Research Article

Measurement of autophagy flux in benign prostatic hyperplasia in vitro

Sung-hee Oh a, Dong-won Lee b, Yong-bok Choi a, Yoo-hyun Lee b, Jeong-sun Ju a, *

a Department of Exercise Science, College of Health Science, The University of Suwon, Bongdam-eup, Hwaseong-si, Gyeonggi-do, 18323, Korea
b Department of Food and Nutrition, College of Health Science, The University of Suwon, Bongdam-eup, Hwaseong-si, Gyeonggi-do, 18323, Korea

Abstract

Background: Recent studies have suggested a novel therapeutic strategy for treatment of benign prostatic hyperplasia (BPH) via modulation of autophagy. However, it is not clear whether autophagy induction or inhibition can render better therapeutic efficacy for BPH treatment because autophagy activation in BPH tissue is not precisely known and still contradictory. The purpose of this study was to examine the levels of autophagy in BPH tissue cells.

Methods: We have analyzed and compared autophagic flux which is defined as a measure of autophagic degradation activity in two human prostate epithelial cell lines, RWPE-1 (normal prostate) and BPH-1 (BPH) using LC3-II turnover assay, to clarify the levels of autophagy in BPH.

Results: The in vitro autophagy flux assays showed that autophagy flux was significantly decreased in BPH-1 cell lines compared with RWPE-1 cells under all three conditions of using the original (~62%), the exchanged (~46%), and the same media (Hank’s balanced salt solution (HBSS), ~40%), and these results were similar to those seen in the prostate of testosterone-induced BPH rats (~50%) (P < 0.05).

Conclusion: It is suggested that defective autophagy, which is decreased autophagy flux in the prostate gland, may be implicated in BPH, and activating autophagy flux of the prostate with BPH may be used as a potential therapeutic target for treating and alleviating BPH disease.

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1. Introduction

Benign prostatic hyperplasia (BPH) is a benign condition of the prostate gland characterized histologically by hyperplastic nodules in the periurethral region and the transition zone of the prostate and clinically by a palpably enlarged prostate and lower urinary tract symptoms (LUTSs).1 BPH prevalence increases with age, and more than half of the male population will have BPH by the time they reach middle age.2 Despite the prevalence of BPH, its pathogenesis is still largely unknown. Several theories have been proposed, which include genetic predisposition, androgen/androgen receptor signaling, embryonic reawakening, stem cell defects, chronic inflammation, imbalance between androgen/estrogen signaling, increased transforming growth factor beta (TGF-β) signaling, and epithelial-mesenchymal transition.3,4

Autophagy is an intracellular degradation system that delivers cytoplasmic constituents to the lysosome via autophagosomes. This process plays a critical role in maintaining intracellular quality control and regulating cellular metabolism and homeostasis. Autophagy is induced by a variety of stress stimuli, such as starvation, reactive oxygen species, protein aggregates, hormone treatment, drugs, and so on.6 Autophagy is regulated by the Atg family of proteins, which are involved in all stages of the autophagy process: induction, autophagosome formation, autophagosome-lysosome fusion, autolysosomal formation, and degradation.7 Autophagy begins with the formation of autophagosomes, which can be determined by visualization of the LC3-II protein because of its localization on the autophagosome membrane. LC3-II derives from a proLC3 30-kDa protein after cleavage by Atg4 to produce the active cytosolic form LC3-I. This in turn is activated by Atg7 and then transferred to Atg3, a second E2-like enzyme, becoming a membrane-bound form, LC3-II, which is degraded by autophagy.8 LC3-II protein levels serve as a readout of an autophagosome number, and measuring the conversion of LC3-I to LC3-II by immunoblot is indicative of autophagic activity.9 Defective autophagy is associated with some human diseases, such as cancer.
neurological disorders, myopathies, infectious diseases, inflammatory diseases, heart diseases, and so on.10

Autophagy has been proposed as a novel therapeutic strategy for BPH treatment in recent studies.11,12,13 However, there are not enough data to support the benefits for the treatment of BPH via autophagy modulation. It is controversial whether autophagy needs to be activated or inhibited to develop a strategy for BPH treatment because it is currently unclear whether autophagy is activated or inhibited in BPH tissue cells. Therefore, the purpose of this study was to investigate the true status of autophagy in BPH tissue cells and find information on how autophagy should be regulated as a therapeutic approach for BPH treatment. We compared the levels of autophagy in RWPE-1 and BPH-1 cell lines using three different conditions of cell culture media: original medium, switched medium, and autophagy-inducing medium.

2. Methods

2.1. Chemicals and antibodies

Colchicine (C9754), bafilomycin A1 (B1793), anti-LC3 polyclonal (L7543) antibody, and antiactin (A2066) antibody were purchased from Sigma-Aldrich. Testosterone propionate was purchased from TCI chemicals. Anti-phospho-S6 (ser235/236) (2211) and anti-S6 ribosomal protein (2217) antibodies were purchased from Cell Signaling Technology. All other reagents were purchased from Sigma-Aldrich.

2.2. Animals

Twenty-two-week-old male Sprague Dawley rats (n=36) with initial weights of 200–225 g were obtained from Samtaco BioKorea (Korea) and used in this study. The animals were housed in polypropylene cages (four per cage) in a controlled atmosphere of 12-h dark/light cycle at a temperature of –22 °C and were provided with food and water ad libitum. The rats were randomly assigned to one of the two groups, either the sham control group (n = 12) or castrated group (n = 24). After 1-week recovery, half of the castrated rats were used for the control (castrated group, n = 12), and another half of the castrated rats (BPH group, n = 12) were subcutaneously injected with corn oil mixed with testosterone propionate (3 mg/kg) daily for 8 weeks; the sham-operated animals were loaded onto nitrocellulose membranes. After blocking in a 5% milk solution (Thermo Fisher Scientific), the sections were stained in hematoxylin for 5 min and washed with water for 5 min. The sections were stained with 0.5% eosin for 30 s, dehydrated with ethanol, and mounted. Slices were observed under a light microscope.

2.4. Measurement of autophagic flux in animals and cultured cells

To measure autophagic flux in the prostate tissue of BPH rats, the animals were treated with saline or colchicine at a dose of 0.4 mg/kg/day intraperitoneally for 2 days before sacrifice. The control rats received an equal volume of saline interperitoneally. The castrated control animals were divided into two groups: castrated plus saline (cast + sal, n = 6) and castrated colchicine (cast + col, n = 6). The sham control animals were also divided into two groups: sham plus saline (sham + sal, n = 6) and sham plus colchicine (sham + col, n = 6). BPH rats were also grouped into two groups: BPH plus saline (BPH + sal, n = 6) and BPH plus colchicine (BPH + col, n = 6). The animals were anesthetized using xylazine and ketamine (0.2 mg/g body weight). The prostate were harvested 48 h after the second dose of colchicine or saline.

To measure in vitro autophagic flux, RWPE-1 and BPH-1 cells, grown on 10-cm dishes, were incubated in their own cell culture medium, exchanged medium, or amino acid and serum-free starvation buffer (Hank’s balanced salt solution (HBSS), Gibco), respectively, and were treated with and without 200 nM bafilomycin A1 (an autophagy inhibitor) for 8 hours. The RWPE-1 cells were divided into two groups: RWPE-1 treated with dimethyl sulfoxide (DMSO) (RWPE-1 + DMSO, n = 8) and RWPE-1 treated with bafilomycin A1 (RWPE-1 + Baf, n = 8). The BPH-1 cells were also divided into two groups: BPH treated with DMSO (BPH-1 + DMSO, n = 8) and BPH-1 treated with bafilomycin A1 (BPH-1 + Baf, n = 8). The detailed method has been described previously described.14,15

2.5. Cell proliferation assay

Cell proliferation was assessed by the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay in accordance with the manufacturer’s instructions (CellTiter 96® Aqueous Cell Proliferation Assay, Promega). In brief, 20 µl of CellTiter 96® Aqueous One Solution was added to each well of the 96-well assay plate containing the samples in 100 µl of culture medium, the plate was incubated at 37 °C for 2 hours, and the absorbance is measured at 490 nm. The experiments were performed in triplicate.

2.6. Immunoblot analysis

Proteins were extracted using ice-cold RIPA lysis buffer containing protease inhibitor cocktail (Sigma-Aldrich), and 20–60 µg of protein was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (10–12% resolving gel). The detailed method has been described previously described.14,15

2.7. Hematoxylin and eosin staining

The prostate tissue sections were prepared as previously described in the study by Ju et al.16 The sections were stained in hematoxylin for 5 min and washed with water for 5 min. The sections were stained with 0.5% eosin for 30 s, dehydrated with ethanol, and mounted. Slices were observed under a light microscope.
2.8. Statistical analysis

Data analyses were conducted using SPSS 22.0. Data are presented as means ± SE and were analyzed by univariate analysis of variance, followed by Fisher’s least significant difference (LSD) post hoc comparisons at p < 0.05.

3. Results

3.1. Prostate weight of BPH animals

There was no significant difference in the body weight of the rats regardless of the 8-week testosterone treatment (Fig. 1A). The testosterone-induced BPH animals had the prostate wet weight of 1.39 ± 0.16 g, which was significantly higher by ~100% than the sham control animals (0.70 ± 0.03 g) (P < 0.05, Fig. 1B). Prostate weight-to-body weight ratio was also significantly increased in the BPH group compared with the sham group (P < 0.05, Fig. 1C). We assessed the changes in the histomorphology of the prostate tissue by hematoxylin and eosin staining. Fig. 1D–F shows the epithelial thickness and the formation of intraluminal polyps in the prostate tissues of the castrated control (d), the sham control (e), and the BPH group (f). The BPH group exhibited marked thickening and hyperplasia with polyp formation in the lining epithelium of the acini compared with the sham group. These data suggest that our BPH model successfully established.

3.2. Autophagy flux in BPH rats

As shown in Fig. 2, colchicine treatment significantly increased LC3-II protein levels in the two groups, both the sham + col and BPH + col groups compared with the sham + sal group, in the prostate of rats. However, the protein levels of LC3-II were significantly lower (~50%) in the BPH + col group than in the sham + col group (P < 0.05, Fig. 2A and B). This result suggests that basal autophagic flux is decreased in the prostate of BPH rats. Similarly, the LC3-II:LC3-I ratio was also decreased in the BPH + col group than in the sham + col group (P < 0.05, Fig. 2A and C). Ribosomal protein S6 (S6) is a downstream target of the mammalian target of rapamycin (mTOR), and autophagy induction can decrease mTOR activation and S6 phosphorylation (ser235/236).17 S6 (ser235/236) phosphorylation was significantly increased in the BPH + col group compared with the sham + col group (P < 0.05, Fig. 2A and D).

3.3. Autophagy flux in RWPE-1 and BPH-1 cells using the original culture medium

The LC3-II turnover assay showed that treatment with bafilomycin A1, an autophagy inhibitor, significantly increased the levels of LC3-II in both RWPE-1 + Baf and BPH-1 + Baf groups compared with the RWPE-1 + DMSO group. However, LC3-II protein levels of the RWPE-1 cells were significantly less increased (~62%) than those of BPH-1 cells when both cells were treated with bafilomycin A1. There was also a similar decrease in the LC3-II:LC3-I ratio in the BPH-1 + Baf group than that in the RWPE-1 + Baf group (P < 0.05, Fig. 2A and C).
Fig. 3. Western blot showed a significant increase in S6 (ser235/236) phosphorylation compared with RWPE-1 cells (P < 0.05, Fig. 3A and E). Cell viability was analyzed using an MTS assay, and the cell proliferation rate was significantly increased in BPH-1 cells compared with RWPE-1 cells (Fig. 3E).

3.4. Autophagy flux in RWPE-1 and BPH-1 cells using the switched culture medium

As shown in Fig. 4, the protein levels of LC3-II were significantly lower (~46%) in the BPH-1 + Baf group than in the RWPE-1 + Baf group (P < 0.05, Fig. 4A and B). The LC3-II:LC3-I ratio was also lower in the BPH-1 + Baf group than in the RWPE-1 + Baf group (P < 0.05, Fig. 4A and C). In Fig. 4D, S6 (ser235/236) phosphorylation was significantly increased in the BPH-1 + DSMO group compared with the RWPE-1 + DSMO group and in the BPH-1 + DMO group compared with the RWPE-1 + Baf group. But S6 (ser235/236) phosphorylation was not significantly increased in the BPH-1 + Baf group compared with the RWPE-1 + Baf group (P < 0.05, Fig. 4A and D).

3.5. Autophagy flux in RWPE-1 and BPH-1 cells using the same culture medium

The protein levels of LC3-II were significantly decreased (~40%) in the BPH-1 + Baf group compared with the RWPE-1 + Baf group (P < 0.05). The LC3-II:LC3-I ratio was also decreased in the BPH-1 + Baf group compared with the RWPE-1 + Baf group (P < 0.05,
Fig. 5. Comparison of autophagy flux in cultured prostate cells, RWPE-1 and BPH-1, in the conditions of original cell culture medium usage. (A) Representative immunoblot images of LC3, phospho-S6 (ser235/236), total S6, or actin. (B) LC3-II:actin, (C) LC3-II:LC3-I, and (D) P–S6:total S6 ratios were quantitated via densitometry from 8 samples as per treatment conditions. Cell proliferation was determined using MTS assay at 16 h after treatment with DMSO or bafilomycin A1 (E). Values are means ± SE (n = 8). *P < 0.05 vs. RWPE + DMSO, #P < 0.05 vs. RWPE + Baf. SE = standard error; DMSO = dimethyl sulfoxide.

4. Discussion

It is certain that treatment of BPH through the manipulation of autophagy cannot be set off until the activation and/or regulatory mechanisms of autophagy in the prostate with BPH are precisely known. Because a method for measuring autophagy flux of patients with BPH has not yet been developed, we measured an in vivo autophagy flux using the testosterone-induced BPH rats to examine activation of autophagy in the prostate with BPH. Autophagic flux, which encompasses the entire process of autophagy, is a more reliable indicator of autophagic activity. The in vivo autophagy flux assay with and without the colchicine administration method described previously is currently one of the most widely and routinely used methods to monitor autophagic flux. The in vivo autophagy flux assay showed that LC3-II protein levels were significantly decreased with the BPH group than with the sham control. The results of the increase in S6 phosphorylation (mTOR activation) confirmed a decrease in autophagy of the prostate of the BPH rats, which was in an mTOR-dependent manner (Fig. 2). While looking at the results of S6 phosphorylation carefully, we started to doubt the results and thought the activation of mTOR in the prostate with BPH was due to the testosterone that had been injected until the last day before being sacrificed to induce BPH rats and not due to BPH disease itself. It is known that androgen deprivation
decreases mTOR activation, which increases autophagy, but testosterone supplementation increases mTOR activation, resulting in the decrease in autophagy. We thought autophagy activation in testosterone-induced BPH rats might be different from that found in patients with BPH, and this animal model does not represent the true autophagy status in BPH disease. The results from the in vivo autophagy flux using testosterone-induced BPH rats in this study were thought to be unconvincing.

Recently, Jiang et al. evaluated the status of autophagy in two human prostate epithelial cell lines, RWPE-1 and BPH-1. The authors showed that basal autophagy was decreased, and autophagy flux was reduced in BPH-1 cells, compared with normal RWPE-1 cells. We compared autophagic flux in the two cell lines using the original culture medium. LC3-II protein levels of the BPH-1 cells were significantly lower than those of the RWPE-1 cells when both cells were treated with bafilomycin A1 (Fig. 3), which were similar to the results found in the study by Jiang et al. However, what we were concerned about was that in these studies, those two cell lines were cultured in different cell culture media containing different growth factors and hormones, which could cause misinterpretation of the precise comparison of autophagy between the two cell lines. The major cell growth factors in the RWPE-1 cell culture medium

**Fig. 4.** Comparison of autophagy flux in cultured prostate cells, RWPE-1 and BPH-1, in the conditions of the switched cell culture medium. (A) Representative immunoblot images of LC3, phosphor-S6 (ser235/236), total S6 or actin. (B) LC3-II:actin, (C) LC3-II:LC3-I, and (D) P-S6:total S6 ratios were quantitated via densitometry from 8 samples as per treatment conditions. Values are means ± SE (n = 8). *P < 0.05 vs. RWPE + DMSO, **P < 0.05 vs. RWPE + Baf. SE = standard error; DMSO = dimethyl sulfoxide.

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were some factors in bovine pituitary extract and epidermal growth factor (EGF), but the BPH-1 cell culture medium contains some factors in fetal bovine serum (FBS), testosterone, and insulin. It has been reported that the growth factor insulin stimulates mTOR and is known as the most potent physiological anabolic agent, and testosterone is related to the PI3K-1/Akt/mTOR signaling pathway that influences the growth, proliferation, and death of prostate cancer cells. Therefore, these two hormones, testosterone and insulin, in the BPH-1 cell culture medium, might have contributed to further increase in mTOR activation and a reduction in autophagy activation than those growth factors in the RWPE-1 cell culture medium in our study as well as in the study by Jiang et al when the levels of autophagy activation between the two cell lines were compared.

To circumvent this misunderstanding of the measurement of autophagy activation in an in vitro system, we exchanged the culture medium with one another and tried to look at the autophagy flux of the two cell lines simultaneously. Unexpectedly, autophagy flux was also significantly decreased in the BPH-1 cells compared with the RWPE-1 cell line (Fig. 4), and this was similar to that seen when the two cell lines were treated using the original culture medium. Next, to see the changes of autophagy flux of the two cell lines under the same medium and under an autophagy-induced condition, both cell lines were treated with the HBSS medium for

Fig. 5. Comparison of autophagy flux in cultured prostate cells, RWPE-1 and BPH-1, in the same condition of autophagy-induced state of starvation using the HBSS medium. Representative immunoblot images of LC3, phospho-S6 (ser235/236), total S6 or actin (A), LC3-II:actin (B), LC3-II:LC3-I (C), and P–S6:total S6 (D) ratios were quantitated via densitometry from 8 samples per treatment conditions. Values are means ± SE (n = 8). *P < 0.05 vs. RWPE + DMSO, #P < 0.05 vs. RWPE + Baf. SE = standard error; HBSS = Hank’s balanced salt solution; DMSO = dimethyl sulfoxide.
8 h. This in vitro autophagy flux assay (Fig. 5) also showed similar results found in the conditions of using both the original and the exchanged media, in which autophagy flux was significantly decreased in the BPH-1 cells compared with the RWPE-1 cells. Our three in vitro experiments convinced us that autophagy flux was reduced in the BPH-1 cells compared with the RWPE-1 cell, and these and the results from the in vivo experiment using testosterone-induced BPH rats suggest that defective autophagy, which is decreased autophagy flux in the prostate tissue, may be associated with the etiology and progression of BPH.

Although we did not measure autophagy flux in patients with BPH directly in this study, we assume that patients with BPH also have reduced autophagy flux in the prostate gland as observed both in the cell culture and the BPH rats in the present study. We support the findings in the study showing that autophagy was suppressed in the prostatic cells of patients with LUTS and BPH. Currently, it is not known how effective it has been in treating BPH by increasing autophagic activity in the prostate tissue of BPH. Autophagy can promote cell death in multiple cell types, which is referred to as autophagic cell death or type II programmed cell death. Impaired autophagy may result in inhibiting autophagic cell death and/or promoting cell survival, leading to increased proliferation of tissue cells. This might have occurred in the present study in which the increased cell proliferation rate was shown in the BPH-1 cells compared with the RWPE-1 (Fig. 3E). It is undetermined how important and significant autophagy is in the cell death process in the prostate tissue of BPH. The decrease in autophagy flux may be implicated in BPH disease based on the current findings, and it would be necessary to normalize defective autophagy in the prostate tissue of BPH and reasonable to say that finding the tactic to activate autophagy flux may be one of the important therapeutic targets for treating and alleviating BPH.

The significance of this study was that this is the first study to reliably and quantitatively measure and demonstrate the status of autophagy flux of BPH disease through both in vivo and in vitro experiments simultaneously using the LC3-II turnover assays. We convincingly determined the autophagic flux in an in vitro system using the two cell lines, RWPE-1 and BPH-1, by manipulating the cell culture conditions. We have found that autophagy flux was decreased in the BPH tissue cells under both basal and induced conditions compared with the normal prostate tissue cells. The application of the in vitro assay model comparing the two cell lines can be expanded to measure other pathophysiological responses, such as inflammation, reactive oxygen species, hormonal-metabolic factors, apoptosis, and so on, in relation to BPH disease. In addition, this in vitro autophagy flux assay can be implemented as a high-throughput screening tool for autophagy-activating drugs to treat BPH.

Conflicts of interest

None declared.

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