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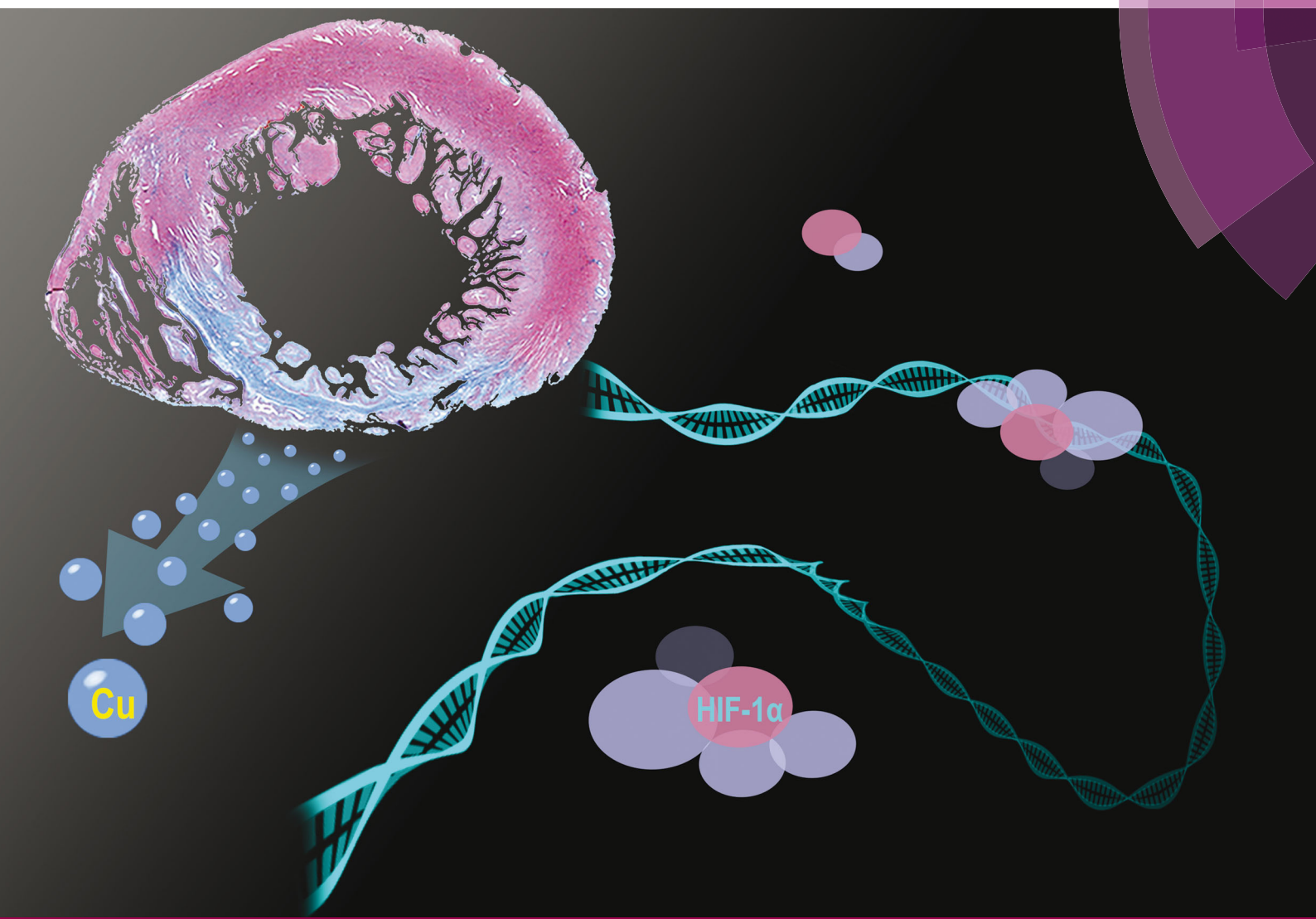

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The association of depressed angiogenic factors with reduced capillary density in the Rhesus monkey model of myocardial ischemia

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Depressed capillary density is associated with myocardial ischemic infarction, in which hypoxia-inducible factor 1α (HIF-1α) is increased. The present study was undertaken to examine changes in the angiogenic factors whose expression is regulated by HIF-1 and their relation to the depressed capillary density in the Rhesus monkey model of myocardial ischemic infarction. Male Rhesus monkeys 2–3 years old were subjected to myocardial ischemia by permanent ligation of left anterior descending (LAD) artery leading to the development of myocardial infarction. Eight weeks after LAD ligation, copper concentrations, myocardial histological changes and capillary density were examined, along with Western blot and immunohistochemical analysis of angiogenic factors and detection of HIF-1 activity. Capillary density was significantly decreased but the concentrations of HIF-1α and HIF-1β were significantly increased in the infarct area. However, the levels of mRNA and protein for VEGF and VEGFR1 were significantly decreased. Other HIF-1 regulated angiogenic factors, including Tie-2, Ang-1 and FGF-1, were also significantly depressed, but vascular destabilizing factor Ang-2 was significantly increased. Copper concentrations were depressed in the infarct area. Copper-independent HIF-1 activity was increased shown by the elevated mRNA level of IGF-2, a HIF-1 target gene. Removal of copper by a copper chelator, tetraethylenepentamine, from primary cultures of neonatal rat cardiomyocytes also suppressed the expression of HIF-1 regulated VEGF and BNIP3, but not IGF-2. The data suggest that under ischemic conditions, copper loss suppressed the expression of critical angiogenic genes regulated by HIF-1, but did not affect copper-independent HIF-1 activation of gene expression. This copper-dependent dysregulation of angiogenic gene expression would contribute to the pathogenesis of myocardial ischemic infarction.

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1. Introduction

Decreased capillary density contributes to the pathogenesis of myocardial ischemic infarction. Thus, promotion of angiogenesis is an important strategy to reduce infarct size and improve recovery from myocardial ischemia.[1] Angiogenesis is a complicated process that involves a variety of factors, such as vascular endothelial growth factor (VEGF) and its receptors VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1); angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2) and their receptor Tie-2; fibroblast growth factor-1 (FGF-1), fibroblast growth factor-2 (FGF-2), neuropilin-1 (NRP-1), and others. The expression of most of these factors is regulated by hypoxia-inducible factor-1 (HIF-1).

HIF-1 is a ubiquitously existing and heterodimeric transcription factor that regulates genes involved in a wide spectrum of cellular events, including angiogenesis, vascular reactivity and remodeling, vasomotor control, glucose and energy metabolism, cell proliferation and viability, and nucleotide metabolism.[2] HIF-1 is composed of HIF-1α and HIF-1β subunits. The HIF-1α subunit is labile while the HIF-1β subunit is constitutively stable in the nucleus. The synthesis and accumulation of HIF-1α is a rate-limiting step for activation of HIF-1.[3–5]

Accumulation of HIF-1α in the ischemic heart has been observed both in human and animal studies.[6–9] Human biopsy specimen studies found increased immunoreactivity of HIF-1α and VEGF in the early phase of acute myocardial ischemia.[6] Autopsy samples from patients with chronic ischemic cardiomyopathy showed an increased level of HIF-1α but not VEGF.[7] In rodent animal models the increased HIF-1α protein level was observed in both acute and chronic phases of myocardial infarction.[8,9] In studies with the rat model, it was found that either systemic ischemia or coronary occlusion led to persistent

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elevation of HIF-1α in cardiomyocytes and stromal cells.[9] However, it has been well documented that in myocardial ischemic infarction, the density of capillary vessels is dramatically decreased and the infarcted myocardium lacks blood supply,[10] leading to the loss of vital cells followed by collagen deposition in the infarcted tissue.

The increase in HIF-1α accumulation along with the depressed capillary density in myocardial ischemia presents a mystery paradigm. The question is: does the increased HIF-1α result in up-regulation of its target angiogenic genes?

It is important, but quite difficult, to study the relationship between the HIF-1α accumulation and the expression of angiogenic genes in humans. Rhesus monkeys serve as an excellent non-human primate animal model and provide a precious opportunity to study human relevant physiological and pathological changes.[11] In particular, Rhesus monkeys are excellent surrogates for humans in cardiovascular disease due to high resemblance of their hearts to humans and close similarity in the distribution of coronary arteries.[12] Our recent studies demonstrated that the Rhesus monkey model of myocardial ischemic infarction recaptured the manifestations in human myocardial infarction.[13–15]

The present study was undertaken employing a Rhesus monkey model of myocardial ischemia induced by left anterior descending (LAD) artery ligation to examine the relationship between the changes in HIF-1 and its regulated gene expression and the depressed capillary density following a long-term ischemia.

2. Methods

2.1 Animals and animal care

Male Rhesus monkeys (Macaca mulatta), aged 2–3 years old and weighed 4.5 to 6.0 kg, were acclimatized to laboratory conditions for a period of at least one month in the Association for Assessment and Accreditation of Laboratory Animal Care accredited facility. All monkeys were handled in strict accordance with good animal practice under supervision of veterinarians and were monitored for evidence of disease and changes in attitude, appetite, or behavior suggestive of illness. All animal procedures were approved by the Institutional Animal Care and Use Committee at the Sichuan University West China Hospital, following the guideline of the US National Institutes of Health.

2.2 Cell culture and treatments

Primary cardiomyocytes were isolated from 1 to 3 day-old SPF-grade Sprague-Dawley neonatal rats (DaShuo, CN) and cultured according to a procedure published previously,[16] with some modifications. Upon isolation, cells were maintained at 37 °C in L-DMEM (GIBCO, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, USA) and 1% penicillin/streptomycin (GIBCO, USA) in a 5% CO_2 incubator. This investigation follows the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). To determine the relationship between copper and HIF-1 activity, cells were treated with varying concentrations of tetraethylenepentamine (TEPA, Sigma, USA) and $CuSO_4$ (Sigma, USA) in FBS-free medium for the time periods indicated in the text. Some cells were incubated under hypoxic (1% O_2 and 5% CO_2) conditions for 6 hours to activate HIF-1 transcription activation.

2.3 Induction of myocardial ischemic infarction

As described in our previous studies,[14,15] the heart of monkeys was exposed *via* the left fourth intercostal thoracotomy. The LAD artery was occluded for 1 min followed by a 5 min reperfusion, and this occlusion-reperfusion cycle was repeated 3 times before the eventual ligation. The sham-operated controls were subjected to the same surgical procedure with the exception of the LAD artery occlusion and ligation.

2.4 Tissue preparation

Eight weeks after surgery, the animals were sacrificed to obtain heart tissue. The infarct area (IA) can be distinguished from non-infarct area by its pale appearance. The border zone (BZ) is defined as the area from 1 mm inside to 3 mm outside the infarct area. The remaining part is defined as the remote area (RA). Heart samples from different areas were preserved in liquid nitrogen for Western blot and RT-PCR analysis and in −80 °C for copper detection. Another portion was embedded in OCT (Leica, Germany) for frozen sectioning. The rest part was fixed with 4% paraformaldehyde and embedded in paraffin as previously described,[17] and then sectioned at 4 μm intervals. The sections were stained with hematoxylin and eosin to observe the structure, and with Masson's trichrome and Sirius red to observe collagen deposition.

2.5 Immunohistochemical staining

Tissue sections were incubated overnight with primary antibodies (anti-HIF-1α, anti-VEGFR-1 and anti-NRP-1, Abcam, USA; anti-HIF-1β and anti-VEGFR-2, Cell Signaling Technology, USA; anti-VEGF, anti-Tie-2, anti-Ang-1, anti-Ang-2, anti-FGF-1 and anti-FGF-2, Santa Cruz, USA) at 4 °C, and were subsequently incubated with secondary anti-mouse or anti-rabbit IgG (Maixin, China) at 37 °C for 10 min according to manufacturer's instruction. Replacement of the primary antibody with PBS served as a negative control.

2.6 Measurement of capillary density

Slides were immunostained with anti-CD31 antibody (Maixin, China) to identify capillaries. Five visual fields of each defined area (IA, BZ, and RA) of each slide were captured under 10× ocular lens and 20× object lens. Each image was viewed by two pathologists independently to reach consensus on an overall estimation.

2.7 Western blot analysis

The protein levels of HIF-1α, HIF-1β, VEGF, VEGFR-1, VEGFR-2, NRP-1, Tie-2, Ang-1, Ang-2, FGF-1 and FGF-2 were determined by Western blot. Equal amounts of protein (30 μg) from each sample were separated by SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Bio-Rad, USA). Membranes were incubated overnight at 4 °C with respective primary antibodies according to the vendor's recommendations. Then the membranes were incubated for 1 h at 37 °C with appropriate secondary antibodies (Santa Cruz, USA). Target proteins were visualized using

a chemiluminescence HRP substrate (Millipore, USA) and analyzed by densitometry using a Quantity One Software.

2.8 Reverse transcription and real time PCR

Total RNA was isolated using Trizol (Invitrogen, 15596-026, USA) as per manufacturer's instructions. 1 μg RNA was used for reverse transcription as per protocols using a Prime Script TMRT reagent kit (TaKaRa, RR037A, Japan). Real-time PCR reaction was performed using a SYBR Premix Ex TaqTM II (TaKaRa, RR820A, Japan). To amplify VEGF, VEGFR-1, Ang-2 and IGF-2 cDNA fragments, the samples were processed using a BIO-RAD CFX96 Real-Time System. Gene expression level was analyzed relative to TBP (for monkey heart tissue) or RPS18 (for primary rat cardiomyocytes) using the $2^{-\Delta\Delta CT}$ method in each sample. Three replicates were run for each sample. The primer sequences were designed using a Primer-BLAST of NCBI (Tables 1 and 2).

2.9 Measurement of copper concentration

Copper concentrations were determined using a graphite furnace atomic absorption spectrophotometer (ICE3500; Thermo, USA). Heart tissue samples were freshly frozen, lyophilized, and digested with nitric acid (HNO_3, Sigma, USA) at 50 °C overnight (10 mg dried tissue with 1 ml nitric acid). Copper concentrations were normalized by dry weight of tissue samples. For the analysis of copper in cardiomyocytes, cells were collected by cell scraper, washed with ice-cold PBS containing 10 mM EDTA (Sigma, USA) to ensure that extracellular copper was completely removed, lysed with 1% SDS solution (Beyotime, CN), and digested with nitric acid at 50 °C overnight. Intracellular copper concentrations were normalized by total protein concentrations.

2.10 Statistical analysis

SPSS was applied to perform the statistical analysis. Data were presented as mean ± SD. One-way ANOVA was used for initial analysis of the capillary density, the Western blot intensity, the HIF-1α positive nuclei, the relative mRNA level of HIF-1 target genes and the copper concentrations, followed by SNK test for difference between groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1 Capillary density in the myocardium after LAD ligation

All animals subjected to LAD ligation for 8 weeks showed left ventricle transmural infarction. As shown in Fig. 1A, the infarct area was located in the left ventricle. Histological sections revealed that this area occupied part of the anterior wall of the left ventricle and the septum. Ventricular wall thinning and chamber dilatation were also observed (Fig. 1A and B).

Masson's trichrome and Sirius red staining showed collagen deposition in different regions of the heart after LAD ligation. Massive fibrosis was shown in the infarcted myocardium by both methods (Fig. 1A and C). The capillary density was significantly decreased in the infarct area in comparison with the sham-operated controls (Fig. 1D).

3.2 Changes in angiogenic factors in the myocardium after LAD ligation

The protein levels of VEGF, VEGFR-1, VEGFR-2, NRP-1, Tie-2, Ang-1, Ang-2, FGF-1, and FGF-2 were determined by Western blot and immunohistochemical method, as categorized as follows.

3.2.1 Factors depressed. The protein levels of VEGF, VEGFR-1, Tie-2, Ang-1, and FGF-1 were decreased significantly in the infarct area compared to the remote area and sham-operated controls determined by Western blot analysis (Fig. 2B), and confirmed by immunohistochemical detection (Fig. 2A). VEGF was mainly localized in endothelial cells and interstitial tissue of myocardium. VEGFR-1, Tie-2, and FGF-1 were expressed in both endothelial cells and cardiomyocytes. In addition, VEGFR-1 was mostly localized in the membrane of cardiomyocytes in sham controls, but in contrast, it was localized in the cytosol of the ischemic cardiomyocytes (Fig. 2A). Ang-1 was mostly in the cytosol of cardiomyocytes (Fig. 2A).

Table 1 RT-PCR primer sequences for monkey heart tissue

Primer	Forward sequence (5′ to 3′)	Reverse sequence (5′ to 3′)
TBP	TGCTCACCCACCAACAGTTT	TGCTCTGACTTTAGCACCTGT
VEGF	GAGCTTCCTACAGCACAACA	CCAGGACTTATACCGGGATTTC
VEGFR-1	GGGTCACATCACCTAACATCAC	CCTTTCTGCTGTCCCAGATTAC
Ang-2	CCACATCAAACTCAGCTAAGGAC	ATAATTGTCCACCCGCCTCC
IGF-2	CGTCACCGTCCCCTGATTG	TCCGATTGCTGGACATCTCT

Table 2 RT-PCR primer sequences for rat cardiomyocytes

Primer	Forward sequence (5′ to 3′)	Reverse sequence (5′ to 3′)
RPS18	GTCCCTAGTGATCCCCGAGA	TGGCCAGAACCTGGCTATACT
BNIP3	TTTAAACACCCGAAGCGCAC	ATGTAGATCCCCAATCCAATGGC
Ang-2	CATGATGTCATCGCCCGACT	AAGGTCAGCGTGTAGATGCC
IGF2	AGAAGCAGAAGAGACGCCCC	ATCCCCATTGGTACCTGGAAGC
VEGF	AATCCTGGAGCGTTCACTGT	ACGCGAGTCTGTGTTTTTGC

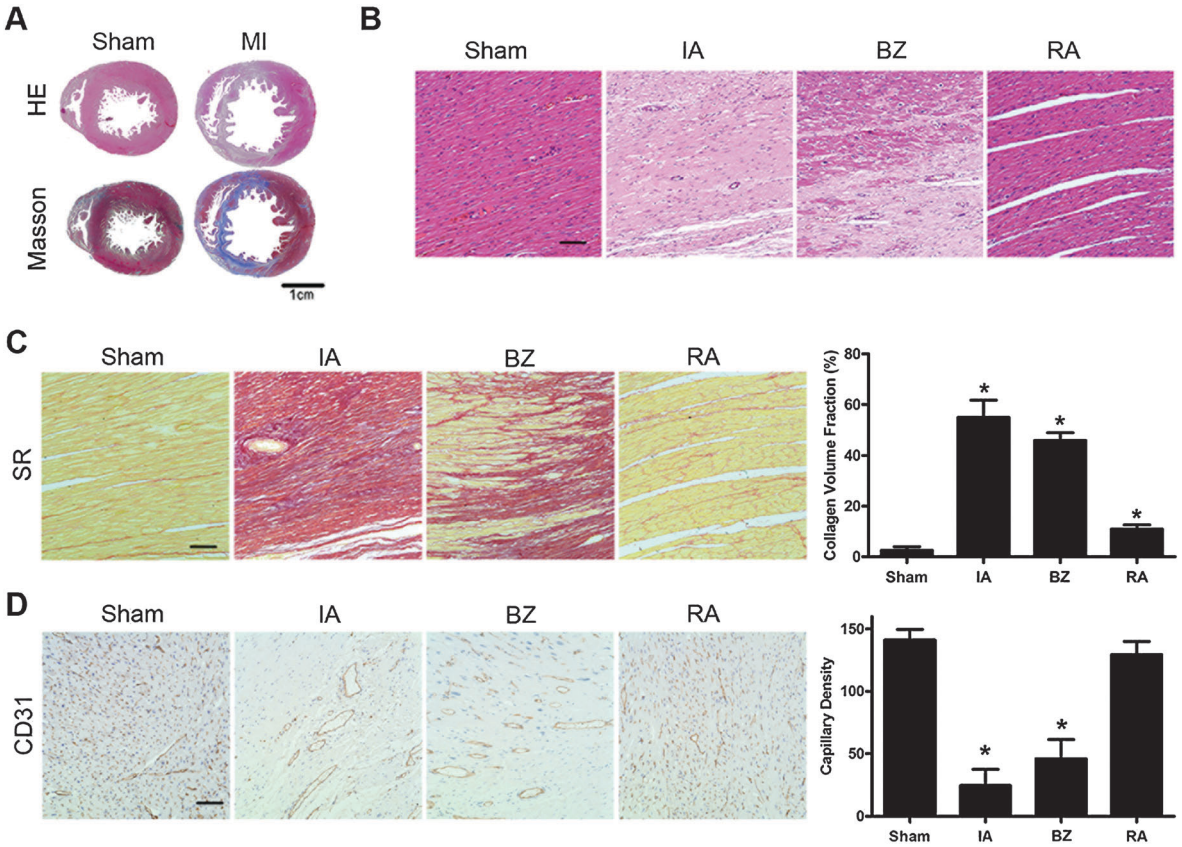

Fig. 1 Change in capillary density. (A) H&E and Masson's trichrome staining of transversely sectioned monkey hearts. Collagen was stained blue on the left ventricular wall, interventricular septum, and right ventricular wall. (B) H&E and (C), Sirius red staining of different areas of LAD ligated hearts. (D) Immunohistochemical staining with antibody against CD31. Bar = 100 μm. Sham: sham operated control (n = 5); MI: 8 weeks after LAD ligation (n = 5); IA: infarct area (n = 4–5); BZ: border zone (n = 4–5); RA: remote area (n = 4–5); *: $P < 0.05$ *versus* sham operated control.

3.2.2 Factors increased. The protein levels of VEGFR-2 and Ang-2 were increased prominently in the infarct area in comparison with the remote area and sham controls, as revealed by both Western blot and immunohistochemical analyses (Fig. 2A and B). VEGFR-2 was only in endothelial cells and Ang-2 was expressed in both endothelial cells and cardiomyocytes (Fig. 2A).

3.2.3 Factors unchanged. The protein levels of NRP-1 and FGF-2 remained unchanged in the infarcted myocardium compared to the remote area and sham controls (Fig. 2). NRP-1 was in the entire myocardium including cardiomyocytes and endothelial cells. FGF-2 was only in the smooth muscle cells of arteries (Fig. 2A).

3.3 Changes in the HIF-1 transcription factor

The immunoreactivity of HIF-1α was increased markedly in cardiomyocytes in the infarct area and border zone (Fig. 3A). The number of HIF-1α positive nuclei increased significantly in these two areas as well (Fig. 3A and C). HIF-1β was localized mostly in the nuclei of cardiomyocytes and endothelial cells. The increased number of HIF-1β positive nuclei was also found in the infarct and border areas (Fig. 3A). The increase in both HIF-1α and HIF-1β in the infarct area was confirmed by Western blot analysis (Fig. 3B).

At the mRNA level, a significant decrease of VEGF and VEGFR-1 was observed in the infarct area compared to the sham-operated controls (Fig. 3D and E). In contrast, the mRNA levels of IGF-2 and Ang-2 were significantly increased (Fig. 3F and G).

3.4 Changes in copper concentrations

Copper is required for HIF-1 activation of the expression of some angiogenic genes.[18–21] Myocardial ischemia is accompanied by the loss of copper.[22,23] As expected, the concentration of copper in the infarct area was significantly reduced compared to the remote area and the sham-operated control, as shown in Fig. 3H.

3.5 Effects of copper chelation by TEPA on HIF-1 regulated gene expression in cardiomyocytes

To further define the role of copper in the regulation of HIF-1 controlled gene expression in the heart, primary cultures of neonatal rat cardiomyocytes were used. As shown in Fig. 4B, intracellular copper concentration was decreased in the presence of TEPA and was increased in the presence of $CuSO_4$. Consistent with our previous study in endothelial cells,[24] TEPA inhibited hypoxia-induced expression of BNIP3 and VEGF (Fig. 4C and D). In contrast, TEPA enhanced IGF-1 and Ang-2 expression (Fig. 4E and F).

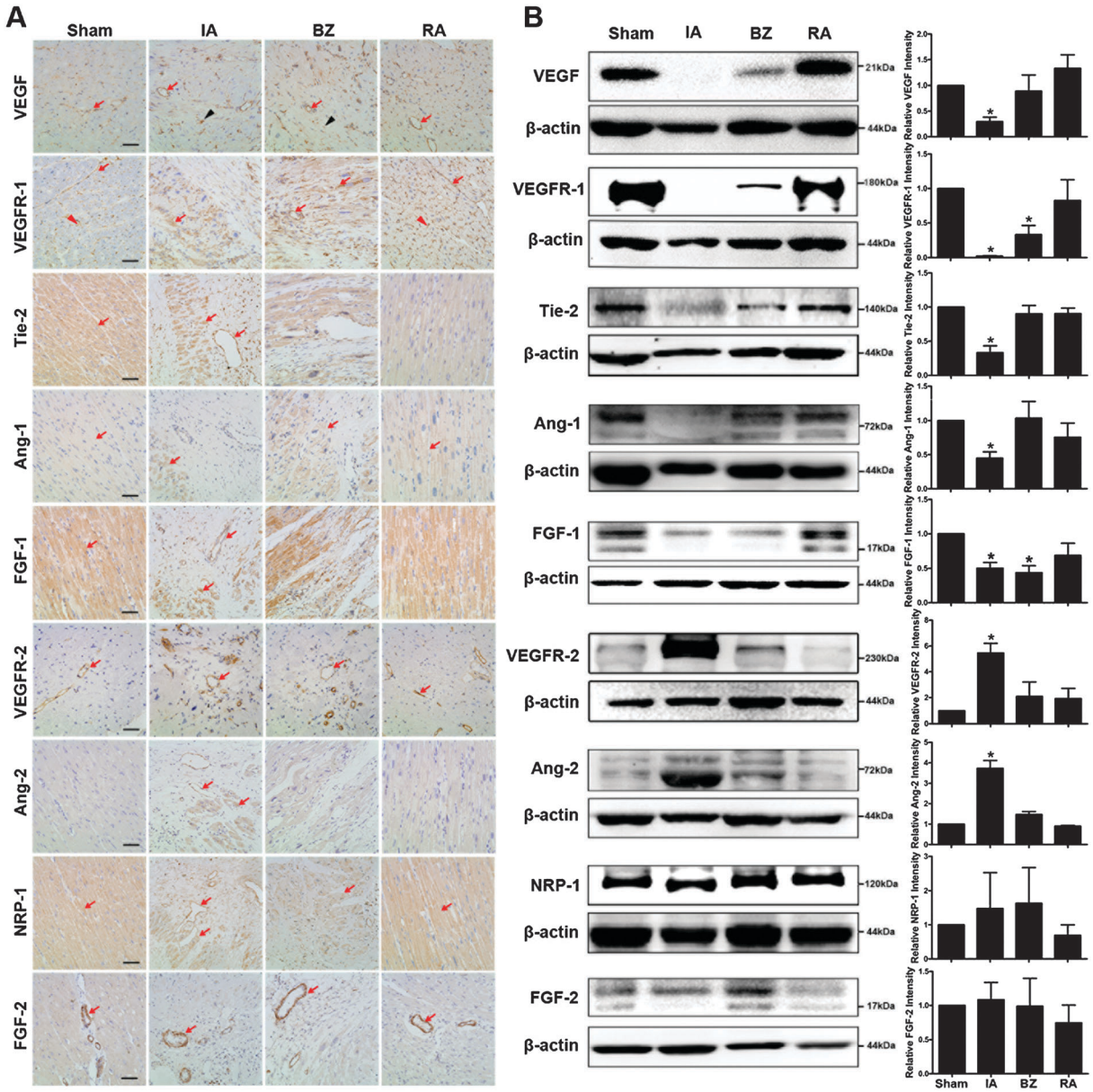

Fig. 2 Changes in angiogenic factors. (A) Immunohistochemical staining of monkey heart sections with antibodies against VEGF, VEGFR-1, VEGFR-2, NRP-1, Ang-1, Ang-2, Tie-2, FGF-1 and FGF-2. VEGF was in endothelial cells (red arrow) and in the extracellular matrix (black arrow head); VEGFR-1 was in the cell membrane (red arrow head) of endothelial cells and cardiomyocytes and in the cytoplasm of cardiomyocytes (red arrow); Tie-2, FGF-1, Ang-2 and NRP-1 were in both cardiomyocytes and endothelial cells (red arrow); Ang-1 was in cardiomyocytes (red arrow); VEGFR-2 was in endothelial cells (red arrow); FGF-2 was in smooth muscle cells (red arrow) of arteries. (B) Protein levels of angiogenic factors determined by Western blot. β-Actin served as a loading control. Blots were analyzed by densitometry using a Quantity One Software. Bar = 50 μm. Sham: sham operated control (n = 3–5); IA: infarct area (n = 3–5); BZ: border zone (n = 3–5); RA: remote area (n = 3–5); *: $P < 0.05$ *versus* sham operated control.

These observations agree with the results obtained from the analysis of ischemic infarct heart of monkeys.

4. Discussion

Both clinical and experimental animal studies demonstrated an increased HIF-1α accumulation in ischemic myocardium. The accumulation of HIF-1α would be expected to cause increased expression of angiogenic genes controlled by this transcription factor, leading to an increase in the capillary density. However, the paradoxical observation is that the increased HIF-1α accumulation is accompanied by depressed capillary density. The data obtained from the present study provide some insights into this paradox.

This study took advantage of the Rhesus monkey model of myocardial ischemia as a surrogate for human patients. The clinical observation that increased HIF-1α accumulation was associated with depressed capillary density in ischemic myocardium was recaptured in the monkey model, verifying this surrogate model for the understanding of the paradox mentioned above.

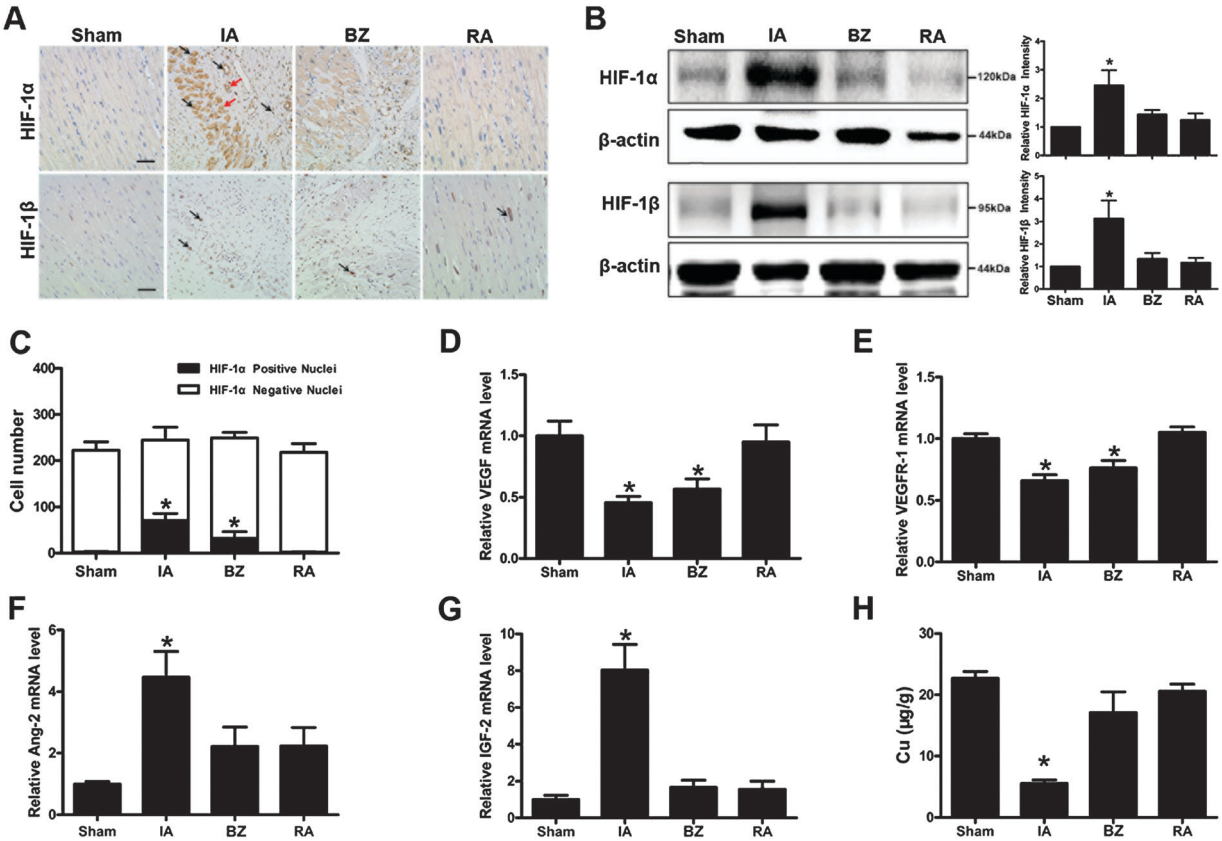

Fig. 3 Changes in HIF-1 proteins, gene expression and copper concentrations. (A) Immunohistochemical staining with antibodies against HIF-1α and HIF-1β. HIF-1α was in the cytoplasm (red arrow) and nuclei (black arrow) of cardiomyocytes and endothelial cells of infarct area; HIF-1β was limited in nuclei of cardiomyocytes (black arrow); (B) protein levels of HIF-1α and HIF-1β determined by Western blot. β-actin served as a loading control. (C) Constituent ratio of HIF-1α positive nuclei cells. (D–G) RT-PCR of VEGF, VEGFR-1, Ang-2 and IGF-2, respectively. (H) Copper concentrations in different regions of the heart. Bar = 50 μm. Sham: sham operated control (n = 3–5); IA: infarct area (n = 3); BZ: border zone (n = 3); RA: remote area (n = 3); *: $P < 0.05$ *versus* sham operated control.

VEGF is critically involved in angiogenesis. Therefore, it is understandable that in the infarct area, the depressed capillary density was associated with reduced protein levels of VEGF. Along with this decrease, the protein level of VEGFR-1 was also depressed. The expression of both VEGF and VEGFR-1 is regulated by HIF-1 transcription factor.[25,26]

On the other hand, the expression of VEGFR-2 was increased. VEGFR-2 is not regulated by HIF-1,[25] but by SP1 and the receptor itself.[27,28] Under hypoxic conditions, SP1 transcription factor is activated.[27] VEGFR-2 is involved in endothelial cell growth and angiogenesis. However, the depressed VEGF level would diminish the activation of this receptor, leading to ultimate depression of angiogenesis.

Other factors whose expressions are regulated by HIF-1, including Tie-2, Ang-1, and FGF-1,[28–31] were also depressed in the infarct area. These factors are all critically involved in angiogenesis. Tie-2 is a receptor of Ang-1 and Ang-2. Ang-1 is expressed by cardiomyocytes and perivascular cells and acts in a paracrine manner on the endothelial cells.[32] In the process of angiogenesis, the binding of Ang-1 to Tie-2 causes phosphorylation of Tie-2 and subsequent vessel stabilization.[32] FGF-1 is involved in the proliferation and migration of endothelial cells in angiogenesis. Administration of FGF-1 has been approached as a therapeutic strategy for limb ischemia.[33] The decreased Tie-2, Ang-1 and FGF-1 in the infarct area would make a contribution to impaired endothelial cell proliferation and depressed angiogenesis.

It was interesting to observe that not all HIF-1 regulated proteins were decreased. Ang-2, whose expression is regulated by HIF-1,[34] was significantly increased in the infarct area. Ang-2 is produced by endothelial cells and acts as an antagonist of Ang-1-mediated Tie-2 activation. The binding of Ang-2 to Tie-2 results in vascular destabilization.[35] The up-regulation of Ang-2 observed in the present study is consistent with previous studies.[31,34] The increased expression of Ang-2 along with decreased Ang-1 would enhance the detrimental effect of Ang-2 under the present experimental conditions.

The other two HIF-1 regulated factors, NRP-1 and FGF-2,[36,37] remained unchanged in the infarct area. NRP-1 is a receptor for VEGF and coordinates the action of VEGFR-2 in the endothelium, playing a role in embryonic and disease-associated angiogenesis.[38] FGF-2 is found to promote endothelial cell proliferation and the physical organization of endothelial cells into tube-like structures.[39] The action of these two factors is to follow endothelial cell growth triggered by VEGF/VEGFR-2 activation.

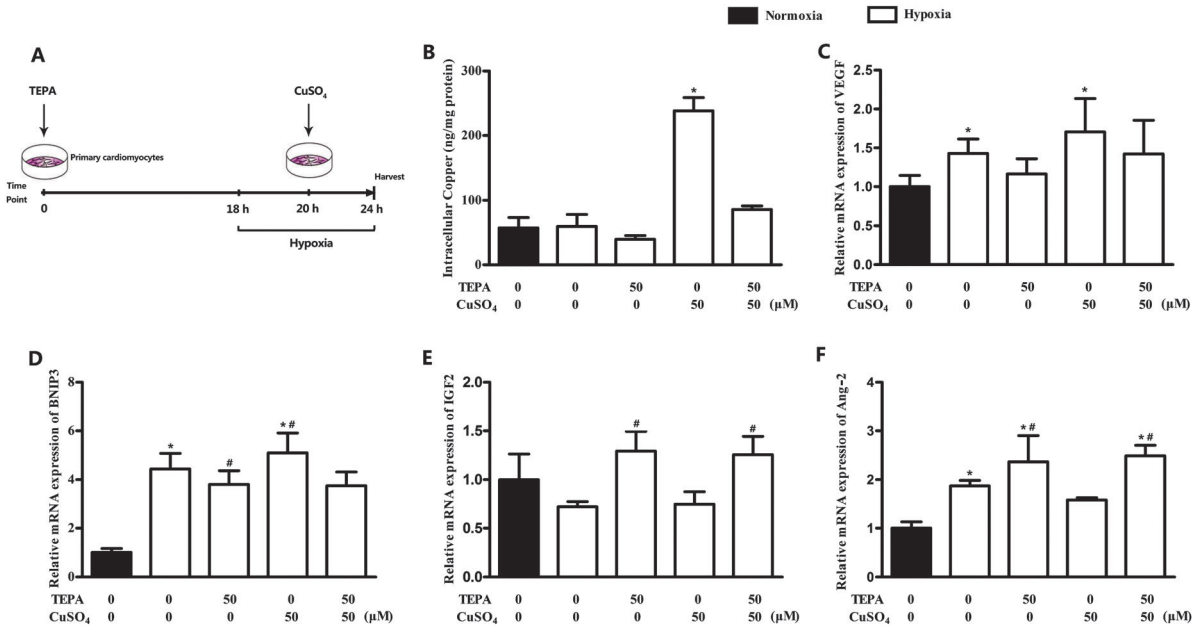

Fig. 4 Effect of copper chelation on HIF-1 activity in primary cardiomyocytes. (A) Outline for the treatment of primary cardiomyocytes. (B) Intracellular copper concentrations after treatment with 50 μM TEPA and/or 50 μM $CuSO_4$ under hypoxic conditions. (C–F) RT-PCR of VEGF, BNIP3, IGF-2 and Ang-2, respectively. *: $P < 0.05$ *versus* normoxia group, #: $P < 0.05$ *versus* hypoxia group.

Therefore, the null reaction of VEGFR-2 would diminish the action of these two factors.

The observations discussed above raise two important questions. First, why was the expression of HIF-1-regulated angiogenic factors depressed while the accumulation of HIF-1α was increased in the infarct area? Second, why was not the expression of all of the HIF-1-regulated genes depressed?

To address the first question, we measured copper concentrations in the ischemic myocardium. Copper is required for HIF-1 transcriptional activity and deprivation of copper from cells by a copper chelator TEPA leads to suppressed expression of VEGF and other genes regulated by HIF-1, but does not affect the accumulation of HIF-1α.[19,21,24,40] However, excessive amount of copper increases the accumulation of HIF-1α by a different mechanism.[41] In the present study, we found that copper concentrations in the infarct area were significantly decreased. Therefore, as a result of depressed copper concentrations, the expression of HIF-1-regulated angiogenic genes was suppressed although the hypoxia-induced accumulation of HIF-1α was increased.

The second question is related to the selectivity of copper regulation of HIF-1 transcriptional activity.[24] Copper is not required for the expression of all HIF-1-regulated genes. Previous studies showed that TEPA treatment suppressed the expression of a group of HIF-1 target genes such as BNIP3 and VEGF, but did not affect other HIF-1 target genes such as IGF-2.[24] In the present study, both the monkey model and primary cultures of cardiomyocytes showed the same result that the expression of IGF-2 was increased but VEGF decreased under the condition of copper deprivation, confirming ① HIF-1 remained active in the infarct area, ② and only copper-dependent expression of HIF-1 regulated genes was suppressed.

It is reasonable to take HIF-1 as a therapeutic target for heart ischemic disease. The beneficial effect of HIF-1 activation on acute myocardial ischemia has been demonstrated by either inhibition of PHD function or transgenic expression of HIF-1α in animal models.[42–45] However, studies have linked the long-term HIF activation to pathogenesis of chronic ischemic cardiomyopathy.[7,46,47] Cardiac-specific VHL deletion in mice caused cardiomyopathy, which was prevented by concomitant deletion of HIF-1α.[46] Long-term cardiac-specific inactivation of both PHD2 and PHD3 and subsequent HIF activation resulted in ischemic cardiomyopathy. These findings raise the issue that long-term HIF activation may not be a safe treatment for chronic ischemic heart disease. The data presented here would help understand this paradox.

Under ischemic conditions, depressed copper concentrations would lead to suppressed expression of some critical factors including VEGF for angiogenesis. On the other hand, the expression of other HIF-1 regulated, but copper-independent, genes would not be affected. This dysregulation of HIF-1-controlled gene expression would eventually lead to detrimental rather than beneficial consequences in the ischemic myocardium. Thus, copper supplementation would be a possible strategy to recover balanced expression of HIF-1-regulated genes as a therapy for chronic myocardial infarction, which will be tested in our future study. In addition, further studies on how copper regulate HIF-1 activity will shed some light on the crosstalk between copper and other metal elements, such as iron, since HIF-1 also controls the expression of genes related to iron metabolism.[48]

In summary, we employed the Rhesus monkey model of myocardial ischemic infarction to recapture clinical observation that HIF-1α accumulation was associated with suppressed angiogenesis and myocardial infarction. The suppressed angiogenesis

was linked to depressed expression of some critical angiogenic factors, such as VEGF and VEGFR-1. The paradox of the increased HIF-1α accumulation and the depressed expression of HIF-1-regulated angiogenic genes would be the result of reduced copper concentrations in the ischemic myocardium. Future work will focus on validation of copper supplementation therapy for angiogenesis to develop clinical application for the treatment of ischemic heart disease.

Author contributions

All authors participated in the design, interpretation of the studies, analysis of the data, and review of the manuscript; WZ, XZ, YX, JC, PH, JZ and HF carried out the experiments; YJK and WZ wrote the manuscript.

Conflict of interest

All authors report no conflict of interest.

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