

Original Research Article

Autophagy inhibition by chloroquine prevents increase in blood pressure and preserves endothelial functions

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Abstract

Purpose: To determine the effects of lysosomal inhibition of autophagy by chloroquine (CHQ) on hypertension-associated changes in the endothelial functions.

Method: Angiotensin II (Ang II)-treated human endothelial cell line EA.hy926 and renovascular hypertensive rats were subjected to CHQ treatment (in vitro: 0.5, 1, and 2.5 μ M; in vivo: 50 mg/kg/day for three weeks). Changes in the protein expressions of LC3b II (autophagosome formation marker) and p62 (autophagy flux marker) were assessed using immunoblotting. Cell migration assay, tubule formation assay (in vitro), and organ bath studies (in vivo) were performed to evaluate the endothelial functions. Hemodynamic parameters were measured as well.

Results: A higher expression of LC3b II and a reduced expression of p62 observed in the Ang II-treated endothelial cells, as well as in the aorta of the hypertensive rats, indicated enhanced autophagy. Treatment with CHQ resulted in reduced autophagy flux (in vitro as well as in vivo) and suppressed Ang II-induced endothelial cell migration and angiogenesis (in vitro). The treatment with CHQ was also observed to prevent increase in blood pressure in hypertensive rats and preserved acetylcholine-induced relaxation in phenylephrine-contracted aorta from the hypertensive rats. In addition, chloroquine attenuated Ang II-induced contractions in the aorta of normotensive as well as hypertensive rats.

Conclusion: These observations indicated that CHQ lowers the blood pressure and preserves the vascular endothelial function during hypertension.

Keywords: Angiotensin II, Autophagy, Chloroquine, Endothelial function, Hypertension, Vascular dysfunction

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INTRODUCTION

Autophagy is a regulated cellular process in which damaged proteins and cell organelles are recycled after their sequestration into autophagolysosomes (bi-membranous intracellu-

lar vesicles) followed by lysosome-dependent degradation [1]. The upregulation of autophagy in cells is characterized by increased autophagosome formation and increased autophagic flux (marked by increased LC3B-II

expression and a decreased p62 expression), and manifests early signs of stress [2].

Enhanced autophagy exerts a detrimental effect during various cardiovascular pathologies, such as severe mitral and tricuspid regurgitation, pressure overload-induced cardiac remodeling, heart failure, atherosclerosis [3], and pulmonary hypertension [4]. Enhanced autophagy is also linked to the progression of renovascular hypertension-associated cardiac remodeling [5]. Angiotensin II (Ang II) plays a crucial role in the pathophysiology of hypertension and has been reported to induce endothelial cell autophagy [6]. Modulation of autophagy in bovine aortic endothelial cells has been demonstrated to alter the endothelial functions. Genetic stimulation of autophagy the overexpression of ATG5 has been observed to increase endothelial cell migration and angiogenesis, while reduced autophagy inhibits endothelial cell migration as well as angiogenesis [7].

The endothelium is a prime target for damage during hypertension. However, limited information is available regarding the role of vascular autophagy in endothelial dysfunction occurring during hypertension. This formed the basis for encouraging the present study. Lysosomal inhibitors of autophagy, such as CHQ and bafilomycin, have been used extensively for the study of autophagy inhibition in various pathologies, including cancer [8] and cardiac hypertrophy [9].

Therefore, the present study was aimed to determine the effects of CHQ-induced lysosomal inhibition of autophagy on the endothelial functions as a response to Ang II-treatment *in vitro* and in the rat model for renovascular hypertension.

EXPERIMENTAL

In vitro cell culture

Human endothelial cell line EA.hy926 [originally developed by C. J. Edgell, and provided as a gift from the University of North Carolina (UNC) Lineberger Comprehensive Cancer Center, USA] was used for conducting the *in vitro* experiments. The cells were cultured in complete Dulbecco's Modified Eagle Medium (DMEM; HiMedia, India) enriched with 10% fetal bovine serum (FBS; HiMedia, India) and 1 % penicillin/streptomycin (HiMedia, India), at 37 °C in 5 % CO₂ atmosphere, until 80 – 90 % confluence was attained. After that, the cells were selected randomly and subjected to the respective treatments in each test.

Cell viability assay

In order to determine the non-cytotoxic concentration of CHQ for the human endothelial cell line EA.hy926, the effect of CHQ on the cell viability was determined using the MTT assay. This assay is based on the metabolic reduction of 3-[4,5-dimethylthiazol-2-y]-2,5-diphenyltetrazolium bromide (MTT). The cells from the cell line EA.hy926 were seeded (2 x 10⁴ cells per well) in the wells of 96-well plates and left undisturbed overnight to allow adherence. Subsequently, the cells in the wells were randomly exposed to various concentrations of CHQ (0.5–10 µM) for 24 h, followed by incubation with MTT at a final concentration of 0.5 mg/L for 2–3 h. After the incubation and the removal of media, dimethyl sodium sulfoxide (150 µL) was added, and the changes in the absorbance at 570 nm were recorded using a microplate reader (BioTek, USA). Data were normalized to the respective controls, and are presented as percentage cell viability.

Endothelial cell migration assay

The effect of CHQ co-treatment on the Ang II-induced migration of the human endothelial cell line was studied using the *in vitro* wound closure assay. The cell line cultured in 6-well plates and having reached 80%–90% confluence was mechanically scratched using a sterile pipette tip to create a cell-free zone. The debris was removed through washing with phosphate-buffered saline (PBS). The culture medium containing 2.5% FBS was added for wound healing.

Subsequently, the cells were randomly assigned and subjected to different treatments: control (untreated) and the Ang II (1 µM) with or without co-exposure to CHQ (0.5 µM, provided ½ h before the Ang II treatment) treatment. After the treatments, phase-contrast images of the scratched area were captured at different time points (0 min, 12 h, and 24 h). The image captured just after the addition of Ang II was considered the image at 0 min time point. The obtained images were analyzed using the Image J software version 1.46r (released by Wayne Rasband, National Institute of Health, USA).

Endothelial capillary tube formation assay in Matrigel

The effect of co-treatment with CHQ (0.5 and 1 µM) on the Ang II (1 µM)-induced angiogenesis was studied using the endothelial capillary tube formation assay [10].

Animals

All the experiments were conducted in accordance with the International Ethical Standards, with prior approval obtained from the Institutional Animal Ethics Committee (IAEC) of CSIR-Central Drug Research Institute (CSIR-CDRI, approval no. IAEC/2017/281). All the animals were handled with care, in accordance with the guidelines provided by National Research Council (USA) [11]. All animals were treated in accordance with Canadian Council on Animal Care (CCAC) guidelines [12]. Male Sprague-Dawley (SD) rats, each weighing 180 – 200 g, were procured from the National Laboratory Animal Center (NLAC), CSIR-CDRI, Lucknow, India, and were housed in polypropylene cages. The rats were maintained under standard environmental conditions comprising 12-h photoperiod, 23 ± 2 °C temperature, and 60%–65% humidity. Feed (dry pellets) and water were available *ad libitum*.

Renovascular model of hypertension

The male SD rat is a widely accepted animal model for renovascular hypertension. The model was developed through partial ligation of the renal artery according to a previously published procedure [13,14]. The rats developed significant hypertension within five weeks. Two weeks after the surgery, the animals were assigned randomly into 3 groups with 5 animals each (n = 5): sham-operated control (Sham), renovascular hypertensive rats (HTN), and CHQ-treated hypertensive rats (HTN + CHQ; 50 mg/kg); and the aorta from all groups were subjected to immunoblotting and organ-bath experiments. The HTN + CHQ group received daily drug treatment beginning from the 3rd week until the end of the 5th-week post-surgery.

Measurement of arterial blood pressure and heart rate in anesthetized rats

Upon completion of the treatment schedule, the rats from each group were anesthetized with urethane (1.25 g/kg; i.p.). The hemodynamic parameters, namely, systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial blood pressure (MAP), and heart rate (H), were recorded by following a previously published invasive method [10].

Immunoblotting

Cell- or tissue-lysates were fractionated on a 12 % SDS-PAGE gel, followed by transfer to a polyvinylidene fluoride membrane. Subsequently, the blots were blocked using 5 % bovine serum

albumin (BSA) prepared in Tris-buffered saline (TBS) containing 0.1 % Tween 20, and incubated overnight with diluted antibodies— anti-p62 (1:1000 dilution; Sigma, USA), anti-LC3B (1:1000 dilution; Cell signaling technology), and anti-beta actin (1:10000 dilution; Sigma, USA). After incubation with the respective antibodies, the membranes were incubated for another 2 h with the respective HP-conjugated mouse or rabbit secondary antibodies, following which they were visualized using ECL western blotting detection reagent. Immunoblot intensity was analyzed using myImageAnalysis software version 2.0 (Thermo Scientific, USA).

Rat aortic rings preparation and tension recording

Soon after the measurement of the hemodynamic parameters, the descending thoracic aorta was isolated, placed in ice-cold Modified Krebs's Henseleit solution (MKHS) [composition (in mM): NaCl, 118; NaHCO₃, 24; KCl, 4; CaCl₂, 1.8; MgSO₄, 1; NaH₂PO₄, 0.43; and glucose, 5.56; pH 7.4], and the adherent adipose and connective tissues were cautiously trimmed off. The transversely cut, 2–3-mm wide aortic rings were mounted individually in a two-chambered organ bath (ADInstruments, Australia) containing MKHS, maintained thermostatically at 37 °C and aerated continuously with carbogen gas. The arterial rings were subjected to passive tension for 60 min, and the tension experiments were conducted using the methods described in a previously published report [15].

Statistical analysis

The data were collected in a blinded manner, and the results were expressed as mean \pm standard error of the mean (SEM). One-way ANOVA, followed by post-hoc analysis with Bonferroni's test, was used for determining significant differences among treatment groups. All the statistical analyses were performed in the GraphPad Prism software version 5.00.28 (GraphPad Software, Inc., San Diego, CA, USA). A probability value of < 0.05 was considered statistically significant.

RESULTS

Chloroquine inhibits angiotensin II-induced upregulation of autophagy in human endothelial cell line, EA.hy926

After 24 h of exposure to CHQ, it was observed that the CHQ concentrations of 0.5, 1, and 2.5 μ M were not toxic for the human endothelial cell

line EA.hy926 (Figure 1 a). Loss of cell viability was observed with the CHQ concentrations of 5 and 10 μM . Therefore, the CHQ concentrations of 0.5, 1, and 2.5 μM were used for further experiments. Treatment of the human endothelial cell line EA.hy926 with Ang II (1 μM) for 24 h was observed to enhance the expression of the LC3B-II/LC3B-I protein (Figure 1 b) and reduce the expression of p62, indicating an increase in the autophagosome formation and the flux of autophagy, respectively.

Co-treatment with CHQ (0.5–2.5 μM) did not result in reduction in the LC3B-II/LC3B-I protein ratio that was increased by Ang II, rather CHQ at a concentration of 2.5 μM was observed to increase this ratio, demonstrating that the Ang II-induced autophagosome formation remained unaffected by the CHQ co-treatment (at 0.5 μM concentration). However, the CHQ co-treatment was observed to significantly increase the expression of p62 in the Ang II-treated endothelial cells in a concentration-dependent manner (0.5 – 2.5 μM), indicating the suppression of Ang II-induced autophagy flux owing to the inhibition of lysosomal function.

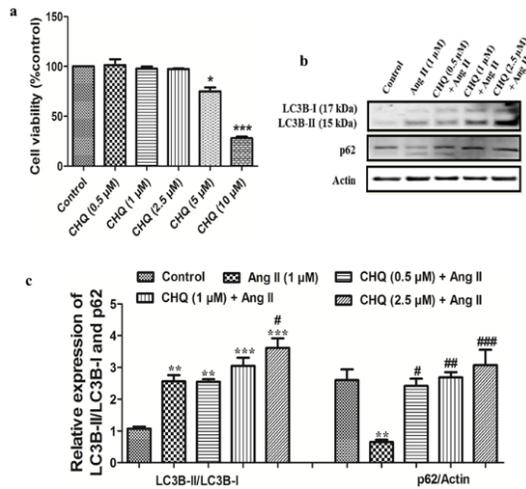


Figure 1: Effect of CHQ exposure for 24 h on (a) the cell viability of human endothelial cell line EA.hy926, and (b and c) on the Ang II (1 μM)-induced LC3B-II and Ang II (1 μM)-reduced p62 expression in the human endothelial cell line EA.hy926. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.005$ vs. control; # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.005$ vs. Ang II-treated Ea.hy926 cells (n = 5)

Chloroquine inhibits angiotensin II-induced endothelial cell migration and angiogenesis

Ang II (1 μM) treatment for 12 h, as well as for 24 h, was observed to significantly induce endothelial cell migration (Figure 2 a) and angiogenesis (marked by increased tubule

formation; Figure 2 b) in the human endothelial cell line EA.hy926. Co-treatment with CHQ at autophagy-inhibiting concentrations (0.5 and 1 μM) significantly suppressed the Ang II-induced endothelial cell migration at 12 h and 24 h. However, CHQ *per se* (1 μM) exerted an effect on endothelial cell migration. In addition, CHQ treatment (at 0.5 and 1 μM concentrations) significantly suppressed both basal and Ang II-induced tubule formation in endothelial cells.

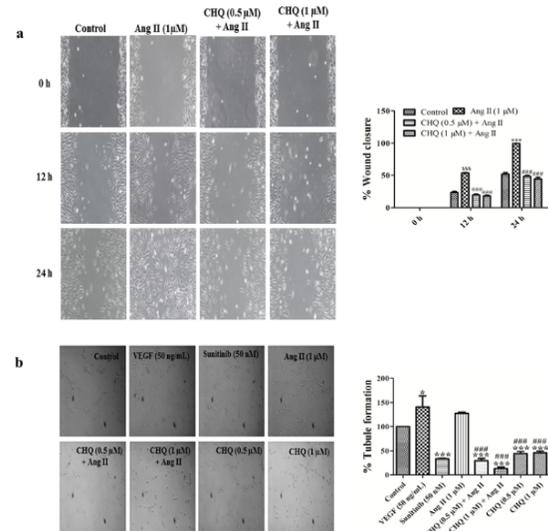


Figure 2: Effect of CHQ on Ang II (1 μM)-induced: (a) cell migration and (b) capillary tubule formation in human endothelial cell line EA.hy926; \$\$\$ $p < 0.005$ vs. Control (12 h); * $p < 0.05$ and *** $p < 0.005$ vs. control (24 h); @@@ $p < 0.005$ vs. Ang II (12 h); ### $p < 0.01$, #### $p < 0.005$ vs. Ang II (24 h)-treated Ea.hy926 cells (n = 5)

Chloroquine prevents a rise in blood pressure in rat model for renovascular hypertension

After the partial ligation of the renal artery, the rats became hypertensive within five weeks after surgery. A significant rise (Figure 3) in the parameters of SBP (150.00 \pm 1.3 mmHg), DBP (94.1 \pm 3.8 mmHg), MAP (112.7 \pm 2.7 mmHg), and H (407.7 \pm 12.8 bpm) was observed compared to the Sham (SBP: 106.9 \pm 3.5 mmHg; DBP: 68.7 \pm 4.3 mmHg; MAP: 81.4 \pm 3.0 mmHg; and H: 339.1 \pm 13.4 bpm). Normalization of these alterations in the hemodynamic parameters was achieved when the renovascular hypertensive rats were treated with CHQ (po, 50 mg/kg) for the duration between the third and the 5th week post-surgery (SBP: 110.4 \pm 4.7 mmHg; DBP: 78.6 \pm 3.7 mmHg; MAP: 90.9 \pm 3.5 mmHg, and H: 296.9 \pm 9.1 bpm).

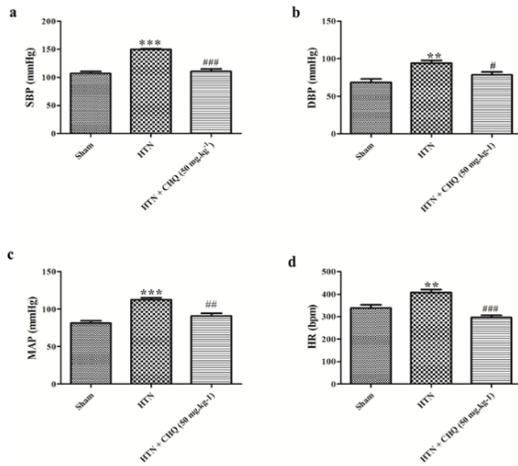


Figure 3: Elevation effect of CHQ on blood pressure in the rat model of renovascular hypertension. **(a)** Systolic blood pressure (SBP), **(b)** diastolic blood pressure (DBP), **(c)** mean arterial pressure (MAP), and **(d)** heart rate (H); *** $p < 0.005$, ** $p < 0.01$ vs. sham; ### $p < 0.005$, ## $p < 0.01$, and # $p < 0.05$ vs. renovascular hypertensive group (n = 5)

Chloroquine inhibits hypertension-induced vascular autophagy

Vascular autophagy was observed to be upregulated in the aorta of the renovascular hypertensive rats compared to the Sham and was characterized by a significant increase in the LC3-II/LC3-I expression and decrease in the p62 expression (Figure 4). CHQ treatment for three weeks was observed to significantly suppress the effect of hypertension on the LC3-II/LC3-I and p62 expressions in the aortic tissue.

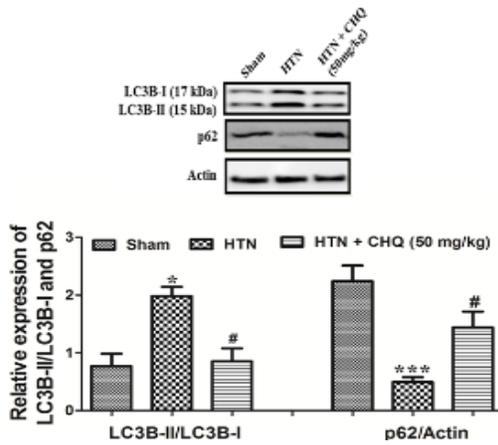


Figure 4: Effect of CHQ treatment on the relative expressions of the markers of autophagy (LC3B-II and p62) in the aorta of renovascular hypertensive rats; *** $p < 0.005$ and ** $p < 0.01$ vs. sham; ### $p < 0.005$, ## $p < 0.01$, and # $p < 0.05$ vs. renovascular hypertensive group (n = 5)

Chloroquine preserved hypertension-impaired endothelial function and suppressed angiotensin II-induced contraction in rat aorta ex vivo

A significant reduction was observed in the acetylcholine-induced relaxation of the PE-contracted aortic rings of the HTN rats, indicating the functional impairment of endothelium. Chloroquine treatment was observed to preserve the endothelial functions in the hypertensive rats (Figure 5 a, b, c, and d). A significant reduction in the aortic tone, induced through a single exposure to Ang II (1 μ M), was observed in the aorta from the Sham or the HTN rats treated with CHQ ex vivo (100 μ M) as well as in the aorta of the CHQ-treated HTN rats (50 mg/kg/day for three weeks; Figure 5 e, f, g, h, i, and j).

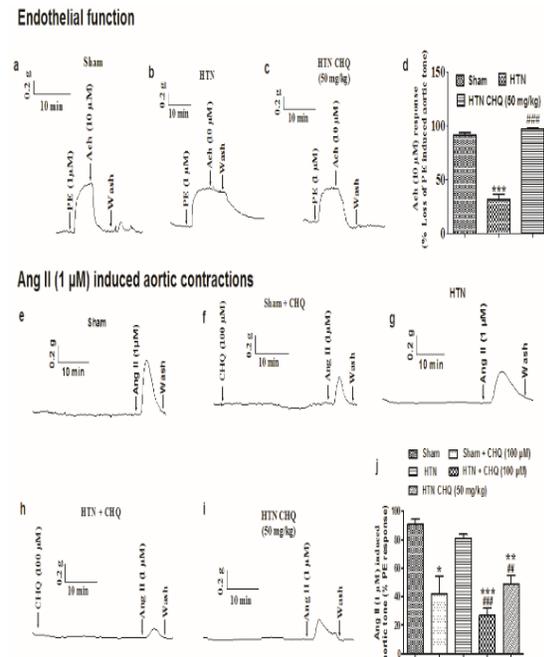


Figure 5: Effect of CHQ on endothelial function impaired because of hypertension **(a, b, c, and d)**, and on Ang II-induced contraction in rat aorta ex vivo **(e, f, g, h, i, and j)**. *** $P < 0.005$, ** $p < 0.001$, and * $p < 0.05$ vs. sham; ### $p < 0.005$, ## $p < 0.001$ vs. renovascular hypertensive group

DISCUSSION

The present work investigated the effect of CHQ-induced lysosomal inhibition of autophagy on the hypertension-associated endothelial dysfunction. The results obtained in the present investigation demonstrated that autophagy has an important role in Ang II-induced endothelial cell migration and angiogenesis, and its inhibition by CHQ provides endothelial protection during hypertension.

Autophagy is a key process in the integrated response to cellular stress and is initiated in the vascular cells of the diseased arteries, including the endothelial cells [2]. In the present work, an increase in autophagy was observed in the endothelial cells upon the Ang II exposure, which was characterized by enhanced LC3-II/LC3-I (autophagosome formation marker) expression and reduced p62 (autophagic flux marker) expression. The Ang II-induced autophagy flux was significantly suppressed upon co-treatment with CHQ, evidenced by the enhanced p62 expression. On the other hand, the effect of Ang II on autophagosome formation remained unaffected upon the CHQ co-treatment, as evidenced by the unaltered Ang II-induced expression of the LC3-II/LC3-I protein in the CHQ-co-treated endothelial cells. These results indicated a late-stage blockage of the autophagy cascade by CHQ. This was consistent with the fact that lysosomotropic agents such as CHQ and bafilomycin block autophagy at its maturation phase by interrupting the fusion of autophagosome with lysosome to form autophagolysosome, while the initial phases of autophagy remain unaffected by these agents [16].

Autophagy has been reported to play a pivotal role in regulating cell migration by directing the focal adhesion turnover and the tensional homeostasis, owing to its fundamental characteristic of compartmentalizing and degrading the cellular constituents [17,18]. In the present work, CHQ significantly suppressed the Ang II-induced endothelial cell migration, which suggested the involvement of autophagy therein. Chloroquine also inhibited both basal and Ang II-induced angiogenesis, which was marked by reduced tubule formation in the endothelial cells. This corroborated the finding of a previous study, that enhanced autophagy increases angiogenesis, while autophagy inhibition suppresses the angiogenesis without affecting the expression of the pro-angiogenic factors such as the vascular endothelial growth factor, platelet-derived growth factor, or integrin αV [7]. It is known that the endothelial cells involved in neovasclogenesis play a crucial role in the pathophysiology of various cardiovascular complications, such as atherosclerosis, aortic aneurysm, stroke, and peripheral vascular diseases [19]. In this context, the results of the present work demonstrated that the enhanced endothelial cell autophagy could be of pathological significance during hypertension. In the present study, prolonged treatment with CHQ prevented the increase in the blood pressure in the renovascular hypertensive rats, which corroborated with a recent study that reported inhibition of

hypertension with the use of CHQ in spontaneously hypertensive rats [20]. Confirming the findings of the *in vitro* investigations conducted in the present study, CHQ also inhibited the upregulation in the vascular autophagy flux in the aorta of the renovascular hypertensive rats, which represent a RAAS-dependent model of hypertension. In contrast, prolonged CHQ treatment suppressed the Ang II-induced autophagosome formation in the aorta of the hypertensive rats, which could be attributed to the reduction in the hemodynamic shear stress [21-23].

Impairment of vasoreactivity is an integral characteristic of hypertension, which also accounts for the increased vascular stress occurring during hypertension [24]. In the present study, sustained inhibition of autophagy by CHQ prevented the hypertension-induced endothelial dysfunction, as evidenced by improvement in the acetylcholine-induced relaxation in the aorta of the CHQ-treated renovascular hypertensive rats. In addition, the chloroquine treatment (*ex vivo* as well as *in vivo*) inhibited the contractions induced by Ang II in the aorta of normotensive as well as hypertensive rats.

Taken together, the findings of the present study demonstrate the therapeutic potential of CHQ-induced lysosomal inhibition of autophagy in hypertension-related vascular pathology and the associated complications. Although Ang II was employed as the effector peptide of RAAS throughout the present study, further investigation is required for deciphering the mechanism underlying the interaction between RAAS and vascular autophagy.

CONCLUSION

The findings of the present study demonstrate that both Ang II and elevated blood pressure, the prime factors governing the vasopathological changes occurring during hypertension, can induce endothelial cell autophagy. Preservation of the endothelial cell functions through lysosomal inhibition of autophagy in Ang II-treated cells or in hypertensive rats highlights its pathological implications in hypertensive vascular disease. In addition, CHQ treatment suppresses the development of hypertension in renovascular hypertensive rats. Thus, autophagy plays an important role in regulating endothelial functions during hypertension, and hence, autophagy inhibition using lysosomotropic agents such as CHQ protects against hypertension-induced endothelial dysfunction.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Moon Jain designed, performed experiments, compiled results, and wrote the manuscript. Hina Iqbal, Pankaj Yadav and Debabrata Chanda conducted the vasoreactivity experiments. Himalaya Singh and Kumaravelu Jagavelu did angiogenesis experiments. Kashif Hanif designed the study, analysed the results and wrote the manuscript.

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