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1314 *Current Neuropharmacology,* **2018**, *16*, **1314-1319**

REVIEW ARTICLE

Autophagy after Subarachnoid Hemorrhage: Can Cell Death be Good?

Wing-Mann Ho[1,2,*], Onat Akyol[2], Haley Reis[3], Cesar Reis[4], Devin McBride[2], Claudius Thomé[1] and John Zhang[2]

[1]Department of Neurosurgery, Medical University Innsbruck, Innsbruck, Austria; [2]Department of Physiology and Pharmacology, Loma Linda University School of Medicine, Loma Linda, USA; [3]Loma Linda University School of Medicine, Loma Linda, USA; [4]Department of Preventive Medicine, Loma Linda University School of Medicine, Loma Linda, USA

ARTICLE HISTORY

Received: April 14, 2017
Revised: July 30, 2017
Accepted: November 21, 2017

DOI:
10.2174/1570159X15666171123200646

Abstract: ***Background*:** Autophagy is a prosurvival, reparative process that maintainsww cellular homeostasis through lysosomal degradation of selected cytoplasmic components and programmed death of old, dysfunctional, or unnecessary cytoplasmic entities. According to growing evidence, autophagy shows beneficial effects following subarachnoid hemorrhage (SAH). SAH is considered one of the most devastating forms of stroke.

***Methods*:** In this review lies in revealing the pathophysiological pathways and the effects of autophagy. Current results from animal studies will be discussed focusing on the effects of inhibitors and inducers of autophagy. In addition, this review discusses the clinical translation of potential neuropharmacological targets that can help prevent early brain injury (EBI) following SAH by incorporating programmed cell death into clinical management.

***Results*:** Published data showed that autophagy mechanisms have a prosurvival effect to reduce apoptotic cell death after SAH. However, if SAH exceeds a certain stress threshold, autophagy mechanisms lead to increased apoptotic cell death, more brain injury, and worse outcome.

***Conclusion*:** Future investigation on the differences and molecular switches between protective mechanisms of autophagy and excessive "self-eating" autophagy leading to cell death is needed to achieve more insight into the complex pathophysiology of brain injury after SAH. If autophagy after SAH can be controlled to lead to beneficial effects only, as the physiological self-control mechanism, this could be an important target for treatment.

Keywords: Subarachnoid hemorrhage, autophagy, cell death, autophagosome, lysosome, neuroprotection, brain injury.

1. INTRODUCTION

Under physiological conditions, autophagy is a cellular defense and survival mechanism involving both the autophagosomal and autolysosomal pathways to process the turnover of proteins. It also eliminates specific cell organelles resulting in regulated death of old, dysfunctional, or unnecessary cytoplasmic entities.

Pathological cellular conditions induce autophagy and crosstalk in the process of apoptosis. Depending on the pathological steps occurring intracellularly, autophagy results in a damaging or protective effect [1, 2]. Various endo- and exogenous stimuli induce autophagy in order to maintain intracellular homeostasis. Dysfunction of this process has been linked to various diseases including cancer, autoimmune diseases, and neurodegenerative diseases [3, 4]. Subarachnoid Hemorrhage (SAH) is a devastating cerebrovascular disease defined as the presence of blood in the subarachnoid space. In most cases, the rupture and subsequent bleeding of an intracranial aneurysm is the source of the blood. Although SAH accounts for only 5% of all strokes, it has a significant impact on individuals and the society. The mortality rates are as high as 45-50% [5] and an estimated 30% of survivors are left with moderate-to-severe disabilities [6]. Despite advances in diagnosis and treatment options of SAH in the last decade, the overall clinical outcome remains disappointing. Therefore, detailed research for more insight into the pathophysiology of EBI after SAH is needed.

2. INVOLVEMENT OF AUTOPHAGY IN EARLY BRAIN INJURY AFTER SAH

After SAH, the autophagy-lysosomal pathway is activated in the ipsilateral frontobasal cortex and lasts up to three days. Autophagy is predominately seen occurring in

*Address correspondence to this author at the Department of Neurosurgery, Medical University Innsbruck, Innsbruck, Austria; Tel: +43 (0)512 504 27452, Fax: +43 (0)512 504 27453; E-mail: wing.ho@tirol-kliniken.at

1570-159X/18 $58.00+.00

neurons 24 hours following SAH, and can be seen on electron microscopy. Findings included cells with multiple membrane cytoplasmic vacuoles, a contracted nucleus, swollen mitochondria, and autophagic vesicles [7]. Endoplasmic Reticulum (ER) stress upregulates antiapoptotic blc-2 protein levels, as well as mcl-1 [8]. Mcl-1 promotes autophagy through its relationship with beclin-1 [9]. Mitochondrial dysfunction is also associated with autophagy through activation of the apoptotic cascade and subsequent elimination of abnormal mitochondria. Electron microscopic studies identified swollen mitochondria with disrupted inner mitochondrial membranes and cristae following SAH. SAH-induced destruction of lysosomal integrity in conjunction with autophagy dysfunction leads to increased autophagosomes with reduced clearance due to the aggregation of damaged mitochondria [10].

Oxidative stress and reactive oxygen species (ROS) production from neuroinflammation have catastrophic effects on proteins, lipids, and nucleic acids. Nuclear translocation of activated nuclear respiratory factor 2 (Nrf2) is required for the expression of inducible and protective cell enzymes such as redoxins and the glutathione system [11]. Additionally, Nrf2 has shown to be an inducer of microtubule-associated protein light chain-3 (LC3) and beclin-1 expression [12]. LC3, a biomarker of the autophagosome, and beclin-1, a bcl-2 interacting protein have critical roles in autophagy.

LC-3 and beclin-1 are considered autophagosomal markers for autophagy. LC3-I is the cytosolic form and LC3-II is linked to the membrane and significantly increases expression during autophagosome formation [7]. Wang and colleagues reported gradually increased levels of autophagy-related proteins such as LC3-II and beclin-1 over 24 hours after SAH. The expression of LC3-II and beclin-1in the basilar artery wall is upregulated during the first 48 hours after SAH [13]. A cytosolic lysosomal degradation enzyme named cathepsin-d is activated through autophagic stimulation inside the cell. Lee and colleagues found that LC3-II, beclin-1, and cathepsin-d gradually increase within 24 hours after SAH induction but decline at 72 hours. Beclin-1 and cathepsin-d are expressed in neurons but not in astrocytes [7]. Downstream of cathepsin, the bcl-2 family is known for its pro- and antiapoptotic properties, and is thought to have an inhibitory effect on beclin-1 dependent autophagy [13]. Mitochondrial bax, a pro-apoptotic member of the bcl-2 family, and cytochrome C release were also found to be elevated 24 hours after SAH in an endovascular perforation model [14]. Cytochrome C is a cofactor for apoptotic protease-activating factor-1 (APAF-1) and triggers formation of the apoptosome and subsequent activation of the initiator and executioner caspases, leading to cell death [15]. When the autophagy blocker 3-methyladenine was applied, autophagy-related genes (Atg) such as Atg5 decreased. In addition, cleaved caspase-3 levels as well as TUNEL positive neurons were upregulated [14]. Studying the pathophysiology following SAH allows researchers to manipulate the expression of pro- and antiapoptotic biomarkers and understand their role in autophagy. Studies aimed at understanding the role of mitochondria in autophagy elucidates treatment options following SAH.

Voltage dependent anion channels (VDAC) are mitochondrial outer membrane protein channels and have been established as main indicators of mitochondrial autophagy [16]. Following SAH, VDAC1 expression increased after 12 and 24 hours and stayed elevated after 48 hours. Additionally, ROS production, cleaved caspase-3, and the LC3-I/LC3-II ratio was elevated 48 hours after SAH. In the same study, VDAC was blocked by siRNA and outcome impairments were similar to the SAH group, while the rapamycin-treated group showed elevated autophagy markers and improvement of functional deficits. Blocking VDAC1 led to inhibition of autophagy and enhanced ROS production and apoptosis. The authors identified neuronal co-localization of VDAC1 and LC3 and proposed that VDAC1-induced mitophagy has a role in autophagy and apoptosis in SAH-induced brain injury. Rapamycin, an autophagy activator, induced VDAC1 and LC3-II expression, thereby alleviating neuronal apoptosis and ROS production 48 hours after SAH [17]. Studies focusing on VDAC1 expression and its impact in cerebral vasospasm after SAH can provide new options for treatment strategies.

EBI following SAH is suggested to be strongly correlated with the neuroinflammation progress. According to previous experimental research on the proinflammatory RAGE (the receptor for advanced glycation end products) pathway, it has a role in autophagy and apoptosis modulation in various brain injury models. Suppression of RAGE signaling markedly augments neuroapoptosis with higher cleaved caspase-3 and bax levels, yet decreased bcl-2 after SAH. Blocking RAGE diminishes LC3-II and beclin-1 upregulation due to SAH and reduces neuroinflammatory cytokines. The multiligand RAGE has a marked regulatory function during the neuroinflammation process, and NF-kβ has a predominant role through RAGE-induced pathways to stimulate neuroinflammatory mediators. The RAGE–NF-kβ signaling pathway contributes in SAH-induced neuroinflammation and therefore EBI [18]. RAGE is predominantly being expressed by neurons and microglia [19]. According to experimental data, the resultant outcome of RAGE signaling on cell survival can be pro- and antiapoptotic. Following SAH, expression of RAGE increases from 12 hours to 72 hours. Furthermore, NF-κβ p65 inhibition was detected as a result of RAGE blocking after SAH and vice versa. A cell culture study by Li and colleagues reported the inhibition of RAGE abolished microglial activation as well as TNF-α, IL-1β and COX-2 release 24 hours after SAH. Additionally, when the RAGE pathway was blocked, cleaved caspase-3 and bax levels were increased, in contrast to diminished autophagy markers LC3 and beclin-1 [20]. These findings support the apoptosis-autophagy balance and feedback after SAH, particularly during EBI, and offer potential targets for ameliorating the consequences of SAH. Deregulation of autophagy-apoptosis feedback and signaling to mitochondria and lysosomes has an essential function during neuroprotection after neuronal injury. However, there are many pending questions concerning the interdependence of neuroapoptosis and autophagy after SAH, especially in EBI and vasospasm periods. Fig. (**1**) provides an overview of molecular pathways associated with autophagy after SAH.The details underlying the pericyte and

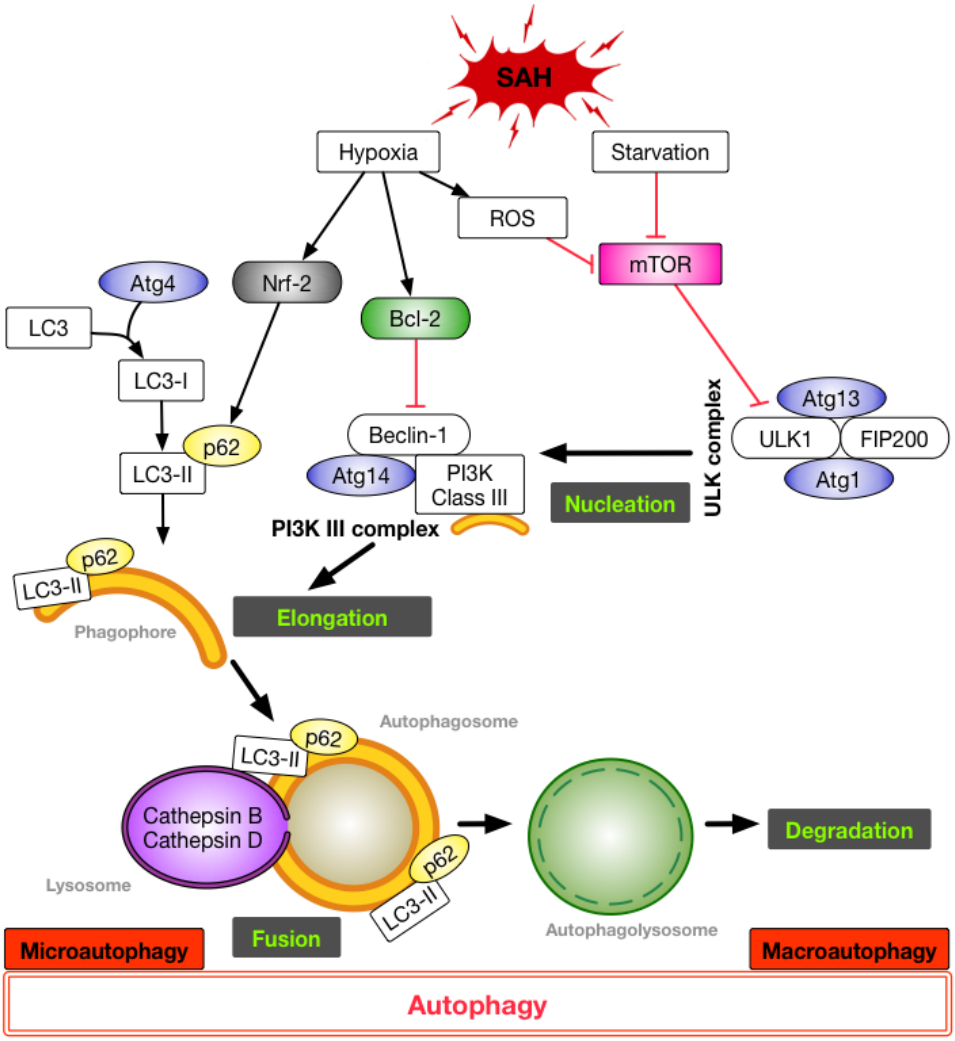

Fig. (1). Autophagy pathways after SAH. Major mechanisms of after SAH are cellular hypoxia and starvation leading to activation of the Nrf-2, Bcl-2, ROS, and inhibition of mTOR activity. Autophagy is initiated via ULK complex induced autophagosome membrane nucleation through PI3K. Elongation and maturation of the phagophore forms the autophagosome. Once the autophagic vesicles are formed and cargo is engulfed, the autophagosome is fused with the lysosome. Unlike this form of chaperone-mediated macroautophagy, there is direct lysosomal microautophagy.

autophagy relationship in SAH-induced brain injury and cerebral vasospasm remains unknown. Future research in the area of autophagy and SAH with an emphasis on pericytes offers promising knowledge, as abnormal pericytic activity causes a defective autophagy process [21].

3. AUTOPHAGY INDUCERS AND INHIBITORS AFTER SAH

Exploring both autophagy inducers and inhibitors provides insight into the delicate balance between too much and not enough programmed cell death concerning autophagy and its effects following SAH.

Previous studies demonstrate that the autophagy pathway is activated after experimental SAH. As previously mentioned, Wang and colleagues demonstrated, that rapamycin (an autophagy activator) and 3-methyladenine (an autophagy inhibitor) regulate EBI after SAH. LC3 and beclin-1 were significantly increased at an earlier stage after SAH and peaked at 24 hours. Rapamycin-treated animals improved on the clinical behavior scale in comparison with vehicle-treated SAH rats. Treatment using 3-methyladenin worsened neurological deficits, and the expression of LC3 and beclin-1 was decreased. The study concluded that activation of autophagy has beneficial effects on EBI development after SAH [13].

In 2013, Zhao *et al*. used a modified endovascular perforating SAH model and rats were given simvastatin 14 days prior to SAH induction and experimentation with autophagy-regulating drugs. Rats were injected with rapamycin, 3-methyladenine, or wortmannin 30 minutes prior to SAH. The neurological scores were decreased and neuronal apoptosis was increased with 3-methyladenine or wortmannin treatment. The combination of rapamycin and prophylactic simvastatin increased autophagy activation and inhibited apoptosis. Autophagy activation reduced translocation of bax from the cytosol into the mitochondrial membrane. The acti-

vation of caspases in the mitochondrial pathway requires outer membrane permeabilization for diffusion of proteins, such as cytochrome C. Autophagy activation effectively ameliorates EBI by inhibiting the mitochondrial apoptotic pathway *via* decreased release of cytochrome C release into the cytosol [22]. The effect of bcl-2 proteins in allowing mitochondrial membrane permeability is a target for preventing apoptosis in EBI.

Another mediator of EBI includes melatonin, a hormone secreted by the pineal gland to help regulate daily body rhythms, such as sleep. Chen and colleagues found that giving melatonin 2 hours after SAH induction reduced caspase-3 activity and decreased apoptotic activity. The treatment increased autophagy biomarkers, including an increase in the LC3-II/LC3-I ratio, indicating melatonin-enhanced autophagy ameliorates apoptotic cell death in rats after SAH. This study further supports that protection against EBI includes reducing bax translocation to the mitochondrial membrane and the subsequent release of cytochrome C into the cytosol [23].

In addition to protecting against pro-apoptotic pathways *via* mitochondrial membrane permeability, ER also plays an important role in EBI. Yan *et al*. investigated the role of ER stress and found that using the ER stress-inducer tunicamycin improved neurological deficits and decreased the expression of pro-apoptotic caspase-3 as well the number of TUNEL-positive cells. Western blot analysis found increased levels of autophagic protein beclin-1 and an increase in the LC3-II/LC3-1 ratio. Analysis also revealed reduced levels of autophagy markers with the use of tauroursodeoxycholic acid, an ER stress inhibitor. This study suggests that ER stress decreased EBI by inhibiting apoptosis and providing neuroprotective effects *via* autophagy activation [24].

Gene transcription regulators contribute to the interaction between apoptosis and autophagy through histone acetylation. Trichostatin A is a pan-histone deacetylase inhibitor found to have neuroprotective properties and used in a study for improving autophagy following SAH. 3-methyladenine, an autophagy inhibitor, was also evaluated. Trichostatin A reduced brain edema and improved neurological deficits 24 hours following SAH. It also increased acetylated histone H3, promoting transcriptional activation. Furthermore, it increased beclin-1 and the LC3-II/LC3-I ratio, suggesting increased activity in conjugation of the autophagosomal marker LC3-II and autophagic activity. Additionally, trichostatin A decreased bax and cleaved caspase-3 in the cortex, showing decreased apoptosis in the ipsilateral basal cortex, while 3-methyladenine reversed the beneficial effects of trichostatin A [25].

Cystatin C is an endogenous cysteine protease inhibitor with the ability to inhibit cathepsins B, H, K, L and S. It exerts its effects over numerous cellular systems, promoting both growth and down-regulation of inflammation [26]. Liu and colleagues evaluated the role of cystatin C and its benefits on EBI and neurobehavioral dysfunction following SAH, as it has been implicated therapeutically for brain ischemia, Alzheimer's disease, and other neurodegenerative disorders [27]. After SAH, they found that LC3 and beclin-1 were significantly increased in the cortex 48 hours after SAH, and were further upregulated with cystatin C therapy. Cystatin C-treated rats showed increased autophagosomes and autolysosomes in neurons, and decreased learning deficits with medium dose concentrations, suggesting its use in attenuating EBI and neurobehavioral dysfunction through autophagy activation [27, 28].

Tertbutylhydroquinone is both an antioxidant and an Nrf2 activator, and provides an additional increase in beclin-1 and LC-3 expression, thereby alleviating brain edema, blood brain barrier disruption, neurologic deficits, and neuronal degeneration 24 hours after SAH. In addition, tertbutylhydroquinone treatment restored bax and bcl-2 expression and reduced caspase-3 cleavage. These findings suggest it exerts a neuroprotective effect and decreases EBI after SAH, independent of Nrf2 activation [12].

To describe mitochondrial dysfunction and autophagy, Chen and colleagues performed an *in-vitro* research study using epigallocatechin-3-gallate, a protein able to convert from the ferric (Fe^{3+}) to ferrous (Fe^{2+}) form by decreasing oxygenated hemoglobin levels. This initiates cytosolic calcium influx after SAH *via* inhibiting voltage-gated calcium channel-dependent calcium entry and mitochondrial calcium uptake. It also opens mitochondrial membrane permeability transition pores and causes mitochondrial depolarization after SAH. When used as pretreatment prior to SAH, epigallocatechin-3-gallate normalizes mitochondrial and cytosolic cytochrome C levels. In this study, the expression of the Atg5 gene, which is essential for autophagy, was found to be reduced after SAH. On the other hand, the expression of autophagosome formation-related genes such as beclin-1 and LC3, were found significantly elevated in the SAH group. This alteration explains the electron microscopy screening of increased numbers of autophagic vacuoles after SAH. Pretreatment with epigallocatechin-3-gallate reversed At5 gene expression, resulting in a decline in autophagosomes and an increase in autolysosomes, along with a reduction in apoptosis [10].

Modulating and controlling autophagy has been implicated as a therapeutic strategy in SAH, and it's been suggested as a neuroprotective mechanism during hemorrhage [29]. Table **S1** provides an overview of substances being investigated and their effects on autophagy and neurological outcome in SAH models.

CONCLUSION

The consequences after SAH are complex and involve the overall pathogenesis and outcomes result from multiple processes. Despite the advances in research, diagnostics, and current treatments of aneurysms and complications, the outcome of patients suffering from SAH remains poor. The major underlying pathophysiological mechanisms are categorized into EBI and delayed brain injury, with both processes leading to poor prognosis. Published studies support that proper functioning of autophagy mechanisms has a prosurvival effect and work to reduce apoptotic cell death after SAH. However, if SAH exceeds a certain stress threshold, autophagy mechanisms lead to increased apoptotic cell death. Therefore, maintaining cell hemostasis and appropriate autophagy activity are necessary for providing beneficial

effects on the patient's outcome after SAH. Future investigation on the differences and molecular switches between protective mechanisms of autophagy and excessive "self-eating" autophagy leading to cell death is needed to achieve more insight into the complex pathophysiology of brain injury and SAH. If autophagy after SAH can be controlled to lead to beneficial effects only, as the physiological self-control mechanism, this could be an important target for treatment. Therefore, achieving a better understanding of the molecular pathways of the disease provides the framework needed to improve the development of more efficient treatment strategies following SAH that utilize the autophagy pathway in improving overall outcomes.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

Declared none.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's web site along with the published article.

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