High Glucose Condition Induces Autophagy in Endothelial Progenitor Cells Contributing to Angiogenic Impairment

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Cardiovascular complications are the major causes of death in patients with diabetes mellitus. Several studies have demonstrated that endothelial progenitor cells (EPCs), adult stem cells contributing to the regeneration of vascular endothelium, are dysfunctional under diabetic condition resulting in impaired peripheral circulation and delayed wound healing. In this study, we investigated the cellular alteration of EPCs under high glucose condition, to elucidate the mechanisms underlying diabetes-associated EPC dysfunction. EPCs were isolated from bone marrow and cultured in normal glucose (5.5 mM)- or high glucose (HG; 30 mM)-containing medium. High glucose treated-EPCs showed decreased ability to form tubule-like networks in Matrigel compared to EPCs under normal glucose, which matched well to the clinical observation of diabetic EPC dysfunction. Conversion of LC3-I to LC3-II was increased in EPCs under HG condition, showing that HG induced autophagy in EPCs. Flow cytometric analysis revealed generation of oxidative stress and disruption of mitochondrial permeability in HG exposed EPCs. Increased mitochondrial oxidative stress was also observed by mitochondria-specific superoxide indicator, MitoSOX™. Taken together, we demonstrated that autophagy and mitochondrial impairment were induced in EPCs under high glucose condition, giving a new insight into the mechanism underlying dysfunction of diabetic EPCs. We hope that our finding can contribute to the development of a new treatment option for cardiovascular complications in diabetic patients.

Key words  endothelial progenitor cell; high glucose; autophagy; oxidative stress; diabetes; mitochondria

In patients with diabetes mellitus, cardiovascular dysfunction is one of the major complications, accounting for up to 80% of deaths. Not only for the mortality, it is also a main cause for the disability in diabetes, where impaired peripheral circulation results in delayed wound healing and lower limb amputation. Nevertheless, the complete mechanisms of the vascular pathogenesis in diabetic condition remain to be established.

The role of endothelial progenitor cells (EPCs) in the vascularization has been identified, suggesting that impaired EPC function may contribute to vascular complications under disease states. Of note, clinical observations demonstrated that the number of circulating EPCs is decreased, and their functions are impaired in diabetes. In vivo diabetic animal studies revealed that EPC functions of mobilization, differentiation and tube formation are disrupted, implying that EPC dysfunction could be critical for defective diabetic angiogenesis. Although several studies have given explanations including increased oxidative stress and impaired nitric oxide signaling, the mechanisms underlying diabetic EPC dysfunction are still largely unknown.

Autophagy, a homeostatic process that involves in organelle recycling and protein degradation, is essential for normal cellular physiology. It is upregulated in response to extra- or intra-cellular signals such as starvation and endoplasmic reticulum (ER) stress, to preserve the balance between biosynthesis and turnover. The importance of autophagy has been highlighted based on the observation that defective autophagy plays a significant role in human pathologies. Nevertheless, autophagy plays a double-sided role in pathological states, and excessive autophagy is linked to cellular dysfunction, preceding cell death. Detrimental intracellular changes such as impaired mitochondrial function, oxidative stress, and accumulation of protein aggregates can result in autophagic cell damage.

Interestingly, impaired function of vascular cells can also be mediated by autophagy. Autophagy in endothelial cells was induced by methylglyoxal, a metabolite specific to diabetes. Despite the important role of EPCs in angiogenesis and vascular regeneration, studies regarding the autophagic regulation in EPCs are very limited. Wang et al. recently observed that hypoxia induced autophagy in EPCs, suggesting the role of autophagy in survival of the transplanted EPCs. To our best knowledge, the autophagic alteration in EPCs under pathological conditions has not been investigated.

Here, we demonstrated that pathological stress of high glucose (HG) condition increased the autophagic marker in bone-marrow derived EPCs (BM-EPCs), which is correlated to the functional impairment. Mitochondrial damage and generation of oxidative stress were increased in HG-exposed EPCs. With this study, we believe that an important evidence for the role of autophagy in EPCs under pathological state of diabetes has been provided.

MATERIALS AND METHODS

Materials  Fluorescein-isothiocyanate (FITC) conjugated lectin was obtained from Vector Laboratories. Acetylated low-density lipoprotein (ac-LDL) from human plasma Dil complex (Dil-ac-LDL), MitoSOX™ Red mitochondrial superoxide indicator (mitoSOX) and 5,5′,6′,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl carboxyanine iodide (JC-1), and 2,7′-dichlorofluorescein diacetate (DCF-DA) were from Invitrogen. Endothelial basal medium-2 (EBM-2) and its

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supplements (EGM-2 Bulletkit) were purchased from Lonza Inc. Growth factor reduced Matrigel was purchased from BD Biosciences, and antibodies against LC3-II and β-actin were obtained from Sigma. Antibody against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was from Millipore. All other reagents were used at the highest available purity.

**Isolation and Characterization of BM-EPCs** All animal procedures were performed according to the guidelines of the Hanyang University Institutional Animal Care and Use Committee (IACUC). BM-EPCs were isolated from tibia and femur, cultured and characterized as previously described.13) After erythrocytes were removed, BM-derived mononuclear cells were washed and cultivated in EGM-2. BM-EPCs were characterized by acLDL and lectin. To establish high glucose condition, excessive 24.5 mM of glucose was added to EGM-2 (5.5 mM glucose) after the initial isolation of BM-EPCs.

**In Vitro Tube Formation Assay** The capacity of EPCs forming tubule-like network was determined using Matrigel.5,13) In brief, Matrigel was solidified, and EPCs were added to Matrigel at 5×10⁴ cells/well. After 10h, the tubes were observed under an inverted microscope (Nikon).

**Reactive Oxygen Species (ROS)** The intracellular and mitochondrial ROS levels in EPCs were evaluated using DCF and mitoSOX by flow cytometry, respectively.14) EPCs were detached by trypsinization and incubated with DCF (20 µM) or mitoSOX (5 µM) for 20 min at 37°C in dark. After incubation, the cells were measured in Guava (Millipore), and analyzed by Guava software 2.6. Cells were considered to be positive when fluorescence intensity was >95% of the signal from NG-exposed control group.

**Western Blot** EPCs were lysed with radioimmunoprecipitation assay (RIPA) lysis and extraction buffer containing protease inhibitor cocktail (Pierce). Thirty microgram/lane were used for standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Western blots were performed by using primary antibodies directed against LC3-II, GAPDH, and β-actin. Bands were visualized with HRP-conjugated secondary antibodies and chemiluminescent substrates (Pierce). Luminescence were captured in ChemiDoc (Bio Rad) and quantified with ImageJ software.

**Quantitative Real Time PCR (qRT-PCR)** Total RNA was extracted from cultured EPCs using RNeasy mini kit (Qiagen), and synthesized cDNA using TaqMan reverse transcription kit (Applied Biosystems). 18S, for: 5′-ACC GCA GCT AGG AAT AAT GGA-3′; rev: 5′-GCC TCA GTT CCG AAA ACC A-3′; and manganese superoxide dismutase (MnSOD), for: 5′-CAC ATT AAC GCG CAG ATC ATG-3′; rev: 5′-CCA GAG CCT CGT GGT ACT TCT C-3′. Quantification of gene copies was carried on the 7500 Real-Time PCR system, using Power SYBR Green master mix (Applied Biosystems). Relative mRNA expressions were calculated by the comparative Ct method, normalized to the endogenous 18S control.

**Mitochondrial Membrane Potential** Changes in mitochondrial membrane potential (Δψm) were determined with the cationic fluorochrome, JC-1, as previously described.15) HG-exposed BM-EPCs were incubated with JC-1 (5 µM) for 20 min

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![Fig. 1. HG-Induced Autophagy in BM-EPCs](image)

(A) BM-EPCs were characterized by triple staining in fluorescence microscopy. (B) Matrigel tube formation were examined in BM-EPCs cultured under normal glucose (NG: 5.5 mM glucose) or high glucose (HG: 30 mM) condition for 7d. The representative photos were shown. (C) After BM-EPCs were cultured for 7d in NG or HG containing media, the conversion from LC3-I to LC3-II or level of GAPDH were examined by Western blot. (A) n=3, scale bar: 100 µm, (B) n=3, scale bar: 500 µm, (C) n=3–5, *p<0.05, **p<0.01 NG vs. HG. Data were expressed as mean±S.E.M. and analyzed using Student’s t-test.
at 37°C. Fluorescence signal from JC-1 aggregates (red) or JC-1 monomers (green) was measured by flow cytometry.

Statistics All obtained values were expressed as mean±S.E.M. Statistical analysis between groups was performed using the Student’s t-test using SPSS. In all cases, a p value of <0.05 was considered significant.

RESULTS

BM-EPCs were characterized by the triple staining of 4',6-diamidino-2-phenylindole (DAPI), the uptake of acetylated LDL and lectin binding (Fig. 1A). Around 90% of cells showed triple positive fluorescence. The morphological shape and the functional ability of tube formation further supported the unique characteristics of EPC population. To investigate the effects of hyperglycemic environment, BM-EPCs were cultured for seven days in normal (NG; 5.5 mM) or high glucose (HG; 30 mM)-containing media. While normal BM-EPCs were capable to form tubule-like network, the number of the tube was significantly decreased in EPCs under HG condition, suggesting that HG-environment impaired the angiogenic function of EPCs (Fig. 1B). There is no difference in EPC function under normo- or hyper-osmotic condition where isosmotic mannitol was added, suggesting that EPC dysfuncition under HG condition is from osmotic pressure (data not shown).

To investigate if autophagic processes occur in BM-EPCs under HG condition, we measured the conversion of LC3-I (microtubule-associated protein 1 light chain 3 form I) to LC3-II, a representative autophagosomal marker. The level of LC3-II was significantly increased in HG-exposed EPCs (Fig. 1C). Interestingly, the protein level of GAPDH, a glycolytic enzyme which is related to autophagic induction in hyperglycemia, was also increased in EPCs under HG condition (Fig. 1C), supporting the autophagic pathways are activated by high glucose in BM-EPCs.

In EPCs, the intrinsic activities of several antioxidant enzymes including MnSOD are higher than other vascular cells, playing key roles in protecting these angiogenic cells against ROS. A recent paper showed that reduced MnSOD activity may be critical in diabetic EPC dysfunction. We investigated if mRNA level of MnSOD in EPCs is decreased under HG condition. The mRNA level of MnSOD was significantly reduced in HG-exposed EPCs (Fig. 2A), suggesting that HG-exposed EPCs may be susceptible to oxidative stress. Notably, the intracellular level of ROS was significantly increased in HG-exposed EPCs (Fig. 2B).

Increased oxidative stress and mitochondrial damage are known to be the important mediators in pathological autophagy. We observed that mitochondrial ROS generation was enhanced in BM-EPCs under HG condition (Fig. 2C), leading to mitochondrial dysfunction as measured by JC-1, which accumulates in mitochondria as red fluorescent aggregates at intact Δψ_m, but exists in green fluorescent monomeric form at decreased Δψ_m (Fig. 2D).

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Fig. 2. Increased Oxidative Stress and Mitochondrial Damage in HG-Exposed BM-EPCs

BM-EPCs were cultured for 7 d in NG or HG containing media. (A) The mRNA level of MnSOD was analyzed by qRT-PCR. (B, C) Intracellular level of cellular (B) or mitochondrial (C) oxidative stress was measured with DCF fluorescence or mitoSOX, respectively, by flow-cytometry. The representative histograms were shown. (D) The change of membrane potential was determined using JC-1. (A) n=3, (B) n=4, (C) n=3, (D) n=6, *p<0.05 NG vs. HG. Data were expressed as mean±S.E.M. and analyzed using Student’s t-test.
DISCUSSION

There is growing interest in the role of autophagy in biological systems. Autophagy has been basically considered as a normal regulatory process for metabolism, growth, and proliferation, allowing cells to survive under nutritional starvation. Recently, it has been known that this regulatory process involves in not only normal cell function, but also autophagic cell damage contributing to the pathogenesis of diverse diseases including aging, cancer, stroke and diabetes. In the present study, we demonstrated that autophagic process occurs in EPC under HG condition. To our best knowledge, this is the first study showing that autophagy could play a key role in EPC function under pathological state such as diabetes. Increased cellular oxidative stress and mitochondrial ROS were observed, along with the decreased mRNA level of MnSOD. With this study, we believe that an important clue for the role of autophagy in EPC dysfunction under diabetes has been provided.

The most novel finding of this study is that over-nutritional environment such as hyperglycemia may lead to autophagy in pathological condition. The traditional concept of autophagy is related to the environmental stress from deprivation of nutrients or growth factors. Here we suggest that excessive intracellular stress resulted from hyper-glucose state may lead to autophagy as a process of cellular damage. A recent review raised an issue regarding alteration of ‘nutrient-sensing’ pathways and subsequent dysregulation of cellular function under diabetic states, focusing on the diabetic nephropathy. The exact role and the regulation of autophagy in diabetes are still controversial. While it is reported that autophagic cell death was enhanced in type-2 diabetes pancreatic beta cells, most studies claimed that impaired autophagy was associated with the accumulation of dysfunctional or damaged cellular components, contributing to cellular dysfunction. In our present study, we reported the increased autophagy in hyper-glycemic environment. The exact role of autophagy in diabetic EPC dysfunction may need to be further elucidated in detail.

It is well established that the cellular responses to stress can modulate autophagy, and increased intracellular stresses such as oxidative stress, ER stress, and mitochondrial damage enhance autophagy. Interestingly, the intrinsic level of antioxidant enzymes including MnSOD is higher in EPCs than other cells, allowing these cells more resistant to oxidative stress, and a loss of tolerance to oxidative stress can play key roles in EPC dysfunction. Here we reported that cellular or mitochondrial ROS generation was increased in HG-exposed EPCs. These observations are consistent with previous literatures, where EPCs in diabetic patients and animal models showed higher oxidative stress. Inhibition of oxidative stress has reversed diabetic EPC dysfunction, suggesting that increased ROS plays key roles in diabetic vascular impairment. The increased level of ROS in EPCs was also detected under HG condition, and here we further demonstrated the enhanced mitochondrial ROS and disruption of mitochondrial membrane potential in HG-exposed EPCs, suggesting its role in HG-induced autophagy in EPCs. Mitochondrial ROS generation and subsequent mitochondrial oxidative damage may modulate mitophagy, a form of micro-autophagy, potentially contributing to autophagic cell death.

In conclusion, we demonstrated increased ROS generation, mitochondrial damage and following autophagic induction in EPCs under HG condition. With this study, we believe that a novel insight into the role of HG-induced autophagy in EPC dysfunction is provided, with an important explanation about the oxidative stress-mediated mitochondrial damage and angiogenic dysfunction in diabetes.

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