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Progression of thanatophagy in cadaver brain and heart tissues

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A B S T R A C T

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

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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)*, ***a**u**to** (self)*, and ***phagy** (eating)*, undergoes the same autophagic processes. However, it is an egnima whether thanatophagic events occur after death in a human

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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

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