Inhibition of Autophagy by Chloroquine Stimulates Nitric Oxide Production and Protects Endothelial Function during Serum Deprivation

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Abstract
Background/Aims: Autophagy plays a fundamental role in cell survival under stress conditions such as nutrient deprivation. Decreased nitric oxide (NO) production, which may contribute to vascular dysfunction, is one of the consequences of autophagy in endothelial cells. The antimalarial drug chloroquine (CLQ) inhibits autophagy by blocking autophagosome formation and has been proposed as adjuvant chemotherapy in other diseases. Methods: Autophagy was induced by serum deprivation in Human Umbilical Vascular Endothelial Cells (HUVEC) as demonstrated by formation of Acidic Vesicular Organelles (AVOs), conversion of Microtubule-associated protein 1 light chain (LC3), and Sequestosome-1 (SQTM1/p62) degradation. Using endothelium-dependent vasorelaxation assays, intracellular NO production in an ex vivo rat aortic ring model pre-constricted with phenylephrine was estimated along with DAF-2 DA cell membrane–permeable NO sensitive fluorescent dye. Results: The inhibition of autophagy by CLQ restored NO levels, protected against superoxide generation and preserved morphology as well as proliferation of HUVEC under serum deprivation. Interestingly, the incubation of rat aortic rings with CLQ resulted in endothelium-dependent relaxation mediated by the increase of NO. Conclusion: These findings emphasize the importance of autophagy in endothelial function and demonstrate the potential use of autophagy inhibitors to protect vascular function during nutrient deprivation.

Introduction
Autophagy is a conserved biological process that involves the sequestration of disposable cellular products and organelles into double-membraned vesicles for degradation by lysosomes [1-4]. Autophagy flux is increased in the endothelial cells after high-glucose or
nutrient deprivation and aortic endothelium of diabetic rat model [5, 6]. An experimental approach to reproduce nutrient deprivation consists of the removal of growth factors or serum deprivation [7, 8]. Endothelial dysfunction involves the loss of production of the main vascular dilation factor NO [9]. Therefore, the regulation of NO bioavailability could determine endothelial function and vascular integrity [10]. Chloroquine (CLQ) is the aminoquinoline drug of choice for the treatment of malaria. The use of CLQ for chemotherapy and in vascular-associated diseases has been proposed [11-13]. CLQ diffuses across cell membranes and accumulates in acidic compartments such as lysosomes, inhibiting autophagosome fusion with lysosomes. We demonstrated here that serum deprivation induces autophagy in HUVEC. The inhibition of autophagy by CLQ restored NO levels, protected against superoxide generation and preserved morphology and proliferation of HUVEC during starvation. However, the incubation of ex vivo aortic rings with CLQ promoted endothelial-dependent vascular relaxation mediated by the increase of NO.

Materials and Methods

Reagents

Acetylcholine (ACHE), acridine orange hydrochloride (AO), bafilomycin A1, calcium ionophore A23187, chloroquine diphosphate (CLQ), diaminofluorescein (DAF), dihydroethidium (DHE) and 3-4,5-dimethylthiazol-2-yl-2, 5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Primary antibodies to light-chain 3 (LC3), sequestosome-1 (SQTM1/P62), caspase-3 (Casp-3), β-actin and their respective secondary antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Cell culture and growth conditions

HUVEC were cultured in DMEM (Dulbecco Modified Eagle’s balanced salt solution medium) supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/ml) and streptomycin (100 μg/ml) at passages 5-15. Cells were grown in a humidified incubator containing 5% CO₂ at 37°C. For serum deprivation, cells were washed with PBS and cultured in DMEM without FBS (serum-free) for 24 hours. Cells at 80% confluence were submitted to the treatment as described in the legends.

Acidic Vesicular Organelles formation detected by flow-cytometry and fluorescence microscope

Autophagy was based on the formation of AVOs stained with acridine orange fluorescent dye. AVOs appear as red fluorescent compartments in autophagic cells while nuclei and cytosol are stained green [14, 15]. After treatment, HUVEC were stained with 1 μg/ml AO for 15 min at room temperature and visualized under inverted fluorescence microscope (Axiovert, Carl Zeiss, Germany) at 40X objective lens magnification. Alternatively, stained cells were collected with trypsin-EDTA and quantified by measuring increases in fluorescence intensity in the FL3 channel in an Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Five thousand events were acquired.

LC3 cleavage, SQTM1/p62 degradation and caspase-3 activation by Western blotting

HUVEC were lysed and 30 μg of protein extract per sample was resolved by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% fat free milk and incubated with primary antibodies to LC3, SQTM1/p62 and caspase-3 overnight at 4°C, followed by incubation in matching secondary antibodies for 2 h at room temperature. The immunoreactive bands were detected by chemiluminescence (ECL prime, GE Health Care, Piscataway, NJ).

Measurement of intracellular NO production

NO production was estimated using DAF-2 DA, a cell membrane–permeable NO sensitive fluorescent dye, which converts to DAF-2 and reacts with NO to form the fluorescent triazole DAF-2T [16]. Prior to treatment, HUVEC were incubated with 5 μM DAF-2 DA for 15 min at room temperature. The increase in fluorescence intensity was monitored at 485 nm excitation and 515 nm emission using a fluorescence microplate reader (SpectraMax Gemini XS, Molecular Devices, Sunnyvale, CA, USA) for 4 hours at 37°C. Fluorescence intensities were normalized based on the number of cells per sample.
Superoxide Production

Superoxide (O$_2^·$) radicals generated in HUVEC under serum deprivation were estimated using dihydroethidium (DHE) dye, which is oxidized by O$_2^·$ to generate fluorescent ethidium bromide [17]. After treatment, HUVECs were collected by trypsinization and incubated with 10 µmol/L DHE for 30 min at room temperature. The fluorescence intensity was determined at 510 nm excitation and 590 nm emission with a fluorescence microplate reader for 24 hours at 37°C. Fluorescence intensities were normalized to the number of cells at each condition.

MTT assay

Cell proliferation was determined based on the reduction of formazan salt. HUVEC were seeded in 96-well plates, treated and incubated with tetrazolium salt at a final concentration of 5 mg/ml for 4 h at 37°C. The dye solution was removed and the salt crystals were then solubilized in DMSO 100% and quantified spectrophotometrically in a microplate reader at a wavelength of 550 nm.

Morphology changes

The changes in morphology of HUVEC as a result of serum deprivation were assessed by branch formation. The incubation of HUVEC in DMEM with no FBS for 24 hours allowed the development of polygonal network structures. The formation of the tubular structures was observed in images of phase contrast microscope. The branches were quantified by counting three randomly selected photographed fields.

Endothelial-dependent vasorelaxation in aortic rings

Endothelium-dependent vascular relaxation was determined ex vivo in the descending rat thoracic aorta as previously reported [18, 19]. Male Wistar rats weighing approximately 200 g were used. Experiments were conducted in accordance with the U.S. National Research Council’s Guide for the Care and Use of Laboratory Animals (National Research Council, 1996). The Institutional Ethics Committee in Animal Research of the Federal University of Sao Carlos (protocol number 012/13) approved the experimental procedures. Cylindrical segments of aortic rings were placed onto two stainless-steel stirrups and connected to an isometric force transducer (DMT instruments) containing Krebs buffer (mM: NaCl 130, KCl 4.7, KH$_2$PO$_4$ 1.2, MgSO$_4$ 1.2, NaHCO$_3$ 14.9, glucose 5.5, and CaCl$_2$ 1.6) at pH 7.4 and gassed with 95% O$_2$ and 5% CO$_2$ to monitor the isometric tension. After an equilibration period of 60 min under resting tension of 1.5 g, the vessels were pre-constricted with phenylephrine (PHE, 0.1 µM). Endothelial integrity was assessed by the degree of relaxation induced by acetylcholine (ACHE 1 µmol/l) in the presence of PHE-induced contractile tone. Rings showing a minimal relaxation response of 80% were selected for the studies. The endothelium was removed by gently rubbing the lumen of the aortic rings for experiments in the absence of endothelium. Confirmation of the absence of endothelium was determined by a lack of a relaxation response to ACHE in aortic rings pre-contracted with PHE. Once the contraction with PHE reached a plateau, the effect of cumulative concentrations of CLQ (0.001 nM to 1 µM) or ACHE (0.001 nM to 1 µM) were determined. The dependence of NO was confirmed by pre-incubation with the NOS inhibitor L-NAME 100 µM for 30 min before CLQ treatment, and evaluation of CLQ-induced relaxation. Data are presented as the percentage of relaxation.

Statistical Analysis

Results were expressed as the mean values ± SD. Data were analyzed by two-way analysis of variance followed by post hoc Tukey-tests for multiple comparisons. A P-value of < 0.05 was considered significant.

Results

Chloroquine inhibits AVOs formation induced by serum deprivation

Autophagosome formation is a marker of late autophagy. HUVEC showed accumulation of AO-stained acidic vacuolar compartments following 24 hours of serum deprivation as demonstrated by increased intensity of red fluorescence (FL3) observed in FACS quantitative analysis (Fig. 1A). CLQ treatment prevented the induction of autophagy by serum deprivation (Fig. 1B). Fluorescence microscopy images also indicated the accumulation of AO (orange/red spots in HUVECs) as well as the inhibition of autophagy by CLQ (Fig. 1C-1E).
Chloroquine accumulates lipidated LC3 and inhibits p62/SQTM1 degradation induced by serum deprivation

The induction of autophagy was confirmed by detection of standard protein markers of autophagy. LC3 lipidation (LC3-II) is a key step in autophagosome formation and autophagy inhibitors are expected to accumulate LC3-II by preventing the degradation of LC3-II-containing autolysosomes [20]. Following western blot analysis, the LC3-II/LC3-I ratio and decrease of SQSTM1/p62 levels show an accumulation of LC3-II by lipidation and inhibition of p62/SQTM-1 degradation by autophagosome proteolysis [21, 22]. In both cases, CLQ was able to inhibit autophagy (Fig. 2A). Apoptosis was observed after serum deprivation as demonstrated by the cleavage of caspase-3; however, CLQ did not cause inhibition (Fig. 2B).

Chloroquine restores NO levels of HUVEC under serum deprivation

The stimulation of NO production by CLQ was evaluated by time-course fluorescence analysis. The formation of the DAF-2T reaction product was monitored in HUVECs cultured in supplemented and serum-free medium and treated with CLQ 10 µmol/L or type (H+)-ATPase inhibitor Bafilomycin A1 (BAF) 10 nmol/L for 240 min. Figure 3A illustrates CLQ did not produce increases in NO production in supplemented cells compared to control cells. However, serum deprivation caused a 58.3% decrease in NO production relative to supplemented cells after 240 min (10.8 ± 4.04 to 4.5 ± 0.68), whereas CLQ led to a 57.1% increase of NO generation relative to starved untreated cells at 240 min (4.5 ± 0.68 to 10.5 ± 2.25, Fig. 3B). BAF did not increase NO production in supplemented cells (Fig. 3C). Ca²⁺ ionophore A23187-mediated NO synthase activation was used as a positive control for the stimulation of NO production.
Chloroquine preserves cell proliferation and morphology and protects against ROS generation induced by serum deprivation

We investigated whether CLQ and BAF rescue cell proliferation affected by serum deprivation. Figure 4A shows that serum deprivation reduced cell proliferation by 24.5% after 24 hours, which was fully restored by CLQ and modestly by BAF at the concentrations of 10 nmol/L and 100 μmol/L respectively. Additionally, serum deprivation caused a 28.3% increase in superoxide generation that was prevented by CLQ (Fig. 4B). Notably, NO has been shown to scavenge superoxide and prevent the impairment of endothelium dependent dilation, enhanced platelet aggregation and intimal proliferation [23, 24]. Morphological changes were observed in HUVECs undergoing autophagy as demonstrated by the formation of branches, whereas cells cultured in supplemented medium maintained a spherical morphology showing no branches. Incubation with CLQ mostly reversed this effect (Fig. 4C).

Chloroquine induces endothelial-dependent vascular relaxation

The impact of NO production in endothelial function was evaluated by vasorelaxation assays using rat isolated aortic rings pre-contracted with PHE (0.1 μM). As shown at Fig. 5A, CLQ induced endothelium relaxation in aortic rings with an intact endothelium (pD2: 10.45 ± 0.13, Emax: 93.37 ± 3.24 n=6), in a concentration-dependent manner. CLQ was also more effective in promoting relaxation than ACHE (CLQ, pD2: 10.45 ± 0.13, n=6 vs ACHE, pD2: 7.91 ± 0.11, n=4; P<0.001) (Fig. 5B). To verify if the relaxation induced by CLQ was endothelium-dependent, a CLQ concentration curve was determined for aortic rings devoid of endothelium. As shown at Fig. 5A, the absence of endothelium abolished the relaxation induced by CLQ (Emax: 4.94 ± 0.24, n=5; P<0.001). The contribution of nitric oxide synthase (NOS) to CLQ-induced relaxation was determined by treating of aorta rings with the non-selective NOS inhibitor L-NAME. L-NAME decreased the potency of CLQ in inducing relaxation (pD2 from: 10.45 ± 0.13 to 7.91 ± 0.11 n=5; p<0.001) without affecting the maximum effect (Emax from: 103.5 ± 4.14 to: 93.37 ± 3.24, n=5).
Fig. 3. CLQ increases NO production under serum deprivation. Monitoring of NO production of DAF-2 stained HUVEC cultured in DMEM supplemented with 10% FBS or in serum-free DMEM treated with CLQ 10 µmol/L (A and B) or BAF 10 nmol/L (C and D) monitored for 4 hours. Controls are indicated as open circles (○), CLQ treatment as filled circles (●) and BAF open squares (□). Ca²⁺ ionophore A23187 was used as positive control (dashed lines). Data are presented as the means ± SD. *P < 0.05 versus control.

Fig. 4. CLQ preserves cell proliferation and morphology and protects against ROS generation under serum deprivation. (A) Proliferation of HUVEC cultured in FBS 10% supplemented and serum-free DMEM treated with CLQ 10 µmol/L or BAF 10 nmol/L for 24 hours assessed by MTT assay. (B) Superoxide generation of DHE-stained HUVEC cultured in FBS 10% supplemented and serum-free DMEM and treated with CLQ 10 µmol/L for 24 hours. (C) Changes in morphology identified by branch formation in phase contrast images of HUVEC under deprivation and treated with CLQ 10 µmol/L for 24 hours. The number of branches was quantified by counting three randomly selected photographed fields. Data are presented as the means ± SD. *P < 0.05 vs FBS 10% supplemented control and #P < 0.05 vs serum-free control.
Discussion

The present work demonstrates autophagy of endothelial cells under serum deprivation and the beneficial effects of autophagy inhibitor CLQ to the endothelial function. As shown by Lum et al. [25], either the withdrawal of growth factors or serum-free medium can trigger autophagy as confirmed by the formation of AVOs and by LC3 lipidation and proteolytic degradation of SQSTM-1/p62. Autophagy is a well-conserved survival mechanism committed to the maintenance of endothelium energy homeostasis under high-glucose or nutrient-deprivation [5, 6]. The involvement of NO in autophagy was demonstrated in previous work showing that autophagy is downregulated following overexpression of nNOS, iNOS, or eNOS [26]. NO is the main vasodilating molecule in the endothelium and participates in many vascular functions including reactivity, platelet adhesion and aggregation. The loss of endothelial NO production leads to the impairment of vasodilation and chronic inflammatory responses [27]. This consequently contributes to the development of cardiovascular diseases such as atherosclerosis, hypertension, and insulin resistance [28]. The first important finding in this study is that the treatment of endothelial cells with CLQ increased NO production. BAF did not reproduce the effects of CLQ on NO production at the highest nontoxic concentration of 10 nmol/L. At higher doses, BAF decreased cell viability, which may reflect the NO-dependent toxic effects of BAF observed in monocytes as demonstrated by its sensitivity to the NO scavengers or nitric oxide synthase inhibitors [29]. Despite the stimulation of NO production could not be seen in cells supplemented with FBS as reported by Ghigo et al. [30], the restoration of NO levels under serum deprivation emphasizes the impact of CLQ on endothelial function during episodes of starvation. To gain insight into the consequences of NO production to endothelial dysfunction, we explored the ability of CLQ to promote endothelium-dependent vascular relaxation in an ex vivo model of vascular reactivity. Surprisingly, CLQ treatment led to potent vasodilation of endothelium-intact rat aortic rings as showed by isometric tension studies. The same effect was not observed in aorta devoid of endothelium. The shift of the relaxation curve in the presence of the NOS inhibitor L-NAME provided substantial evidence that the effect of CLQ was mediated by an increase in NO production (Fig. 5). Serum deprivation also increases superoxide generation, which is the primary driver of autophagy during starvation [31, 32]. In fact, our data indicate that serum deprivation elevated ROS generation in agreement with a prior observation that ROS is required for induction of autophagy in endothelial cells [33]. NO has been reported to be a free radical scavenger [34], and its loss may result in an accumulation of superoxide [35], leading to reperfusion-induced mucosal injury [36, 37]. Additionally, decreased NO during
starvation is a consequence of increased in superoxide during endothelial dysfunction in aging [38]. Notably, inhibition of autophagy by CLQ also prevented ROS. Hypoxia and serum depletion also induce of tube formation in endothelial cells in an autophagy-dependent manner [39]. The formation of branches after serum deprivation further confirmed the protective effect of CLQ on the morphology of endothelial cells. Finally, CLQ did not inhibit apoptosis induced by serum deprivation and dramatically decreased cell viability at higher concentrations. In fact, CLQ promotes autophagy-independent chemosensitization [40] and increases apoptosis in melanoma cells sensitized by nutrient deprivation [41]. The ability of CLQ to downregulate AMPK not mimicked by autophagy inhibitors or LC3b shRNA emphasizes such observations [42]. Together, the presented data show the ability of CLQ to modulate NO production and protect endothelial function, emerging evidence for the role of autophagy in vascular pathophysiology.

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**Disclosure Statement**

The authors have no conflict of interest to report.

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