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Intracellular acidosis via activation of Akt-Girdin signaling promotes post ischemic angiogenesis during hyperglycemia

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ABSTRACT

Aims: The impaired angiogenesis is the major cause of diabetic delayed wound healing. The molecular insight remains unknown. Previous study has shown that high glucose (HG) activates Na^+/H^+ exchanger 1 (NHE1) and induces intracellular alkalinization, resulting in endothelial dysfunction. The aim of this study is to investigate whether activation of NHE1 in endothelial cells by HG damages the angiogenesis in vitro and in vivo.
Methods and results: We used western blot to detect the phosphorylations of both Akt and Girdin, and pH-sensitive BCECF fluorescence to assay NHE1 activity and pHi value, respectively. The angiogenesis was evaluated by measuring the number of tube formation in vitro, and blood perfusion by laser doppler and neovascularization by staining CD31 in vivo. Our results indicated that induction of intracellular acidosis (IA) increased p-Akt and p-Girdin in human umbilical vein endothelial cells (HUVEC). HG activated NHE1 and increased pHi value in a time-dependent manner, associated with the decreased phosphorylations of both Akt and Gridin, while inhibition of NHE1 by amiloride abolished the HG-induced reductions of p-Akt and p-Girdin. However, silence of Akt by siRNA transfection or pharmacological inhibitors (wortmannin and LY294002) bypassed IA-induced Girdin phosphorylation. Overexpression of constitutively active Akt abolished HG-reduced Girdin phosphorylation. In addition, upregulation of Akt or inhibition of NHE1 remarkably attenuated HG-impaired tube formation in HUVEC. In vivo study revealed that amiloride dramatically rescued hyperglycemia-delayed blood perfusion and neovascularization by augmenting ischemia-induced angiogenesis.
Conclusion: IA promotes ischemia-induced angiogenesis via Akt-dependent Girdin phosphorylation in diabetic mice.

1. Introduction

Cardiovascular complications are the leading cause of morbidity and mortality in patients with diabetes mellitus, which is characterized as endothelial dysfunction [1–3]. In particular, diabetes is associated with a poor outcome after vascular occlusion partially attributed to the impaired neovascularization [4]. Angiogenesis plays a critical role in the neovascularization, which was impaired in diabetes [5,6]. The molecular mechanism responsible for delayed-angiogenesis in diabetes remains largely unknown, which limits effective therapeutic interventions in clinic investigations.

All eukaryotes contain an intracellular fluid in which pH value is known as the intracellular pH (pHi) value. There are numerous mechanisms that can cause the alteration of pHi value, including metabolic acid production, leakage of acid across plasma and organelle membranes and membrane transport processes [7]. The pHi value regulates many cellular functions such as metabolism and cell proliferation. As a consequence, the regulation of pHi value within narrow limits is critical for maintaining the normal functions in cells.

Abbreviations: BCECF, 2-carboxyethyl-5(6)-carboxyfluorescein; CA, constitutively active; FCS, fetal calf serum; GFP, green fluorescence protein; HG, high glucose; HUVEC, human umbilicus vein endothelial cells; IA, intracellular acidosis; NG, normal glucose; NHE1, Na^+/H^+ exchanger 1; pHi, intracellular pH; siRNA, small interference RNA; STZ, Streptozotocin.

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The Na^+/H^+ exchanger 1 (NHE1) is expressed ubiquitously in the plasma membrane of mammalian cells and exchanges intracellular H^+ for extracellular Na^+ to regulate pHi value. Several pathological factors, such as advanced glycation end products and TNF alpha, activate NHE1 to induce intracellular alkalinization and result in cell dysfunctions [8,9]. Inhibition of NHE1 via intracellular acidosis has been shown to produce cardioprotective effects against diabetic nephropathy and hypertension-induced cardiomyopathy [10]. Our previous study has also indicated that cariporide, a selective NHE1 inhibitor, reversed endothelial dysfunction induced by high glucose (HG) and inhibited the adhesion of monocytes to endothelial cells [11–13], suggesting the important role of NHE1 in vascular complications in diabetes.

NHE1 has been demonstrated to be involved in angiogenesis [14]. However, the molecular mechanisms by which NHE1 activation promotes ischemia-induced angiogenesis in diabetes remains poorly elucidated. Thus, the aim of the present study is to establish the molecular signaling insights by which NHE1 activation delays angiogenesis in response to hindlimb ischemia. Our results revealed that inhibition of hyperglycemia-activated NHE1 by amiloride via induction of intracellular acidosis (IA) enhances ischemia-induced angiogenesis via Akt-dependent Girdin phosphorylation in diabetic mice.

2. Methods and materials

An expanded Methods and materials section is available in the Online Supplement.

2.1. Animals

Male wild-type (C57BL6) mice, 8–12 weeks of age, 20–25 g, were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were housed in temperature-controlled cages with a 12-h light-dark cycle and given free access to water and normal chows. All animal procedures conform to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes or the NIH guidelines. The animal protocol was reviewed and approved by the University of Shandong, Animal Care and Use Committee.

2.2. Induction of IA by using the NH_4Cl pulse method

As described in previous study [15], cultured human umbilical vein endothelial cells (HUVEC) were initially bathed in bicarbonate-free Tyrode solution consisting of 137 mM NaCl, 5.4 mM KCl, 1.0 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM HEPES (pH 7.4) and 10 mM glucose for 90 min in room air at 37 °C. Then the IA was induced by exposure to bicarbonate-free Tyrode solution containing 30 mM NH_4Cl for 5 min, followed by washout of NH_4Cl with Tyrode solution without Na^+ or containing amiloride.

2.3. Measurement of pHi value

The pH_i value was measured in HUVEC by using the pH-sensitive fluorescent dye BCECF as previously described [16].

2.4. Establishment of mouse hind limb ischemia model

We used a mouse model of revascularization as described previously [6]. For establishing the severe ischemia model, the entire left femoral artery and vein in control or diabetic mice was ligated under anaesthesia with an injection of 100 mg/kg sodium pentobarbital. Laser Doppler Blood Flowmetry (LDBF; Lisca AB, Linkoping, Sweden) was used to assess the extent of hindlimb blood flow in mice. After left femoral artery ligature, mice showing <70% of blood flow reduction were excluded from this study. On the 28th postoperative day, we performed LDBF analysis over the legs and feet.

2.5. Capillary density analysis

As described previously [6], capillary density in adductor muscle was analyzed to obtain specific evidence of vascularity at the level of microcirculation by immunohistochemical staining with CD31 monoclonal antibody.

2.6. Statistical analysis

Data are reported as mean ± SEM. All data were analyzed with a 1-way ANOVA followed by Bonferroni *post-hoc* analyses, except for those data obtained from the concentration/time courses, which were analyzed with repeated-measures ANOVA. A two-sided $P < 0.05$ was considered as significant.

3. Results

3.1. IA activates Akt by increasing serine 473 phosphorylation in endothelial cells

Cell survival and function are regulated via the close control of pHi value by NHE1 [17]. We first hypothesized that Akt, which is a key mediator of tyrosine kinase receptor signaling for endothelial cell proliferation and growth, is regulated by the decrease of pHi value. IA was induced by NH_4Cl pulse plus inhibition of NHE1 by Na^+-free buffer as shown in Fig. 1A. The physiologic pHi value is 7.21 in HUVEC balanced with HCO_3^--free Tyrode solution for 90 s. Incubation of HUVEC with 30 mM NH_4Cl for 5 s caused a little increase of pHi value. Washout of NH_4Cl with Na^+-free Tyrode solution but not Tyrode solution remarkably decreased pHi value. In addition, Na^+-free Tyrode solution time-dependently decreased pHi value (Fig. 1B). This indicated that NH_4Cl pulse is effective to induce IA in HUVEC, consistent with previous reports [11].

The phosphorylation of Akt at serine 473 (p-Akt), which represents Akt activity [18], was detected by Western blot. In Fig. 1C, Na^+-free Tyrode solution but not Tyrode solution dramatically increased Akt ser473 phosphorylation. As well as the alteration of pHi value, Na^+-free Tyrode solution increased Akt ser473 phosphorylation (Fig. 1D) and its activity (Fig. 1E) in a time-dependent manner. The effects of Na^+-free Tyrode solution on Akt ser473 phosphorylation were imitated by inhibition of NHE1 by amiloride (Fig. 1F), further supporting that IA increases Akt phosphorylation and activity in endothelial cells.

3.2. IA increases Girdin serine phosphorylation in endothelial cells

The phosphorylated Girdin detaches from the plasma membrane with actin filaments, leading to the rearrangement of the actin cytoskeleton and cell migration [19]. We then investigated whether IA activates Girdin by increasing serine phosphorylation. Due to the lack of commercial anti-phospho-Girdin antibody, we detected Girdin serine phosphorylation by incubating total cell lysates with total anti-Girdin antibody followed staining with total anti-phospho-serine antibody. This method is used for all Girdin serine phosphorylations (p-Girdin) in this study. As depicted in Fig. 2A, it is Na^+-free Tyrode solution (Lane c′) but not Tyrode solution (Lane c) increased total Girdin serine phosphorylations, similar to Akt ser473 phosphorylation (Fig. 1C). The time-dependent Girdin phosphorylation was also determined in Fig. 2B. Na^+-free Tyrode solution time-dependently increased Girdin phosphorylation as indicated times. Amiloride mirrored the time-dependent effects of Na^+-free Tyrode solution on Girdin phosphorylation (Fig. 2C) as well as Akt phosphorylation (Fig. 1F). Taking these data together, it indicates that the decrease of pHi value increases Girdin phosphorylation in endothelial cells.

3.3. Akt is required for IA-induced Girdin phosphorylation in HUVEC

Previous studies have shown that Girdin is directly phosphorylated by Akt [20]. We then examined whether Akt is essential for IA-induced Girdin phosphorylation in HUVEC. As shown in Fig. 2D, HUVEC were incubated with Akt inhibitor wortmannin (1 μM) or LY294002 (1 μM) throughout the whole time of IA (from Tyrode to NH_4Cl to amiloride Tyrode). Both wortmannin and LY294002 blocked the increase of Girdin phosphorylation induced by IA.

To further confirm whether the effect of wortmannin or LY294002 is Akt-specific, we used specific-target siRNA transfection to silence Akt protein expression. HUVEC were transfected with control siRNA and Akt siRNA for 48 h, and then IA was induced by NH_4Cl pulse method. As shown in Fig. 2E, in control and control siRNA-transfected HUVEC, IA increased Girdin phosphorylation by 2–3 folds. However, IA failed to increase Girdin phosphorylation in HUVEC transfected with Akt

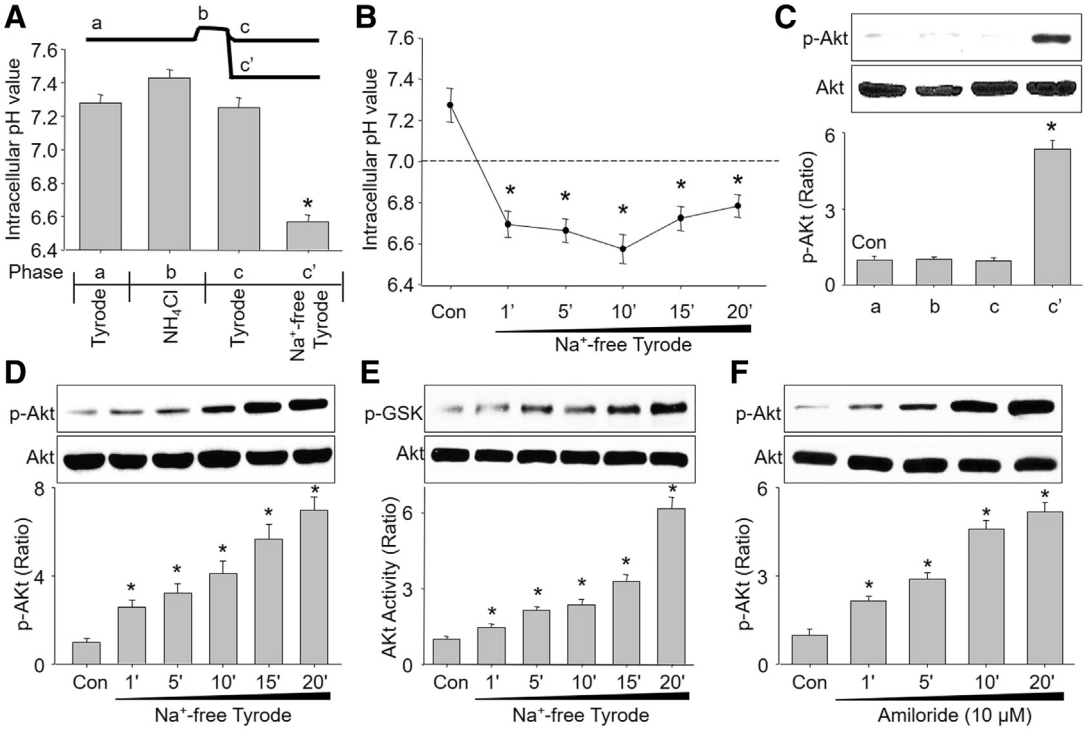

Fig. 1. IA increases Akt phosphorylation at serine 473 in HUVEC. (A) The IA in HUVEC was induced by washout of NH_4Cl with Na^+-free Tyrode solution. pHi value was detected by BCECF fluorescence. a, HCO_3^--free Tyrode solution; b, HCO_3^--free Tyrode solution plus NH_4Cl; c, HCO_3^--free Tyrode solution plus NH_4Cl plus HCO_3^--free Tyrode solution; c′, HCO_3^--free Tyrode solution plus NH_4Cl plus with Na^+-free Tyrode solution. N is 5 in each group. $^*P < 0.05$ vs. a, b, or c. (B) IA was induced by washout of NH_4Cl with Na^+-free Tyrode solution as indicated times in HUVEC. N is 5 in each group. $^*P < 0.05$ vs. control. (C) Phosphorylation of Akt at ser473 was detected by Western blot as analyzed in A. This blot shown is a representative of 5 independent experiments. $^*P < 0.05$ vs. a, b, or c. (D and E) IA was induced by washout of NH_4Cl with Na^+-free Tyrode solution as indicated times in HUVEC. Phosphorylation (D) and activity (E) of Akt were detected by Western blot and commercial kit, respectively. N is 5 in each group. $^*P < 0.05$ vs. control. (F) IA was induced by washout of NH_4Cl with Tyrode solution containing 10 μM amiloride as indicated times. Akt phosphorylation was detected by Western blot in total cell lysates. This blot shown is a representative blot of 5 independent experiments. $^*P < 0.05$ vs. control.

siRNA, suggesting that IA-induced Girdin phosphorylation requires Akt in HUVEC.

3.4. HG activates NHE1 and induces intracellular alkalinization in HUVEC

Diabetes-delayed angiogenesis is through multiple signal pathways [21]. We thought whether high ambient glucose via NHE1 activation attenuates angiogenesis. To test this notion, we determined whether HG alters NHE1 activity and pHi value in HUVEC. As shown in Fig. 3A and B, HG (30 mM D-glucose) significantly increased both NHE1 activity and pHi value in a time dependent manner.

3.5. HG reduces both Akt and Girdin phosphorylations in HUVEC

We next detected if HG alters Akt and Girdin phosphorylations in cultured HUVEC. Confluent HUVEC were treated with varying times of HG from 30 s to 8 h. As shown in Fig. 3C and D, Akt and Girdin phosphorylations were gradually decreased beginning from 2 h after incubation with 30 mM HG. HG treatment did not alter total protein levels of Akt or Girdin, implying that HG-reduced phosphorylation of Akt or Girdin was not due to the altered expression of total proteins.

3.6. NHE1 activation is essential for HG-induced reductions of p-Akt and p-Girdin in HUVEC

In order to investigate whether HG-reduced the levels of p-Akt and p-Girdin are mediated by NHE1 activation, we determined the effects of NHE1 inhibitor, amiloride, on the levels of p-Akt and p-Girdin in HG-treated HUVEC. As shown in Online Fig. S1A, HG-enhanced NHE1 activity is dramatically abolished by amiloride treatment in a dose-dependent manner, suggesting that HG activated amiloride-sensitive NHE1.

Next we examined whether inhibition of NHE1 ablated the reductions of p-Akt and p-Girdin levels in HG. In Online Fig. S1B and C, treatment of HUVEC with amiloride significantly reversed HG-reduced levels of p-Akt and p-Girdin. These data indicate that HG-induced reductions of both p-Akt and p-Girdin are NHE1-dependent.

3.7. Overexpression of constitutively active Akt attenuates HG-decreased Girdin phosphorylation in endothelial cells

It is worth to examining whether upregulation of Akt reverses HG-reduced Girdin phosphorylation. As shown in Online Fig. S1D, Akt protein is highly expressed in constitutively active Akt (Akt-CA) infected HUVEC but not in GFP-infected HUVEC. HG inhibited the Girdin phosphorylation in GFP-infected HUVEC, but not in Akt-CA infected HUVEC. In addition, Akt-CA alone caused a light rise of p-Girdin in normal glucose condition. Together, it suggested that HG-reduced Girdin phosphorylation is Akt-dependent.

3.8. NHE1 inhibition or Akt activation enhances tube formation in HG-treated endothelial cells

Tube formation of endothelial cells is critical to angiogenesis, which is involved cell migration and proliferation. We then tested if inhibition of NHE1 reversed HG-impaired tube formation in HUVEC. Cells were treated with HG in presence or absence of amiloride, and tube formation was assayed by matrix gel. As shown in Online Fig. S2A and B, HG inhibited the tube formation of endothelial cells. However, treatment of HUVEC with amiloride maintained the ability of HUVEC to form the tube in HG condition. As expected, gain-function of Akt by gene

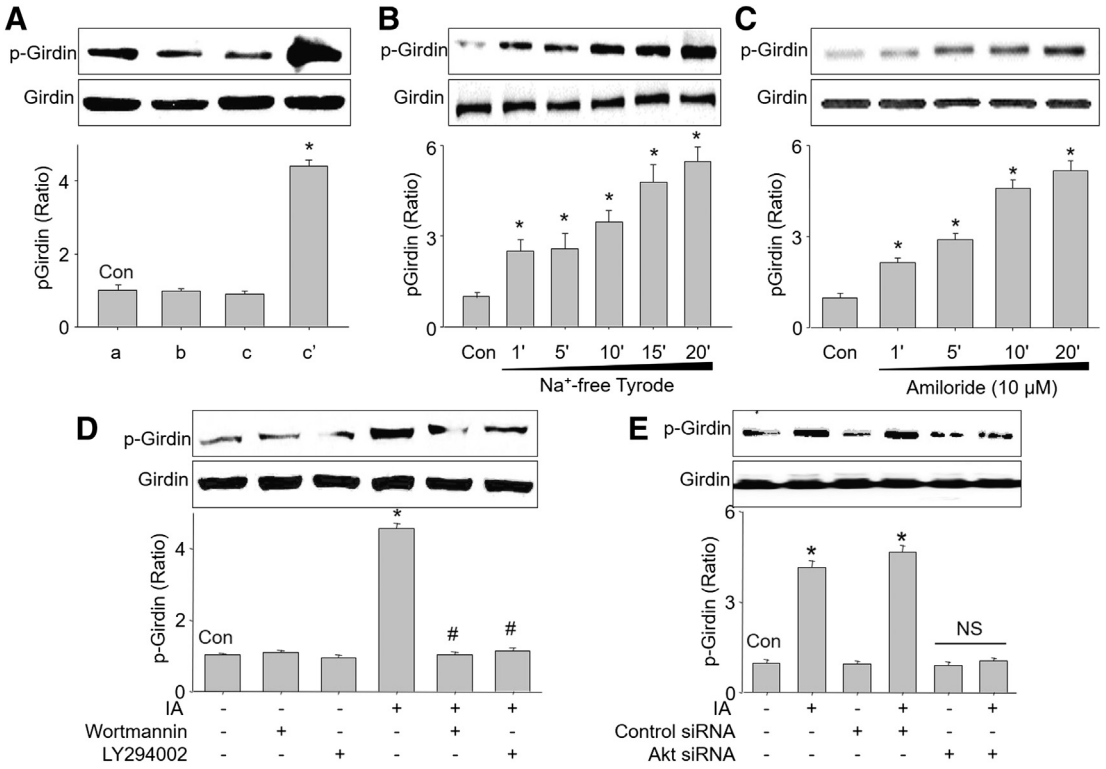

Fig. 2. IA increases Girdin phosphorylation in endothelial cells, which is Akt-dependent. (A) The IA in HUVEC was induced by washout of NH_4Cl with Na^+-free Tyrode solution. Serine phosphorylation of Girdin was detected by immunoprecipitation with antibodies to Girdin followed by western blotting with antibodies to phosphorylated serine. a, HCO_3^--free Tyrode solution; b, HCO_3^--free Tyrode solution plus NH_4Cl; c, HCO_3^--free Tyrode solution plus NH_4Cl plus HCO_3^--free Tyrode solution; c′, HCO_3^--free Tyrode solution plus NH_4Cl plus with Na^+-free Tyrode solution. This blot shown is a representative picture of 5 independent experiments. $^*P < 0.05$ vs. a, b, or c. (B and C) IA was induced by washout of NH_4Cl with (B) Na^+-free Tyrode solution or (C) Tyrode solution containing 10 μM amiloride in different times in HUVEC. p-Girdin was detected by Western blot. N is 5 in each group. $^*P < 0.05$ vs. control. (D) IA was induced in HUVEC after 30-min pre-incubation with or without Akt inhibitor, wortmannin (1 μM) or LY294002 (1 μM). Cell lysates were subjected to detect p-Akt and p-Girdin by Western blot. The blot is a representative of 5 independent experiments. $^*P < 0.05$ vs. control, $^\#P < 0.05$ vs. IA alone. (E) HUVEC were transfected with control siRNA and Akt siRNA. After induction of IA, cells were subjected to detect pGirdin by Western blot. The blot is a representative of 5 independent experiments. $^*P < 0.05$ vs. control or control siRNA. NS is indicated as no significant difference.

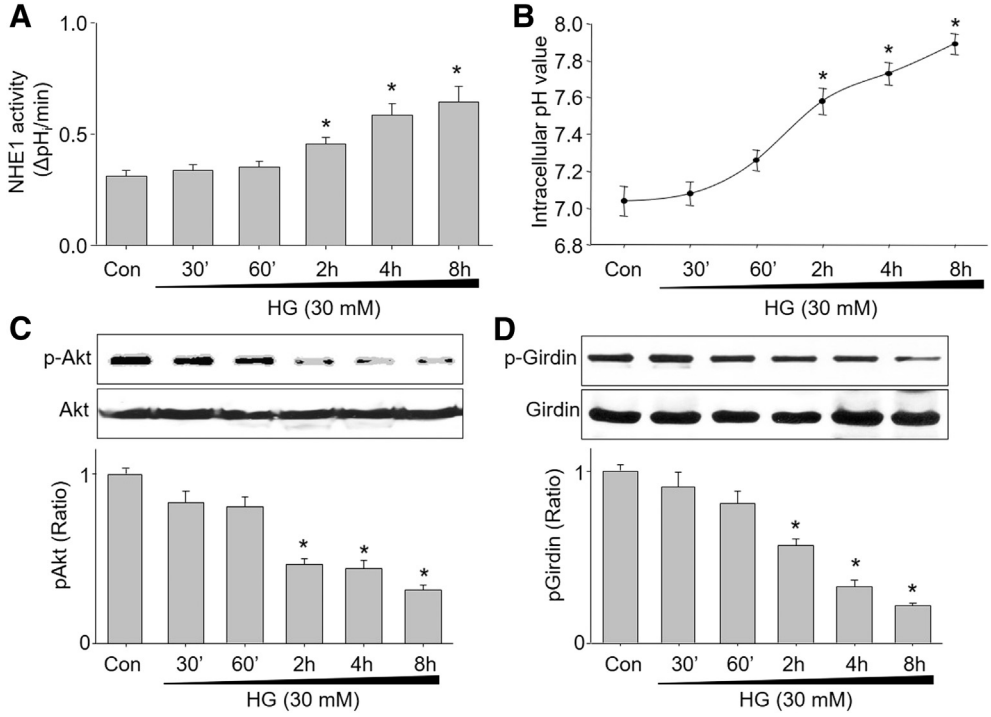

Fig. 3. HG time-dependently activates NHE1, increases pHi value, and inhibits the Akt and Girdin phosphorylations in HUVEC. HUVEC were incubated with D-glucose (30 mM) for 0.5, 1, 2, 4, and 8 h. The detections of (A) NHE1 activity by NH_4Cl pulse method, (B) pHi value by BCECF fluorescence, (C) pAkt, and (D) pGirdin by Western blot were performed, respectively. N is 5 in each group. $^*P < 0.05$ vs. control.

overexpression also promoted tube formation in HG-treated cells (Online Fig. S2C and D). This appearance was not seen in GFP-infected cells, indicating the specific effect of Akt on promoting tube formation. All these data suggest that HG via NHE1/Akt signaling impaired tube formation in endothelial cells.

3.9. Inhibition of NHE1 by amiloride promotes blood perfusion and angiogenesis in diabetic hindlimb following ischemia

Hyperglycemia-impaired angiogenesis contributes to vascular complications, such as diabetic foot and delayed wood healing [22]. Finally, we tested whether activation of NHE1 is involved in the ischemia-induced angiogenesis in diabetic mice. CD31 positive expression was detected by IHC represents angiogenesis. As shown in Fig. 4A–D, STZ-induced hyperglycemia dramatically reduced blood perfusion and damaged angiogenesis following ischemia, compared to non-diabetic mice. Amiloride alone did not change ischemia-induced angiogenesis and blood perfusion in control mice, but significantly augments the blood perfusion and angiogenesis in diabetic mice.

We also detected the effects of amiloride on phosphorylations of Akt and Girdin, and NHE1 activity in vivo. As depicted in Online Fig. S3A, the phosphorylations of Akt and Girdin were decreased by hyperglycemia in mice hindlimb after ischemia, as well as increased NHE1 activity (Online Fig. 3B). Importantly, all these phenotypes induced by hyperglycemia were reversed by amiloride, suggesting that hyperglycemia-impaired angiogenesis depends on NHE1/pHi/Akt/Girdin signaling.

4. Discussion

In the present study, we provided the first evidences that hyperglycemia activates NHE1 to induce intracellular alkalinization, which increases both Akt and Girdin phosphorylations and consequent impairment of tube formation. IA induced by inhibition of NHE1 reversed the detrimental effects of hyperglycemia on angiogenesis (Online Fig. 3C). This mechanism not only uncovers a molecular mechanism by which pHi value regulates endothelial cell network in diabetes, but also provides a novel target for exploring drugs against impaired angiogenesis.

The major discovery of this study is that pHi value regulates Akt phosphorylation via ser473 phosphorylation in endothelial cells. The relationship between Akt and NHE1 is so controversy. NHE1 is reported to be an Akt substrate necessary for actin filament reorganization by growth factors via direct phosphorylation of NHE1 serine 648 [23]. On the contrary, NHE1 recruits ezrin/radixin/moesin proteins to regulate Akt-dependent cell survival and inhibition of NHE1 markedly reduced Akt activity in left ventricular hypertrophy in the Goto-Kakizaki rat model of type 2 diabetes [24]. Our results clearly support that the decrease of pHi value induced by IA increases Akt phosphorylation and Akt-dependent signaling. Although our observations provided supports on pHi-dependent regulation of Akt phosphorylation in endothelial cells, the molecular mechanisms by how pHi regulates Akt phosphorylation need to be further investigated.

Another discovery is that diabetes-impaired angiogenesis is NHE1 (or pHi value) dependent. Angiogenesis is a vital process for embryological growth, tissue development, and wound healing in damaged tissues [1]. This process requires several biochemical and physiological factors

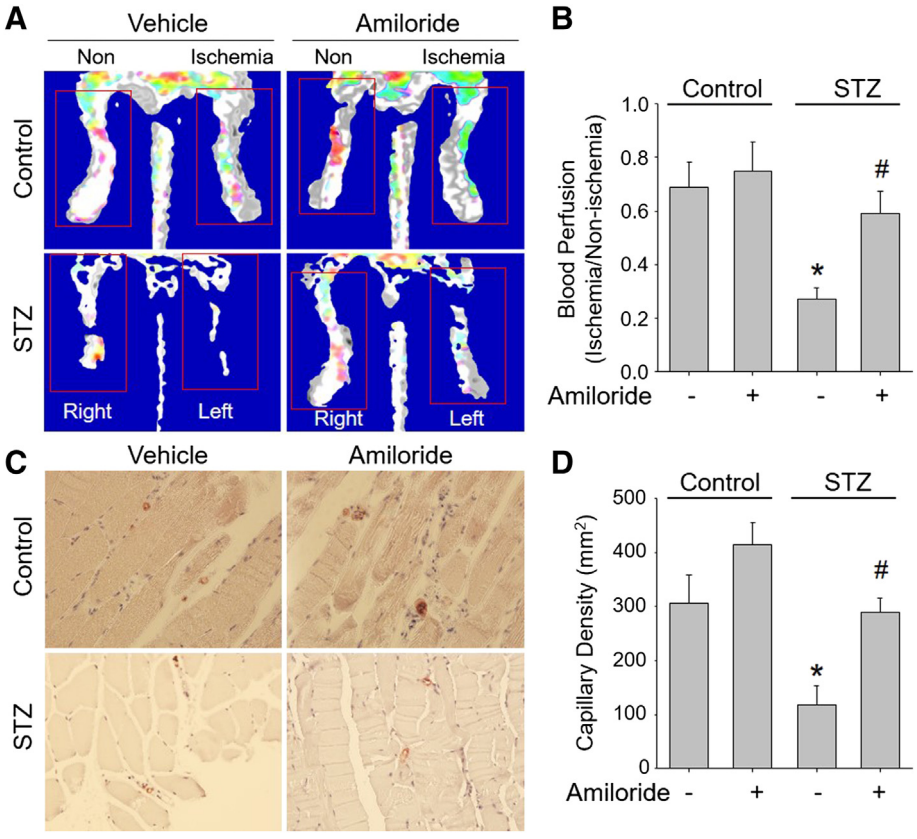

Fig. 4. Inhibition of NHE1 by amiloride administration promotes revascularization following hindlimb ischemia in diabetic mice. All diabetic and control mice were received with or without amiloride administration (1 mg/kg body weight daily) beginning at 7 days before surgery and kept the treatment during the whole experiments. At the end of experiments, ischemic/normal blood flow ratio in hind limb was performed by laser Doppler in A and quantitative analysis was shown in B. A low-perfusion signal (dark blue) was observed in the ischemic hindlimb (left leg) of STZ-injected mice, whereas a high-perfusion signal (white to red) was detected in non-ischemic hindlimb (left leg) in control mice after 28 postoperative days. The area surrounded by a red line was chosen to calculate blood flow. Neovascularization was determined by detecting CD31 by IHC in C and capillary density on the 28th postoperative day in mice adductor muscle was calculated in D. N is 5–10. $^{*}P < 0.05$ vs. control, $^{\#}P < 0.05$ vs. STZ.

to stimulate vessel sprouting and remodeling of the primitive vascular network, which in turn establishes stable and functional blood vessel networks. There are several angiogenic factors, such as VEGFs, FGFs, angiopoietins, PDGF, TGF, which are involved in stimulation, promotion, and stabilization of new blood vessels [1]. Inconsistently, VEGF expression has been shown to be downregulated by a decrease in pHi value induced by blocking Na^+/H^+ antiport [25,26]. We would explain that different species of cells would contribute to this discrepancy. They used human myeloid K562 cells, while we used endothelial cells in this study.

A limitation of this study is that amiloride may non-selectively affect the other possible ion transport mechanisms, e.g. Na^+-Ca^{2+} exchange. In our previous studies, we have demonstrated that in vitro or in vivo high glucose produced a cariporide-sensitive activation of NHE in endothelial cells [12,13,15], indicating that high glucose may dominantly activate NHE1 in endothelial cells. As concerned to how glucose increases NHE1 activity, we thought that a phosphorylation-dependent increase in the activity of NHE1 may be involved because PKC is one of the kinases responsible for this phosphorylation [27], while the de novo biosynthesis of DAG, as a strong activator of PKC, is potentiated under hyperglycemia [28].

It is questionable whether the STZ-treated animal model is a good model for high glucose in the cell culture. STZ is known to destroy islets of Langerhans in the pancreas [29], therefore the induced diabetes in the animals resembles insulin-dependent type 1 diabetes, rather than non-insulin dependent diabetes. Cardio- and Cerebra-vascular complications of diabetes are characteristic for type 2 diabetes or insulin resistance [30]. It is better to use animal models of type 2 diabetes or obesity [31], rather than STZ-induced diabetic model.

Another issue is whether any other transcriptional pathway is activated by H^+, which links pHi value to the formation of capillaries tubes in HUVECs, besides Girdin phosphorylation. We speculated not only Akt is activated by H^+, calpain-eNOS pathway may contribute to endothelial dysfunction because this pathway is also dysregulated by high glucose [11]. AMP-activated protein kinase, prostacyclin synthase, microRNAs including miR-133 and miR-199, as the important regulators of endothelial cell functions, may be involved in this biological process [32–36].

In summary, the present study proposes a role of NHE1 in the tissue response of angiogenesis to ischemic stress in diabetes. Specifically, when angiogenesis is induced by ischemia in tissues, NHE1 is activated by hyperglycemia to enhance the intracellular alkalinization, which inactivates Akt via unknown mechanism. Akt suppression serves to maintain low levels of Girdin phosphorylation, which is ultimately not enough for a normal angiogenesis (Online Fig. 3C). Further delineation of these proposed mechanisms will be necessary before a complete understanding of this process is achieved. Our study also suggests that amiloride or its analogues may be a new drug to treat angiogenesis-related peripheral vascular diseases, such as diabetes foot and delayed wood healing.

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Conflicts of interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijcard.2018.08.028.

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