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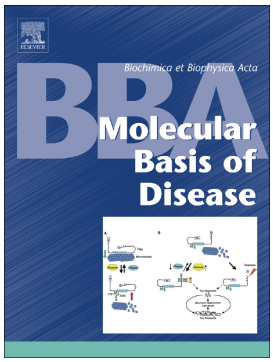

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Title

Extracellular histones activate autophagy and apoptosis via mTOR signaling in human endothelial cells

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Abstract

Circulating histones have been proposed as targets for therapy in sepsis and hyperinflammatory symptoms. However, the proposed strategies have failed in clinical trials. Although different mechanisms for histone-related cytotoxicity are being explored, those mediated by circulating histones are not fully understood. Extracellular histones induce endothelial cell death, thereby contributing to the pathogenesis of complex diseases such as sepsis and septic shock. Therefore, the comprehension of cellular responses triggered by histones is capital to design effective therapeutic strategies. Here we report how extracellular histones induce autophagy and apoptosis in a dose-dependent manner in cultured human endothelial cells. In addition, we describe how histones regulate these pathways via Sestrin2/AMPK/ULK1-mTOR and AKT/mTOR. Furthermore, we evaluate the effect of Toll-like receptors in mediating autophagy and apoptosis demonstrating how TLR inhibitors do not prevent apoptosis and/or autophagy induced by histones. Our results confirm that histones and autophagic pathways can be considered as novel targets to design therapeutic strategies in endothelial damage.

Highlights

1. Extracellular histones induce autophagy and apoptosis in HUVEC, in a dose dependent manner
2. Histones impair the expression of upstream regulators of mTOR-mediated pathways
3. TLR inhibition, by itself, does not prevent apoptosis induced by histones in HUVEC

Keywords

Circulating Histones; Autophagy; Apoptosis; Toll-Like receptors; endothelial cells

Abbreviations

PTMs, post-translational modifications; PAMPs, pathogen-associated molecular patterns; TLRs, Toll-like receptors; HUVEC, human umbilical vein endothelial cells; AMPK, AMP-activated protein kinase; Akt: serine/thPGI2, prostacyclin; TXA2, thromboxane A2; COX-2, prostaglandin-endoperoxide synthase 2; PGIS, prostacyclin synthase; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; ULK1, unc-51 like autophagy activating kinase 1; mTOR, mammalian target of rapamycin; LC3 (LC3-I and -II), Microtubule-associated protein 1A/1B-light chain 3.

1. Introduction

Histones are essential proteins that participate in the conformation of chromatin and in the regulation of gene expression. Histones contribute to epigenetic regulation by means of post-translational modifications (PTMs) in the histone tails. Surprisingly, as early as in the 1950's James G. Hirsch described the activity of histones as antimicrobials [1]. In recent years, it has been shown that extracellular histones are toxic to cultured mouse intestinal epithelial cells in serum-free medium [2] and that they induce apoptosis in renal tubular epithelial cells [3] as well as in lymphocytes [4]. Furthermore, it has been demonstrated that severely damaged tissues release high amounts of nucleosomes (the basic structural unit of chromatin composed by histones and DNA) as well as free histones into the bloodstream, aggravating the clinical features of a trauma [5]. In fact, Abrams *et al*. demonstrated that circulating histones act as mediators for distant organ damage (i.e. lungs) through interaction with membrane phospholipids, causing cellular calcium influx. Corroborating those observations, histones released into the bloodstream were found in patients with severe blunt trauma, pancreatitis, and sepsis [5, 6], and also in disseminated intravascular coagulation [7]. Based on these pathological properties, extracellular nucleosomes and histones are considered pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). These molecules are able to activate the innate immune system through Toll-like receptors (TLRs). In this scenario, the deregulation of the innate immune system triggered by the presence of PAMPs may also produce alterations in the endothelium [8]. Moreover, TLRs are also found in endothelial cells, being involved in the physiopathology of sepsis by regulating and activating inflammatory signaling, coagulopathy and vascular permeability [9]. Once activated by TLRs, endothelial cells were found to secrete mediators including cytokines, chemokines, reactive oxygen species (ROS) and nitric oxide, promoting also the secretion of cell surface proteins such as ICAM-1 [8]. Furthermore, inflammatory insults are known to activate innate immunity and propagate organ damage through the recognition of extracellular DAMP molecules. Therefore, considering the role of extracellular histones as PAMPs and DAMPs, and the multiple pathways which they can activate, they have been proposed as candidate biomarkers for diagnostic of sepsis, and consequently as novel therapeutic targets in human diseases [10]. Importantly, interactions between histones and TLRs have been related to cell death in some cell types [11, 12], but there is still scarce information about the signaling cascade mediated by extracellular histones through TLRs in human umbilical vein endothelial cells (HUVEC).

The endothelium mediates the release of vasoactive mediators that control the vascular tone and is also involved in the maintenance of a balance between pro- and anti-apoptotic signals. Perturbation of this equilibrium may contribute to the pathogenesis of diverse diseases [13-16]. Histones have been detected in plasma of mice, baboons, and human patients with sepsis [6, 17], and they have been shown to induce endothelial cell death during septic shock; however, there is limited information regarding the mechanism by which endothelial cells die. Endothelial apoptotic cells become pro-coagulant and pro-adhesive [18], resulting in changes in the integrity and function of the endothelium, and thereby participating in the pathogenesis of a variety of human diseases [16]. We have recently demonstrated in HUVEC that extracellular histones stimulate the release of endothelial-dependent mediators through an up-regulation in the COX-2-PGIS-PGI2 pathway, which involves a COX-2-dependent superoxide production that downregulates eNOS and NO bioavailability, thereby contributing to endothelial cell dysfunction [19].

Therefore, if extracellular histones are cytotoxic towards the endothelium [6], they should probably mediate cellular responses contributing to worsening of the disease. As discussed above, several therapeutic strategies have failed in fighting sepsis and septic shock from a molecular approach [20], highlighting the possibility that elucidation of the mechanisms involved in histone-mediated cytotoxicity may contribute to a deepest and more detailed comprehension of the complex pathogenesis of sepsis. Hence, a fine dissection of the endothelial cellular responses triggered by histones will open the path to the design of therapeutic strategies, using circulating histones as prognosis biomarkers in those diseases where they are present. Here we describe the molecular landscape of histone-mediated autophagy and apoptosis in endothelial cells, providing new clues on the mechanisms underlying the complications of sepsis and septic shock.

2. Material and methods

2.1. Cell culture and experimental design

Primary human umbilical vein endothelial cells (HUVEC) cultures were obtained from human umbilical cord from *Hospital Clínico Universitario* of Valencia as previously described [21]. Umbilical cords were obtained following the principles outlined in the Declaration of Helsinki and were approved by the Ethical Committee of Clinical Research of the INCLIVA, *Hospital Clínico Universitario of Valencia.* Written informed consent was obtained from all donors. Briefly, umbilical veins were treated with 1% collagenase (Life Technologies, Carlsbad, CA, USA) and HUVEC were cultured in endothelial growing media (EGM)-2 (Lonza, Barcelona, Spain) that contains Endothelial cell basal medium-2 and the following growth supplements: human Epidermal Growth Factor (hEGF), 0.1%; Vascular Endothelial Growth Factor (VEGF 0.1%; R3-Insulin-like Growth Factor-1 (R3-IGF-1), 0.1%; Ascorbic Acid, 0.1%; Hydrocortisone, 0.04%; human Fibroblast Growth Factor-Beta (hFGF-β), 0,4%; Heparin (0.1%); Fetal Bovine Serum (FBS), 2%; Gentamicin/Amphotericin-B (GA), 0.1%.

HUVEC from passages 3 to 5 were used in this study. When they reached 80% of confluence, media was changed and cells were exposed during 4 h to different calf thymus (CT) histone concentrations (Sigma-Aldrich, St. Louis, MO, USA): 10, 25, 50 or 100 µg/mL prepared in PBS pH 7.4 (Gibco, MS, USA). Lysosomal inhibitors used for the study of autophagy activation were 20 mM NH4Cl plus 0.1 mM leupeptin (treated during the last hour and a half) to inhibit LC3-II degradation as described in Rubinsztein et. al. [22]. For TLR inhibition, compunds used were: 3 µM CLI-095 as TLR-4 antagonist (Invivogen, Tolouse, France); 0.7 µM iODN, as TLR-7/9 antagonist (Enzo Life Science, Farmingdale, NJ, USA); and OxPAPC 25 µg/mL (30 µM) (Sigma-Aldrich), as TLR-2 and TLR-4 antagonist. Compounds were added to HUVEC 1 h before histone treatments.

2.2. Western blot

After histone treatment cells were scraped on ice in lysis buffer [20mM Hepes pH 7.4, 1% Triton X-100, 100mM NaCl, 50mM NaF, 10mM β-glycerophosphate, 1mM phenylmethylsulfonyl fluoride (PMSF), 1mM sodium orthovanadate, protease inhibitor cocktail (Roche Diagnostics, Barcelona, Spain)]. Proteins were denatured using the sample buffer (Tris 40mM, EDTA, bromophenol blue 0,01%, sucrose 40%, SDS 4%, β-

mercaptoethanol 10%) and heated to 95°C for 5 min. Afterwards, samples were electrophoresed in a 12% SDS-PAGE and transferred onto nitrocellulose or PVDF membrane (Whatman GmbH, Dassel, Germany).

After transference, the membrane was blocked with milk 5% or BSA 5% (in the case of phosphorylated proteins) in TBS-Tween (TBST) for 1 h. Afterwards, the membranes were incubated with specific primary antibodies recognizing LC3 (NB100-2220; Novus Biologicals, Abingdon, UK), Lamp2 (ab25631; Abcam; Cambridge, UK), Bax (GTX109683; GeneTex; Irvine, USA), Bcl-2 (2870), p70S6 kinase (9202), phospho-p70S6 kinase (Thr389, #9205), AMPK (#2532), phospho-AMP kinase (Thr172, #2535), Akt (9272), phospho-Akt (Ser473, 9271), phospho-p53 (Ser15, #9284), ULK1 (ab167139, Abcam; Cambridge, UK), phospho-ULK1 (Ser555, #5869), mTOR (#2972), phospho-mTOR (Ser2448, #2971) all from Cell Signalling Technology (Leiden, The Netherlands), sestrin 2 (sc-101249,Santa Cruz Biotech; Dallas, USA), beta-actin (A-2066, Sigma-Aldrich; St Louis, USA). Blots were probed with anti-mouse or anti-rabbit IgG-HRP secondary antibody. Thereafter, the blots were washed again with TBST and further incubated for 1 h with a secondary mouse, rabbit or goat antibody conjugated with horseradish peroxidase-linked. The membrane was incubated at room temperature and constant agitation. Finally, the membrane was washed 3x5 min with TBST. To observe protein signals, Chemioluminiscent ECL Western Blotting Detection reagents (GE Healthcare, Hatfield and Hertfordshire, UK) was added onto the membrane, 1 ml/membrane, and membranes were revealed by an image reader LAS-4000 (General Electric Healthcare, Uppsala, Sweden). Signal density was analysed with ImageJ software (NIH Image, National Institutes of Health, Bethesda, MD, USA).

2.3. **Apoptosis determination**

Apoptosis was determined by Annexin-V kit (Immunostep, Salamanca, Spain) following the manufacturer specifications. Culture media was recovered together with HUVEC previously exposed to desired treatments. Cells were resuspended in Annexin-V buffer and stained with Annexin-V-FITC and propidium iodide (PI) for 15 min in the dark. Then, stained cells were analyzed by flow cytometry by using a FACS-Verse cytometer (Beckton Dickinson, San Jose, CA, USA) and Infinicyt software (Cytognos, Santa Marta de Tormes, Salamanca, Spain). Apoptotic cells were also determined by fluorescence microscopy. Adhered HUVEC were stained with Annexin-V-PE (Immunostep, Salamanca, Spain) for 15 min. Counter staining was achieved by incubating cells with DAPI (Life Technologies, San Diego, CA, USA). Images were

obtained with an inverted fluorescence microscope Nikon Eclipse Ti (40x magnification).

2.4. **Confocal microscopy**

Confocal images were acquired using a Leica TCS-SP8 X confocal laser scanning unit (Leica Microsystems, Wetzlar, Germany). All images are maximum intensity projection from 10 optical slices in Z-dimension. HUVEC were seeded in 2 cm^2 Lab-TeK II chambered cover glass (Nunc, Thermo Fischer Scientific, Waltham, MA, USA). The number of autophagic vacuoles was measured using immunofluorescence staining of endogenous LC3. Briefly, fixed cells with 4% paraformaldehyde in PBS for 10 min at room temperature and quenched with 75 mM NH4Cl and 25 mM glycine for 10 min, permeabilized with 0.3% Triton-X100 and blocked with blocking buffer (10 mg/ml de BSA, 4 % de FBS, 0.1 % de Triton-X100). Cell were incubated overnight at 4 ºC with anti-LC3 antibody (dilution 1:50) (Novus, NB100-2220). Coverslips were washed four times with PBS and incubated with secondary antibody solution consisting of AlexaFluor488-conjugated donkey anti-rabbit IgG secondary antibody (Invitrogen, 1:500) for 1 h, at RT. After extensively washing with PBS, the coverslips were mounted using DAPI Fluoromount-G (SouthernBiotech; Birmingham, AL, USA). Lysosomes were stained with LysoTracker Red DND-99 50x10-9M (Molecular Probes, Carlsbad, CA, USA) at 37 ºC for 30 min. The number of fluorescent dots corresponding to autophagolysosomes per cell were counted (at least 10 HUVEC cells) using ImageJ (National Institutes of Health) [23], as previously described by one of the authors [24].

2.5. **Statistical analysis**

For the statistical analysis of the results, the mean was taken as the measurement of the main tendency, while standard deviation was taken as the dispersion measurement. One-way analysis of variance was conducted in order to explore significant differences between groups. When an interaction effect was found, multiple comparisons using the Tukey post-hoc test was performed. The significance has been considered at *P<0.05, **P<0.01 and ***P<0.001, as indicated in each case. GraphPad Prism v5.0 (GraphPad Software, San Diego, CA, USA) was used for statistical analysis and graphic representations.

3. Results

3.1. Extracellular histones induce autophagy and apoptosis in a dose-dependent manner in HUVEC

In order to assess the dose-dependent activation of cellular damage, we analyzed the effect of increasing concentrations of extracellular histones, ranging from 10 to 100 µg/mL. It has been previously described that circulating histones can damage mammalian cells. Abrams *et al*. found that the concentration of circulating histones ranges between 10 to 230 µg/mL within 4 h after lung injury in humans [5]. Therefore, we wondered whether extracellular histones were able to damage human endothelial cells (HUVEC). We observed that cell viability, apoptosis, and cell morphology of HUVEC were gradually affected by histone concentration. Cell viability, measured by flow cytometry, decreased scarcely at 50 µg/mL but the decrease was more evident when cells were incubated with 100 µg/mL of histones (Figure 1A). The analysis of apoptosis by flow cytometry using Annexin-V-PE showed a significant increase in apoptotic cells at both 50 and 100 µg/mL (Figure 1B), as corroborated by fluorescence microscopy of cells using the same Annexin-V-PE staining; besides, there was a clear alteration of cell morphology and an increased number of dead cells (Figure 1C and 1D, respectively).

Autophagy is a cellular degradation process induced in response to many types of stress, but also a cellular mechanism used to promote cell survival, as cells defective in autophagy undergo apoptosis [25]. Thus, we wondered whether autophagy could be triggered after incubation with extracellular histones. To analyze the different steps of autophagy, assays were performed in the presence or absence of lysosomal inhibitors. As a marker of autophagy, we measured the LC3-II form, mammalian homolog of yeast Atg8 [26], which serves as an indicator of autophagosome formation progress. Western blot analysis showed an increase in the 16 KDa form of LC3 (LC3-II), indicating the activation of autophagy from 25 to 100 µg/mL of extracellular histones when using lysosomal inhibitors (Figure 2A). Immunofluorescence of LC3 (Figure 2B) further demonstrated the formation of autophagosomes in HUVEC exposed to extracellular histones.

To clarify the specific concentration of extracellular histones that promotes the increase of lysosomal mass, we measured the levels of Lamp2 (Figure 3A). This protein is localized primarily in late endosomes and lysosomes [27] and acts as a receptor for the selective degradation of proteins in the lysosome [28]. Thus, Lamp2 is necessary for the proper fusion of lysosomes with autophagosomes in the late stage of the

autophagic process to form autophagolysosomes [25]. Our results show that Lamp2 levels increased from 25 to 50 μg/mL of histones, being the highest expression at 100 μg/mL (Figure 3A). Accordingly, the number of lysosomes, as indicated by lysotracker staining, increased in a dose-dependent manner from 25 to 100 μg/mL of histones (Figure 3B). These data support the notion that extracellular histones increase autophagosomes/autophagolysosomes formation in a dose-dependent manner.

3.2. Extracellular histones activate autophagy by affecting different upstream mTOR regulators

Since multiple upstream signals contribute to the activation of autophagy, we analyzed which specific pathways were activated in response to the stimulus produced by extracellular histones in HUVEC.

The mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase that negatively regulates autophagy [29, 30]. One of the upstream pathways involved in activation of mTOR-mediated autophagy is controlled by extracellular signal-regulated kinase 1/2 (ERK1/2) [31, 32], but we did not find any significant increase in the phosphorylation of ERK1/2 in response to extracellular histones (data not shown). Other upstream pathways involved in autophagy activation are mediated by Sestrin2, an inductor of LC3-II [31, 32]. Western blot analysis showed that expression of Sestrin2 was increased according to the concentration of extracellular histones, showing highest expression at 50 and 100 μg/mL. Furthermore, we found an increase in the phosphorylation of Thr172 in AMPK (pho-AMPK) (see Figure 4A and 4B) that correlated with the increased levels of Sestrin2. AMPK is involved in processes that synergize to activate autophagy, both by direct activation of ULK1 and impairment of the mTOR-dependent inhibition of ULK1 [33]. In addition, the phosphorylation of AKT (Ser473) decreased inversely with the concentration of histones, which might result in blocking the activation of mTOR and thus activating autophagy [34] (Figure 4A and 4B). Furthermore, a decrease in the phosphorylation of p70SK6 (a protein downstream to mTOR) at Thr389 was detected, suggesting again that autophagy is activated in HUVEC in response to a challenge of 25 to 100 μg/mL of extracellular histones. Taken together, our results indicate that this increase in autophagy responds to an inhibition of mTOR-mediated pathways, as corroborated by the decrease in the phosphorylated levels of mTOR observed by Western blot analysis (Figure 4A).

3.3. High concentrations of extracellular histones inversely affect the levels of pro- and anti-apoptotic proteins

To study the relationship between apoptosis and autophagy, we evaluated the levels of the anti-apoptotic protein Bcl-2, the pro-apoptotic protein Bax, and the key regulator p53, which regulates both autophagy and apoptosis by either activating Sestrin2 or Bax, respectively [35]. We found that apoptosis was promoted by 50 and 100 µg/mL of extracellular histones, correlating with an increase Bax levels, and a decrease in Bcl-2 levels (Figure 4C and 4D). It is known that phosphorylation of p53, among other factors, produces a p53-dependent up-regulation of Bax [36]. In our results, we found pho-p53/Bax-dependent activation of apoptosis at 100 µg/mL, which in turn may repress the expression of Bcl2 [37, 38]. Furthermore, the activation of Bax may be promoted by downregulation of Bcl-2, and it has been proposed that the Bax/Bcl2 ratio is critical to direct cells towards apoptosis [39, 40], accordingly to what we observe at 50 and 100 µg/mL (Figure 4D) and again corroborating our results obtained by flow cytometry (Figure 1B).

3.4. TLR2, TLR4 and TLR7/9 inhibitors modulate apoptosis and autophagy signals in HUVEC

Since the role of histones regulating TLR-induced inflammation is not fully understood [41], we evaluated whether the use of TLR inhibitors (TLR2/TLR4 inhibitor OxPAPC (30µM), TLR7/TLR9 inhibitor iODN (0.7µM), and TLR4 inhibitor CLI-095 (3µM)) could affect the endothelial responses mediated by extracellular histones, either activating or inhibiting autophagy and apoptosis. We found increased percentage of apoptosis plus necrosis in HUVEC when cells were pre-incubated with TLR4 inhibitor (20.61±3.03) and TLR2/TLR4 inhibitor (22.56±8.08), but not when HUVEC were incubated with TLR7/TLR9 inhibitor, as compared to control conditions (10.44±4.29) (Figure 5A and Table 1). Using 50 µg/mL of extracellular histones the percentage of cells in apoptosis plus necrosis increased (30.17±3.04) when cells were pre-incubated with TLR4 inhibitor and TLR2/TLR4 inhibitor (17.45±0.01) when compared to HUVEC incubated only with histones (16.63±4.45) (Figure 5A and Table 1). Finally, when cells were incubated at 100 µg/mL of extracellular histones, TLR4 inhibitor or TLR2/TLR4 inhibitor further increased apoptosis and necrosis (Figure 5A and Table 1). In this regard, when cells were incubated with 100 µg/mL of extracellular histones, the percentage of HUVEC in apoptosis plus necrosis increased (36.58±9.04) after incubation with TLR4

inhibitor or TLR2/TLR4 inhibitor (45.38±6.58), as compared to HUVEC incubated only with 100 µg/mL of extracellular histones (21.93±3.27) (Figure 4A and Table 1).

We also analysed cell apoptosis and necrosis produced by TLR inhibitors by comparing the effects of increasing histone concentrations. Results showed that 100 µg/mL of extracellular histones significantly increase HUVEC apoptosis plus necrosis (comparing 100 µg/mL vs. 0 µg/mL and vs. 50 µg/mL) in HUVEC cells previously incubated with any of the TLR inhibitors (Table 2). These results also showed that TLR2/TLR4 inhibitor OXPAPC was the most cytotoxic TLR inhibitor in presence of 100 µg/mL histones, whereas TLR4 inhibitor CLI-095 was the least cytotoxic (Table 1 and Table 2), which suggests that the inhibition of TLR2 can further compromise viability of HUVEC.

When we analysed the effect of TLR inhibitors on autophagy by measuring the levels of LC3-II, we observed that OxPAPC, iODN and CLI-095 have the capacity to increase autophagy by themselves, and subsequent addition of extracellular histones did not produce any effect beyond that produced by TLR inhibitors (Figure 5B and Figure 5C). These results suggest that TLR inhibition, by itself, does not prevent autophagy activation nor apoptosis in HUVEC when cells are challenged with extracellular histones.

4. **Discussion**

The information regarding the mechanism by which endothelial cells die as a consequence of circulating histones - a process that occurs during a septic episode - is scarce. Histones have been detected in plasma of human patients with sepsis and septic shock reaching levels higher than 70 µg/mL [6, 17, 42]. Since several therapeutic strategies have failed in treating sepsis and septic shock [43], we consider relevant to characterize the mechanisms mediated by extracellular histones [20, 44].

Extracellular histones can damage HUVEC through different histone-activated mechanisms which are dependent on the concentration of extracellular histones, as described by Abrams *et al*. after lung injury [5], and as we also observed in our experiments [19]. Here we show that histones induce different cellular responses that lead the cells towards activation of autophagy and/or apoptosis, depending on the concentration of extracellular histones. These pathways proceed through different mechanisms that regulate the balance between autophagy and apoptosis in relation to different effectors. p53 can play a key role by regulating autophagy and apoptosis via

Bax/Bcl-2 [45] and autophagy via sestrin2-driven pathways [35]. Autophagy has been described as a cell survival mechanism through suppression of apoptosis [46]. However, its key role as a stress signaling mechanism [47-49] makes autophagy a relevant process that can direct cells either to death or to survival. In fact, when autophagy is highly activated, it represents a pre-requisite for apoptosis and is related with programmed cell death in a non-apoptotic way [50]. The cross-regulation between cell survival, apoptosis and autophagy was evident in our studies, which showed that histones direct the cells to autophagy through Sestrin2 up-regulation and through a decrease in AKT activation. Histone concentrations above 25 µg/mL also promote phosphorylation of AMPK and dephosphorylation of p70S6K, contributing to autophagy upregulation. However, at concentrations of 50 µg/mL or higher, although autophagy was still activated, extracellular histones induced apoptosis in a p53-Bax-dependent manner. A representative scheme of the interlinkage between apoptotic and autophagic pathways affected by extracellular histones, as suggested by our experiments, is summarized in Figure 6. Our results, hence, reveal that the progress of endothelial cell death in response to extracellular histones depends on histones concentration, in a delicate balance between autophagy and apoptosis.

These two mechanisms are closely related pathways [51]. Even the pro-survival functions of autophagy can produce deleterious effects that lead to cell death [52]. In addition, autophagy has been shown to be an important component of the innate immune response [53], in which Toll-like receptors (TLRs) are essential key contributors [54, 55]. Importantly, the endothelial autophagy system represents a novel immune regulatory mechanism that may be exploited therapeutically, in order to ameliorate the deleterious effects of deregulated autophagy and apoptosis in a variety of disorders in which levels of circulating histones are increased. However, histone concentrations above 50 µg/mL direct cells to apoptosis, which may produce endothelial activation that in turn may alter vascular homeostasis, as we have recently shown [19]. The development of TLR antagonists is of current interest for their application and potential use in therapy, being TLR4 and TLR2 the favourite targets for developing anti-sepsis drugs [56]. In fact, histones can bind to TLR2 and TLR4 [57], and more recently it has been proposed that histones produce upregulation of procoagulant tissue factor (TF), partly mediated by TLR2 and TLR4 [58]. However, some discrepancies have been found using these therapeutic approaches in mice and humans following the treatment of TLR inhibitors [56, 59]. Our results indicate that the use of TLR inhibitors increases apoptosis. Some reports suggested the role of OxPAPC, a TLR2/TLR4 inhibitor, in apoptosis. Shimamoto A., et al. described that the

use of Eritoran, a TLR4-inhibitor, was able to decrease the expression of inflammatory cytokines and the inflammatory response, but it was unable to inhibit apoptosis in C57BL/6 mice during myocardial ischemia-reperfusion injury [60]. Furthermore, Ulbrich *et al*. reported that OxPAPC did not influence rotenone-induced apoptosis in SH-S5Y5, a human neuroblastoma cell line [61]. In other studies, Yvan-Charvet l. *et al*. showed the effect of OxPAPC activating macrophage apoptosis [62]. Importantly, the literature agrees with the multiple effects of OxPAPC on protective, inflammatory and pro-apoptotic pathways, suggesting the multiple functions and mechanisms mediated by TLRs. Following this line of evidence, our results show that the concentration of circulating histones is relevant for the activation of cell death mechanisms in HUVEC, and that TLRs are important elements in the modulation of cell death responses. In this regard, the broad diversity of pathways mediated by TLRs suggests that besides their role in mediating anti-microbial defences, they can also induce apoptosis [63]. The synergistic effect we observed on apoptosis, when TLRs were inhibited before histone treatment, reinforces the idea of the importance of these receptors in mediating cell pro-survival by promoting autophagy. This fact is reinforced by results previously described by Wagner et al., in which TLR2 inhibition induces ERK1/2 and AKT signaling [64], which we know are relevant upstream pathways activating autophagy. Importantly, TLRs are involved in detection of PAMPs and microbes, initiating innate immune responses, and linking innate and adaptive immunity, as proposed by Takeda and Akira [65].

Our study shows that extracellular histones produce endothelial autophagy and cell apoptosis, which are dependent on the concentration of extracellular histones. Although further studies are required to thoroughly elucidate the effect of circulating histones on endothelial cells, our observations could have major implications for a variety of acute inflammatory disorders that are characterized by endothelial dysfunction, and that cause organ injury and failure, such as sepsis. In addition, we recently demonstrated that extracellular histones induce concentration-dependent changes in the two main vasoactive mediators, prostanoids and NO, resulting in a decrease in NO levels and a shift in prostanoids release [19], which is in agreement with the present results on Akt phosphorylation, since Akt activity positively correlates with NO generation in endothelial cells [66].

In addition to Akt, AMPK is a key gene controlling several cellular functions such as cell growth, sensor of cell energy and cell survival [67, 68]. Indeed, AMPK overexpression has been reported as a crucial event preventing endothelial cell apoptosis [69]. In fact,

AMPK activation can mediate mTOR inhibition [70] and, therefore, finally increasing autophagy in endothelial cells.

The presence of circulating histones can be used as a biomarker to be considered during the evolution of a septic process [42] and could explain why TLRs inhibitors such as Eritoran have failed in clinical trials [59]. Furthermore, the effect observed using inhibitors of TLRs in which apoptosis is exacerbated when TLRs are inhibited, supports the clinical observations that toll-like receptor-2 (TLR2) polymorphisms are linked to increased risk of developing septic shock and increased mortality [71]. Our results agree with this hypothesis, because the inhibition of TLRs increased apoptosis in HUVEC when exposed to extracellular histones. Future studies might confirm whether any intervention in the autophagy pathway (activating or inhibiting) serves to modulate the pathogenic process of sepsis. This may provide novel therapeutic avenues for sepsis and septic shock, and harbours special relevance to avoid the pleiotropic effects of histones in endothelial cells and preventing the formation of a thrombotic environment [58].

5. **Conclusions**

Histones have been detected in plasma of human patients with sepsis inducing different histone-activated mechanisms which are dependent on the concentration of these nuclear proteins. Histones produce damage to endothelial cells, thereby they may contribute to sepsis progression towards septic shock. Here we show that histones induce different cellular responses that commit the cell to autophagy and/or apoptosis. Furthermore, the results obtained demonstrate that the inhibition of TLRs increased apoptosis in HUVEC when cells were challenged with extracellular histones. Therefore, the results reveal the role of histones as new mediators participating in the control of autophagy and/or apoptosis, which could serve to improve survival in sepsis. In turn, histones may provide not only new biomarkers of sepsis-associated damage progression, as we demonstrated recently [42], but also novel therapeutic strategies to avoid the pleiotropic effects produced in endothelial cells, such as the increase of cell death and the formation of a thrombotic environment, among others. These results reinforce the necessity of developing combined multi-target therapeutic strategies that face the multiple and diverse molecular pathways that are participating in sepsis and septic shock.

Conflict of Interest Statements

JSIC, CA, JLGG, DPC, CBB, CRM, SN, CH, and FVP declare no competing financial interests.

Authors contributions

Conception and study design: JLGG, CH, SN and FVP. Experiment performing and acquisition: JSIC, CA, JLGG, DPC, CBB, CRM, SN. Statistical analysis: EMGL. Drafting manuscript: JLGG, CRM, CA, SN, CH, and FVP. All authors approved the final version of manuscript. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Figure legends

Figure 1. Extracellular histones decrease cell viability and induce apoptosis. HUVEC were exposed to increasing concentrations of histones (0-100 μg/mL) for 4 h. (**A**) Percentage of living cells and (**B**) apoptotic cells were measured by flow cytometry from five independent experiments and represented from a total of 4.000 events to each sample. The significance has been considered from the value of 0 μg/mL samples in each condition (***P*<0.01 and ****P*<0.001). (**C**) Bright field images recorded by Nikon's inverted eclipse Ti microscope (magnification x100) reveal alterations in cell morphology. (**D**) Cell apoptosis was further corroborated by labeling HUVEC with Annexin-V-PE. Counterstaining was achieved by DAPI staining. Images are representative of five independent experiments recorded by Nikon's inverted eclipse Ti microscope (magnification x40).

Figure 2. Analysis of autophagy flux in HUVEC at different concentration of extracellular histones. Protein extracts from cultured HUVEC incubated at different concentrations of histones (0-100 μg/mL) during 4 h were loaded on SDS-PAGE gels and analyzed by Western blotting using anti-LC3 and anti-β-actin as a loading control (**A**) To study autophagy flux cellular extracts were obtained without (left panel) or with (central panel) lysosomal inhibitors (20 mM NH4Cl plus 0.1 mM leupeptin). Densitometry of LC3-II bands from three independent experiments with lysosomal inhibitors are shown at the right. The significance has been considered from the value of 0 μg/mL samples in each condition (***P*<0.01 and ****P*<0.001). (**B**) Fluorescence microscopy images of LC3 in HUVEC incubated with histones (0-100 μg/mL). Maximum projection images derived from 10 Z axis planes are shown. High magnification of representative cells is shown in the inset. Dots correspond to autophagosomes. Images are representative of 3 independent experiments recorded by Leica TCS SP8 X microscope (magnification x40).

Figure 3. Analysis of autophagolysosome formation in HUVEC at different concentrations of extracellular histones. (**A**) Levels of LAMP2, a lysosomal marker, from HUVEC treated with different concentrations of extracellular histones (0-100 μg/mL) during 4 h were analyzed by Western blotting. (**B**) Lysosomal mass detected by confocal microscopy of HUVEC incubated with histones (0-100 μg/mL) and analysed using Lysotracker Red 50 nM. Images are maximum projection derived from 10 Z axis planes and are representative of 3 independent experiments recorded by Leica TCS SP8 X microscope (magnification x40). Histogram shows the mean value of the

number of lysosomes per cell (dots per cell were counted in at least 10 HUVEC cells using ImageJ software).

Figure 4. Analysis of regulators of autophagy and apoptosis in HUVEC at different concentrations of extracellular histones.

Upstream and downstream signals to mTOR mediated by proteins involved in the activation of autophagy in HUVEC incubated with extracellular histones (0-100 µg/mL). (**A**) Representative images of Western blots for β-actin, Sestrin2, pho-AMPK (T172), AMPK, pho-ULK1 (S555), ULK1, pho-Akt (S473), Akt, pho-mTOR (S2448), mTOR, pho-P70S6K (T389) and P70S6K. (**B**) Densitometry analysis of Sestrin2, pho-AMPK/AMPK, pho-ULK1/ULK1, pho-Akt/AKT, and pho-P70S6K/P70S6K ratios. Data represent the mean from three independent experiments and statistical significance refers to the value of control samples (***P*<0.01 and ****P*<0.001). (**C**) Regulation of apoptosis was analyzed by Western blotting using the anti-apoptotic marker Bcl-2 and pro-apoptotic markers pho-p53 (S15) and Bax. (**D**) Densitometry analysis of Bax/Bcl-2 ratio to evaluate apoptosis flux. Data represent the mean from three independent experiments and statistical significance refers to the value of control samples (**P*<0.05 and ***P*<0.01).

Figure 5. Effect of TLR inhibitors on apoptosis and autophagy in HUVEC exposed to different concentration of extracellular histones. (A) TLR inhibitors (30 µM OxPAPC, an inhibitor of TLR-2 and TLR-4, 0.7 µM iODN, a TLR-7/9 antagonist, and 3 µM of CLI-095, a TLR-4 antagonist) in HUVEC exposed to extracellular histones. Apoptosis and necrosis was assessed by flow cytometry analysis by labelling HUVEC using Annexin-V and Propidium iodide. Data represent the mean from five independent experiments and statistical significance refers to the value of control samples (HUVEC without incubation of extracellular histones nor TLR inhibitors (**P*<0.05 and ***P*<0.01). Mean±SD for viable, apoptotic and necrotic cells and p values for each condition analysed are shown in Table 1 and Table 2. **(B)** Western blotting using anti-LC3 in presence of lysosomal inhibitors to analyse autophagy activation in HUVEC treated with 50 µg/mL histones and incubated with TLR inhibitors. **(C)** Western blotting using

anti-LC3 in presence of lysosomal inhibitors to analyse autophagy activation in HUVEC incubated with 100 μg/mL histones in presence of TLR inhibitors. Densitometric analyses from three independent experiments of LC3-II normalized levels versus β-actin determined by Western blotting, are shown in the bottom panel. The significance has been considered from the value of 0 μg/mL samples in each condition (*P<0.05, **P<0.01 and ***P<0.001)

Figure 6. Influence of extracellular histones on autophagy and apoptosis regulators. Scheme summarizing the levels of different autophagy and apoptosis regulators after treatment with extracellular histones, showing the relationships among them, and the net result in autophagy activation (left) or apoptosis activation (right). Treatments between 10 and 100 μg/mL extracellular histones result in both an increase in sestrin2 levels (with the consequent increase in phospho-AMPK and phospho-ULK1), and a decrease in phospho-Akt, resulting in an inactivation of mTOR, which correlates with a decrease in the phosphorylation of p70S6K, all of it leading to autophagy activation. On the other side, only under exposition to 100 μg/mL of histones levels of phospho-p53 increase, as well as the ratio Bax/Bcl2, leading to activation of apoptotic pathways. Moreover, an increase in p53 activation could also contribute to maintenance of autophagy through sestrin2 (dashed arrow).

Table 1. Effect of extracellular histones on HUVEC pre-incubated with TLR inhibitors. Table shows the % of viable, apoptotic, necrotic and apoptotic+necrotic cells. A One-way ANOVA tests were conducted in order to explore significant differences into each group, established in base of extracellular histones concentration and comparing the effect of TLR inhibitors. For comparisons with *p* values lower than 0.05, Tukey post-hoc tests were performed. Mean±SD and data *p* value for each condition and comparison consisting in five independent experiments is shown.

[Histone]	Condition	% Viable cells			% Apoptotic cells			% Necrotic cells			% Apoptotic+Necrotic cells		
		Mean	SD	p value	Mean	SD	p value	Mean	SD	p value	Mean	SD	p value
0 µg/µL	**Control**	89.44	4.23	-	9.66	3.91	-	0.77	0.59	-	10.44	4.29	-
	OxPAPC	77.44	8.09	**0.010**	20.69	8.81	**0.026**	1.86	081	**0.026**	22.56	8.08	**0.010**
	iODN	84.83	5.48	>0.05	13.94	5.73	>0.05	1.24	0.77	>0.05	15.13	6.13	>0.05
	CLI-95	79.39	3.04	**0.001**	19.13	3.64	**0.006**	1.48	0.77	>0.05	20.61	3.03	**0.001**
50 µg/µL	**Control**	82.96	4.84	-	15.65	4.82	-	1.08	0.57	-	16.63	4.45	-
	OxPAPC	82.55	0.014	>0.05	14.83	1.66	>0.05	2.61	1.67	>0.05	17.45	0.014	**0.044**
	iODN	86.68	5.33	>0.05	12.00	6.22	>0.05	1.32	1.40	>0.05	13.32	5.33	>0.05
	CLI-95	69.83	3.35	**0.005**	29.65	3.11	**0.003**	0.52	0.23	>0.05	30.17	3.04	**0.002**
100 µg/µL	**Control**	78.50	2.99	-	19.64	2.28	-	2.29	1.22	-	21.93	3.27	-
	OxPAPC	54.61	6.58	**0.010**	40.44	6.06	**0.011**	4.94	2.89	>0.05	45.38	6.58	**0.011**
	iODN	73.11	8.14	>0.05	21.91	5.07	>0.05	4.98	3.23	>0.05	26.89	8.13	>0.05
	CLI-95	61.43	6.49	**0.010**	34.19	9.80	>0.05	2.39	0.26	>0.05	36.58	9.80	**0.033**

Table 2. Effect of extracellular histones on HUVEC pre-incubated with TLR inhibitors. Table shows the % of viable, apoptotic, necrotic and apoptotic+necrotic cells. One-way ANOVA tests were conducted in order to explore significant differences into each group, established in base of TLR inhibitors and comparing the effect of different extracellular histones concentration. For comparisons with *p* values lower than 0.05, Tukey post-hoc tests were performed. Mean±SD and data *p* value for each condition and comparison consisting in five independent experiments is shown.

Toll-like receptors inhibitor	[Histone] µg/µL	% Viable cells					% Apoptotic cells					% Necrotic cells					% Apoptotic+Necrotic cells				
		Mean	SD	P value (*vs.* 0)	p value (*vs.* 50)	P value (vs. 100)	Mean	SD	P value (*vs.* 0)	p value (*vs.* 50)	P value (vs. 100)	Mean	SD	P value (*vs.* 0)	p value (*vs.* 50)	P value (vs. 100)	Mean	SD	P value (*vs.* 0)	p value (*vs.* 50)	P value (vs. 100)
OxPAPC	0	77.40	8.09	-	n.s	0.005	20.69	8.81	-	n.s	0.013	1.86	0.80	-	n.s	n.s	22.56	8.08	-	n.s	0.005
	50	82.55	0.01	n.s	-	0.005	14.83	1.66	n.s	-	0.010	2.61	1.67	n.s	-	n.s	17.45	0.01	n.s	-	0.005
	100	54.61	6.58	0.005	0.005	-	40.44	6.06	0.013	0.010	-	4.93	2.89	n.s	n.s	-	45.38	6.58	0.005	0.005	-
iODN	0	84.82	5.48	-	n.s	0.036	13.93	5.73	-	n.s	n.s	1.24	0.77	-	n.s	0.028	15.17	5.33	-	n.s	0.036
	50	86.68	5.33	n.s	-	0.027	12.00	6.22	n.s	-	n.s	1.32	1.40	n.s	-	0.049	13.32	5.33	n.s	-	0.027
	100	73.11	8.14	0.036	0.027	-	21.91	5.07	n.s	n.s	-	4.98	3.23	0.028	0.049	-	26.89	8.13	0.036	0.027	-
CLI-095	0	79.39	3.04	-	n.s	0.003	19.13	3.40	-	n.s	0.042	1.48	0.77	-	n.s	n.s	20.61	3.34	-	n.s	0.031
	50	69.83	3.51	n.s	-	n.s	29.65	3.11	n.s	-	n.s	0.52	0.23	n.s	-	0.013	30.17	3.34	n.s	-	n.s
	100	61.44	6.49	0.003	n.s	-	34.19	9.80	0.042	n.s	-	2.39	0.26	n.s	0.013	-	36.58	9.80	0.031	n.s	-

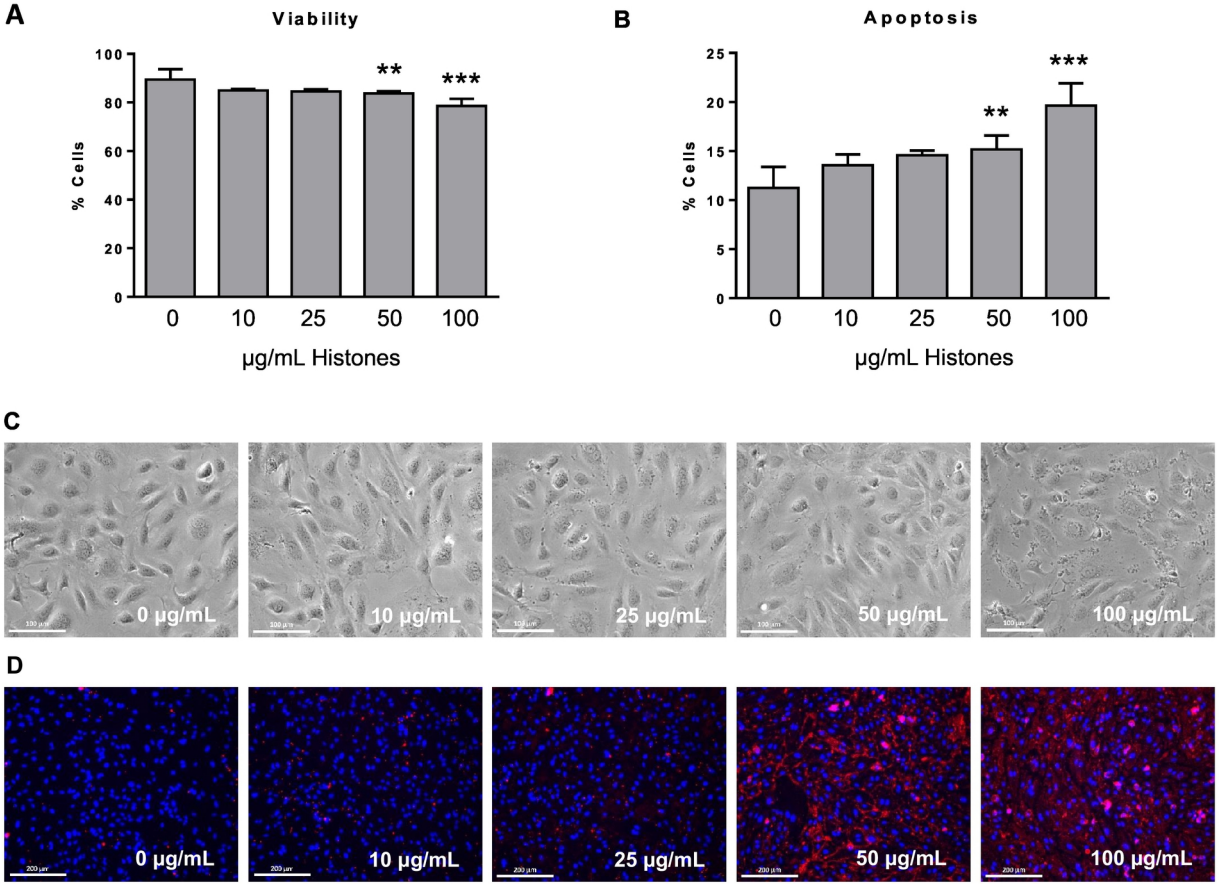
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Figure 1

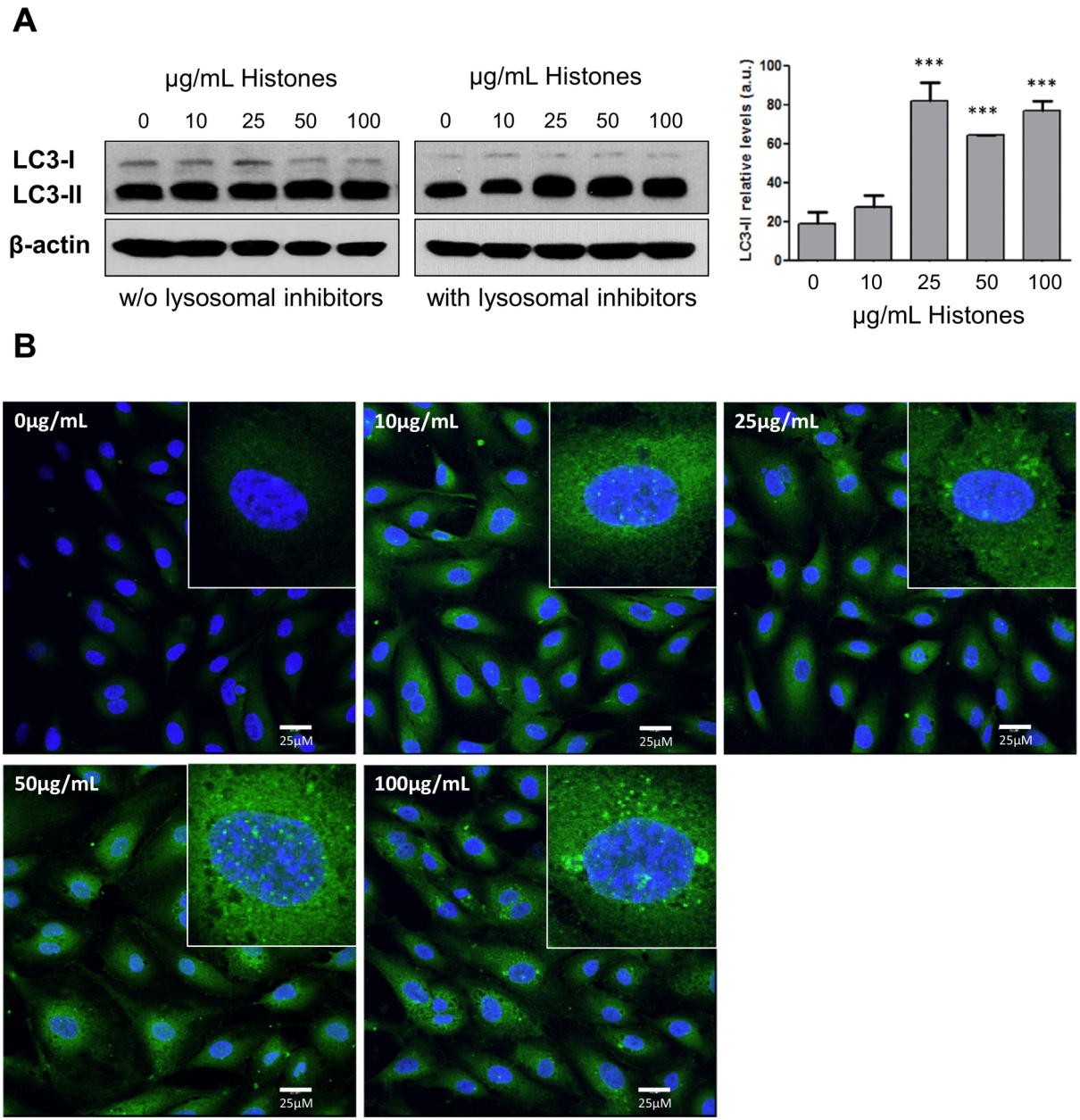
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Figure 2

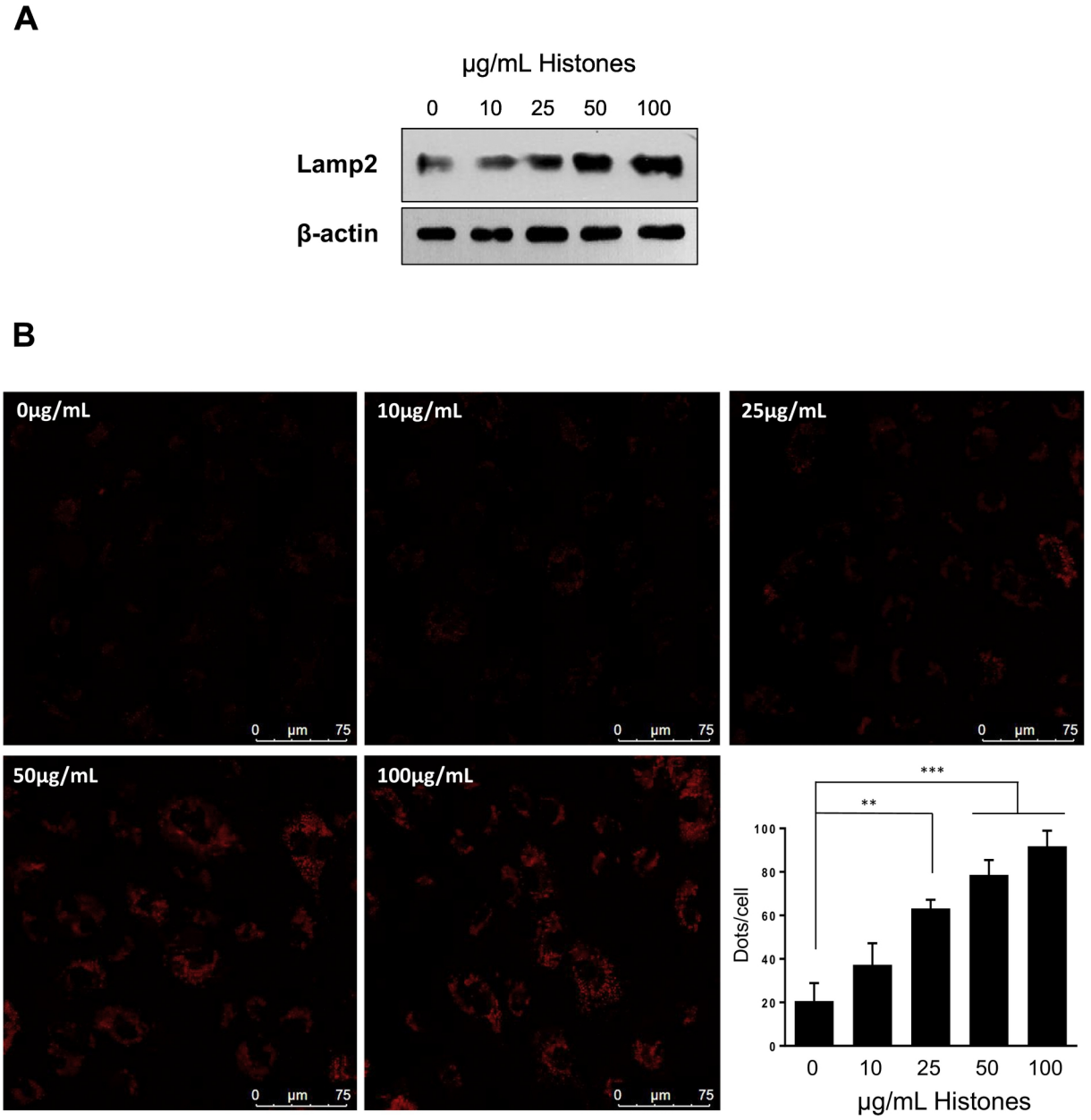
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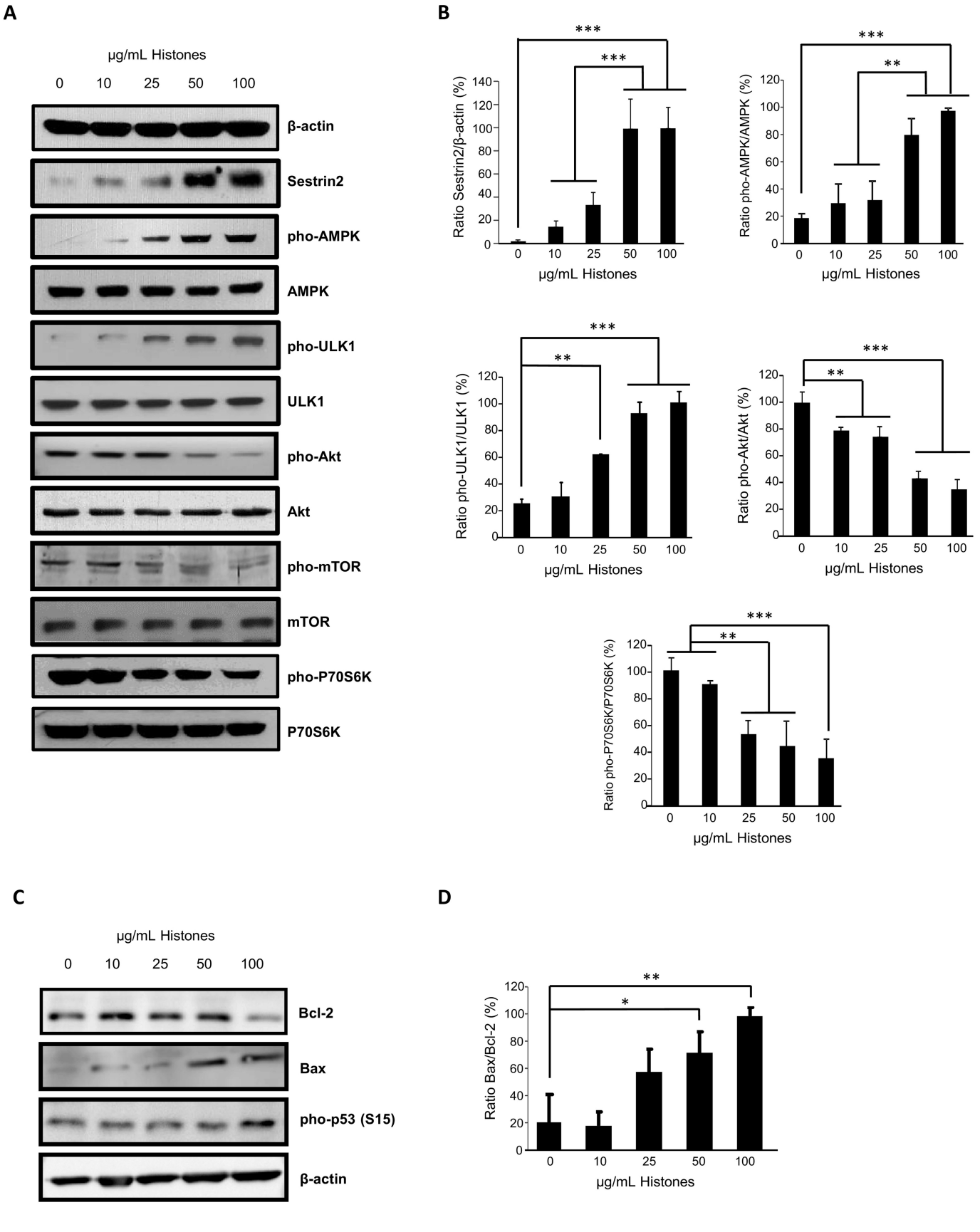
Figure 3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mTOR
pho-P70S6K
P70S6K
B
Ratio Sestrin2/β-actin (%)
Ratio pho-AMPK/AMPK (%)
Ratio pho-ULK1/ULK1 (%)
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Ratio pho-P70S6K/P70S6K (%)
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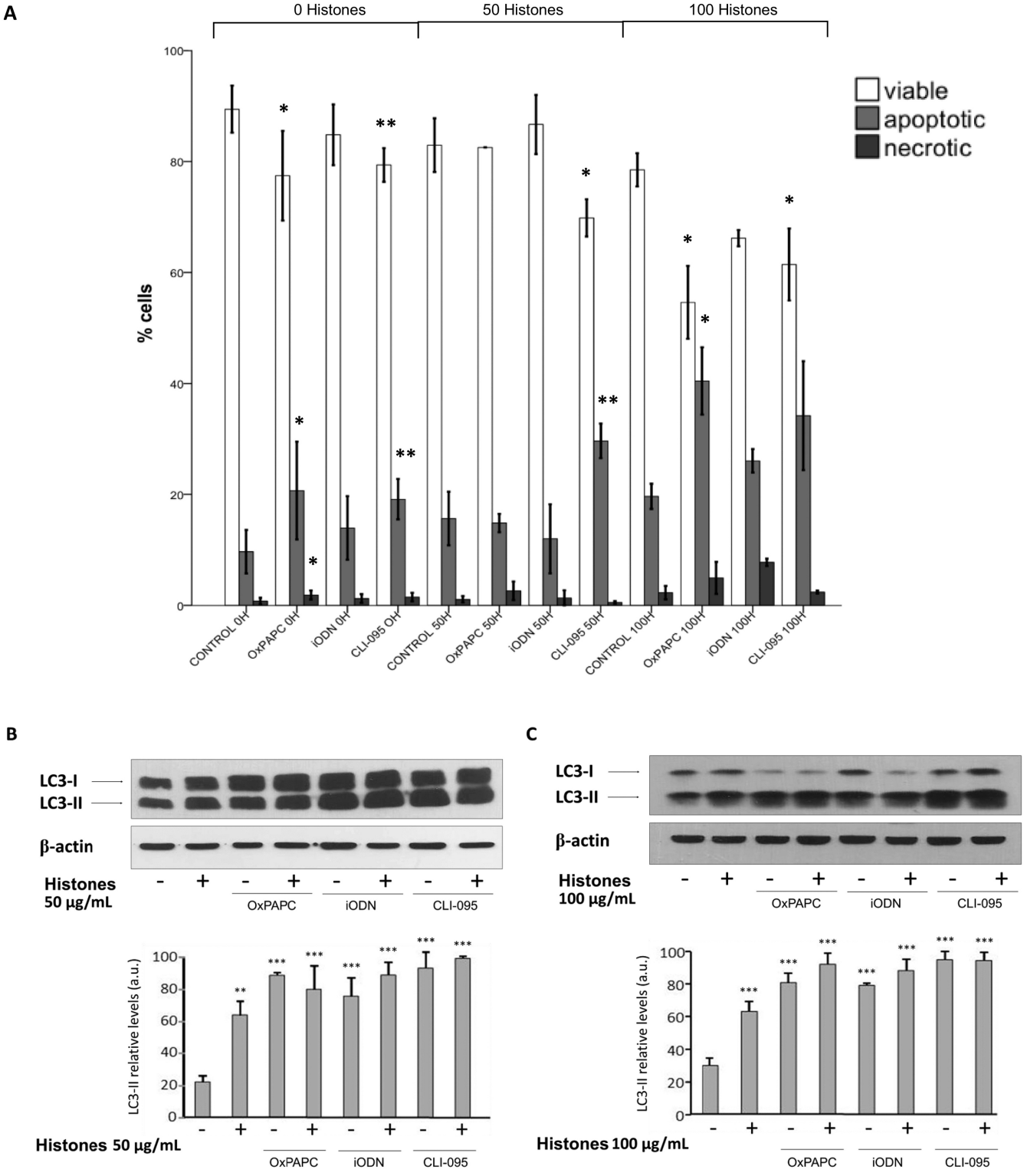
Figure 4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CLI-095 50H'
CONTROL 100H'
OxPAPC 100H'
iODN 100H'
CLI-095 100H'
B
LC3-I
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β-actin
Histones 50 µg/mL
OxPAPC
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CLI-095
LC3-II relative levels (a.u.)
Histones 50 µg/mL
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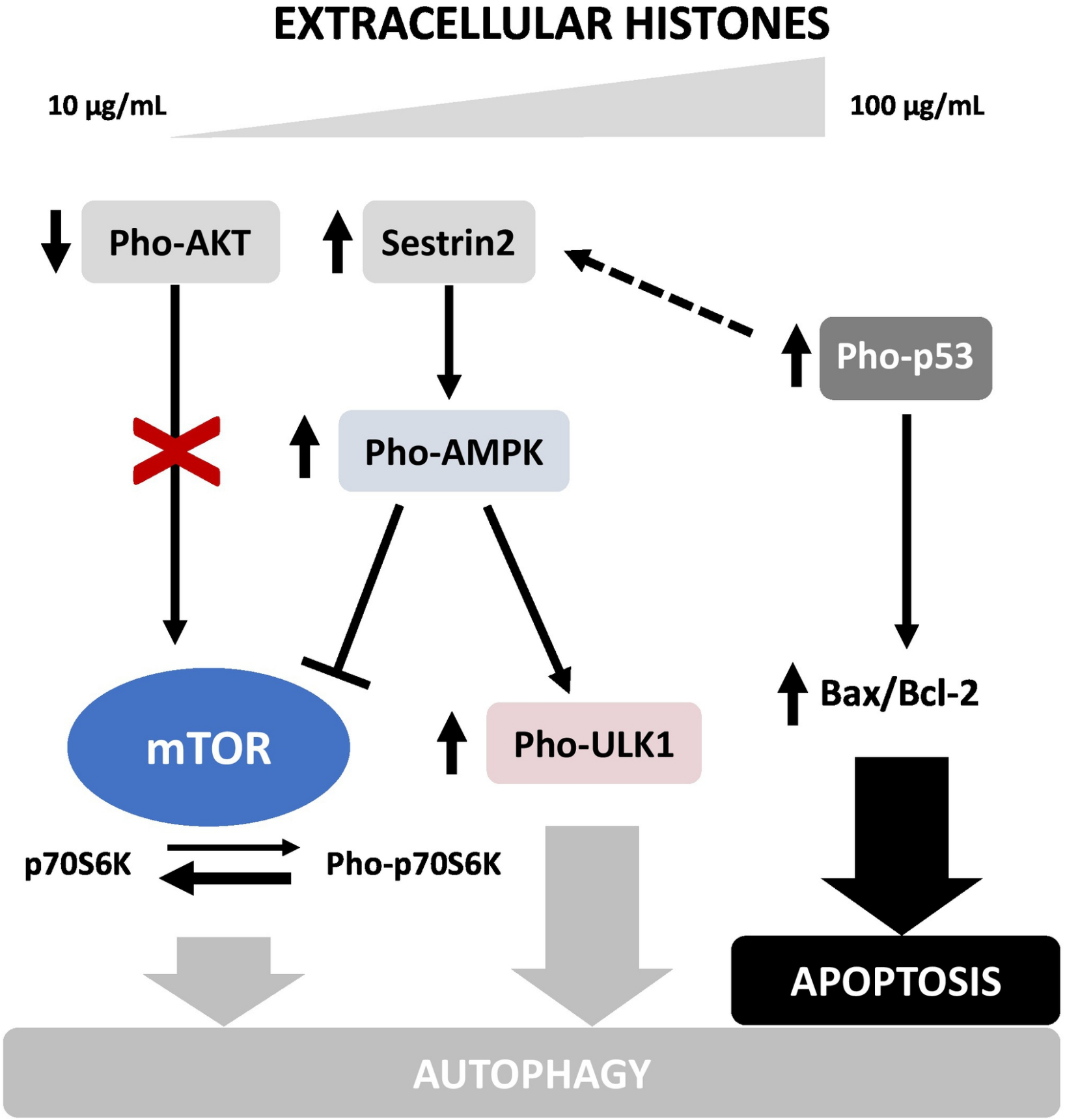
LC3-II relative levels (a.u.)
Histones 100 µg/mL

Figure 5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p70S6K
Pho-p70S6K
APOPTOSIS
AUTOPHAGY

Figure 6