Biochemistry and Biophysics Reports 5 (2016) 152–159

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

Progression of thanatophagy in cadaver brain and heart tissues

Gulnaz T. Javan [a,*], Insu Kwon [b], Sheree J. Finley [a], Youngil Lee [b]

[a] Forensic Science Program, Physical Sciences Department, Alabama State University, Montgomery, AL, United States
[b] Department of Exercise Science and Community Health, University of West Florida, Pensacola, FL, United States

ARTICLE INFO

Article history:
Received 18 June 2015
Received in revised form
12 November 2015
Accepted 16 November 2015
Available online 18 November 2015

Keywords:
Thanatophagy
Coronary heart disease
Postmortem interval
Cadaver

ABSTRACT

Autophagy is an evolutionarily conserved catabolic process for maintaining cellular homeostasis during both normal and stress conditions. Metabolic reprogramming in tissues of dead bodies is inevitable due to chronic ischemia and nutrient deprivation, which are well-known features that stimulate autophagy. Currently, it is not fully elucidated whether postmortem autophagy, also known as thanatophagy, occurs in dead bodies is a function of the time of death. In this study, we tested the hypothesis that thanatophagy would increase in proportion to time elapsed since death for tissues collected from cadavers. Brain and heart tissue from corpses at different time intervals after death were analyzed by Western blot. Densitometry analysis demonstrated that thanatophagy occurred in a manner that was dependent on the time of death. The autophagy-associated proteins, LC3 II, p62, Beclin-1 and Atg7, increased in a time-dependent manner in heart tissues. A potent inducer of autophagy, BNIP3, decreased in the heart tissues as time of death increased, whereas the protein levels increased in brain tissues. However, there was no expression of BNIP3 at extended postmortem intervals in both brain and heart samples. Collectively, the present study demonstrates for the first time that thanatophagy occurs in brain and heart tissues of cadavers in a time-dependent manner. Further, our data suggest that cerebral thanatophagy may occur in a Beclin-1- independent manner. This unprecedented study provides potential insight into thanatophagy as a novel method for the estimation of the time of death in criminal investigationsAbstract: Autophagy is an evolutionarily conserved catabolic process for maintaining cellular homeostasis during both normal and stress conditions. Metabolic reprogramming in tissues of dead bodies is inevitable due to chronic ischemia and nutrient deprivation, which are well-known features that stimulate autophagy. Currently, it is not fully elucidated whether postmortem autophagy, also known as thanatophagy, occurs in dead bodies is a function of the time of death. In this study, we tested the hypothesis that thanatophagy would increase in proportion to time elapsed since death for tissues collected from cadavers. Brain and heart tissue from corpses at different time intervals after death were analyzed by Western blot. Densitometry analysis demonstrated that thanatophagy occurred in a manner that was dependent on the time of death. The autophagy-associated proteins, LC3 II, p62, Beclin-1 and Atg7, increased in a time-dependent manner in heart tissues. A potent inducer of autophagy, BNIP3, decreased in the heart tissues as time of death increased, whereas the protein levels increased in brain tissues. However, there was no expression of BNIP3 at extended postmortem intervals in both brain and heart samples. Collectively, the present study demonstrates for the first time that thanatophagy occurs in brain and heart tissues of cadavers in a time-dependent manner. Further, our data suggest that cerebral thanatophagy may occur in a Beclin-1-independent manner. This unprecedented study provides potential insight into thanatophagy as a novel method for the estimation of the time of death in criminal investigations

1. Introduction

Autophagy is a unique catabolic membrane signaling process that targets cellular constituents and nutrient storage reservoirs, and plays a critical role in cell survival in live cells under stress conditions (i.e., nutrient deprivation and ischemia) [1,2]. According to Efeyan et al. (2015), depletion of critical nutrients is possibly the most effective inducer of autophagy [3]. It is a plausible notion that "Thanatophagy," a term we derived from a combination of the Greek terms, ***than****atos (death)*, ***auto*** *(self)*, and ***phagy*** *(eating)*, undergoes the same autophagic processes. However, it is an enigma whether thanatophagic events occur after death in a human

* Corresponding author.
E-mail addresses: gjavan@alasu.edu (G.T. Javan), ikwon@uwf.edu (I. Kwon), sfinley@alasu.edu (S.J. Finley), ylee1@uwf.edu (Y. Lee).

http://dx.doi.org/10.1016/j.bbrep.2015.11.013

body. Autophagy is the normal, non-apoptotic activity of living, antemortem cells to sustain basal conditions not only under stress stimuli such as nutrient depletion [4,5], but also etoposide (an anticancer drug) [6] and growth factor reduction [7,8]. The autophagic process involves the sequestration of cargo receptors, bulk phagocytosis, and lysis of misfolded cytosolic proteins and dysfunctional organelles to maintain basal homeostasis [4].

Autophagy has co-evolved through a network of several mammalian proteins as a quality control mechanism to guard the cell from damage due to toxic cellular components and damaged organelles. Proteomic analysis of autophagy networks in human cells undergoing basal autophagic conditions demonstrates the interaction of a network of 409 proteins [9]. Recent studies have demonstrated that autophagy under nutrient deprivation is controlled by two different conserved proteins: i) the inactivation by mammalian target of rapamycin (mTOR) or ii) activation by AMP-activated protein kinase (AMPK) [10,11]. After initiation, other proteins of the linkage of the human autophagy system include Beclin-1 which dissociates from B-cell lymphoma 2 (Bcl-2) to form a complex with Class III phosphatidylinositol 3-kinase (PI3K)/vacuolar protein sorting 34 (class III PI3 kinase/Vps34). This complex is activated by Unc-51-like Kinase 1 (ULK1) which controls early steps in autophagosome formation by inducing the nucleation of the phagophore [12]. Another protein, namely, the autophagy-related protein 7 (Atg7), an autophagy rate-limiting enzyme, conjugates phosphatidylethanolamine to the microtubule-associated protein 1 light chain 3-I (LC3-I), which results in the formation of the microtubule-associated protein 1 light chain 3-II (LC3-II). The LC3-II protein is bound to an oligomeric Atg12-Atg5-Atg-16 complex and facilitates the conjugation of autophagosomes. It is LC3-II that directly interacts with autophagy adapter proteins containing LC3-interacting receptors (i.e., BNIP3, NIX, and p62) [13–15]. Given this essential function of LC3-II in autophagy, it has been used as a defining protein marker of autophagy [16,17]. In this regard, recent studies have suggested that since the proteotoxic stress response protein, p62, is degraded with its cellular substrates in lysosomes, analyses of both LC3-II and p62 proteins are often performed in autophagic investigations [18].

Although the exact mechanism has not been fully elucidated, overexpression of Bcl-2 19-kDa interacting protein 3 (BNIP3) has been shown to be a strongly positive modulator of autophagy in cells [4,19–21]. For instance, studies have demonstrated that during hypoxia, expression of BNIP3 is upregulated, and the protein promotes autophagy by dislodging Bcl-2 from the Bcl-2/Beclin-1 complex [19]. Further, functional activity of BNIP3 in autophagy has been reported in several cardiovascular diseases such as hypertrophy, heart failure, and ischemic heart diseases [22], and in neuronal cells [23–25].

The study of gene expression in internal organs of cadavers, termed thanatotranscriptome by Javan et al. (2015), has been performed [26]. Nevertheless, there is a paucity of studies to establish the link between thanatophagy and postmortem functions for the investigation of time of death in humans. A study by Zhou et al. (2011) revealed that autophagy was elevated in the brain tissues sampled from cadavers of human immunodeficiency virus-(HIV)-infected patients diagnosed with HIV-1 encephalitis, compared to cadaver brain tissue from infected persons with no clinical signs of the encephalitis or the control taken from HIV-uninfected brain [27].

Extensive studies have focused on antemortem autophagy of cytoplasmic constituents; however, relatively little research that details the mechanisms of autophagy of cellular components after death has been performed. Therefore, in this current study, we examined the levels of thanatophagy by measuring autophagic protein markers in the brain and heart tissues of four cadavers whose cause of death was coronary artery disease. The results of Western blot analysis demonstrated that thanatophagy increased in both (brain and heart) tissues in association with postmortem interval (PMI) and cerebral thanatophagy may occur in a Beclin-1-independent manner.

2. Material and methods

2.1. Cadaver cases

Adult cadavers were kept at 1 °C in the morgue of the Alabama Department of Forensic Sciences Medical Laboratory in Montgomery, AL. Cardiac and cerebral tissue samples were collected from four corpses of victims of coronary artery disease. The metadata collected for each corpse were the cadaver age at death, sex, weight, height, cause of death, PMI, and ambient temperature upon autopsy (Table 1). Only samples with known times of death, obtained from official Daily Crime Logs generated and certified by local police departments, were used for this study.

All procedures were in accordance with institutional guidelines and approved by the Alabama State University Institutional Review Board (IRB) under number: 2013CMST004A.

2.2. Tissue collection and homogenization

The medico-legal autopsy was performed in a clinical laboratory area at 20 °C. The dissection of portions of the brain and heart tissues was performed using a sterile scalpel and transferred into labeled polyethylene bags. After collection, the samples were transported on ice to Alabama State University (within 10 min) in an insulated container and stored in a freezer at −80 °C until the time of analysis. The tissues were homogenized using a glass tissue grinder (Fisher Scientific, Pittsburgh, PA) in ice-cold tissue lysis buffer (pH 7.4) containing 50 mM Tris–HCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100 with complete protease inhibitor (Roche Applied Science, Indianapolis, IN), and phosphatase inhibitor (Roche Applied Science, Indianapolis, IN). The tissue homogenates were incubated on ice for 30 min, and then the tissue lysates were collected by centrifugation at 14,000 rpm for 20 min.

2.3. Western blotting

The proteins in the tissue lysates were separated by SDS-PAGE (10% Bis-Tris precast gel with MES running buffer) (Life Technology, Carlsbad, CA) under reduced conditions and transferred to nitrocellulose membrane. The membrane was blocked with 5% nonfat milk dissolved in Tris-buffered saline containing 5% Tween-20 (TBS-T) for 1.5 h at room temperature and subsequently incubated with designated antibodies overnight at 4 °C. The primary antibodies used in the study

Table 1
A summary of the metadata collected for each corpse used in the thanatophagy study. Postmortem interval (PMI) was obtained from Daily Crime Logs generated by local police departments.

Case #	Age (years)	Sex	Weight(kg)	Height (cm)	Cause of Death	PMI (hours)	Ambient Temp. (°C)
1	55	M	112	198	Coronary artery disease	6	27
2	17	M	88	185	Coronary artery disease	16	18
3	65	M	100	191	Coronary artery disease	36.5	19
4	48	F	154	160	Coronary artery disease	58	30.5

were: LC3 (Cell Signaling Technology, Danvers, MA), p62 (Cell Signaling Technology, Danvers, MA), Beclin-1 (Cell Signaling Technology, Danvers, MA), Atg7 (Cell Signaling Technology, Danvers, MA), GAPDH (Cell Signaling Technology, Danvers, MA), and BNIP3 (Abcam, San Francisco, CA). The membrane was then washed three times for 15 min with TBS-T and incubated for 1 h at room temperature with the designated secondary antibodies which were goat anti-mouse IgG, HRP conjugated or goat anti-rabbit IgG, HRP conjugated (Life Technology, Carlsbad, CA). The membrane was washed and the designated protein bands were detected using SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Rockford, IL) and were quantified using densitometry software (Image Studio, LI-COR Biosciences, Lincoln, NE).

3. Results

3.1. Cardiac thanatophagy occurs in a PMI-dependent manner

To examine whether thanatophagy was induced in a PMI-dependent manner, we measured levels of LC3-I and LC3-II in heart tissues collected at different time of death (i.e., 6 h, 16 h, 36.5 h, and 58 h). Western blotting and its quantification data revealed that as PMI elapsed, levels of LC3-II were significantly increased (Fig. 1A and B). To confirm whether the increase in LC3-II is mediated by enhanced autophagic flux, the levels of p62 which is reported to be co-degraded with autophagosomes were measured, and the results demonstrated that p62 was increased in a PMI-dependent fashion. Since BNIP3 has been reported to be a potent inducer of autophagy and is upregulated by hypoxia, we measured the protein levels and found that BNIP3 was inversely regulated compared to LC3-II and p62.

3.2. Upregulation of cardiac thanatophagy is mediated by an enhanced initiation process

Beclin-1 and Atg7 are critical proteins for the nucleation and elongation process of autophagosome formation. To explore the question of whether Beclin-1 and Atg7 are linked to the induction of thanatophagy, their levels were measured. The results demonstrated that, similar to LC3-II, both proteins were upregulated in a PMI-dependent manner. Beclin-1 peaked at a PMI of 36.5 h and was maintained until 58 h (Fig. 2A for blots and 2B and 2C for quantification of Beclin-1 and Atg7, respectively).

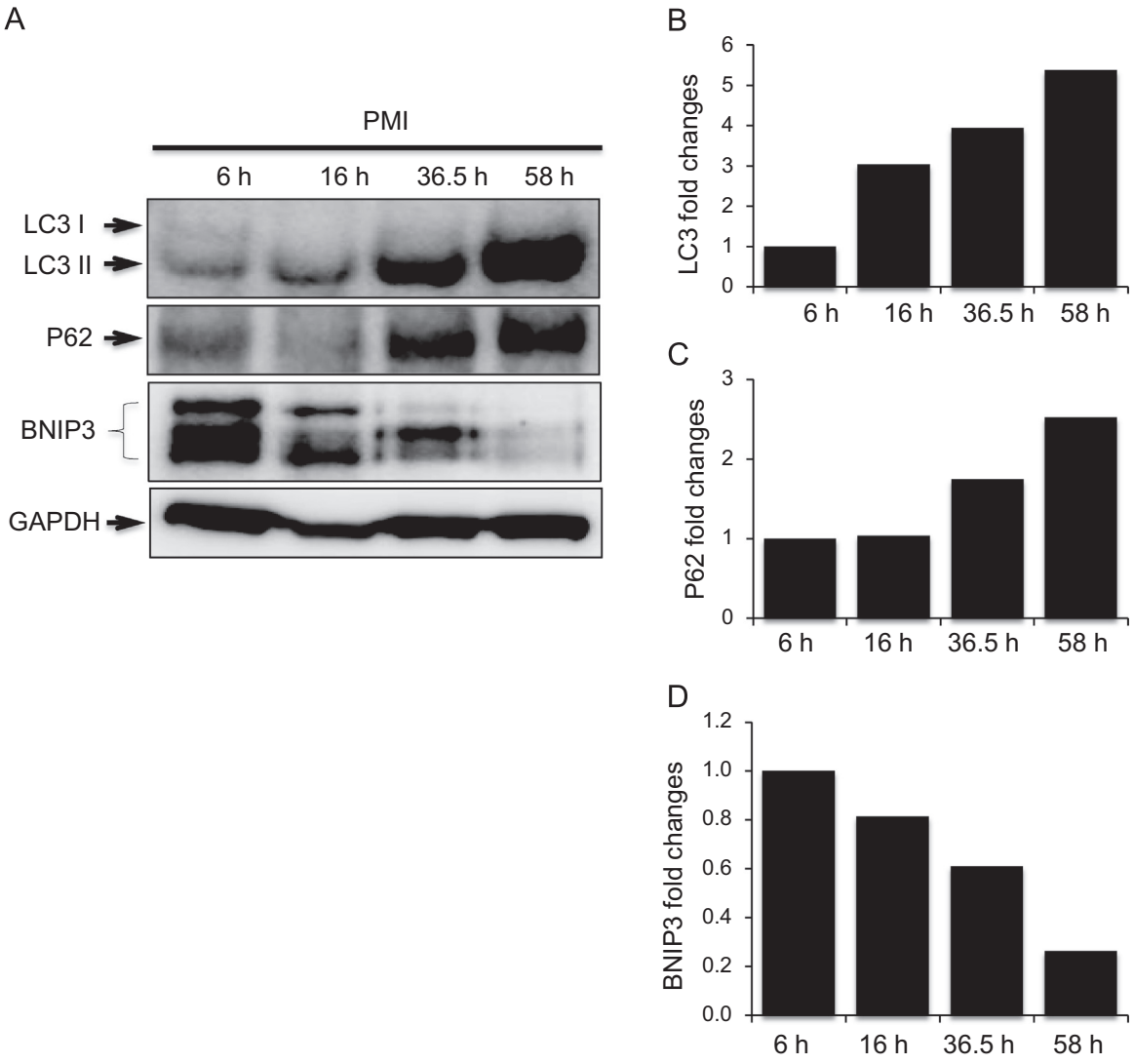

Fig. 1. Cardiac thanatophagy occurs in a PMI dependent manner, but BNIP3 is inversely related to thanatophagy. (A) Western blot analysis of LC3, p62, BNIP3 in dead human hearts at various PMI. (B) Quantitation of LC3/GAPDH. (C). Quantitation of p62/GAPDH. (D) Quantitation of BNIP/GAPDH. PMI: postmortem interval.

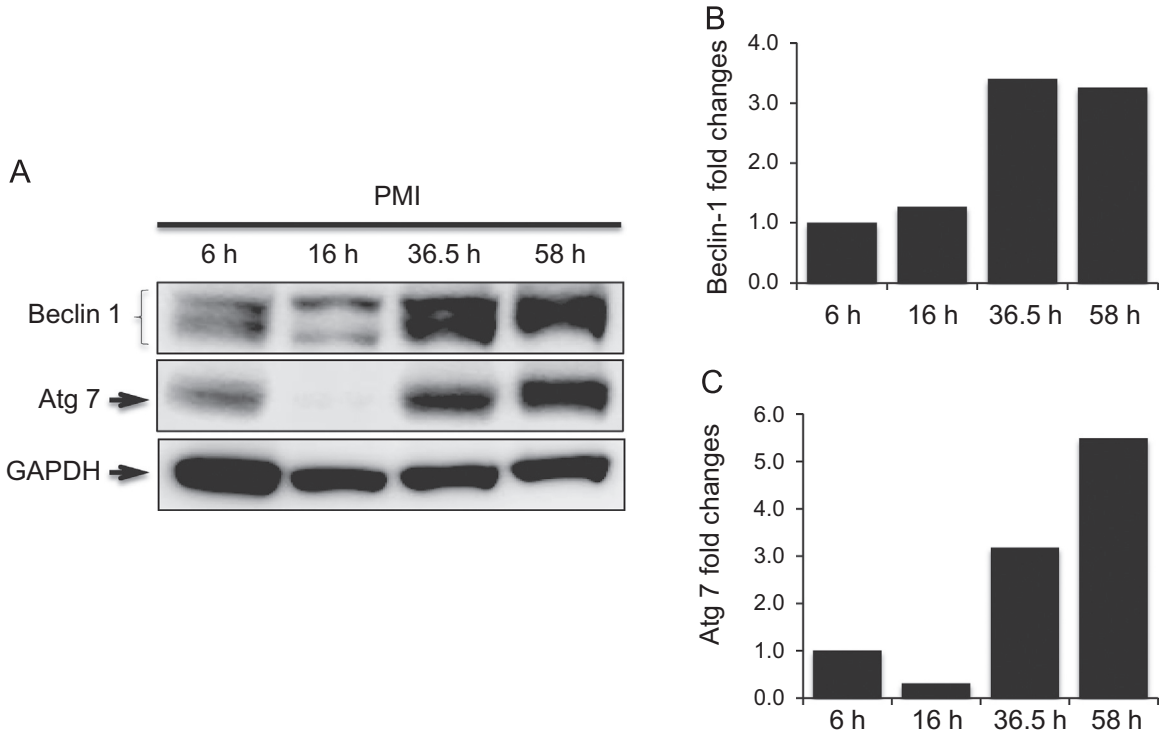

Fig. 2. As PMI elapses, Beclin-1 and Atg7 increase. (A) Western blot analysis of Beclin-1 and Atg7 in postmortem hearts at various PMI. (B) Quantitation of Beclin-1/GAPDH. (C) Quantitation of Atg7/GAPDH.

3.3. Cerebral thanatophagy occurs in PMI-dependent manner

Given the fact that the brain tissues also participate in autophagy under nutrient deprivation or hypoxia, whether thanatophagy occurs in a tissue-specific manner was examined at various times of death. The postmortem brain showed increased levels of LC3-II in a PMI-dependent manner (Fig. 3A: top panel and 3B). Similarly, levels of p62 also increased as PMI elapsed (Fig. 3A: second panel from top and Fig. 3C). Next, BNIP3 was examined and the results demonstrated that this protein was elevated, except at a PMI of 58 h in which no expression of BNIP3 was observed (Fig. 3A: third panel from top and Fig. 3D).

3.4. Upregulation of cerebral thanatophagy is mediated independent of Beclin-1

To explore a question of whether an increase in thanatophagy is induced by promoting initiation and elongation of autophagosomes, Beclin-1 and Atg7 protein levels, were measured. Unexpectedly, the levels of Beclin-1 tend to decrease as PMI elapsed, with Beclin-1 drastically dropping to its lowest level at a PMI of 58 h (Fig. 4A: top panel and Fig. 4B). On the other hand, expression of Atg7 was substantially increased at PMIs of 16 h and 58 h.

4. Discussion

Antemortem autophagy is well recognized as a key self-degradation process in mammalian brain and heart cells and is an essential survival mechanism that protects against the accumulation of toxic products. However, there is a paucity of investigations elucidating the networks that regulate thanatophagy (autophagy in dead human bodies). In forensic science, there is a need for more valid and reliable methods for the accurate determinate of the time of death. The induction of thanatophagy in cadaver brain and heart tissues could potentially represent a suitable target to estimate PMI. Thus, a thorough understanding of cellular and molecular changes and characterization in tissues of dead bodies is crucial for the identification of new techniques for criminal investigations.

Studies have shown that autophagy is a highly specific process for the maintaining of cellular equilibrium in the heart of living bodies under normal and stress conditions [21,28]. Extreme stress conditions such as ischemia (eventually anoxia), hemodynamic stress, and nutrient deprivation ensue within four minutes after death [29]. Increased autophagy is frequently detected in the myocardial tissues of living patients with acute and chronic ischemia, heart failure, and dilated cardiomyopathy [13,28]. Studies have demonstrated that autophagy is essential in removing dysfunctional mitochondria in heart tissue, and the clearing of other damaged organelles and toxic protein aggregates in tissues. Mitochondrial fission and fusion are important processes in regulating cell survival and death in the myocardium. Disruptions in antemortem autophagy result in the accumulation of mitochondria as well as a decline in various cardiovascular pathological functions [21]. Autophagy in live cells is an essential self-defense mechanism for sustaining cell function as deletion of cardiac-specific autophagy genes in mice heart caused rapid deterioration of cardiac function leading to cardiomyopathy [22]. Nevertheless, prior to the current study, it is undefined whether thanatophagy occurs and which proteins and signaling networks contribute to postmortem autophagy, thanatophagy (Table 2).

In theory, it is conceivable that antemortem and postmortem autophagic proteins exhibit functional similarities and autophagic processes are homologous and applicable to thanatophagy. For example, LC3-II is universally considered as a defining protein marker of autophagy [21,30–33]. Consistent with other reports from nutrient deprivation and ischemia research, we observed a significant accumulation of LC3-II in the brain and heart of postmortem subjects who died from coronary artery disease. An escalation of LC3-II levels or the ratio of LC3-I to LC3-II can accumulate not only by the increased activity of autophagosome

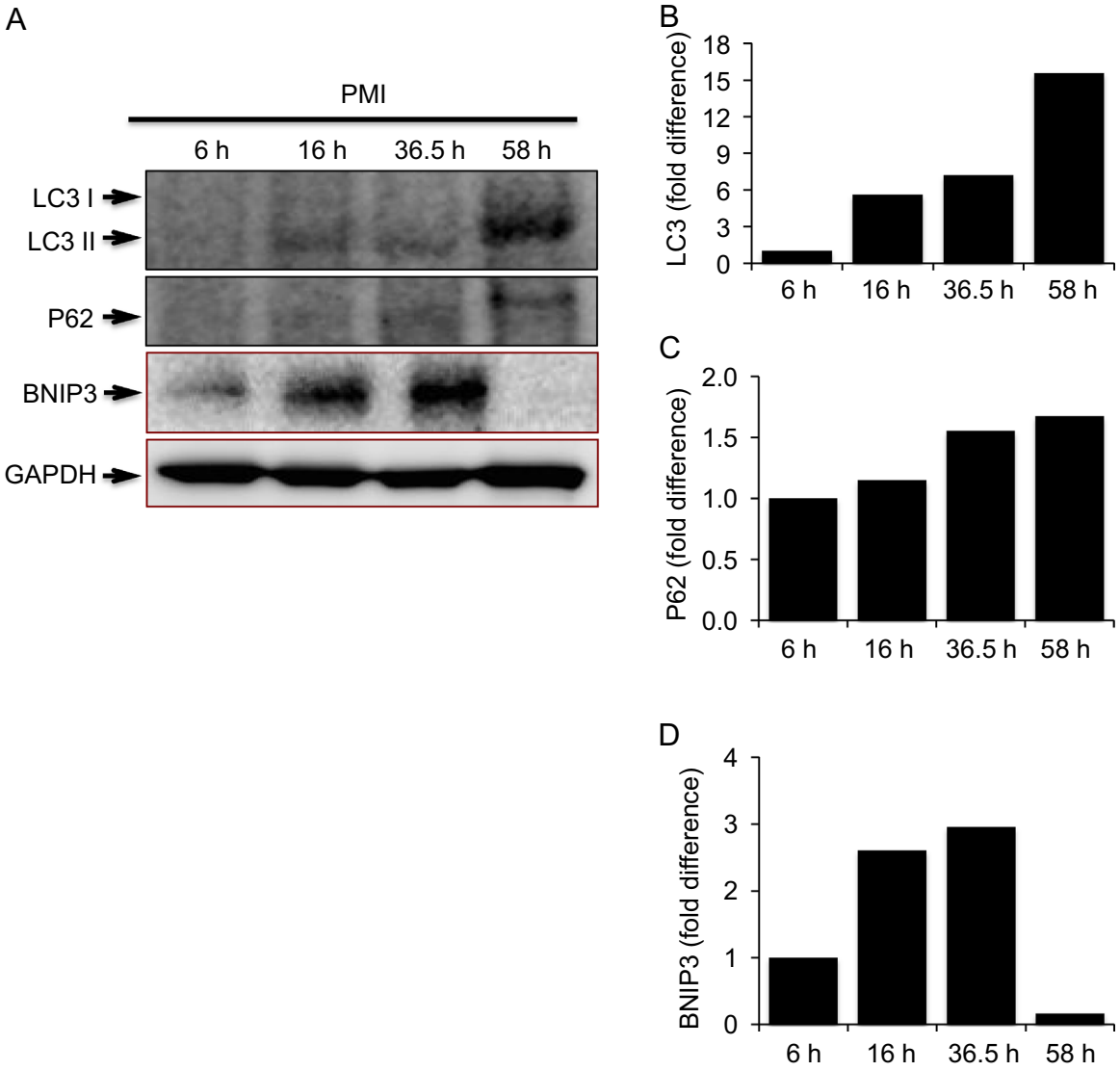

Fig. 3. Cerebral thanatophagy occurs in a PMI dependent manner. (A) Western blot analysis of LC3, p62, BNIP3 in dead human brain at various PMI. (B) Quantitation of LC3/GAPDH. (C). Quantitation of p62/GAPDH. (D) Quantitation of BNIP/GAPDH.

formation, but also by the impaired fusion process between autophagosome and lysosome. Our data does not confirm whether thanatophagy is caused by increased autophagic flux or by disturbance of the fusion process. Recent studies show that autophagic flux (i.e., input and output) can be examined by measuring LC3-II in the presence and absence of lysosomal protease inhibitors [17]. This study measured levels of an autophagy adaptor protein, p62 as an alternative marker of autophagy flux, which is co-degraded with LC3-II [16]. Consequently, there was no observed reduction of p62 levels in both brain and heart, but rather, they were increased in a PMI-dependent manner. Apparently, our results raised the possibility that accumulated LC3-II levels may be due to suppressed autophagic degradation rather than increased autophagy. Activity of lysosomal vacuolar ATPase constantly pumps protons utilizing ATP which is interrupted by a deficiency of ATP. This deficiency is due to prolonged hypoxia and nutrient starvation conditions in the corpse. Thus, the result is an accumulation of LC3-II as well as p62. However, we are not excluding the possibility that p62 may be transcriptionally upregulated via NF-E2-related factor 2 (NRF2) [34].

BNIP3 is upregulated during hypoxia and promotes autophagy [35,36] by displacing Bcl-2 from the Bcl-2/Beclin-1 complex [19]. In addition, translocation of BNIP3 to the outer membrane of mitochondria and subsequent dimerization of BNIP3 promote mitochondrial autophagy [37]. Interestingly, our data show that BNIP3 in heart tissues was reduced; whereas, it was increased in brain tissues as PMI elapsed. The expression of BNIP3 in both tissues was drastically reduced at a PMI of 58 h. These findings suggest that BNIP3-mediated thanatophagy induction occurs in a tissue-specific manner, BNIP3-dependent in the brain but independent in the heart. However, on the basis of our observation showing almost no BNIP3 expression at a PMI of 58 h with the highest levels of LC3-II, we are open to the possibility that BNIP3 may not be linked to thanatophagy.

Approximately 30 various genes involved in mammalian autophagy have been identified [4]. Among those gene products, Beclin-1 (the mammalian orthologue of yeast Atg6) is a crucial member of the autophagy network because it promotes the formation of the autophagy-regulating macromolecular complex along with the Vps34 [12]. The interaction between Beclin-1 and Vps34 triggers the autophagic activity of Vps34, resulting in the establishment of nucleation of the phagophore [10]. Multiple lines of recent evidence demonstrated that Beclin-1 plays a pivotal role in promoting autophagy in the heart [38]. Concordant with

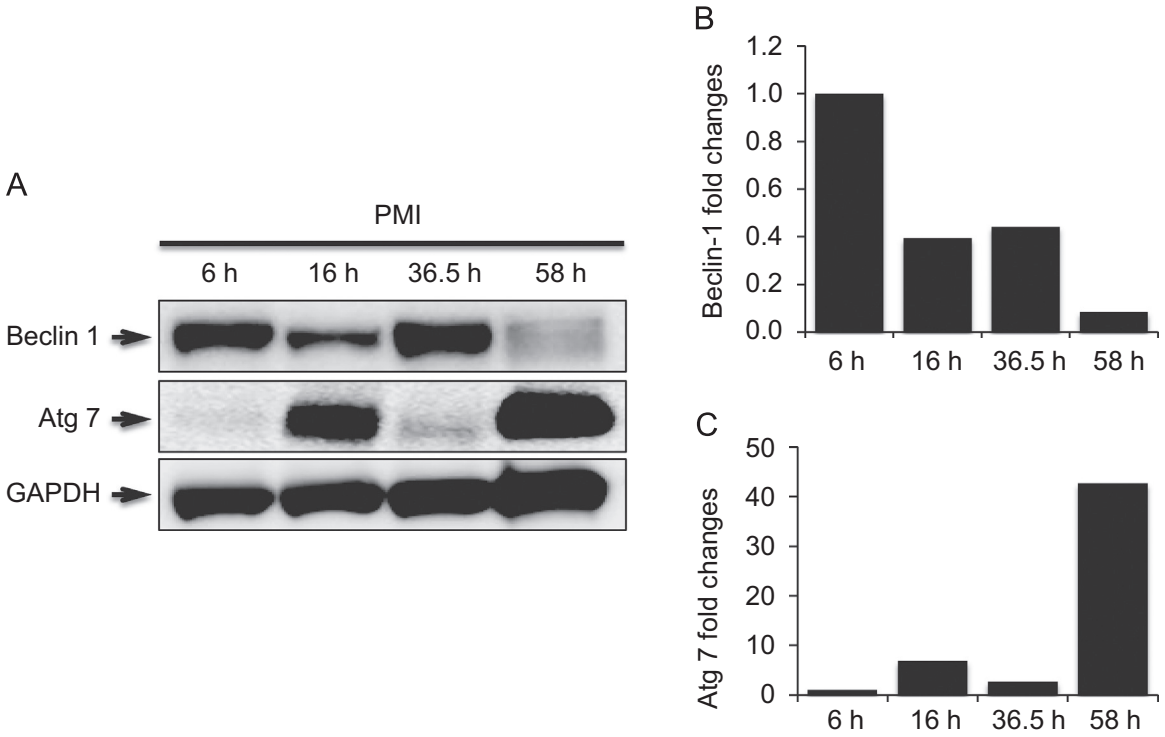

Fig. 4. Cerebral Beclin-1 deceases as PMI elapses, but Atg7 increases. (A) Western blot analysis of Beclin-1 and Atg7 in postmortem brains at various PMI. (B) Quantitation of Beclin-1/GAPDH. (C) Quantitation of Atg7/GAPDH.

previous studies, our data also demonstrated a drastic escalation of Beclin-1 in hearts as PMI elapsed which is in parallel with LC3-II levels. Inversely, Beclin-1 in brain samples deceased as PMI elapsed, with Beclin-1 dropping to the lowest level at a PMI of 58 h despite the highest level of LC3-II at the same PMI. Although Beclin-1 is linked to elevated autophagy in many cell types and tissues, a recent study demonstrated Beclin-1-independent autophagy (noncanonical), showing that siRNA-mediated knockdown of Beclin-1 in neuronal cells did not prevent cannabinoid receptor 1 inhibition-induced autophagy [39]. This study is consistent with our observation that thanatophagy in the brain of corpses increases dependent upon participation of Beclin-1.

Our results also establish thanatophagy in the brain and the heart from cadavers as a novel indicator of the time of death. Consequently, these findings suggest that thanatophagy may play an important role and may be a potential method in medico-legal science.

Atg7 is a substantial protein for the formation of autophagosomes via LC3 processing [40]. Hiebel et al. (2014) showed that siRNA-mediated Atg7 knockdown inhibited autophagy when stimulated [39]. We found that Atg7 increased in a PMI-dependent fashion in hearts and similar trends were observed for LC3-II and Beclin-1. Atg7 in the brain also increased in a PMI-dependent manner except at a PMI of 16 h, with its level peaking at 58 h in which the level of LC3-II was highest. These data suggest that Atg7 in the brain may provide a crucial function in cerebral thanatophagy.

Metabolic reprograming in tissues of dead human bodies is inevitable due to chronic ischemia (eventually anoxia) and nutrient deprivation. This reprogramming may lead the tissues into immediate hibernation by which a cellular ATP demand is suppressed. Thus, ATP required for maintaining minimum cellular function accumulates. Since the phenomenon is a well-known feature that stimulates autophagy, the presence of thanatophagy is not suppressed, at least in the initial stages of death. However, it is unclear whether thanatophagy continues as a function of postmortem interval and if it can be utilized as a possible method to determine precise PMI.

4.1. Introduction of a new term

This study introduces the term “thanatophagy,” which refers to the autophagy occurring in human cells after a person dies. We recognize that there are other terms used to indicate the activity of autophagy: kinetophagy [40], mitophagy [41], macroautophagy [42]. These three terms are not suitable for this study due to the fact that the tissues were sampled after the death of the study cases and represents postmortem autophagy, or thanatophagy.

In order to fully substantiate the existence and rate of thanatophagy, studies engaging a longitudinal experimental design, in which specimens are sampled from the same cadaver at different time points, are apropos. Generally, the study design in autophagy investigations using mammalian models involves sacrificing and destructive dissection (e.g. replicate mice, rat, and swine) at different time points or as well as cell lines maintained in tissue culture [41–43]. Studies of human autophagy use cultured tissue samples collected from actual cases [27]. However, the present study uses whole, human corpses from real forensic cases actively undergoing medico-legal investigations. Since real, whole-cadaver cases were used, it is virtually impossible to “design” an experiment in which brain and heart tissues are collected at different time point from the same subject. Thus, due to legal ramifications, our thanatophagy study necessitates a special status and entails a cross-sectional experimental study design. Thanatophagy conceivably occurs at different rates in the internal organs of cadavers. Nevertheless, we proved the hypothesis that thanatophagy increases in proportion to time elapsed since death for various cadavers using brain and heart tissues. The cadaver variability and disease etiology could plausibly determine the rate of thanatophagy and would warrant future studies. Our study involved the unprecedented use of criminal cadavers. A limitation to this type of study is that only postmortem tissues were analyzed due to the fact that antemortem sampling is impractical to execute.

In summary, our results demonstrate that thanatophagy occurs in a PMI-dependent manner in both brain and heart tissues and

Table 2
A comparison of the current study's thantophagy results with select autophagy studies involving brain and heart tissues [44-52].

Autophagic Proteins	Current Study		Previous Studies		References
	Brain	Heart	Brain	Heart	
LC3	Proportional increase in expression as PMI increased	Proportional increase in expression as PMI increased	Significantly increase in brains with HIV-1 encephalitis	Increase elicited by hypoxic adaptation in myoblast cells of rats	[27,44]
p62	Proportional increase in expression as PMI increased	Proportional increase in expression as PMI increased	Induced expression during apoptosis and proteasomal inhibition in neuronal cells	Significant increase in human ischemic and dilated cardiomyopathy	[45,46]
Beclin-1	Proportional decrease in expression as PMI increased	Proportional increase in expression as PMI increased	Decrease in expression in human brain tumors Significant increase in brains with HIV-1 encephalitis	Significant up-regulation in the rat hearts after ischemic adaptation	[27,44,47,48]
Atg7	Proportional increase in expression as PMI increased	Proportional increase in expression as PMI increased	Significant increase in brains with HIV-1 encephalitis	Triggered an increase in induction of basal autophagy in cultured cardiomyocytes	[27,49]
BNIP3	Proportional decrease in expression as PMI increased	Proportional decrease in expression as PMI increased	Up-regulation in stroke and hypoxia	Strong up-regulation in response to hypoxia in rat cardiac myocytes	[50-52]

Key: represents thanatophagic or autophagic proteins that are up-regulated; represent down-regulated proteins.

that BNIP3 may not be associated with the induction of cardiac and cerebral thanatophagy. Our study also suggests that while cardiac thanatophagy may occur in association with Beclin-1, cerebral thanatophagy takes place independent of Beclin-1. To our knowledge, this is the initial study that uses corpse brain and heart tissues, applies autophagic knowledge to thanatophagy, and explores a potential method to use thanatophagy for PMI. Future studies would include a mechanistic animal model of the time of death to investigate PMI-mediated thanatophagy to provide novel insight into developing a new forensic method.

Author contributions

GTJ collected the cadaver samples; GTJ and YL developed the experimental design; YL, GTJ and IK performed experimental methods; GTJ, YL, and SJF analyzed the data; and all participated in writing the manuscript.

Acknowledgments

This work was supported by National Science Foundation (NSF) grant HRD 1401075 and Forensic Sciences Foundation Lucas Research Grant Program.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2015.11.013.

References

[1] N. Hosokawa, T. Hara, T. Kaizuka, C. Kishi, A. Takamura, Y. Miura, S. Iemura, T. Natsume, K. Takehana, N. Yamada, et al., Nutrient-dependent mTORC1 association with the ULK1–Atg13–FIP200 complex required for autophagy, Mol. Biol. Cell. 20 (2009) 1981–1991.
[2] N. Gurusamy, I. Lekli, N.V. Gorbunov, M. Gherghiceanu, L.M. Popescu, D.K. Das, Cardioprotection by adaptation to ischaemia augments autophagy in association with BAG-1 protein, J. Cell. Mol. Med. 13 (2009) 13373–13387.
[3] A. Efeyan, W.C. Comb, D.M. Sabatini, Nutrient-sensing mechanisms and pathways, Nature 517 (2015) 302–310.
[4] B. Ravikumar, S. Sarkar, J.E. Davies, M. Futter, M. Garcia-Arencibia, Z.W. Green-Thompson, M. Jimenez-Sanchez, V.I. Korolchuk, M. Lichtenberg, S. Luo, et al., Regulation of mammalian autophagy in physiology and pathophysiology, Physiol. Rev. 90 (2010) 1383–1435.
[5] A. Nakai, O. Yamaguchi, T. Takeda, Y. Higuchi, S. Hikoso, M. Taniike, S. Omiya, I. Mizote, Y. Matsumura, M. Asahi, et al., The role of autophagy in cardiomyocytes in the basal state and in response to hemodynamic stress, Nat. Med. 13 (2007) 13619–13624.
[6] B.S. Xie, H.C. Zhao, S.K. Yao, D.X. Zhuo, B. Jin, D.C. Lv, C.L. Wu, D.L. Ma, C. Gao, X. M. Shu, Z.L. Ai, Autophagy inhibition enhances etoposide-induced cell death in human hepatoma G2 cells, Int. J. Mol. Med. 27 (2011) 599–606.
[7] J.J. Lum, D.E. Bauer, M. Kong, M.H. Harris, C. Li, T. Lindsten, C.B. Thompson, Growth factor regulation of autophagy and cell survival in the absence of apoptosis, Cell 120 (2005) 237–248.
[8] B. Levine, Eating oneself and uninvited guests: autophagy-related pathways in

cellular defense, Cell 120 (2005) 159–162.
[9] C. Behrends, M.E. Sowa, S.P. Gygi, J.W. Harper, Network organization of the human autophagy system, Nature 466 (2010) 68–76.
[10] L. Shang, X. Wang, AMPK and mTOR coordinate the regulation of Ulk1 and mammalian autophagy initiation, Autophagy 7 (2011) 924–926.
[11] J. Kim, M. Kundu, B. Viollet, K.L. Guan, AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1, Nat. Cell. Biol. 13 (2011) 132–141.
[12] N. Furuya, J. Yu, M. Byfield, S. Pattingre, B. Levine, The evolutionarily conserved domain of Beclin 1 is required for Vps34 binding, autophagy and tumor suppressor function, Autophagy 1 (2005) 46–52.
[13] W.X. Ding, H.M. Ni, M. Li, Y. Liao, X. Chen, D.B. Stolz, G.W. Dorn 2nd, X.M. Yin, Nix is critical to two distinct phases of mitophagy, reactive oxygen species-mediated autophagy induction and Parkin-ubiquitin-p62-mediated mitochondrial priming, J. Biol. Chem. 285 (2010) 27879–27890.
[14] R.A. Hanna, M.N. Quinsay, A.M. Orogo, K. Giang, S. Rikka, Å.B. Gustafsson, Microtubule-associated protein 1 light chain 3 (LC3) interacts with Bnip3 protein to selectively remove endoplasmic reticulum and mitochondria via autophagy, J. Biol. Chem. 287 (2012) 19094–19104.
[15] T. Johansen, T. Lamark, Selective autophagy mediated by autophagic adapter proteins, Autophagy 7 (2011) 279–296.
[16] D.J. Klionsky, H. Abeliovich, P. Agostinis, D.K. Agrawal, G. Aliev, D.S. Askew, M. Baba, E.H. Baehrecke, B.A. Bahr, A. Ballabio, et al., Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes, Autophagy 4 (2008) 151–175.
[17] N. Mizushima, T. Yoshimori, How to interpret LC3 immunoblotting, Autophagy 3 (2007) 542–545.
[18] P. Jiang, N. Mizushima, LC3- and p62-based biochemical methods for the analysis of autophagy progression in mammalian cells, Methods (2014), http://dx.doi.org/10.1016/j.ymeth.2014.11.021, pii: S1046-2023(14)00384-3.
[19] H. Zhang, M. Bosch-Marce, L.A. Shimoda, Y.S. Tan, J.H. Baek, J.B. Wesley, F. J. Gonzalez, G.L. Semenza, Mitochondrial autophagy is an HIF-1-dependent adaptive metabolic response to hypoxia, J. Biol. Chem. 283 (2008) 10892–10903.
[20] L.M. Lindqvist, M. Heinlein, D.C. Huang, D.L. Vaux, Prosurvival Bcl-2 family members affect autophagy only indirectly, by inhibiting Bax and Bak, Proc. Natl. Acad. Sci. USA 111 (2014) 8512–8517.
[21] Y. Lee, H.Y. Lee, R.A. Hanna, Å.B. Gustafsson, Mitochondrial autophagy by Bnip3 involves Drp1-mediated mitochondrial fission and recruitment of Parkin in cardiac myocytes, Am. J. Physiol. Heart Circ. Physiol. 301 (2011) H1924–H1931.
[22] J. Oyabu, O. Yamaguchi, S. Hikoso, T. Takeda, T. Oka, T. Murakawa, H. Yasui, H. Ueda, H. Nakayama, M. Taneike, et al., Autophagy-mediated degradation is necessary for regression of cardiac hypertrophy during ventricular unloading, Biochem. Biophys. Res. Commun. 441 (2013) 787–792.
[23] Y.L. Hu, M. DeLay, A. Jahangiri, A.M. Molinaro, S.D. Rose, W.S. Carbonell, M. K. Aghi, Hypoxia-induced autophagy promotes tumor cell survival and adaptation to antiangiogenic treatment in glioblastoma, Cancer Res. 72 (2012) 1773–1783.
[24] J. Pratt, B. Annabi, Induction of autophagy biomarker BNIP3 requires a JAK2/STAT3 and MT1-MMP signaling interplay in Concanavalin-A-activated U87 glioblastoma cells, Cell. Signal 26 (2014) 917–924.
[25] A. Salminen, K. Kaarniranta, A. Kauppinen, J. Ojala, A. Haapasalo, H. Soininen, M. Hiltunen, Impaired autophagy and APP processing in Alzheimer's disease: the potential role of Beclin 1 interactome, Prog. Neurobiol. 106 (2013) 33–54.
[26] G.T. Javan, I. Can, S.J. Finley, S. Soni, The apoptotic thanatotranscriptome associated with the liver of cadavers, Forensic Sci. Med. Pathol. (2015), http://dx.doi.org/10.1007/s12024-015-9704-6, in press.
[27] D. Zhou, E. Masliah, S.A. Spector, Autophagy is increased in postmortem brains of persons with HIV-1-associated encephalitis, J. Infect. Dis. 203 (2011) 1647–1657.
[28] Y. Lee, H.Y. Lee, A.B. Gustafsson, Regulation of autophagy by metabolic and stress signaling pathways in the heart, J. Cardiovasc. Pharmacol. 60 (2012) 118–124.
[29] A.A. Vass, Beyond the grave-understanding human decomposition, Microbiol. Today 28 (2001) 190–192.
[30] I. Tanida, T. Ueno, E. Kominami, LC3 conjugation system in mammalian autophagy, Int. J. Biochem. Cell. Biol. 36 (2004) 2503–2518.
[31] I. Tanida, T. Ueno, E. Kominami, LC3 and autophagy, Methods Mol. Biol. 445 (2008) 77–88.
[32] N. Hariharan, Y. Ikeda, C. Hong, R.R. Alcendor, S. Usui, S. Gao, Y. Maejima, J. Sadoshima, Autophagy plays an essential role in mediating regression of hypertrophy during unloading of the heart, PLOS One 8 (2013) e51632.
[33] L. Yan, J. Sadoshima, D.E. Vatner, S.F. Vatner, Autophagy in ischemic preconditioning and hibernating myocardium, Autophagy 5 (2009) 709–712.
[34] S. Schlossarek, D.R. Englmann, K.R. Sultan, M. Sauer, T. Eschenhagen, L. Carrier, Defective proteolytic systems in Mybpc3-targeted mice with cardiac hypertrophy, Basic Res. Cardiol. 107 (2012) 235.
[35] A. Hamacher-Brady, N.R. Brady, S.E. Logue, M.R. Sayen, M. Jinno, L. A. Kirshenbaum, R.A. Gottlieb, A.B. Gustafsson, Response to myocardial ischemia/reperfusion injury involves Bnip3 and autophagy, Cell. Death Differ. 14 (2007) 146–157.
[36] S. Thongchot, P. Yongvanit, W. Loilome, W. Seubwai, K. Phunicom, W. Tassaneeyakul, C. Pairojkul, W. Promkotra, A. Techasen, N. Namwat, High expression of HIF-1alpha, BNIP3 and PI3KC3: hypoxia-induced autophagy predicts cholangiocarcinoma survival and metastasis, Asian Pac. J. Cancer Prev. 15 (2014) 5873–5878.
[37] M. Band, A. Joel, A. Hernandez, A. Avivi, Hypoxia-induced BNIP3 expression and mitophagy: in vivo comparison of the rat and the hypoxia-tolerant mole rat, Spalax ehrenbergi, FASEB J. 23 (2009) 2327–2335.
[38] Y. Matsui, H. Takagi, X. Qu, M. Abdellatif, H. Sakoda, T. Asano, B. Levine, J. Sadoshima, Distinct roles of autophagy in the heart during ischemia and reperfusion: roles of AMP-activated protein kinase and Beclin 1 in mediating autophagy, Circ. Res. 100 (2007) 914–922.
[39] C. Hiebel, T. Kromm, M. Stark, C. Behl, Cannabinoid receptor 1 modulates the autophagic flux independent of mTOR- and BECLIN1-complex, J. Neurochem. 131 (2014) 484–497.
[40] N. Mizushima, T. Yoshimori, Y. Ohsumi, The role of Atg proteins in autophagosome formation, Annu. Rev. Cell. Dev. Biol. 27 (2011) 107–132.
[41] J. Fields, W. Dumaop, E. Rockenstein, M. Mante, B. Spencer, I. Grant, R. Ellis, S. Letendre, C. Patrick, A. Adame, E. Masliah, Age-dependent molecular alterations in the autophagy pathway in HIVE patients and in a gp120 tg mouse model: reversal with beclin-1 gene transfer, J. Neurovirol. 19 (2013) 89–101.
[42] K. Gao, G. Wang, Y. Wang, D. Han, J. Bi, Y. Yuan, T. Yao, Z. Wan, H. Li, X. Mei, Neuroprotective effect of simvastatin via inducing the autophagy on spinal cord injury in the rat model, Biomed. Res. Int. (2015), http://dx.doi.org/10.1155/2015/260161.
[43] X. Zhang, M.E. Gibson, Z.L. Li, X.Y. Zhu, K.L. Jordan, A. Lerman, L.O. Lerman, Autophagy portends the level of cardiac hypertrophy in experimental hypertensive swine model, Am. J. Hypertens. (2015), http://dx.doi.org/10.1093/ajh/hpv057, in press.
[44] N. Gurusamy, I. Lekli, N.V. Gorbunov, M. Gherghiceanu, L.M. Popescu, D.K. Das, Cardioprotection by adaptation to ischaemia augments autophagy in association with BAG-1 protein, J. Cell. Mol. Med. 13 (2009) 373–387.
[45] E. Kuusisto, A. Salminen, I. Alafuzoff, Ubiquitin-binding protein p62 is present in neuronal and glial inclusions in human tauopathies and synucleinopathies, Neuroreport 12 (2001) 2085–2090.
[46] R. Cortés, M. Portolés, E. Roselló-Lletí, L. Martínez-Dolz, L. Almenar, A. Salvador, M. Rivera, Nuclear changes and p62 expression in ischemic and dilated cardiomyopathy, Rev. Esp. Cardiol. 60 (2007) 1319–1323.
[47] C. Miracco, E. Cosci, G. Oliveri, P. Luzi, L. Pacenti, I. Monciatti, S. Mannucci, M. C. De Nisi, M. Toscano, V. Malagnino, S.M. Falzarano, L. Pirtoli, P. Tosi, Protein and mRNA expression of autophagy gene Beclin 1 in human brain tumours, Int. J. Oncol. 30 (2007) 429–436.
[48] H. Zhu, L. He, Beclin 1 biology and its role in heart disease, Curr. Cardiol. Rev. 11 (2015) 229–237.
[49] J.S. Pattison, J. Robbins, Autophagy and proteotoxicity in cardiomyocytes, Autophagy 7 (2011) 1259–1260.
[50] Z. Zhang, X. Yang, S. Zhang, X. Ma, J. Kong, BNIP3 upregulation and EndoG translocation in delayed neuronal death in stroke and in hypoxia, Stroke 38 (2007) 1606–1613.
[51] K.M. Regula, K. Ens, L.A. Kirshenbaum, Inducible expression of BNIP3 provokes mitochondrial defects and hypoxia-mediated cell death of ventricular myocytes, Circ. Res. 91 (2002) 226–231.
[52] L.A. Kubasiak, O.M. Hernandez, N.H. Bishopric, K.A. Webster, Hypoxia and acidosis activate cardiac myocyte death through the Bcl-2 family protein BNIP3, Proc. Natl. Acad. Sci. USA 99 (2002) 12825–12830.