Autophagy Induction by Cyclic Stretch and Its Effect on Cytokines in Retinal Pigment Epithelial Cells

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Research Article

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Abstract

Background: Strong evidence of the correlation between age-related macular degeneration (AMD) and vitreomacular interface abnormality (VMIA). Meanwhile, as a crucial mechanism of retinal pigment epithelial (RPE) cells' homeostasis, autophagy induction by cyclic stretch appears to be particularly significant.

Methods: Cultured ARPE-19 cells were subjected to cyclic stretch (20% elongation, 1HZ) for 1h, 2h, 6h, 12h,24h and 48h by FX-5000 Tension System. Then, we observed the expression levels of LC3I, LC3II, Beclin-1, SQSTM1/p62, LAMP-1, mTOR and phosphorylated mTOR(pmTOR), AMPK and pAMPK, NADPH oxidase 4 (NOX4), and vascular endothelial growth factor (VEGF) in RPE cells under stretch by western blot and immunofluorescence.

Results: We found autophagic proteins mostly induced by cyclic stretch in a time-dependent fashion via mTOR suppression and AMPK activation, except for SQSTM1/p62. 3-Methyladenine(3-MA), an inhibitor for autophagy, could reduce the up-regulation of autophagy due to cyclic stretch, leading to higher level of VEGF release after 24h cyclic stretch. Rapamycin could narrow the increase degree of VEGF and NOX4 by cyclic stretch by raise autophagic level in RPE cells.

Conclusion: Stretch might induce autophagy in RPE cells by mTOR or AMPK pathway. Autophagy might play the protective function for RPE cells away from mechanical stress derived from VMIA-related AMD.

Background

Age-related macular degeneration (AMD), which was considered as a main cause of visual impairment in older people worldwidely, is a chronic and progressive ocular fundus disease concerned by many of ophthalmologists[1]. On the basis of the angiogenesis and vascular exudation, late-stage AMD was divided into "dry" and "wet" AMD[2]. Although the application of anti-vascular endothelial growth factor (VEGF) injection promotes many of the patients gain better a visual prognosis, there were still no efficient methods for non-sensitive wAMD patients, along with dry AMD ones.[3]

On the other hand, as the age of the population increases, the vitreous body was gradually liquefied until detached with retina. However, some people appeared abnormal detachment, which manifested vitreomacular interface abnormality (VMIA), which was proved as a related factor in AMD[4,5], and a negative factor for benefiting from anti-VEGF injection[6]. Furthermore, the abnormal detachment between vitreous body and macular retina could form the mechanical stretch through the whole retina until to retinal pigment epithelium (RPE) cells, which was confirmed to play a vital role in the development of AMD[7]. In previous studies, we have shown mechanical stretch could induce RPE cells to express AMD-related cytokines, like VEGF, reactive oxygen species (ROS), interleukin-related immune proteins and cytoskeleton[8–11]. Therefore, mechanical stretch which applied to RPE cells did show effects on AMD progress.

At the same time, autophagy, a decisive mechanism of AMD which mentioned these years, showed correlations with mechanical stretch in other diseases or homeostasis in cells, tissues and organs[12–14]. Similarly, studies on trabecular meshwork indicated mechanical stretch will induce autophagy in cells by macrophagy pathways[15–17]. Therefore, whether cyclic stretch, which mentioned in our previous studies, could affect RPE's autophagy, and the mechanism of altering cellular autophagy by cyclic stretch needs further study.

The purpose of this article is to explain whether cyclic stretch could affect cellular autophagy in RPE cells over time. After confirming the alteration, we continue to explore the pathway cyclic stretch change cellular autophagy. Furthermore, the relationship between VEGF, NOX4 and autophagy will discuss then. In conclusion, cyclic stretch could stimulate autophagy in RPE cells at a late period, which could decline by the autophagy inhibitors. The alteration in autophagy might influence VEGF and NOX4 by mTOR and AMPK pathways.

Methods

Culture of ARPE-19

The ARPE-19 cells (ATCC cop) were cultivated in 25 cm^2 cell culture flask (Corning, New York, USA) with culture medium consisted of DMEM/F12 medium (Gibco) with 10% FBS (Gibco) at 37℃ in a humidified of 95% air and 5% CO_2. The culture medium was replaced every 48 hours, and cell passage was applied when cell density is over 80%.

Cyclic stretch on cells

At the time ARPE-19 cells meets cell passaging conditions, the cells were passaged on six-well BioFlex plates coated by collagen-I (Flexcell International Corporation, Burlington, CA, USA) with DMEM/F12+10% FBS for one or two days until cells growed adhering to the elastic membrane and the cells density is over 70-80%. Then we replaced the medium to DMEM/F12 with no FBS for another 12 hours for preparing cyclic stretch. After that, the plates were placed onto the Flexcell FX-5000T Tension System (Flexcell International Corporation, Burlington, CA, USA) for cyclic stretch. The concrete parameter for the system was 1Hz for 20% elongation amplitude with 1:1 stretch:relaxation ratio for 1h, 2h, 6h, 12h, 24h and 48 hours. Along with the control plates, the experimental ones were incubated in the 37℃,5% CO_2 environment. 3-Methyladenine(3-MA) (MedChemExpress, Monmouth Junction, NJ, USA) , which is a PI3K inhibitor for autophagy suppression, and Rapamycin (MedChemExpress), which is a mTOR inhibitor for autophagy activation, were both applied for pathway investigation and consequences.

Western blot analysis

After suffering from the cyclic stretch, ARPE-19 cells were extracted by RIPA buffer (Thermofisher, Waltham, USA) with protease inhibitor cocktail (Thermo) and phosphatase inhibitor cocktail (Thermo). The total protein of these samples was measured by BCA protein assay kit (Thermo). 10% and 12%

precast polyacrylamide gels by Sodium dodecyl sulphate-polyacrylamide gel electroporesis (SDS-PAGE) (Thermo) were applied according to protein molecular weight of these proteins. Afterwards, polyvinylidene difluoride (PVDF) membranes were used for blotting from gels and soaked into 5% no-fat milk for blocking. The membranes were incubated with primary antibodies: LC3II/I(1:2000, Abcam, Cambridge, UK, EPR18709), SQSTM1/p62(1:10000, Abcam, ab109012), Beclin-1(1:2000, Abcam, ab207612), LAMP-1(1:1000, Abcam, ab24170), GAPDH(1:5000, Abcam, ab8245), NOX4(1:2000, Abcam, ab133303), VEGF(1:1000,CST, Danvers, USA, No.2463), MTOR(1:1000, CST, No.2983), p-MTOR(1:1000, CST, No.5536), ERK(1:1000, CST, No.4965), AMPK(1:1000, CST, No.5831), pAMPK(1:1000, CST, No.50081). The primary antibodies were diluted into Tris-Buffered Saline with Tween-20 (TBST) with 5% no-fat milk and incubated within PVDF membrane overnight at 4℃. Then, the membranes were washed 3 times for 5 minutes each time with TBST for cleaning primary antibodies and incubated with a secondary goat-anti-rabbit IgG antibody (1:10000, Gene-Protein Link, Beijing, China) for 1h at room temperature. At last, the ECL chemiluminescent substrate (Thermo) was taken into use for display and analyze blotting signals by BIO-RAD Quantity One Imaging software (Millopore, USA).

Immunofluorescence

After cyclic stretch, the culture medium was aspirated from the wells and phosphate buffered saline (PBS) was used for washing 3 times. 4% paraformaldehyde was applied for immobilization. 0.1% Triton-X for permeability and membranes were disposed by blocking buffer (abcam) in 30min. The primary antibody, LC3b (1:1000, abcam, EPR18709) was used for incubation at 4℃ overnight, and next day the cells incubated in fluorescent secondary antibody (1:10000, Gene-Protein Link) for 90min at room temperature. The cells then were covered by Mounting Medium with DAPI and observed by a confocal laser scanning microscope (Leica TCS-NT, Germany),

Statistical analysis

All data were expressed as the mean±SEM. Three samples were measured for each condition in order for avoiding errors. Bonferroni’s multiple comparison test were used for statistical significance ($P<0.05$) (SPSS 23.0). For groups comparison, two-tailed paired t-test was applied.

Results

Autophagy is upregulated in RPE cells by cyclic stretch in vitro

Under 20% elongation and 1HZ cyclic stretch, the expression ratio of LC3II/I was significantly raised at 12h ($p<0.01$). At 24h and 48h during the stretch, LC3II/I was still increased compared to the control group($p<0.001$). At the same time, Beclin-1 started to significantly up-regulate at 12h, LAMP-1 at 12h, and pAMPK/AMPK ratio at 6h ($p<0.01$, respectively), while pmTOR/mTOR ratio showed decrease at 6h until to 48h respectively ($p<0.01$), However, SQSTM1/p62 didn’t change during the period of cyclic stretch

(p>0.05) (Fig.1). After 24h of stretch, the expression of LC3II/I was appeared in RPE cells compared to control group. (Fig.2)

3-Methyladenine could inhibit the upregulated autophagy in RPE cells after cyclic stretch, which cause VEGF over-expression after cyclic stretch

Under 20% elongation and 1HZ cyclic stretch for 24h, 3-MA could reduce significantly the ratio of LC3II/I, LAMP-1, Beclin-1, SQSTM1/p62 ($p<0.01$, respectively) at the concentration of 10mM 3-MA compared to control groups with no 3-MA in DMEM/F12. At the same time, VEGF release showed uptrend at the concentration of 5mM and 10mM 3-MA compared to control groups ($p<0.01$, $p<0.005$), however, NOX4 showed no change with concentration alteration ($p>0.1$) (Fig. 3).

Rapamycin could induce autophagy in RPE cells at early period in cyclic stretch, leading to reduce the protein expression level of VEGF and NOX4

In order to induce autophagy in advance, we pre-conditioned RPE cells 1%FBS in DMEM/F12 with Rapamycin for 24h, and BioFlex Plate was located on the Flexcell Tension System for cyclic stretch. After 20% elongation and 1HZ stretch for 24h, compared to RPE cells without Rapamycin, significant increase was shown in the ratio of LC3II/I at 100nM($p<0.01$); SQSTM1/p62 at 100nM($p<0.05$); Beclin-1 at 25nM, 50nM and 100nM ($p<0.01$, $p<0.05$, $p<0.005$, respectively); LAMP-1 at 100nM($p<0.01$), pAPMK/AMPK ratio at 50nM and 100nM ($p<0.01$, $p<0.001$, respectively), significant decrease was shown in pmTOR/mTOR ratio at 50nM and 100nM($p<0.01$, $p<0.01$, respectively); NOX4 at 100nM ($p<0.01$); VEGF at 50nM and 100nM($p<0.01$, $p<0.01$, respectively) (Fig. 4)

Discussion

Several clinical researches believed that vitreomacular interface abnormality (VMIA) might be related to AMD's occurrence and progress by mechanical stretch from vitreous to RPE layers through the whole retina[4–7]. Our laboratory found mechanic stretch induced by Flexcell system could induce oxidative stress by mitochondrial and NADPH oxidase, induce changes in morphological, actin-cytoskeleton and raise the expression level of cytokines in RPE cells which refers to apoptosis[8–11]. At the same time, Autophagy in RPE cells had proven as a vital mechanism for age-related macular degeneration for degrading reactive oxygen species by oxidative stress [18]and photoreceptor outer segment phagocytosis[19,20]. Its abnormality might induce redundant inpaired cargo and lipofuscin accumulation, and reduced disturbed waste clearance[21]. And it could be induced by mechanical stretch in recent researches. Xu et al. found autophagy could keep bone homeostasis by resistance to cyclic stretch[22]. Besides, Lin et al. believed autophagy involved in cardiac hypertrophy through AT1 receptor-mediated activation of p38MAPK by cyclic stretch models[23]. In a word, autophagy possesses the peculiarity of mechanical sensitivity, and cyclic stretch might alter autophagic capacity in RPE cells.

Indeed, in our study, autophagic flex, which is represented by the expression level of LC3II, was raised at 6h and keep rising until to 48h in a high level. Meanwhile, Beclin-1, which was participated in autophagic induction and phagophore, and LAMP-1, which was represented for process of autophagy fusion, were both up-regulated and MTOR, which was considered as a main regulatory target for autophagy, declined, while SQSTM1/p62 showed no change during the cyclic stretch process. We further showed that the induction of autophagy in RPE cells by cyclic stretch could be inhibited by 3-Methyladenine (3-MA), and a rising protein expression level of VEGF was appeared with the inhibitor after stretch. Furthermore, in condition of autophagic stimulator (Rapamycin)cultivation combined with cyclic stretch, VEGF and NOX4 were down-regulated opposed to control group. To our knowledge this is the first study on analysis for induction of autophagy in RPE cells by cyclic stretch. Flexcell Tension System was applied for mimic mechanical stretch to RPE layers, 20% elongation and 1HZ were applied broadly in recent studies. Qi et al. found cyclic stretch could induce autophagy elevation in vascular smooth muscle cells by mTOR (mammalian target of Rapamycin) pathway[24], King et al reported breast cancer cells suffered from mechanical compression by mTOR pathway[25,26] also, while Liton et al. found no change in trabecular meshwork cells[27]. Based on previous reports, autophagic induction is not only due to mechanical signal stimulation direct to cytoskeleton, but also due to damaged components by excessive mechanical stress indirectly. In our previous studies, actin cytoskeleton, IL-8, IL-6, VEGF, mitochodrial and NADPH oxidase were all induced by cyclic stretch[8–11]. Therefore, we should have ample reason to assumed that cyclic stretch could induce autophagy in RPE cells.

In our study, indeed, mTOR was suppressed by cyclic stretch at 12h until 48h. mTOR, which is a vital regulator for autophagy, could phosphorylate autophagic proteins for autophagy imitation and autophagosome nucleation and nuclear translocation prevention[28]. Meanwhile, as proven by Vion et al[29], autophagy was induced by mechanical stretch in mTOR and AMPK pathway, which identically elevated in RPE cells by mechanical stretch in our study. It's worth noting that SQSTM1/p62 didn't decrease during the period, which be similar to Liton's[15]. They also found no change for SQSTM1/p62 after mechanical stretch, for the reason that SQSTM1/p62 decrease seemed to less relevant to LC3-II accumulation, though SQSTM1/p62 was also a critical indicator for autophagy machinery. Additionally, LC3-associated phagocytosis (LAP) might be also considered due to: 1. The autophagic proteins have a conspicuous non-canonical role for endocytic/phagocytic pathway which called LC3- associated phagocytosis (LAP), LAP play a vital role in the homeostasis and phagocytosis for RPE cells[30]; 2. Evidence in recent studies showed no relationship between SQSTM1 and LC3 expression when LAP happens[31]. However, cyclic stretch induces whether macroautophagy or LAP or other forms of autophagy such as chaperone-mediated autophagy (CMA) needs further study. Therefore, mechanical stretch could induce autophagy in RPE cells in mTOR and AMPK pathway. The high expression of autophagic flux represented stress protective function of RPE cells from traction.

Autophagy is considered to be an important mechanism for maintaining cellular homeostasis, and it is now generally believed that under conditions of exposure to adverse external cellular stimuli, cellular autophagy levels are first upregulated in response to external stress conditions, enhancing the enzymatic

resolution of misfolded proteins within the cell[21]. When external stress is extended, the cellular autophagy level decreases instead due to the excessive accumulation of autophagosome, thus leaving the cell lacking the ability to remove cellular wastes[32]. Especially in the case of retinal pigment epithelial cells, for example, its autophagy level also assumes the function of phagocytosis of photoreceptor outer segments[33], and when a decrease in autophagy level occurs, this decrease in phagocytic digestion is manifested by lipid deposits and thus derived to the early stages of age-related macular degeneration[20,30]. However, the impact of altered autophagy in cells under stress interventions is still controversial. Some studies have suggested that autophagic upregulation of cells under stress conditions are necessary for disease development[34,35], while others have suggested that the upregulation is a self-protective mechanism of cells[15,36]. To investigate the effects of increased autophagy in RPE cells by mechanical stretch, on the one hand, the autophagy inhibitor 3-MA was used to inhibit autophagy, and a significant increase in VEGF expression was found in RPE cells; on the other hand, the autophagy activator Rapamycin was used, and the cells were given 24 hours of traction after pre-raising the autophagy level of RPE cells, and it was found that the NOX4 and VEGF expression was significantly decreased in RPE cells compared to the control group In our previous study, NOX4[11] and VEGF[8], which were two vital cytokines for AMD progression, were both up-regulated by cyclic stretch. Although RPE cells which pre-treated by Rapamycin still showed less upgraded expression of NOX4 and VEGF after 24h stretch, lower expression of them compared to control ones was also demonstrated by Western Blot, which means moderately elevated autophagy for RPE cells might reduce the negative influence by cyclic stretch. Kauppinen et al. found Resvega, which was seen as an activator for autophagy, could reduce VEGF release by IL-1β diminish level[37]. Mitter et al. believed that autophagy for RPE cells was crucial for resistance to oxidative stress, autophagic imperfection might aggravate damage in AMD[38]; Chen et al. found enhancing autophagic activity can alleviate oxidative stress in AMD and protect RPE and photoreceptor cells from progressive degenerations[39]. It is believed that NFE2L2, PGC-1, AMPK and mTOR pathways is vital to improve our comprehending of the regulatory mechanisms in autophagy that alleviate oxidative stress and assuage the progress of AMD[18]. Therefore, Rapamycin might act on mTOR or AMPK pathways for improving resistance to cyclic stretch, so as to VEGF and NOX4 lower expression compared with control group. However, the molecular mechanism for how autophagy affects VEGF secretion and NOX4 expression needs additional study.

This study is still having several limitations. First, ARPE-19 cells are only a cellular model for research, it has less representation for RPE cells in healthy people. Second, due to the limitation of light transmission of Flexcell plates, transmission electron microscope for autophagosome was lack, and gene upregulation/down regulation by qPCR for VEGF and ROS was lack either. Third, the regulatory mechanism between mechanical stretch, autophagy and AMD-related cytokines required further study.

Conclusions

In conclusion, cyclic stretch could induce autophagy in RPE cells, manifested as LC3-II, Beclin-1 and LAMP-1 expression. mTOR and AMPK pathway might be involved in this stretch-induced autophagy in

RPE cells. The elevated autophagy level could help RPE cells away from oxidative stress and VEGF accumulation, which might related to VIMA-related AMD intervention and treatment.

Declarations

Acknowledgements

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Availability of data and materials

The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

Involved in the design of the study (WL); conduct of the study (ZYW, XDL, SW, JXZ, QL and BYQ); collection, management, and analysis of the data (ZYW,XDL and BYQ); preparation of the manuscript (ZYW); and critical revision of the manuscript (WL, YPY, XHY, LZL, KZ, SW, JXZ,QL). All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

All authors declare that they have no conflicts of interest regarding this submission.

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Figures

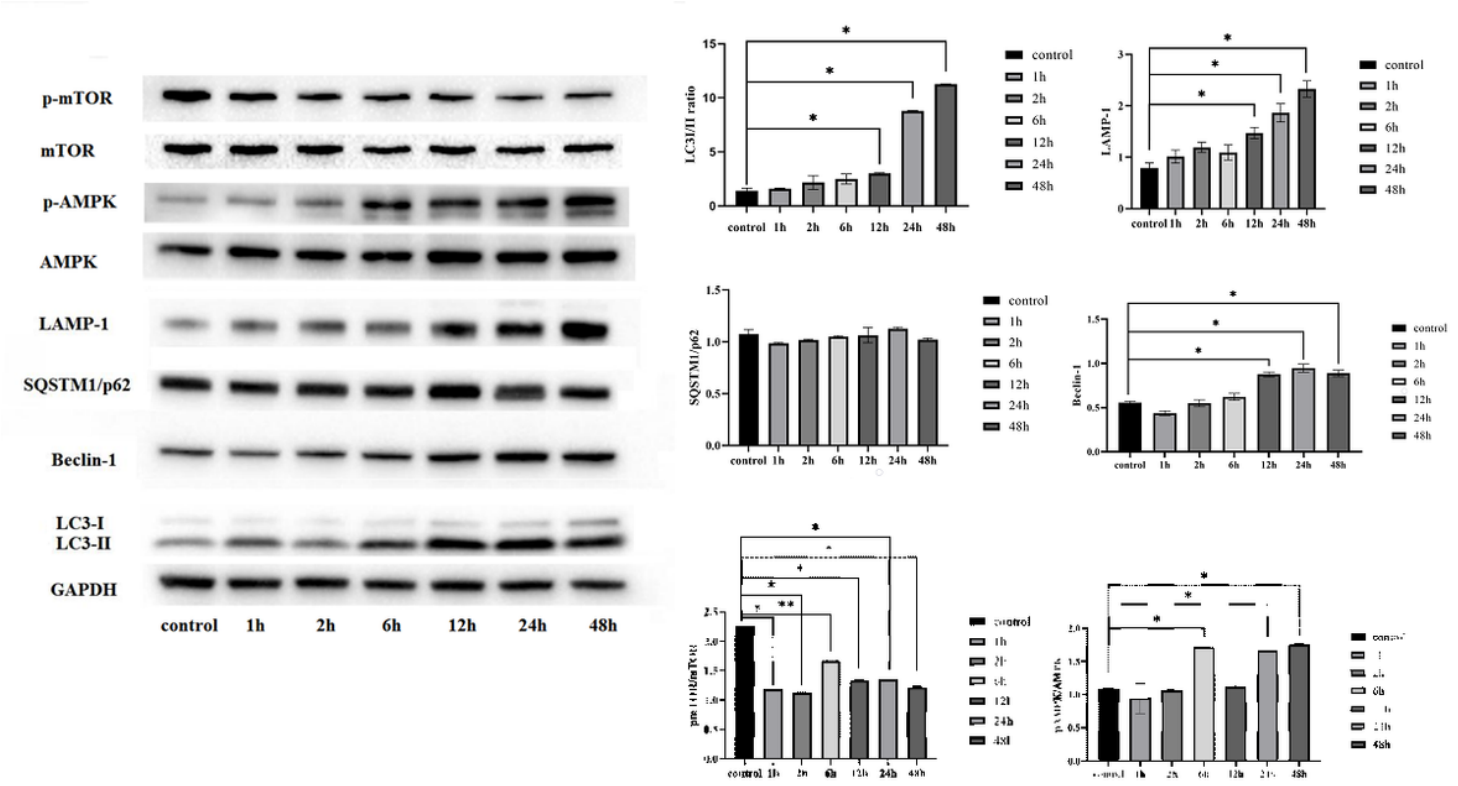

Figure 1

Autophagy-related proteins expression under cyclic stretch in ARPE-19 cells based on Western Blotting. The expression ratio of LC3II/I, LAMP-1 and Beclin-1 were significantly increased at 12h,24h, and 48h; mTOR phosphorylation suppression started at 1h until to 48h; AMPK phosphorylation activation were occurred at 6h, 24h and 48h. *:p<0.01 **:p<0.05

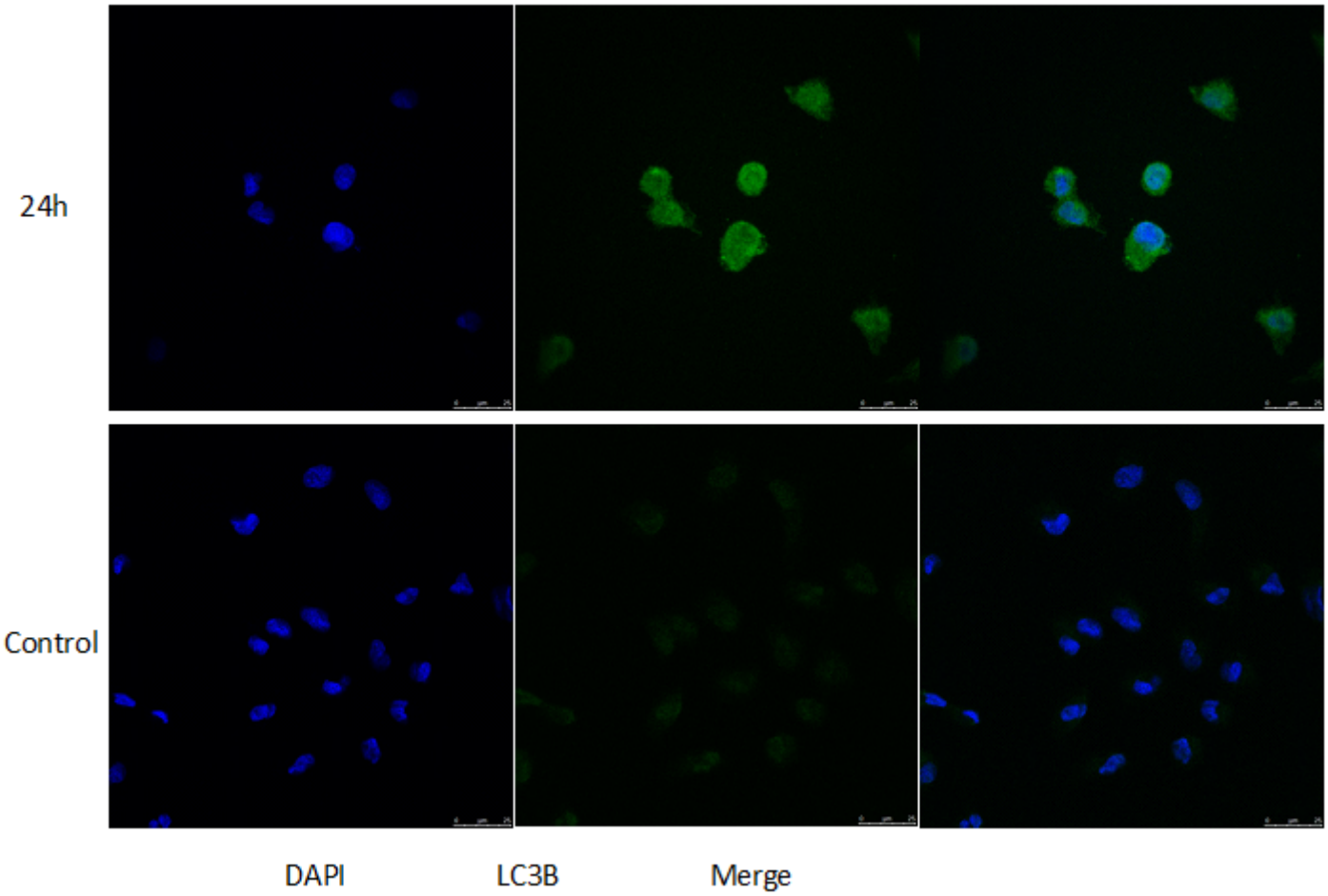

Figure 2

Strained LC3B protein under cyclic stretch for 24h in RPE cells. LC3B proteins, which showed basic expression level for cell homeostasis (control group). Mechanical stress could induce LC3B protein expression at 24h significantly (24h group)

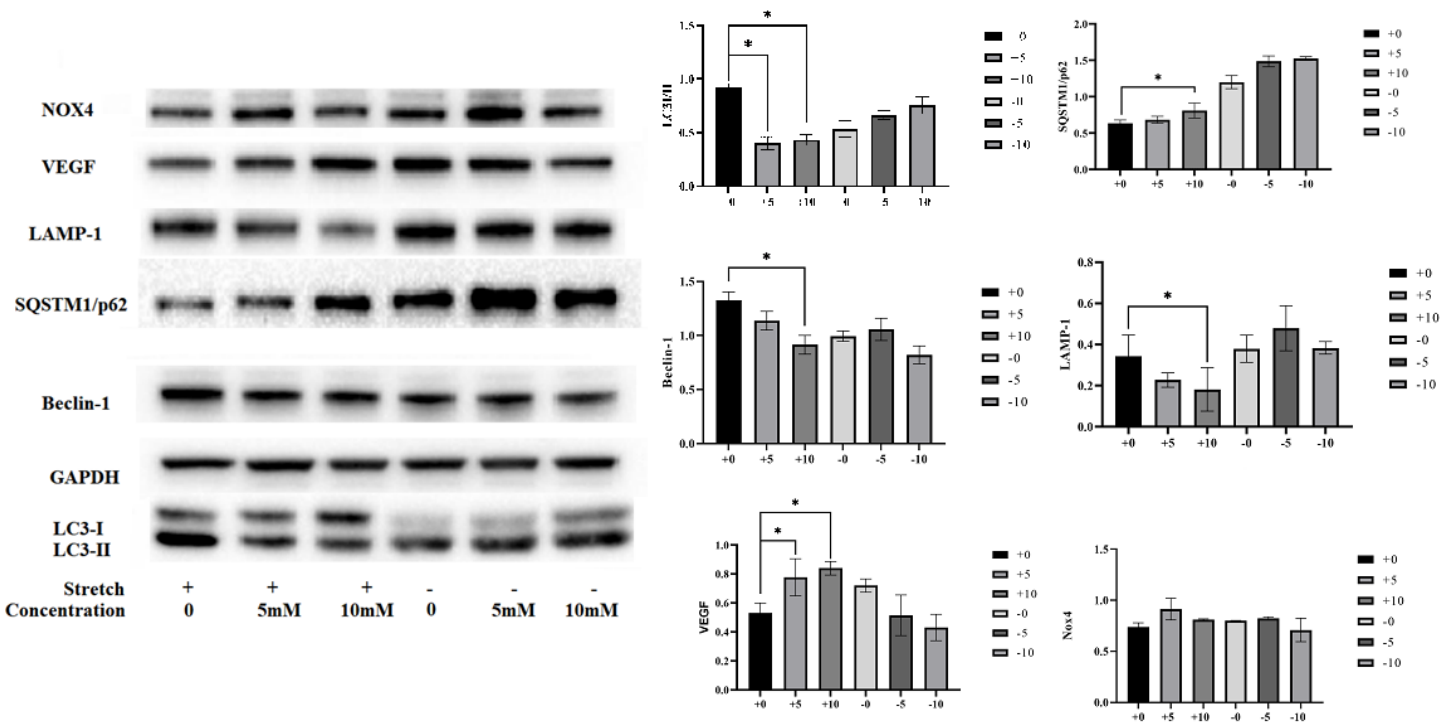

Figure 3

Autophagy-related proteins ,VEGF and NOX4 expression with 3-Methyladenine(3-MA) co-cultivation under cyclic stretch for 24h in ARPE-19 cells based on Western Blotting.At the concentration of 10mM, LC3II/I, SQSTM1/p62, Beclin-1 and LAMP-1 were significantly decreased by inhibitor of autophagy. At the same time, VEGF showed significant increase at a concentration of 5mM and 10mM, while NOX4 showed no change at any concentration. *: p<0.01

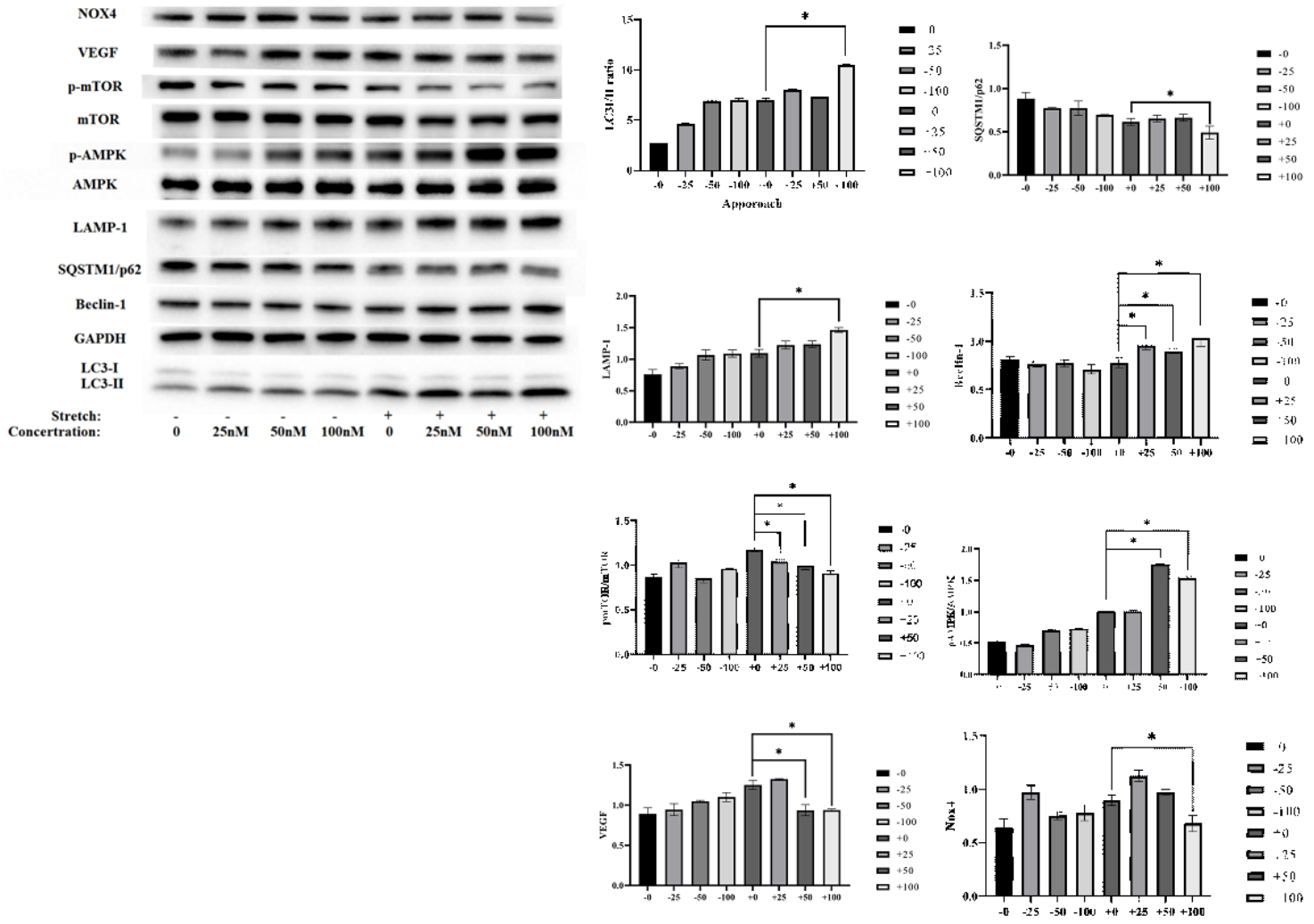

Figure 4

Autophagy-related proteins, VEGF and NOX4 expression with Rapamycin pre-cultivation for 24h following cyclic stretch for another 24h in ARPE-19 celss based on Western Blotting. All autophagy-related proteins showed significant increase at a concentration of 100nM, SQSTM1/p62 and mTOR phosphorylation suppression were both decrease in a concentration-dependent fashion. At the same time, VEGF and NOX4 showed significant decrease at a concentration of 100nM. *:p<0.01