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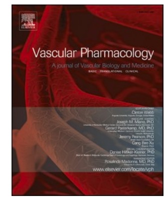

B-blockers activate autophagy on infantile hemangioma-derived endothelial cells *in vitro*

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A R T I C L E I N F O

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A B S T R A C T

The mechanisms underlying the success of propranolol in the treatment of infantile hemangioma (IH) remain elusive and do not fully explain the rapid regression of hemangiomatous lesions following drug administration. As autophagy is critically implicated in vascular homeostasis, we determined whether β-blockers trigger the autophagic flux on infantile hemangioma-derived endothelial cells (Hem-ECs) *in vitro*.

Material and methods: Fresh tissue specimens, surgically removed for therapeutic purpose to seven children affected by proliferative IH, were subjected to enzymatic digestion. Cells were sorted with anti-human CD31 immunolabeled magnetic microbeads. Following phenotypic characterization, expanded Hem-ECs, at P2 to P6, were exposed to different concentrations (50 μM to 150 μM) of propranolol, atenolol or metoprolol alone and in combination with the autophagy inhibitor Bafilomycin A1. Rapamycin, a potent inducer of autophagy, was also used as control. Autophagy was assessed by Lysotracker Red staining, western blot analysis of LC3BII/LC3BI and p62, and morphologically by transmission electron microscopy.

Results: Hem-ECs treated with either propranolol, atenolol or metoprolol displayed positive LysoTracker Red staining. Increased LC3BII/LC3BI ratio, as well as p62 modulation, were documented in β-blockers treated Hem-ECs. Abundant autophagic vacuoles and multilamellar bodies characterized the cytoplasmic ultrastructural features of autophagy in cultured Hem-ECs exposed *in vitro* to β-blocking agents. Importantly, similar biochemical and morphologic evidence of autophagy were observed following rapamycin while Bafilomycin A1 significantly prevented the autophagic flux promoted by β-blockers in Hem-ECs.

Conclusion: Our data suggest that autophagy may be ascribed among the mechanisms of action of β-blockers suggesting new mechanistic insights on the potential therapeutic application of this class of drugs in pathologic conditions involving uncontrolled angiogenesis.

1. Introduction

Since their introduction, the therapeutic use of beta (β)-blockers has been expanded to include hypertension, cardiac arrhythmias, myocardial infarction, thyrotoxicosis, essential tremors, situational anxiety, migraine headaches, kidney disease and glaucoma [1–3].

Intriguingly, several studies have demonstrated that β-blockers in combination with standard therapies possess anticancer effect and improve survival of cancer patients, opening a new field of investigations on the role of these drugs in neoplastic diseases [4–6]. In addition, the observation that propranolol exerts anti-proliferative effect on infantile hemangioma (IH) [7], a benign vascular neoplasm resulting

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from the abnormal proliferation of endothelial cells (EC) and angiogenesis, also favors the hypothesis that β-blockers possess unexpected therapeutic properties. Although the treatment of IH with propranolol has shown remarkable results, side effects, such as hypoglycemia, bronchial hyperreactivity, hyperkaliemia, diarrhea, sleep disturbance, and insomnia have been reported [8–13]. Moreover, the issue whether propranolol can cross the blood-brain barrier remains under debate [14,15]. Other β-blockers are employed for the treatment of IH, including atenolol and timolol, leaving systemic glucocorticoids and surgical intervention as a second-line therapy [16–19].

The mechanisms underlying the activity of β-blockers on IH are largely unknown and potentially include local vasoconstriction, downregulation of angiogenic growth factors and matrix metalloproteinases excretion, and decrease proliferation and induction of apoptosis of ECs [20–23].

There is a growing interest on the role of autophagy in the modulation of normal and pathologic vascular system and, according to the literature, particular attention has been paid on how autophagy regulates EC biology [24–28]. Autophagy is a subcellular, reparative and multistep process critically involved in preserving cellular homeostasis and, by removing cytoplasmic components and unwanted proteins, represents a life-sustaining tool. Three main types of autophagy have been described: chaperone-mediated autophagy (CMA), microautophagy, and macroautophagy (hereafter referred to as autophagy). Autophagic processes occur under basal condition although are also stimulated by various *noxae* like starvation, hypoxia, reactive oxygen species (ROS), microorganisms, viruses or pharmacological agents (*i.e.* rapalogs) [29–31]. Dysfunction in the autophagy pathway has been implicated in an increasing number of clinical disorders including infections, inflammatory processes, cancer, neurodegeneration, metabolic as well as cardiovascular diseases [24,32–34].

The aim of the present study was to determine whether autophagy is involved on the therapeutic efficacy of β-blockers in IH. Thus, the *in vitro* effects of propranolol hydrochloride, a non-cardioselective β-adrenergic receptor blocker, atenolol or metoprolol-tartrate (both specific β1-adrenoreceptor blockers) were tested on primary cultures of hemangioma-derived ECs (Hem-ECs). The induction of autophagy by β-blocking agents was extensively investigated at both biochemical and morphologic levels. We provide evidence that hemangioma-derived ECs undergo autophagy upon β-blockers exposure, supporting the hypothesis that modulation of autophagy by β-adrenergic receptors blockade may be involved in restoration of angiogenesis in vascular anomalies.

2. Materials and methods

2.1. Tissue sampling

Proliferative infantile hemangioma (IH) tissues from seven children (age < 12 months), enrolled from 2018 to 2021, were obtained from the Unit of Pediatric Surgery, Ospedale dei Bambini of Parma. Patients were enrolled after parents' informed consent to the employment of biologic samples for research purpose. Samples were collected under sterile condition and transferred to the Cell Culture Laboratory, Pathology Section of the University Hospital of Parma, within 30 min from resection. IH tissues were sampled by the medical staff ensuring the priority of their use for diagnostic purposes. The procedure was approved by the Local Ethics Committee (129/2018/OSS/AOUPR) and in accord with principles listed in the Helsinki declaration.

2.2. Endothelial cell lines

Under sterile conditions, fragments of hemangioma specimens were washed several times with phosphate buffer saline (PBS), finely minced using surgical scissors, and subjected to enzymatic digestion for 60 min at 37 °C with 1 mg/ml collagenase/dispase solution (Roche; 11097113001). The resulting digestion product was filtered using a 100 μm cell strainer (BD Biosciences, 352360) to remove aggregates. The harvested cells were washed, seeded on 6-well plate (Corning; 3516) pre-treated with collagen solution type 1 (Merck, C8919), and cultured in complete medium composed of Endothelial Cell Growth Medium (ready to use; Cell Application, Inc.; 211–500), plus 100 U/ml penicillin, and 100 μg/ml streptomycin (Merck, P4333).

After 24 h, non-adherent cells and debris were removed, whereas the adherent cell population was washed with PBS and cultured in fresh complete medium until 70–80% confluence. Primary cell cultures, consisting of a heterogeneous adherent cell population, were recovered with trypsin/EDTA (Merck, T3924) and sorted with anti-human CD31 immunolabeled magnetic microbeads, according to manufacturer's recommendations (CD31 MicroBeads Kit; Miltenyi Biotec, 130–091-935). Harvested $CD31^{pos}$ cells, defined as hemangioma-derived endothelial cells (Hem-ECs), were cultured in complete medium and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Two cell lines were used at passages 2 to 6 for the experiments. Cells were tested for mycoplasma contamination, at the beginning and during all experiments, by a detection kit according to the manufacturer's protocol (PCR Mycoplasma Detection Kit; abm; G238) and 4′,6-Diamidino-2-phenylindole (DAPI; Merck, D8417).

2.3. Immunofluorescence / immunocytochemistry

Immunofluorescence assays were performed on Hem-EC lines cultured on chamber slides (BD Biosciences, 354108) pre-treated with collagen solution type 1. When 80% confluence was reached, cells were fixed in 4% paraformaldehyde in PBS (pH = 7.4) for 15 min at room temperature (RT). After washing, monolayers were incubated with 20% goat serum (Merck, G9023) for 30 min at RT to block unspecific bindings. Cells were then incubated with the following unconjugated primary antibodies: mouse anti-human CD31 (1:60; 30 min 37 °C; DAKO, M0823) and rabbit anti-human von Willebrand factor (vWF) (1:200; overnight 4 °C; DAKO, A0082). Anti-mouse and -rabbit IgG FITC-conjugated secondary antibodies (1:70; 60 min 37 °C; Merck, F4018 and F9887) were employed, respectively to reveal the epitope. Nuclei were counterstained by DAPI (0.5 mg/ml, 15 min at RT). Coverslips were mounted with Vectashield (Vector Laboratories, H-1000) and samples were analyzed by a fluorescence microscope (Olympus BX60) connected with a digital camera (QICAM) (QImaging, Surrey, BC, Canada).

GLUT1 was investigated by immunoperoxidase. As above, after fixation, 3% hydrogen peroxide solution was applied for 10 min on chamber slides containing Hem-ECs and then cells were incubated with rabbit anti-human GLUT1 (ready to use, 37 °C; 30 min; Ventana-Roche). The immunoreaction was revealed by IHC Detection Kit-Micropolymer (Abcam; ab236466) according to manufacturer's recommendations. Hematoxylin was used as nuclear counterstain. Following the application of coverslips, slides were analyzed under an optical microscope (Olympus BX60) connected to QICAM camera.

Negative controls were represented by exposing cell preparations to the same immunostaining procedure but avoiding primary antibodies.

2.4. Chemical reagents

Propranolol hydrochloride (Prop; P0884), atenolol (Ate; A7655), metoprolol-tartrate (Meto; M5391), rapamycin (Rapa; R0395), and bafilomycin A1 (BafA1; B1793) were purchased from Merck and reconstituted and stored according to the manufacturer's instructions.

2.5. Cell viability assay

Ninety-six-well tissue culture flat-bottom plates (Corning, 3595) were coated with collagen solution type 1. Hem-ECs were seeded at 4×10^3 cells per well in 100 μl of complete medium. After adhesion, endothelial cells were exposed to various β-blockers concentrations (50,

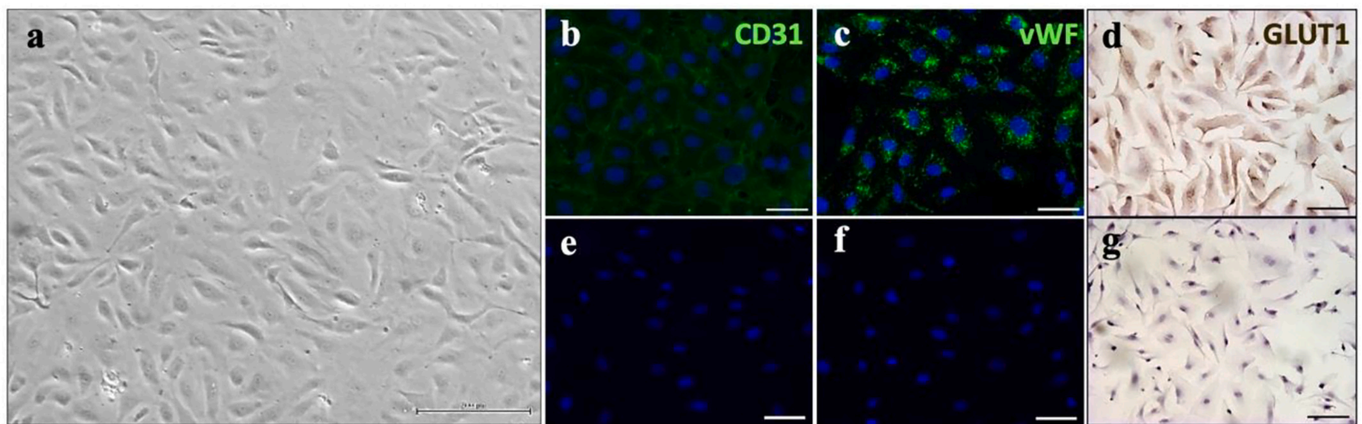

Fig. 1. Hemangioma-derived ECs (Hem-ECs) morphology and immunophenotype. (a): phase contrast image of a primary culture of Hem-ECs following immunomagnetic sorting with CD31 microbeads, illustrating a confluent monolayer with cobblestone-like formation suggestive of endothelial phenotype. The surface expression of the pan-endothelial marker CD31 (b) and the dot-like cytoplasmic labelling of von Willebrand factor (c) in Hem-ECs are documented by green immunofluorescence. Blue fluorescence corresponds to DAPI staining of nuclei. (d): the expression of GLUT1 (brownish) in Hem-ECs is shown by immunoperoxidase. Nuclei are counterstained by light hematoxylin. Negative controls for CD31, vWF and GLUT1, using the same immunostaining procedure but avoiding primary antibodies, are shown in e, f, and g, respectively. Scale bars: a = 200 μm; b, c, e, f = 50 μm; d, g = 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

75, 100, and 150 μM). After 24 h, 100 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide at 1 mg/ml (MTT; Merck, M2128) were added to each well. Plates were further incubated at 37 °C for 2 h. At the end of incubation, the formazan was dissolved using 100 μl of dimethyl sulfoxide (DMSO; Merck, D2650) per well. The optical density was measured with a microplate reader (Victor™X4; Perkin Elmer).

All experiments were carried out twice and five replicates for each condition were made and data expressed as viability percentage.

2.6. Western blot analysis

Thirty μg of proteins lysates from Hem-EC monolayers untreated (CTRL = control vehicle) and treated, for 6 h, with 75 μM Prop ±50 nM BafA1; 100 μM Ate ±50 nM BafA1, 100 μM Meto ±50 nM BafA1; 50 nM Rapa ±50 nM BafA1; or 50 nM BafA1 alone, were resolved by SDS-PAGE and transferred to Nitrocellulose membranes (ThermoFisher Scientific). Membranes were incubated with: 1:1000 rabbit anti-SQSTM1/p62 (#5114S Cell Signaling Technology, Beverly, MA, USA); 1:1000 rabbit anti-LC3B (#2775S Cell Signaling Technology, Beverly, MA, USA); 1:1000 mouse anti-GAPDH (sc-137179, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Blots were then washed and incubated with anti-mouse or anti-rabbit antibodies at 1:5000 dilution (#926–68020 IRDye®680LT Goat anti-Mouse IgG and #926–32211 IRDye®800CW Goat anti-Rabbit IgG - LI-COR, Biosciences, Lincoln, NE, USA). The bands signals were acquired by Licor Odyssey Scanner Infrared Imager (LI-COR Biotechnology Lincoln, NE, USA) and quantified using ImageJ software.

2.7. Lysotracker red probe

Hem-EC lines were cultured on chamber slides pre-treated with collagen solution type 1. Confluent cells were either untreated or treated with 75 μM Prop ±50 nM BafA1; 100 μM Ate ±50 nM BafA1, 100 μM Meto ±50 nM BafA1; 50 nM Rapa ±50 nM BafA1; or 50 nM BafA1 alone. Rapamycin is a potent inducer of autophagy, conversely bafilomycin A1 is autophagy inhibitor, because is a vacuolar-type H + -translocating ATPase (V-ATPase) and a lysosomal inhibitor. After 6 h, Hem-ECs were labeled with 75 nM of the acidotropic dye LysoTracker Red DND-99 (LTRed; Invitrogen #L7528; 1 mM stock solution in DMSO) for 90 min at 37 °C, according to manufacturer's recommendations. Slides were immediately analyzed by EVOS FL fluorescence microscope (Thermo Scientific, Waltham MA, USA) with a filter set at 590 nm fluorescence emission and representative images were captured.

2.8. TEM analysis

Hem-ECs were cultured in complete medium (control) or exposed to 75 μM propranolol ±50 nM BafA1; 100 μM atenolol ±50 nM BafA1; 100 μM metoprolol ±50 nM BafA1; 50 nM rapamycin ±50 nM BafA1; or 50 nM bafilomycin A1 alone for 6 h in 75 cm^2 flasks (BD Biosciences, 351036) pre-treated with collagen solution type 1. A detailed methodology of TEM analysis has been previously reported by our laboratory [35]. In brief, cellular monolayers were harvested, fixed and included. At least 10 cells from each treatment were examined under a transmission electron microscope (Philips EM 208S) to detect subcellular features and representative images were captured.

2.9. Statistical analysis

All experiments were done in duplicate. One-way analysis of variance (ANOVA) with Holm-Sidak's *post hoc* test was used. Data are reported as mean ± standard error (SE). Statistical significance was set at $*p < 0.05$ and $\# \ p < 0.001$.

3. Results

Hem-ECs harvested following enzymatic digestion and immunomagnetic sorting were cultured, expanded, and initially identified as EC according to morphologic criteria (Fig. 1A). To ensure the purity of recovered cells, immunocytochemistry was performed to assess the expression of the pan-endothelial marker CD31 and von Willebrand (vWF) factor. As shown in Fig. 1B and C, Hem-ECs displayed remarkable expression of both endothelial associated antigens. In addition, Hem-ECs consistently expressed GLUT1 (Fig. 1D). IH cell lines were tested as mycoplasma-free during all experimental procedures (data no shown).

3.1. Cell viability

Exposure to β-blockers for 24 h affected Hem-ECs viability, as measured by MTT assay (Fig. 2). The negative effect on cell survival was dose-dependent, reaching statistical significance at 75 μM propranolol and 100 μM atenolol or metoprolol ($p < 0.05$).

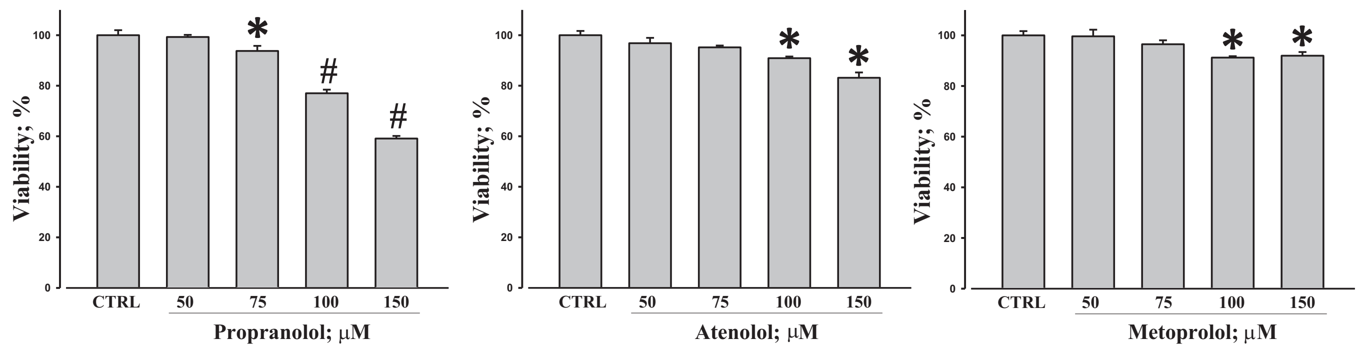

Fig. 2. Viability assay. Representative histograms from MTT assays on Hem-ECs treated with increasing dose of each beta-blocker for 24 h. Percent viability with respect to untreated cells (CTRL) is reported as mean ± standard error (SE); $^*p < 0.05$ *vs* control; $\#p < 0.001$ *vs* control. Holm-Sidak's test.

3.2. Autophagy-related proteins

To document the autophagic flux, western blot analysis was employed to assess the conversion of cytosolic LC3-I into LC3-II and p62 expression in Hem-ECs after 6 h exposure to 75 μM propranolol, 100 μM atenolol, or 100 μM metoprolol (Fig. 3). As indicated by an increased LC3-II/LC3-I ratio, propranolol, atenolol, and metoprolol were able to modulate, lipidate, and cleave LC3. Moreover, p62 was downregulated in Hem-ECs exposed to experimental concentrations of all three β-blockers, clearly documenting activation of the autophagic flux. Accordingly, rapamycin exerted a similar effect on LC3 cleavage which was associated with p62 downregulation. In accordance with the literature, BafA1 was able to inhibit β-blockers- and rapamycin- induced autophagic flux, as documented by accumulation of LC3-II and lack of p62 decay, respectively [36,37].

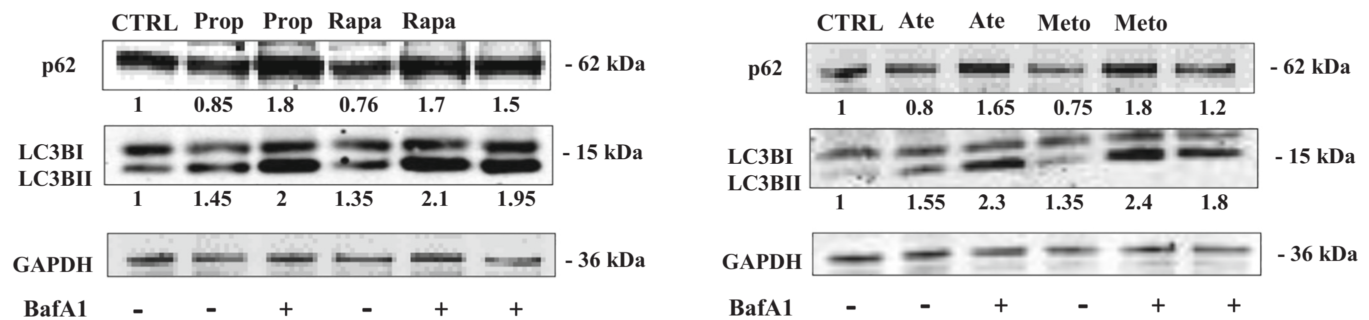

Fig. 3. Western blot assay. Immunoblotting analysis of LC3 and p62 proteins on cultured Hem-ECs either untreated (CTRL) or exposed for 6 h to propranolol (Prop, 75 μM), atenolol (Ate, 100 μM) or metoprolol (Meto, 100 μM). The effects on LC3 and p62 expression by rapamycin (Rapa, 50 nM) or the addition of bafilomycin A1 (BafA1, 50 nM) to each Hem-ECs culture condition are also shown. Densitometric values normalized *vs* control (GAPDH) are reported at the bottom of each corresponding blot.

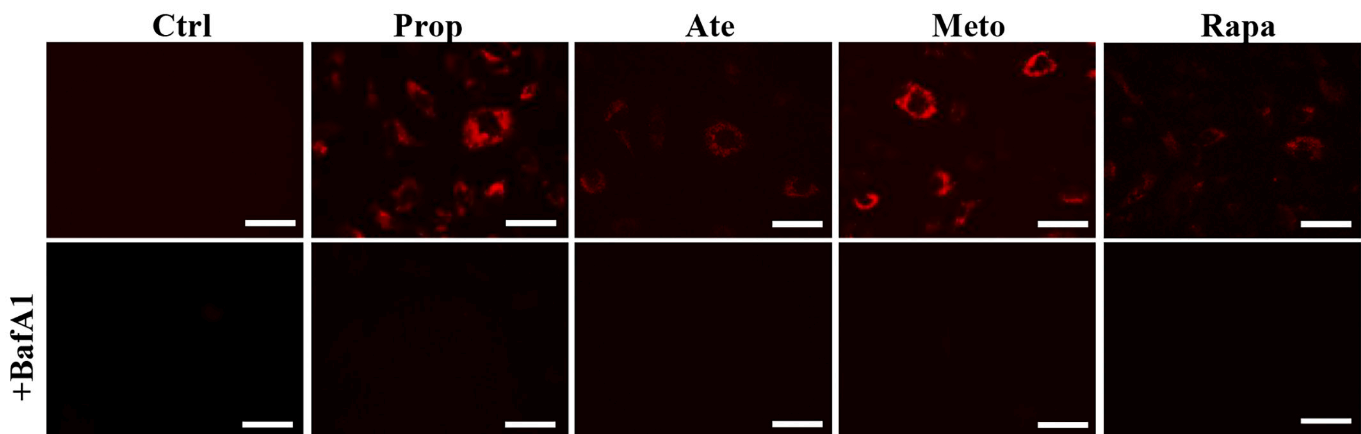

Fig. 4. LysoTracker® Red DND-99 staining. Upper panels: induction of autophagy in cultured Hem-ECs exposed to 6 h propranolol (Prop, 75 μM), atenolol (Ate, 100 μM) or metoprolol (Meto, 100 μM) is illustrated by the strong cytoplasmic red fluorescence of autolysosomes as detected by LysoTracker. The absence of fluorescent signals in control (CTRL) untreated Hem-ECs and the expected positive fluorescent labelling after exposure to rapamycin (Rapa, 50 nM) are also shown. Lower panels: Hem-ECs did not display LysoTracker-positive lysosomes following the addition of Bafilomycin A1 (BafA1, 50 nM) to each Hem-EC culture condition. Scale bars: 25 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

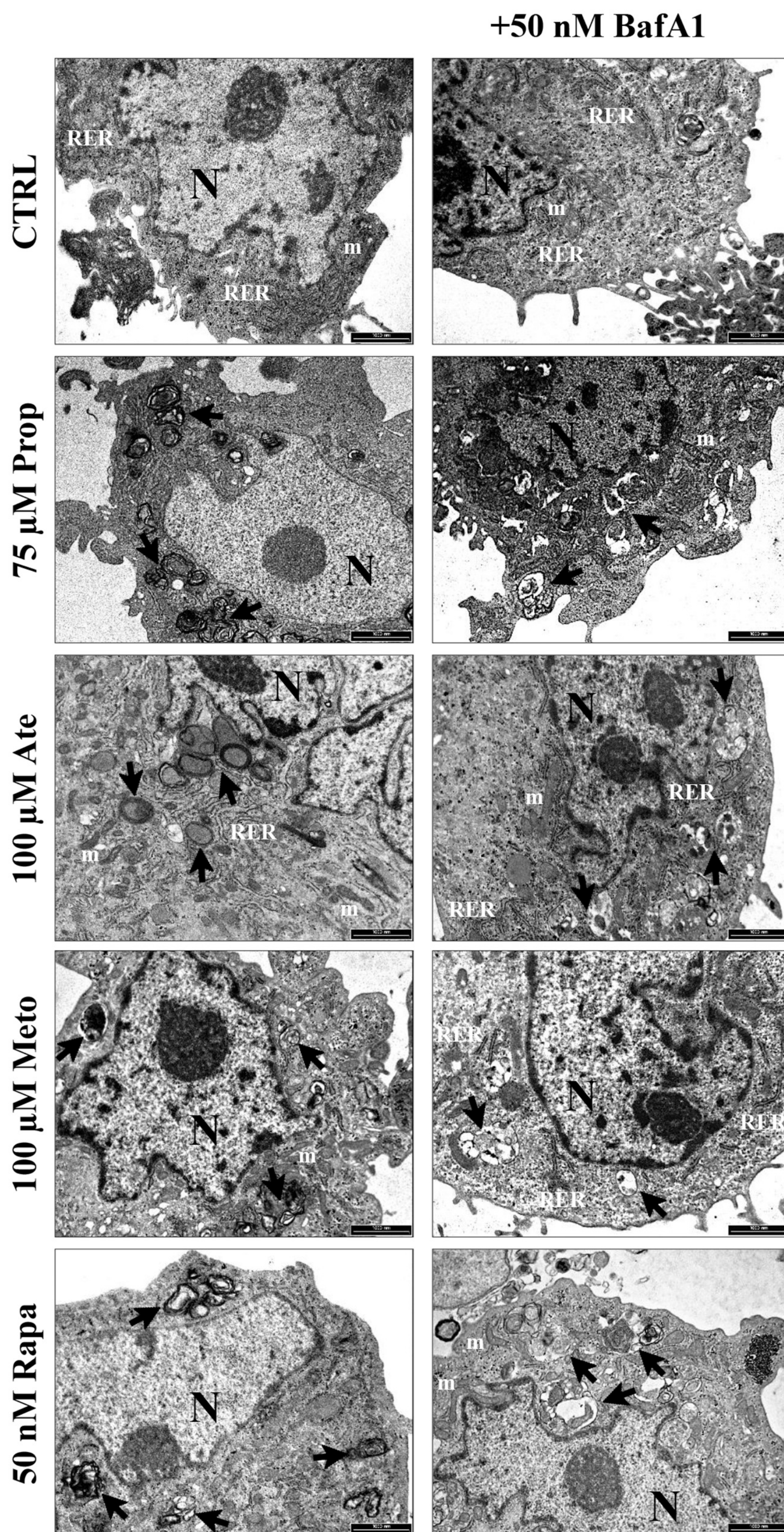

Fig. 5. Transmission Electron Microscopy. Representative microphotographs illustrating the ultrastructural features of Hem-ECs either untreated (CTRL) or exposed for 6 h to propranolol (Prop), atenolol (Ate) or metoprolol (Meto) at the indicated μM concentrations. The effects on cell morphology of rapamycin (Rapa) or the addition of bafilomycin A1 (BafA1, 50 nM) to each experimental culture condition are also shown. RER = rough endoplasmic reticulum; m = mitochondria; N = nucleus; arrows point to autophagic vacuoles and multilamellar bodies. Scale bars = 1000 nm.

3.3. Lysotracker acidotropic staining

To ascertain the induction of autophagy by β-blockers, we performed acidotropic staining with LysoTracker Red dye and observed a significant increase in the number of acidic vesicles, indicative of autophagosome/lysosome fusion in Hem-ECs (Fig. 4). These fluorescent patterns induced by all tested β-blocking agents were not detectable after the addition of 50 nM bafilomycin A1, a known inhibitor of autophagosomes and lysosome fusion. Conversely, acidotropic fluorescent signals were clearly displayed by Hem-ECs treated with 50 nM

rapamycin while absent in cells exposed to 50 nM BafA1 alone (Fig. 4) [37].

3.4. TEM analysis

Finally, TEM analysis was employed as the gold standard method to document autophagy in biological samples [36]. The ultrastructural features of Hem-ECs cultured for 6 h in the absence or presence of β-blockers (75 μM Prop, 100 μM Ate, or 100 μM Meto) ± 50 nM BafA1 were evaluated. The effects of 50 nM rapamycin ±50 nM BafA1, or BafA1 (50 nM) alone on Hem-ECs morphology were also investigated (Fig. 5).

Untreated cells showed normal organelles distribution and morphology, characterized by preserved mitochondria and abundant rough endoplasmic reticulum (RER) in perinuclear area, characterizing healthy ECs. Interestingly, several autophagic vacuoles with dense amorphous contents, unrecognizable cargo, or multi-lamellar membrane structures were present in Hem-ECs treated with 75 μM propranolol, 100 μM atenolol, or 100 μM metoprolol. Similar autophagic findings were detected in Hem-ECs treated with 50 nM rapamycin. According to the literature, 50 nM bafilomycin A1 blunted the autophagic flux induced by β-blockers or rapamycin and was associated with enlarged non-functioning autophagosome [37,38].

4. Discussion

The discovery that a non-selective β-blocker, propranolol, was able to determine complete involution of hemangiomatous lesions in cardiopathic kids supports the contention that this class of drugs possesses properties yet to be explored [7]. Since then, treatment of IH by propranolol and more recently, topical atenolol, has become popular worldwide and positive results have been repeatedly reported [16–19]. Atenolol offers a comparable or superior efficacy with respect to propranolol with fewer side effects (sleep disturbance, bronchospasm and hypoglycemia) and more-favorable posology [19]. However, the mechanisms underlying the efficacy of β-blockers remain elusive and proposed explanations are unable to fully clarify this issue [20–23]. Results of the present study provide evidence that autophagy may be included among the biological effects of β-blockers on hemangioma-derived endothelial cells.

Autophagy is a subcellular and reparative multistep process of great relevance in maintaining cellular homeostasis thereby involved in various physiologic processes, such as embryogenesis, development, differentiation, and aging [39,40]. In addition, autophagy contributes to the innate and adaptive immune responses [41] and regulates vWF processing by endothelial cells [42].

Excessive or deficient autophagy processes have been implicated in an increasing number of human illnesses like infectious diseases, inflammation, neurodegeneration, metabolic disease, cancer, as well as cardiovascular diseases [26,43].

Autophagy has been generally ascribed as a protective cellular process [44] also in reason of its anti-inflammatory properties and specifically on ECs by interfering with oxidized low-density lipoprotein-induced injury and apoptosis [45,46]. Importantly, inflammation and autophagy are reciprocally regulated as inflammation profoundly affects autophagy-associated transcriptional program which includes hypoxia inducible factor-1 (HIF-1), JUN, signal transducers, and activators of transcription (STAT) 1 and STAT3 [47] while in turn autophagy acts as a negative regulator of inflammasomes [48]. How and to what extent β-adrenergic receptor blockade interferes with this complex and finely regulated machinery in normal and neoplastic angiogenesis remains uncovered.

An increasing number of recent observations have underlined the importance of autophagy in cardiovascular pathophysiology. Studies have focused on the role of autophagy in the myocardium, for example during ischemia/reperfusion in which it can be either beneficial or harmful [49]. Furthermore, rising data from the literature have tried to link autophagy to vascular disorders as atherosclerosis, aneurysm, arterial aging, vascular stiffness, and chronic venous disease [25,28,50–52]. Moreover, endothelial-to-mesenchymal transition, a widely recognized detrimental event associated with severe vascular and valvular damage, may be prevented by the activation of autophagic pathways [53]. Whether this additional protective mechanism of autophagy is modulated by β-adrenergic receptors is unknown and may represent a future area of intense scrutiny.

Although intensively investigated, a pivotal uncertain question is whether the autophagic process may represent a therapeutic target. Better understanding of the role of autophagy in vascular biology is the next goal to validate druggable steps and exploit their therapeutic potential on clinical ground. However, the highly fine tuning of the process makes this goal difficult to accomplish [52,54–56].

Numerous *in vivo* e *in vitro* studies have employed animal models and normal and cancer cell lines, to explore the function of autophagy by testing different compounds and stressors. Results still remain controversial, indicating that the activation of autophagy is likely a double-edged sword [56,57].

Although not tested here, an attractive hypothesis on the beneficial effects of β-blockers on IH through the induction of EC autophagy may be advanced by the observation that VEGFA is a natural autophagic substrate. Indeed, autophagic inhibitors, as BafA1 and chloroquine, may lead to VEGFA accumulation and *in vivo* starvation experiments in mice have shown at cardiac and aortic sites increased VEGFA expression which was blunted by chloroquine administration [58]. This finding might be of particular relevance when applied to tumor angiogenesis and the oncogenic potential of VEGFA. Along this line, the tumor suppressive properties of decorin, a soluble small leucine-rich proteoglycan and a novel anti-cancer protein, are attributed to an increased rate of VEGFA clearance through the promotion of the autophagic flux [59]. In partial support of our hypothesis is the recently documented reduction of blood VEGFA levels in patients affected by IH undergoing propranolol treatment [60], although further validation is necessary before providing conclusive statements.

When autophagy is triggered, mature autophagosomes fuse with lysosomes, and finally degrade the sequestered cargo. The conversion of cytosolic microtubule-associated protein 1 light chain 3 (LC3)-I into autophagosome-specific LC3-II have been widely used as evidence of autophagy. Up-regulation of LC3II/LC3I ratio and decreased p62 protein levels were detected here in Hem-EC lines exposed to propranolol, atenolol, or metoprolol. Conversely, the autophagy flux was inhibited by bafilomycin A1, as confirmed by the LysoTracker assay. Taken together, all these molecular findings, supported by TEM analysis, provide evidence that autophagy may be a new potential mechanism by which beta-blockers may restore vascular anomalies.

The use of old drugs and their subsequent evolution have unexplored clinical potential, partly due to the lack of appropriate experimental animal models (*i.e.* infantile hemangioma) and, on the other, unexpected pharmacological effects.

While proposing a new aspect of the clinical activity of β-blocking agents, several limitations of the present work must be acknowledged. First, potentially targetable molecular pathways underlying β-blockers induced autophagy were not tested as we did not provide *in vivo/ex vivo* evidence on the role of autophagy and its modulation in IH. Transgenic mouse models are warranted to cover the actual drawbacks affecting the IH scenario.

An additional weakness, restraining the translational significance of our study, is the lack of functional tests on ECs and of data challenging β-blockers with new clinically valuable autophagy inducers [61] or other drugs displaying clinical meaningful properties through modulation of autophagy [62].

Further studies on cultured HemECs and tissue samples from patients affected by proliferative and involuted IH are undergoing and will prospectively achieve more informative mechanistic insights.

5. Conclusion

Our *in vitro* study proposes, for the first time, autophagy as a plausible pivotal mechanism of action of propranolol and atenolol, actually employed as first-line therapy in IH.

This hypothesis is in line with recent observations documenting that activated autophagy pathways may be involved in the recovery of morbid conditions [51,63–65], such as altered angiogenesis in infantile hemangioma.

This field of research represents an exciting challenge to optimize and expand druggable targets endowed with the potential to change the natural history of the disease and improve the actual clinical management of IH.

Author contributes

Conceptualization: Lorusso B, Lagrasta C, Cerasoli G; Data curation: Lorusso B, Lagrasta C, Quaini F, Roti G; Investigation & Methodology: Lorusso B, Falco A, Frati C, Madeddu D, Nogara A, Graiani G, Gherli A, Cerretani E, Caputi M, Corradini E, Pilato FP, Gnetti L; Supervision & Validation: Lagrasta C, Quaini F, Roti G; Writing - original draft: Lorusso B; Figures – Lorusso B, Falco A, Frati C, Madeddu D, Cerretani E; Caputi M; Writing - review & editing: Lorusso B, Lagrasta C, Quaini F, Graiani G.

All authors approved the final version of manuscript.

Authors statement

All the authors have read and approved the revised manuscript entitled "*β-blockers activate autophagy on infantile hemangioma-derived endothelial cells in vitro*" sent for publication on Vascular Pharmacology.

Declaration of Competing Interest

The authors declare no conflict of interest.

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