**XBP1 mRNA Splicing Triggers an Autophagic Response in Endothelial Cells through BECLIN-1 Transcriptional Activation**

Received for publication, August 28, 2012, and in revised form, November 13, 2012. Published, JBC Papers in Press, November 13, 2012, DOI 10.1074/jbc.M112.412783

Andriana Margariti1, Hongling Li1, Ting Chen1, Daniel Martin1, Gema Vizzay-Barreña1, Saydul Alam1, Eirini Karamariti1, Qingzhong Xiao1 Anna Zampetaki1, Zhongyi Zhang2, Wen Wang2‡, Zhixin Jiang3†, Chan Gao5, Benyu Ma5, Ye-Guang Chen5, Gillian Cockerill5, Yanhua Hu5, Qingbo Xu5, and Lingfang Zeng1‡

From the Cardiovascular Division, King’s College London BHF Centre, 125 Coldharbour Lane, London SE5 9NU, United Kingdom, the Department of Cardiology, First Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou 310003, China, the Centre for Ultrastructural Imaging, King’s College London, Guy’s Campus, London WC2R 2LS, United Kingdom, the Centre for Clinical Pharmacology, William Harvey Research Institute, Barts and The London School of Medicine and Dentistry, Queen Mary University of London, London E1 4NS, United Kingdom, the School of Engineering and Materials Science, Queen Mary University of London, London E1 4NS, United Kingdom, the Cardiovascular Research Group, University College London, London SW17 0RE, United Kingdom, the Centre Laboratory, 305th Hospital of the People’s Liberation Army, Beijing 100017, China, the State Key Laboratory of Bio-membrane and Membrane Biotechnology, School of Life Sciences, Tsinghua University, Beijing 100084, China, and the Department of Cardiovascular Science, St. George’s University of London, London SW17 0RE, United Kingdom

---

**Background:** Apoptosis and autophagy are two closely related systems that induce cell death.

**Results:** X-box-binding protein 1 (XBP1) mRNA splicing regulates BECLIN-1 transcriptional activation, a fundamental player in the initiation of autophagy.

**Conclusion:** XBP1 splicing induces an autophagic response in endothelial cells.

**Significance:** XBP1 could be used as an important pharmacological target that can regulate the autophagic machinery and endothelial cell death.

---

Sustained activation of X-box-binding protein 1 (XBP1) results in endothelial cell (EC) apoptosis and atherosclerosis development. The present study provides evidence that XBP1 mRNA splicing triggered an autophagic response in ECs by inducing autophagosome vesicle formation and markers of autophagy BECLIN-1 and microtubule-associated protein 1 inducing autophagic vesicle formation and markers of mRNA splicing triggered an autophagic response in ECs by reduced cell proliferation, irreversible growth arrest, and apoptosis. Nonetheless, a growing body of evidence suggests that elucidation of the signaling pathways regulating autophagy may provide novel therapeutic approaches in the prevention or treatment of atherosclerosis (1). Recent studies have reported that autophagy becomes dysfunctional in atherosclerosis, and its deficiency promotes atherosclerosis in part through inflammasome hyperactivation (2). Moreover, it has also been shown that macrophage autophagy plays a protective role in advanced atherosclerosis (3), whereas transmission electron microscopy of smooth muscle cells in the fibrous cap of advanced plaques revealed ultrastructural features of autophagy, such as vacuolization and formation of myelin figures (4). Endothelial cell (EC) dysfunction or death are also key events in the pathophysiology of the vessel wall and the development of atherosclerosis (5, 6). However, the regulation of autophagy in ECs in atherosclerotic plaques has not been fully elucidated yet. Autophagy is a multistep catabolic process, which has been shown to be involved in a variety of pathophysiological conditions (1). During the autophagic process, long-lived proteins and organelles are sequestered in a double membrane-bound autophagosome (1) and degraded via lysosome (7–11). Under physiological con-

---

1 To whom correspondence may be addressed. Tel.: 44-20-7848-5322; Fax: 44-20-7848-5296; E-mail: andriana.margariti@kcl.ac.uk.

2 To whom correspondence may be addressed. E-mail: lingfang.zeng@kcl.ac.uk.

3 The abbreviations used are: EC, endothelial cell; ER, endoplasmic reticulum; MOI, multiplicity of infection; nt, nucleotide(s); CREBP, cAMP-response element-binding protein.
**XBP1 Triggers Autophagy in Endothelial Cells**

Adenoviral Gene Transfer—Ad-FLAG-XBP1s and Ad-FLAG-XBP1u were created and amplified as described previously (12). The FLAG tag is located in the N terminus. Empty vector-derived Ad-null virus was used as control virus. For adenoviral gene transfer, confluent human umbilical vein endothelial cells were infected with Ad-XBP1s or Ad-XBP1u at a multiplicity of infection (MOI) of 5 for 6 h followed by replacement with complete growth medium and further culture for the time indicated.

**Transmission Electron Microscopy**—Human umbilical vein endothelial cells were infected with Ad-null, Ad-XBP1u, and Ad-XBP1s viruses at 5 MOI for 48 h, or ECs were infected with non-target (shNT) or XBP1-targeting (shXBP1) shRNA lentivirus for 48 h following by 24 h of endostatin. The cells were grown to confluence on 13-mm diameter glass coverslips. For transmission electron microscopy, cells on coverslips were fixed with 4% paraformaldehyde, PBS for 15 min and permeabilized with 0.1% Triton X-100, PBS for 10 min, followed by rinsed several times with 0.1M phosphate buffer, followed by postfixation with 1% (w/v) osmium tetroxide in 0.1M phosphate buffer (pH 7.3) for 20 min at 4 °C. The coverslips were rinsed (10 min) in phosphate buffer and dehydrated in a graded series of ethanol to 100%. The coverslips were then flooded with TAAb epoxy resin and left to infiltrate for 4 h at room temperature. To section in the plane of the monolayers, the coverslips were embedded by inverting them (monolayer side down) onto a capsule (TAAb) overfilled with resin and then polymerized for 24 h at 70 °C. Ultrathin sections (70–90 nm) were prepared using a Reichert-Jung Ultracut E ultramicrotome, mounted on 150-mesh copper grids, contrasted using uranyl acetate and lead citrate, and examined on an FEI Tecnai 12 transmission electron microscope operated at 120 kV. Images were acquired with an AMT 16000M digital camera.

**RNA Extraction and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Real-time PCR—RNA extraction, RT-PCR, and real-time PCR were performed as described previously (23). One μg of RNA was reverse transcribed into cDNA with random primer by MMLV reverse transcriptase (Promega). 20–50 ng of cDNA (relative to RNA amount) was amplified by standard PCR with TaqDNA polymerase (Invitrogen) or real-time PCR. The primers for each gene are as follows: for LC3β, 5'-TGTCGGCAGTTTACGTCAAGAGCAGCA-3' and 5'-TTCAAAACAGAGAAGGTCGGCTA-3'; for XBP1, 5'-CCTTTGTAGTTGAGAACCCAGGAG-3' and 5'-GGTCCAAGTTGTCAGGATGTCGTC-3'; for BECLIN-1, 5'-AGGTTGAGAAAGCGGAGACACG-3' and 5'-ATGTCACGTTCAGAATGTCGTC-3'; for IRE1α, 5'-GAAGGTCGAGTTCGCCAGGCGTTC-3' and 5'-AGGAGAAGGTCATTGAGTCGTC-3'; for E2F1, 5'-AGGTGTGTCAGCTGAGATGTCGTC-3' and 5'-TGGGGTATGAGCGAGGTCGTC-3'; for E2F2, 5'-TGGGGTATGAGCGAGGTCGTC-3'. PCR primers for real-time PCR were designed using Primer Express software (Applied Biosystems). Indirect Immunofluorescence Assay—ECs were seeded on collagen I-coated slides 24 h before infection with Ad-XBP1s or Ad-XBP1u viruses at 5 MOI. Three days later, the slides were fixed with 4% paraformaldehyde, PBS for 15 min and permeabilized with 0.1% Triton X-100, PBS for 10 min, followed by incubation with primary and secondary antibodies, as described previously (22). Briefly, incubation with the primary antibodies, FLAG (goat), BECLIN-1 (rabbit), LC3β (rabbit), and XBP1 (rabbit), was performed for 1 h at 37 °C. The bound primary antibodies were revealed by incubation with an ALEXA 546-conjugated donkey anti-goat IgG and an FITC-conjugated swine anti-rabbit IgG at 37 °C for 30 min. Cells were...
counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma), mounted with Fluoromount-G (DAKO, Denmark), and examined under an SP5 confocal microscope (Leica, Germany). Magnification in the figures is indicated by the scale bars.

**Immunoblotting**—Cells were harvested and washed with cold PBS, resuspended in lysis buffer (25 mM Tris-Cl, pH 7.5, 120 mM NaCl, 1 mM EDTA, pH 8.0, 0.5% Triton X-100) supplemented with protease inhibitors (Roche Applied Science), and lysed by ultrasonication (twice, 6 s each) (Branson Sonifier 150), as described previously (22). The protein concentration was determined using the Bio-Rad protein assay reagent. 50 μg of whole lysate was applied to SDS-PAGE and transferred to Hybond PVDF membrane (GE Healthcare), followed by a standard Western blot procedure. The bound primary antibodies were detected by the use of horseradish peroxidase (HRP)-conjugated secondary antibody (both obtained from GE Healthcare) to precipitate the endogenous XBP1. Normal IgG was used as a control. Immunocomplexes were collected the following morning using Protein G-agarose/salmon sperm DNA beads for 1 h. The beads were washed with ChIP dilution buffer and precleared with Protein G-agarose/salmon sperm DNA beads for 1 h. Subsequently, fresh medium in the absence or presence of endostatin (20 ng/ml) was added, and the cells were harvested 48 h later.

**Chromatin Immunoprecipitation Assay**—ECs (1 × 10^6 cells) were infected with Ad-XBP1s or Ad-XBP1u at 5 MOI for 6 h and further cultured for 48 h, or normal ECs were used for endogenous assays. The chromatin immunoprecipitation assay was performed with a commercial kit (EZ ChIP, Millipore) according to the protocol provided and as described previously (12, 24). In brief, cells were treated with 1% (v/v) formaldehyde at room temperature for 10 min and then quenched with glycine at room temperature. The medium was removed, and cells were harvested in lysis buffer. Following a short incubation on ice, chromatin was sheared by sonication. The sheared samples were diluted into 0.9 ml of ChIP dilution buffer and precleared with Protein G-agarose/salmon sperm DNA beads for 1 h. Subsequently, immunoprecipitation was conducted with anti-FLAG antibody (to precipitate XBP1s and XBP1u and their bound chromatin) or XBP1 antibody (Santa Cruz Biotechnology, Inc.) to precipitate the endogenous XBP1. Normal IgG was used as a control. Immunocomplexes were collected the following morning using Protein G-agarose/salmon sperm DNA beads pelleted by centrifugation and washed with low salt buffer, high salt buffer, and Tris-EDTA buffer (25 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 7.2) to remove any nonspecific binding. The immunocomplexes were eluted from the beads using 200 μl of elution buffer (100 mM NaHCO₃, 1% SDS), and the cross-links of the protein-DNA complexes were reversed by an overnight incubation of the elute products at 65 °C. A total of 2 μl of proteinase K (10 μg/μl) was subsequently added to the solution, and samples were incubated at 45 °C for 1 h. DNA was then purified using the spin columns provided. Aliquots of chromatin were also analyzed before immunoprecipitation and served as an input control. BECLIN-1 gene promoter sequences were amplified by PCR with primer sets 1–4: set 1, 5′-AACCCTCACGGCTCTTATTGGAGT-3′ and 5′-AGAGGCAAACATTAGGGAGAAGCGA-3′; set 2, 5′-TGTTGTCTACCGTCTGAGCCTGGCAG-3′ and 5′-ACTCCCTTCTTAAGGTCCCACCCTC-3′; set 3, 5′-ACCAGCCCGCTAGTTT-3′ and 5′-TTCACAGGGAAAGTGTAAGAGCCCAAA-3′; set 4, 5′-TGTTCTCGAACTCTGACCCGGTATG-3′ and 5′-ATCCACGTTCATCCCCAGCTACTC-3′. The PCR products were analyzed in 2% agarose gel, and images were assessed with the BioSpectrum AC imaging system and Vision- WorksLS software.

**Luciferase Activity Assay**—The BECLIN-1 promoter (bp −206 to −910) region was amplified by PCR with a primer set of 5′-CAGCATCTCTAGTGTTCTGTGAGATGGG-3′ and 5′-GCCGTTAAGCTTACTCCTCAATAAGGCGGTG-3′ and cloned into the Xhol/HindIII sites of pGL3-Luc reporter vector and verified by DNA sequencing. Additionally, pGL3-Luc-BECLIN-1 plasmid was digested with the restriction enzymes PmlI and StuI, and a truncated construct lacking the XBP1s-specific binding site was generated. In brief, ECs were transfected with full-length or truncated pGL3-Luc-BECLIN-1 (0.33 μg/well) together with expression plasmid (0.16 μg/well) encoded from XBP1s and XBP1u. pGL3-Luc Renilla (0.1 μg/well) was included in all transfection assays as an internal control, and pShuttle2 vector was also used as a mock control. Luciferase and Renilla (Promega) activity assays were run 24 and 48 h after transfection using a standard protocol. A relative luciferase unit was defined as the ratio of luciferase activity to Renilla activity, with that of control set as 1.0.

**Ex Vivo Experiments**—For the generation of XBP1 EC conditional knock-out (XBP1eko) mice, an 11.5-kb DNA fragment encompassing XBP1 gene exons 1–5 was isolated from the mouse genome. Two LoxP sites were inserted into the promoter and intron 2 regions, respectively. An FRT-flanked Neo cassette was inserted upstream of the second LoxP site in the intron 2 for positive clone selection. Recombinant 129Sv/Pas ES cell clones were injected into recipient blastocysts isolated from pregnant C57BL/6 females. The injected blastocysts were then reimplanted into OF1 pseudopregnant females and allowed to develop to term. The F1 generation of chimeras was cross-bred with CAG-Cre deleter to create global knock-out XBP1 mice, whereas they were cross-bred with FLPe transgenic mice to create XBP1eko mice (25, 26). All of the gene cloning, ES cell culture and selection, and the XBP1ΔloxP/ΔloxP mouse creation were performed by genOway. The following breeding procedures were performed in the King’s College London animal facility. All animal experiments in this study were performed according to protocols approved by the Institutional Committee for Use and Care of Laboratory Animals. Arterial vessels from aorta were isolated from wild type and XBP1eko mice and cut into 2 × 2-mm sections. The sections were incubated with M199 supplemented with 10% FBS in the presence or absence of endostatin (20 ng/ml) for 24 h. The segments were fixed with 4%
paraformaldehyde, followed by immunofluorescence staining with anti-XBP1, anti-CD31, and anti-LC3β antibodies, as described in the supplemental material. The segments were mounted on a slide with the vessel lumen facing up. Images were taken under an SP5 confocal microscope (Leica). Magnification is indicated in the figures by scale bars.

Statistical Analysis—Data are expressed as the means ± S.E. and were analyzed with a two-tailed Student’s t test for two groups or pairwise comparisons. A value of p < 0.05 was considered to be significant.

RESULTS

Sustained Activation of XBP1 mRNA Splicing Induces Autophagy in ECs—In the present study, we tested the hypothesis that XBP1 splicing induced autophagy in ECs. The study was initiated by infected ECs with XBP1 spliced form adenovirus (Ad-XBP1s), where a number of structures that could be either droplets or autophagic vacuoles were observed (Fig. 1, A–C).

No such structures or very few of them were observed in ECs infected with unspliced XBP1 form (Ad-XBP1u) or null control (Ad-null) (Fig. 1, A–C). Oil Red O staining demonstrated that these structures were not oil droplets. To explore whether these structures had an autophagic phenotype, ECs were infected with Ad-XBP1s, Ad-XBP1u, or Ad-null viruses for 48 h, and samples were prepared and observed using transmission electron microscopy. The results confirmed the presence of autophagosomes in ECs infected with Ad-XBP1s (scale bars, 2 μm (D–F) and 0.5 μm (G–I)). The overexpression of the unspliced and spliced XBP1 is shown by conventional (J) and real-time PCR. *, p < 0.05 (K). Data presented are representative of or an average from three independent experiments. Error bars, S.E.

FIGURE 1. Sustained activation of XBP1 splicing induces autophagy in ECs. A–C, ECs were infected with Ad-null, Ad-XBP1u, and Ad-XBP1s viruses at 5 MOI for 6 h and cultured for 48 h, followed by hematoxylin staining (scale bars (A–C), 25 μm). D–I, ECs were infected with Ad-null, Ad-XBP1u, and Ad-XBP1s viruses at 5 MOI for 48 h and grown to confluence on 13-mm diameter glass coverslips. The cells were fixed with 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer, and ultrathin sections (70–90 nm) were prepared and examined on an FEI Tecnai 12 transmission microscope showing the presence of autophagosomes in ECs infected with Ad-XBP1s (scale bars, 2 μm (D–F) and 0.5 μm (G–I)). The overexpression of the unspliced and spliced XBP1 is shown by conventional (J) and real-time PCR. *, p < 0.05 (K). Data presented are representative of or an average from three independent experiments. Error bars, S.E.
cell, more fields have been examined, as shown in Fig. 2B, and the percentage of the XBP1 and LC3β double-positive cells has been calculated and found to be around 95% (Fig. 2C). Additional experiments have revealed that low levels of XBP1s overexpression are sufficient to trigger an autophagic response in ECs by increasing the expression of BECLIN-1.
and LC3β (Fig. 2D). Importantly, when ECs were infected with Ad-XBP1s and treated with chloroquine, an inhibitor of the fusion of autophagosomes with lysosomes, a further increase in LC3βII expression level was observed (Fig. 2E). These results further confirm that XBP1s triggers an autophagic response in ECs.

Overexpression of XBP1s Increases Autophagy Gene Expression—To verify that XBP1 mRNA splicing induces autophagy, XBP1s and XBP1u were overexpressed in ECs by adenoviral gene transfer, and the expression of autophagy genes was assessed at the mRNA and protein levels. Real-time quantitative RT-PCR showed that XBP1s increases BECLIN-1 and LC3β autophagy genes when compared with control virus-infected cells or Ad-XBP1u-infected cells in a time course experiment (Fig. 3A). Routine RT-PCR confirmed the overexpression of the two isoforms of XBP1 in ECs (Fig. 3B). XBP1 mRNA splicing was detected by routine RT-PCR, followed by PstI digestion. There is a PstI site in the 26-nucleotide intron of XBP1u but not in XBP1s mRNA. Digestion of the RT-PCR product with a PstI restriction enzyme allows XBP1s (not digested) and XBP1u (digested into two smaller bands) to be distinguished. Western blot analysis showed induced expression of BECLIN-1 and autophagosome-specific isoform LC3βII, indicating activation of autophagy (Fig. 3C). Furthermore, immunofluorescence staining revealed that Ad-XBP1s increased BECLIN-1 expression in ECs (Fig. 3D). These results suggest that XBP1 splicing is involved in the induction of the autophagy gene transcription in ECs.
BECLIN-1 Deficiency in ECs Abolishes the XBP1-induced Autophagy Response—To shed light on the mechanisms involved in XBP1s-induced autophagy, further experiments were performed to explore the effect of XBP1s on BECLIN-1 expression. BECLIN-1 has been shown to play an important role in the vesicle nucleation during the initial steps of autophagy (27). Importantly, when BECLIN-1 was knocked down by shRNA in ECs, the induction of autophagy mediated by XBP1 was ablated because autophagic vacuole structures were not observed (Fig. 4A). Further experiments revealed that knockdown of BECLIN-1 abolished XBP1-induced LC3βII expression at the protein level (Fig. 4B and C, quantification). Recent reports have shown that I4-3-3T and E2F1 regulate the BECLIN-1 gene and initiate autophagy (28). Therefore, we
XBP1 Triggers Autophagy in Endothelial Cells

explored whether XBP1s could regulate BECLIN-1 through 14-3-3T and E2F1. However, neither XBP1s nor XBP1u could regulate 14-3-3T and E2F1 gene expression (supplemental Fig. S1), suggesting that XBP1s-expressed BECLIN-1 expression is not through these two genes. Because XBP1s is a transcription factor, we hypothesized that it may directly regulate BECLIN-1 gene expression at the transcriptional level. Luciferase assays demonstrated that overexpression of XBP1s induced the expression of the BECLIN-1 promoter in ECs (Fig. 4E). This transcriptional regulation of BECLIN-1 was specific for the XBP1s and not for the XBP1u (Fig. 4E). These results demonstrate that knockdown of BECLIN-1 abolished XBP1-induced autophagy, highlighting the importance of BECLIN-1 in induction of autophagy mediated by XBP1s.

XBP1s Directly Binds to the BECLIN-1 Gene Promoter—To investigate whether XBP1s directly binds to the BECLIN-1 promoter region, chromatin immunoprecipitation assays were performed with anti-FLAG antibody. A map of the BECLIN-1 promoter showing the relative regions covered by the primer sets 1–4 is illustrated in Fig. 4D. As expected, a binding of XBP1s to the BECLIN-1 gene promoter was detected, which locates at region −537 to −755 nt upstream of the transcription initiation site (Fig. 4F). Moreover, the binding of XBP1s to the BECLIN-1 gene promoter was verified in experiments with antibody against endogenous XBP1 (Fig. 4G). Further experiments were performed to explore whether the direct binding of XBP1s to the BECLIN-1 promoter is essential for BECLIN-1 transcription. A truncated construct of the BECLIN-1 promoter lacking the −537 to −755 region was created. Transient transfections with the intact and the truncated pGL3-Luc-BECLIN-1 promoter constructs were performed. As shown in Fig. 4H, deletion of the −537 to −755 region abolished XBP1s-induced BECLIN-1 reporter gene expression. These results suggest that XBP1s mRNA splicing triggers an autophagic response in ECs through BECLIN-1 transcriptional activation by direct binding to the −537 to −755 nt region in BECLIN-1 gene promoter.

Endostatin Induces Autophagy in ECs through IRE1α-mediated XBP1 mRNA Splicing—Angiogenesis inhibitors, such as endostatin, are reported to induce autophagy in ECs (29). In this study, ECs treated with endostatin induced autophagy, as demonstrated by the appearance of autophagic vacuole structures (Fig. 5A). Further experiments revealed that endostatin induced the expression levels of genes related to autophagy at both the RNA level in a time course experiment (Fig. 5B) and at the protein level (Fig. 5C) after 24 h upon endostatin stimulation. Importantly, when ECs were treated with endostatin in the presence of chloroquine, an accumulation of LC3βII was observed (Fig. 5D). To determine whether XBP1s mRNA splicing is involved in the endostatin-induced autophagy process, the effect of endostatin on XBP1s mRNA splicing was assessed. Endostatin treatment robustly increased XBP1s splicing but had no effect on the total XBP1 mRNA level (Fig. 5E). XBP1s mRNA undergoes unconventional splicing through the phosphorylation of IRE1α (17, 18). Therefore, we further tested whether endostatin could alter the phosphorylation status of IRE1α. Indeed, endostatin induced IRE1α phosphorylation, as revealed by Western blot analysis (Fig. 5F). These results suggest that endostatin can activate XBP1 mRNA splicing through IRE1α phosphorylation. To investigate whether XBP1s mRNA splicing is necessary for endostatin-induced autophagy gene expression, ECs were infected with non-target (shNT) or XBP1s-targeting (shXBP1s) shRNA lentiviruses for 72 h and then treated with endostatin for 4 h. Real-time PCR analysis demonstrated that knockdown of XBP1 abolished endostatin-induced autophagy gene expression (Fig. 5G). Similarly, knockdown of IRE1α also suppressed endostatin-induced autophagy-related gene expression (Fig. 5H and I). These results suggest that IRE1α-mediated XBP1 mRNA splicing is necessary for endostatin-induced autophagy in ECs. In order to address the important question of whether knockdown of XBP1 could inhibit the induction of autophagy, ECs were infected with Non-target (shNT) or XBP1s-targeting (shXBP1s) shRNA lentiviruses for 48 h, followed by 24-h endostatin treatment. The cells were fixed, and samples were prepared to be observed under a transmission electron microscope. It is shown that knockdown of XBP1 by shRNA abolished the presence of autophagosomes in ECs mediated by endostatin treatment (Fig. 5J). Moreover, the binding of XBP1s to the BECLIN-1 gene promoter was verified by Western blot analysis (Fig. 5F). These results reveal that knockdown of XBP1 abolished the induction of autophagy mediated by endostatin, highlighting the important role of XBP1s in the induction of autophagy.

XBP1 Deficiency in ECs Abolishes the Endostatin-induced Autophagy Response—The above data have demonstrated that XBP1 mRNA splicing is involved in autophagy response in cultured ECs. To further explore the role of XBP1 in autophagy in intact endothelium, we generated a conditional null allele of XBP1 in endothelial cells. Targeting of XBP1 was performed by introducing LoxP sites into the promoter and intron 2 regions through homologous recombination (supplemental Fig. S2A). The introduction of a LoxP site into the promoter region does not affect XBP1 transcription. The mutation mediated by Cre recombinase deletes a 2.2-kb DNA fragment comprising part of the promoter and the exons 1 and 2, totally ablating XBP1 transcription. Germ line transmission was detected by Southern blot (supplemental Fig. S2B), and deletion of XBP1 was confirmed by PCR at the genomic level (supplemental Fig. S2C and D). XBP1neo-loxP mice were bred to CAG-Cre (25) transgenic mice, which express Cre recombinase ubiquitously, allowing for the generation of XBP1−/− mice. XBP1loxP/loxP mice were bred to Tie2-Cre (26) transgenic mice, which express Cre recombinase in ECs and bone marrow progenitor cells, allowing for the generation of EC conditional knock-out mice (Tie2Cre/XBP1loxP/loxP). Supplemental Fig. S2D shows the genotypes of the littersmates from the cross-breeding of Tie2Cre/XBP1loxP/loxP and Tie2Cre/XBP1loxP/loxP (XBP1eko) mice. The XBP1eko mice can survive, showing defects in angiogenesis.4 To this end, ex vivo experiments were performed on arterial vessel sections isolated from wild type and XBP1eko mice in which XBP1 is specifically disrupted in ECs. Arterial vessels were isolated from wild type and XBP1eko mice, dissected into 2 × 2-mm sections, and incubated with M199 supplemented.

4 L. Zeng, Q. Xiao, H. Li, A. Margariti, D. Martin, S. Alam, A. Zampetaki, Y. Hu, Q. Xu, unpublished data.
with 10% FBS in the absence or presence of endostatin for 24 h, followed by immunofluorescence staining with anti-XBP1, anti-CD31 (Fig. 7A), and anti-LC3β (Fig. 7B) antibodies. As expected, no XBP1 can be detected in ECs on vessels from XBP1eko mice (Fig. 7A, bottom). Importantly, XBP1 deficiency not only reduced the basal level of LC3β (Fig. 7B, left panels) but also abolished the endostatin-induced LC3β expression (Fig. 7B, right panels). In XBP1eko mice, a low level of LC3β could be detected after endostatin treatment, indicating that endostatin-induced LC3β expression and autophagy response can also be activated in XBP1-independent pathways. These results confirm that XBP1 is involved in endostatin-induced autophagy.

**DISCUSSION**

The findings of this study reveal that XBP1 mRNA splicing is implicated in the induction of autophagy in ECs through transcriptional regulation of BECLIN-1. In line with this finding, endostatin activated autophagic gene expression through XBP1 mRNA splicing in a Ire1α-dependent manner. Targeting of XBP1 or Ire1α by shRNA in ECs ablated endostatin-induced autophagy, confirming XBP1 is involved in the endostatin-induced autophagy pathway.
autophagy, whereas XBP1 deficiency in ECs reduced the basal level of LC3β expression and ablated response to endostatin stimulation in XBP1 EC conditional knock-out (XBP1eko) mice.

Among the causes that stimulate autophagy are intracellular stress conditions and hypoxia (11, 30). In response to ER stress, XBP1 mRNA undergoes unconventional splicing through IRE1α, giving rise to a spliced form (17, 18). A number of previous reports have already highlighted the link of ER stress response with the induction of autophagy (31–34). For instance, Yorimitsu et al. (35) have shown that ER stress induces autophagy, which can protect against cell death. Furthermore, hypoxia has been reported to activate an autophagy-lysosomal degradation pathway, and unfolded protein response enhances the capacity of hypoxic tumor cells to carry out autophagy and induces cell survival (36). Recent evidence is also providing mechanistic insights into this concept by demonstrating that activating transcription factor 4 (ATF4) is required for ER stress and hypoxia-induced expansion of autophagy through direct binding to a cyclic AMP-response element binding site in the LC3β promoter. This results in LC3β up-regulation and implication in cell survival (37). In contrast, inhibition of ER stress by knockdown of IRE1 inhibited autophagy and adipogenesis (38). In the present study, we provide novel findings that suggest that XBP1s, a crucial signal transducer of ER stress response, is involved in the induction of autophagy in ECs, providing insight into EC survival.

Autophagy has also been activated in response to angiogenesis inhibitors (39). Endostatin, which is a well characterized inhibitor of angiogenesis that induces apoptosis (29, 40), has been shown to induce autophagy in ECs (40) and increase BECLIN-1 expression through β-catenin- and Wnt-mediated signaling pathways (29, 41). In the present study, we provide evidence that endostatin stimulation is implicated in autophagic gene expression through XBP1 mRNA splicing in an IRE1α-dependent manner. When XBP1 or IRE1α was targeted by shRNA in ECs or in XBP1eko mice, the endostatin-induced autophagic gene expression and autophagosome for-
mation were ablated. These results further support the notion that XBP1s is involved in the regulation of autophagy in ECs.

BECLIN-1 is an essential autophagic protein, which has a crucial role in the initial stages of autophagy (27). Previous studies have shown that BECLIN-1 levels and autophagic vesicle formation were regulated by Bcl-2 and Bcl-xL (29), which are important players in a cell’s decision to progress to an apoptotic or necrotic death (27, 42–44). In order to shed light on the mechanistic insights into the regulation of autophagy by XBP1 splicing in ECs, further experiments revealed that activation of XBP1 splicing triggers BECLIN-1 transcriptional induction through directly binding to the BECLIN-1 promoter at the region of nt −537 to −755. A number of potential transcription factor binding sites in the region of nt −537 to −755 have been identified using TFMATRIX software, including CREBP. Therefore, XBP1s may function as homodimer or heterodimer with another transcription factor, such as CREBP. Importantly, further experiments have demonstrated that XBP1s did not

FIGURE 7. XBP1 deficiency in ECs ablates the endostatin-induced autophagy. Arterial vessels were isolated from wild type and XBP1eko mice and dissected into 2 × 2-mm sections. The sections were incubated with M199 supplemented with 10% FBS in the absence or presence of endostatin for 24 h, followed by immunofluorescence staining with anti-XBP1, anti-CD31 (A), and anti-LC3β (B) antibodies. Note that XBP1 deficiency reduces the basal level of LC3β expression and its response to endostatin stimulation. Data presented are representative images from 20 views. Scale bar, 25 μm.
XBP1 Triggers Autophagy in Endothelial Cells

induce transcriptional activation of a truncated construct of the pGL3-Luc-BECLIN-1 promoter lacking the −537 to −755 region, providing further insights into the BECLIN-1 transcriptional activation mechanism through XBP1s. XBP1 has been reported to regulate histone H4 acetylation (45), whereas XBP1s is also a target of acetylation and deacetylation mediated by p300 and SIRT1 (sirtuin 1), respectively (46). In addition, it has been recently shown that the acetylation status of the ER is regulated by IRE1/XBP1, by controlling the influx of acetyl-CoA through the membrane transporter AT-1, which results in the regulation of autophagy (47). Thus, XBP1s may control the transcriptional activation of BECLIN-1 through recruiting other co-factors that induce acetylation and protein stability and/or inhibit deacetylation in a cell-dependent manner. Further experiments will elucidate these pathways and enhance our knowledge on this aspect.

Vidal et al. (50) have recently reported that targeting XBP1 protects against Huntington disease through the regulation of FoxO1 and autophagy. The authors also showed that XBP1 deficiency enhanced the severity of spinal cord injury in a mouse model (48), whereas it did not alter prion pathogenesis (49), which indicates that the XBP1-regulated autophagy pathway contributes only to certain diseases with distinct outputs (50). Therefore, based on those findings and taking into account our data, we can now describe important aspects in the regulation of autophagy, for example, the activation of XBP1 splicing has a precise role in the autophagy, and autophagy-mediated by XBP1 splicing in ECs is cell type-dependent. For instance, activation of XBP1 splicing in vascular smooth muscle cells does not induce autophagic response.

Evidence is accumulating that autophagy occurs in advanced atherosclerotic plaques (51). For instance, in advanced atherosclerosis, the role of autophagy in macrophages (3) and vascular smooth muscle cells (52) has been shown. In addition, Ox-LDL can also activate autophagy (53) in ECs through the LC3β/BECN1 pathway, leading to the degradation of ox-LDL through lysosomes (54), whereas induction of autophagy promotes angiogenesis by activating the vascular endothelial growth factor (55). Therefore, autophagy may be defined as “a final attempt for survival” based on an adaptive response to fight against cellular stress. However, it remains elusive whether autophagy is a harmful or a protective mechanism in atherosclerotic plaques. The key questions are “how long” and “at what levels” autophagy can be activated without causing detrimental consequences (56, 57). Recent studies have shown that caspase activation regulates the extracellular export of autophagic vacuoles, which reveals that apoptosis and autophagy are two closely related and tightly regulated processes (58). We have previously shown that sustained activation of XBP1 results in EC apoptosis and atherosclerosis development (12). In the present study, we provide evidence that XBP1s triggers an autophagy response in ECs. Thus, it seems that the tight regulation of the expression levels and duration of splicing activation of key molecules, such as XBP1s, could determine the threshold of the autophagic responses through regulation of precise mechanisms in a cell-specific manner. Cross-breeding of the mouse model for atherosclerosis apolipoprotein E with XBP1eko mice is currently in progress, and it will undoubtedly help to answer these questions and demonstrate the role of XBP1s in the regulation of autophagy in ECs and the development of atherosclerosis. Further studies will elucidate whether XBP1 mRNA splicing could be used as an important pharmacological target that can regulate the autophagic machinery.

In summary, endostatin and other stimuli activate IRE1α phosphorylation, which in turn removes the 26-nt intron from XBP1 mRNA, causing the open reading frame shift and giving rise to the spliced isoform. The spliced XBP1 translocates into the nucleus and binds as homodimers or heterodimers to the BECLIN-1 gene promoter region at −537 to −755 nt upstream of the transcription initiation site, leading to the activation of BECLIN-1 transcription, which mediates activation of LC3βII and in turn induces autophagic response, leading to EC survival or apoptosis.

REFERENCES
1. Martinet, W., and De Meyer, G. R. (2008) Autophagy in atherosclerosis. Curr. Atheroscler. Rep. 10, 216–223
2. Razani, B., Feng, C., Coleman, T., Emanuel, R., Wen, H., Hwang, S., Ting, J. P., Virgin, H. W., Kastan, M. B., and Semenkovich, C. F. (2012) Autophagy links inflammasomes to atherosclerotic progression. Cell Metab.
In situ hybridization studies suggest a role for the basic region-leucine zipper protein hXBPI in exocrine gland and skeletal development during mouse embryogenesis. Dev. Dyn. 197, 146–156

Reimold, A. M., Atkin, C., Perkins, A., Friend, D. S., Zhang, L., Horton, H. F., Scott, A., Orkin, S. H., Byrne, M. C., Grusby, M. J., and Glimcher, L. H. (2000) Proteasome inhibitors disrupt the unfolded protein response in myeloma cells. Proc. Natl. Acad. Sci. U.S.A. 100, 9946–9951

Lee, K., Tirasophon, W., Shen, X., Michalak, M., Ting, J. P., and Glimcher, L. H. (1990) A new member of the leucine zipper class of proteins that binds to the HLA DR beta promoter. Science 247, 1581–1584

Sriburi, R., Jackowski, S., Mori, K., and Brewer, J. W. (2004) XBPI. A link between the unfolded protein response, lipid biosynthesis, and biogenesis of the endoplasmic reticulum. J. Cell Biol. 167, 35–41

Lee, A. H., Iwakoshi, N. N., Anderson, K. C., and Glimcher, L. H. (2000) An essential role in liver development for transcription factor XBP-1. Mol. Cell Biol. 20, 4422–4429

Lee, A. H., Iwakoshi, N. N., Anderson, K. D., and Glimcher, L. H. (2005) Autophagy as a bulk protein degradation system. It plays various roles. Tanpakushitsu Kakusan Koso 49, 1029–1032

Levine, B., and Kroemer, G. (2007) Self-eating and self-killing. Cross-talk between autophagy and apoptosis. Nat. Rev. Mol. Cell Biol. 8, 741–752.

Wang, B., Ling, S., and Lin, W. C. (2012) 14–3-3tau regulates Beclin 1 and is required for autophagy. PLoS One 5, e10499.

Nguyen, T. M., Subramanian, I. V., Xiao, X., Ghosh, G., Nguyen, P., Kelekar, A., and Ramakrishnan, S. (2009) Endostatin induces autophagy in endothelial cells by modulating Beclin 1 and b-catenin levels. J. Cell Mol. Med. 13, 3687–3698

Gozuacik, D., and Kimchi, A. (2004) Autophagy as a cell death and tumor suppressor mechanism. Oncogene 23, 2891–2906

Fouillet, A., Levet, C., Virgone, A., Robin, M., Pour Din, R., Rieusset, J., Bédal, E., Ovize, M., Tourret, M., Natif, S., and Mollereau, B. (2012) ER stress inhibits neuronal death by promoting autophagy. Autophagy 8, 915–926

Ghavami, S., Yeganefar, B., Stelmack, G. L., Kashani, H. H., Sharma, P., Cunnington, R., Rattan, S., Bathe, K., Klonisch, T., Dixon, I. M., Freed, D. H., and Halayko, A. J. (2012) Apoptosis, autophagy, and ER stress in mevalonate cascade inhibition-induced cell death of human arial fibroblasts. Cell Death Dis. 3, e330

Vidal, R. L., and Hetz, C. (2012) Crossstalk between the UPR and autophagy pathways contribute to handling cellular stress in neurodegenerative disease. Autophagy 8, 970–972.

Rovetta, F., Stacchiotti, A., Consiglio, A., Cadei, M., Grigolato, P. G., Lavazza, A., Rezzani, R., and Aloe, M. F. (2012) ER signaling regulation drives the switch between autophagy and apoptosis in NRK-52E cells exposed to cisplatin. Exp. Cell Res. 318, 238–250

Yorimitsu, T., Nair, U., Yang, Z., and Klionksy, D. J. (2006) Endoplasmic reticulum stress triggers autophagy. J. Biol. Chem. 281, 30299–30304

Rouschop, K. M., van den Beucken, T., Dubois, L., Niessen, H., Bussink, J., Savelkoul, K., Keulers, T., Muijic, H., Landuyt, W., Vonck, J. W., Lambin, P., van der Kogel, A. I., Koritzinsky, M., and Wouters, B. G. (2010) The unfolded protein response protects human tumor cells during hypoxia through regulation of the autophagy genes MAP1LC3B and ATG5. J. Clin. Invest. 120, 127–141

Rzymski, T., Milani, M., Pike, L., Buffa, F., Mellor, H. R., Winchester, L., Pires, I., Hammond, E., Rougouis, I., and Harris, A. L. (2010) Regulation of autophagy by ATP7 in response to severe hypoxia. Oncogene 29, 4424–4435

Younce, C. and Kolattukudy, P. (2012) MCP-1-induced protein promotes adipogenesis via oxidative stress, endoplasmic reticulum stress and autophagy. Cell Physiol. Biochem. 30, 307–320

Nguyen, T. M., Subramanian, I. V., Kelekar, A., and Ramakrishnan, S. (2007) Kringle 5 of human plasminogen, an angiogenesis inhibitor, induces both autophagy and apoptotic death in endothelial cells. Blood 109, 4793–4802

Chau, Y. P., Lin, S. Y., Jerzy, J. H., and Tai, M. H. (2003) Endostatin induces autophagic cell death in EAhy926 human endothelial cells. Histol. Histopathol. 18, 715–726.

Gao, C., Cao, W., Bao, L., Zuo, W., Xie, G., Cai, T., Fu, W., Zhang, J., Wu, W., Zhang, X., and Chen, Y. G. (2010) Autophagy negatively regulates...
XBP1 Triggers Autophagy in Endothelial Cells

Wnt signalling by promoting Dishevelled degradation. Nat. Cell Biol. 12, 781–790
42. Oberstein, A., Jeffrey, P. D., and Shi, Y. (2007) Crystal structure of the Bcl-XL-Beclin 1 peptide complex. Beclin 1 is a novel BH3-only protein. J. Biol. Chem. 282, 13123–13132
43. Pattingre, S., and Levine, B. (2006) Bcl-2 inhibition of autophagy. A new route to cancer? Cancer Res. 66, 2885–2888
44. Pattingre, S., Tassa, A., Qu, X., Garuti, R., Liang, X. H., Mizushima, N., Packer, M., Schneider, M. D., and Levine, B. (2005) Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. Cell 122, 927–939
45. Tao, R., Chen, H., Gao, C., Xue, P., Yang, F., Han, J. D., Zhou, B., and Chen, Y. G. (2011) Xbp1-mediated histone H4 deacetylation contributes to DNA double-strand break repair in yeast. Cell Res. 21, 1619–1633
46. Wang, F. M., Chen, Y. J., and Ouyang, H. J. (2011) Regulation of unfolded protein response modulator XBP1s by acetylation and deacetylation. Biochem. J. 433, 245–252
47. Pehar, M., Jonas, M. C., Hare, T. M., and Puglielli, L. (2012) SLC33A1/AT-1 protein regulates the induction of autophagy downstream of IRE1/XBP1 pathway. J. Biol. Chem. 287, 29921–29930
48. Valenzuela, V., Collyer, E., Armentano, D., Parsons, G. B., Court, F. A., and Hetz, C. (2012) Activation of the unfolded protein response enhances motor recovery after spinal cord injury. Cell Death Dis. 3, e272
49. Hetz, C., Lee, A. H., Gonzalez-Romero, D., Thielen, P., Castilla, J., Soto, C., and Glimcher, L. H. (2008) Unfolded protein response transcription factor XBP-1 does not influence prion replication or pathogenesis. Proc. Natl. Acad. Sci. U.S.A. 105, 757–762
50. Vidal, R. L., Figueroa, A., Court, F. A., Thielen, P., Molina, C., Wirth, C., Caballero, B., Kiffin, R., Segura-Aguilar, J., Cuervo, A. M., Glimcher, L. H., and Hetz, C. (2012) Targeting the UPR transcription factor XBP1 protects against Huntington’s disease through the regulation of FoxO1 and autophagy. Hum. Mol. Genet 21, 2245–2262
51. Schrijvers, D. M., De Meyer, G. R., and Martinet, W. (2011) Autophagy in atherosclerosis. A potential drug target for plaque stabilization. Arterioscler. Thromb. Vasc. Biol. 31, 2787–2791
52. Hu, P., Lai, D., Lu, P., Gao, J., and He, H. (2012) ERK and Akt signaling pathways are involved in advanced glycation end product-induced autophagy in rat vascular smooth muscle cells. Int. J. Mol. Med. 29, 613–618
53. Han, Q., Zhang, Y. L., You, S. J., Liu, H. H., Cao, Y. J., Chen, R., and Liu, C. F. (2011) [Autophagy of human vascular endothelial cells by oxidized low-density lipoprotein. Involvement of oxidative stress but no oxidized low density lipoprotein-1]. Zhonghua Yi Xue Za Zhi 91, 2216–2220
54. Zhang, Y. L., Cao, Y. J., Zhang, X., Liu, H. H., Tong, T., Xiao, G. D., Yang, Y. P., and Liu, C. F. (2010) The autophagy-lysosome pathway. A novel mechanism involved in the processing of oxidized LDL in human vascular endothelial cells. Biochem. Biophys. Res. Commun. 394, 377–382
55. Du, J., Teng, R. J., Guan, T., Eis, A., Kaul, S., Konduri, G. G., and Shi, Y. (2012) Role of autophagy in angiogenesis in aortic endothelial cells. Am. J. Physiol. Cell Physiol. 302, C383–C391
56. Debnath, J., Baehrecke, E. H., and Kroemer, G. (2005) Does autophagy contribute to cell death? Autophagy 1, 66–74
57. Levine, B., and Yuan, J. (2005) Autophagy in cell death. An innocent convict? J. Clin. Invest. 115, 2679–2688
58. Sirois, I., Groleau, J., Pallet, N., Brassard, N., Hamelin, K., Londono, I., Pshezhetsky, A. V., Bendayan, M., and Hébert, M. J. (2012) Caspase activation regulates the extracellular export of autophagic vacuoles. Autophagy 8, 927–937