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Original article

Endothelial deletion of mTORC1 protects against hindlimb ischemia in diabetic mice *via* activation of autophagy, attenuation of oxidative stress and alleviation of inflammation

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ABSTRACT

Peripheral arterial disease (PAD) complicated with diabetes mellitus (DM) still remains a thorny issue due to lack of effective strategies. Our previous study has demonstrated that inhibition of mTORC1 protected adipose-derived stromal cells from hindlimb ischemic injury in PAD mice. However, whether inhibition of mTORC1 could protect against PAD in diabetes mellitus and the underlying mechanisms remained elusive. In this study, we employed endothelial-specific *raptor* (an essential component of the mTORC1 signaling complex) knockout (KO) mice (Tie2-mTORC1^{ko}) to investigate whether and how mTORC1 downregulation could alleviate hindlimb ischemic injury in diabetic mice. Tie2-mTORC1^{ko} mice and their wild-type littermates were intraperitoneally injected with streptozocin to induce type 1 diabetic model, after which the hyperglycemic mice were randomly allocated to sham operation or PAD operation (femoral artery ligation). The restoration of hindlimb blood perfusion and recovery of limb functions were improved in diabetic Tie2-mTORC1^{ko} PAD mice with significant improvements of autophagy, angiogenesis and vascular integrity as well as attenuation of apoptosis, inflammation and oxidative stress. *In vitro*, high glucose combining with hypoxia/serum deprivation treatment (HG + H/SD) significantly triggered apoptosis, reactive oxygen species generation and inflammation while inhibited autophagy and tube formation in HUVECs. The effect could be accentuated and attenuated by mTORC1 over-expression (*TSC2* siRNA) and mTORC1 silencing (*raptor* siRNA), respectively. Moreover, autophagy inhibitor 3-MA could simulate the effects of *TSC2* siRNA while autophagy inducer rapamycin could mimic the effects of *raptor* siRNA, suggesting that the beneficial effects of mTORC1 deletion were associated with autophagy induction. In conclusion, our present study demonstrates that endothelial mTORC1 deletion protects against hindlimb ischemic injury in diabetic mice possibly *via* activation of autophagy, attenuation of oxidative stress and alleviation of inflammation. Therapeutics targeting mTORC1 may therefore represents a promising strategy to rescue limb ischemia in diabetes mellitus.

Abbreviations: 3-MA, 3-methyladenine; 3-NT, 3-nitrotyrosine; AI, apoptosis index, α-SMA, α-smooth muscle actin; bFGF, basic fibroblast growth factor; BM, bone marrow; CCK-8, cell counting kit-8; CLI, critical limb ischemia; DAPI, 49, 6-diamidino-2-phenylindole; DCFH-DA, 2, 7-Dichlorodi -hydrofluorescein diacetate; DHE, Dihydroethidium; DM, diabetes mellitus; ELISA, enzyme-linked immunosorbent assay; HG, high glucose; H/SD, hypoxia and serum deprivation; HUVEC, human umbilical vein endothelium cell; IL-1β, Interleukin-1β; IL-6, interleukin-6; IOD, integral optical density; KO, knockout; LC3, microtubule-associated protein 1 light chain3; LDPI, laser doppler perfusion imaging; MMP, mitochondrial membrane potential; mTOR, mechanistic target of rapamycin complex 1; NO, nitric oxide; PAD, peripheral artery disease; PBS, phosphate buffer solution; POD, post operational day; PR, perfusion ratio; raptor, regulatory-associated protein of mammalian target of rapamycin; ROS, reactive oxygen species; SEM, scanning electron microscope; sICAM-1, soluble intercellular cell adhesion molecule-1; siRNA, small interfering RNA; STZ, streptozocin; sVCAM-1, soluble VCAM-1; TEM, Transmission electron microscope; TNF-α, tumor necrosis factor-alpha; TSC2, Tuberous sclerosis complex 2; TUNEL, Terminal deoxy-nucleotidyl transferase-mediated dUTP nick end labeling; ULK1/2, Unc51-like kinase1/2; VAM-1, Vascular cell adhesion protein 1; VEGF, vascular endothelial growth factor

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1. Introduction

Peripheral arterial disease (PAD) is often manifested as intermittent claudication and ranks as the leading cause of nontraumatic amputation [1]. The presence of diabetes mellitus (DM) multiplied the prevalence and severity of PAD [2]. A variety of factors were implicated in the exacerbation of PAD by DM, among which endothelial dysfunction was the most prominent one [3–6]. The elevated glucose levels in DM not only induced endothelial cell senescence [7,8], but also leaded to impaired mobilization of endothelial progenitor cells from the bone marrow [9], inhibition of angiogenesis [10], and even direct injury and death of endothelial cells [11,12]. In this regard, improving endothelial dysfunction is pertinent to the therapy of PAD patients with concurrent DM.

Autophagy (or self-eating) was described as a lysosomal degradation pathway that scavenges protein aggregates and damaged organelles, thereby maintaining intracellular homeostasis under various physiological and pathological conditions [13]. Impairment of autophagy is observed in endothelial cells, and is implicated in the pathogenesis of DM, including DM-induced endothelial dysfunction [14]. Meanwhile, oxidative and subsequent nitrosative damage of the myocardium and vasculature was conceived as the primary mechanisms contributing to pathologic alterations in diabetic cardiovascular complications [15]. Furthermore, the interplay between autophagy and oxidative stress was frequently correlated with cell survival or cell death in various diseases including endothelial oxidative damage in DM, and modulation of autophagy has been shown to attenuate endothelial oxidative damage, improve endothelial dysfunction and facilitate tissue repair especially therapeutic angiogenesis [16–18]. In addition, inflammation, another crucial mechanism leading to endothelial injury in diabetic context, is often closely related to oxidative stress and exacerbates oxidative stress-induced endothelial damage [19].

Nutrient-sensing pathways including the mechanistic target of rapamycin complex 1 (mTORC1) pathway have been recognized as pivotal factors that regulate autophagy in diabetic organs. Activation or restoration of autophagy by inhibiting the mTORC1 pathway in renal cells has been demonstrated to effectively ameliorate advanced diabetic nephropathy [20]. On the other hand, our previous research has proved that inhibition of mTORC1 with rapamycin protected transplanted adipose-derived stromal cells from hindlimb ischemic injury *via* suppressing inflammatory response [21]. Therefore, we hypothesized that endothelial deletion of mTORC1 might activate autophagy, thereby protecting against hindlimb ischemia in diabetic mice. To examine this hypothesis, we utilized endothelial-specific *raptor* (an essential component of the mTORC1 signaling complex) knockout (KO) mice to characterize the effects of mTORC1 deletion on hindlimb ischemia in diabetic mice with a focus on autophagic activity, inflammatory response and oxidative stress injury in endothelial cells.

2. Methods

2.1. Experimental animals

To generate mice with endothelial cell-specific raptor deletion, mice with loxP-flanked (floxed, fl) raptor alleles (C57BL/6 background; commercially purchased from Jackson Laboratory, Stock Number: 013188) were mated with mice expressing a Cre recombinase-estrogen receptor fusion protein ER(T2) under control of the endothelial receptor tyrosine kinase (Tie2) promoter (C57BL/6 background; commercially purchased from Jackson Laboratory, Stock Number: 004128). Genotyping was performed by PCR. The endothelial cell-specific mTORC1 deletion data was presented in supplementary figure. Age- and gender-matched littermates (6–8 weeks, 20–25 g) were used throughout the study.

2.2. Animal model and treatment

The mice were randomly divided into 6 groups: (1) wild-type Sham group (Sham); (2) wild-type PAD group (PAD); (3) wild-type DM group (DM); (4) wild-type DM + PAD group (DM + PAD); (5) Tie2-raptor^{KO} + DM group (Raptor$^{-/-}$ + DM); (6) Tie2-raptor^{KO} + DM + PAD group (Raptor$^{-/-}$ + DM + PAD)(n = 15 for each group). Diabetes was induced in group (3), (4), (5) and (6) mice according to our previous study [22]. Briefly, Tie2-raptor^{KO} mice and their wild-type littermates were subjected to intraperitoneal injection of streptozotocin (STZ) (50 mg/kg; dissolved in 0.1 mol/l citrate buffer, pH 4.5) daily for 5 consecutive days, mice were then maintained for another 3 months followed by examination of random blood glucose levels. Mice with random blood glucose over 11.1 mmol/L were defined as having diabetes. PAD model was operated in group (2), (4) and (6) mice. The surgical procedure was described as we have previously described with minor modifications [23,24]. In brief, PAD was induced by ligating and excising the left femoral artery with all superficial and deep branches. Sham-operated mice received incision without artery ligation. All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals by the National Institute of Health (NIH Publication No. 85-23, revised 1985 and updated 2011). The protocol was approved by the Fourth Military Medical University ethics review board (XJLL2015065).

2.3. Cell culture and drug treatment

The cell line of human umbilical vein endothelial cells (HUVECs) were purchased from American Type Culture Collection (ATCC) center (Menassas, VA, USA) and cultured in Dulbecco's Modified Eagle Medium (DMEM)(Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% FBS and 1% penicillin–streptomycin in a humidified 5% CO_2, 37 ℃ incubator (Thermo, MA, USA). Experiments on HUVECs were carried out at the 3–7 passages. HUVECs cultured in medium containing 33.3 mmol/L glucose for 24 h were served as the high glucose group (HG group), while those cultured in 5.5 mmol/L glucose for equivalent period of time were conceived as control group. After transfected with or without small interfering RNA, HUVECs were then exposed to a 24-h high glucose medium or control medium in the presence or absence of the autophagy inhibitor 3-methyladenine (3-MA,10 mM, Sigma-Aldrich, St. Louis, MO, USA) or the autophagy inducer rapamycin (Rapa, 100 n M, Sigma-Aldrich,St. Louis, MO, USA), followed with or without a 12 h hypoxia and serum deprivation (H/SD) in the presence or absence of 3-MA or rapamycin.

2.4. Hypoxia/serum deprivation injury

Cultured HUVECs were stimulated with hypoxia/serum deprivation (H/SD) injury to mimic *in vivo* ischemic injury as previously described [25]. Briefly, after siRNA transfection and/or high glucose culturing, HUVECs were washed with PBS, and cultured in Hanks buffer (GIBCO BRL, Grand Island, NY, USA). After that, HUVECs were incubated in an anoxic chamber (95% N_2 /5% CO_2) (Thermo Fisher Scientific, Waltham, MA, USA) at 37 ℃ for 12 h. In the control and high glucose only group, HUVECs were maintained at normoxia (95% air, 5% CO_2) for equivalent periods of time.

2.5. Serial laser Doppler perfusion imaging of hindlimbs

Laser Doppler perfusion imaging (LDPI) was used to serially monitor the blood perfusion recovery of the ischemic hindlimbs. Briefly, mice were placed in supine position on a 37.4–38.0 ℃ heating pad and then imaged using an analyzer (PeriScan-PIM3 Perimed AB, Sweden). The blood flux was quantified using perfusion ratio (ratio of average LDPI index of ischemic to nonischemic) by LDPI win 3.1.3 (Perimed AB, Sweden).