Roles of p300 and cyclic adenosine monophosphate response element binding protein in high glucose-induced hypoxia-inducible factor 1α inactivation under hypoxic conditions

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ABSTRACT
Aims/Introduction: Given the high prevalence of diabetes and burn injuries worldwide, it is essential to dissect the underlying mechanism of delayed burn wound healing in diabetes patients, especially the high glucose-induced hypoxia-inducible factor 1 (HIF-1)-mediated transcription defects.

Materials and Methods: Human umbilical vein endothelial cells were cultured with low or high concentrations of glucose. HIF-1α-induced vascular endothelial growth factor (VEGF) transcription was measured by luciferase assay. Immunofluorescence staining was carried out to visualize cyclic adenosine monophosphate response element binding protein (CREB) localization. Immunoprecipitation was carried out to characterize the association between HIF-1α/p300/CREB. To test whether p300, CREB or p300+CREB co-overexpression was sufficient to rescue the HIF-1-mediated transcription defect after high glucose exposure, p300, CREB or p300+CREB co-overexpression were engineered, and VEGF expression was quantified. Finally, in vitro angiogenesis assay was carried out to test whether the high glucose-induced angiogenesis defect is resuable by p300 and CREB co-overexpression.

Results: Chronic high glucose treatment resulted in impaired HIF-1α-induced VEGF transcription and CREB exclusion from the nucleus. P300 or CREB overexpression alone cannot rescue high glucose-induced HIF-1α transcription defects. In contrast, co-overexpression of p300 and CREB dramatically ameliorated high glucose-induced impairment of HIF-1α-mediated VEGF transcription, as well as in vitro angiogenesis. Finally, we showed that co-overexpression of p300 and CREB rectifies the dissociation of HIF-1α-p300-CREB protein complex in chronic high glucose-treated cells.

Conclusion: Both p300 and CREB are required for the function integrity of HIF-1α transcription machinery and subsequent angiogenesis, suggesting future studies to improve burn wound healing might be directed to optimization of the interaction between p300, CREB and HIF-1α.

INTRODUCTION
There have been abundant studies about burn injuries from electric heating pads, foot spas and water baths, and these burn injuries have been considered as important hazards, particularly to diabetic patients¹. With the rapidly increasing prevalence of diabetes worldwide, evaluating the effect of hyperglycemia on burn wound healing seems to be critical in order to reduce the type 2 diabetes mellitus-related social cost and patient suffering.
Delayed wound healing of burn injuries in diabetic patients, which often results in extended hospital stay and therapeutic cost, has been well documented by numerous reports, and confirmed in different animal models. However, the mechanisms responsible for delayed burn wound healing in diabetic patients are far less well understood.

Wound healing is a dynamic, interactive process involving coagulation, inflammation, tissue formation and remodeling. Neovascularization, which is an essential component of wound healing, is triggered by the hypoxia-induced expression of critical angiogenic factors, such as vascular endothelial growth factor (VEGF). Recently, Sokho Kim et al. reported that VEGF expression decreases in burn-injured skin of diabetic mice. It has also been reported by different research groups that hyperglycemia might inhibit VEGF production from endothelial cells.

It is well-known that expression of VEGF is controlled by hypoxia-inducible factor 1 (HIF-1) at the transcriptional level. HIF-1 functions as a master regulator of adaptive responses to hypoxia/ischemia, which often exist during wound healing. The core HIF-1 transcription machinery is composed of a highly-regulated α-subunit and a constitutively expressed β-subunit. Co-factors including p300, cyclic adenosine monophosphate response element binding protein (CREB)-binding protein and CREB are also essential for optimal functions of HIF-1 transcription activity.

Recent studies showed that the functions of HIF-1 transcription machinery in multiple types of cells might be affected by environmental chronic high glucose in vitro and in vivo. For example, Catrina et al. showed that hyperglycemia impairs hypoxia-induced HIF-1 accumulation in human dermal fibroblasts. Bento et al. reported that HIF-1α expression level in ARPE-19 cells (an immortal retinal pigment epithelial cell line) decreased when the cells were treated with high glucose for 10 days. Some studies came to different conclusions that the effects of high glucose on tissues and cells might be complex and tissue-specific. For example, Li et al. reported that the expression levels of HIF-1α and VEGF of rat retinal Müller cells increased after high-glucose treatment.

Other than regulating HIF-1 protein expression, several studies showed that high glucose can also affect the transcription activity of HIF-1 complex. Katavetin et al. showed that high glucose reduces the hypoxia-response element (HRE) pathway activation both in immortalized rat proximal tubular cells and in ischemic kidney of diabetic rats. Thangarajah et al. found that high glucose induces impairment of HIF-1 transcription activity when p300 (one of the co-factors of HIF-1 complex) fails to bind to HIF-1α as a result of covalent modification of p300 by the dicarbonyl metabolite methylglyoxal. More importantly, Duscher et al. showed that deferoxamine can improve neovascularization and healing of diabetic ulcers when HIF-1α transcription activity was rescued.

Another co-factor of HIF-1 complex, CREB, might also be important in HIF-1 transcription machinery. CREB was first reported in 1987 as a transcription governor for the somatostatin gene. Wu et al. showed that CREB is crucial for promoting prostate cancer metastasis by inducing VEGF transcription through HIF-1-dependent mechanism. In our current study, we tried to identify the role of CREB/p300 in high glucose-induced impairment of HIF-1 transcription activation.

**MATERIALS AND METHODS**

**Cell culture**

Human umbilical vein endothelial cells (HUVECs) were purchased from ATCC (Manassas, Virginia, USA) and maintained in vascular cell basal medium together with endothelial cell growth kit (from ATCC). For high-glucose treatment, D-glucose was added into regular medium to make 10 or 25 mmol/L in total. For normoxia culture, cells were maintained at 37°C in a humidified chamber supplied with 5% CO2. For hypoxia culture, a low-oxygen incubator was used in which oxygen concentration was kept at 5%. Cells were cultured under normoxic conditions for 4 weeks in medium supplied with different concentrations of glucose (5.5, 10 or 25 mmol/L). After that, if without any specific indication, cells were cultured under hypoxic conditions for 24 h before they were used in the following experiments (Figure S1). Low glucose (LG) and high glucose (HG) represents 5.5 and 25 mmol/L glucose, respectively.

**Plasmid construction**

The deoxyribonucleic acid (DNA) fragment encoding human p300 protein was amplified from another plasmid purchased from Addgene (#23252; Cambridge, Massachusetts, USA), and inserted into pCDH-EF1-T2A-puro vector to make pCDH-EF1-p300-T2A-puro plasmid. The DNA fragment that encodes CREB protein was amplified from complement DNA of HUVECs by nested polymerase chain reaction (PCR). The outer primers are: 5′-GGGAGAAAATGACTCCACACCCGAAGA and 5′-GTCCTT CAGGGGTGCGCCCAAGC. The inner primers are: 5′-GC CGGATATCATGACCATTGAGATCTGAGG and 5′-GGCG GGATCCATATGACTTATGTGGGCAGT.

**Lentiviral production and infection**

Lentiviral particles were prepared following the manufacturer’s manual (System Biosciences, Palo Alto, California, USA). HUVECs were plated, infected with virus and drug selected with puromycin or G418. All the gene transduction and drug selection processes were carried out in HUVECs under regular culture condition. After the transduced genes were stably expressed, the cells were cultured under the appropriate conditions described in Figure S1.

**Western blot**

Protein samples were separated in 8% (or 12%) sodium dodecyl sulfate polyacrylamide gel electrophoresis gels and transferred onto polyvinylidene difluoride membranes. The primary antibodies used are: anti-HIF-1α antibody (1:1,000; Abcam,
Cambridge, Massachusetts, USA), anti-β-actin antibody (1:25,000; Abcam), anti-VEGF antibody (1:1,000; Abcam), anti-p300 antibody (1:1,000; Santa Cruz, Santa Cruz, California, USA), anti-CREB antibody (1:500; Santa Cruz) and anti-Histone H3 antibody (1:2,000; Millipore, Billerica, Massachusetts, USA).

Quantitative reverse transcription PCR
Treated cells were collected and ribonucleic acid (RNA) was extracted using the Trizol method. The quantitative reverse transcription PCR (qRT–PCR) reactions were carried out using the One-Step RT–PCR kit (TaqMan, Waltham, Massachusetts, USA) with specific probes for HIF-1 (Hs02957097), VEGF (Hs00900055) and glyceraldehyde-3-phosphate dehydrogenase (Hs0420697).

Luciferase activity assay
The HRE containing the 5′-flanking sequence of the VEGF gene (~1,014 to –1, relative to the transcription start site) was amplified by PCR reaction using the genomic DNA extracted from HUVECs. The primers used here are: 5′-GCGCGCTAGCCCTGCTCCCTTTGGGTTTTG-3′ and 5′-GCGCTCGAGGGCTACCAGCGAGCTTTT-3′. A luciferase reporter plasmid containing the above fragment was constructed. Briefly, pGL3-basic plasmid (Promega, Madison, WI, USA) was used as the vector. The fragment was inserted to produce pGL3-HRE plasmid. Then, 2 μg of pGL3-HRE plus 0.5 μg of pSV-β-galactosidase was transfected into 5 × 10^6 HUVECs by electroporation. The remaining cells were plated in dish for 24 h. The luciferase activity of the cell lysates was measured using Firefly Luciferase Reporter Gene Assay Kit (Beyotime Biotechnology, Nanjing, China), and normalized against the β-galactosidase activity of the same group.

Immunoprecipitation
Cells were collected and washed with phosphate-buffered saline twice. After that, the cell pellet was resuspended in buffer A (10 mmol/L HEPES-KOH, 10 mmol/L KCl, 1.5 mmol/L MgCl2, 0.34 mol/L sucrose, 10% glycerol, Triton-X 100 to 0.1%, pH 7.5), incubated in ice and then centrifuged. The supernatant was transferred to new tubes and the protein concentrations were measured. The primary antibody against the targeted protein was added to nucleus lysate and was incubated with agitation for 3 h at 4°C. Subsequently, protein A/G conjugated beads were added, and the mixture was incubated with agitation for 1 h. After a gentle wash, the proteins were obtained by boiling the beads in sodium dodecyl sulfate sample buffer. Finally, the proteins were quantified with Western blot. Proteinase inhibitors were added to all the buffers used in this protocol.

Immunofluorescence staining
The cells were mounted on coverslips. The cells were fixed and permeabilized. The slides were incubated with phosphate-buffered saline containing 4% of bovine serum albumin for 30 min. Subsequently, primary antibody solution was added onto the slides. After incubation for 1 h, the secondary antibody was added onto the slides for 30 min. Nuclei were stained with DAPI solution. Slides were visualized under a fluorescent microscope (NIKON ECLIPSE E600; Nikon, Melville, New York, USA), and pictures were taken at ×400.

In vitro angiogenesis assay
In vitro angiogenesis assay was carried out by using BD Matrigel Matrix (BD Bioscience, San Jose, California, USA). Briefly, matrix was added to each well of a 96-well plate, and incubated at 37°C for 1 h to solidify. A suspension of HUVECs (30,000 cells) in growth medium were seeded into each well. After 18 h of incubation at 37°C, images were captured and tube formation was scored as follows: a three-branch point event was scored as one. Less branching indicated a decreased level of angiogenesis.

RESULTS

Chronic high-glucose treatment impairs HIF-1α-induced VEGF transcription
To identify the impacts of chronic high-glucose exposure to HIF-1α-induced VEGF expression in hypoxic conditions that mimics the scenarios for burn wound healing in diabetic patients, HUVECs were first cultured under normoxic conditions in medium with 5.5, 10 and 25 mmol/L D-glucose for 4 weeks, and then cultured under hypoxic conditions for 24 h (Figure S1). Cell lysates were collected for quantification of HIF-1α and VEGF protein expression. Results showed that VEGF protein was significantly downregulated at both high-glucose concentrations, whereas HIF-1α remained largely unchanged after chronic high-glucose exposure (Figure 1a). Consistently, qRT–PCR data showed that chronic glucose exposure impaired full induction of hypoxia-stimulated VEGF messenger RNA (mRNA) transcription in a concentration-dependent manner, whereas hypoxia-induced activation of HIF-1α mRNA transcription remained unchanged (Figure 1b,c). Given the established role of HIF-1α as a transcriptional governor for VEGF, our data argue that the full presence of HIF-1α is not sufficient for VEGF induction under hypoxic conditions. As expected, data showed HIF-1α-mediated VEGF transcription under hypoxic conditions is significantly downregulated after chronic glucose exposure as manifested by decreased luciferase activities (Figure 1d), indicating a defect in HIF-1α transcription machinery that governs VEGF response to hypoxia.

P300 overexpression cannot rescue impairment of HIF-1α-mediated VEGF transcription responding to hypoxia after chronic glucose exposure
As the next step, we sought to characterize the defects that result in the impaired HIF-1α-mediated VEGF transcription...
responding to hypoxia after chronic glucose exposure. Given the essential roles of p300 in HIF-1 transcription machinery, the affinity between p300 and HIF-1α was first measured through immunoprecipitation. As shown in Figure 2a, chronic high-glucose exposure resulted in a significantly decreased association between p300 and HIF-1α under hypoxic conditions (Figure 2a). An interesting question follows: what if p300 protein is overexpressed (Figure 2b), is it sufficient to rectify the binding between HIF-1α and p300? Similar to Figure 2a, chronic high-glucose exposure resulted in decreased p300-HIF-1α binding despite the existence of overexpressed p300, showing that p300 overexpression alone is not sufficient to rescue impairment of HIF-1-mediated VEGF transcription (Figure 2c). Consistently, we found neither VEGF mRNA expression nor HIF-1-mediated VEGF transcription activity can be rescued by p300 overexpression (Figure 2d,e).

Chronic high-glucose exposure results in dissociation of HIF-1α and CREB
Given the previous finding showing p300 itself is not sufficient to drive HIF-1 transcription machinery, it remains intriguing to identify a molecule that is modifiable and exerts a pivotal regulatory role on HIF-1α-mediated transcription activities. In light of the notion that CREB has been implicated in HIF-1α transcriptional complex, we sought to identify the roles of CREB in chronic high-glucose exposure-induced impairment of HIF-1α-mediated transcription. As shown in Figure 3a, chronic high-glucose exposure resulted in a significant decrease of CREB signaling in the nucleus, indicating the lack of CREB protein at the potential transcription sites, which is consistent with subsequent western blot data showing a decreased CREB level only in the nucleus, but not in the cytoplasm, after chronic high-glucose exposure (Figure 3b,c). As several previous studies reported that phosphorylation of CREB is required for its
translocation from the cytoplasm into the nucleus to exert its transcriptional regulatory activities, we further tested the levels of phosphorylated CREB in these cells. As expected, phosphorylated CREB also significantly decreased in the nuclei of high-glucose treated HUVECs (Figure 3d). Excitingly, we also found that the association between CREB and HIF-1α was significantly decreased after chronic high-glucose exposure, which suggests that the dissociation between HIF-1α with CREB could
be one of the key defects responsible for impaired HIF-1α transcriptional activities. As the next step, we tried to overexpress CREB in HUVECs, and found that CREB overexpression is not sufficient to rescue the impaired HIF-1α transcriptional activities (Figure S2).

**Co-overexpression of p300 and CREB rescues HIF-1α transcriptional activities and angiogenesis after chronic high-glucose exposure**

Given that either p300 or CREB alone is not sufficient to rescue impaired HIF-1α-mediated transcription, it seems intriguing to identify whether simultaneous overexpression of p300 and CREB might restore the function of HIF-1 transcription machinery. To do this, we first engineered p300 and CREB co-overexpression in HUVECs (Figure 4a). Initially, we found that CREB overexpression restored its protein localization in the nucleus (Figure 4b). More interestingly, p300 and CREB co-overexpression resulted in significantly increased binding of p300 and CREB to HIF-1α after chronic high-glucose exposure (Figure 4c). In addition, we carried out a similar experiment in cells cultured with regular medium (5.5 mmol/L of glucose), and found that p300 and CREB co-overexpression had no obvious effect on the protein complex (Figure S3). More importantly, co-overexpression of p300 and CREB leads to restoration of VEGF mRNA transcription and protein expression, which is initially impaired with chronic high-glucose exposure (Figure 4d,e). Not surprisingly, we were also able to observe that restoration of *in vitro* angiogenesis after p300 and CREB co-overexpression, which might be associated with restored VEGF expression (Figure 5).

**DISCUSSION**

Although there have been a significant number of studies that tried to elucidate the underlying pathways involved in burn wound healing, very few have been translated to effective therapeutic regimens. In a recent review of the advances in burn wound care, Rose et al.24 showed that thermal wound produgs targeting different inflammatory signaling molecules, such as transforming growth factor-β, platelet-derived growth factor and interleukin-10, were discontinued at different stages of clinical development. More recently, cell or tissue engineering-based experimental therapies have emerged with the advances of modern stem cell or bioengineering technology. These studies were relatively focused on scar-free wound healing, and there is a relative lack of evidence for the development of delayed healing or even non-healing of burn wound injuries in diabetes patients25-27. The limited success of current studies, especially for diabetic burn wound patients, calls for the development of a novel therapeutic regimen by targeting new candidate molecules.
Emerging evidence has shown that HIF-1α and VEGF play pivotal roles in different stages of burn wound healing. The interplay between HIF-1 and pro-angiogenic factors is a cornerstone for almost every step during capillary formation under hypoxia. Among all the pro-angiogenic factors, VEGF is the master regulator of angiogenesis, given the fact that angiogenesis initiated by hypoxia or HIF-1 is mostly VEGF-dependent. Basically, hypoxia-induced HIF-1 accumulation directly targets and upregulates VEGF transcription, which in turn binds to its receptors, enhances other pro-angiogenic factor expression, such as phosphatidylinositol glycan anchor biosynthesis class F or fibroblast growth factors, and ultimately stimulates capillary sprouting.

Based on these notions, the present study was initially directed to dissect the key defects in inducing VEGF expression involved in hypoxia. The first question is that whether chronic high-glucose exposure impairs hypoxia-induced HIF-1α expression, which is considered to be the initial event in the signaling axis. As shown in Figure 1a and b, the results showed that hypoxia-induced HIF-1α accumulation directly targets and upregulates VEGF transcription, which in turn binds to its receptors, enhances other pro-angiogenic factor expression, such as phosphatidylinositol glycan anchor biosynthesis class F or fibroblast growth factors, and ultimately stimulates capillary sprouting.

Based on these notions, the present study was initially directed to dissect the key defects in inducing VEGF expression involved in hypoxia. The first question is that whether chronic high-glucose exposure impairs hypoxia-induced HIF-1α expression, which is considered to be the initial event in the signaling axis. As shown in Figure 1a and b, the results showed that hypoxia-induced HIF-1α expression itself is not affected by chronic high-glucose exposure. Instead, we found that hypoxia-induced VEGF expression is significantly impaired after chronic high-glucose treatment. If HIF-1α, like in most other studies, serves as the master regulator of VEGF, our data strongly argue that the defects responsible for hyperglycemia-induced impairment of VEGF expression and subsequent angiogenesis originated from HIF-1-mediated transcription, but regulation of HIF-1α expression seems not to be pivotal in this scenario. As a logic chasing down approach, we felt it imperative to identify whether HIF-1 transcription machinery is implicated in this defect. As shown in Figure 1c and d, the data strongly suggested that HIF-1-mediated transcription of VEGF mRNA is significantly impaired, which seems to be a reasonable explanation for the defects we observed above.

The next key question seems to be how HIF-1α transcriptional activities are impaired. In many scenarios, HIF-1α binds to a significant number of partner molecules and forms transcription activation complexes. Among those partners, p300/CBP complex has been frequently implicated in HIF-1α-driving scenarios, which include angiogenesis and malignancy. CBP/p300 itself functions as a potent histone acetyltransferase, which governs chromatin structure remodeling and increases its accessibility to other protein regulators.

The association/dissociation dynamics between p300 and HIF-1α are essential for normoxic/hypoxic regulation. When oxygen tension is normal, HIF-1α is rapidly degraded through a series of signaling events that include hydroxylation of specific proline residues, ubiquitination and proteasomal degradation of the monomer. Under hypoxic conditions, HIF-1α dimerizes with the HIF-1β subunit in the nucleus, which facilitates the recruitment of p300

Figure 5 | The effects of co-overexpression of p300 and cyclic adenosine monophosphate response element binding protein (CREB) on in vitro angiogenesis of human umbilical vein endothelial cells with high-glucose (HG; 25 mmol/L) treatment. (a) The representative pictures of in vitro angiogenesis assay on human umbilical vein endothelial cells with indicated treatment. (b) Quantification of (a) (mean ± standard deviation, n = 3). IgG, immunoglobulin G.

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In contrast, there are fewer data available for the interaction between CREB and HIF-1α. The present data strongly argue that sufficient existence of CREB protein in the nucleus is pivotal for HIF-1α-induced VEGF expression. Classically, CREB proteins are phosphorylated by a diverse panoply of kinases, such as protein kinase A and Ca2+/calmodulin-dependent protein kinases at residue serine 133. On phosphorylation, CREB transfers from cytoplasm into the nucleus and forms a complex with transcriptional coactivators and binds to 5′ upstream of the cyclic adenosine monophosphate response elements promoter. Commonly, CREB proteins dimerize with each other through interactions between the hydrophobic leucine residue located at the inner edge of the alpha helix that makes up a leucine zipper motif. A recent study showed that CREB binds to HIF-1 response elements within the promoter of plasminogen activator inhibitor-1 promoter, and thereby regulates the glucagon action. More interestingly, Wu et al. suggested another model for binding of CREB with HIF-1α in transcription induction based on their own data and several other studies. They suggested that CBP/p300 functions as a physical bridge between CREB and HIF-1α to form the functional protein complex. The present data argue that CREB localization to the nucleus, together with the full presence of p300, is critical to drive HIF-1α-mediated VEGF induction, which is essential for subsequent angiogenesis and wound healing. However, we should admit that whether this action is mediated by direct binding between HIF-1α, p300 and CREB remains uncertain. In particular, the physical binding between HIF-1α and p300 seems to be more likely given the current body of literature knowledge, whereas CREB might exert its role in HIF-1α transcription action machinery through binding to CBP/p300 complex.

In short, our data strongly argues that prolonged high-glucose exposure (which mimics burn wound healing for diabetes patients) impairs the functional integrity of HIF-1α transcription machinery as manifested by impairment of VEGF expression, which is thought to be partially mediated by CREB dislocalization from the nucleus. Interestingly, simultaneous overexpression of p300 and CREB greatly restores the HIF-1α-mediated VEGF induction. Although we agree that the pathophysiological events underlying delayed burn wound healing in diabetes patients are complicated, and the in vitro experiments might not fully mimic the real scenarios in vivo, the present results suggested that future studies to improve burn wound healing for diabetes patients might be directed to optimization of the interaction between p300, CREB and HIF-1α as a transcription complex.

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DISCLOSURE

The authors declare no conflict of interest.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article:

**Figure S1** | The basic scheme of cell culture in this current research.

**Figure S2** | Cyclic adenosine monophosphate response element binding protein (CREB) over-expression cannot rescue high glucose-induced impairment in hypoxia-inducible factor 1 (HIF-1)-mediated transcription.

**Figure S3** | The effect of p300 and cyclic adenosine monophosphate response element binding protein (CREB) co-overexpression on cells cultured in regular medium.