Hypoxia-induced autophagy in endothelial cells: a double-edged sword in the progression of infantile haemangioma?

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Aims

The aim of this study was to investigate the precise role of hypoxia-induced autophagy in endothelial cells, and whether it contributes to the distinctive progression of infantile haemangioma (IH).

Methods and results

The endothelial cells (EOMA and HUVECs) were cultured under hypoxic conditions for indicated times (0–72 h). The results showed that short exposure of the endothelial cells to hypoxia resulted in increased cell survival and proliferation, accompanied by occurrence of autophagy. Prolonged hypoxia-induced autophagy, correlating with increased cell death, was also detected afterwards. Correspondingly, autophagy inhibition prevented the enhanced cell survival and proliferation capacity, advanced the occurrence of cell-death in early hypoxic stage, and meanwhile attenuated the ability of prolonged hypoxia in cell-death induction. Moreover, our data demonstrated that the functional transformation of hypoxia-induced autophagy, pro-survival to pro-death, was rigorously regulated by the switch between hypoxia-inducible factor-1α (HIF-1α) and mammalian target of rapamycin (mTOR) pathways. Importantly, we also revealed the activation levels of HIF-1α and mTOR, as well as the autophagy status during the progression of IH.

Conclusion

This study unmasks the functional switch between HIF-1α and mTOR in regulating hypoxia-induced autophagy in endothelial cells and, more importantly, indicates its potential role in the progression of IH.

Keywords

Infantile haemangioma • Hypoxia • Autophagy • HIF-1α • mTOR

1. Introduction

Infantile haemangioma (IH), the most common tumour of infancy, affects ~10% of infants of mixed European descent by the age of 1 year.¹–³ IH has been well characterized by its distinctive presentation, namely rapid growth during infancy followed by subsequent regression in early childhood.⁴ This lesion is usually harmless; however, ~10% of IHs are destructive, disfiguring, and even vision- or life-threatening.⁵,⁶ So far, the precise pathogenesis of IH is still far from clear. It is therefore essential to discover novel molecular events underlying the pathological progression of this disease, which may allow for early detection and more importantly developing new strategies for therapeutics.

Hypoxia is a common environmental stress involved in numerous pathophysiological conditions. Several pieces of evidence at molecular cellular and clinical levels have suggested a possible role of hypoxia in IH pathogenesis. The predilection for IH formation was found non-randomly near the lines of fusion between mesenchymal growth centres ("placodes"),⁷,⁸ which are more likely to suffer hypoxia during embryonic development. Moreover, studies also demonstrated that during delivery, the infant might experience some degree of hypoxia as a consequence of the birthing process,⁹ and this could be an important trigger in the growth of IH. In accordance, a pale or blanched area of skin, which indicates local hypoxia and ischaemia, may be usually observed prior to the rapid growth of IH.⁶ On the
other hand, the transcription factor hypoxia-inducible factor-1α (HIF-1α), which plays an essential role in the maintenance of oxygen homeostasis, has also been reported to express and activate in IH.1 In addition, HIF-1α has been implicated in the regulation of angiogenic factors during IH progression, such as VEGF-A, matrix metalloproteinase 9 (MMP-9), and stromal cell-derived factor-1α (SDF-1α).4,9 All these discoveries have provided important clues for understanding the potential role of hypoxia in IH. However, the precise role of hypoxia in IH, especially during the involuting phase, has not been fully clarified yet.

Autophagy or ‘self-eating’; a lysosome-dependent degradation process with essential functions in immunity, development, tumorigenesis, and cellular homeostasis, has currently entered the research spotlight.10–12 Autophagy could be regulated by a variety of pathological processes. Hypoxic stress is one such process during which autophagy often serves as an important mediator.13,14 Basal autophagy generally functions as a cellular housekeeper by turnover and recycling of proteins and organelles.15 However, when cells are exposed to unfavourable stimulus, such as hypoxia (low oxygen) or anoxia (no oxygen), autophagy is rapidly activated as an adaptive response.16,17 Previous studies18,19 have indicated that autophagy could facilitate the removal of damaged proteins and organelles, and meanwhile serve to maintain energy metabolism and macromolecular synthesis to sustain cell viability under the stress induced by hypoxia. However, if prolonged, hypoxia-induced elevated autophagy may reversely lead to programmed cell death.14 Moreover, it has also been revealed that the pro-survival autophagy may involve the activation of HIF-1α/Bcl2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) pathway, while the pro-death autophagy is predominantly due to blockade of adenosine monophosphate-activated protein kinase (AMPK)-dependent mTOR.20 In addition, previous studies21,22 have identified hypoxia-induced autophagy as a process that can be activated during vascular disorders, including ischaemia—reperfusion injury of the heart and other organs, cardiomyopathy, myocardial injury, and atherosclerosis. More importantly, we find particularly intriguing is how IH, a kind of vascular disorder as well, inovulates spontaneously following a rapid proliferative stage. All these evidences raise our attention to the pathophysiological roles of hypoxia-induced autophagy in IH. Thus, we sought to investigate whether hypoxia-induced autophagy may contribute to the distinctive progression of IH, and whether the functional switch between HIF-1α and mTOR signalling pathways is involved in this process.

2. Methods

2.1 Cell culture

The mouse haemangiendothelioma cell line EOMA23 were obtained from the American Type Culture Collection (ATCC) and were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. Human umbilical vein endothelial cells (HUVECs) grown in EGM-2 were isolated from human umbilical cord veins by collagenase treatment as described previously24 and used in passages 2–7. The investigation conforms to the principles outlined in the Declaration of Helsinki. The study was approved from the review board of the Ethics Committee of Hospital of Stomatology, Wuhan University. When cells were grown to 70% confluence, they were cultured for 0–72 h in Anoxomat chambers (Part Microbiology, Lichtenvoorde, Netherlands) for severe hypoxia (0.1% O₂), physiological hypoxia (5% O₂), or normoxia (21% O₂).

2.2 Determination of apoptosis

Apoptosis induction by hypoxic incubation was determined according to our previous procedures25 as follows: (a) morphological evaluation by Hoescht staining; (b) quantitation of cells in sub-G1 DNA content by flow cytometry following staining with PI; (c) quantitation of cytoplasmic histone-associated DNA fragments with Cell Death Detection ELISA PLUS kit; and (d) western blot analyses for Bax/Bcl-2 ratio and Poly (ADP-ribose) polymerase (PARP) cleavage.

2.3 Evaluation of autophagy

Autophagy activation under hypoxic conditions was determined according to our previous procedures26 as follows: (a) transmission electron microscopy for appearance of membranous vacuoles (autophagosomes); (b) detection of acidic vesicular organelles (AVOs); (c) detection of punctate LC3 by LC3 immunofluorescence or EGFP-LC3 transfection; (d) western blot analyses for autophagy-related gene expression and LC3 cleavage; (e) measurement of autophagic flux by LC3 turnover assay.

2.4 Specimens, immunohistochemistry, and immunofluorescence

Twenty IH specimens, and control specimens including 20 venous malformations, 20 pyogenic granulomas, 10 normal dental pulps, 6 samples of normal skin resected during the repair of cleft lip, were collected at Hospital of Stomatology, Wuhan University. All specimens were fixed in buffered 4% paraformaldehyde and embedded in paraffin. The procedures conform to the principles outlined in the Declaration of Helsinki. The study was approved from the review board of the Ethics Committee of Hospital of Stomatology, Wuhan University. The diagnosis of IH was performed as we described earlier,27,28 and is described in full in Supplementary material online, Table S1.

2.5 Statistical analysis

All data were expressed as mean ± SEM of three independent experiments. One-way analysis of variance (ANOVA) and Student–Newman–Keuls were used for statistical analysis. P < 0.05 was considered significantly different.

An expanded Methods section is available in the Supplementary material.

3. Results

3.1 Bi-directional role of hypoxic stress in endothelial cells

As shown in Supplementary material online, Figure S1A, compared with normoxia (21% O₂), physiological hypoxia (0–60 h; 5% O₂) and short exposure (0–36 h) to severe hypoxia (0.1% O₂) obviously promote the growth ability of both HUVECs and EOMA cells. However, prolonged severe hypoxia (48–72 h; 0.1% O₂) reversely inhibited the growth of both HUVECs and EOMA cells. We then determined the cell viability after hypoxic treatment. The data revealed that prolonged incubation under severe hypoxia for 48 and 72 h significantly decreased the cell viability in both HUVECs and EOMA cells (see Supplementary material online, Figure S1B), suggesting the cytotoxicity exerted by prolonged severe hypoxia. Based on the above findings, 0.1% O₂ concentration was selected for hypoxic culture in the following experiments in order to further unmask the bi-directional role of hypoxia in endothelial cells.
3.2 Prolonged hypoxia induces apoptosis in endothelial cells

To determine whether the cytotoxicity exerted by prolonged severe hypoxia was due to apoptosis induction, morphological observation was firstly performed under microscope. As the data shown, after exposure to severe hypoxia for 48 or 72 h, several apoptotic morphological features such as apoptotic bodies, cell shrinkage, and chromatin condensation were observed in both HUVECs and EOMA cells by Hoechst 33258 staining assays (Figure 1A). To verify hypoxia-induced apoptosis in endothelial cells, the cell cycle analysis was then performed. The results showed that, in both HUVECs and EOMA cells, sub-G1 DNA contents were significantly increased at 48 or 72 h when exposed to 0.1% O2 (Figure 1B). These results were further confirmed by the DNA fragmentation assay that prolonged incubation (48 and 72 h) under 0.1% O2 elicited obvious formation of DNA fragments in both HUVECs and EOMA cells (Figure 1C). The data also showed that both the ratio of Bax/Bcl-2 and cleavage of PARP in endothelial cells were significantly increased after prolonged exposure to 0.1% O2 (Figure 1D), confirming the apoptosis induction by prolonged severe hypoxia. In contrast, we did not find significantly increased cell apoptosis until 72 h when the endothelial cells were cultured under normoxic conditions (data not shown). At the same time, the expression levels of survivin, Bcl-2, Bax, and cleaved-PARP in EOMA cells were determined by western blotting. In all cases, at least three independent experiments were performed.

**Figure 1** Both HUVECs and EOMA cells were cultured under severe hypoxia (0.1% O2) for 6, 12, 24, 48, and 72 h. (A) The cell apoptosis was initially measured by Hoechst 33258 staining. (B) The sub-G1 peak (apoptosis peak) was detected using flow cytometry with PI staining. (C) DNA fragmentation was quantified using the Cell Death Detection ELISAPLUS assay. *P < 0.05, **P < 0.01 compared with the controls. (D) The expression levels of survivin, Bcl-2, Bax, and cleaved-PARP in EOMA cells were determined by western blotting. In all cases, at least three independent experiments were performed.
time, we also noticed that the expression level of survivin was up-regulated at the early stage under severe hypoxic conditions (Figure 1D), which is consistent with the above findings that short exposure to severe hypoxia may promote the survival and growth ability in endothelial cells.

### 3.3 Hypoxic stress activates autophagy in endothelial cells

To explore the participation of autophagy in hypoxic regulation in endothelial cells, we next evaluated the activation status of autophagy in both HUVECs and EOMA cells when cultured under hypoxic conditions. As shown in Figure 2A, exposure of HUVECs cells to severe hypoxia resulted in the appearance of membranous vacuoles (AVs) resembling autophagosomes, as well as formation of punctate LC3 and yellow-orange AVOs at 12 h, and persisted to 72 h, with a peak at about 24 h. On the other hand, the similar results were also found in the EOMA cell line (Figure 2B). However, incubation under normoxia did not cause obvious endothelial cell autophagy (data not shown). In addition, the expression of autophagy-related genes, including Atg5-Atg12 complex, Beclin1, and cleaved-LC3, was also not shown. In addition, the expression of autophagy-related genes, including Atg5-Atg12 complex, Beclin1, and cleaved-LC3, was also correspondingly found to increase in both HUVECs and EOMA cells under hypoxic conditions (Figure 2C). To further distinguish whether the autophagosomal accumulation is due to a block in down-stream steps, we then performed LC3 turnover assay by western blot analysis using the lysosomal inhibitor Bafilomycin A1 to measure the effect of hypoxia on autophagic flux. The results demonstrated that the difference in LC3-II levels in the presence and absence of Bafilomycin A1 is much larger under hypoxic conditions when compared with the normoxic controls, indicating that autophagic flux is indeed increased by hypoxia (Figure 2D). Collectively, these observations suggested that hypoxic stress activated autophagy in endothelial cells.

### 3.4 Hypoxia-induced autophagy plays a bi-directional role in endothelial cells

Since Atg5 and Beclin1 are essential proteins for autophagy activation, either Atg5 or Beclin1 deficiency can significantly interrupt autophagy. DN-Atg5\(K^{130R}\) is a dominant-negative mutant of Atg5, in which Lys130 of Atg5 was replaced with Arg, and has been previously shown to inhibit its conjugation with Atg12 and suppress vacuole formation. 29 Expression of Beclin1 could also be specifically knocked down by shRNA targeting Beclin1. 30 Therefore, in order to determine the precise role of autophagy in hypoxia-exposed endothelial cells, autophagy inhibition by DN-Atg5\(K^{130R}\) and Beclin1-shRNA transfection was then performed. As the results shown, both DN-Atg5\(K^{130R}\) and Beclin1-shRNA significantly prevented the expression of cleaved-LC3 and correspondingly blocked the autophagy activation induced by hypoxia in both HUVECs (Figure 3A) and EOMA cells (Figure 3B). Then, we further proceeded to investigate the effects of DN-Atg5\(K^{130R}\) and Beclin1-shRNA on endothelial cell survival and death when cultured under hypoxic conditions. The results revealed that inhibition of autophagy weakened the bi-directional effects of hypoxic stress on endothelial cells. On the one hand, inhibition of autophagy blocked the growth promotion by hypoxia, and meanwhile induced more apoptotic cells at a relatively early stage (Figure 3C and D). On the other hand, DN-Atg5\(K^{130R}\) and Beclin1-shRNA transfection also significantly prevented the cell apoptosis induced by prolonged hypoxia in both HUVECs and EOMA cells (Figure 3C). The data from western blotting also confirmed our above findings that both the increased survivin expression at the early hypoxic stage and the cleavage of PARP at the prolonged stage were prevented by autophagy inhibition (Figure 3E). All the results indicated that autophagy might bi-directionally regulate endothelial cells under hypoxia.

### 3.5 Functional rotation between HIF-1α/BNIP3 and AMPK/mTOR signalling pathways regulates hypoxia-induced autophagy in endothelial cells

To further explore the underlying molecular mechanisms, we then tested the possible involvement of HIF-1α and mTOR signalling pathways. Our data showed that the protein expression level of HIF-1α was rapidly up-regulated at 2 h when exposed to hypoxia and persisted to 12 h (Figure 4A). The expression of BNIP3, a downstream target by HIF-1α, also showed the same tendency (Figure 4A). On the other hand, the activity of mTOR and its target protein S6 were not altered until 8 h under hypoxic stress. They were decreased at 12 h with the same down-regulated tendency of HIF-1α/BNIP3, but a little earlier (Figure 4A). However, another mediator of hypoxic stress, HIF-2α, did not show the similar change tendency (Figure 4A). Meanwhile, we observed that the expression of p-AMPK was significantly up-regulated much earlier at 6 h, suggesting an energy stress (Figure 4A). On the other hand, we found that their expression levels were not altered under normoxic conditions until 72 h (data not shown). To further determine the involvement of HIF-1α and mTOR pathways, and meanwhile elucidate their relationship, transfection of HIF-1α-siRNA and CA-mTOR were then performed. As the results shown, HIF-1α-siRNA only prevented early hypoxic exposure-induced autophagy activation, but not that mediated by prolonged hypoxia (Figure 4B). A contrary result was observed after CA-mTOR transfection (Figure 4B). Furthermore, we found that HIF-1α-siRNA significantly prevented the cell growth promotion mediated by early hypoxic exposure, and led to early cell apoptosis, but it did not affect the activity of AMPK/mTOR/S6 (Figure 4C–E). On the contrary, CA-mTOR transfection enhanced the cell growth promotion by early hypoxic exposure, and meanwhile effectively impaired cell apoptosis induced by prolonged hypoxia, and it also prevented the down-regulation of HIF-1α/BNIP3 at the late stage (Figure 4C–E), indicating that mTOR may serve as an potential up-stream regulator of HIF-1α. Taken all the above findings into consideration, we may draw a conclusion that the functional rotation between HIF-1α/BNIP3 and AMPK/mTOR signalling pathways plays an important role in hypoxia-induced autophagy in endothelial cells.

### 3.6 HIF-1α and mTOR-dependent autophagy activation in infantile IH

To investigate whether hypoxia-induced autophagy may contribute to the progression of IH, and whether the functional rotation between HIF-1α and mTOR signalling pathways is involved in this process, we next explored the autophagy activation status in 20 IH tissues through detecting the expression levels of Beclin1 and Atg5. As the results from immunohistochemistry shown, positive stainings for both Beclin1 and Atg5 were detected in all of the IH specimens, with specific location in endothelial cells which were signed as CD34-positive staining in serial sections (Figure 5A). In contrast, the cytoplasmic immunostainings for Beclin1 and Atg5 were nearly not detectable in the blood vessels of venous malformation,
Figure 2  (A) Hypoxia-induced appearance of membranous vacuoles (AVs), punctate pattern of LC3 (EGFP-LC3), formation of yellow-orange AVOs in HUVECs were detected using the described methods. (B) Hypoxia-induced punctate pattern of LC3 (EGFP-LC3) and formation of yellow-orange AVOs in EOMA cells were also determined. (C) The expression levels of Atg5-Atg12 complex, Beclin1 and LC3, were detected by western blotting in both HUVECs and EOMA cells. (D) The difference in LC3 expression levels between normoxic and hypoxic conditions with or without Bafilomycin A1 was compared by western blotting. At least three independent experiments were performed in all cases.
Figure 3 Hypoxia-induced autophagy in both HUVECs (A) and EOMA cells (B) was suppressed using transfection with DN-Atg5K130R or Beclin1-shRNA, and further confirmed by western blotting and punctate LC3 detection (LC3 Immunofluorescence). Suppression of hypoxia-induced autophagy prevented the enhanced cell survival, and advanced the occurrence of cell death in early hypoxic stage, and meanwhile attenuated the ability of prolonged-hypoxia in cell-death induction in both HUVECs (C) and EOMA cells (D). The cell growth and cell death were determined by the CTG luminescent assay and Cell Death Detection ELISA$^{\text{PLUS}}$ assay, respectively. $^*P < 0.05$, $^{**}P < 0.01$ compared with untreated controls. (E) The expression levels of survivin and cleaved-PARP in transfected EOMA cells under severe hypoxic condition were detected by western blotting. In all cases, three independent experiments were performed.
pyogenic granulomas, normal dental pulp, and normal skin tissues (see Supplementary material online, Figure S2A). Moreover, the results of immunofluorescence histochemistry also confirmed the concurrent expression of Beclin1 and Atg5 in IH, as evidenced by the co-localization of these two molecules in endothelial cells (Figure 5B). Then the staining scores of Beclin1 and Atg5 in proliferative IH tissues were evaluated against involuting and involuted IH tissues, and the data showed that there was no significant difference between proliferative and involuting stages for Beclin1 and Atg5 expression (see Supplementary material online, Table S2). However, the possible contrary functions for autophagy activation in proliferative and involuting IHs were still unmasked through co-staining with survivin and cleaved-PARP. The results revealed that, in the proliferative IHs, positive staining of Beclin1 was mostly located in the endothelial cells with high survivin expression; while in the involuting IHs, Beclin1 expression was almost detected in the cleaved-PARP-positive IH cells (Figure 5C and 5D). The above findings suggested that the autophagy activation in IH might be a double-edged sword: pro-survival in the proliferative stage, while pro-death in the involuting stage.

To unmask the nature of the molecular mechanism behind the autophagy activation in IH, we then evaluated the expression

![Figure 4](image-url)

**Figure 4** (A) The expression levels of the HIF-1α/BNIP3 and AMPK/mTOR signalling pathways in EOMA cells were detected by western blotting. (B) Knock down of HIF-1α in EOMA cells using HIF-1α-siRNA attenuated formation of punctate LC3 (LC3 Immunofluorescence) in early hypoxic stage (at 24 h), while CA-mTOR\(^{L1460P}\) transfection weakened the autophagy activation in prolonged hypoxic condition (at 72 h) without affecting it in the early hypoxic stage. (C and D) Suppression of HIF-1α impaired cell growth, promoted cell death in EOMA cells when cultured under hypoxic condition, while CA-mTOR\(^{L1460P}\) transfection enhanced the cell viability and prevented cell death in EOMA cells under hypoxic stress. *\(P < 0.05, **P < 0.01\) compared with untreated controls. (E) The expression levels of the HIF-1α/BNIP3 and AMPK/mTOR signalling pathways, as well as survivin, cleaved-PARP, and LC3 were detected by western blotting in transfected EOMA cells when cultured under hypoxic condition. At least three independent experiments were performed in all cases.
Figure 5 (A) Immunohistochemical staining of Atg5, Beclin1, and CD34 in IH tissues. (B) Triple-labelling immunofluorescence staining for Atg5, Beclin1, and CD34 in IH tissues. (C) Triple-labelling immunofluorescence staining for survivin, Beclin1, and CD34 in IH tissues. (D) Triple-labelling immunofluorescence staining for cleaved-PARP (C-PARP), Beclin1, and CD34 in IH tissues.
Figure 6 (A) Immunohistochemical staining of p-mTOR, HIF-1α, and CD34 in IH tissues. (B) Triple-labelling immunofluorescence staining for p-mTOR, HIF-1α, and CD34 in IH tissues. (C) Triple-labelling immunofluorescence staining for p-mTOR, HIF-1α, and Beclin1 in IH tissues. (D) The mRNA expression levels of HIF-1α, BNIP3, Beclin1, and Atg5 in three proliferating and three involuting IH tissues were analysed by real-time PCR. (E) Tissue lysates from three venous malformations, as well as three proliferating and three involuting IH tissues, were analysed by western blotting.
level and/or activation status of HIF-1α and mTOR in IH tissues. Using immunohistochemistry (Figure 6A) and immunofluorescence (Figure 6B) analyses, we found that both HIF-1α and p-mTOR were over-expressed in the proliferative stage, but declined in the involuting stage (see Supplementary material online, Table S2, P < 0.05). However, no specific activation of HIF-2α in IH tissues was detected (data not shown). When co-stained with Beclin1, it was revealed that Beclin1 expression in the proliferative IHs was almost co-located with positive HIF-1α expression, while that in the involuting IHs was mostly detected in the mTOR-inactivated endothelial cells (Figure 6C), indicating that the autophagy activation in proliferative IHs might be HIF-1α dependent, while the autophagy activation in involuting IHs was probably due to mTOR inhibition. On the other hand, the HIF-1α and p-mTOR immunostainings were nearly not detectable in the control tissues (see Supplementary material online, Figure S2B).

In order to strengthen the above findings in IH tissues, we further carried out the quantitative assays to evaluate the specific expression and activation of HIF-1α and mTOR, as well as Beclin1 and atg5 in IH tissues. The results showed that both the mRNA (Figure 6D) and protein expression (Figure 6E) levels of HIF-1α were significantly higher in proliferative IH tissues, when compared with the involuting specimens. Meanwhile, the data also revealed that the activation status of mTOR in proliferative IH tissues was higher when compared with those in venous malformation tissues, while significantly declined in involuting IH tissues, correlated with the up-regulated expression of p-AMPK and cleaved-PARP, as well as the down-regulation of HIF-1α expression (Figure 6E). On the other hand, although showing no difference between proliferative and involuting stages, the data revealed the specific expression of Beclin1 and atg5 in IH tissues when compared with the tested venous malformations (Figure 6E). These findings again confirmed that autophagy activation may play essential roles during the progression of IHs, and this process may be rigorously regulated by the switch between HIF-1α and mTOR pathways.

4. Discussion

In the present study, we implicated the endothelial autophagy process in IH for the first time, and demonstrated that it might play a bi-directional role in the distinctive progression of IH. Moreover, we also revealed that the contrary functions of autophagy in IH are, respectively, dependent on the HIF-1α and mTOR signalling pathways, two important regulators under hypoxic stress, which was consistent with the very recent findings that rapamycin, the specific inhibitor of mTOR, and also a well-known autophagy inducer, could be effective in treating IH.17–23

Actually, previous studies have implicated the existence of hypoxic stress, as well as its promotive roles in the proliferative stage of IH,4 despite of still poorly known underlying mechanisms. In the present study, we also implicated a hypoxic environment in proliferative IHs as demonstrated by the high expression level of HIF-1α, a major regulator of the cellular response to hypoxia. Additionally, we also indicated the potential role of hypoxia in the involution of IH, for the first time (see Supplementary material online, Figure S3B). Our results indicated that the role of hypoxic stress in IH should be persistent and bi-directional.

In order to provide more evidences for our above hypothesis, further studies using HUVECs and EOMA cells were carried out. As expected, we found the bi-directional effects of hypoxic stress on endothelial cells in vitro. The data showed that short exposure to severe hypoxia effectively promoted the growth ability of both HUVECs and EOMA cells, while prolonged severe hypoxia significantly inhibited the endothelial cell growth and meanwhile decreased the cell viability. Moreover, our results also revealed that the decreased cell viability by prolonged severe hypoxia was due to apoptosis induction, as demonstrated by Hoechst staining, cell cycle analysis, DNA fragments detection, as well as test for apoptosis-related protein expression. At the same time, we also noticed an increase in the expression level of survivin at the early stage of hypoxic incubation, suggesting enhanced survival and growth ability. Therefore, based on the above findings, we can draw a conclusion that hypoxia may bi-directionally regulate the survival and death of endothelial cells.

We next turned to explore the underlying events by which hypoxia bi-directionally regulates the survival and death of endothelial cells. It has already been shown that hypoxia could activate autophagy in multiple cell types,20,34,35 including endothelial cells.36,37 Autophagy has been described as an evolutionarily conserved pathway that mediates cellular degradation through the action of lysosomes.15,38 Importantly, autophagy itself is paradoxical that can both protect and impair cell survival depending on the environment changes,15 which, in our opinion, should include the hypoxic environment changes. Thus, we then proceeded to determine whether the paradoxical autophagy activation was involved in the bi-directional role of hypoxic stress in endothelial cells. Initially, we detected the autophagy activation status in IH tissues through detecting the expression levels of Beclin1 and Atg5, two essential autophagy-related genes. As the results, these two proteins were specifically detected in IH specimens, but not the blood vessels of control tissues. Moreover, the contrary functions for autophagy activation in proliferative and involuting IH were still unmasked through co-staining with survivin and cleaved PARP, suggesting that the autophagy activation in IH might be a double-edged sword: pro-survival in the proliferative stage, while pro-death in the involuting stage.

Additionally, the in vitro studies further confirmed that short exposure to hypoxia effectively activated autophagy in endothelial cells rather than apoptosis, which is consistent with the indication by previous study.17,37 And, more importantly, we, for the first time, revealed the essential role of autophagy in the bi-directional effects of hypoxia on endothelial cells. By employing the genetic inhibition of autophagy, we determined that inhibition of autophagy significantly blocked the growth promotion by short hypoxic exposure, and meanwhile induced more apoptotic cells at a relatively early stage. On the other hand, autophagy suppression also significantly prevented the apoptosis induced by prolonged hypoxia. All these above findings promote us to conclude that hypoxia may probably exert its bi-directional effects on endothelial cells through activating autophagy with contrary functions, pro-survival or pro-death, due to the exposure period.

To the best of our knowledge, this is the first study demonstrating the bi-directional roles of hypoxia-induced autophagy in endothelial cells in one research system. Importantly, both the in vivo and in vitro data indicated that the paradoxical autophagy activation by hypoxic stress in endothelial cells was related to the HIF-1α and mTOR signalling pathways, but not HIF-2α, which was recently indicated as a brake to the autophagy accelerator function of HIF-1α and suppressed autophagy in chondrocyte.39 However, the
mechanisms which transformed excess protective autophagy into detrimantal autophagy, as well as the mechanisms which connected endothelial cell autophagy and apoptosis are still unclear. Previous studies have implicated several key machinery molecules in the regulation of both autophagy and apoptosis, such as Beclin1, Atg5, Bax, etc. \(^{40,41}\) Notably, in our present study, both Beclin1 and Atg5 were involved in the regulation of hypoxia-induced autophagy. Inactivation of Beclin1 and Atg5 could significantly prevent the inducement of autophagy by hypoxic stress, and meanwhile effectively reduce the occurrence of apoptotic cell death. These results not only demonstrated essential roles of Beclin1 and Atg5 in regulation of hypoxia-mediated autophagy and apoptosis in endothelial cells, but also provide additional evidence to indicate that hypoxia-induced autophagy and apoptosis are interrelated. Nevertheless, whether hypoxia-induced autophagy could directly execute cell death and by which mechanism Beclin1 and Atg5 connects hypoxia-induced autophagy and apoptosis is still to be elucidated yet.

In summary, in this study, we found that short exposure to hypoxia may induce HIF-1alpha/BNIP3-dependent autophagy, this type of autophagy may promote endothelial cell survival and growth; but if the hypoxia stress is prolonged, the autophagy activation may turn to be AMPK/mTOR dependent, and it is probably cell damaging and may cause further programmed endothelial cell death. Taken into account, the results that autophagy is activated in both proliferative and involuting IHs but with contrary functions, pro-survival or pro-death, depend on HIF-1alpha and mTOR signalling pathways, respectively, we may draw a conclusion that hypoxia-induced autophagy can bi-directionally regulates the survival of endothelial cells, and more importantly it might be a double-edged sword in the distinctive progression of IH.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

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