50 μ m. B&C: Quantification of angiogenesis and proliferation index, showing that the increase in capillary density and endothelial cellular proliferation by ischemia can be prevented by CaMKII inhibitor KN93. D: Western blot analysis for CD31 expression in heart. The analysis data were from 6 mice. *: $P < 0.05$, **: $P < 0.01$ compared with sham group. #: $P < 0.05$, ##: $P < 0.01$ compared with ischemia group. PECAM: platelet endothelial cell adhesion molecule.

Fig. 3. Analysis of RT-qPCR in ischemia (A), ischemia + KN92 (B) and ischemia + KN93 (C) groups. CaMKII and VEGF mRNA levels measured at day 0, 1, 3, 5, and 7 of periodic ischemia. CaMKII levels increased after ischemia and peaked after 5 days ischemia in the ischemia, ischemia + KN92, and ischemia + KN93 group. VEGF expression increased in the ischemia and ischemia + KN92 groups but unchanged in the ischemia + KN93 group. All values are mean $\pm$ SEM from 5 independent experiments. *: $p < 0.05$, **: $p < 0.01$ compared with Day 0.

Fig. 4. Protein expression levels of CaMKII, p-CaMKII and VEGF in the ischemia, ischemia + KN93 and ischemia + KN92 groups measured at day 0, 1, 3, 5, and 7 of periodic ischemia. CaMKII expression gradually increased in all groups. Phosphorylation level of CaMKII and VEGF increased remarkably in the ischemia and ischemia + KN92 groups. CaMKII inhibition by KN93 eliminated the ischemia-induced VEGF expression. Data presented as mean $\pm$ SEM were from 5 independent experiments. *: $P < 0.05$, **: $P < 0.01$ compared with Day 0.

CaMKII inhibition suppressed CMECs proliferation and migration induced by hypoxia in vitro

To understand the role of CaMKII in regulating ischemia-induced angiogenesis, we further investigated the effect of CaMKII inhibition on cardiac microvascular endothelial cells *in vitro*. CMECs were isolated (Fig. 5A) and identified by CD31 immunofluorescent staining (Fig. 5B). The CD31 positive ratio of cells reached 95% during the third generation. The proliferation of CMECs was assessed by the CCK-8 assay (Fig. 5C), we found that KN92 has no effect on cell proliferation in the normoxia group or hypoxia group (Table 3) while KN93 inhibited CMECs proliferation in a concentration-dependent manner in both normoxia (IR% = 1.3 ± 1.9 , 7.3 ± 1.4 , 8.5 ± 2.2 , 16.4 ± 1.2 for $2 \mu\text{M}$, $5 \mu\text{M}$, $10 \mu\text{M}$ and $20 \mu\text{M}$ KN93, respectively) and

Table 3. The effect of KN92 on CMEC proliferation. Data were from 6 independent experiments and expressed as mean $\pm$ SEM

Concentration of KN92	0 μM	2 μM	5 μM	10 μM	20 μM
Normoxia	0 ± 1.18	0.4 ± 0.63	1 ± 1.04	0.8 ± 0.33	1.1 ± 0.72
Hypoxia	0 ± 1.17	0.2 ± 1.73	0.1 ± 1.94	0.1 ± 0.38	1.5 ± 0.51

Fig. 5. KN93 inhibited the proliferation and migration of CMECs *in vitro*. CMECs were isolated (A) and then identified by CD31 immunofluorescent staining (B). Scale bar = $50 \mu\text{m}$. The CCK-8 assay was conducted to reflect the proliferation of CMECs indirectly (C). KN93 inhibited the CMEC proliferation in a concentration-dependent manner with a greater inhibition in the hypoxia than the normoxia condition. Migration of CMECs was inhibited by CaMKII inhibitor KN93 but not by its inactive analogue KN92 (D). Data were from 6 independent experiments and expressed as mean $\pm$ SEM. **: $P < 0.01$, compared with blank medium group in normoxia condition. #: $P < 0.01$, compared with blank medium group in hypoxia condition.

Fig. 6. Hypoxia-induced VEGF expression was suppressed by CaMKII inhibition. A: Representative Western blots showing expression of CaMKII, p-CaMKII and VEGF in CMECs. B: Analysis of protein levels of CaMKII, p-CaMKII and VEGF in CMECs in the condition of hypoxia and normoxia with treatment of KN93 or KN92, respectively. Data were from 6 independent experiments and expressed as mean $\pm$ SEM. *: $P < 0.05$, **: $P < 0.01$ compared with normoxia, #: $P < 0.05$, ##: $P < 0.01$ compared with hypoxia.

hypoxia ($IR\% = 2.5 \pm 1.1$, 8.5 ± 1.5 , 13.4 ± 2.9 , 23.9 ± 1.3 for $2\mu M$, $5\mu M$, $10\mu M$ and $20\mu M$ KN93, respectively) groups, with a much greater inhibition rate in the hypoxia group. We further explored the effect of KN93 on CMEC migration by transwell assay. As shown in Fig. 5D, hypoxia induced remarkable increase in cell migration (53.8 ± 5.9 vs 185.9 ± 19.9 , $p < 0.01$). As expected, $2\mu M$ KN93 dramatically suppressed CMEC migration (185.9 ± 19.9 vs 65.7 ± 7.8 , $p < 0.01$). These results demonstrated that CaMKII plays a key role in regulating the proliferation and migration of cardiac microvascular endothelial cells.

CaMKII inhibition attenuated VEGF expression in CMECs

We have detected the protein levels of CaMKII, p-CaMKII and VEGF in CMECs. As shown in Fig. 6, the levels of phosphorylated CaMKII and VEGF expression were increased after 24 hours hypoxia, but this increase has been attenuated by KN93. Interestingly, KN93 also reduced the basal VEGF protein level, indicating that a basal level of CaMKII activity is required to maintain VEGF expression in the normoxic condition. The total CaMKII expression did not change in hypoxic condition. Unlike KN93, KN92 had no effects on the expression of CaMKII, p-CaMKII and VEGF.

Discussion

In this study, we have demonstrated that (1) the periodic transient ischemia-induced significant coronary angiogenesis with increased expression levels of CaMKII, p-CaMKII and VEGF in the ischemic myocardium. (2) CaMKII inhibition by KN93 down-regulated the expression of VEGF and thus inhibited the ischemia-induced coronary angiogenesis. (3) In line with the *in vivo* results, CaMKII inhibition suppressed CMEC proliferation and migration in hypoxia condition. (4) Ischemia increased CaMKII expression in myocardium but not in the endothelial cells.

CaMKII is a known pro-arrhythmic molecule and a key mediator of heart failure [20, 21]. CaMKII also participated in artery remodeling through vascular smooth muscle cells [22]. In the present study, we have revealed an important role of CaMKII activation in the ischemia-induced coronary angiogenesis. Previous studies have reported that chronic ischemia has a direct association with coronary angiogenesis, but the underlying molecular mechanisms are unclear [23, 24]. It has been shown that CaMKII inhibition blunted tumor response to hypoxia [17] and that CaMKII is likely the upstream signal of the hypoxia inducible factor-1 alpha (HIF-1 α) in macrophage and some cancer cell lines [14-16]. Indeed, oxidative stress and calcium entry caused by repetitive ischemia can both activate CaMKII [25-27]. Our experiment showed a clear association between CaMKII activation and coronary angiogenesis induced by repetitive ischemia. CaMKII inhibition by KN93 abrogated the ischemia-induced

CaMKII activation, down-regulated VEGF expression and eliminated coronary angiogenesis. However, KN93 did not decline cardiac function in mice subjected to the repetitive ischemia (data not shown). A possible explanation is that in the setting of ischemia, cardiac CaMKII is excessively activated, which stimulates cardiac angiogenesis to improve cardiac perfusion but meanwhile reduces myocyte function (by inducing sarcoplasmic reticulum calcium leak [18, 28]). A myocyte-specific CaMKII inhibition is therefore desirable.

VEGF is a known key mediator for the ischemia-induced angiogenesis. Previous studies have demonstrated that VEGF expression is essential in coronary collateral growth [29, 30]. VEGF gene delivery increased microvascular density in the myocardial infarct zone [8]. The primary promoters of VEGF are HIF-1 α and activating protein-1 (AP-1). CaMKII was able to up-regulate the expression of HIF-1 α in macrophage [14] and retinal Müller cells [31], and increase AP-1 protein level in osteosarcoma [15]. However, there was study showed that CaMKII inhibitor had no effect on HIF-1 α induced VEGF production in rheumatoid synovial fibroblast [32]. In hepatoma cells, CaMKII has been shown to up-regulate VEGF expression directly [16]. Interestingly, in hippocampal neurons and retinal endothelial cells, CaMKII was activated by VEGF via increasing Ca²⁺ influx [33-35]. In consistent with this, our data indicated that inhibition of CaMKII decreased expression of VEGF and suppressed CMECs proliferation and migration and *in vivo* coronary angiogenesis. These findings suggested that CaMKII activation promotes coronary angiogenesis likely through up-regulating VEGF. In fact, increasing of VEGF expression was detected in limb ischemia, [36] experimental MI [37] and ischemia-induced coronary collateral growth models [29, 30]. However, in a mouse model of coronary ischemia established by Lavine et al [38], neither mRNA nor protein level of VEGF increased. We speculate that this inconsistency may be related to the stimulus frequency and duration of ischemia. In Kory's model, ischemia was induced by only 3 ischemia episodes in a week, while we established a model by 5 minutes ischemia per 8 hours for one week. This pattern avoids myocardial necrosis and ensures a frequent onset of hypoxia to stimulate the VEGF expression. Moreover, this chronic repetitive transient ischemia is more close to the clinical stable angina caused by coronary stenosis.

Stimulation of coronary collateral formation provides a broad prospect for the treatment of CAD patients [39-41]. Various therapeutic angiogenesis attempts focused on VEGF and FGF to induce coronary collateral growth have been reported in recent decades [10, 42, 43], but none of them really works in the patients. Our findings revealed a novel mechanism underlying the repetitive ischemia-induced coronary angiogenesis which highlight the important role of CaMKII herein.

In this study, we have demonstrated that microvascular density was significantly reduced by inhibition of CaMKII activation. However, even treated with KN93, coronary angiogenesis has not been fully suppressed, indicating that other signal pathways may also be involved in coronary angiogenesis. In addition, the expression of CaMKII didn't change during hypoxia *in vitro*, but increased markedly in the ischemic myocardium. This finding indicates that besides CMECs, myocardial environment may be important for coronary angiogenesis. For instance, the increase in CaMKII activity, as a key trigger of ischemia-induced coronary angiogenesis, may largely derive from the ischemic myocardium.

Conclusion

Our data revealed a novel mechanism for the coronary angiogenesis. Chronic repetitive ischemia induces CaMKII activation which triggers VEGF expression and thereby promotes coronary angiogenesis.

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Y.W. and J.C. conceived and designed the project and experiments. Z.C. performed the experiments. Z.C., Q.D. and C.Q. analyzed the data. Z.C., B.L. and Y.W. wrote and revised the paper. All authors reviewed the manuscript.

Disclosure Statement

The authors declare no competing financial interests.

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