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Original Article

Metformin inhibits angiogenesis in endothelial progenitor cells through inhibiting MMP2, MMP9 and uPA expression via AMPK-mTOR-autophagy pathway

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Abstract: Objective: The aim of this study was to investigate the effect of metformin on endothelial progenitor cells (EPCs) angiogenesis under physiology condition and to explore the possible mechanisms. Methods: EPCs were treated with metformin and angiogenesis of EPCs were evaluated by capillary tube formation assay in Matrigel. Moreover, we also assessed AMPK-mTOR-autophagy pathway to explore the possible mechanisms. Results: Metformin treatment could significantly down-regulate metal matrix Proteinase 2 (MMP2), MMP9 and urokinase-type plasminogen activator (uPA) expression, and subsequently decrease angiogenesis of EPCs. Increased levels of phospho-AMPK and LC3II expression, as well as decreased phospho-mTOR, contribute to this phenomenon. Down-regulated autophagy by autophagy protein 5 siRNA could reverse the effect exerted by metformin. Conclusions: Our results here showed that metformin could inhibit EPCs angiogenesis through inhibiting the expression of MMP2, MMP9 and uPA via AMPK-mTOR-autophagy pathway.

Keywords: Metformin, endothelial progenitor cells, tube formation, angiogenesis, autophagy

Introduction

Angiogenesis is a central physiological process in growth and development and has been shown to be an excellent therapeutic target for the treatment of cardiovascular disease (CVD) [1]. In traditionally, it was considered that angiogenesis is sprouted from mature endothelium. But recently Asahara et al [2] found that endothelial progenitor cells (EPCs) could incorporate into sites of active angiogenesis and played an important role in postnatally therapeutic angiogenesis which had been evaluated by many subsequent pre-clinical and clinical studies [3-5]. According to previous studies, EPCs could promote angiogenesis not only by directly incorporate into new vessels but also via indirect mechanisms [6, 7]. Cell-based therapy using EPCs have been represented as one of the promising regenerative medicine [8].

Angiogenesis deficiency is a key pathological characteristic of diabetes microvascular complications in peripheral tissues [9], which may partly be associated with alterations in EPCs physiology [10, 11]. In diabetes mellitus (DM) patients, the number of EPCs was decreased [12-14] and the functions of EPCs were impaired [13, 15, 16]. Thus, augmentation of the number and improvement of the functions of EPCs might be a potential therapeutic target of diabetic vascular complications [17]. Metformin, a biguanide derivative, is widely used in clinical as a drug for type 2 DM. It can improve the numbers and function of circulating EPCs in type 2 DM [18]. Besides, in the previous studies, metformin played a paradoxical role in angiogenesis of endothelial cells and some cancer cells [19-24]. But the exact role of metformin on angiogenesis conducted by EPCs has not been elaborated well enough.

To investigate the exact role of metformin on angiogenesis of EPCs, we conducted capillary tube formation assays in Matrigel. We found that metformin could decrease the numbers of tube formation. We speculated that autophagy enhanced by metformin might be involved in

the regulation mechanisms. As autophagy was attenuated by autophagy protein 5 (ATG5) siRNA, angiogenesis inhibition by metformin was neutralized.

Materials and methods

Antibodies and reagents

Antibodies for phospho-AMPK, AMPK, LC3B were from Cell Signaling Technology (Danvers, MA, USA). Antibodies for urokinase-type plasminogen activator (uPA), Metal Matrix Proteinase (MMP) 2, MMP9, Atg5 and p62 were from Abcam Inc (Cambridge, MA, USA). Antibody for phospho-mTOR was from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA). Antibody for vascular endothelial growth factor (VEGF) was for ProteinTech Company (Chicago, IL, USA). Metformin and antibodies for β-actin were purchased from Sigma-Aldrich (St.Louis, MO, USA).

Isolation and characterization of rat EPCs

The study protocol was approved by the ethics committee of the Second Affiliated Hospital of Soochow University, Suzhou, China. Rat EPCs were isolated according to the previous description [25-28]. Briefly, bone marrow was obtained by flushing tibiae and femurs of Sprague-Dawley rats (80-100 g, from Experimental Animal Center of Soochow University, Suzhou, China). Mononuclear cells, isolated from bone marrow by density gradient centrifugation using Lymphocyte Separation Medium-LSM™ (MP Biomedicals, USA), were seeded onto 25-mm^2 cell culture dishes (Corning, Corning, NY, USA) at a density of 2.5×10^6 cells/cm^2 and cultured with endothelial cell basal medium-2 (EGM-2MV; Lonza, Walkersville, MD, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA) and maintained in a 37°C, 5% CO_2 incubator. After culturing for 4 days, medium was changed as fresh EGM-2 MV. The identification of EPCs was determined by surface expression of VEGFR2, CD133 and CD34. Cells within 5 passages were used for following experiments. To evaluate the effect of metformin, identified EPCs were incubated with 1 mM metformin [27].

Autophagy protein 5 (Atg5) knockdown using siRNA

ATG5 Knockdown was performed as previously described [28]. SiRNA against ATG5 (ATG siRNA), and control siRNA were obtained from (Shanghai GenePharma Co., Ltd). The target sequences are sense: 5'-UUC UUC GAA CGU GUC ACG UTT-3'; antisense: 5'-ACG UGA CAC GUU CGG AGA ATT-3'. After reaching 50% confluence, EPCs were transfected with 30 nmol/L ATG siRNA or control siRNA, by use of Lipofectamine 2000 (Invitrogen, Carsland, CA, USA) in serum-free medium (EBM-2MV), according to the manufacturer's instruction. Medium change with fresh EGM-2MV was performed at 6 hours after transfection, and the cells were analyzed 48 hrs after transfection.

Capillary tube formation assay in Matrigel

The in vitro endothelial tube formation assay was performed as previously described [26, 29] . Briefly, 50 μL of thawed Matrigel (BD Bioscience, Germany) was spread to a 96-well plate and allowed to polymerize at 37°C for 30 min. Normal EPCs or metformin treated EPCs were suspended in FBS-free EBM-2 MV medium and seeded in each well at a concentration of 2×10^4 cells/well. Cells were incubated at 37°C with or without the supplement of the metformin. After 12 hours, capillary tube formation in Matrigel was observed under a light microscope to assess the formation of capillary-like structures. The total numbers of the formed tubes, which represent the degree of angiogenesis *in vitro*, were scanned and quantified in three randomly picked fields per well (× 10 magnification). The results were expressed as mean number of tubes compared with the control groups.

For the group of Atg5 siRNA neutralizing autophagy, EPCs were transfected with Atg5 siRNA and then treated with metformin. Compared with normal EPCs which were treated with metformin alone, cells were seeded in each well at a concentration of 2×10^4 cells/well.

Western-blot analysis

EPCs (1×10^6 cells) were lysed in RIPA buffer, followed by high-speed centrifugation and bicinchoninic acid quantification. Cellular proteins were separated bysodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidenedifluoride membranes. After blocking with 5% Bovine Serum Albumin Tris buffered Saline-Tween 20 (TBS-T), membranes were incubated with primary anti-

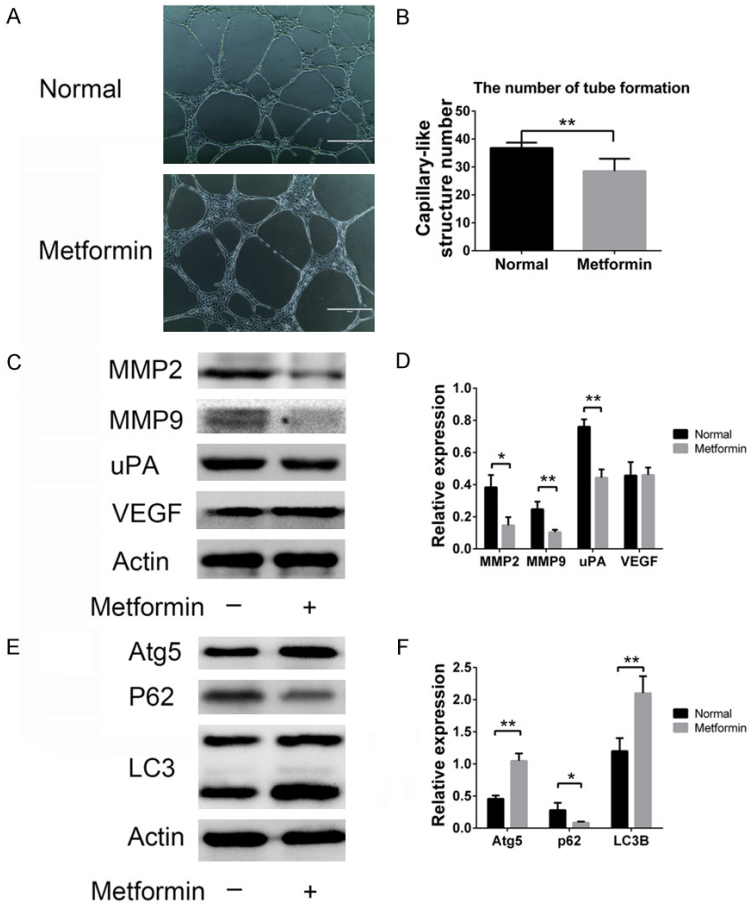

Figure 1. Effect of metformin on endothelial progenitor cells (EPCs) angiogenesis. A, B: Metformin significantly decreased the number of tube formation of EPCs. Representative pictures of tube formation in normal and metformin groups (× 10 Magnification). C, D: Metformin reduced the expression of matrix metalloproteinases 2 (MMP2), MMP9 and urokinase-type plasminogen activator (uPA) but had no effects on vascular endothelial growth factor (VEGF). E, F: When EPCs were treated with metformin, the expression of Atg5 and LC3B were enhanced while the expression of the selective autophagy adaptor sequestosome-1 (p62) was inhibited. Values were normalized to beta-actin protein (means ± SE; n=3).

bodies against AMPK, phospho-AMPK, phospho-mTOR, LC3B, P62, Atg5, VEGF, uPA, MMP2 and MMP9. Appropriate horseradish peroxidase-conjugated secondary antibodies were applied. β-actin (Sigma) was used as the loading control. The protein bands were detected with EZ-ECL Chemiluminescence Detection Kit (Biological industries, Kibbutz, Israel) on Gene Gnome XRQ system (Syngene Ltd, UK).

Statistical analyses

All statistical analyses were carried out using SPSS v21 (SPSS, Chicago, IL). Data are presented as mean ± standard deviation (SD) if normal distribution was met. Student's t-test was utilized to examine differences two group comparison. These data met the requirement of Huynh-Feldt and were analyzed by one-way analysis of variance (ANOVA). Repeated measurement data were correlated if *P* value was less than 0.05, and were analyzed using ANOVA. The comparison of measurement data between two groups were performed using one-way ANOVA. P<0.05 was considered as statistically significant.

Results

Metformin decreased EPCs tube formation ability

Capacity of tube formation in Matrigel was used to evaluate in normal and metformin treated EPCs. It was found that metformin could inhibit formation of capillary-like structures in Matrigel (**Figure 1A**). There was a significant difference in the number of tubes between the two groups (**Figure 1B**, P<0.05). This effect could be reduced when treated together with Atg5 siRNA (**Figure 2A** and **2B**). There was also a significant difference in the number of tube formation between the two groups (**Figure 2B**, P<0.05).

Metformin regulates EPCs tube formation through influencing uPA, MMP2 and MMP9 but not VEGF

We examined the expression of MMP2, MMP9, uPA and VEGF in EPCs with or without metformin treatment in EPCs. The results showed that decreased expression of MMP2, MMP9 and uPA in EPCs treated with metformin (**Figure 1C** and **1D**), which indicating a decreased activity of gelatinase and fibrinolysis. But expression of VEGF was enhanced (**Figure 1C** and **1D**). As shown in **Figure 1E** and **1F**, the expression of Atg5 and LC3B were enhanced while the expression of the selective autophagy adaptor

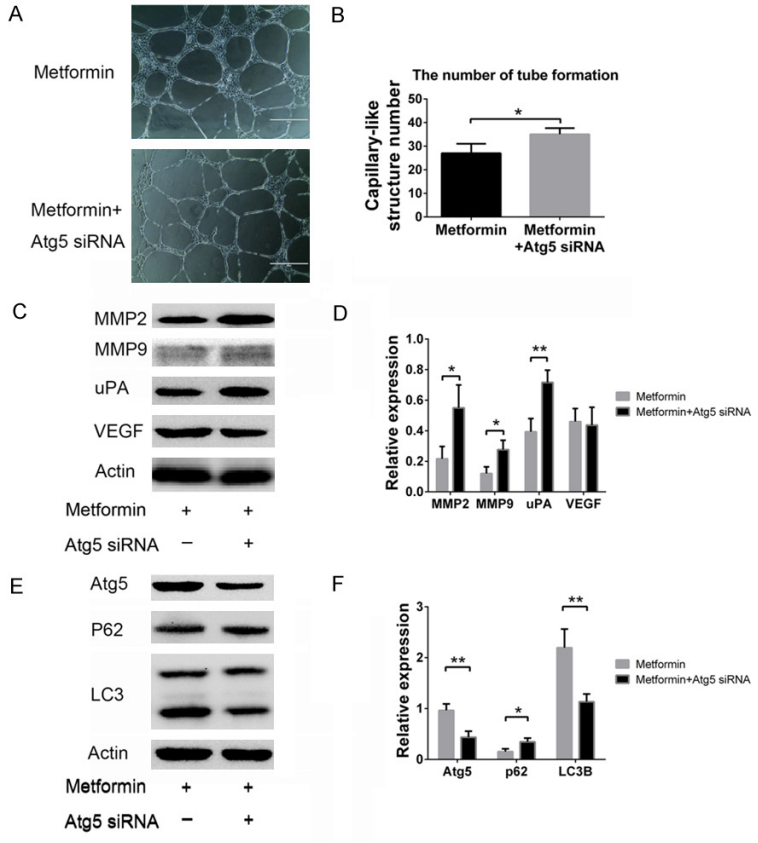

Figure 2. Autophagy down-regulation could reverse the inhibition role of metformin in tube formation. A, B: Inhibition of tube formation regulated by metformin was attenuated by Atg5 knockdown. C, D: Compared with metformin treated alone, Atg5 knockdown could promote the expression of matrix metalloproteinases 2 (MMP2), MMP9 and urokinase-type plasminogen activator (uPA). E, F: Autophagy promoted by metformin could be attenuated by Atg5 knockdown. Values were normalized to beta-actin protein (means ± SE; n=3).

sequestosome-1 (p62) was inhibited. These results showed that metformin could promote autophagy in EPCs. We speculated that metformin attenuated tube formation by inhibiting MMP2, MMP9 and uPA via autophagy pathway. Thus we added Atg5 siRNA into the group of metformin to verify this hypothesis. It was found that down-regulated could reverse the inhibition of metformin in the expression of MMP2, MMP9 and uPA which was consistent with the role in tube formation (**Figure 2**).

Metformin regulates tube formation via AMPK-mTOR-autophagy pathway

Metformin is both the agonists of AMPK and autophagy [30-32]. Besides, AMPK activation could subsequently inhibit mTOR. This could promote autophagy further. Thus, we speculated that AMPK-mTOR-autophagy pathway might play an important role in inhibition of EPCs tube formation. As seen from the **Figure 3**, metformin could enhance phosphorylation of AMPK but attenuate phosphorylation of mTOR. And then LC3 and Atg5 expressions were promoted. Consistently, MMP2, MMP and uPA expressions were inhibited. Inhibiting autophagy by Atg5 siRNA could neutralize this phenomenon.

Discussion

It has been demonstrated that EPCs can promote angiogenesis in a paracrine fashion by secretion of angiogenic factors for bone-marrow progenitors mobilization and mature ECs activation [33, 34]. In the present study, we found metformin could inhibit the function of angiogenesis in EPCs under physiology condition. This may attribute to the down-regulated MMP2, MMP9 and uPA. AMPK-mTOR-autophagy pathway may be involved in this phenomenon.

MMPs, especially MMP9 and MMP2, has been implicated to promote cell migration and tube formation [35, 36], which was considered as the potent triggers and contributors of the angiogenic switch. Heissig et al showed that MMP-2 and MMP-9 played an important role in focal angiogenesis in response to VEGF stimulation [37]. uPA, which is highly expressed in EPCs [38], plays an important role in the whole process of angiogenesis [39]. Previous studies showed that angiogenesis induced by VEGF was also depended on uPA [40-43]. Therefore, in spite of the promoted expression of VEGF, reduction of MMP2, MMP9 and uPA might contribute to the effect of metformin on EPCs in physiology condition.

Metformin is one of the agonist of AMP-activated protein kinase (AMPK) pathway [30]. Upon the latter activation, AMPK phosphorylates and inhibits mTOR and subsequently promote autophagy [31, 32]. In the previous studies, autophagy pathways had been implicated

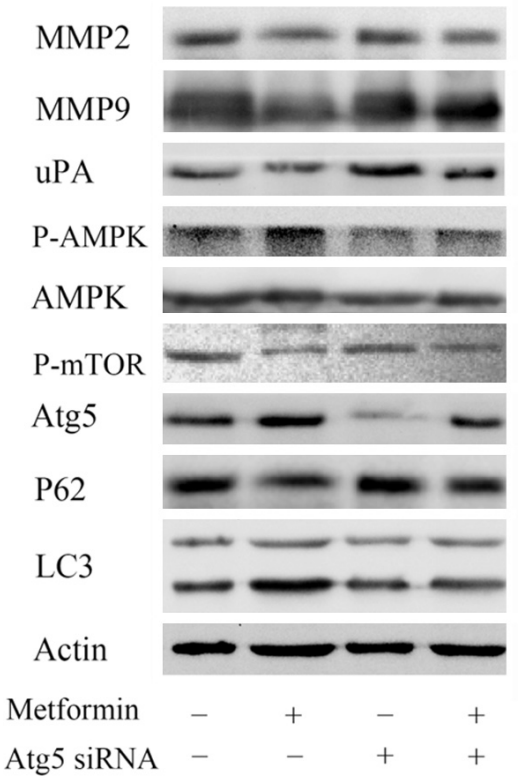

Figure 3. Metformin regulates endothelial progenitor cells (EPCs) angiogenesis via AMPK-mTOR-autophagy pathway. Western blot analysis of MMP2, MMP9, p-AMPK, AMPK, p-mTOR, Atg5, LC3 and p62 expression after metformin and/or Atg5 siRNA treatment.

to exert paradoxical effects on angiogenesis [44-46]. Ramakrishnan S, et al [44] and Kim, K. W, et al [45] found that autophagy could inhibit angiogenesis. But Du, J. et al [47] found that angiogenesis was positive correlation with autophagy. In the present study, we found that metformin could enhance autophagy significantly. And then the expressions of MMP2, MMP9 and uPA were down-regulated. Conversely, when autophagy was attenuated by Atg5 siRNA, angiogenesis inhibition by metformin was neutralized. Consistently, the down-regulated expressions of MMP2, MMP9 and uPA were reversed.

There are a few limitations in this study. First, we only collected the normal healthy subjects to examine the effect of metformin on normal EPCs. The effect of metformin on the EPCs derived from patients with DM has not been examined yet. Second, the function of the metformin treated EPCs in vivo was not elucidated yet. Appropriate animal model studies should been taken to verify the function change of EPCs in vivo. Therefore, further studies were needed to address these problems.

In conclusion, our results here showed that metformin could inhibit the angiogenesis of EPCs. AMPK-mTOR-autophagy pathway, which could inhibit the expression of MMP2, MMP9 and uPA, was involved in the regulation mechanisms.

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Disclosure of conflict of interest

None.

Authors' contribution

WL, AQ and FL contributed in generating experimental data. WL, LZ, YZ and XL contributed in discussion and reviewed/edited manuscript. WL, KJ, XL wrote the manuscript and drew the figures.

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