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## Restoration of Autophagy in Endothelial Cells from Patients with Diabetes Mellitus Improves Nitric Oxide Signaling

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### Abstract

**Background**—Endothelial dysfunction contributes to cardiovascular disease in diabetes mellitus. Autophagy is a multistep mechanism for removal of damaged proteins and organelles from the cell. Under diabetic conditions, inadequate autophagy promotes cellular dysfunction and insulin resistance in non-vascular tissue. We hypothesized that impaired autophagy contributes to endothelial dysfunction in diabetes mellitus.

**Methods and Results**—We measured autophagy markers and endothelial nitric oxide synthase (eNOS) activation in freshly isolated endothelial cells from diabetic subjects (n=45) and non-diabetic controls (n=41). p62 levels were higher in cells from diabetics (34.2±3.6 vs. 20.0±1.6, P=0.001), indicating reduced autophagic flux. Bafilomycin inhibited insulin-induced activation of eNOS (−21±5% vs. 64±22%, P=0.003) in cells from controls, confirming that intact autophagy is necessary for eNOS signaling. In endothelial cells from diabetics, activation of autophagy with spermidine restored eNOS activation, suggesting that impaired autophagy contributes to endothelial dysfunction (P=0.01). Indicators of autophagy initiation including the number of LC3-bound puncta and beclin 1 expression were similar in diabetics and controls, whereas an autophagy terminal phase indicator, the lysosomal protein Lamp2a, was higher in diabetics. In endothelial cells under diabetic conditions, the beneficial effect of spermidine on eNOS activation was blocked by autophagy inhibitors bafilomycin or 3-methyladenine. Blocking the terminal stage of autophagy with bafilomycin increased p62 (P=0.01) in cells from diabetics to

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### Author Contributions

J.L.F. conducted experiments, wrote the manuscript. M.H. recruited participants. N.F. recruited participants and carried out patient visits. B.F. carried out patient visits and conducted several experiments. R.B.F. reviewed/edited the manuscript. E.A.L., B.D.B., and M.A.D. performed vascular testing and aided in patient visits. M.G.F., N.G., and O.S.S. reviewed/edited the manuscript. J.A.V. and N.M.H. conceived the overall experimental strategy and design and directed the studies.

### Disclosures

None.

a lesser extent than in cells from controls ( $P=0.04$ ), suggesting ongoing, but inadequate autophagic clearance.

**Conclusion**—Inadequate autophagy contributes to endothelial dysfunction in patients with diabetes and may be a target for therapy of diabetic vascular disease.

### Keywords

autophagy; endothelial cells; diabetes mellitus

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Subjects with type 2 diabetes mellitus have increased risk for cardiovascular disease that persists despite aggressive control of glucose, cholesterol, and blood pressure.<sup>1, 2</sup> Endothelial dysfunction contributes to the pathogenesis of cardiovascular disease in diabetes, and an improved understanding of the responsible mechanisms could lead to new approaches for therapy.<sup>3</sup> Diabetes is associated with excess production of reactive oxygen species by complexes of the mitochondrial electron transport chain and other enzymes in endothelial cells that decrease the bioactivity of nitric oxide, activate pro-inflammatory signaling pathways, and cause damage to cellular proteins and organelles.<sup>4</sup> Since damaged mitochondrial enzymes produce more oxidants, failure of quality control mechanisms could exacerbate oxidative stress and cellular dysfunction.<sup>5, 6</sup>

As is illustrated in Figure 1, autophagy is a multistep mechanism for the clearance of damaged proteins and organelles from the cell.<sup>7, 8</sup> Components are tagged for degradation through ubiquitination and are linked to LC3 through the adapter protein p62. During initiation of autophagy, LC3-I is lipidated to form LC3-II, which targets the damaged material to the developing double-membrane autophagosome. Beclin 1, the mammalian homolog of yeast Atg6, is part of the lipid-PI3 kinase complex that coordinates autophagosome formation.<sup>9, 10</sup> The autophagosome fuses with a lysosome to produce the autophagolysosome, a process that is mediated in part by SNARE proteins, including Rab7 and Lamp2a.<sup>11, 12</sup> The contents of the autophagolysosome are degraded by cathepsins. p62 is degraded along with the targeted organelles and proteins, while LC3 may be degraded or recycled back into the cytosol.<sup>7, 8</sup>

The pattern of autophagy proteins in cells reflects the state of autophagy. Activation of autophagy results in the accumulation of LC3-bound puncta, consistent with conversion of LC3-I to LC3-II, and decreased p62 reflecting degradation in the autophagolysosome. Impairment of autophagy at the initiation stage is characterized by a loss of LC-3-bound puncta, decreased LC3-II, and increased p62. Failure of the terminal phases of autophagy, including autophagosome-lysosome fusion or cargo degradation, is characterized by a normal or increased number of puncta, increased LC3-II, and increased p62 in the cell, reflecting an inability to clear autophagosomes and degrade p62.

Recent studies link autophagy and diabetes mellitus. Activators of autophagy such as exercise and calorie restriction improve insulin sensitivity.<sup>7, 8</sup> Reduced autophagy contributes to insulin resistance in traditional insulin-responsive tissues such as liver, skeletal muscle, and adipose tissue.<sup>13, 14</sup> In pancreatic beta cells, diabetic conditions are associated with inadequate autophagic clearance and lysosome function leading to impaired insulin

secretion.<sup>15</sup> Little is known, however, about autophagy in vascular tissue or its contribution to diabetic vascular disease, particularly in human subjects. In the present study, we hypothesized that impaired autophagy contributes to endothelial dysfunction in diabetes. We sought to determine the state of autophagy in freshly isolated endothelial cells from patients with autophagy and to evaluate whether autophagy contributes to endothelial insulin signaling and nitric oxide production.

## METHODS

### Study Subjects

We enrolled adults with type 2 diabetes mellitus, defined as fasting glucose  $\geq 126$  mg/dL, hemoglobin A1C  $\geq 6.5\%$  or ongoing treatment for diabetes. Similarly-aged non-diabetic adults with fasting glucose below 100 mg/dL served as controls. Each subject made a study visit for collection of blood and endothelial cells and non-invasive testing of endothelial function. Subjects fasted overnight prior to the visit. Vasoactive medications were withheld for 24 hours prior to study. Blood glucose and lipid levels were measured in the Boston Medical Center Clinical Laboratory. The protocol was approved by the Boston Medical Center Institutional Review Board and all study subjects provided written informed consent.

### Vascular Function Testing

Brachial artery flow-mediated vasodilation was measured as previously described.<sup>16</sup> In brief, vascular ultrasound was used to measure brachial artery diameter and blood flow velocity before and one minute following induction of reactive hyperemia by five minutes of upper arm occlusion with a narrow gauge blood pressure cuff. Hyperemic flow increases shear stress at the endothelial surface, activates endothelial nitric oxide synthase (eNOS), and induces dilation of the conduit brachial artery. We also assessed non-endothelium dependent dilation by measuring brachial diameter before and three minutes after sublingual nitroglycerin (0.4 mg).

### Venous Endothelial Cell Biopsy and Cell Activation Experiments

Peripheral vein endothelial cells were collected as previously described.<sup>17-20</sup> Briefly, a 0.018 inch J-wire (Arrow International, Reading, PA) was inserted into a forearm vein through a 20 or 22 gauge intravenous catheter and used to gently rub the endothelial surface. Endothelial cells were recovered from the wire in red blood cell lysis/dissociation buffer, centrifuged, and fixed onto poly-L-lysine coated slides (Sigma, St. Louis, MO) using 4% paraformaldehyde. Slides were dried, and frozen at  $-80^{\circ}\text{C}$  prior to staining and immunofluorescence imaging to measure protein expression as described below. Under all conditions with the exception of spermidine treated cells, freshly isolated venous endothelial cells from the human subjects were fixed within two hours following biopsy. For the spermidine experiment, endothelial cells were treated with 3 mM spermidine or vehicle (endothelial growth medium-2 without growth factors) for 6 hours following which the cells were incubated an additional 18 hours in media with 5% serum.

For some experiments, insulin-mediated eNOS activation was examined by measuring the levels of phosphorylated eNOS (serine 1177) in cells treated with 10 nM insulin (Sigma

Aldrich, St. Louis, MO) or vehicle (endothelial growth medium-2 without growth factors, Lonza Inc, Walkersville, MD) for 30 minutes prior to fixation. We also performed insulin stimulation experiments and measured expression of autophagy proteins after incubation with 10 nM bafilomycin A<sub>1</sub> (LC Laboratories, Woburn, MA) for 60 minutes to inhibit autophagy by blocking autophagolysosome acidification, or 18 hours after 6 hours of spermidine (3 mM) treatment to globally activate autophagy. Finally, we activated eNOS with 10 nM insulin for 30 minutes and measured nitric oxide production using the fluorescent probe DAF-2DA (EMD Millipore, Billerica, MA) as previously described.<sup>21</sup>

### Quantification of Protein Levels in Freshly Isolated Endothelial Cells

Protein levels in freshly isolated endothelial cells were quantified using immunofluorescence microscopy.<sup>18–20</sup> Slides were stained with one of the following primary antibodies for one hour, unless otherwise indicated: p62 (1:100 dilution; Abnova, Cambridge, MA), beclin 1 (1:300 dilution; Novus Biologicals, Littleton, CO), LC3B (1:100; Abcam, Cambridge, MA), phosphorylated Ser1177 eNOS (1:200 dilution; Millipore), eNOS (1:100 dilution; BD Transduction Laboratories, San Diego, CA), Rab7 (1:100 dilution for 4 hours; Abcam), Lamp2a (1:100 dilution; Abcam), Atg7 (1:200 dilution; Sigma Aldrich, St. Louis, MO) and acetylated Lys9 histone 3 (1:100 dilution for 4 hours; Cell Signaling, Beverly, MA). The fluorescence intensity was quantified in 20 endothelial cells and averaged for each condition. To control for batch-to-batch variability, fluorescence intensity was normalized to the intensity in human aortic endothelial cells (HAECs), which were stained simultaneously. Final intensity was calculated by dividing the average fluorescence intensity for the subject sample by the average fluorescence intensity of the HAEC sample and multiplying by 100. The intensity is expressed in arbitrary units (AU). All quantification was performed blinded to subject identity and diabetes status. Confocal images of selected proteins assessed by immunofluorescence were taken for illustrative purposes using a Leica SP5 microscope (Solms, Germany).

### Western Blots

Following lysis of HAECs in RIPA buffer containing proteinase and phosphatase inhibitors, protein levels were quantified utilizing BioRad's (Hercules, CA) Dc Protein assay, per manufacturer instructions. For all Western blots, 30 µg of protein was loaded onto 4–20% gradient gels and run at 100 mV, transferred to PVDF membranes, and blocked in 3% BSA/TBS-T for 30 minutes. The blots were probed against LC3A/B (1:1000 dilution; Abcam, Cambridge, MA) and β-actin (1:1000 dilution; Cell Signaling, Danvers, MA) overnight in 3% BSA/TBS-T at 4°C. All blots were imaged using a LAS-4000 Luminescent Image Analyzer by Fuji (Tokyo, Japan) and data was normalized to β-actin.

### Cell Culture

To gain further insight into the links between altered autophagy and eNOS signaling in diabetes, we used HAECs exposed to physiological (5 mM) and elevated (30 mM) glucose concentrations for 24 hours. HAECs were purchased from Lonza, Inc and maintained with the EGM-2 bullet kit containing 5 mM glucose. Expression of autophagy proteins and insulin-mediated activation of eNOS was assessed by immunofluorescence, as described above. To block autophagy and to probe the level of autophagic flux, HAECs were incubated

for 60 min with 10 nM bafilomycin A<sub>1</sub>.<sup>22, 23</sup> To determine whether activating autophagy would reverse glucose-induced endothelial dysfunction, HAECs were incubated with 30 mM glucose in EGM-2 media for 24 hours and then treated with 3 mM spermidine (Sigma Aldrich) for six hours followed by 18 additional hours of 30 mM glucose exposure before fixing cells for protein expression or examining insulin-induced eNOS activation as described above. In addition, insulin-induced eNOS activation was assessed following treatment with autophagy inhibitors bafilomycin A<sub>1</sub> (10 nM) or 3-methyladenine (3-MA; 10 mM; Sigma Aldrich) during insulin stimulation for one hour following spermidine treatment. Spermidine activates autophagy via a general increase in the expression of autophagy-related genes, which was confirmed by RT-qPCR, as described below.

### Assessment of LC3-bound Puncta

In addition to measuring LC-3 protein levels in freshly isolated endothelial cells, we also assessed autophagy status by counting the number of LC3-bound puncta, which reflects autophagosome formation. For HAEC's, we counted the number of cells with more than twenty LC3-bound puncta per cell. Cells were stained with antibody against LC3B (1:100; Abcam, Cambridge, MA) as described above. An investigator blinded to subject status or cell culture condition counted puncta in twenty cells calculated the mean. To assess reproducibility, puncta count was performed twice in a blinded fashion (n=10). The coefficient of variation was 2.4%.

### Endothelial Cell Gene Expression

For gene expression studies, HAECs were trypsinized and the cell pellets were prepared per manufacturer's instructions for gene expression using the Fastlane Cell cDNA kit (Qiagen, Germantown, MD) and stored at -80°C until processed further. RNA synthesis was completed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) and quantitative real-time PCR reactions were performed using TaqMan gene expression assays (Applied Biosystems). Data were normalized to GAPDH and compared using the  $\Delta\Delta C_t$  method. Gene expression results are expressed as fold change relative to the control group as indicated in the figure legend.

### Statistical Analysis

Statistical analyses were carried out using SPSS version 19.0 (IBM Corp, Armonk, NY). Data are expressed as mean±standard deviation unless otherwise noted. Variables were evaluated for normality by the Shapiro-Wilk test. Variables with normal distribution were compared using unpaired t-test, paired t-tests, or chi-square tests for two-group comparisons or repeated-measures ANOVA for multi-treatment comparisons. Variables that were not normally distributed were compared with the Mann-Whitney U test to compare diabetics and controls or the Wilcoxon Signed Rank test for paired samples. Spearman correlation coefficients were used to correlate clinical characteristics, measures of vascular function, and expression of autophagy proteins. A two-tailed  $P<0.05$  was considered to be statistically significant.

## RESULTS

### Study Subjects and Vascular Function

We enrolled 45 subjects with diabetes mellitus and 41 controls. As shown in Table 1, the two groups were similar in age and gender. There was a modest preponderance of black subjects in the diabetic group ( $P=0.047$ ). As expected, the diabetic subjects were taking medications to lower blood glucose and had metabolic abnormalities, including higher fasting glucose, hemoglobin A1C, triglycerides, and body mass index. Diabetics had lower total and LDL cholesterol levels, likely reflecting the greater use of cholesterol-lowering medications.

In regard to vascular function, brachial artery flow-mediated dilation was lower in the diabetics ( $P<0.001$ ), suggesting conduit artery endothelial dysfunction. Nitroglycerin-mediated dilation also was lower ( $P=0.047$ ) suggesting a concurrent defect in the vasodilator function of vascular smooth muscle.

### Expression of Autophagy Proteins in Endothelial Cells from Study Subjects

As shown in Figure 2A, p62 levels were higher in endothelial cells isolated from subjects with diabetes compared to controls ( $34.2\pm 3.6$  vs.  $20.0\pm 1.6$ ,  $P=0.001$ ), suggesting an overall impairment of autophagy.

As shown in Figure 2B, the number of LC3-bound puncta ( $19\pm 2$  vs.  $22\pm 2$  per cell,  $P=0.28$ ) and total LC3 levels ( $35.2\pm 6.0$  vs.  $38.1\pm 4.2$  AU,  $P=0.76$ ) did not differ between the diabetics and the non-diabetics, respectively, suggesting that impaired autophagosome formation does not explain the impairment of autophagic flux. As shown in Figure 3A, beclin 1 levels did not differ between diabetics and non-diabetics ( $57.7\pm 8.9$  vs.  $48.1\pm 6.3$  AU,  $P=0.52$ ), further arguing against a defect in the initiation stage of autophagy, which would be expected to be associated with lower beclin 1 expression.<sup>10</sup> In contrast, Atg7 levels were higher in endothelial cells from diabetic subjects compared to non-diabetic subjects ( $67.2\pm 10.7$  vs.  $44.6\pm 4.2$  AU,  $P=0.03$ ) (Figure 3B).

In addition, expression of the lysosomal protein Lamp2a, which plays a role in autophagosome-lysosome fusion, was higher in the endothelial cells from diabetic subjects compared to non-diabetics ( $16.7\pm 1.6$  vs.  $11.8\pm 1.2$  AU,  $P=0.03$ , Figure 3D). Expression of Rab7a did not differ between groups ( $25.5\pm 3.3$  vs.  $23.1\pm 3.6$  AU,  $P=0.65$ , Figure 3C). Together these findings suggest that there may be a compensatory alteration in factors regulating autophagy in the diabetic endothelium.

### Effects of High Glucose Concentration on the Expression of Autophagy Proteins

The pattern of autophagy protein expression in HAEC's exposed to 30 mM glucose mimicked the pattern observed in endothelial cells freshly isolated from subjects with type 2 diabetes mellitus. As shown in Figure 4A, in HAEC's exposed to 30 mM glucose, p62 expression was higher ( $P<0.001$ ) whereas the ratio of LC3II (activated form of LC3) to LC3I (inactive form of LC3) ( $P=0.85$ ) was similar (Figure 4B). The number of LC3 puncta ( $P=0.89$ ) and total LC3 expression ( $P=0.88$ ) were also similar in cells exposed to high and physiological glucose levels (Supplemental Figure 1). As shown in Figure 4C, gene

expression of LAMP2A (P=0.03) and cathepsin B (P=0.001) message was higher and there was a trend for higher Rab7a (P=0.06) in cells exposed to 30 mM glucose. In contrast, expression of beclin 1 and LC3 did not differ between normal and high glucose. In addition, lysosome number (P=0.99) and acidification (P=0.67) were similar in HAEC's exposed to high and physiological glucose levels (Supplemental Figure 2A and Figure 2B, respectively). As in the cells from diabetic subjects, these data are consistent with a possible alteration of the terminal phase or overall level of autophagy in HAEC's exposed to high glucose.

### **Inhibiting Autophagy Induces Endothelial Dysfunction**

As shown in Figure 5A, insulin-induced eNOS activation was profoundly impaired in endothelial cells isolated from diabetic subjects compared to non-diabetics ( $-21.7 \pm 5.1\%$  vs.  $64 \pm 22\%$ , respectively, P=0.003). This decrease in eNOS activation was likely not due to differences in total eNOS levels as we have previously reported similar levels of eNOS in endothelial cells from non-diabetics and diabetics.<sup>18</sup> Bafilomycin inhibits the terminal phases of autophagy by inhibiting lysosomal acidification and autophagosome-lysosome fusion, thus preventing degradation of autophagolysosome contents.<sup>22, 23</sup> In cultured endothelial cells, LysoSensor levels were reduced with bafilomycin treatment under glucose conditions confirming previous studies suggesting that bafilomycin inhibits lysosome acidification (5mM, P=0.002) (30mM glucose, P=0.04) (Supplemental Figure 2B).

Incubation of cells from non-diabetic subjects with bafilomycin for 60 minutes (Figure 5B), increased p62 ( $20.7 \pm 7.9$  to  $29.9 \pm 4.2$  AU, P=0.01), consistent with an inhibition of autophagy. Bafilomycin treatment inhibited insulin-induced eNOS activation ( $64.7 \pm 22\%$  to  $-47.8 \pm 8.1\%$ , P=0.04), producing a phenotype similar to cells from diabetic subjects (Figure 5C versus 5A). As shown in Supplemental Figure 3, bafilomycin did not change the basal level of eNOS phosphorylation ( $15.9 \pm 3.2$  vs.  $13.7 \pm 0.8$  AU, P=0.89).

### **Activating Autophagy Reverses Endothelial Dysfunction in Diabetes**

Spermidine is a polyamine compound that activates autophagy through deacetylation of specific residues on histone 3 leading to the upregulation of genes related to multiple stages of the autophagic process.<sup>10, 24-26</sup> As shown in Supplemental Figure 4A, 3 mM spermidine incubation for 6 hours decreased nuclear levels of acetylated Lys9 histone 3 (P=0.004) in HAEC's exposed to 30 mM glucose. Under these conditions, spermidine also increased expression of multiple autophagy genes, including Atg5, Atg12, beclin 1, BNIP3L, p62, and PINK (all P<0.05) with trends (P<0.10) for increased ATG4B, cathepsin B, Rab7a, and LAMP2A (Supplemental Figure 4B).

In endothelial cells from diabetic patients, treatment with spermidine improved insulin-mediated eNOS phosphorylation (P=0.01, Figure 6A), consistent with the possibility that augmenting autophagy improves eNOS activation in diabetes. Similarly, spermidine treatment reversed the impairment in insulin-mediated eNOS phosphorylation induced by high glucose exposure in HAEC's (P=0.04, Figure 6B vs Supplemental Figure 5A and 5B). Co-treatment with autophagy inhibitors bafilomycin or 3-MA prevented the beneficial effect of spermidine on eNOS activation in HAEC's exposed to high glucose suggesting that the

effects of spermidine on eNOS signaling are mediated through autophagy (Figure 6B). Further, activating autophagy with spermidine restored insulin-induced nitric oxide production ( $P=0.04$ , Figure 6C) in HAEC's exposed to high glucose, suggesting that enhanced eNOS activation was associated with higher nitric oxide bioavailability. Spermidine had no significant effect on eNOS phosphorylation ( $P=0.72$ ) or nitric oxide production ( $P=0.72$ ) in cells exposed to 5 mM glucose arguing against a non-specific effect (Supplemental Figures 6A and 6B, respectively). Collectively, these findings suggest that activation of autophagy with spermidine reverses endothelial dysfunction associated with diabetes and thus implicates impaired autophagy as a mechanism of endothelial dysfunction in this setting.

As shown in Supplemental Figure 6D, total eNOS protein expression increased following spermidine incubation in cells exposed to 30 mM glucose ( $P=0.03$ ) but not 5 mM glucose ( $P=0.23$ ). As shown in Supplemental Figure 6C, this change in total eNOS protein was accompanied by an increase in basal (unstimulated) phosphorylated eNOS ( $P=0.04$ ). These changes would not explain our findings, since an increase in the basal level of phosphorylated eNOS or NO production would tend to decrease rather than increase the insulin-induced changes in these measures of endothelial function.

### Diabetes is Associated with Inadequate Autophagic Flux

Bafilomycin blocks lysosome acidification and autophagolysosome maturation.<sup>22, 23</sup> Since p62 is normally degraded along with the contents of the autophagosome, monitoring p62 levels following bafilomycin exposure provides insight into the state of autophagic flux. If the terminal stages of autophagy are completely blocked, bafilomycin should have no effect on p62 levels. In contrast, an increase in p62 following bafilomycin exposure under diabetic conditions would support the contention that autophagolysosome maturation and clearance are ongoing, but inadequate. As shown in Figure 7A, bafilomycin increased p62 levels in endothelial cells from diabetic subjects ( $P=0.01$ ), providing support for the latter mechanism of impaired autophagic flux. However, the increase in p62 following bafilomycin treatment was lower in endothelial cells from subjects with diabetes as compared to controls (Figure 7B) consistent with lower autophagic flux in the diabetic endothelium ( $P=0.04$ ).

### Clinical Characteristics, Vascular Function, and Autophagy Proteins

As noted, there was a modest preponderance of black subjects in the diabetic group. After adjusting for black race, the observed differences in p62 ( $P<0.001$ ), Lamp2A ( $P=0.04$ ), and insulin-mediated eNOS phosphorylation ( $P=0.002$ ) remained statistically significant, suggesting that race does not account for the findings.

When adjusting for the use of renin-angiotensin system inhibitors, the differences in flow-mediated dilation and autophagy (p62) remain significant between control and diabetic subjects ( $P<0.001$ ,  $P=0.002$ , respectively).<sup>27</sup>

Finally, we investigated whether vascular function related to autophagy proteins in freshly isolated endothelial cells. There was a trend for an inverse correlation between p62 and reactive hyperemia ( $R= -0.42$ ,  $P=0.096$ ). There were no significant correlations between the other measures of vascular function and autophagy proteins.



## DISCUSSION

The present study provides evidence that inadequate autophagy contributes to endothelial dysfunction in diabetes. We observed higher p62 levels in endothelial cells from diabetics, indicating impaired autophagy. The lack of a difference in LC3 puncta count in cells from diabetics and non-diabetic controls and in cells exposed to high and low glucose suggests that the initiation phase is intact, while the increased levels of Lamp2a and cathepsin B under diabetic conditions implicates a defect at the terminal phase of autophagy. The finding that bafilomycin increases p62 to a lesser extent in cells from diabetics compared to non-diabetics suggests that autophagic clearance is ongoing, but reduced. We also showed a link between autophagy and endothelial function. Inhibiting autophagy in cells from healthy controls impairs eNOS activation to an extent comparable to that observed in cells from diabetics. In endothelial cells from patients with diabetes, activation of autophagy with spermidine restores insulin-mediated eNOS activation in endothelial cells from patients with diabetes and enhanced nitric oxide production in cultured endothelial cells in high glucose conditions. Restoration of eNOS activation by spermidine in cultured endothelial cells was blocked by autophagy inhibitors, bafilomycin and 3-MA, suggesting an autophagy-dependent mechanism. Taken together, these findings implicate inadequate autophagic flux as a mechanism of endothelial dysfunction in diabetes.

Prior studies have shown disordered autophagy in traditional insulin target tissues in experimental models diabetes. In the liver, mouse models of insulin resistance and obesity are associated with inadequate autophagy, and inhibiting autophagy by genetic deletion of Atg7 or Atg5 induces insulin resistance.<sup>28, 29</sup> As was recently reviewed, inadequate autophagy also contributes to ectopic fat deposition, including the development of hepatic steatosis.<sup>30</sup> In skeletal muscle, exercise prevents the development of insulin resistance and associated metabolic abnormalities in mice consuming a high fat diet, but the benefits of exercise are blocked in a genetically modified mouse model with an inability to induce autophagy in response to stress.<sup>31</sup> In adipose tissue, autophagy is required for adipogenesis and, interestingly, excess autophagy may contribute to excessive release of free fatty acids from lipid droplets and promote cellular toxicity.<sup>30</sup> In pancreatic islet cells, inadequate autophagic flux and abnormalities of lysosomal function under diabetic conditions reduce insulin secretion and, thus, may contribute to the pathogenesis and progression of type 2 diabetes.<sup>8, 15, 32</sup>

In the present study, we now provide evidence linking impaired autophagy to endothelial dysfunction in diabetes mellitus. Importantly, we demonstrate the presence of inadequate autophagy in vascular cells taken from patients with clinical diabetes. These findings add to the accumulating evidence that altered autophagy affects the endothelium in other disease states. Aging is strongly associated with endothelial dysfunction, and a recent study showed elevated p62 and reduced beclin-1 in freshly isolated endothelial cells from older subjects.<sup>10</sup> Those results suggest impairment in the initiation phase of autophagy in aging and potentially implicate a different mechanism from our findings in cells from diabetics. In that study, beclin-1 expression correlated with endothelium-dependent vasodilation in the forearm, further supporting the idea that impaired autophagy initiation relates to endothelial dysfunction in aging. In an animal model of arterial aging, impaired autophagy was

associated with endothelial dysfunction, and activators of autophagy, including spermidine and trehalose, restored endothelial dependent vasodilation, decreased oxidative stress, and attenuated inflammation.<sup>10, 33</sup> The beneficial effects of spermidine on vascular function and endothelial phenotype in this model were lost when Atg12, a key initiator of autophagy, was knocked down suggesting that the effects are autophagy-driven.<sup>10, 28</sup> The present study goes beyond the work of LaRocca and colleagues. In addition to showing differences in basal levels of autophagy proteins in freshly isolated endothelial cells, we studied functional responses to activators and inhibitors of autophagy to link inadequate autophagy to endothelial dysfunction in diabetes mellitus. Further, we demonstrate that induction of autophagy improves eNOS signaling in endothelial cells taken from patients with diabetes. We have previously reported in two published studies that eNOS activation measured by the phosphorylation of the activation site Ser1177 in endothelial cells freshly isolated from non-diabetics and diabetic subjects correlates with flow-mediated vasodilation. Thus, improvements in eNOS activation are linked to clinically relevant measures of endothelial function. Our work in the present study supports future studies to investigate whether systemic treatment with autophagy activators improve endothelial function in patients with diabetes.

In addition to eNOS activation, recent studies suggest that autophagy plays a protective role for other aspects of endothelial function. For example, activation of autophagy maintains cell viability in endothelial cells exposed hemin<sup>34</sup> and advanced glycation end-products<sup>35</sup>. Furthermore, the polyphenolic compound curcumin increased endothelial cell survival following hydrogen peroxide exposure in a manner that depended on activation of autophagy via inhibition of mTOR and activation of FOXO1.<sup>36</sup> Autophagy has been shown to regulate the release of von Willibrand factor from endothelial cells<sup>37</sup> and angiogenic activity as reflected by endothelial sprouting, proliferation, and tube formation.<sup>38, 39</sup> Finally, autophagy is important for clearance of oxidized low-density lipoprotein from endothelial cells, and thus may be relevant to atherogenesis.<sup>40</sup> Consistent with this possibility, p62 gene expression was higher in sections of human aorta containing fatty streaks compared to unaffected sections, although that investigation did not specifically examine the endothelium.<sup>41</sup> Thus, there is a growing body of evidence that autophagy is important for multiple aspects of endothelial function, but there has been a paucity of studies that examined these issues in human subjects.

Combining the present findings with the results of our recent studies using the same methodology suggests a mechanism to explain how inadequate autophagy may promote endothelial dysfunction. We previously observed higher nitrotyrosine levels and mitochondrial fragmentation in endothelial cells freshly isolated from subjects with type 2 diabetes and higher mitochondrial ROS production and mitochondrial fragmentation in endothelial cells exposed to high glucose.<sup>18, 19</sup> Abnormal insulin-induced eNOS activation under diabetic conditions was linked to increased oxidative stress and was reversed by a scavenger of reactive oxygen species and by inhibiting mitochondrial fusion, which dramatically reduces mitochondrial ROS production.<sup>18, 19</sup> As has been previously observed in pancreatic islet cells,<sup>42</sup> these results suggest a problem with mitochondrial quality control and impaired autophagic clearance, leading to the accumulation of dysfunctional organelles that produce excess ROS and promote endothelial dysfunction. Excess ROS production by

mitochondria has been shown to not only scavenge nitric oxide but also alter kinase signaling pathways that may be relevant in eNOS activation.<sup>4, 5</sup>

The observed pattern of autophagy protein expression (higher p62, unchanged LC3 puncta, increased Lamp2a) and the responses to spermidine (improved endothelial function) all suggest that autophagosome formation is intact or even increased and that there is a defect in the terminal phase of autophagy in endothelial cells under diabetic conditions. This situation may occur when there is a state of cellular stress with a high burden of damaged organelles and macromolecules, leading to activation of autophagosome formation and ongoing, but inadequate clearance from the cell. Our findings comparing the response to bafilomycin in diabetics compared to non-diabetics suggest that autophagic flux is lower in the diabetic endothelium and may be inadequate to clear the burden of organelles and macromolecules.

Our study has a number of limitations. The diabetic subjects had concomitant risk factors that might have contributed to our findings and the relatively modest sample size reduces the power to perform multivariable analyses to distinguish the relative importance of the metabolic risk factors associated with diabetes mellitus. We observed a trend for an inverse correlation between p62 levels and reactive hyperemia that might have been significant with a larger sample size. We examined venous endothelial cells rather than arterial endothelial cells, which may be more relevant to atherosclerosis. We point out, however, that venous endothelial cells are exposed to the same systemic risk factors in diabetes, and previous studies have shown correlated findings in arterial and venous endothelial cells collected with this methodology.<sup>17, 43</sup> Additionally, the number of endothelial cells obtained using the venous endothelial cell biopsy precludes the use of some methodology commonly utilized to study the mechanisms of autophagy impairment. As with all pharmacologic agents, spermidine likely has additional non-autophagy related cellular effects that may influence endothelial function. However, previous studies have shown that the beneficial effects of spermidine on vascular function appear to be specific to alterations in autophagy as knock down of Atg12 in endothelial cells in an aging model lose this beneficial effect.<sup>10, 42</sup> A concentration of 30 mM (540 mg/dL) was used to induce acute diabetic-like conditions in cultured HAEC's but acute higher glucose levels may differ in effect from the chronic elevations with greater variability observed in patients with diabetes. The range of fasting blood glucose in the diabetic subjects was 84–268 mg/dL (4.6 mM– 14.9 mM) and it is likely that post-prandial levels are considerably higher and may influence endothelial phenotype. Regulation of p62 levels involves both autophagic degradation and transcription modulation. Taken together with the findings using autophagy modulators, differential p62 expression in the diabetic endothelium appears to reflect inadequate autophagy. Finally, autophagy is a highly regulated and complex mechanism, and additional work will be required to determine the precise alterations in diabetes that influence eNOS signaling and other functions of the vascular endothelium.

In conclusion, we observed inadequate autophagy in endothelial cells isolated from subjects with diabetes mellitus and cultured cells exposed to a high glucose concentration. Furthermore, we showed that intact autophagy is required for eNOS signaling in cells from healthy subjects and that generalized activation of autophagy reverses endothelial dysfunction under diabetic conditions in cells exposed to high glucose. These findings

suggest that inadequate autophagy is a contributing mechanism for endothelial dysfunction in diabetes mellitus. Given the importance of endothelial dysfunction for the pathogenesis and clinical expression of atherosclerotic cardiovascular disease, these results are likely to be clinically relevant.<sup>44</sup> Interventions directed toward restoring normal autophagic flux, particularly therapies directed toward the terminal phases of autophagy and autophagic clearance may have therapeutic potential.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

### Sources of Funding

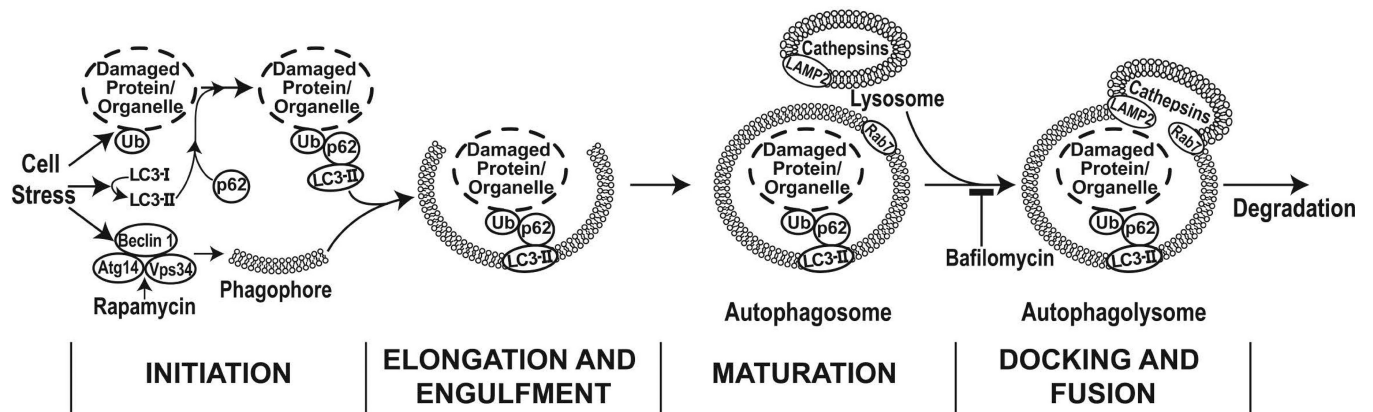
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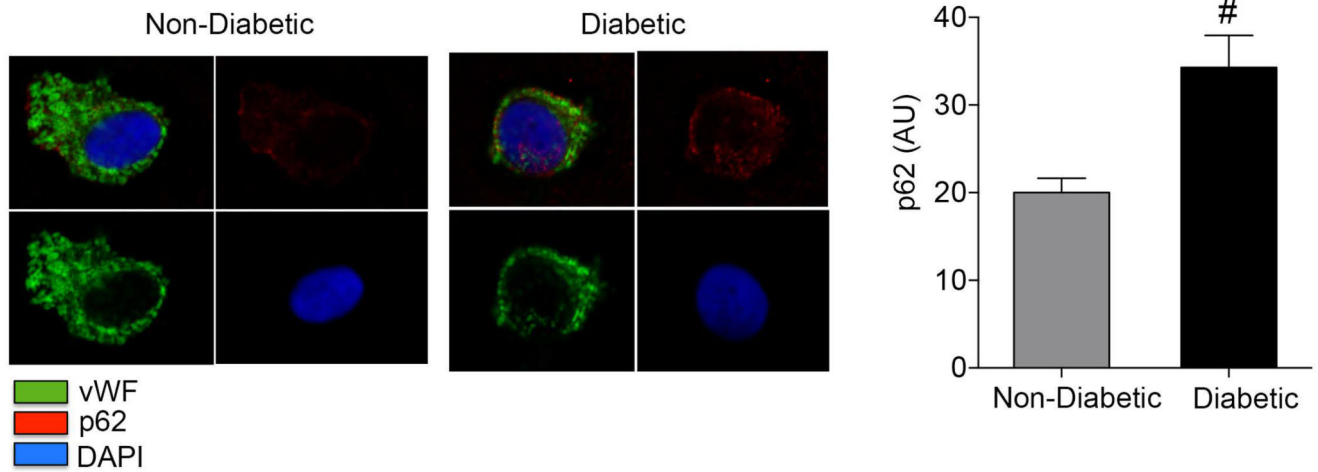
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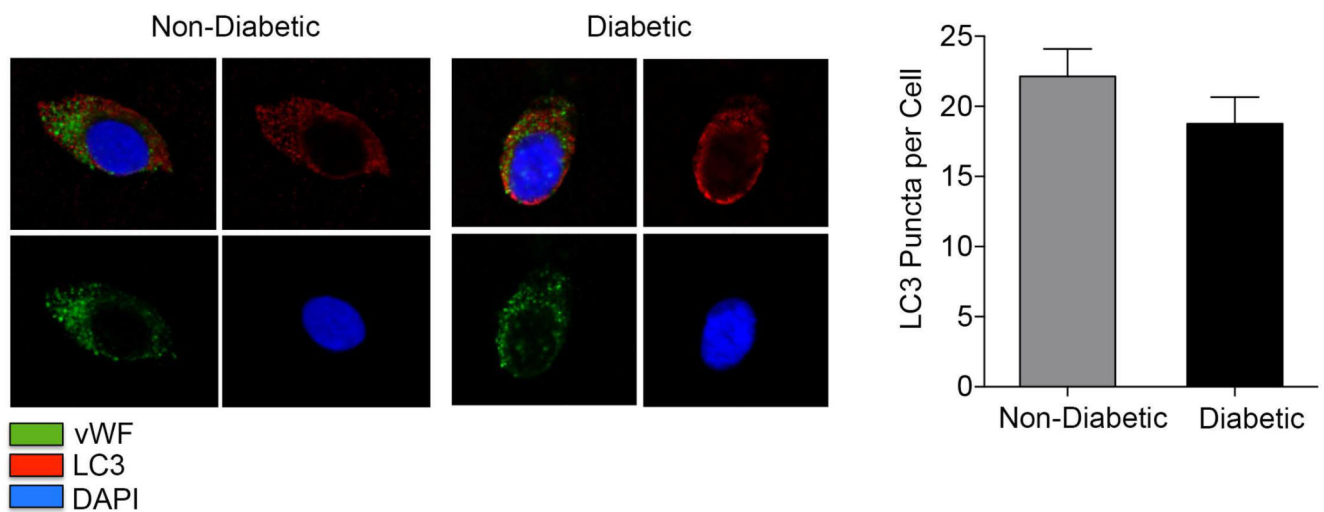
**Figure 1.**

Cellular stress induces autophagy, a multi-step process that serves to remove damaged and dysfunctional cellular components and organelles.

A



B

**Figure 2.**

p62 and LC3 in freshly isolated human endothelial cells. **A:** Venous endothelial cells were isolated from healthy subjects and diabetic subjects as outlined in the methods. Endothelial cells from diabetic subjects (n=11) and non-diabetic controls (n=16) were stained for p62. Representative confocal images show higher levels of p62 (red channel) in a diabetic compared to a non-diabetic participant. Also shown are vWF (green channel), DAPI (blue channel) and merged images (upper left). Pooled data shows that diabetic subjects have higher levels of p62 staining compared to non-diabetic subjects ( $\#P<0.01$ ). **B:** Representative confocal images show LC3 puncta in freshly isolated endothelial cells from a non-diabetic and a diabetic. Also shown are vWF (green channel), DAPI (blue channel) and merged



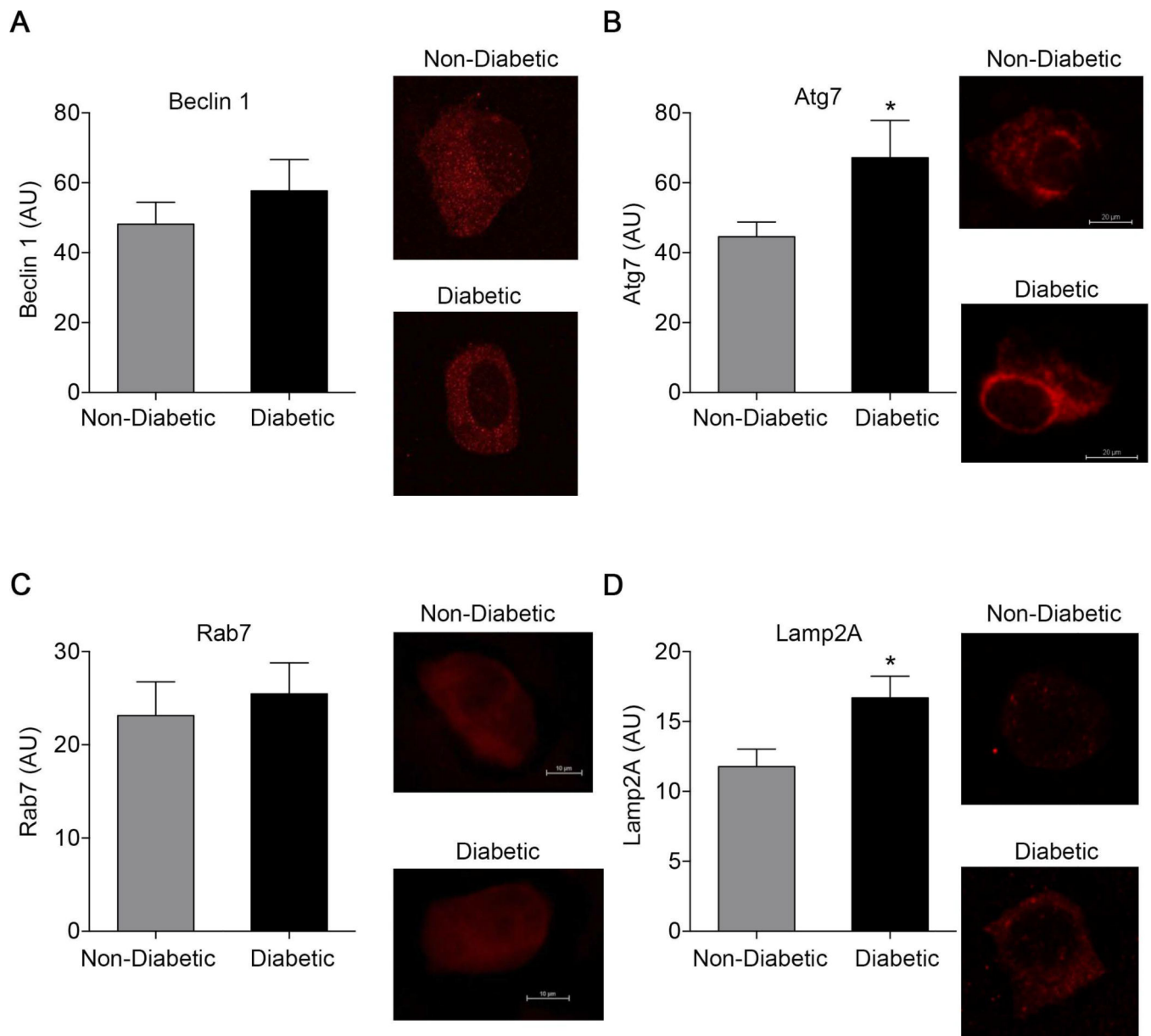
images (upper left). There were no differences in the number of LC3 puncta per cell in freshly isolated endothelial cells from diabetics (n=9) and non-diabetics (n=13).

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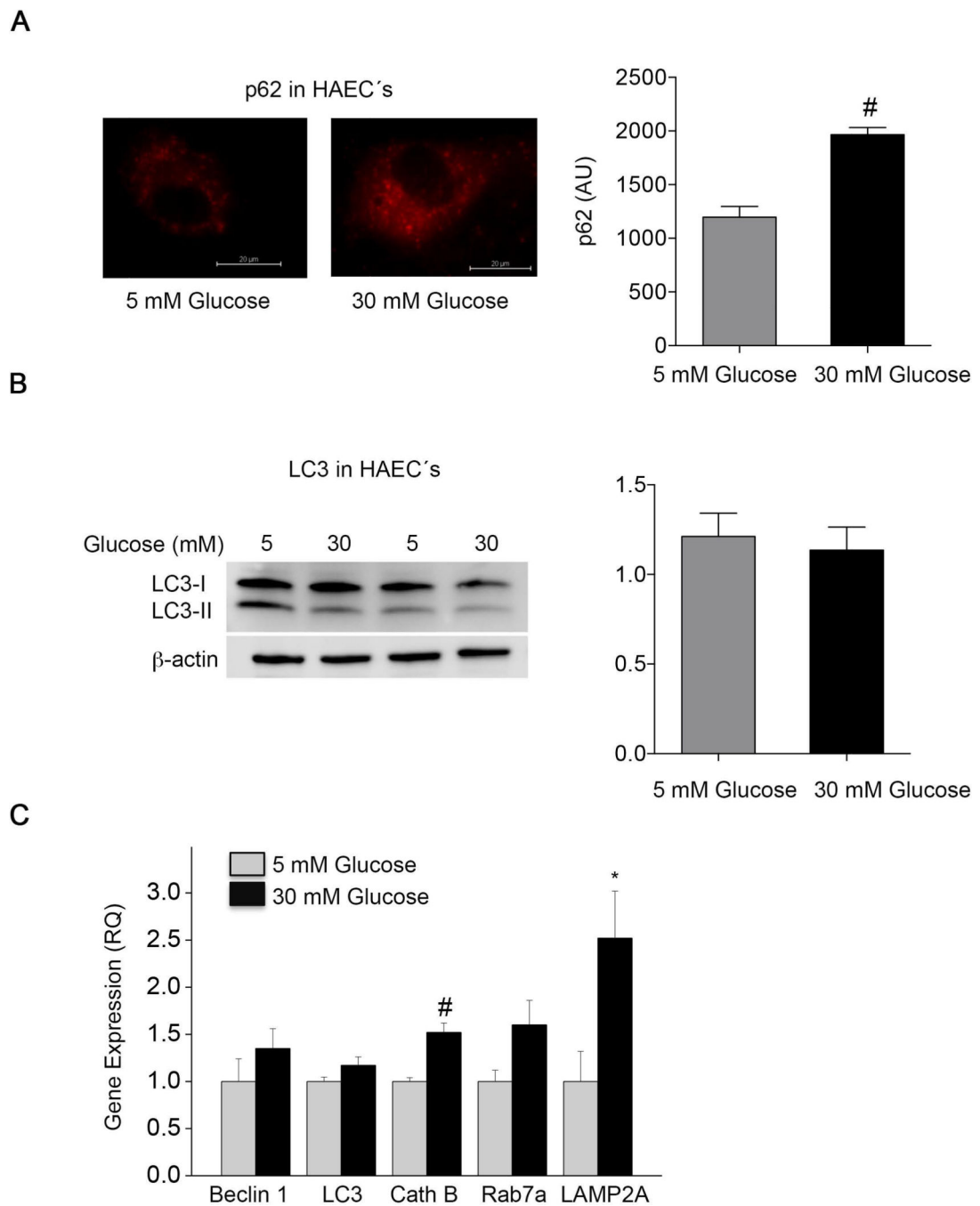
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**Figure 3.**

Beclin 1, Atg7, Lamp2A, and Rab7 in freshly isolated human endothelial cells. **A:** No differences in beclin 1 levels were observed in freshly isolated endothelial cells from non-diabetic (n=10) and diabetic subjects (n=10) assessed by immunofluorescence. **B:** Atg7 levels were higher in endothelial cells from diabetic subjects (n=8) compared to non-diabetics (n=11) \* $P<0.05$ . **C:** No differences were observed in Rab7 levels (n=11 non-diabetics and n=8 diabetics). **D:** Lamp2A levels were higher in endothelial cells from diabetic subjects (n=15) compared to non-diabetics (n=12) \* $P<0.05$ .

**Figure 4.**

Autophagy markers in human aortic endothelial cells (HAEC's) under diabetic conditions. **A:** p62 was increased in HAEC's exposed to 30 mM glucose compared to cells exposed to 5 mM glucose ( $\#P < 0.01$ ). **B:** LC3II/LC3I was similar between HAEC grown under 5 mM and 30 mM glucose. **C:** Quantitative RT-PCR of cultured cells showed that HAEC grown under high glucose expressed higher levels of LAMP2A ( $*P < 0.05$ ) and cathepsin B (cath B,  $\#P < 0.01$ ) compared to HAEC grown under 5 mM glucose. There were no differences in

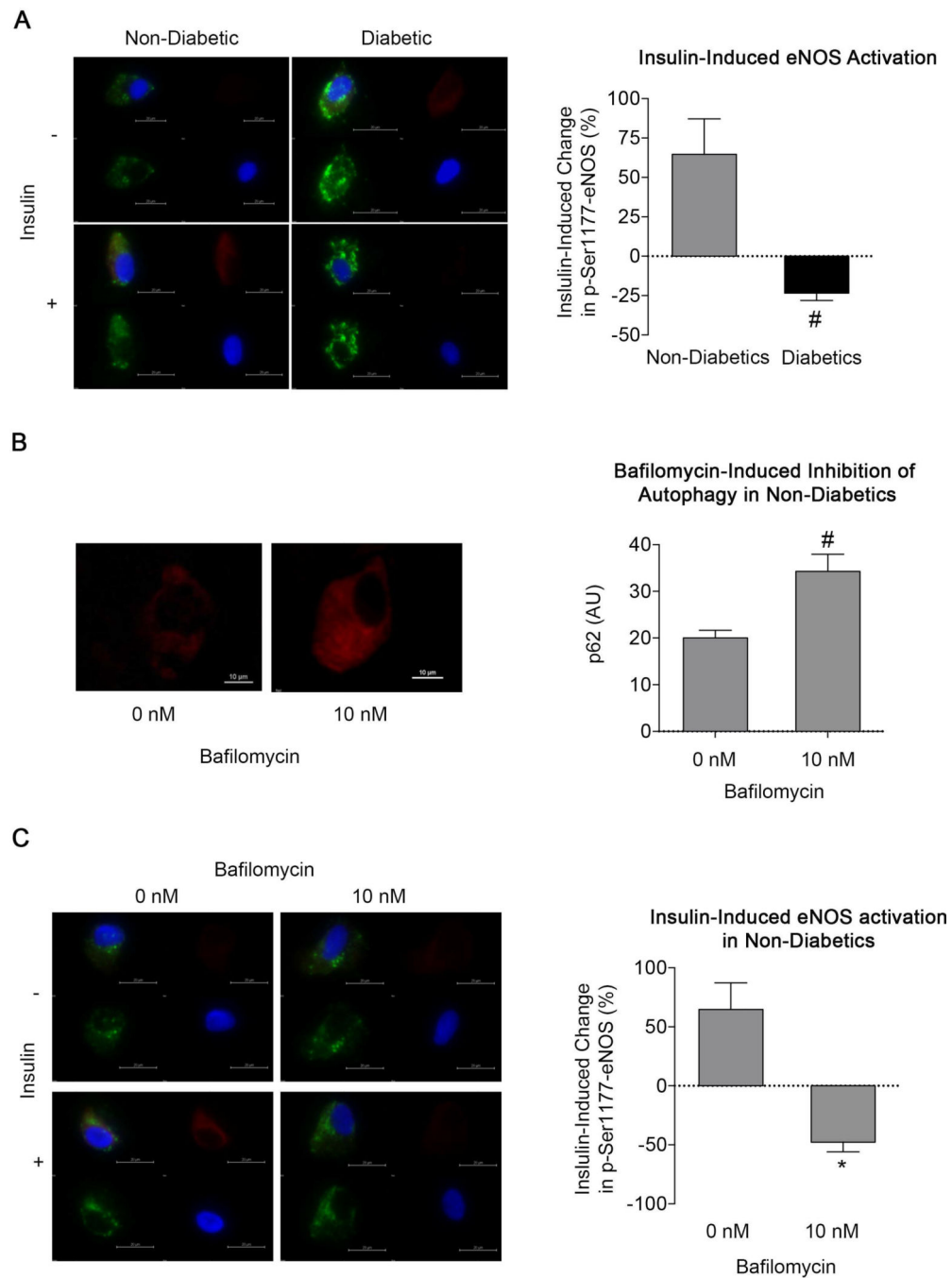
expression of beclin 1 or LC3 (data expressed as relative quantification, RQ). Data are shown as mean±SEM for 5 experiments.

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**Figure 5.** Inhibition of autophagy in endothelial cells from non-diabetics impairs endothelial function. **A:** Insulin-induced activation of eNOS by phosphorylation at Ser1177 (red channel) was measured in endothelial cells from non-diabetics and diabetics. Also shown are vWF (green channel), DAPI (blue channel) and merged images (upper left). Cells from diabetic subjects displayed impaired activation ( $\#P < 0.01$ ). **B:** Treatment of endothelial cells isolated from non-diabetic subjects ( $n = 6$ ) with 10 nM bafilomycin for 60 minutes increased p62 levels, indicating impaired autophagy ( $\#P < 0.01$ ). **C:** Bafilomycin impaired insulin-induced eNOS

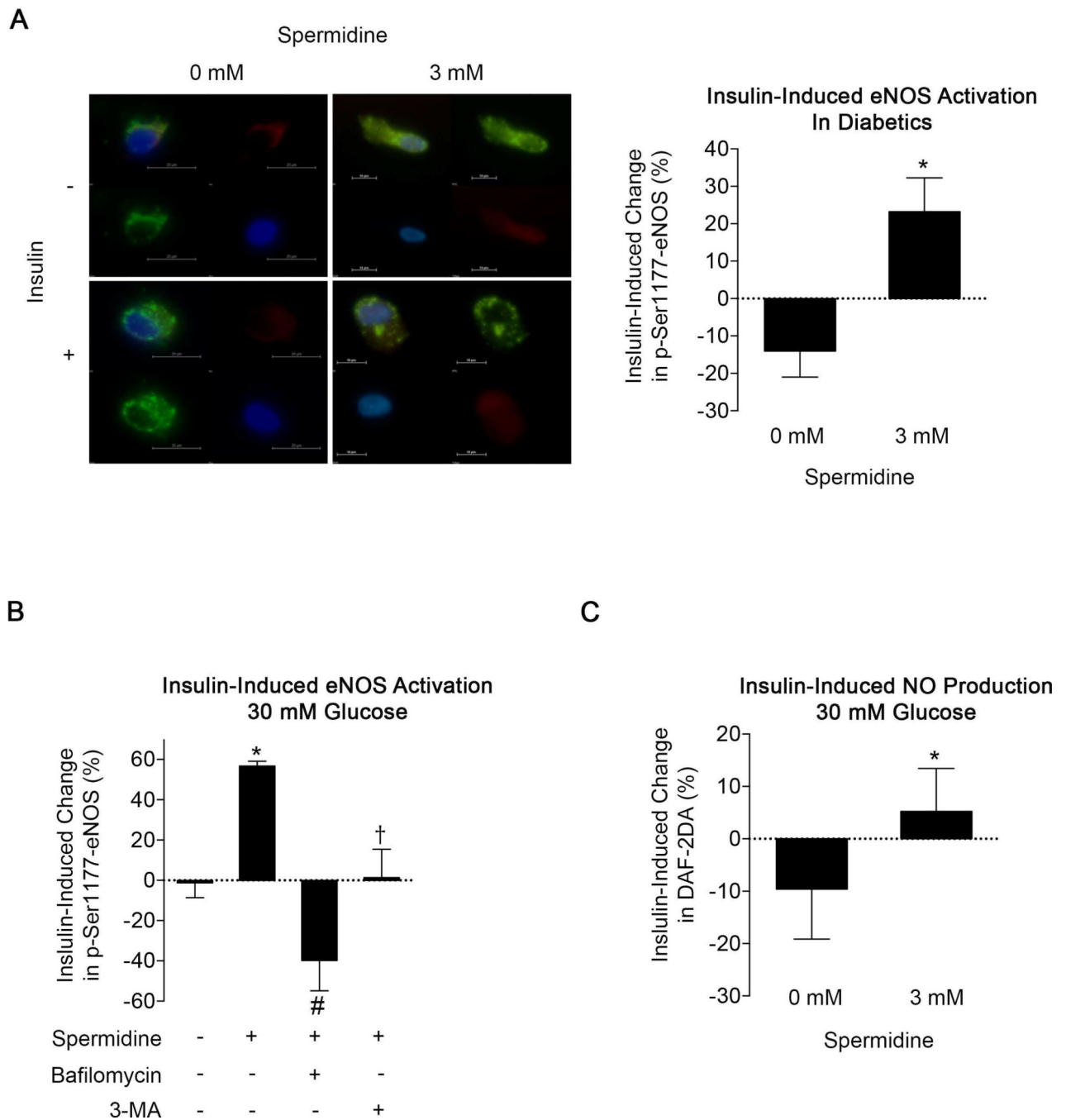
activation (red channel) in endothelial cells from non-diabetics ( $*P<0.05$ ). Also shown are vWF (green channel), DAPI (blue channel) and merged images (upper left).

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**Figure 6.**

Autophagy activation with spermidine restored eNOS activation and nitric oxide production.

**A:** Treatment of freshly isolated endothelial cells from diabetic subjects ( $n=5$ ) with spermidine for 24 hours improves insulin-induced eNOS activation (red channel,  $\#P$  0.01). Also shown are vWF (green channel), DAPI (blue channel) and merged images (upper left). **B:** Insulin-induced activation of eNOS at Ser1177 was assessed by immunofluorescence in HAEC's under high glucose in the presence and absence of spermidine treatment. Treatment of HAEC's under high glucose with spermidine restored insulin-induced eNOS activation,

which was blocked by autophagy inhibitors bafilomycin and 3-MA (RM-ANOVA  $P=0.004$ ,  $*P<0.05$  versus control,  $\#P 0.01$  versus spermidine,  $\dagger P 0.01$  versus spermidine). C: Spermidine improved nitric oxide production in HAEC's exposed to high glucose ( $*P<0.05$ ). Data are shown as mean $\pm$ SEM for 4 experiments.

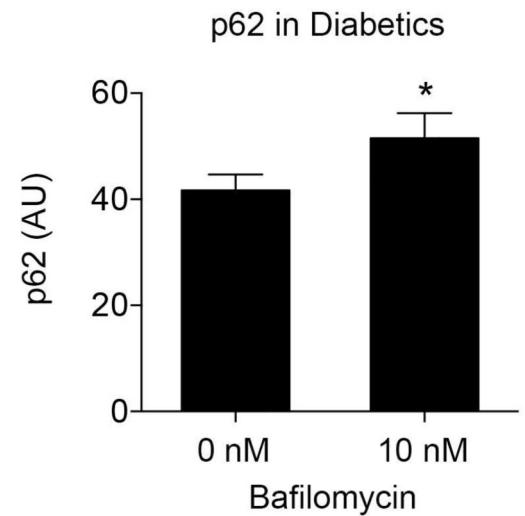
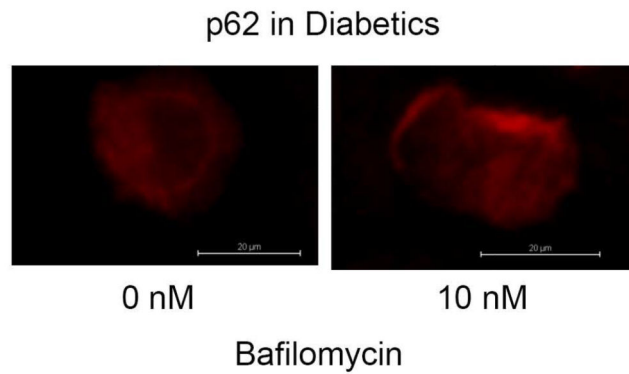
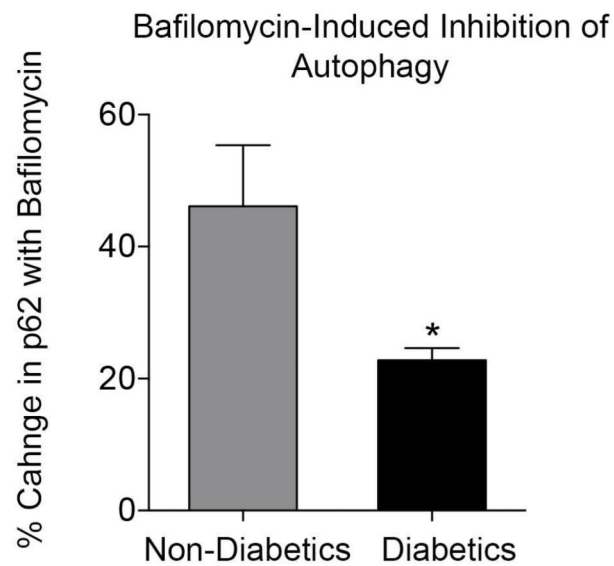
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**A****B****Figure 7.**

Autophagic flux is ongoing, but inadequate in endothelial cells from diabetic subjects. **A:** Bafilomycin increased p62 levels in endothelial cells from diabetic subjects assessed by immunofluorescence (n = 6, #P 0.01). **B:** Augmentation of p62 levels with bafilomycin treatment was higher in endothelial cells non-diabetics (n=5) compared to cells from diabetics (n=8, \*P<0.05).

**Table 1**

## Clinical Characteristics and Vascular Function

	Control (n=41)	Diabetic (n=45)
Clinical characteristics		
Age (yrs)	52±8	54±11
Female sex, n (%)	24 (59%)	27 (60%)
Black race, n (%)	15 (37%)	26 (58%)*
Body mass index, kg/m <sup>2</sup>	28.9±5.2	34.3±7.4*
Total cholesterol, mg/dL	196±37	180±39*
LDL cholesterol, mg/dL	124±31	104±36*
HDL cholesterol, mg/dL	49±14	43±11*
Triglycerides, mg/dL	117±60	174±120*
Fasting glucose, g/dL	91±11	174±85*
Hemoglobin A1c, %	5.4±0.4	8.2±2.0*
Systolic blood pressure, mmHg	126±18	133±14
Diastolic blood pressure, mmHg	77±10	78±10
Lipid lowering therapy, n (%)	6 (15%)	21 (47%)*
Metformin therapy, n (%)	0	27 (60%)*
Sulfonylureas, n (%)	0	10 (22%)*
Thiazolidinediones, n (%)	0	1 (2%)
Insulin therapy, n (%)	0	18 (40%)*
Antiplatelet therapy, n (%)	4 (10%)	16 (36%)*
ACE inhibitor or ARB therapy, n (%)	5 (12%)	22 (49%)*
Vascular function		
Baseline brachial diameter, mm	4.13±0.76	4.41±0.75
Brachial artery flow-mediated dilation, %	9.7±4.6	5.8±3.2*
Nitroglycerin-mediated dilation, %	15.6±7.7	10.7±5.9*
Reactive hyperemia, ml/min	903±298	892±318

\*P<0.05 by unpaired t test or chi square test.

Data are mean ± SD.

LDL=low density lipoprotein, HDL=high density lipoprotein, ACE=angiotensin-converting enzyme, ARB=angiotensin receptor blocker.